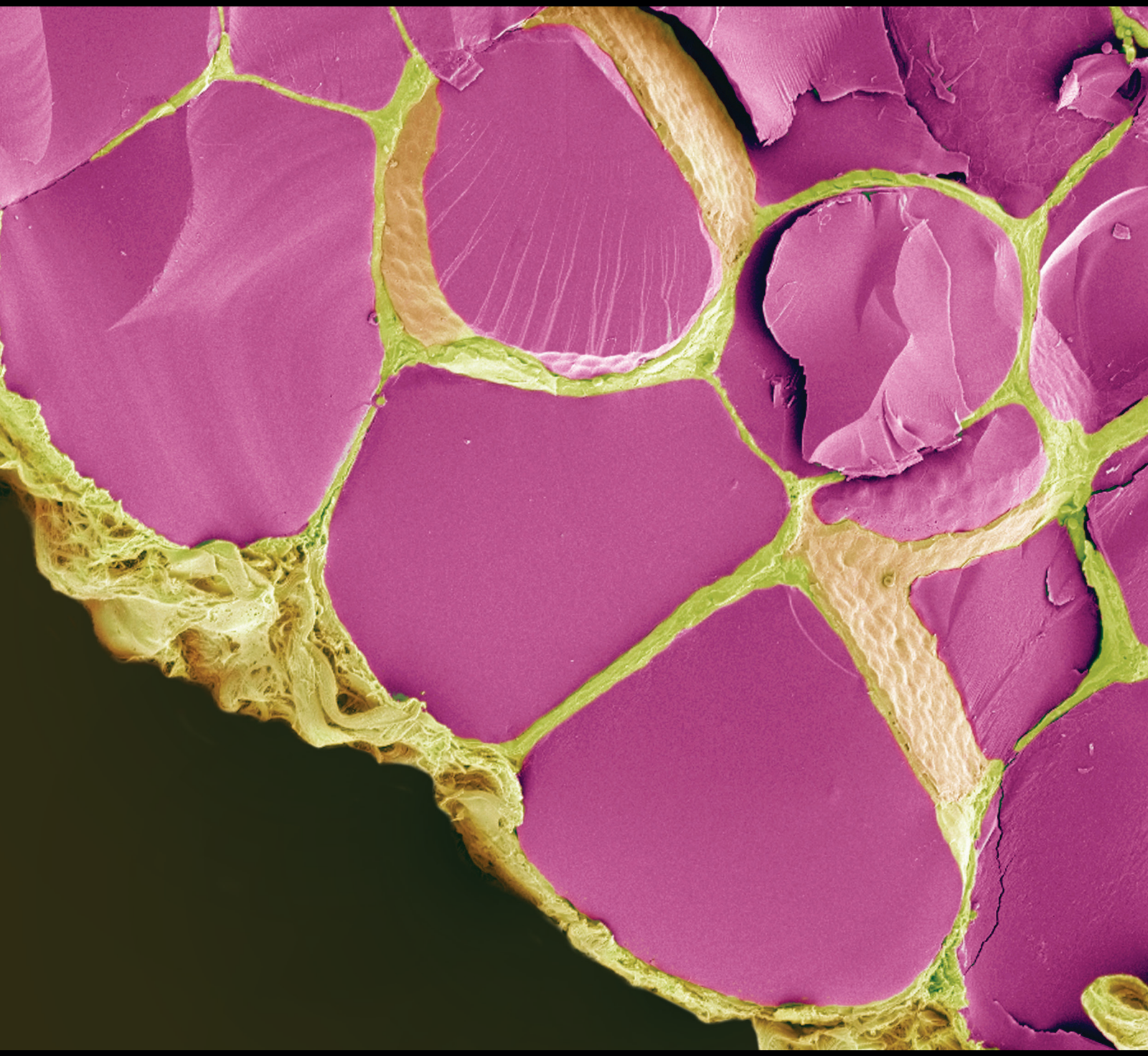
International Journal of Endocrinology

Diabetes Mellitus 2014

Guest Editors: Ilias Migdalis, David Leslie, Anastasia Mavrogiannaki, Nikolaos Papanas, Paul Valensi, and Helen Vlassara



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Editorial Board

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Editorial

Diabetes Mellitus 2014

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The ongoing progress in diabetes mellitus is reflected in new treatment algorithms [1], new oral hypoglycaemic agents [2], new tests for the early diagnosis of complications [3], and improved organisation of healthcare resources [4]. The present special issue is devoted to the recent progress. The articles cover 4 thematic areas: the main area is diabetic complications, while the other 3 are treatment, metabolism, and miscellaneous issues.

(a) Diabetic Complications. Complications of diabetes remain a major area needing improvement [4, 5]. J. Lowe et al. in their paper entitled "The Guyana Diabetes and Foot Care Project: Improved Diabetic Foot Evaluation Reduces Amputation Rates by Two-Thirds in a Lower Middle Income Country" reported an overall 68% reduction in major amputations after initiation of a multi-expert intervention programme in Guyana: below-knee amputations were reduced by 80%, while above-knee amputations remained unchanged. These results are very encouraging.

Two papers "Vascular Effects of Dietary Advanced Glycation End Products" by A. Stirban and D. Tschöpe and "Diabetes, Endothelial Dysfunction, and Vascular Repair: What Should a Diabetologist Keep His Eye on?" by V. Altabas shed more light on diabetes-induced vascular injury and the underlying mechanisms, including the effect of advanced glycation end products (AGEs) [6], depletion of nitric oxide (NO), and endothelial apoptosis.

Diabetic kidney function and diabetic nephropathy have been the object of some articles in this special issue. In their work titled "Inverse Levels of Adiponectin in Type 1 and Type 2 Diabetes Are in Accordance with the State of Albuminuria," S. Ljubic et al. found that adiponectin increased in type 1 (T1DM) but decreased in type 2 diabetes (T2DM) with deterioration of albuminuria. These interesting results enrich our knowledge on the role of adiponectin as a risk marker of vascular disease in diabetes [7], while the difference between the two diabetes types calls for additional investigation. In 19 patients with diabetic nephropathy receiving angiotensin converting enzyme inhibitors or angiotensin II receptor blockers, S. M. Lee et al. evaluated the effect of omega-3 fatty acids (3 g per day) versus olive oil on proteinuria in their study entitled "Effect of Omega-3 Fatty Acid on the Fatty Acid Content of the Erythrocyte Membrane and Proteinuria in Patients with Diabetic Nephropathy." Neither treatment succeeded in reducing proteinuria, possibly due to concomitant angiotensin converting enzyme inhibitor/angiotensin II receptor blocker treatment and to the absence of very severe proteinuria at baseline.

Including 150 patients with mild-to-moderate diabetic nephropathy, A. P. Silva et al. in their paper entitled

"Low Magnesium Levels and FGF-23 Dysregulation Predict Mitral Valve Calcification as well as Intima Media Thickness in Predialysis Diabetic Patients" found that reduced magnesium and high levels of fibroblast growth factor-23 were independent predictors of mitral valve calcification and of increased carotid intima media thickness. These findings are a valuable contribution to the search for risk markers of vascular calcification in chronic kidney disease [8]. "The Blocking on the Cathepsin B and Fibronectin Accumulation in Kidney Glomeruli of Diabetic Rats" is a study by A. Wyczalkowska-Tomasik et al., who compared enalapril, losartan, enalapril plus losartan, spironolactone, and no treatment in terms of the activity of cathepsin B accumulation in the glomeruli. Enalapril plus losartan treatment increased glomerular activity of cathepsin B compared to untreated diabetic rats. Spironolactone increased this activity as well. These results need to be seen in the context of renin-angiotensin-aldosterone system inhibition as a therapeutic strategy for diabetic nephropathy, but caution is needed when extrapolating to humans.

Other groups dealt with coronary artery disease (CAD) and cardiac structure. K. Lalić et al. in their study entitled "Altered Daytime Fluctuation Pattern of Plasminogen Activator Inhibitor 1 in Type 2 Diabetes Patients with Coronary Artery Disease: A Strong Association with Persistently Elevated Plasma Insulin, Increased Insulin Resistance, and Abdominal Obesity" reported reduced diurnal fluctuation of plasminogen activator inhibitor-1 in patients with T2DM and CAD, along with increased insulin resistance, suggesting that these perturbations may be of relevance for the accelerated atherosclerosis in such patients. In their paper titled "The Difference Quantity of Urinary Peptides between Two Groups of Type 2 Diabetic Patients with or without Coronary Artery Disease," G. Fu et al. examined T2DM patients and noted significant differences in the expression of peptides (fragments of isoform 1 of fibrinogen alpha chain precursor, prothrombin precursor, and inter-alpha-trypsin inhibitor heavy chain H4) between those with CAD and those without CAD, suggesting that we need to consider how such differences may be practically utilised in the search for CAD biomarkers. Similarly, E. C. Pereira et al. in their article entitled "Predictive Potential of Twenty-Two Biochemical Biomarkers for Coronary Artery Disease in Type 2 Diabetes Mellitus" found that 8 biomarkers (methionine, nitrate plus nitrite, n-acetyl-β-glucosaminidase, BMI, LDL, HDL, reduced glutathione, and L-arginine/asymmetric dimethyl-L-arginine) were associated with CAD in T2DM. Finally, K. Ziros et al. in their experimental study entitled "The effect of a low glycemic index diet on cardiac structure in an experimental model of diabetic rats" examined streptozotocin-induced diabetic rats and found greater area, perimeter and length of collagen fibres in heart vessels among those under normal glycaemic index diet as compared with those under low glycaemic index diet, indicating the importance of the glycaemic index of experimental diets in the study of complications in experimental works.

S. T.-H. Chiang et al. in their study entitled "Investigation of the Protective Effects of Taurine against Alloxan-Induced Diabetic Retinal Changes via Electroretinogram and Retinal Histology with New Zealand White Rabbits" demonstrated that taurine supplement reduced both hyperglycaemia and retinal electrophysiological changes in alloxan-induced diabetic rabbits. In a more practical setting, A. Jotic et al. in their article entitled "Decreased Insulin Sensitivity and Impaired Fibrinolytic Activity in Type 2 Diabetes Patients and Nondiabetics with Ischemic Stroke" identified the levels of insulin, plasminogen activator inhibitor-1, and insulin sensitivity as independent predictors of ischaemic stroke in patients with and without T2DM. The final study about complications is entitled "Role of the Insulin-Like Growth Factor Type 1 Receptor in the Pathogenesis of Diabetic Encephalopathy" by D. Zhang et al. The authors researched into diabetic encephalopathy, a condition including perturbations in cognition, cerebral signal conduction, neurotransmission, and other functions, in primary rat PC-12 cells. The authors found lower glucose metabolism and abnormally high expression of insulin-like growth factor-1 receptor in the diabetic encephalopathy model. The defect of insulin-like growth factor-1 receptor improved glucose utilisation and insulin sensitivity. These novel experimental results add to the accumulating experience on the role of insulin and insulin-like growth factor-1 in cognition and memory [9].

(b) Treatment. G. Rombopoulos et al. in their 24-week observational study of 659 metformin-treated T2DM patients titled "Treatment Compliance with Fixed-Dose Combination of Vildagliptin/Metformin in Patients with Type 2 Diabetes Mellitus Inadequately Controlled with Metformin Monotherapy: A 24-Week Observational Study" showed equal hypoglycaemic effect but higher compliance rates for the fixed vildagliptin plus metformin combination tablet versus the free-dose combination therapy with these two agents. I. Migdalis et al. performed a cost analysis of the treatment for T2DM in Greece ("The Cost of Managing Type 2 Diabetes Mellitus in Greece: A Retrospective Analysis of 10-Year Patient Level Data 'The HERCULES Study'"). Of note, the largest part of total expenditure (48%) was for management of comorbidities, while pharmaceutical treatment comprised 35.9% and antidiabetic treatment only 14.9%. The highest cost was seen in obese men with a long diabetes duration and those with poor education. These useful results have obvious implications in the face of Greek economic crisis and its impact on the treatment of diabetic complications [10].

In their article entitled "Sitagliptin: Is It Effective in Routine Clinical Practice?" R. M. Dallumal et al. collected data from medical records of 457 T2DM patients. In the majority of these, sitagliptin was added to other antidiabetic agents, commonly metformin and/or sulfonylurea. The authors documented a −0.8% reduction in glycated haemoglobin within the first 6 months of sitagliptin treatment. However, no further improvement was seen during the next 6 treatment months. These observations are useful, but inhibitors of dipeptidyl peptidase 4 (DPP-4) usually maintain their efficacy longer [1], and so the authors need to look at longer follow-up data. H. Z. Huri et al. in their study entitled "Factors Associated with Utilization of Dipeptidyl-4 Inhibitors in Patients with Type 2 Diabetes Mellitus: A Cross-Sectional Retrospective Study" looked at retrospective data from 299

subjects taking either sitagliptin or vildagliptin. Of these, 95% received combination therapy. Age < 65 years ($p = 0.049$), no beta-blocker therapy ($p = 0.045$), and no aspirin therapy ($p = 0.008$) were significantly associated with choice of DPP-4 inhibitors as antidiabetic therapy. These observations reflect prescription patterns in the authors' country.

A. M. L. Martín et al. in their paper titled "Breaking Therapeutic Inertia in Type 2 Diabetes: Active Detection of In-Patient Cases Allows Improvement of Metabolic Control at Midterm" provided very interesting evidence that hospitalisation on the surgical ward may serve as an opportunity for detection of poor glycaemic control and prompt consultation with a diabetologist. Approximately half of the patients were reevaluated at 3–6 months, and a significant ($p < 0.004$) reduction in glycated haemoglobin was seen (mean reduction: –1.1%).

H.-W. Lin and C.-H. Tseng published "A Review on the Relationship between SGLT2 Inhibitors and Cancer." Their conclusion was that the relationship between inhibitors of sodium-glucose cotransporter 2 and cancer is not conclusive, calling for larger databases and longer patient follow-up.

(c) Metabolism. This section covers various areas of metabolism. M. Acevedo et al. in their study entitled "Comparison of Lipoprotein-Associated Phospholipase A2 and High Sensitive C-Reactive Protein as Determinants of Metabolic Syndrome in Subjects without Coronary Heart Disease: In Search of the Best Predictor" observed that high sensitivity C-reactive protein (hsCRP) and Lipoprotein-associated phospholipase A2 (Lp-PLA2) were predictors of the metabolic syndrome in patients without CAD.

K. Toulis et al. in their study titled "Thyroid Autoimmunity in the Context of Type 2 Diabetes Mellitus: Implications for Vitamin D" examined an elderly population with frequent vitamin D deficiency and showed that the presence of T2DM increased the likelihood of thyroid autoimmunity. J. Tian et al. in their article entitled "Trends in the Levels of Serum Lipids and Lipoproteins and the Prevalence of Dyslipidemia in Adults with Newly Diagnosed Type 2 Diabetes in the Southwest Chinese Han Population during 2003–2012" demonstrated alarmingly high rates of dyslipidaemia, CAD, and cerebrovascular disease in newly diagnosed T2DM Chinese patients, emphasising the need for early diagnosis and aggressive management.

In their study entitled "Effects of Aerobic Exercise Based upon Heart Rate at Aerobic Threshold in Obese Elderly Subjects with Type 2 Diabetes," G. P. Emerenziani et al. provided evidence that aerobic exercise improved physical fitness, heart function, and metabolism in obese elderly T2DM patients. A. S. Moghaddam et al. in their paper titled "The Effects of Soy Bean Flour Enriched Bread Intake on Anthropometric Indices and Blood Pressure in Type 2 Diabetic Women: A Crossover Randomized Controlled Clinical Trial" showed no significant effects of soy bread on anthropometric indices and blood pressure in T2DM. M. Rodríguez-Cruz et al. in their paper titled "Evidence of Insulin Resistance and Other Metabolic Alterations in Boys with Duchenne or Becker Muscular Dystrophy" found high rates of obesity and insulin resistance in boys with these two muscular dystrophies independent of corticosteroid treatment, while insulin resistance appeared to have a genetic component as well. In the experimental work "Resistance to the Beneficial Metabolic Effects and Hepatic Antioxidant Defense Actions of Fibroblast Growth Factor 21 Treatment in Growth Hormone-Overexpressing Transgenic Mice," R. K. Boparai et al. noted resistance to the beneficial metabolic effects of fibroblast growth factor 21 in these transgenic animals.

(d) Miscellaneous. This final section covers diverse research fields. E.-H. Lee et al. published "Psychometric Properties of the Diabetes Management Self-Efficacy Scale in Korean Patients with Type 2 Diabetes," providing a Korean version of the Diabetes Management Self-Efficacy Scale, which proved reliable for clinical use in their country. M. H. Yang et al. in their article "Do Behavioral Risk Factors for Prediabetes and Insulin Resistance Differ across the Socioeconomic Gradient? Results from a Community-Based Epidemiologic Survey" reported that the association between waist circumference and insulin resistance was independent of socioeconomic status, but its association with prediabetes was only significant in the highest socioeconomic level. These observations are interesting in terms of the interplay between societal and metabolic factors [11] and need further exploration. R. de M. B. Marques et al. in their article "Relative Validity and Reproducibility of a Quantitative Food Frequency Questionnaire for Adolescents with Type 1 Diabetes: Validity of a Food Frequency Questionnaire" showed adequate validity and reproducibility of a quantitative food frequency questionnaire for T1DM adolescents. A. Spirkova et al. in their work titled "Treated Autoimmune Thyroid Disease Is Associated with a Decreased Quality of Life among Young Persons with Type 1 Diabetes" found that thyroxin-treated autoimmune thyroid disease (but not coeliac disease) was associated with reduced quality of life in children with T1DM. These findings increase our insight into the intricate psychological issues needing careful consideration in the modern management of T1DM [12].

In the Norwegian study "The Chromosome 9p21 CVD- and T2D-Associated Regions in a Norwegian Population (The HUNT2 Survey)," Ø. Helgeland et al. confirmed the association of variants of *CDKN2B* on chromosome 9p21 in patients with T2DM and CAD. F. A. F. Da-Mata et al. in their paper "Prevalence of Self-Reported Diabetes and Its Associated Factors: A Population-Based Study in Brazil" reported a 10.1% prevalence of diabetes in the adult Brazil population. Age 35–65 years, hypertension, respiratory and cardiovascular disease, and pain/discomfort were significantly associated with diabetes. P. Mesquita et al. found a surprisingly high (20.6%) frequency of orthostatic hypertension in elderly patients with T2DM in their article entitled "Prevalence of Orthostatic Hypertension in Elderly Patients with Type 2 Diabetes." Last but not least, A. S. Peacock et al. in their paper entitled "A Randomised Controlled Trial to Delay or Prevent Type 2 Diabetes after Gestational Diabetes: Walking for Exercise and Nutrition to Prevent Diabetes for You" documented that the use of a pedometer and a nutrition programme was successful in achieving both weight loss and

increased physical activity over a 3-month period in women with prior gestational diabetes mellitus, cherishing the hope that this approach might contribute to the prevention of T2DM in later life.

Conclusions. This special issue testifies to the ongoing progress in diabetes research and care. Naturally, it has been impossible to cover all areas showing progress, for example, the role of microcirculation [13] and bariatric surgery [14]. More importantly, the challenge remains how much of and how often this new knowledge can be utilised in everyday clinical practice.

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References

[1] S. E. Inzucchi, R. M. Bergenstal, J. B. Buse et al., "Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes," *Diabetes Care*, vol. 38, no. 1, pp. 140–149, 2015.

[2] K. Pafili and N. Papanas, "Luseogliflozin and other sodium-glucose cotransporter 2 inhibitors: no enemy but time?" *Expert Opinion on Pharmacotherapy*, vol. 16, no. 4, pp. 453–456, 2015.

[3] N. Papanas and D. Ziegler, "New vistas in the diagnosis of diabetic polyneuropathy," *Endocrine*, vol. 47, no. 3, pp. 690–698, 2014.

[4] C. A. Manu, O. G. Mustafa, M. Bates et al., "Transformation of the multidisciplinary diabetic foot clinic into a multidisciplinary diabetic foot day unit: results from a service evaluation," *The International Journal of Lower Extremity Wounds*, vol. 13, no. 3, pp. 173–179, 2014.

[5] Z. T. Bloomgarden, "Cardiovascular disease, neuropathy, and retinopathy," *Diabetes Care*, vol. 32, no. 6, pp. e64–e68, 2009.

[6] H. Vlassara and G. E. Striker, "Advanced glycation endproducts in diabetes and diabetic complications," *Endocrinology and Metabolism Clinics of North America*, vol. 42, no. 4, pp. 697–719, 2013.

[7] G. K. Shetty, P. A. Economides, E. S. Horton, C. S. Mantzoros, and A. Veves, "Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes," *Diabetes Care*, vol. 27, no. 10, pp. 2450–2457, 2004.

[8] M. Noordzij, E. M. Cranenburg, L. F. Engelsman et al., "Progression of aortic calcification is associated with disorders of mineral metabolism and mortality in chronic dialysis patients," *Nephrology Dialysis Transplantation*, vol. 26, no. 5, pp. 1662–1669, 2011.

[9] C.-C. Huang, C.-C. Lee, and K.-S. Hsu, "The role of insulin receptor signaling in synaptic plasticity and cognitive function," *Chang Gung Medical Journal*, vol. 33, no. 2, pp. 115–125, 2010.

[10] K. Aloumanis and N. Papanas, "Greek financial crisis: consequences in the healthcare of diabetes and its complications," *Hippokratia*, vol. 18, no. 1, pp. 4–6, 2014.

[11] I. Migdalis, D. Leslie, N. Papanas, P. Valensi, and H. Vlassara, "Diabetes mellitus," *International Journal of Endocrinology*, vol. 2014, Article ID 108419, 6 pages, 2014.

[12] R. Whittemore, S. Jaser, A. Chao, M. Jang, and M. Grey, "Psychological experience of parents of children with type 1 diabetes: a systematic mixed-studies review," *Diabetes Educator*, vol. 38, no. 4, pp. 562–579, 2012.

[13] I. Pham, M.-T. Nguyen, P. Valensi et al., "Noninvasive study of coronary microcirculation response to a cold pressor test," *European Journal of Clinical Investigation*, vol. 45, no. 2, pp. 135–143, 2015.

[14] A. J. Thomas, H. A. Bainbridge, J. L. Schone et al., "Recruitment and screening for a randomized trial investigating Roux-en-Y gastric bypass versus intensive medical management for treatment of type 2 diabetes," *Obesity Surgery*, vol. 24, no. 11, pp. 1875–1880, 2014.

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Research Article

The Cost of Managing Type 2 Diabetes Mellitus in Greece: A Retrospective Analysis of 10-Year Patient Level Data "The HERCULES Study"

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Objective. This study aimed to estimate the mean annual cost of treating type 2 diabetes mellitus patients (T2DM) including complications and comorbidities in Greece. *Design.* A noninterventional retrospective study was based on patient level data analysis (bottom-up approach) from medical records, with at least 10-year-follow-up data. *Results.* The total annual cost per patient for managing diabetes in Greece was estimated at € 7,111 and was, statistically significantly, higher for patients with inadequate glycemic control (Hba1c > 7%) versus patients with adequate control (Hba1c = 7%) (€ 7,783 versus € 6,366, resp.; $P = 0.017$). This was mainly attributed to difference in CV hospitalizations between groups 14/111 versus 4/100, respectively, OR = 3.46 (95% CI: 1.10–10.9) for inadequately controlled patients. The largest component of cost was management of comorbidities, accounting for 48% of costs, and pharmaceutical treatment at 35.9% while only 14.9% was attributed to diabetes treatment per se. Obese men and patients with poor education are the groups with higher treatment costs. *Conclusions.* This is the first study to capture all cost components and the real burden of diabetes in Greece. Comorbidities were found to account for almost half of total cost, significantly higher in nonoptimally controlled diabetes patients.

1. Introduction

Diabetes mellitus (DM) is a chronic condition primarily defined by the level of hyperglycemia giving rise to risk of microvascular and macrovascular damage [1, 2].

Type 2 diabetes mellitus (T2DM) comprises 90% of people with diabetes around the world and is largely the result of excess body weight and physical inactivity [3]. A recent Greek study in a large representative rural, urban, and suburban population showed that T2DM was associated with advancing age, obesity, exposure to smoke, and low socioeconomic status [4].

T2DM has become an epidemic [5] and affects about 6% of the adult population in the western world [6]. In Greece, the projected prevalence of T2DM in 2002 was 7.6% in men and 5.9% in women [7]. Two other studies estimated the prevalence of diabetes among adult urban and rural populations in Greece: for the urban population it was estimated at 8.2% (men, 8.5%; women, 7.8%) in 2002 and 9.5% (men, 9.7%; women, 9.3%) in 2006 [8]; for the rural population, the prevalence of diabetes was estimated at 7.8% in 2002 [9].

There is an increasing trend in the prevalence of diabetes; the study by Wild and colleagues showed that the "diabetes epidemic" will continue even if levels of obesity remain constant [10]. Therefore, this trend becomes even more worrying since the prevalence of obesity, the primary risk factor of T2DM, also exhibits an increasing trend [11, 12].

Despite many advances in its treatment over the past few decades, T2DM remains a serious public health problem and is a growing burden on global economies [13]. It is associated with reduced life expectancy; in 2004, an estimated 3.4 million people died from consequences of high fasting blood sugar [14]. The World Health Organization's (WHO) projections show that diabetes will be the 7th leading cause of death in 2030 [15].

In addition, T2DM is associated with significant morbidity and low quality of life (QoL) due to specific diabetes-related microvascular complications, increased risk of macrovascular complications (ischemic heart disease, stroke, and peripheral vascular disease), blindness, renal failure, and amputations [1, 16, 17]. According to a recently published Greek study, patients with poorer glycemic control score significantly lower QoL levels compared to their well-controlled counterparts [18].

T2DM is also a very costly disease. The American Diabetes Association (ADA) estimated the total cost of diabetes in the US at $174 billion in 2007, including $116 billion in excess medical expenditures and $58 billion in reduced national productivity [19]. The total direct medical cost of T2DM in eight European countries was estimated at € 29 billion per year (at an average annual cost per patient of € 2,834) [20]. The INSTIGATE study showed that the mean total direct costs per patient in five European countries increased in the 6-month follow-up period, compared with the 6-month period prior to insulin initiation, and ranged from € 577 in Greece to € 1402 in France. In all countries, the breakdown of total direct costs by expenditure category varied considerably across countries, reflecting differences in resource use patterns, prices of medical resources, and different health care systems [21]. In Greece, the annual cost of treating diabetes had been estimated by Athanasakis and colleagues [22]. In addition, a more recent study estimated the mean costs associated with the management of T2DM, after initiating insulin therapy [23]. The aforementioned studies did not include hospitalization, comorbidities, and complications cost.

The primary objective of the study was to estimate the mean annual cost of T2DM treatment in Greece, based on medical records of patients with at least a 10-year history of T2DM. In addition, this study explored the association of total cost of diabetes with HbA1c levels, after controlling for a set of demographic and socioeconomic parameters, in order to identify the determinants and key cost drivers of diabetes.

2. Patients and Methodology

2.1. Description of Study Design. A noninterventional retrospective study was conducted between June 30, 2011, and June 1, 2012, in four diabetes centers operating in public hospitals. The four participating diabetes centers were among the 25 official diabetes centers in Greece and had sufficient databases with a patient follow-up of at least 10 years in order to be able to collect the necessary data retrospectively.

Patients were recruited during their routine visit for the management of their T2DM. Adult T2DM patients receiving any type of antidiabetic treatment at least for 10 years before recruitment who have performed at least one visit per year to the diabetes center throughout the last decade with a complete patient file and adequate medical data according to the evaluation schedule were considered as eligible to participate. Eligibility was assessed by the study investigators, who were medical staff of the aforementioned diabetes centers. Eligible patients willing to participate had to sign an informed consent in order to be enrolled. The study protocol was approved by the Institutional Review Board prior to study initiation (April 2011).

An electronic Case Report Form (eCRF) was developed in order to collect the data necessary for the analysis. The eCRF was completed for each of the patients only once and recorded data, based on patients 10-year medical history and on demographic characteristics, personal information, medical HbA1c measurements, complications of T2DM (all micro- and macrovascular complications), comorbidities, as well as resource use data (visits to physicians/outpatient visits, frequency and duration of hospitalization, pharmaceutical treatments, and laboratory tests), and work-loss days due to diabetes (absenteeism). Medical history data and laboratory test results were retrieved from medical records. If demographic or nonlaboratory data (absenteeism and number/duration of hospitalizations) were missing, they were collected through direct interviews with the patients conducted by the investigators during the same visit.

According to the study protocol, patients recruited in the study into two subgroups in a predefined 1 : 1 ratio, based on their mean HbA1c level over the last 10 years (at least one HbA1c measurement per year was a prerequisite). If the average HbA1c was equal or less than 7, the patient was categorized as adequately controlled, whereas if the average HbA1c was over 7, the patient was categorized as inadequately controlled. In order to achieve the 1 : 1 ratio, HBA1c measurement was uploaded in the eCRF system upon patient recruitment and participating investigators were informed on the number of patients recruited in each subgroup.

In order to estimate total costs for the management of diabetes and its comorbidities, unit costs were assigned to resource use data collected from patient files and related interviews. Pharmaceutical costs were retrieved from publicly available sources, hospitalization costs from Diagnosis Related Groups (DRGs) tariffs, cost for specialist visits, and examination costs from National Health Care System of Greece (NHS) price list [24–26]. Only direct costs were included in the study. All costs were estimated from the NHS perspective in 2013 prices (€).

2.2. Statistical Analysis. Statistical analysis was conducted with SPSS version 19.0. Descriptive analysis was used to describe the continuous and categorical data of patients. Bivariate and multivariate analyses were conducted to identify the sociodemographic and clinical parameters that mostly influence the cost, at a statistical significance level of 0.05. Bivariate analysis was performed using nonparametric tests (Mann-Whitney and Kruskal-Wallis). Due to the nonnormal distribution of the cost data, all costs were

Table 1: Demographic characteristics.

	Total ($N = 211$)	HbA1c ≤ 7 ($N = 100$)	HbA1c > 7 ($N = 111$)	P value
Age (years)				
Mean	72.9	73.6	72.3	N/S
(SD)	(8.1)	(7.5)	(8.6)	
Gender				
Males	106 (50.2%)	51 (51.0%)	55 (49.5%)	N/S
Females	105 (49.8%)	49 (49.0%)	56 (50.5%)	
BMI (at time of recruitment)				
Normal/thin (<25)	37 (17.5%)	26 (26.0%)	11 (9.9%)	0.022
Overweight (25–30)	93 (44.1%)	43 (43.0%)	50 (45.0%)	N/S
Obese (>30)	81 (38.4%)	31 (31.0%)	50 (45.0%)	0.039
Years since first diagnosis				
Mean	21.2	20.0	22.3	N/S
(SD)	(7.5)	(7.6)	(7.2)	
HbA1c				
Mean	7.3	6.6	8.0	
(SD)	(1.0)	(0.4)	(0.8)	

BMI: body mass index; SD: standard deviation.

Table 2: Comorbidities.

	Total ($N = 211$)	HbA1c ≤ 7 ($N = 100$)	HbA1c > 7 ($N = 111$)	P value
Dyslipidemia	170/211 (80.6%)	80/100 (80.0%)	90/111 (81.1%)	0.739
Hypertension	176/211 (83.4%)	78/100 (78.0%)	98/111 (88.3%)	0.069
Coronary artery disease	51/211 (24.2%)	24/100 (24.0%)	27/111 (24.3%)	0.959
Stroke	15/211 (7.1%)	5/100 (5.0%)	10/111 (9.0%)	0.257
Other (not related to T2DM)	149/209 (71.3%)	71/99 (71.7%)	78/110 (70.9%)	0.897

logarithmically transformed, which allowed for the use of parametric methods and resulted in regression models with better goodness of fit. The variables investigated in the bivariate and multivariate analyses were age, gender, disease control level (HbA1c), body mass index (BMI) at recruitment, comorbidities, and complications.

3. Results

3.1. Patient Characteristics. A total of 211 patients were enrolled in the study, of which 100 (47.4%) were categorized as adequately controlled and 111 (52.6%) as inadequately controlled. Patient characteristics were similar in the two subgroups with the exception of BMI and are presented in Table 1. Inadequately controlled patients were more likely to be obese compared to adequately controlled counterparts ($P < 0.05$).

Regarding comorbidities, patients with high HbA1c levels were more likely to suffer from hypertension but the difference was not statistically significant (88.3% versus 78.0%, $P = 0.069$). Moreover, the difference in the prevalence of all other comorbidities between the two groups was not statistically significant (Table 2).

The most common diabetes complications of study population were diabetic retinopathy (37%) and cardiovascular events (coronary artery disease including myocardial infarction and heart failure) (31%). Other complications included peripheral vascular disease (18%), diabetic neuropathy (17%), renal impairment (10%), and stroke (8.5%).

Regarding antidiabetic treatment, patients with high HbA1c levels were on average prescribed more medication than those in the adequately controlled group ($P < 0.01$). The duration of antidiabetic treatment was comparable between the two patient subgroups ($P = 0.375$) (Table 3).

There was a statistically significant difference ($P < 0.001$) in favour of patients on long and short-acting insulin of the adequately controlled group (Table 3). Differences in the use of all other antidiabetic agents (alpha-glucosidase inhibitors, biguanides, DPP4 inhibitors, glitazones, GLP-1 analogues, meglitinides, and sulphonylureas) were not statistically significant.

Only 46 (21.8%) of the 211 patients reported that they had been hospitalized during the period under consideration. Patients were on average admitted to hospitals 1.4 times per year for the treatment of diabetes and its complications, with a mean duration of hospitalization of 6.3 days. CV hospitalizations were 3.46 times greater (OR) for inadequately

Table 3: Pharmaceutical treatment: antidiabetic and comorbidities therapies.

	Total ($N = 211$)	HbA1c ≤ 7 ($N = 100$)	HbA1c > 7 ($N = 111$)	P value
Number of antidiabetic agents				
Mean (SD)	3.4 (1.5)	3.1 (1.4)	3.7 (1.5)	<0.01
Duration of antidiabetic therapy (years)				
Mean (SD)	7.6 (3.8)	7.1 (3.4)	8.1 (4.1)	0.375
Number of comorbidities treatments				
Mean (SD)	5.5 (3.0)	5.4 (3.2)	5.6 (2.8)	0.630
Antidiabetic agents (n)				
Alpha-glucosidase inhibitors	22/211 (10.4%)	15/111 (13.5%)	7/100 (7.0%)	0.187
Biguanides	180/211 (85.3%)	93/111 (83.8%)	87/100 (87.0%)	0.643
DPP4 inhibitors	59/211 (28.0%)	26/111 (23.4%)	33/100 (33.0%)	0.163
Glitazones	39/211 (18.5%)	23/111 (20.7%)	16/100 (16.0%)	0.481
GLP-1 analogues	9/211 (4.3%)	4/111 (3.6%)	5/100 (5.0%)	0.739
Long-acting insulin	129/211 (61.1%)	87/111 (78.4%)	42/100 (42.0%)	<0.001
Rapid-acting insulin	99/211 (46.9%)	68/111 (61.3%)	31/100 (31.0%)	<0.001
Meglitinides	45/211 (21.3%)	22/111 (19.8%)	23/100 (23.0%)	0.693
Sulphonylureas	137/211 (64.9%)	71/111 (64.0%)	66/100 (66.0%)	0.869

Table 4: Resource use associated with hospitalization, complications, and medical care.

	Total ($N = 211$)	HbA1c ≤ 7 ($N = 100$)	HbA1c > 7 ($N = 111$)	P value
Number of visits to a specialist* per year				
Mean (SD)	3.2 (0.9)	3.1 (0.9)	3.3 (0.9)	0.259
Number of admissions to hospital per year				
Mean (SD)	1.4 (1.1)	1.2 (0.4)	1.5 (1.2)	0.528
Duration of hospitalization (days per hospitalization)				
Mean (SD)	6.3 (3.9)	6.6 (4.1)	6.2 (3.8)	0.831
Number of complications over the last 10 years				
Mean (SD)	2.1 (1.7)	2.2 (2.2)	2.0 (1.3)	0.732
Duration of complications (years)				
Mean (SD)	6.5 (4.1)	5.8 (3.6)	6.8 (4.4)	0.511

* At the diabetes center.

controlled patients (95% CI: 1.10–10.9) versus controlled patients with 14/111 (12.6%) and 4/100 (4%), respectively. CV hospitalizations (myocardial infraction, heart failure, by-pass, pulmonary edema, angioplasty, and stroke) and difference in stroke incidence between groups justify the total cost difference of controlled and uncontrolled patients. The total number and duration of hospitalizations were comparable between the two subgroups, with small differences not being statistically significant (Table 4).

Number of visits to a specialist in the diabetes center was on average 32.2 over the last 10 years, with no statistically significant differences between the two subgroups ($P = 0.259$). Although CV hospitalizations and stroke incidence were different between subgroups still the total number and duration of complications due to diabetes were not statistically significantly different between the two groups.

3.2. Costs. The total annual cost per patient for managing diabetes was estimated at € 7,111 (SD = 4,323) excluding disability pensions. The largest component of this cost was management of comorbidities, accounting for 48% (€ 3,353)

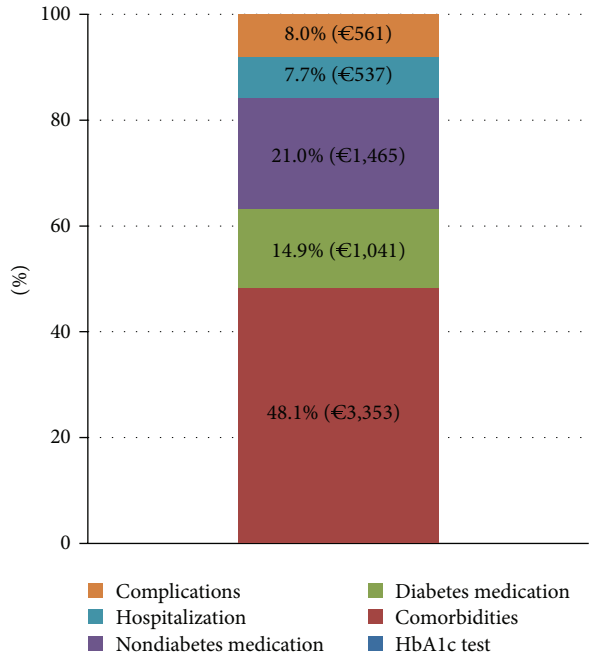

Figure 1: Breakdown of the total cost of diabetes management. Note: cost of HbA1c test is 0.2% (€ 13) of total cost; disability pensions are excluded.

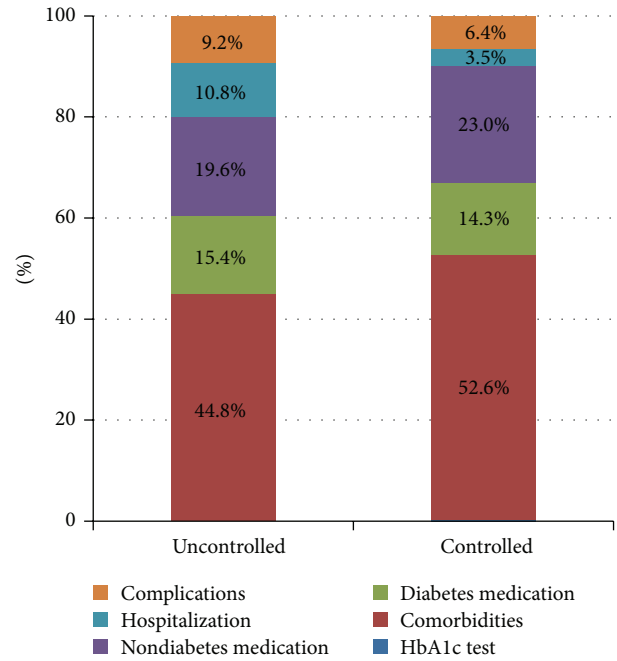

Figure 2: Breakdown of total cost of adequately versus inadequately controlled patients. Note: cost of HbA1c test is 0.2% of total cost in both subgroups; disability pensions are excluded.

of total costs, while pharmaceutical treatment (including diabetes-related and nondiabetes medication) accounted for 35.9% (€ 2,506). Antidiabetic agents accounted only for 14.9% (€ 1,041) of total cost (Figure 1).

For adequately controlled patients, the total cost per year was estimated at € 6,366, while the cost of inadequately controlled patients was estimated at € 7,783, with their difference being statistically significant ($P = 0.017$). Differences in costs of hospitalization and complications between the two subgroups were not statistically significant ($P = 0.091$). Figure 2 presents the breakdown of adequately versus inadequately controlled patients' costs.

Bivariate analyses showed that inadequately controlled patients cost is on average € 1,417 more per year compared to the optimally controlled group ($P = 0.017$). Male patients showed higher costs than females (Table 5); the same applies to overweight or obese patients compared to normal weight. In addition, there was no statistically significant difference found between patient subgroups relating to employment, marital status, and monthly income level.

Existence of comorbidities vastly increases annual costs ($P < 0.01$). In particular, diabetes patients with coronary artery disease (CAD) were found to have the highest yearly cost (€ 11,662), followed closely by those with stroke (€ 11,366).

The relationship between the total cost of managing diabetes, the patient characteristics (demographics, personal information, and income level), and T2DM control level was explored through a multivariate analysis, with cost as a dependent variable and all other parameters as explanatory variables. The results of this analysis showed that healthcare for men with diabetes costs € 1,277 more per year, in comparison to healthcare for women with diabetes, and also that treating obese diabetics costs € 1,460 more per year in comparison to treating patients in other BMI groups. In addition, low education level (up to primary school) patients with diabetes cost € 2,341 more on average in comparison to better educated patients, while adding a year in the diabetes history of a patient increases his/her annual cost by almost € 90.

4. Discussion

The current study aimed to estimate the total cost of T2DM in Greece, based on a dataset of patients being followed up for at least 10 years. The main finding is that the average annual cost of diabetes treatment was € 7,111. The estimates of the total cost of diabetes are much higher than those reported by previous Greek studies. In particular, Athanasakis and colleagues estimated the annual per patient cost at € 1,297 per patient ranging from € 982 to € 1,566 for adequately controlled and inadequately controlled patients, respectively [22]. However, the latter study included only treatment cost and not costs of diabetic complications and comorbidities which, combined, are the largest component of the total cost (56%), as shown in the present study. For similar reasons, the current results deviate from the cost estimates of the study of Aloumanis et al., presenting a range between € 496 before initiation of insulin treatment and € 573 after initiating insulin therapy [23].

The current findings are in accordance with estimates of other international studies, which also show high per patient costs for the management of diabetes. In particular, the

Table 5: Average annual costs by patient subgroups.

	Mean annual cost per patient	SD	*P* value
HbA1c			
HbA1c > 7	7,783.3	4,549.0	0.017
HbA1c ≤ 7	6,366.6	3,948.7	
Gender			
Women	6,470.5	2,677.0	0.037
Men	7,747.2	5,428.5	
BMI			
Normal/thin (<25)	5,837.7	3,911.9	<0.01 (versus obese)
Overweight (25–30)	6,903.8	4,491.2	<0.01 (versus obese)
Obese (>30)	7,932.8	4,183.6	—
Education			
Primary school	8,104.2	4,649.9	—
Secondary school	5,640.2	2,575.1	<0.01 (versus primary)
High school	6,817.1	3,885.2	<0.01 (versus primary)
University	6,405.9	4,731.1	<0.01 (versus primary)
Comorbidities			
Dyslipidemia	7,889.6	4,290.1	<0.01 (versus CAD)
Hypertension	7,847.0	4,257.3	<0.01 (versus CAD)
Coronary artery	11,662.4	5,592.9	—
Stroke	11,366.4	8,389.2	<0.01 (versus CAD)
Other	7,699.8	4,349.3	<0.01 (versus CAD)

BMI: body mass index; CAD: coronary artery disease.

American Diabetes Association study estimated that people with diagnosed diabetes incur average medical expenditures of about $13,700 per year, of which about $7,900 is attributed to diabetes [27]. In addition, 23% of the medical costs are used to directly treat diabetes, while 50% of the costs are used to treat the portion of chronic complications that are attributed to diabetes [19]. This is comparable to our finding that treating comorbidities accounts for 48% of total costs.

The present study is the first one in Greece to have captured most of the cost components and therefore it may more accurately reflect the real burden of diabetes in the Greek health care setting. Estimating indirect costs were beyond the scope of this study; however, if indirect cost was included, estimates of total costs of T2DM would have been even higher.

The study also showed that the cost of treating inadequately controlled patients was statistically significantly higher (€ 7,783) than the cost associated with treating adequately controlled patients (€ 6,366), a finding which is also consistent with the literature [22]. From a recent published Greek study performed on 6,631 T2DM patients, the majority of the sample (59%) was inadequately controlled, leading to an additional burden of the national health care budget [18]. Another interesting finding of this study is that, concerning the cost of treatment, with the exception of the insulin, no statistically significant difference was observed between the two groups. This could be attributed to the legacy effect due to limited treatment options during the first decade of the diabetes history of study sample (mean T2DM duration about 20 years in the study population).

The current study had several limitations, one of which is the 10-year retrieval of data which might be underreported in patients file. Additionally, recall bias of such a long period might also be considered as a limitation. Based on the abovementioned, the lack of statistical significance in hospitalization and absenteeism between the two subgroups (adequately and inadequately controlled) could be partly attributed to recall bias arising from the fact that data on hospitalization were retrieved through interviews. The same applies to complication costs as that could be attributed to the retrospective design of the study. However, the abovementioned limitations could be considered as symmetric across the two subgroups and therefore of minimal effect.

5. Conclusions

This study is the first aiming to capture all cost components and better reflect the real burden of diabetes in the Greek setting. The cost of managing diabetes in Greece is high and is statistically significantly higher for inadequately controlled versus adequately controlled patients, attributed mainly to difference in CV hospitalizations and numbers of stroke. Obese men with a long diabetes history and patients with lower educational levels are the subgroups found to have higher treatment cost. The management of comorbidities constitutes a major cost component, accounting for 48% of total costs, while T2DM-related pharmaceutical treatment accounted only for 14.9%. Since comorbidities account for almost half of diabetes expenditure and inadequately controlled patients have significantly higher costs than adequately controlled, any efforts to contain diabetes burden should be aiming at preventing complications and comorbidities in order to reduce costs of managing diabetes.

Conflict of Interests

G. Rombopoulos and M. Hatzikou are Novartis Hellas employees, I. Migdalis, C. Manes, N. Kypraios, and N. Tentolouris received compensation as trial investigators from Novartis Hellas.

References

[1] World Health Organization, "Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia," 2006.

[2] World Health Organization, Diabetes: Fact sheet No 312, 2013, http://www.who.int/mediacentre/factsheets/fs312/en/index.html.

[3] World Health Organization, *Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications. Part 1: Diagnosis and Classification of Diabetes Mellitus*, World Health Organization, Geneva, Switzerland, 1999.

[4] N. Tentolouris, A. Andrianakos, G. Karanikolas et al., "Type 2 diabetes mellitus is associated with obesity, smoking and low socioeconomic status in large and representative samples of rural, urban, and suburban adult Greek populations," *Hormones*, vol. 11, no. 4, pp. 458–467, 2012.

[5] M. Stumvoll, B. J. Goldstein, and T. W. van Haeften, "Type 2 diabetes: principles of pathogenesis and therapy," *The Lancet*, vol. 365, no. 9467, pp. 1333–1346, 2005.

[6] H. King, R. E. Aubert, and W. H. Herman, "Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections," *Diabetes Care*, vol. 21, no. 9, pp. 1414–1431, 1998.

[7] D. B. Panagiotakos, C. Pitsavost, C. Chrysohoout, and C. Stefanadis, "The epidemiology of type 2 diabetes mellitus in Greek adults: the ATTICA study," *Diabetic Medicine*, vol. 22, no. 11, pp. 1581–1588, 2005.

[8] A. Gikas, A. Sotiropoulos, D. Panagiotakos et al., "Rising prevalence of diabetes among Greek adults: findings from two consecutive surveys in the same target population," *Diabetes Research and Clinical Practice*, vol. 79, no. 2, pp. 325–329, 2008.

[9] A. M. Melidonis, S. M. Tournis, M. K. Kompoti et al., "Increased prevalence of diabetes mellitus in a rural Greek population," *Rural and Remote Health*, vol. 6, no. 1, p. 534, 2006.

[10] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[11] World Health Organization, *Obesity 1998: Preventing and Managing the Global Epidemic*, World Health Organization, Geneva, Switzerland, 1998.

[12] C. L. Ogden, M. D. Carroll, L. R. Curtin, M. A. McDowell, C. J. Tabak, and K. M. Flegal, "Prevalence of overweight and obesity in the United States, 1999–2004," *The Journal of the American Medical Association*, vol. 295, no. 13, pp. 1549–1555, 2006.

[13] The International Diabetes Federation. IDF Diabetes Atlas, 2009.

[14] World Health Organization, *Global Health Risks. Mortality and Burden of Disease Attributable to Selected Major Risks*, World Health Organization, Geneva, Switzerland, 2009.

[15] World Health Organization, *Global Status Report on Noncommunicable Diseases 2010*, 2011.

[16] P. Hogan, T. Dall, and P. Nikolov, "Economic costs of diabetes in the US in 2002," *Diabetes Care*, vol. 26, pp. 917–932, 2003.

[17] P. Zimmet, K. G. M. M. Alberti, and J. Shaw, "Global and societal implications of the diabetes epidemic," *Nature*, vol. 414, no. 6865, pp. 782–787, 2001.

[18] G. Rombopoulos, M. Hatzikou, D. Latsou, and J. Yfantopoulos, "The prevalence of hypoglycemia and its impact on the quality of life (QoL) of type 2 diabetes mellitus patients (The HYPO Study)," *Hormones*, vol. 12, no. 4, pp. 550–558, 2013.

[19] American Diabetes Association, "Economic costs of diabetes in the U.S. In 2007," *Diabetes Care*, vol. 31, no. 3, pp. 596–615, 2008.

[20] B. Jönsson, "Revealing the cost of Type II diabetes in Europe," *Diabetologia*, vol. 45, no. 6, pp. S5–S12, 2002.

[21] S. Jones, C. Castell, A. Goday et al., "Increase in direct diabetes-related costs and resource use in the 6 months following initiation of insulin in patients with type 2 diabetes in five European countries: data from the INSTIGATE study," *ClinicoEconomics and Outcomes Research*, vol. 4, no. 1, pp. 383–393, 2012.

[22] K. Athanasakis, M. Ollandezos, A. Angeli, A. Gregoriou, M. Geitona, and J. Kyriopoulos, "Estimating the direct cost of type 2 diabetes in Greece: the effects of blood glucose regulation on patient cost," *Diabetic Medicine*, vol. 27, no. 6, pp. 679–684, 2010.

[23] K. Aloumanis, M. Benroubi, S. Sourmeli, and V. Drossinos, "Clinical outcomes and costs for patients with type 2 diabetes mellitus initiating insulin therapy in Greece: two-year experience from the INSTIGATE study," *Primary Care Diabetes*, vol. 7, no. 3, pp. 235–242, 2013.

[24] Greek Ministry of Health, *Drug Price Bulletin 05/06/13*, 2014, http://www.moh.gov.gr/.

[25] Greek Ministry of Health, Diagnosis Related Groups March, 2012, http://www.moh.gov.gr/articles/health/domes-kai-draseis-gia-thn-ygeia/kwdikopoihseis/709-kleista-enopoihmena-noshlia-1.

[26] FEK 157/91 issue A, http://www.et.gr/index.php.

[27] American Diabetes Association, *Economic Costs of Diabetes in the U.S. in 2012. Diabetes Care*, American Diabetes Association, Alexandria, Va, USA, 2013.

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Research Article

Prevalence of Orthostatic Hypertension in Elderly Patients with Type 2 Diabetes

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Background. The aim of the present study was to determine the prevalence of orthostatic hypertension (OHT) in elderly patients with type 2 diabetes and its relation to metabolic and echocardiographic parameters. *Methods*. This was an analytical cross-sectional study in 97 patients normotensive or hypertensive. OHT was defined as a ≥10 mmHg increase in systolic blood pressure after four minutes in the standing position. *Results*. The prevalence of OHT was 20.6%. The mean body mass index was significantly higher in patients with OHT than in those without it (29.80 ± 4.10 versus 27.51 ± 3.98 kg/m^2; $P = 0.026$). There were no statistically significant differences between the two groups for other metabolic parameters. Among the 68 patients who had an echocardiographic examination 27% of those with OHT had an increase in their left atrial volume index (LAVi) compared with 75% of those who did not have OHT ($P = 0.004$). The mean LAVi of patients with OHT was significantly lower than that of those without OHT (26.27±6.37 versus 32.65±7.54, resp.; $P = 0.011$). *Conclusion*. We found a high prevalence of orthostatic hypertension and a lower left atrial volume indexed in the patients with orthostatic hypertension.

1. Introduction

Autonomic cardiac neuropathy is associated with significant morbidity and mortality in diabetics, the risk being greater in those with hypertension [1]. Some reports in the literature suggest that orthostatic hypertension occurs in some forms of autonomic dysfunction and in primary chronic conditions such as type 2 diabetes mellitus, elderly hypertensive individuals, and peripheral neuropathy [2].

The prevalence and clinical significance of orthostatic hypertension in diabetic patients are as yet not well understood, unlike postural hypotension, which is a common feature of advanced autonomic neuropathy in type 2 diabetes mellitus (T2DM) [3, 4].

The difficulty in determining the actual prevalence of orthostatic hypertension is due to several factors, such as the varying definitions used and differences between the populations that have been studied to date. There are studies that define orthostatic hypertension as a rise in systolic blood pressure (SBP) of 5 mmHg [5], while others define it as an increase in SBP of 20 mmHg [6]. Still others define it as an increase >140 mmHg in systolic and/or 90 mmHg in diastolic blood pressure (DBP) after standing up [3].

In hypertensive patients, the prevalence and clinical significance of orthostatic hypertension also remain largely undetermined [6, 7]. A recent study evaluated the association of orthostatic hypertension with cardiovascular disease (CVD) and damage to the target organ in 4,711 hypertensive and 826 normotensive patients. After controlling for confounders such as age and gender OHT was independently associated with peripheral arterial disease (OR 1.36, 95% CI 1.05 to 1.81; $P < 0.05$) and stroke (OR 1.76, 95% CI 1.27 to 2.26; $P < 0.01$) [6].

In a study of 277 Japanese males with DM2, including 90 hypertensive patients and 128 nondiabetic males matched by age, excluding users of antihypertensive medications,

the prevalence of orthostatic hypertension, in normotensive and hypertensive diabetics, was significantly higher than in the controls (12.8 versus 1.8%; $P < 0.01$, for normotensive patients, and 12.6 versus 11.1%, nonsignificant for hypertensive patients) [3]. A population-based study revealed a positive correlation of postural hypotension/hypertension and orthostatic dizziness in adults (≥20 yr) of all age groups, defining OHT as a postural increase in SBP ≥20 mmHg and that age ($P < 0.001$) and systolic blood pressure in the supine position ($P = 0.023$) were correlated with orthostatic hypertension in a statistically significant manner, with the risk for this condition increasing in adults from the age of 40 yr [8].

In view of the lack of published data on the prevalence of orthostatic hypertension in elderly patients with diabetes, this study was designed to determine the prevalence of orthostatic hypertension and its possible relation to metabolic factors and echocardiographic parameters in a population referred for treatment to an outpatient endocrine clinic.

2. Subjects and Methods

Approximately 4,800 patients spontaneously sought the outpatient endocrinology clinic at Agamenon Magalhães Hospital within six months of data collection. Assuming a 12% prevalence of orthostatic hypotension in normotensive and hypertensive diabetics, a sample of 375 patients with an error rate of 2.5% was calculated. However only 97 patients 60 years and older were evaluated, all of whom were diagnosed with type 2 diabetes mellitus. The patients were normotensive or hypertensive, receiving either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers and/or a calcium channel blocker. The exclusion criteria were as follows: patients with type 1 diabetes mellitus; those reporting the chronic use of diuretics, beta blockers or alpha blockers for treatment of hypertension; heart failure patients with systolic ejection fraction ≤35%, or functional class III or IV as established by the New York Heart Association; patients with atrial fibrillation; patients suffering from terminal chronic renal disease undergoing hemodialysis; patients with cancer at any site except the skin (nonmelanoma); those with rheumatic diseases (rheumatoid arthritis or arthrosis of the hands and feet); patients with limitations in the supine position; and those with Parkinson's disease or tremors of the extremities.

The patients answered a questionnaire after signing the informed consent form. The questionnaire consisted of age, sex, physical examination with measurement of weight, height, waist circumference, and hip circumference, physical exam-ınation of the feet (tactile, thermal and vibratory sensitivity, presence or not of ulcer, and test monofilament), medications used, diagnosis of arterial hypertension, and time since diagnosis of diabetes mellitus. They were also evaluated for the presence of comorbidities, based on their previous diagnosis of cardiovascular disease due to acute myocardial infarction or coronary artery bypass graft (with bypass surgery or stenting) coronary angiography with stenosis of at least ≥50%, ischemic stroke, ≥50% stenosis of the carotid, or previous peripheral arterial disease.

Patients underwent a thorough physical examination, including measurement of systolic blood pressure (SBP) and diastolic blood pressure (DBP) with an automatic device (Omron Health Care, Inc., Kyoto, Japan), weight, height, body mass index (BMI), waist circumference, and hip circumference.

Blood pressure was measured with the patient seated and again after four minutes in the standing position, using an automatic device (Omron Health Care, Inc., Kyoto, Japan). Two measurements were taken consecutively in seated and standing with the arm supported at heart level in both positions, the mean of the two being considered for purposes of assessment.

Blood samples were collected after 12 hours of fasting to determine serum levels of glucose, glycated hemoglobin, total cholesterol and fractions (LDL-cholesterol and HDL-cholesterol), triglycerides, and creatinine.

Urine samples were collected for creatinine and protein determination.

Laboratory tests were performed using an autoanalyzer, Johnson & Johnson, USA, and for HbA1C, Fugion, USA.

Patients underwent echocardiography (Philips Envisor USA, 2005), conducted by an examiner blinded to which patients had orthostatic hypertension. Measurements were made for left atrial volume and left ventricular mass.

2.1. Definition of Concepts of Variables Studied

(i) Orthostatic hypertension was defined as a ≥10 mmHg increase in systolic blood pressure after 4 minutes in the standing position, compared with the sitting position.

(ii) According to the recommendations of the Committee of the American Society of Echocardiography (ASE) 2005 [9] the left atrial volume index (LAVi) was measured, which is the left atrial volume divided by the body surface area. Normal volumes were considered to be from 16 to 28 mL/m^2. LAVi greater than 28 mL/m^2 was considered to be altered. The left ventricle mass index (LVMi) was measured, which is the mass of the left ventricle divided by the body surface area. The normal female value was from 43 to 95 g/m^2, slightly abnormal between 96 and 108 g/m^2, moderately abnormal between 109 and 121 g/m^2, and critically abnormal ≥122 g/m^2. For male it ranged from 49 to 115 g/m^2, slightly abnormal between 116 and 131 g/m^2, moderately abnormal between 132 and 148 g/m^2, and critically abnormal ≥149 g/m^2.

The study was approved by the Agamenon Magalhães Hospital Ethics in Research Committee (registration CAAE-.0114.0.236.000-11).

2.2. Statistical Analysis. In the statistical analysis the paired Student's t-test was used for numeric variables, Pearson's chi-square test for association of two categorical variables, or Fisher's exact test when it was not possible to use the chi-square test, and the Student's t-test demonstrated both equal and unequal variances. Verification of the hypothesis of equality of variances was performed using Levene's F-test

and the correlation analysis using the F-test (ANOVA). Considering the confidence interval of 95%, the index of significance was set at $P < 0.05$. The software used was version 17 of the SPSS.

3. Results

Ninety-seven patients were studied with ages ranging from 60 to 88 years (median, 68.97 yr, SD ± 6.80), of whom 30.9% (30 patients) were males. The mean time since the diagnosis of diabetes was 12.23 years (±8.34 years). Mean glycated hemoglobin was 7.9% (±1.71%). 59 (60.8%) patients were taking statins and 33 (34%) patients used insulin. Of the 97 patients, 7 (7.2%) had an ulcer on foot, 34 (24.7%) had negative monofilament test, 35 (36.1%) had a change (decreased or absent) in vibration sensitivity, 39 (40.2%) had changes (decreased or absent) in thermal sensitivity, and 11 had changes (decreased or absent) of tactile sensitivity. The majority of patients in this study had hypertension (77; 79.4%), were overweight or obese (77; 79.3%), with a mean BMI of 27.95 kg/m^2 (Table 1). The prevalence of orthostatic hypertension was 20.6% (20 patients) (Table 1) with most patients being overweight or obese (BMI ≥ 25 kg/m^2) (19 patients, 52.5%; $P = 0.111$) (Table 2). BMI was significantly higher in individuals with OHT than in those without (29.80 ± 4.10 versus 27.51 ± 3.98 kg/m^2, $P = 0.026$). No statistically significant association was seen between orthostatic hypertension and abnormal physical examination of the feet. Of the orthostatic hypertension patients, 17 (22.7%) had a diagnosis of systemic arterial hypertension ($P = 0.551$) (Table 2). Of the 68 patients who did an echocardiographic examination, bearing in mind that 29 patients did not have the examination, 27% (3 patients) from those with OHT had an increase in their left atrial volume index (iLAV) compared with 75% (43 patients) of those without OHT ($P = 0.004$). The mean iLAV of patients with OHT was significantly lower than those without it (26.27 ± 6.37 versus 32.65 ± 7.54, resp., $P = 0.011$). We examined the interaction of BMI with the iLAV and found that the correlation persisted (−0.092; $P = 0.452$) (Table 3).

Table 1: Clinical, laboratorial, and echocardiography characteristics of studied subjects.

Variable	Mean
Age (years)	68.97 ± 6.80
Gender	
Male	30.9%
Female	69.1%
Diabetes duration (years)	12.23 ± 8.34
BMI (kg/m^2)	27.95 ± 4.06
Waist circumference (cm)	98.45 ± 12.43
Hip circumference (cm)	101.71 ± 9.85
Fasting glucose (mg/dL)	143.20 ± 56.41
HbA1C (%)	7.90 ± 1.71
MDRD (mL/min/1.73 m^2)	91.85 ± 25.98
HDL (mg/dL)	52.91 ± 22.73
LDL (mg/dL)	97.95 ± 31.95
Triglycerides (mg/dL)	136.61 ± 58.75
Medications	%
Statin	60.8
ACE inhibitor	49.5
ARB	23.7
CCB	19.6
Aspirin	52.6
Metformin	76.3
Sulfonylurea	32
Insulin	34
Comorbidities	
Hypertension	79.4
Stroke	4.1
Myocardial infarction	3.1
CABG	2.1
PVD	1
Left atrial volume index (mL/m^2)	121.77 ± 22.81
Left ventricular mass index (g/m^2)	31.60 ± 7.64
Orthostatic hypotension (DSBP > 20 and/or DDBP > 10 mmHg)	3.1%
Orthostatic hypertension (DSBP ≥ 10 mmHg)	20.6%

ACE: angiotensin converting enzyme.
ARB: angiotensin II receptor blockers.
CCB: calcium channel blockers.
CABG: coronary artery bypass grafting.
PVD: peripheral vascular disease.
BMI: body mass index.
DSBP: differential systolic blood pressure.
DDBP: Differential diastolic blood pressure.

4. Discussion

Available data shows a strong association between obesity and hypertension. In the Framingham study, the relative risk of hypertension in overweight men and women was 1.46 and 1.75, respectively, after adjusting for age [10].

Data on OHT are not well characterized in the literature and its real clinical significance is unknown, although some studies carried out in recent years have suggested that orthostatic hypertension may be a new cardiovascular risk factor [11].

Orthostatic hypertension can vary from a simple incidental finding during a physical examination to a significant increase in blood pressure, resulting in baroreflex deregulation [2]. However its precise mechanism remains unclear [11].

Initial studies involving orthostatic hypertension showed that individuals with this condition had a greater decrease in cardiac output in the upright position, with an increase in venous blood flow in the lower extremities, and higher levels of plasma norepinephrine after standing up [12, 13]. The hypothesis was that the accumulation of venous blood leads to a reduction in cardiac output and that the response would be an increase in sympathetic activity, leading to elevated DBP. This hypothesis of venous pooling in the feet and subsequent decreased cardiac output initially seems paradoxical. Maybe in patients with orthostatic hypertension

Table 2: Evaluation of the occurrence of orthostatic hypertension (OHT) according to clinical data.

Variable	Occurrence of OTH		Total		P value	RP (CI of 95%)
	N	%	N	%		
Diabetes duration (years)						
<10	9	27.3	33	100.0		1.25 (0.48 to 3.26)
10 to 19	5	17.9	28	100.0	$P^{(1)} = 0.677$	0.82 (0.27 to 2.49)
>19	5	21.7	23	100.0		1.00
Group total	**19**	**22.6**	**84**	**100.0**		
BMI						
Overweight	8	29.6	27	100.0		**
Obese	11	22.9	48	100.0	$P^{(1)} = 0.111$	**
Normal	1	5.0	20	100.0		**
HBP						
Yes	17	22.7	75	100.0	$P^{(2)} = 0.551$	1.51 (0.49 to 4.65)
No	3	15.0	20	100.0		1.00
Left atrial volume index (mL/m^2)						
Abnormal	3	6.5	46	100.0	$P^{(1)} = 0.004^*$	1.00
Normal	8	36.4	22	100.0		5.58 (1.64 to 19.00)
Left ventricular mass index (g/m^2)						
Severe	4	16.0	25	100.0		**
Moderate	3	20.0	15	100.0	$P^{(1)} = 0.408$	**
Slightly above normal	—	—	11	100.0		**
Normal	4	23.5	17	100.0		**
Group total	**11**	**16.2**	**68**	**100.0**		

*Significant association at the 5.0% level.
**Impossible to determine due to the occurrence of null and very low incidence rates.
(1) Using Fisher's exact test.
(2) Using Pearson's chi-square test.
BMI: body mass index.
HBP: high blood pressure.

Table 3: Statistics on left atrium volume according to BMI classification.

Statistics	BMI classification			P value
	Normal	Overweight	Obese	
(i) Mean	34.84	30.06	31.62	
(ii) Median	32.74	30.17	30.76	
(iii) Standard deviation	8.85	6.01	8.62	$P^{(1)} = 0.118$
(iv) Minimum value	21.35	17.93	19.62	
(v) Maximum value	55.60	39.47	60.08	

(1) F-test (ANOVA).

the central sympathetic excitation is pathologically excessive. This process could take place in an environment of partial dysautonomia involving venous capacitance or in individuals with pathological deregulation in the brainstem or centers involved in autonomic control. Why some patients experience orthostatic hypotension while others demonstrate orthostatic hypertension in this situation remains unclear [2].

In the present study orthostatic hypertension was found to be present in 20.6% of the elderly diabetic patients. In the literature the prevalence of orthostatic hypertension varies greatly, depending on the definition used and the population studied. In a study of Japanese men with diabetes, the prevalence of orthostatic hypertension was 12.8% in normotensive patients and 12.6% in hypertensives ones, using an increase in SBP of the 20 mmHg or more after 3 minutes in the supine position to define orthostatic hypertension [6]. In another study in young adults, orthostatic hypertension was defined as an increase of 5 mmHg in SBP upon standing, and the prevalence of orthostatic hypertension was 16.2% [5]. Regarding type 2 diabetic patients, the prevalence of orthostatic hypertension, defined as blood pressure (BP) <140/90 mmHg in the supine position, and BP measured again after 1 minute standing of 140/90 mmHg or more, the prevalence of HTO was 12.6% [3].

In our study, with a population consisting of patients aged 60 years and more, we found no association between orthostatic hypertension and increase in age. However, there are some reports in the literature in which older people have a higher occurrence of orthostatic hypertension [8]. Likewise, others have found that a complication of orthostatic hypertension in diabetic patients may be associated with increased

serum triglyceride concentrations [2]. Similar findings were not shown to be statistically significant in this study.

In a multivariate analysis, high BMI and not being treated with insulin were independent factor associated with OHT.

The literature consulted indicates an increased occurrence of orthostatic hypertension in elderly patients with hypertension [14]. However, in the present study on diabetic patients, no significant association was observed between orthostatic hypertension and high blood pressure.

Increased left atrial volume is often reported with age and with a variety of cardiovascular disorders [15], as in hypertensive patients [16]. In our study the occurrence of orthostatic hypertension and increases in left atrial volume index were not substantiated. Our finding of a lower iLAV in patients with OHT is due to unknown mechanism. One possibility is that our patients with OHT had a significant high BMI but had a lower iLAV, suggesting that OHT may not be a harmful phenomenon. They should therefore not be submitted to an intensive control of blood pressure, considering that the results of the ACCORD BP trial in diabetics [17] fail to show any reduction in the rate of major fatal or nonfatal cardiovascular events, even with an intensive control of their blood pressure.

A study on orthostatic changes in blood pressure and target organ damage showed no significant correlation between left ventricle mass index and the occurrence of orthostatic hypertension [14], no such association been observed, as in the present study.

Our study had three main limitations: the small size of the sample; the fact that 29 patients did not undergo echocardiography exam because it had to be done on a different day from the clinical examination of the patient; and the lack of a control group.

5. Conclusion

We found a high prevalence of orthostatic hypertension in elderly patients with type 2 diabetes. High BMI and not being treated with insulin were independent factors associated with OHT. A left atrial volume indexed was found significantly lower suggesting that OHT may not be a harmful phenomenon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] I. Istenes, K. Keresztes, Z. Hermányi et al., "Relationship between autonomic neuropathy and hypertension-are we underestimating the problem?" *Diabetic Medicine*, vol. 25, no. 7, pp. 863–866, 2008.

[2] D. Robertson, "Orthostatic hypertension: the last hemodynamic frontier," *Hypertension*, vol. 57, no. 2, pp. 158–159, 2011.

[3] M. Yoshinari, M. Wakisaka, U. Nakamura, M. Yoshioka, Y. Uchizono, and M. Iwase, "Orthostatic hypertension in patients with type 2 diabetes," *Diabetes Care*, vol. 24, no. 10, pp. 1783–1786, 2001.

[4] E. A. Jarmuzewska, R. Rocchi, and A. A. Mangoni, "Predictors of impaired blood pressure homeostasis during acute and sustained orthostasis in patients with type 2 diabetes," *Panminerva Medica*, vol. 48, no. 1, pp. 67–72, 2006.

[5] R. J. Thomas, K. Liu, D. R. Jacobs Jr., D. E. Bild, C. I. Kiefe, and S. B. Hulley, "Positional change in blood pressure and 8-year risk of hypertension: the CARDIA study," *Mayo Clinic Proceedings*, vol. 78, no. 8, pp. 951–958, 2003.

[6] X.-H. Fan, Y. Wang, K. Sun et al., "Disorders of orthostatic blood pressure response are associated with cardiovascular disease and target organ damage in hypertensive patients," *American Journal of Hypertension*, vol. 23, no. 8, pp. 829–837, 2010.

[7] S. Vardan, N. E. Hill, K. G. Mehrotra, S. Mookherjee, and H. Smulyan, "Hormonal response to orthostasis in elderly people with systemic systolic hypertension," *Coronary Artery Disease*, vol. 9, no. 9, pp. 597–601, 1998.

[8] J.-S. Wu, Y.-C. Yang, F.-H. Lu, C.-H. Wu, and C.-J. Chang, "Population-based study on the prevalence and correlates of orthostatic hypotension/hypertension and orthostatic dizziness," *Hypertension Research*, vol. 31, no. 5, pp. 897–904, 2008.

[9] R. M. Lang, M. Bierig, R. B. Devereux et al., "Recommendations for chamber quantification: a report from the American Society of Echocardiography's guidelines and standards committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology," *Journal of the American Society of Echocardiography*, vol. 18, no. 12, pp. 1440–1463, 2005.

[10] P. W. Wilson, R. B. D'Agostino, L. Sullivan, H. Parise, and W. B. Kannel, "Overweight and obesity as determinants of cardiovascular risk; the Framingham experience," *Archives of Internal Medicine*, vol. 162, pp. 1867–1872, 2002.

[11] K. Kario, "Orthostatic hypertension—a measure of blood pressure variation for predicting cardiovascular risk," *Circulation Journal*, vol. 73, no. 6, pp. 1002–1007, 2009.

[12] D. H. P. Streeten, J. H. Auchincloss Jr., G. H. Anderson Jr., R. L. Richardson, F. D. Thomas, and J. W. Miller, "Orthostatic hypertension: pathogenetic studies," *Hypertension*, vol. 7, no. 2, pp. 196–203, 1985.

[13] D. H. P. Streeten, G. H. Anderson Jr., R. Richardson, and F. D. Thomas, "Abnormal orthostatic changes in blood pressure and heart rate in subjects with intact sympathetic nervous function: evidence for excessive venous pooling," *Journal of Laboratory and Clinical Medicine*, vol. 111, no. 3, pp. 326–335, 1988.

[14] K. Eguchi, K. Kario, S. Hoshide et al., "Greater change of orthostatic blood pressure is related to silent cerebral infarct and cardiac overload in hypertensive subjects," *Hypertension Research*, vol. 27, no. 4, pp. 235–241, 2004.

[15] G. P. Aurigemma, J. S. Gottdiener, A. M. Arnold, M. Chinali, J. C. Hill, and D. Kitzman, "Left atrial volume and geometry in

healthy aging the cardiovascular health study," *Circulation: Cardiovascular Imaging*, vol. 2, no. 4, pp. 282–289, 2009.

[16] S. Eshoo, D. L. Ross, and L. Thomas, "Impact of mild hypertension on left atrial size and function," *Circulation: Cardiovascular Imaging*, vol. 2, no. 2, pp. 93–99, 2009.

[17] ACCORD Study Group, "Effects of intensive blood-pressure control in type 2 diabetes mellitus," *The New England Journal of Medicine*, vol. 362, no. 17, pp. 1575–1585, 2010.

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Research Article

Treatment Compliance with Fixed-Dose Combination of Vildagliptin/Metformin in Patients with Type 2 Diabetes Mellitus Inadequately Controlled with Metformin Monotherapy: A 24-Week Observational Study

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Objective. To evaluate the differences in treatment compliance with vildagliptin/metformin fixed-dose versus free-dose combination therapy in patients with type 2 diabetes mellitus (T2DM) in Greece. *Design*. Adult patients with T2DM, inadequately controlled with metformin monotherapy, (850 mg bid), participated in this 24-week, multicenter, observational study. Patients were enrolled in two cohorts: vildagliptin/metformin fixed-dose combination (group A) and vildagliptin metformin free-dose combination (group B). *Results*. 659 patients were enrolled, 360 were male, with mean BMI 30.1, mean T2DM duration 59.6 months, and mean HbA1c at baseline 8%; 366 patients were assigned to group A and 293 to group B; data for 3 patients was missing. In group A, 98.9% of patients were compliant with their treatment compared to 84.6% of group B. The odds ratio for compliance in group A versus B was (OR) 18.9 (95% CI: 6.2, 57.7; $P < 0.001$). In group A mean HbA1c decreased from 8.1% at baseline to 6.9% ($P < 0.001$) at the study end and from 7.9% to 6.8% ($P < 0.001$) in group B. *Conclusions*. Patients in group A were more compliant than patients in group B. These results are in accordance with international literature suggesting that fixed-dose combination therapies lead to increased compliance to treatment.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic, progressive disease. As glycemic control deteriorates over time, treatment intensification with the addition of multiple oral antihyperglycemic agents is often required in patients inadequately controlled with monotherapy [1]. Polypharmacy and complexity of the treatment regimens are associated with poor adherence to treatment, which in turn is associated with inadequate glycemic control [2–4]. On the other hand, the use of a fixed-dose combination of agents with complementary mechanisms of action is associated with improved patient compliance and adherence to treatment, as well as better glycemic control [5, 6].

Vildagliptin is a potent and selective oral dipeptidyl peptidase-4 inhibitor that improves glycemic control in patients with T2DM by increasing both the α-cell and β-cell responsiveness to glucose [7, 8]. In numerous clinical trials, combination therapy with vildagliptin and metformin has demonstrated a better efficacy and safety profile with good gastrointestinal tolerability than high-dose metformin monotherapy [9, 10]. A single-pill combination of vildagliptin/metformin has been approved in the European Union and across many countries in the world for the treatment of patients with T2DM inadequately controlled with metformin alone [11]. In the present study, we evaluated the differences in the treatment compliance with vildagliptin/metformin fixed-dose combination and vildagliptin (50 mg bid) added to metformin

(free-dose combination) therapy in patients with T2DM in Greece.

2. Materials and Methods

This was a 24-week, multicenter, observational study. Patients aged >18 years with T2DM and inadequate glycemic control with metformin monotherapy (850 mg bid) were eligible to participate in the study. Patients were enrolled in two cohorts on 1 : 1 ratio, according to everyday clinical practice: those receiving either vildagliptin/metformin fixed-dose combination pill (hereafter referred to as the fixed-dose combination group) or vildagliptin (50 mg bid) added to metformin (850 mg bid) (hereafter referred to as the free-dose combination group).

Patients with a history of type 1 diabetes, end stage renal disease, undergoing hemodialysis, congestive heart failure, and pregnant or lactating women were excluded from the study. In order to assess the treatment compliance, investigators were asked to complete a compliance questionnaire by interviewing patients both at the baseline (visit 1) and final visit 3 (24 weeks after baseline) (Table 1). Patients were considered compliant if they did not miss any drug dose or no more than 2 doses per week, received the correct dosage of the medication, and did not interrupt their treatment. Treatment compliance was assessed from the compliance questionnaire, and the difference in compliance between the treatment groups was reported. In addition to the questionnaire, investigator collected clinical, demographic, and relevant medical history data including comorbidities and complications. At the baseline visit, each patient was given a diary to record their medication intake on a daily basis. The patient was asked to return this diary to the physician at the final visit.

The study was designed and conducted in accordance with the applicable local regulations and with the ethical principles laid down in the Declaration of Helsinki. A written, informed consent was requested from each patient before enrollment in the study.

2.1. Efficacy and Safety Assessments. The primary objective was to compare the percentage of patients compliant with their prescribed therapy. Secondary objectives of the study were to assess the changes in the levels of HbA1c from the baseline until the end of the study (day 0 to 6 months after) and to assess the safety and tolerability profile of vildagliptin.

2.2. Statistical Analysis. Assuming 60% of the patients on fixed-dose combination therapy were compliant and a difference in the treatment groups of 12%, 320 patients per treatment group were required to provide 90% power at a significance level of 5%. The primary variable, difference in compliance between the two treatment groups, was assessed using a multiple binary logistic regression model and adjusted for age, sex, comorbidities, concomitant medications, duration of T2DM, whether patients remembered the names of their medications for T2DM, difficulties in ingestion, and clinical laboratory test results.

Table 1: Compliance questionnaire.

Variable	
Does the therapy affect the daily activities of the patient?	In acute degree
	In some degree
	No
Taking their medication at the same time every day?	Yes
	No
Does the patient have difficulties in swallowing the medication?	Yes
	No
How important do you consider that the therapy is in order to treat the disease?	Very important
	Important
	Of some importance
Have they missed any dose of the treatment?	Today
	Yesterday
	Last week
	Last 2 weeks
	Last month
	Not one dose
Percentage of medication received last month.	Mean
	SD
Does the patient remember the commercial names of the medications?	Yes
	No
Total number of daily tablets for the treatment of T2DM.	Mean
	SD
How often do they forget to take their treatment for T2DM.	Never/almost never
	1-2 times a month
	1 time in a week
	>1 time in a week
	Almost every day
Compliance.	Yes
	No

The odds ratio (OR) with 95% confidence intervals (CI) was also calculated from the multiple binary logistic regression analysis. Change in HbA1c from baseline to end of study was analyzed using an analysis of covariance model (AN.CO.VA.). All adverse events (AEs) and serious adverse events (SAEs) were recorded and monitored, along with their severity and relationship to the study drug.

3. Results

Patient demographics and baseline characteristics were generally comparable between the two treatment groups (Table 2). Of the 659 patients enrolled, 366 (55.5%) were assigned to the fixed-dose combination group and 293 (44.5%) to the free-dose combination group; data for 3 patients were missing. Overall, 54.4% of patients were men, mean age was 61.9 years, mean body mass index (BMI) was 30.1 kg/m^2, and mean baseline HbA1c was 8.0%. About 9% of patients were taking other concomitant medications and 16% of patients had comorbidities, of which 70% of patients had hypertension, 59% had dyslipidemia, and 12% had ischemic heart disease.

Table 2: Patient baseline and demographic characteristics.

	Free-dose combination group $N = 293$	Fixed-dose combination group $N = 366$	Total $N = 659$
Age, years	62.0 (9.6)	61.9 (8.7)	61.9 (9.1)
60–75, *n* (%)	141 (48.1)	192 (52.5)	334 (50.5)
>75, *n* (%)	23 (7.8)	16 (4.4)	39 (5.9)
Gender, men, *n* (%)	152 (51.9)	205 (56.0)	360 (54.4)
Body mass index, kg/m^2	29.6 (3.9)	30.4 (4.04)	30.1 (4.0)
Duration of T2DM, months	55.4 (51.1)	62.4 (55.2)	59.6 (54.2)
Mean HbA1c, %	7.9 (0.8)	8.1 (0.9)	8.0 (0.8)
Comorbidity, yes, *n* (%)	54 (18.4)	49 (13.4)	104 (15.7)
Concomitant treatments, yes, *n* (%)	28 (9.6)	31 (8.5)	60 (9.1)

T2DM, type 2 diabetes mellitus; data are presented as mean (SD) unless otherwise specified.

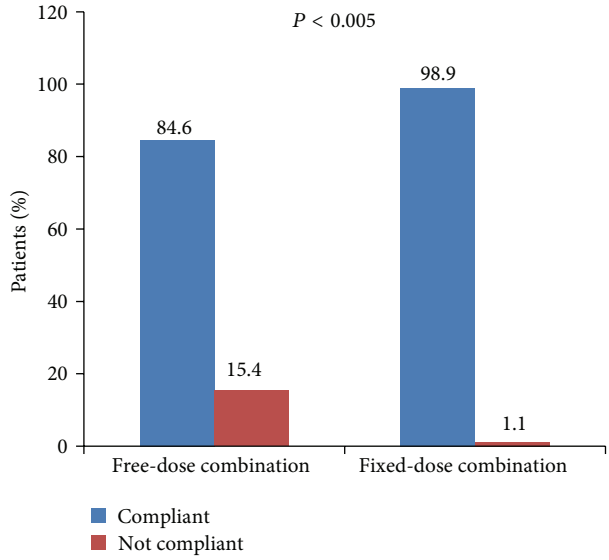

Figure 1: Percentage of patients compliant to treatment (logarithmic regression model, chi-square test).

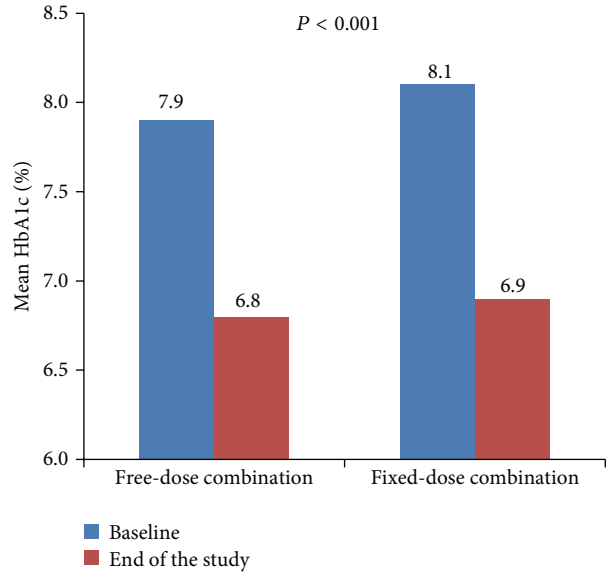

Figure 2: Mean HbA1c (%) at baseline and at end of study (logarithmic regression model, chi-square test).

Overall, 92.6% of patients were compliant with their prescribed therapy according to the definition of compliance used in this study.

The percentage of patients compliant with treatment in the fixed-dose combination group was 98.9% compared with 84.6% in the free-dose combination group before adjusting for confounding factors (Figure 1). The OR for compliance in the fixed-dose combination group versus the free-dose combination group was 18.9 (95% CI: 6.2, 57.7; $P < 0.001$) after adjusting for confounding factors. Patients who remembered the names of their medications were five times more likely to comply with their treatment than patients who did not remember the names of their medications. Patients who experienced difficulty in swallowing their medications were 31.3 times less likely to comply with their treatment compared with patients who did not experience any difficulty in swallowing their medications. The model was also tested for goodness of fit to the data of the study using the Hosmer and Lemeshow that proved that the model had a good fit to the study data; P value = 0.619 (Table 3).

The mean HbA1c decreased from a baseline of 8.1% to 6.9% in the fixed-dose combination group and from 7.9% to 6.8% in the free-dose combination group; the change was statistically significant from baseline to study end in both groups but not between groups (Figure 2). No serious AEs were reported during the study.

4. Discussion

Management of T2DM is complex due to multiple factors such as competing comorbidities, resistance to pharmacotherapy, reluctance to increase the dosage and/or the number of medications, low socioeconomic or educational status, and lack of adherence to lifestyle modifications [12]. All the above factors lead to poor treatment compliance. One practical way to enhance compliance in patients with multiple

Table 3: Log regression model on compliance and confounding factors.

Variables	B	P value	Exp(B)	95% CI for Exp(B)	
				Lower	Upper
Treatment (fixed versus free combination)	2,938	0.001	18,887	6,178	57,738
Medication recall visit 3	1,609	0.001	4,997	2,432	10,264
Swallowing difficulties visit 3	−2,574	0.001	0,076	0,032	0,184
Constant	3,488	0.220	32,728		

comorbidities and receiving concomitant medications is to simplify the treatment regimen with fixed-dose combinations. Results from meta-analysis of clinical trials showed that fixed-dose combinations reduce the risk of noncompliance and improve compliance with treatment compared with free-dose combination regimens [13, 14].

As treatment compliance may influence the overall glycemic control as well as progression of the disease, findings from this study may prove to be useful when assessing treatment strategies for diabetes mellitus. In the present observational study, more number of patients in the fixed-dose combination group were found to be compliant to the treatment (OR 18.9, 95% CI: 6.2, 57.7; $P < 0.001$) compared with the free-dose combination group. This is consistent with the findings from a meta-analysis of seven studies that reported 10% to 13% higher treatment adherence with fixed-dose combination of medications than with free-dose combinations [5]. In this study, patients who did not remember the names of their medications and those who experienced difficulty in swallowing their medications were less likely to comply with their treatment, suggesting that simple names for medications and pill size could help in improving the compliance with medication. The mean HbA1c decreased from a baseline of 8.1 to 6.9% in the fixed-dose combination group and from 7.9% to 6.8% in the free-dose combination group. The observed HbA1c drop in the present study is consistent with the results reported from a large clinical trial ($-0.9 \pm 0.1\%$) which assessed the efficacy and safety of vildagliptin add-on to metformin [15]. Of note, although there were differences with respect to treatment compliance between the fixed-dose and free-dose combinations, these did not result in a difference in efficacy. The results from the present study showed that the combination of two oral antihyperglycemic agents with complementary mechanisms of action offers benefits of consistent glycemic control and helps to improve medication compliance. In addition, there were no new safety signals observed with either fixed-dose or free-dose combinations of vildagliptin and metformin which was generally consistent with the previously reported tolerability profile of vildagliptin as add-on therapy to metformin [8].

The present study has certain limitations that need to be considered while interpreting the results. Only a few patients completed the diaries on a daily basis which resulted in inadequate data for additional analysis and, further, the 6-month follow-up period might be considered a short duration for the measurement of compliance and its effect on efficacy. Moreover, it should be added that the method assessing compliance (interview) is not as accurate as the pill count method or the microprocessor method.

In conclusion, patients on vildagliptin/metformin fixed-dose combination were more compliant with their treatment when compared with patients on free-dose combination. Taking into account that T2DM is a chronic disease, it is important to emphasize that its management should be a part of a health policy plan, and priority should be given to therapies with proven effectiveness and safety as well as fixed-dose combinations that improve patients' compliance.

Conflict of Interests

Grigorios Rombopoulos and Magdalini Hatzikou are employees of Novartis Hellas S.A., Athens, Greece; Athanasios Athanasiadis has no conflict of interests and Professor Moyses Elisaf received compensation as trial investigator from Novartis Hellas.

Authors' Contribution

Grigorios Rombopoulos was involved in designing the study protocol; Magdalini Hatzikou and Athanasios Athanasiadis have analyzed the data and Moyses Elisaf performed the final review of the study outcomes report. All authors were involved in providing inputs and reviewing of the paper.

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References

[1] S. E. Inzucchi, R. M. Bergenstal, J. B. Buse et al., "Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD)," *Diabetes Care*, vol. 35, no. 6, pp. 1364–1379, 2012.

[2] J. M. Schectman, M. M. Nadkarni, and J. D. Voss, "The association between diabetes metabolic control and drug adherence in an indigent population," *Diabetes Care*, vol. 25, no. 6, pp. 1015–1021, 2002.

[3] M. Pladevall, L. K. Williams, L. A. Potts, G. Divine, H. Xi, and J. E. Lafata, "Clinical outcomes and adherence to medications measured by claims data in patients with diabetes," *Diabetes Care*, vol. 27, no. 12, pp. 2800–2805, 2004.

[4] R. A. Vigersky, "An overview of management issues in adult patients with type 2 diabetes mellitus," *Journal of Diabetes Science and Technology*, vol. 5, no. 2, pp. 245–250, 2011.

[5] V. Hutchins, B. Zhang, R. L. Fleurence, G. Krishnarajah, and J. Graham, "A systematic review of adherence, treatment satisfaction and costs, in fixed-dose combination regimens in type 2

diabetes," *Current Medical Research and Opinion*, vol. 27, no. 6, pp. 1157–1168, 2011.

[6] S. Han, K. Iglay, M. J. Davies, Q. Zhang, and L. Radican, "Glycemic effectiveness and medication adherence with fixed-dose combination or coadministered dual therapy of antihyperglycemic regimens: a meta-analysis," *Current Medical Research and Opinion*, vol. 28, no. 6, pp. 969–977, 2012.

[7] B. Balas, M. R. Baig, C. Watson et al., "The dipeptidyl peptidase IV inhibitor vildagliptin suppresses endogenous glucose production and enhances islet function after single-dose administration in type 2 diabetic patients," *The Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 4, pp. 1249–1255, 2007.

[8] B. Ahrén, J. E. Foley, and E. Bosi, "Clinical evidence and mechanistic basis for vildagliptin's action when added to metformin," *Diabetes, Obesity and Metabolism*, vol. 13, no. 3, pp. 193–203, 2011.

[9] E. Bosi, F. Dotta, Y. Jia, and M. Goodman, "Vildagliptin plus metformin combination therapy provides superior glycaemic control to individual monotherapy in treatment-naive patients with type 2 diabetes mellitus," *Diabetes, Obesity and Metabolism*, vol. 11, no. 5, pp. 506–515, 2009.

[10] C. Filozof, S. Schwartz, and J. E. Foley, "Effect of vildagliptin as add-on therapy to a low-dose met formin," *World Journal of Diabetes*, vol. 1, no. 1, pp. 19–26, 2010.

[11] Novartis, Eucreas. Summary of Product Characteristics (UK).

[12] M. P. Hermans, C. Brotons, M. Elisaf, G. Michel, E. Muls, and F. Nobels, "Optimal type 2 diabetes mellitus management: the randomised controlled OPTIMISE benchmarking study: baseline results from six European countries," *European Journal of Preventive Cardiology*, vol. 20, no. 6, pp. 1095–1105, 2013.

[13] S. Bangalore, G. Kamalakkannan, S. Parkar, and F. H. Messerli, "Fixed-dose combinations improve medication complaince: a meta-analysis," *American Journal of Medicine*, vol. 120, no. 8, pp. 713–719, 2007.

[14] L. Blonde and Z. T. San Juan, "Fixed-dose combinations for treatment of type 2 diabetes mellitus," *Advances in Therapy*, vol. 29, no. 1, pp. 1–13, 2012.

[15] E. Bosi, R. P. Camisasca, C. Collober, E. Rochotte, and A. J. Garber, "Effects of vildagliptin on glucose control over 24 weeks in patients with type 2 diabetes inadequately controlled with metformin," *Diabetes Care*, vol. 30, no. 4, pp. 890–895, 2007.

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Clinical Study

The Guyana Diabetes and Foot Care Project: Improved Diabetic Foot Evaluation Reduces Amputation Rates by Two-Thirds in a Lower Middle Income Country

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Background. Type 2 diabetes is the fourth leading cause of death in Guyana, South America. A complex, interprofessional, quality improvement intervention to improve foot and diabetes care was rolled out in two phases. *Methods & Findings*. Phase 1: Establishment of an Interprofessional Diabetic Foot Center (DFC) of Excellence to improve foot care and reduce diabetes-related amputations at the national referral hospital. Phase 2: Regionalization to cover 90% of the Guyanese population and expansion to include improved management of diabetes and hypertension. Fourteen key opinion leaders were educated and 340 health care professionals from 97 facilities trained. Eight centers for the evaluation and treatment of foot ulcers were established and 7567 people with diabetes evaluated. 3452 participants had foot screening and 48% were deemed high risk; 10% of these had undocumented foot ulcers. There was a 68% reduction in rate of major amputations ($P < 0.0001$); below knee amputations were decreased by 80%, while above knee amputations were unchanged. An increased association of diabetes with women (F/M = 2.09) and increased risk of major amputation in men [odds ratio 2.16 (95% CI 1.83, 2.56)] were documented. *Conclusions*. This intervention improved foot care with reduction in major amputations sustained over 5 years.

1. Introduction

While substantial research has demonstrated the potential for preventing the adverse outcomes of type 2 diabetes [1], the increase in the number of people with diabetes (PWD) has outpaced the response of health systems [2, 3]. This incongruity is particularly marked in low and middle income countries (LMIC) where 80% of deaths from diabetes occur [4].

The estimated adult prevalence of diabetes in Guyana was 15.5% in 2011 [5] and in 2008 diabetes was the fourth leading cause of death [6]. Prior to 2008, diabetic foot complications were the most common admitting diagnosis to the surgical ward of the national referral and teaching hospital, Georgetown Public Hospital Corporation (GPHC) [7], with 42% having a lower extremity amputation (LEA) [8]. Scoping visits by Canadian expert team members participating in a recently introduced surgery residency program [9] confirmed that diabetic foot complications were the most common reason for admission to the surgical units of GPHC. Care was uncoordinated, with lack of a systems approach (no screening for high risk feet/ulcers, practice in silos, overaggressive debridement without adequate vascular assessment, no plantar pressure redistribution, and narrow spectrum or missed antimicrobial doses). The high burden of disease (30% inpatient population), prolonged stay, and frequent readmissions with poor patient outcomes resulted in staff demoralization. Surgical debridement took place either

on poorly lit and unsanitary wards or had to compete with other surgical emergencies in the operating rooms. There was inappropriate reliance on major amputations instead of limb salvage. Local health care leaders were keen to address this problem and entered into a partnership with Canadian surgeons and wound/foot care experts to develop the Guyana Diabetes and Foot Care Project (GDFP) 2008–2013.

The Phase 1 goal was to create health system changes in evaluation and management to improve foot care in PWD and reduce diabetes-related LEA at GPHC, while Phase 2 expanded this to 6 administrative regions, comprising 90% of the population, and added training in the management of diabetes and hypertension.

2. Materials and Methods

Clinical activities resided within the Guyanese public health system and staff and resource costs were paid by Ministry of Health (MoH). Multilevel knowledge to action (K2A) cycles was utilized to identify the challenges facing the Guyanese health care system and develop the intervention. Both process and clinical outcomes were monitored. Participants were all persons with type 2 diabetes presenting to the GPHC or to regional facilities with personnel trained in the project, and the care provided to them was based on the decision of these personnel and patient wishes. Patient data was entered into a ministry approved database and identifiable personal information, apart from sex and age, was withheld from the authors. Since this was a quality improvement (QI) project, run under the auspices of the Ministry of Health of Guyana in public health facilities, approval of an ethics committee was not required. Project oversight and coordination was provided by steering committees, meeting regularly, with both Canadian and Guyanese members, including Ministry of Health officials.

2.1. Project Model. The key interventions are detailed elsewhere [10]. In brief, the intervention comprised development of a key opinion leader (KOL) team, this following a train the trainer model. The KOLs were trained using well established Canadian training programs: the International Interprofessional Wound Care Course (IIWCC) [11] and the International Diabetes Federation approved Michener Institute Diabetes Educator course. Health systems change was facilitated through networking with key stakeholders to establish foot care centres and embed practice change. Key opinion leaders (KOLs) attended these training programs; then trained primary health care workers through iterative 3-day workshops on basic foot and wound care using the screening tool and referral criteria. All trainings were interprofessional with doctors, nurses, medex (doctor equivalents), and rehabilitation specialists learning together. The Canadian training programs were supplemented by on-site skills training and reflective practice to develop local expertise as well as supported by continued mentoring from Canadian experts.

2.2. Diabetic Foot Evaluation and Management. Given the limited local resources, it was important to allocate the available resources effectively, and this was facilitated by using clinical screening tools to recognize loss of protective sensation, and identification of the patient at high risk of ulceration or amputation. The simplified 60-second screening tool was developed [12]. The highest risk individuals were then referred to the national Diabetic Foot Center (DFC) at the Georgetown Public Hospital Corporation (GPHC) [13] for more intensive surveillance, education on foot care foot wear and smoking cessation, debridement of callus linked to the use of protective footwear and orthotic devices, improved glycemic control, and the treatment of foot ulcers/complicating infections [14]. Absence and cost of wound care products used for diabetic foot ulcer care in high income countries led to adaptation of more cost effective wound care practices, and commercially available (Darco) wound care sandals were prescribed at a fraction of the price. In the absence of any foot specialists, principles of plantar pressure redistribution (PPR) therapy were taught to rehabilitation assistants, orthotic, prosthetic, and cast room technicians. In Phase 2, the previously listed methods were applied to build capacity across the country, and the foot care program was expanded to 6 Guyanese administrative regions. A 3-day training program on diabetes and hypertension management was added with these components introduced throughout the project regions and HbA1c testing introduced into the public system. A project database was designed to capture the more complex project outcomes with clerks appointed and trained in data entry at each center.

2.3. Targeted Outcomes. Targeted process outcomes were the establishment of a National Centre of Excellence in foot care at GPHC and 7 regional foot care centers, project tools accepted and used by the MoH, measurement of HbA1c and blood pressure for people with diabetes, identification of the high risk foot using the simplified 60-second screening tool, and appropriate referral to regional or national DFCs. Targeted clinical outcomes included reduction in major LEA at GPHC and measurement of the proportion of PWD with HbA1c <9% (75 mmol/mol) and BP <160/95. Diabetic foot admissions at GPHC were determined using admission books on surgical wards and amputations from operating room records. Audits of the admissions book on surgical floors were undertaken to identify patients with diabetic foot complications from 2006 to 2010 because a review of chart coding based records found it to be inaccurate.

2.4. Statistical Analysis. Continuous variables were summarized using means (SD) and median (IQR) and tested using two sample t-tests and paired t-tests as appropriate. Although the t-test is robust to nonnormality, since some data was mildly skewed, we verified the results using a nonparametric Wilcoxon test and found similar results. Categorical data was reported using frequency and percent and tested using the Chi-Square statistic. Odds ratios were also examined for comparisons between groups and the Breslow-Day statistic was used to test for the homogeneity of odds ratios. Time series analysis was employed to examine the effect of the intervention on the number of amputations after adjusting for

autocorrelation and is presented in Figure 1. Autocorrelation and partial autocorrelation plots indicated a good fit. The augmented Dickey-Fuller test and Ljung-Box test indicated good fitting and no concern due to stationary or white noise [15–17].

3. Results and Discussion

3.1. Educational Outcomes. Key opinion leader (KOL) team: A total of 16 trainees (7 doctors, 1 medex, 4 nurses, 3 rehabilitation specialists, and 1 diabetic foot care worker) participated in the International Interprofessional Wound Care Course in 5 cohorts; 14 completed the course and 10 are currently working in the KOL team. The KOL team then trained a total of 340 other Guyanese health care professionals (F/M = 1.8) (Phase 1: 65 HCP in 4 workshops; Phase 2: 275 HCP in 18 workshops). These professionals staff 8 DFCs and 89 health facilities providing chronic disease care.

3.2. Clinical Outcomes

3.2.1. Foot Screening. The simplified 60-second screening tool was developed in Guyana [12] and implemented to screen 3452 persons and 643 completed a follow-up screen. 48% had at least one abnormality and were classified as high risk. A reliability study confirmed the utility of this tool [18], which was adopted by the MoH to be used throughout Guyana.

3.2.2. Patient Database. From July 2010 to March 2013, 7567 PWD were assessed with F/M = 2.09 [19]. As of March 2010, there were a cumulative 6075 patient visits to the GPHC foot center, an average of 13.6/day. As of March 2013, 1186 patients (F/M = 1.60) with foot ulcers have been treated at regional DFCs; there have been over 20,776 visits for dressing care. HbA1c testing was successfully introduced to the public system and a tool to ensure appropriate use of this limited resource was implemented. Since April 2010, 4062 PWD have had HbA1c testing of whom 62% had an HbA1c <9% (75 mmol/mol). The average HbA1c was 8.56% (SD ± 2.85) {70 mmol/mol; SD ± 28 mmol/mol} with women having significantly higher values than men 8.66 (SD ± 2.89) {71 mmol/mol; SD ± 31.6 mmol/mol} versus 8.31 (SD ± 2.77) {67 mmol/mol; SD ± 30.3 mmol/mol}; $P = 0.0001$. Mean HbA1c was 13% higher in patients with foot complications with 44% having HbA1c over 9% (75 mmol/mol). The average blood pressure in 814 PWD was 134 mmHg systolic and 82 mmHg diastolic. 16% of patients had blood pressure greater than either 160 systolic or 95 diastolic. 30% had blood pressure greater than either 140 systolic or 90 diastolic. 649 persons (80%) were on treatment for hypertension. There was not enough power to detect a change in BP or HbA1c over time as too few subjects had recurrent measures. Change in these outcomes is likely to be incremental rather than sudden.

3.2.3. Diabetes-Related Major Lower Extremity Amputations at GPHC. In the 42 months before the DFC opened, the mean monthly number of amputations was 7.95 (SD ± 4.05) and this fell significantly to 3.89 (SD ± 2.30) in the 54 months after the DFC opened through to December 2012 ($P < 0.0001$). This represents a 51% decrease and translates to a saving of 219 limbs from July 2008 to December 2012. The time series analysis (Figure 1) demonstrated a significant decrease in the number of amputations (4.32/month (95% CI 2.40, 6.24); $P < 0.0001$) coincident with the commencement of the project. The opening of the DFC overlapped an ongoing postgraduate surgical training programme [9]. While an apparent rise in major amputation numbers during months 24–41 may have been associated with this increase in surgical capacity, this did not preclude the observed reduction in monthly amputations subsequent to DFC operation.

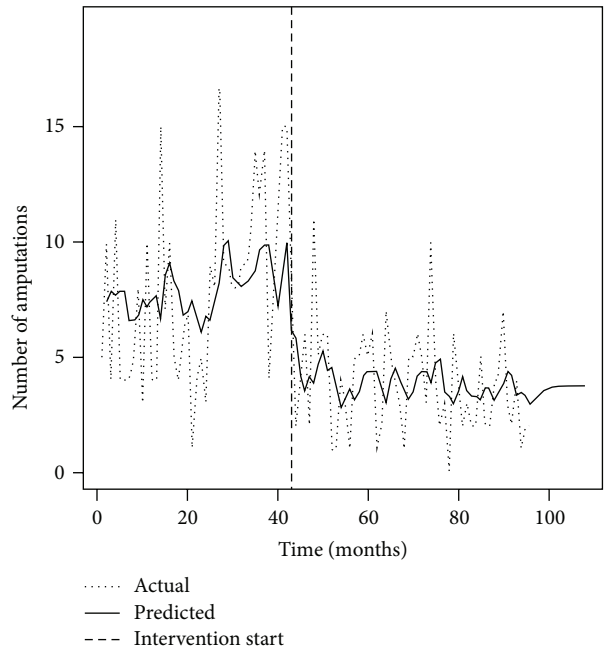

Figure 1: Time series analysis of diabetes-related major amputations at Georgetown Public Hospital Corporation 2005–2012.

Of even greater significance is the marked reduction in proportion of inpatients with diabetic foot complications subjected to major amputation (Table 1) despite a doubling of the rate of diabetic foot admissions at GPHC. The average monthly admissions rose from 21.2 before the DFC opened to 42 in the first 22 months of operation. The proportion of inpatients subjected to a major amputation during this period fell from 41.4% to 11.9% ($P < 0.0001$). A Poisson regression model was used to examine the rate of amputations adjusted for patient volume and after intervention the risk of having an amputation decreased by 68.6% (95% CI 53.9%, 78.5%) as compared to before intervention.

The number of specific types of major amputations, their means, and medians are shown in Table 2. The sums are less than the total number of amputations reported because the type was not specified in some cases. There was no significant difference ($P = 0.07$) in the mean number of above knee amputations (AKA) after intervention as compared to before, while there was a significant difference in below knee amputations (BKA) ($P < 0.0001$).

Table 1: Diabetic foot (DF) admissions and amputation rates at Georgetown Public Hospital Corporation.

Variable	Before DFC (30 months)	After DFC (22 months)	Analysis
DF admissions (ward records)	633	924	
Number of amputations	262	110	
Average monthly proportion DF patient with major amputation	41.4%	11.9%	$P < 0.0001$

Table 2: Major amputations by type at Georgetown Public Hospital Corporation.

Variable	Before intervention	After intervention	Test statistic	P value
Time in months	42	48		
N above knee amputations	124	113*		
Mean (SD)	2.95 (2.44)	2.13 (1.81)	−1.82 (t)	0.07
Median (IQR)	2 (1–4)	2 (1–3)	1.47 (Z)	0.14
N below knee amputations	166	41*		
Mean (SD)	3.95 (2.64)	0.77 (1.05)	−7.35 (t)	<0.0001
Median (IQR)	3 (2–5)	0 (0-1)	6.82 (Z)	<0.0001

*Represents total with available dates. One AKA and 3 BKAs were not dated.

The changes in the frequency of AKAs and BKAs before and after the intervention give an indication of the limitations of this kind of project focused on primary care. While BKAs showed an 80% and significant reduction after the DFC was opened, AKAs showed no change. Currently there is no vascular surgical capacity in Guyana to treat vascular insufficiency, a common comorbidity in diabetic foot complications. We suggest that patients with both diabetic foot complications and uncorrected vascular insufficiency are more likely to require AKAs. This service gap could explain the lack of decline in AKAs after intervention. It would also speak to the need for developing a vascular surgical capacity in resource-constrained settings, if limb salvage in the diabetic foot is to be optimized. Figure 1 illustrates a plateau effect on amputations after intervention and it may be that this is the best that can be achieved without further resources (e.g., vascular surgery and renal dialysis).

3.3. Sex-Based Differences in Type 2 Diabetes and Amputations. We have already reported on the divergence from global averages of the sex ratios of type 2 diabetes in Guyana and have estimated that the odds ratio for women compared to men is 2.486 (95% CI 2.442, 2.531, $P < 0.0001$) [19]. The sex-based risks for diabetes-related amputations in Guyana are reversed. There were 544 major amputations (278 in women and 266 in men) over 8 years with F/M of 1.05. Since few regional hospitals in Guyana have surgical capacity and most diabetic foot problems are referred to GPHC, virtually all diabetes-related LEAs in Guyana are being performed at that hospital. To calculate the sex-based relative risks we assumed that any amputations outside GPHC follow the same distribution for sex and type and that the number of persons with diabetes remained constant over the 2005–2012 periods. Since the estimated prevalence of diabetes in women is twice that in men, the odds ratio of amputations for males as compared to females during the entire study period is 2.16 (95% CI 1.83, 2.56; $P < 0.0001$). The reasons for the increased risk of amputations in men are unknown but may be related to an increased risk of ulceration due to social factors (occupational hazards, smoking) or failure to seek medical attention. The odds ratios for amputations in men compared to women increased from 1.86 (95% CI 1.50, 2.31) before the intervention to 2.73 (95% CI 2.08, 3.58) after the intervention ($P = 0.015$). We tested whether AKA and BKA amputation rates differed between males and females before versus after the intervention and found that the odds ratios did not change before versus after intervention (OR = 0.9959 {95% CI 0.6576, 1.4682}).

3.4. System Change. The MoH embraced the model, which is described in detail elsewhere [10], and approved in the new *MOH Strategic Plan 2013–2020: Integrated Prevention and Control of Non Communicable Disease in Guyana* [20]. Despite the many challenges facing the MoH, a significant change in the approach to evaluation of the diabetic foot and diabetes management occurred.

4. Conclusions

We demonstrated that it is possible to introduce the best practice methods to evaluate for the high risk foot in people with diabetes and achieve sustained improvements in evaluation and care of foot ulcers. After the project began GPHC achieved a marked and sustained reduction both in major amputation numbers and in the proportion of inpatients with diabetic foot complications requiring major amputation. That this reduction occurred almost immediately after project commencement suggests that surgeons embraced the importance of maintaining limb integrity. Change was likely sustained by provision of new alternate methods and dedicated clinic spaces for treatment based on context specific practice guidelines. Vascular surgery capacity is essential to maximize limb salvage.

Translating clinical guidelines and QI principles into practice, in both the developed and developing world is challenging. In low and middle income countries (LMIC) the

challenge is to deploy interventions that are cost saving or cost effective. This requires empirical research in a variety of contexts. Our project contributes to this research. One of our next steps is to investigate the transferability of our model to another limited resource setting.

Abbreviations

AKA: Above knee amputation
BKA: Below knee amputation
BP: Blood pressure
CI: Confidence interval
CIDA: Canadian International Development Agency
CME: Continuous medication education
DFC: Diabetes and foot center
F/M: Female to male ratio
GDFP: Guyana Diabetes and Foot Care Project
GPHC: Georgetown Public Hospital Corporation
HbA1c: Glycosylated hemoglobin
HCP: Health care professional
IDF: International Diabetes Federation
IIWCC: International Interprofessional Wound Care Course
K2A: Knowledge to action
KOL: Key opinion leader
LEA: Lower extremity amputation
LMIC: Low and middle income countries
MoH: Ministry of Health
PPR: Plantar pressure redistribution
PWD: Person with diabetes
QI: Quality improvement
SD: Standard deviation.

Conflict of Interests

The funding organizations were independent of the design and conduct of the study, the collection, management, analysis, and interpretation of the data, or the preparation of the paper. Drs. Brian Ostrow, R. Gary Sibbald, and Julia Lowe received travel funding to Guyana from Guyana Diabetes and Foot Care Project. Drs. R. Gary Sibbald and Julia Lowe received honoraria for their participation. Dr. Gerald Lebovic's institution received payment for statistical analysis.

Authors' Contribution

R. Gary Sibbald, Brian Ostrow, and Julia Lowe wrote the first draft of the paper. Julia Lowe, R. Gary Sibbald, Nashwah Y. Taha, Gerald Lebovic, Brian Ostrow, Madan Rambaran, RK, Indira Bhoj, and Carlos Martin contributed to the writing of the paper. Julia Lowe, R. Gary Sibbald, Nashwah Y. Taha, Gerald Lebovic, Brian Ostrow, Madan Rambaran, Indira Bhoj, and Carlos Martin read and met ICMJE criteria for authorship. Julia Lowe, R. Gary Sibbald, Nashwah Y. Taha, Gerald Lebovic, Brian Ostrow, Madan Rambaran, Indira Bhoj, and Carlos Martin agree with paper results and conclusions.

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References

[1] American Diabetes Association, "Standards of medical care in diabetes—2010," *Diabetes Care*, vol. 33, supplement 1, pp. S11–S61, 2010.

[2] J. B. Saaddine, M. M. Engelgau, G. L. Beckles, E. W. Gregg, T. J. Thompson, and K. M. V. Narayan, "A diabetes report card for the United States: quality of care in the 1990s," *Annals of Internal Medicine*, vol. 136, no. 8, pp. 565–574, 2002.

[3] Canadian Diabetes Association, *An Economic Tsunami: The Rising Costs Of Diabetes in Canada*, Canadian Diabetes Association, 2009, http://www.diabetes.ca/documents/get-involved/FINAL_Economic_Report.pdf.

[4] C. D. Mathers and D. Loncar, "Projections of global mortality and burden of disease from 2002 to 2030," *PLoS Medicine*, vol. 3, no. 11, article e442, 2006.

[5] International Diabetes Federation, *IDF Diabetes Atlas*, International Diabetes Federation, Brussels, Belgium, 5th edition, 2011, http://www.idf.org/diabetesatlas.

[6] Ministry of Health Statistics Unit, *Statistical Bulletin 2008*, Ministry of Health, Georgetown, Guyana, 2009, http://www.statisticsguyana.gov.gy/pubs.html.

[7] B. Ostrow, C. Martin, and M. Rambaran, "Clinicians work to enhance diabetic foot program in Guyana," *Advances in Skin & Wound Care*, vol. 20, no. 12, pp. 640–641, 2007.

[8] K. Newark, S. Scotland, O. Seepersaud, and N. Persaud, "Lower extremity amputations in diabetic patients with foot ulcers at Georgetown Public Hospital Corporation (2003–2006)," *West Indian Medical Journal*, vol. 57, supplement 2, article 27, 2008, Chronic Diseases II, Conference Abstract 29.

[9] B. H. Cameron, M. Rambaran, D. P. Sharma, and R. H. Taylor, "International surgery: the development of postgraduate surgical training in Guyana," *Canadian Journal of Surgery*, vol. 53, no. 1, pp. 11–16, 2010.

[10] J. Lowe, R. G. Sibbald, N. Y. Taha et al., "The Guyana diabetes and foot care project: a complex quality improvement intervention to decrease diabetes-related major lower extremity amputations and improve diabetes care in a lower middle income country," *PLoS Medicine*. In press.

[11] R. G. Sibbald and H. Orsted, "The international interdisciplinary wound care course at the University of Toronto: a 4-year evolution," *International Wound Journal*, vol. 1, no. 1, pp. 34–37, 2004.

[12] R. G. Sibbald, E. A. Ayello, A. Alavi et al., "Screening for the high-risk diabetic foot: a 60-second tool," *Advances in Skin and Wound Care*, vol. 25, no. 10, pp. 465–476, 2012.

[13] B. Ostrow, K. Y. Woo, and R. G. Sibbald, "The Guyana diabetic foot project: reducing amputations and improving diabetes care in Guyana," *WCET Journal*, vol. 30, no. 4, pp. 28–32, 2010.

[14] N. Singh, D. G. Armstrong, and B. A. Lipsky, "Preventing foot ulcers in patients with diabetes," *Journal of the American Medical Association*, vol. 293, no. 2, pp. 217–228, 2005.

[15] D. A. Dickey and W. A. Fuller, "Distribution of the estimators for autoregressive time series with a unit root," *Journal of the American Statistical Association*, vol. 74, no. 366, pp. 427–431, 1979.

[16] U. Helfenstein, "Box-Jenkins modelling in medical research," *Statistical Methods in Medical Research*, vol. 5, no. 1, pp. 3–22, 1996.

[17] G. M. Ljung and G. E. P. Box, "On a measure of lack of fit in time series models," *Biometrika*, vol. 65, no. 2, pp. 297–303, 1978.

[18] G. Woodbury, R. G. Sibbald, B. Ostrow, and J. Lowe, "Interrater reliability of simplified 60-second diabetic foot screen tool and creation of the simplified 60-second diabetic foot screen tool," *PLoS ONE*. Submitted.

[19] B. Ostrow, R. G. Sibbald, N. Y. Taha, and J. Lowe, "Sex disaggregated diabetes prevalence study in Guyana needed," *Diabetes Research and Clinical Practice*, vol. 106, no. 2, article e17, 2014.

[20] Pan American Health Organization (PAHO) and World Health Organization, *Launch of Strategic Plan 2013–2020: Integrated Prevention and Control of Non-communicable Diseases (NCD) in Guyana*, World Health Organization, Georgetown, Guyana, 2013, http://www.paho.org/guy/index.php?option=com_content&view=article&id=180:launch-of-strategic-plan-2013-2020-integrated-prevention-and-control-of-non-communicable-diseases-ncds-in-guyana&catid=498:guy.01-reducing-the-burden-of-disease:-communicabl&Itemid=194.

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Research Article

Comparison of Lipoprotein-Associated Phospholipase A2 and High Sensitive C-Reactive Protein as Determinants of Metabolic Syndrome in Subjects without Coronary Heart Disease: In Search of the Best Predictor

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High sensitivity C-reactive protein (hsCRP) is a marker of metabolic syndrome (MS) and cardiovascular (CV) disease. Lipoprotein-associated phospholipase A2 (Lp-PLA2) also predicts CV disease. There are no reports comparing these markers as predictors of MS. *Methods*. Cross-sectional study comparing Lp-PLA2 and hsCRP as predictors of MS in asymptomatic subjects was carried out; 152 subjects without known atherosclerosis participated. Data were collected on demographics, cardiovascular risk factors, anthropometric and biochemical measurements, and hsCRP and Lp-PLA2 activity levels. A logistic regression analysis was performed with each biomarker and receiver operating characteristic (ROC) curves were constructed for MS. *Results*. Mean age was 46 ± 11 years, and 38% of the subjects had MS. Mean Lp-PLA2 activity was 185 ± 48 nmol/mL/min, and mean hsCRP was 2.1 ± 2.2 mg/L. Subjects with MS had significantly higher levels of Lp-PLA2 ($P = 0.03$) and hsCRP ($P < 0.0001$) than those without MS. ROC curves showed that both markers predicted MS. *Conclusion*. Lp-PLA2 and hsCRP are elevated in subjects with MS. Both biomarkers were independent and significant predictors for MS, emphasizing the role of inflammation in MS. Further research is necessary to determine if inflammation predicts a higher risk for CV events in MS subjects.

1. Introduction

Metabolic syndrome (MS) is a group of cardiovascular (CV) risk factors including abdominal obesity, hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL-C), dysglycemia, and high blood pressure (BP) [1]. This condition is associated with a proinflammatory and prothrombotic condition [2]. Numerous studies have shown that a significant increase in systemic inflammatory markers, such as high sensitivity C-reactive protein (hsCRP), interleukin-6, and tumor necrosis factor-α (TNF-α), among others, is observed in subjects with MS [3].

High sensitivity C-reactive protein is a good predictor of MS and is strongly associated with abdominal obesity [4]. Moreover, this biomarker predicts CV events in healthy people as well as in those with atherosclerotic disease [5, 6]. It has been reported that hsCRP is elevated in an urban population of Santiago, Chile, with MS, and there is a clear correlation between MS, hsCRP, and subclinical atherosclerosis in this population [7]. Thus, the clustering of CV risk factors and inflammation observed in MS increase the risk of atherosclerosis.

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a recently described inflammatory marker [8]. It is an enzyme produced by macrophages that hydrolyzes phospholipids of oxidized low-density lipoprotein (LDL), releasing oxidized fatty acids and lysophosphatidylcholine, which are potent proinflammatory and prooxidative molecules [8]. Since oxidized LDL is mostly located in the intima of the artery, the products of Lp-PLA2 activity are generated mainly in the vascular wall. Therefore, these mediators play an important role in inflammation at the vascular level. Given that atherosclerosis is an inflammatory disease which begins in the vascular wall, Lp-PLA2 may have a prominent role in its pathophysiology [8].

Both Lp-PLA2 and hsCRP are associated with coronary and cerebrovascular atherosclerotic disease in populations with and without a history of CV events [9–12]. Furthermore, Lp-PLA2 is related to CV risk factors, with a strong correlation with LDL, as well as various components of MS (e.g., abdominal obesity) [13]. The vast majority of published studies do not show any association between Lp-PLA2 and hsCRP; hence, these inflammatory markers have been considered to be independent of each other. There are no reports in literature that compare these markers as predictors of MS.

The objective of this study was to analyze and compare the levels of Lp-PLA2 and hsCRP as predictors of MS in subjects without atherosclerotic disease.

2. Materials and Methods

This was a descriptive cross-sectional study in 152 subjects (69 women), without history of atherosclerotic disease, who were recruited in two preventive cardiology centers of Santiago between October 2011 and June 2012: Hospital Clínico de la Pontificia Universidad Católica de Chile and Hospital de la Previsión de Carabineros de Chile. Subjects between 18 and 70 years old were included. Exclusion criteria were (a) history of coronary heart disease and/or carotid or peripheral vascular disease; (b) lipid-lowering therapy (statins, ezetimibe, fibrates, nicotinic acid, and omega 3); (c) use of oral contraceptives or hormone replacement therapy; (d) chronic intake of anti-inflammatory medicines (steroidal and nonsteroidal); (e) acute and/or chronic inflammatory diseases; and (f) pregnancy.

The investigation protocol was approved by the ethics committees of both institutions. Subjects were contacted by telephone and invited to participate by nurses or doctors in charge of the study.

After signing the written, informed consent, patients were subjected to clinical data collection and anthropometric and laboratory measurements, all detailed below.

2.1. Data Collection. During the visit to the centers, subjects were interviewed about demographics, medical history, education, physical activity, family medical history, and intake of medications.

2.1.1. Anthropometric and Laboratory Measures. Weight, height, body mass index (BMI), and hip and waist circumferences were measured. The latter was measured in the midpoint between the last rib and the iliac crest. Blood pressure was measured following the recommendations of the Seventh Joint National Committee [14], with the subject sitting and after 5 minutes of rest.

Venous blood samples were taken after 12-hour fasting for determining the following biochemical parameters:

(i) *Lp-PLA2*: samples for analysis of Lp-PLA2 activity were frozen and stored at −70°C. Levels of Lp-PLA2 activity were determined by enzymatic method (DIADEXUS, USA), Cobas 8000 analyzer, c702 module. Prior to the analysis, precision and trueness of the enzymatic test were assessed locally, and a calibration curve was constructed, which was sent to the international center for approval. Following the approval, the analysis of the subjects' samples was performed.

(ii) *Total cholesterol (total-C)*: colorimetric enzymatic method, Roche Diagnostics Cobas analyzer Cobas 8000, c702 module.

(iii) *HDL-C*: colorimetric homogeneous enzymatic method, Roche Diagnostics Cobas, Cobas 8000 analyzer, c702 module.

(iv) *Triglycerides*: white colorimetric enzymatic method with glycerol, Roche Diagnostics Cobas, Cobas 8000 analyzer, c702 module.

(v) *LDL cholesterol (LDL-C)*: calculated by Friedewald formula.

(vi) *Blood glucose*: enzymatic method (hexokinase), Roche Diagnostics Cobas, Cobas 8000 analyzer, c702 module.

(vii) *Creatinine*: Jaffé method, Roche Diagnostics Cobas, Cobas 8000 analyzer, c70 module.

(viii) *hsCRP*: nephelometric method on BN ProSpec analyzer, Siemens (detection limit 0.16 mg/L).

(ix) *Fibrinogen*: Clauss method in ACL Top 500 analyzer, Instrumentation Laboratory.

2.2. Variables. Hypertensives were subjects who had a prior diagnosis according to the JNC 7 [14], with or without pharmacologic therapy, and/or those with an average blood pressure ≥140/90 mm Hg. Dyslipidemics were subjects who had LDL-C level ≥130 mg/dL, HDL-C level <40 mg/dL in men, <50 mg/dL in women, or non-HDL-C level ≥160 mg/dL

Table 1: Demographic data and prevalence of traditional cardiovascular risk factors in the total sample divided by gender.

	Total ($n = 152$)	Men ($n = 83$)	Women ($n = 69$)	P
Age (years)	46 ± 11	45 ± 11	47 ± 11	NS
Educational level (years)	13 ± 3	14 ± 2	13 ± 4	NS
Dyslipidemia (%)	62	68	55	NS
Hypertension (%)	30	31	29	NS
Smoking (%)	31	31	30	NS
Diabetes (%)	5	7	3	NS
Physical inactivity (%)	78	76	80	NS
Overweight (%)	37	41	33	NS
Obesity (%)	33	34	32	NS
Family history of CHD (%)	13	10	16	NS

Data are presented as mean ± SD or percentage.
CHD: coronary heart disease; NS: not significant.

in the laboratory assessment. Subjects were considered diabetics if they had a prior diagnosis, with or without drug treatment, and/or if they had a fasting blood glucose ≥126 mg/dL during the study, according to the American Diabetes Association criteria. Current smokers were those subjects who smoked daily during the last month, and ex-smokers were those subjects who had at least six consecutive months without smoking. The recent harmonized criteria were used for the diagnosis of MS, which includes a waist circumference ≥90 cm in men and ≥80 cm in women for Latin American populations [1].

2.3. Statistical Analysis. The software R 2.15.2 was used for statistical analysis. A logistic regression analysis was performed with each biomarker, adjusted for age and gender. Smooth receiver operating characteristic (ROC) curves were constructed with cubic splines for MS (area under the curve C value = 0.50 implies a predictive value equivalent to chance). Comparisons of means (expressed as mean ± standard deviation [SD]) are based on the analysis of variance and linear regression models.

3. Results

This study included 152 subjects (45% women) with a mean age of 46 ± 11 years. Table 1 shows demographic data and prevalence for CV risk factors. Prevalence rate for hypertension, diabetes, dyslipidemia, and current smoking was 30%, 5%, 62%, and 31%, respectively, with no significant differences observed between genders. Prevalence of MS was 38% (57 subjects) in the total sample with no significant differences between genders: 42% and 32% in men and women, respectively. Table 2 provides anthropometric and laboratory measures from all subjects divided by gender. Men had significantly higher waist circumference ($P < 0.01$), systolic and diastolic BP ($P < 0.0001$), and creatinine levels ($P < 0.0001$) than women. Women had higher HDL-C levels ($P < 0.0001$).

Table 2: Anthropometric measurements and laboratory data for all subjects divided by gender.

	Total ($n = 152$)	Men ($n = 83$)	Women ($n = 69$)	P
BMI	28 ± 4	28 ± 4	28 ± 5	NS
Waist circumference (cm)	93 ± 11	95 ± 10	90 ± 11	<0.01
Waist ≥ 90/80 cm (men/women), n (%)	115 (76%)	61 (74%)	54 (79%)	NS
SBP (mm Hg)	119 ± 15	124 ± 14	113 ± 16	<0.0001
DBP (mm Hg)	76 ± 13	81 ± 11	70 ± 13	<0.0001
Blood glucose (mg/dL)	91 ± 24	93 ± 29	89 ± 16	NS
Total-C (mg/dL)	208 ± 44	209 ± 47	207 ± 39	NS
HDL-C (mg/dL)	52 ± 15	46 ± 13	58 ± 14	<0.0001
LDL-C (mg/dL)	128 ± 36	131 ± 38	126 ± 34	NS
Non-HDL-C (mg/dL)	154 ± 43	158 ± 43	148 ± 42	NS
Creatinine (mg/dL)	0.8 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	<0.0001
hsCRP (mg/L)	2.1 ± 2.2	2.2 ± 2.2	2.0 ± 2.2	NS
Lp-PLA2 (nmol/mL/min)	185 ± 48	201 ± 49	166 ± 38	<0.0001

Values expressed as mean ± SD, except where indicated.
BMI: body mass index; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; hsCRP: high sensitive C-reactive protein; LDL-C: low-density lipoprotein cholesterol; Lp-PLA2: lipoprotein-associated phospholipase A2; NS: not significant; SBP: systolic blood pressure; SD: standard deviation; total-C: total cholesterol.

Mean level of hsCRP was 2.1 ± 2.2 mg/L, and there were no significant differences between men and women (Table 2). Likewise, there were no significant differences in the prevalence of high levels of hsCRP (defined as >2 mg/L) between genders (39% in men and 36% in women). Of note, hsCRP was significantly and directly correlated with BMI, waist circumference, blood glucose, systolic BP, non-HDL-C, and fibrinogen, and it was inversely and significantly correlated with HDL-C (Table 3).

Mean level of Lp-PLA2 activity was 185 ± 48 nmol/mL/min in the total sample with significant differences between men and women: 201 nmol/mL/min and 166 nmol/mL/min ($P < 0.0001$), respectively (Table 2). Lp-PLA2 was significantly and directly correlated with BMI, waist circumference, diastolic BP, LDL-C, non-HDL-C, and plasma creatinine; HDL-C was inversely and significantly correlated with Lp-PLA2. No significant correlations were found between Lp-PLA2 and blood glucose, systolic BP, hsCRP, and fibrinogen (Table 3). No differences were found between Lp-PLA2 activity of active smokers and nonsmokers.

As shown in Table 4, both Lp-PLA2 and hsCRP were significantly higher in subjects with MS than in those without MS. In subjects with MS, Lp-PLA2 was 198 ± 45 nmol/mL/min versus 180 ± 48 nmol/mL/min in subjects without MS ($P = 0.03$). Similarly, hsCRP was 4.1 ± 3.3 mg/L in subjects with MS versus 2.2 ± 3.2 mg/dL in those without MS ($P = 0.0001$). Both biomarkers significantly increased with the number of MS components: Lp-PLA2 levels increased from

Table 3: Correlation coefficients of Lp-PLA2 with demographic variables, lipid factors, blood glucose, blood pressure, hsCRP, and fibrinogen.

	Lp-PLA2			hsCRP	
Variable	Correlation coefficient	P	Variable	Correlation coefficient	P
BMI	0.20	0.02	BMI	0.54	<0.0001
Waist	0.28	<0.001	Waist	0.48	<0.0001
Blood glucose	0.05	0.56	Blood glucose	0.17	<0.05
SBP	0.09	0.25	SBP	0.23	<0.01
DBP	0.18	0.03	DBP	0.15	0.07
LDL-C	0.62	<0.001	LDL-C	0.15	0.07
HDL-C	−0.45	<0.001	HDL-C	−0.25	<0.01
Non-HDL-C	0.66	<0.001	Non-HDL-C	0.27	<0.001
hsCRP	−0.04	0.67	Lp-PLA2	0.40	<0.0001
Fibrinogen	−0.02	0.79	Fibrinogen	0.40	<0.0001
Creatinine	0.32	<0.001	Creatinine	0.02	NS

BMI: body mass index; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; hsCRP: high sensitive C-reactive protein; LDL-C: low-density lipoprotein cholesterol; Lp-PLA2: lipoprotein-associated phospholipase A2; NS: not significant; SBP: systolic blood pressure.

Table 4: Mean levels of hsCRP and Lp-PLA2 by number of metabolic syndrome components according to harmonized criteria[1] in the total sample.

Total (n = 152)	NO MS (0–2 RF) (n = 95)	MS (3 or more RF) (n = 57)	P
LpPLA2 levels (nmol/mL/min)	180 ± 48	198 ± 45	0.03
hsCRP (mg/L)	2.2 ± 3.2	4.1 ± 3.3	<0.0001

hsCRP: high sensitive C-reactive protein; Lp-PLA2: lipoprotein-associated phospholipase A2; MS: metabolic syndrome; RF: risk factors.

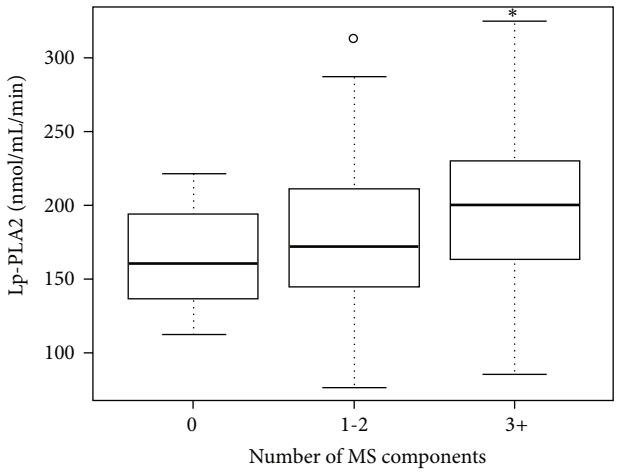

Figure 1: Levels of lipoprotein-associated phospholipase A2 by number of metabolic syndrome components. $^*P < 0.01$ versus 0 MS components. LpPLA2: lipoprotein-associated phospholipase A2; MS: metabolic syndrome.

163 nmol/mL/min to 198 nmol/mL/min ($P < 0.01$) and hsCRP increased from 1.5 mg/L to 4.1 mg/L ($P = 0.04$) when subjects with 0 and ≥3 components of MS were compared (Figures 1 and 2).

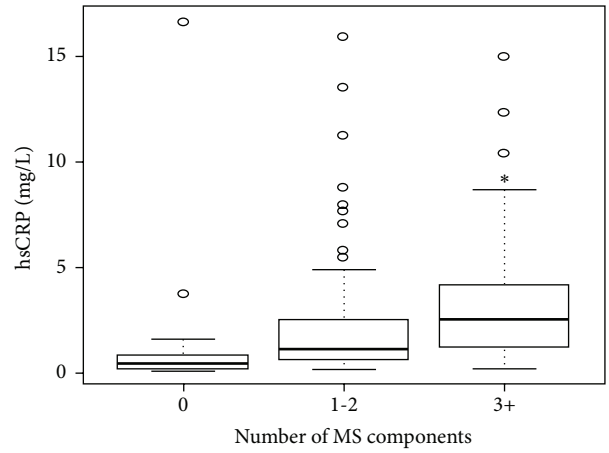

Figure 2: Mean levels of high sensitive C-reactive protein by number of metabolic syndrome components. $^*P = 0.04$ versus 0 MS components. hsCRP: high sensitivity C-reactive protein; MS: metabolic syndrome.

The results of the logistic regression analysis for determining MS, which included both biomarkers adjusted for age and gender, were as follows: odds ratio (OR) for LpPLA2: 1.02 (confidence interval [CI]: 1.00–1.02, $P = 0.03$) and OR for hsCRP: 2.5 (CI: 1.65–3.80, $P < 0.0001$). Figure 3 shows the ROC curves for both biomarkers adjusted for age and gender: Lp-PLA2, C value = 0.66 [0.57–0.74] and hsCRP, C value = 0.73 [0.65–0.81]. According to this analysis, although hsCRP shows a better area under the curve, from a statistical point of view, it cannot be shown that it is better than Lp-PLA2, as the CIs of both biomarkers overlap.

4. Discussion

In this study, we have demonstrated in a population without atherosclerotic disease that levels of Lp-PLA2 activity and hsCRP are elevated in subjects with MS. Both biomarkers

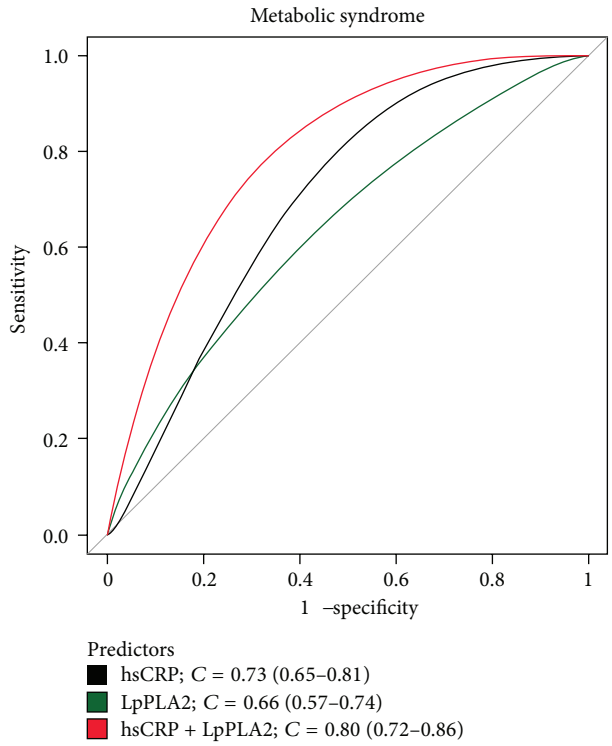

Figure 3: ROC curves for lipoprotein-associated phospholipase A2 (Lp-PLA2), high sensitivity C-reactive protein (hsCRP), and both together adjusted by age and gender.

significantly increased with the number of metabolic risk factors, and both were shown to be independent and statistically significant predictors of MS. These results highlight the role of inflammation in MS.

The MS corresponds to a clustering of CV and metabolic risk factors and inflammation. In our country, MS prevalence is approximately 30%, and it is occurring at younger ages, probably due to the overweight, obesity, and insulin resistance epidemic. Although, the pathophysiologic mechanism involved in MS is not entirely clear, it has been shown that it confers an increased risk for diabetes and atherosclerotic CV disease [9, 15].

Inflammation plays a fundamental role in the development of MS. Recent studies have reported that the presence of MS and inflammation (determined by hsCRP) confers more CV risk than the presence of MS alone [16, 17]. High sensitivity C-reactive protein is also a predictor for the development of MS and diabetes [18]. In our country, we have previously reported that subjects with MS and elevated hsCRP have more subclinical atherosclerosis and carotid atherosclerotic plaques [7].

It is well known that atherosclerosis is an inflammatory disease [19]. Therefore, many inflammatory markers related to MS have been investigated in the search of a possible explanation for the elevated CV risk associated with it. The most studied biomarker is hsCRP; however, there are new biomarkers associated with atherosclerotic disease that may be more specific to vascular inflammation. Among them is Lp-PLA2, an enzyme that acts on oxidized LDL and produces strong inflammatory and oxidative mediators of the intima. Epidemiologic and clinical studies have shown an association between atherosclerotic disease and Lp-PLA2 concentration, in a similar way as reported with hsCRP [9–11, 13].

In this study, we have demonstrated that both biomarkers, Lp-PLA2 and hsCRP, predicted MS with a similar power. Regarding the association of hsCRP with MS, this study confirms the available evidence showing that levels of hsCRP are increased in subjects with MS [16, 17, 20]. Our results also support the association between hsCRP and overall/visceral obesity, HDL-C, non-HDL-C, and glycemia. Similar to the findings of Ballantyne and colleagues [10], we demonstrated that hsCRP is not associated with Lp-PLA2, suggesting that their inflammation pathways are different from a physiologic point of view. Conversely, hsCRP was related to fibrinogen, also an acute phase protein that has a similar mechanism of production by the liver. hsCRP is produced by the liver, primarily by the action of interleukin-6, a cytokine that increases in the conditions of visceral obesity and insulin resistance. Based on this evidence, although hsCRP is an important inflammatory marker, it is unspecific.

Activity levels of Lp-PLA2 were also significantly higher in subjects with MS in our study. These findings are similar to those from the Bruneck Study in 2009 [12]. In that study, Tsimikas et al. found significant associations between Lp-PLA2 and LDL-C, non-HDL-C, HDL-C, and homeostasis model assessment insulin resistance (HOMA-IR), whereas correlations with BMI and waist circumference were weaker. Our results are identical to those reported by these authors regarding lipid factors and obesity. However, we did not find any direct correlation with blood glucose, and we did not measure insulin resistance. Furthermore, Lp-PLA2 was not correlated to BP and smoking in our study, which was seen in the Bruneck Study. Lastly, we found no association between Lp-PLA2 and fibrinogen, which confirms that Lp-PLA2 inflammatory pathway is not through acute phase reaction.

It is important to analyze the relationship between Lp-PLA2 and LDL-C and non-HDL-C (atherogenic cholesterol). After adjusting for gender and age, these correlations became even more significant. Our population had an average LDL-C level of 128 mg/dL, which suggests that, even at moderate levels of LDL-C, subjects with MS had elevated activity of this enzyme. This result could be because many subjects with MS have primarily small and dense LDL molecules, which are prone to oxidation and therefore they are substrate for Lp-PLA2. The inflammation produced by Lp-PLA2 is primarily in the vascular level, because lysophosphatidylcholine and oxidized fatty acids, generated after Lp-PLA2 action over oxidized LDL, induce adhesion molecules and other cytokines in the same vascular wall. Thus, it can be hypothesized that, unlike hsCRP (which occurs as a result of inflammation caused by other cytokines from the visceral fat), Lp-PLA2 is directly involved in the inflammatory process, and, subsequently, the atherogenic process of the plaque. Thus, Lp-PLA2 could be a more specific marker of atherosclerotic events in patients with MS. Confirmation of this hypothesis must be demonstrated by studies of CV events in patients with

MS, who have these biomarkers measured, and with findings adjusted for all the risk factors associated with hsCRP and Lp-PLA2.

In this regard, it should be noted that a meta-analysis evaluating the clinical use of hsCRP has shown that this biomarker adds little in the prognosis of CV risk, when adjusting by the risk factors involved in MS [21]. Conversely, the fact that Lp-PLA2 was weakly related to these metabolic factors should preserve its power as a biomarker. However, the latter has not been easy to confirm. The reasons for not having completely cleared Lp-PLA2 prognosis utility are based primarily on the different techniques used for its measurement, which makes it impossible to compare the results of different studies.

In this study, we did not expect to find the same power for hsCRP and Lp-PLA2 as predictors of MS. We expected hsCRP to be a better predictor, given its close association with metabolic risk factors included in MS. However, from a statistical point of view (as demonstrated by the ROC curves), both had a similar level of prediction. These results suggest that Lp-PLA2 is related to MS through other pathways that are not fully known and most likely not associated with the metabolic risk factors. We know that Lp-PLA2 is linked through oxidized LDL, which is elevated in subjects with MS, although it cannot be perceived due to the moderate levels of LDL-C observed in the subjects of our study. The explanation is that Lp-PLA2 is associated with "oxidized" LDL, and, for measuring "oxidized" LDL in the clinic, it required more expensive and sophisticated methods not frequently used in clinical practice.

Since MS is highly prevalent in our population, our results could provide knowledge about nontraditional risk factors that could help to identify which patients with MS must be treated earlier and more aggressively. This is important, because currently there is a pharmacologic inhibitor of Lp-PLA2 activity, darapladib, which is being investigated in subjects with atherosclerotic disease. In addition, another study is currently investigating the use of a monoclonal antibody against interleukin-1 for the intervention against high hsCRP. If these studies have positive results, the measurement and targeting of these biomarkers might become important components of clinical management of patients. Finally, it must be noted that we found no studies in literature that compare Lp-PLA2 and hsCRP as predictors of MS in the general population.

Our study has some limitations: (1) it is a cross-sectional study, and hence causality inferences cannot be made; (2) it was done in a small, nonrandom sample of subjects; (3) we did not include HOMA-IR, which could explain the relationship of insulin resistance with MS and inflammatory markers; (4) finally, we only determined Lp-PLA2 activity instead of mass. This decision was subject to the availability of the laboratory determination in our country. However, currently, it is the most used technique, and it has shown the best correlation with MS [13].

The strengths of the study include the following: (1) the recruitment of subjects was performed prospectively; (2) subjects with lipid-lowering therapy and women with hormone replacement therapy were excluded, among others; (3) this study has validated the measurement of Lp-PLA2 in our country.

In conclusion, Lp-PLA2 and hsCRP are good determinants of MS. Prospective studies must confirm which of the markers or if both markers are causally related to the atherosclerotic consequences observed in subjects with MS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mónica Acevedo and Paola Varleta contributed equally to this work.

Acknowledgment

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References

[1] K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy et al., "Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity," *Circulation*, vol. 120, no. 16, pp. 1640–1645, 2009.

[2] P. Dandona, A. Aljada, A. Chaudhuri, P. Mohanty, and R. Garg, "Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation," *Circulation*, vol. 111, no. 11, pp. 1448–1454, 2005.

[3] P. Libby, P. M. Ridker, and A. Maseri, "Inflammation and atherosclerosis," *Circulation*, vol. 105, no. 9, pp. 1135–1143, 2002.

[4] H. S. Park, J. Y. Park, and R. Yu, "Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-α and IL-6," *Diabetes Research and Clinical Practice*, vol. 69, no. 1, pp. 29–35, 2005.

[5] P. M. Ridker, E. Danielson, F. A. Fonseca et al., "Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial," *The Lancet*, vol. 373, no. 9670, pp. 1175–1182, 2009.

[6] P. M. Ridker, D. A. Morrow, L. M. Rose, N. Rifai, C. P. Cannon, and E. Braunwald, "Relative efficacy of atorvastatin 80 mg and pravastatin 40 mg in achieving the dual goals of low-density lipoprotein cholesterol <70 mg/dl and C-reactive protein <2 mg/L: an analysis of the PROVE-IT TIMI-22 trial," *Journal of the American College of Cardiology*, vol. 45, no. 10, pp. 1644–1648, 2005.

[7] M. Acevedo, P. Arnaiz, R. Corbalán et al., "Modificación del grosor intima-media carotídeo según factores de riesgo clásicos y sindrome metabólico con o sin inflamación," *Revista Chilena de Cardiología*, vol. 28, pp. 337–348, 2009.

[8] P. P. Toth, P. A. McCullough, M. S. Wegner, and K. J. Colley, "Lipoprotein-associated phospholipase A_2: role in atherosclerosis and utility as a cardiovascular biomarker," *Expert Review of Cardiovascular Therapy*, vol. 8, no. 3, pp. 425–438, 2010.

[9] The Lp-PLA2 Studies Collaboration, "Lipoprotein-associated phospholipase A_2 and risk of coronary disease, stroke, and mortality: collaborative analysis of 32 prospective studies," *The Lancet*, vol. 375, no. 9725, pp. 1536–1544, 2010.

[10] C. M. Ballantyne, R. C. Hoogeveen, H. Bang et al., "Lipoprotein-associated phospholipase A_2, high-sensitivity C-reactive protein, and risk for incident coronary heart disease in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study," *Circulation*, vol. 109, no. 7, pp. 837–842, 2004.

[11] C. J. Packard, D. S. J. O'Reilly, M. J. Caslake et al., "Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease," *The New England Journal of Medicine*, vol. 343, no. 16, pp. 1148–1155, 2000.

[12] S. Tsimikas, J. Willeit, M. Knoflach et al., "Lipoprotein-associated phospholipase A2 activity, ferritin levels, metabolic syndrome, and 10-year cardiovascular and non-cardiovascular mortality: Results from the Bruneck study," *European Heart Journal*, vol. 30, no. 1, pp. 107–115, 2009.

[13] M. Persson, J.-Å. Nilsson, J. J. Nelson, B. Hedblad, and G. Berglund, "The epidemiology of Lp-PLA_2: distribution and correlation with cardiovascular risk factors in a population-based cohort," *Atherosclerosis*, vol. 190, no. 2, pp. 388–396, 2007.

[14] A. V. Chobanian, G. L. Bakris, H. R. Black et al., "The seventh report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 report," *Journal of the American Medical Association*, vol. 289, no. 19, pp. 2560–2572, 2003.

[15] S. M. Grundy, J. I. Cleeman, S. R. Daniels et al., "Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute scientific statement," *Circulation*, vol. 112, no. 17, pp. 2735–2752, 2005.

[16] P. M. Ridker, J. E. Buring, N. R. Cook, and N. Rifai, "C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women," *Circulation*, vol. 107, no. 3, pp. 391–397, 2003.

[17] P. M. Ridker, P. W. F. Wilson, and S. M. Grundy, "Should C-reactive protein be added to metabolic syndrome and to assessment of global cardiovascular risk?" *Circulation*, vol. 109, no. 23, pp. 2818–2825, 2004.

[18] T. S. Han, N. Sattar, K. Williams, C. Gonzalez-Villalpando, M. E. J. Lean, and S. M. Haffner, "Prospective study of C-reactive protein in relation to the development of diabetes and metabolic syndrome in the Mexico City diabetes study," *Diabetes Care*, vol. 25, no. 11, pp. 2016–2021, 2002.

[19] R. Ross, "Atherosclerosis—an inflammatory disease," *The New England Journal of Medicine*, vol. 340, no. 2, pp. 115–126, 1999.

[20] D. Aronson, P. Bartha, O. Zinder et al., "Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome," *International Journal of Obesity*, vol. 28, no. 5, pp. 674–679, 2004.

[21] S. Tsimikas, J. T. Willerson, and P. M. Ridker, "C-reactive protein and other emerging blood biomarkers to optimize risk stratification of vulnerable patients," *Journal of the American College of Cardiology*, vol. 47, no. 8, supplement, pp. C19–C31, 2006.

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Research Article

Do Behavioral Risk Factors for Prediabetes and Insulin Resistance Differ across the Socioeconomic Gradient? Results from a Community-Based Epidemiologic Survey

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To examine whether behavioral risk factors associated with diabetes (diet, BMI, waist circumference, physical activity, and sleep duration) are also related to both prediabetes and insulin resistance (IR), we used data from Boston Area Community Health (BACH) Survey (2010–2012, $n = 3155$). Logistic and linear regression models were used to test the association of lifestyle factors with prediabetes status, insulin resistance, and prediabetes or insulin resistance. All regression models were stratified by education and income levels (to examine whether risk factors had differential effects across socioeconomic factors) and adjusted for age, gender, race/ethnicity, family history of diabetes, and smoking status. We found that large waist circumference was consistently associated with higher levels of insulin resistance (IR) and increased odds of prediabetes. While the association between large waist circumference and IR was consistent across all levels of SES ($P < 0.001$), the association between large waist circumference and prediabetes was only statistically significant in the highest socioeconomic strata with odds ratios of 1.68 (95% CI 1.07–2.62) and 1.88 (95% CI 1.22–2.92) for postgraduate degree and income strata, respectively. There was no association between diet, physical activity, sleep duration, and the presence of multiple risk factors and prediabetes or IR within SES strata.

1. Introduction

The prevalence of type 2 diabetes continues to rise and currently affects over 25 million Americans and is estimated to reach 439 million adults worldwide by 2030 [1, 2]. Along with this increase, the prevalence of prediabetes is also rising, affecting approximately one in three U.S. adults aged ≥ 20 years (an estimated 79 million individuals) [3, 4]. Furthermore, it is estimated that, among U.S. adults with undiagnosed diabetes or impaired fasting glucose, the proportion of adults with insulin resistance increased from 24.8% to 31.1% during the time periods of 1988–1994 to 1999–2002 [5].

Prediabetes is indicated when blood glucose or hemoglobin A1c (A1c) levels are higher than normal, but not yet high enough to be classified as diabetes. Prediabetes raises the risk of type 2 diabetes by 3- to 10-fold and it is estimated that up to 70% of people with prediabetes may develop type 2 diabetes during their lifetime [3, 6, 7]. A study conducted by Geiss et al. [8] estimates 30% of the U.S. adult population had prediabetes in 2005-2006, but only 7% were aware that they had the condition. Insulin resistance (IR) is a condition in which the body produces insulin but does not use it effectively. When people have insulin resistance, glucose builds up in the blood instead of being absorbed by the cells, leading to type 2 diabetes or prediabetes.

The American Diabetes Association (ADA) (April 4) [9] considers both prediabetes and insulin resistance precursors to type 2 diabetes. Both the ADA and the National Heart Lung and Blood Institute's National Cholesterol Education

Program (NCEP) [10] suggest the risk factors for type 2 diabetes, prediabetes, and IR are similar and include the following (not comprehensive): obesity, physical inactivity, family history of diabetes, race/ethnicity, high blood pressure, large waist circumference, and high triglycerides. Notably, socioeconomic status is usually not included but is an important focus of this paper.

Socioeconomic status is a complex construct, broadly based on relative income, education, and occupation—all of which have been repeatedly associated with lifestyles and behavioral risk factors. In this analysis, we use income and education as indicators of SES, since occupation has been shown to be highly correlated with education [11, 12]. Numerous studies have identified low physical activity [13–15], low diet quality [14, 16, 17], and, more recently, poor sleep quality, as associated with diabetes [18–22]. However, it is uncertain whether these factors equally affect prediabetes and diabetes risk across the SES gradient. That is, most previous studies statistically adjust for education, income, or some other marker of SES [23–25]. But to develop well-targeted and likely effective primary and secondary interventions it is necessary to understand how modifiable risk factors influence risk of diabetes among the different SES groups. Studies have shown that while health programs and therapies exist to manage prevention of diabetes and its complications, these programs are underutilized among those in low socioeconomic groups [26–28].

While there are many risk factors contributing to diabetes, this study seeks to identify those most likely to improve the effectiveness of primary and secondary prevention initiatives. Coupled with the under-utilization of diabetes prevention programs among those with low SES [26–28], there is a potential benefit to identifying modifiable diabetes risk factors that may contribute to prediabetes among those with low SES and to examine the different effects of diabetes risk factors operating among different SES levels.

Therefore, the objectives of this analysis are

(1) within and across each level of SES, to examine the associations between known behavioral risk factors for diabetes (low diet quality, poor sleep, low physical activity, and high waist circumference) and both prediabetes and IR;

(2) to examine these modifiable factors collectively (presence of two or more risk factors) for their potential impact on the prevalence of prediabetes and IR within and across the SES gradient.

2. Materials and Methods

2.1. Participants. The Boston Area Community Health (BACH) Survey is a longitudinal cohort study of residents of Boston, MA, aged 30–79 years at baseline (March 2002–June 2005). Detailed methods have been described elsewhere [29, 30]. Briefly, a stratified two-stage cluster sample was used to recruit an approximately equal number of participants by gender, race/ethnicity (Black, Hispanic, White), and age group (30–39, 40–49, 50–59, 60–79). 5,502 adults participated in baseline BACH I (1767 Black, 1876 Hispanic, 1859 White; 2301 men, 3201 women). Follow-up surveys were collected at two time points approximately 5 (BACH II 2006–2010) and 7 (BACH III 2010–2012) years later. For BACH III, completed interviews were obtained for 3155 individuals (1184 men; 1971 women). In all surveys, data were collected during a two-hour interview in English or Spanish, after obtaining written informed consent. The study was approved by New England Research Institutes' Institutional Review Board. Analyses for this paper use data from the most recent interview, BACH III (2010–2012).

2.2. Sociodemographic Lifestyle Assessment Measures. Education, based on years of education completed, is composed of four categories: <high school, high school or GED, some college, or college or advanced degree. Income was collected as total annual income and categorized into three categories: <$20,000, $20,000–$49,999, or $50,000+. Physical Activity was measured using the Physical Activity for the Elderly (PASE) scale and categorized into low, moderate, or high [31]. Anthropometric measures-Trained field Interviewers directly measured the subject's height (cm), weight (kg), from which BMI was calculated (kg/m^2), and waist circumference (cm). Waist circumference was further dichotomized into high waist circumference defined as ≥88 cm for women and ≥102 cm for men [32, 33]. BMI was dichotomized using the cut-off point of 25 kg/m^2 that defines overweight in the current WHO classification.

Diet was measured with the Block Food Frequency Questionnaire (FFQ) administered in English or Spanish. The FFQ has been validated to obtain data on usual dietary intake over the past year [34]. We calculated an overall healthy eating score using the USDA and AHA guidelines for healthy eating [35, 36]. The healthy eating score is composed of FFQ data on average daily intake of sodium (g), vegetables (servings/day), fruits (servings/day), meats/beans (serving/day), grains (servings/day), fiber (g), and saturated fat (g). Participants were classified into three possible diet score groupings: poor (score 0–2 points), intermediate (score 2–5 points), or high (score 5–7 points). The overall healthy eating index for this sample ranged from 0 to 5 indicating that no one met criteria for high (healthiest) healthy eating score. Therefore, for analyses, we created 3 groups (low/medium/high) based on off tertile cut-points. Each individual component of the diet score was also examined separately.

Sleep duration was measured using self-reported sleep patterns in the previous month with the question "How many hours of actual sleep do you get during the night?" and grouped as follows: <6.5 hours, 6.5–<8 hours, and ≥8 hours to create low, intermediate, and high duration of sleep.

Multiple risk factors measures were created by combining diet, sleep, and activity scores. The presence of two factors is defined as having poor diet (lowest tertile) and low sleep duration (<6.5 hours). The three-risk-factor measure is defined as the presence of low physical activity, poor diet, and low sleep duration. Lastly the four-risk-factor measure is defined as the presence of high BMI (≥25) or high waist circumference (≥88 cm women and ≥102 cm men) and presence of the three risk factors mentioned above.

2.3. Major Outcome Measures. Diagnosed type 2 diabetes was determined by self-report (Have you ever been told by a doctor or other health professional that you have type 2 diabetes?). Medication inventory confirmed over 80% of the self-reported cases of diabetes. Insulin resistance was measured using the homeostasis model assessment (HOMA) index where the product of fasting glucose and fasting insulin was taken and then divided by 405 [37]. Insulin resistance was calculated only in subjects that had fasting glucose collected and has an analytic sample size of 2359. Prediabetes is defined among those subjects with no diabetes (type 1 or type 2) and no undiagnosed diabetes defined as fasting glucose >125 or HbA1c ≥6.5. Subjects were considered prediabetic if they have fasting glucose 100–125 or HbA1c 5.7–6.4 [38]. Subjects with prediabetes are compared to diabetes unaffected subjects—that is, those without diabetes (self-reported type 1 or 2 or undiagnosed) and with no prediabetes leaving an analytic sample size of 2175. A third outcome combining prediabetes and insulin resistance was created to capture the presence of prediabetes or insulin resistance among nondiabetic subjects ($N = 2175$). To define insulin resistance, we used the NHANES study cut-point of ≥2.73 [39].

2.4. Statistical Analysis. Bivariate associations between health factors and education were examined with Wald F Chi-Square tests for categorical measures and Wald F test P value from linear regression models for continuous measures.

Logistic regression models were utilized to examine the association of lifestyle/behavioral health factors with prediabetes and the combined outcome of insulin resistance or prediabetes. Linear regression models were constructed to examine the association between lifestyle and behavioral factors on insulin resistance. Due to positive skew, insulin resistance was log transformed in final regressions. In all regression models we stratified by education level or income group and adjusted for age, gender, race/ethnicity, family history of diabetes, and smoking status. Regression models were examined for the overall healthy eating score as well as each of the 7 subscales but results are presented only for the overall score. Analyses of diet were adjusted for total caloric intake to minimize measurement error by over/underreporting [40]. Separate unstratified regression models were examined to test for interactions between lifestyle and behavioral factors and SES measures income and education.

All analyses were performed with SAS callable SUDAAN 11.0 [41] using sampling weights and stratification measures to account the complex survey design of BACH. Multiple imputation was used to reduce bias resulting from missing data for all exposure covariates using multivariate imputation by chained equations (MICE) in R [3]. Briefly, MICE imputes missing values with estimated predictions from regression models that reflect the relationships observed in the data, while considering the complex survey sampling design. Age, race, and their interaction were included as predictors in each of the imputation models. In addition, mice selected important predictors for each variable and these were also included in the model. Less than 5% of the covariates (age, gender, education, race/ethnicity, and income) were imputed [42]. Fifteen multiple imputation datasets were created stratified by race/ethnicity by gender.

3. Results and Discussion

The study population characteristics are shown in Table 1. Among the total sample, 28% had normal blood glucose levels (without self-reported diabetes and not prediabetic). In the sample without diabetes ($n = 2379$) 65% were prediabetic and 69% were prediabetic or IR. The geometric mean of the HOMA-IR measure was 1.9 (SE 1.0). Among those with prediabetes, over half were White and reported medium levels of physical activity and 46% reported a family history of diabetes. In subjects with prediabetes or IR, 45% were male and reported a family history of diabetes. The mean waist circumference for those with prediabetes or IR is the highest at 96 cm compared to those unaffected or with prediabetes alone. In general, mean diet FFQ scores (total score and subscales) were comparable across groups.

Similar patterns were observed in the bivariate associations between study population characteristics with educational level and income (data not shown). Educational level is significantly associated with most measures except for total FFQ score. Males were more likely to have higher education (postgraduate) compared to female (51% versus 49%, $P = 0.04$). The <HS group also reported the highest rates of current smoking and family history of diabetes compared to all other education levels ($P \leq 0.0001$ in all comparisons). Regarding diabetes outcomes, subjects in the highest education group had higher rates of being in the unaffected group (34% versus 12%, $P \leq 0.0001$) and lower rates of reporting prediabetes (61% versus 80%, $P \leq 0.0001$) and prediabetes or IR (65% versus 83%, $P = 0.006$) compared to the lowest education group. Gender, current smoking, family history and diabetes, and the diabetes outcomes were associated with income with similar patterns.

In unstratified models there were no significant interactions seen between education and income with lifestyle/behavioral factors (data not shown). Table 2 displays the results of the insulin resistance regressions and Figure 1 plots mean HOMA-IR for significant predictors. Insulin resistance is associated with BMI and waist circumference across all levels of education and income (P ranges from 0.02 to ≤0.0001, Table 2, Models 2 and 3). The magnitudes of association with BMI and waist circumference on IR are similar across all levels of education and income. Those with BMI ≥ 25 have on average a higher mean log IR score of 0.20 compared to those with BMI < 25. Similarly, those with a high waist circumference have higher mean IR scores anywhere between 0.22 and 0.33 points higher than those with a smaller waist circumference. The healthy eating score, other diet components, physical activity, and sleep duration were not associated with insulin resistance. In addition, presence of multiple risk factors was not predictive of insulin resistance.

The covariate adjusted geometric means for insulin resistance in the BMI ≥ 25 group are nearly twice as high

Table 1: Characteristics of BACH-3 analytic sample ($N = 3155$), overall and by fasting glucose/diabetes status.

		Fasting glucose/diabetes status			
	Total $N = 3155$	Unaffected $N = 641$ (27.7%)	Prediabetes*** $N = 1533$ (64.6%)	Prediabetes or insulin resistant $N = 1627$ (69.2%)***	Insulin resistance HOMA model* $N = 2359$ Mean (SE) = 1.9 (1.0)
Age, y mean SE	54.0 (0.5)	50.8 (0.8)	53.7 (0.6)	53.5 (0.6)	0.05
Gender, %					
Male	1184 (46.5%)	241 (47.8%)	559 (43.9%)	599 (44.5%)	2.04 (1.05)
Female	1971 (53.5%)	399 (52.2%)	973 (56.1%)	1028 (55.5%)	1.73 (1.05)
BMI, mean SE	29.5 (0.2)	27.6 (0.3)	29.3 (0.3)	29.4 (0.3%)	0.41
Waist circumference, cm mean SE	97.0 (0.5)	91.9 (1.0)	95.8 (0.7)	96.2 (0.6%)	0.45
Education					
<HS	618 (7.9%)	76 (3.4%)	246 (7.2%)	259 (7.0%)	2.24 (1.09)
HS or GED	948 (24.4%)	173 (21%)	454 (23.1%)	483 (23.1%)	2.24 (1.06)
Some college	671 (20.5%)	139 (18.2%)	332 (21%)	354 (21.2%)	2.06 (1.07)
College or advanced	916 (47.1%)	251 (57.4%)	499 (48.7%)	531 (48.8%)	1.60 (1.05)
Income					
<$20,000	1338 (27.0%)	204 (19.1%)	577 (23.8%)	615 (24.5%)	2.43 (1.07)
$20,000–$49,999	914 (25.1%)	181 (21.2%)	472 (27.1%)	498 (26.7%)	1.97 (1.05)
>$50,000	902 (48.0%)	93 (59.7%)	482 (49.2%)	514 (48.8%)	1.68 (1.07)
Race/ethnicity					
Black	1026 (27.1%)	140 (13.8%)	504 (29.1%)	531 (28.6%)	2.30 (1.06)
Hispanic	1036 (12.2%)	195 (11.6%)	475 (12.5%)	507 (12.7%)	1.96 (1.08)
White	1093 (60.7%)	305 (74.6%)	553 (58.4%)	589 (58.7%)	1.69 (1.05)
Physical activity					
Low	1244 (32.2%)	185 (25%)	546 (30.7%)	580 (30.8%)	2.14 (1.06)
Medium	1492 (51.2%)	346 (57%)	768 (52.3%)	812 (51.9%)	1.75 (1.05)
High	417 (16.6%)	109 (18%)	218 (17%)	235 (17.3%)	1.75 (1.08)
Smoking status					
Never	1386 (45.2%)	295 (48.5%)	685 (46.3%)	727 (46.3%)	1.83 (1.05)
Former	1160 (36.2%)	231 (35.6%)	541 (35%)	573 (34.8%)	1.85 (1.05)
Current	608 (18.6%)	113 (15.9%)	306 (18.7%)	327 (18.9%)	2.03 (1.09)
Total Diet Quality score**	0.67 [0.02, 1.42]	0.68 [0.03, 1.43]	0.69 [0.03, 1.43]	0.69 [0.04, 1.43]	−0.02
Sleep duration					
<6.5 HR	1477 (41.9%)	238 (32.7%)	706 (41.4%)	740 (40.6%)	2.08 (1.05)
6.5–<8.0 HR	881 (32.4%)	221 (41%)	443 (31.5%)	473 (31.7%)	1.61 (1.06)
≥8.0 HR	795 (25.7%)	181 (26.3%)	383 (27.2%)	414 (27.7%)	1.89 (1.06)
HOMA-IR*	1.86 (1.05)	1.27 (1.05)	1.86 (1.04)	2.68 (0.12)	NA
Family history of diabetes	1686 (46.5%)	276 (36.8%)	751 (45.7%)	792 (45.2)	2.09 (1.05)

*Geometric means are presented for categorical variables and weighted Pearson's correlations are presented for continuous variables. Insulin resistance was collected on subjects with fasting glucose.
**Median [interquartile range] presented for skewed predictors.
***Conducted among $N = 2175$ nondiabetic subjects.

compared to the BMI < 25 group within the lowest education level <HS or GED (Figure 1). The same magnitude in mean differences between the BMI categories is also seen in the HS or GED and some college group. Geometric HOMA-IR means are also higher in those with BMI ≥ 25 compared to BMI < 25 within income levels. Across both education and income groups, HOMA-IR geometric means are lower as one moves into higher educated or income groups. The geometric means for those with BMI ≥ 25 are 2.8, 2.2, and 1.8 for the income groups <$20,000, $20,000–$49,000, and $50,000+, respectively. With regard to waist circumference, higher mean HOMA-IR values are observed among subjects

Table 2: Associations (Beta estimate [95% CI]) between diet quality, sleep duration, physical activity and waist circumference and insulin resistance, by socioeconomic status level.

	Education level Beta estimate [95% CI][A] $N = 2359$*				Income Beta estimate [95% CI][A] $N = 2359$*		
	<HS $N = 403$	HS or equiv. $N = 698$	College or equiv . $N = 509$	Postgrad $N = 750$	<$20,000 $N = 939$	$20,000–$49,999 $N = 682$	$50,000+ $N = 738$
Individual factor							
Model 1[C]: Total diet quality (lower tertile versus upper and middle tertiles)	0.04 [−0.13, 0.22]	0.01 [−0.12, 0.14]	0.08 [−0.04, 0.20]	−0.01 [−0.11, 0.08]	0.002 [−0.13, 0.14]	0.03 [−0.08, 0.13]	0.02 [−0.07, 0.11]
Model 2: BMI (≥25 versus <25)	**0.22 [0.04, 0.40]**[B]	**0.23 [0.10, 0.35]**[B]	**0.24 [0.10, 0.38]**[B]	**0.19 [0.11, 0.28]**[B]	**0.25 [0.13, 0.37]**[B]	**0.22 [0.12, 0.33]**[B]	**0.19 [0.10, 0.28]**[B]
Model 3: Waist circumference, cm mean SE (Larger: ≥88 for women, ≥102 for men, versus smaller)	**0.33 [0.19, 0.47]**[B]	**0.28 [0.19, 0.37]**[B]	**0.27 [0.16, 0.39]**[B]	**0.22 [0.14, 0.30]**[B]	**0.27 [0.15, 0.38]**[B]	**0.26 [0.17, 0.36]**[B]	**0.23 [0.15, 0.30]**[B]
Model 4: Physical activity (low versus med/high)	0.13 [−0.02, 0.27]	0.10 [−0.00, 0.21]	−0.01 [−0.14, 0.12]	0.05 [−0.03, 0.14]	0.02 [−0.08, 0.13]	0.05 [−0.04, 0.15]	0.05 [−0.04, 0.14]
Model 5: Sleep length/quality (low versus med/high)	0.05 [−0.12, 0.22]	0.05 [−0.06, 0.15]	0.03 [−0.09, 0.14]	0.03 [−0.05, 0.11]	0.06 [−0.12, 0.24]	0.04 [−0.09, 0.17]	0.01 [−0.14, 0.15]
Two factors							
Low diet and sleep scores	0.05 [−0.18, 0.28]	0.04 [−0.13, 0.22]	0.07 [−0.12, 0.25]	−0.03 [−0.17, 0.12]	0.07 [−0.15, 0.29]	0.16 [−0.09, 0.41]	0.18 [−0.09, 0.46]
Three factors							
Low diet and sleep scores, plus low activity	0.12 [−0.13, 0.36]	0.11 [−0.13, 0.34]	0.18 [−0.06, 0.42]	0.05 [−0.22, 0.32]	0.07 [−0.15, 0.29]	0.16 [−0.09, 0.41]	0.18 [−0.09, 0.46]
Four factors							
Low diet, sleep, activity, plus high BMI or waist (select the relevant one)	0.13 [−0.13, 0.38]	0.11 [−0.12, 0.35]	0.22 [−0.05, 0.49]	0.11 [−0.18, 0.41]	0.09 [−0.15, 0.32]	0.19 [−0.08, 0.45]	0.23 [−0.07, 0.52]

*These analyses exclude subjects that did not have fasting glucose available. The column totals do not add up due to rounding from multiple imputations.

[A]From multivariable models adjusting for age, gender, race/ethnicity, smoking, and family history of diabetes.

[B]In bold, $P \leq 0.05$.

[C]Dietary models are adjusted for age, gender, race/ethnicity, total caloric intake, smoking, and family history of diabetes.

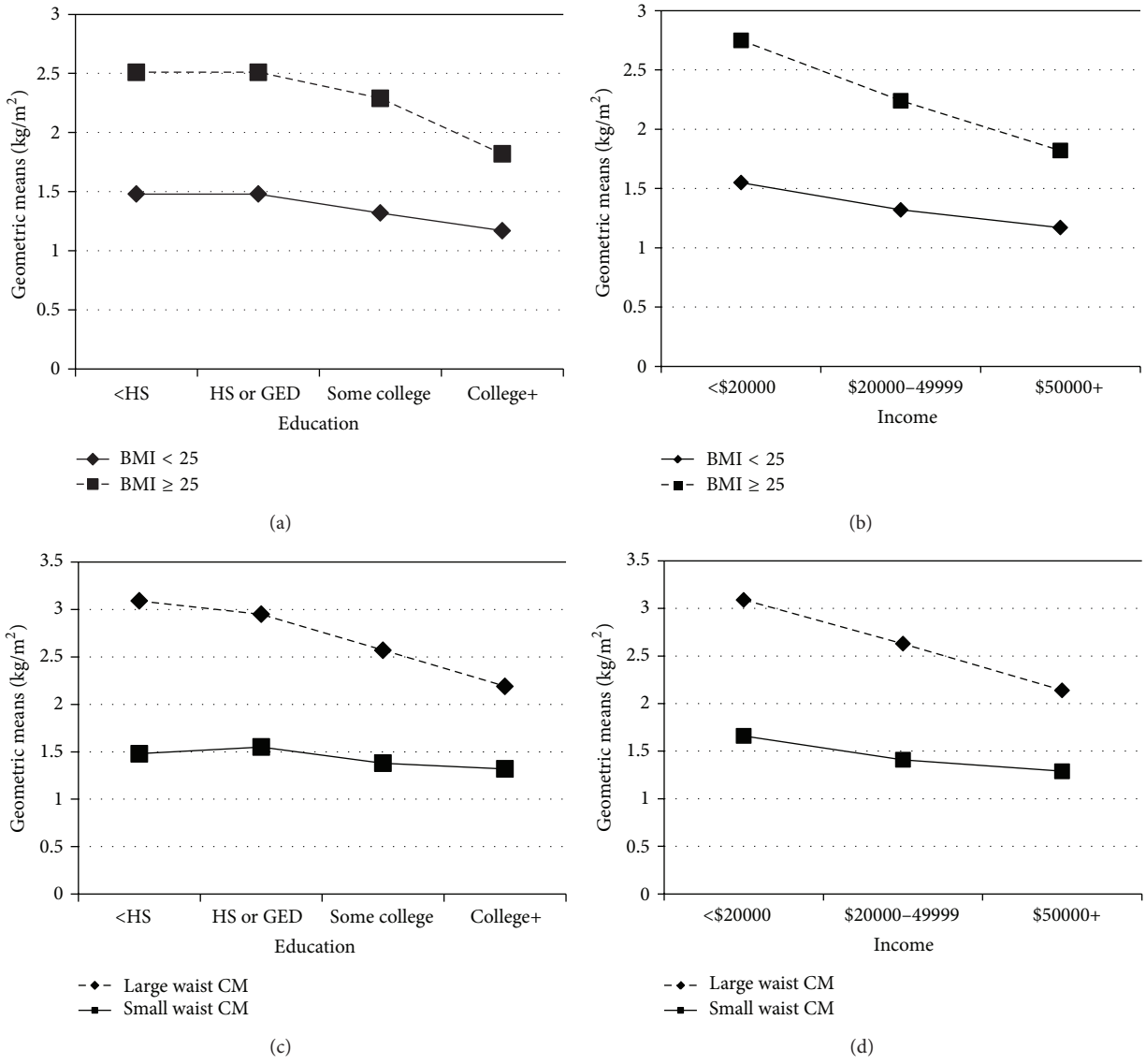

Figure 1: Geometric means of BMI and waist circumference by education and income strata.

with large waist cm compared to small waist cm within all levels of education and income ($P \leq 0.0001$ in all models). These differences are nearly threefold.

Few significant associations were seen in logistic regression models of prediabetes (Table 3). Large waist circumference was significantly associated with increased odds of prediabetes in the college or advance degree group such that those with large waist circumference were 1.7 times as likely to develop prediabetes compared to those with lower waist circumference (OR 1.68, 95% CI: 1.07–2.62). Similarly for income, subjects with large waist cm were nearly twice as likely to have prediabetes compared to those of lower waist cm in the medium and high income strata with OR 2.13 (95% CI: 1.10–4.10), and OR 1.88 (95% CI: 1.22–2.92) for medium and high income respectively. As with insulin resistance, diet, physical activity, sleep duration, and the presence of multiple risk factors were not associated with prediabetes.

Figures 2 and 3 display the results of the prediabetes or IR logistic regression models for strata of education and income, respectively. We see similar patterns to the insulin resistance and prediabetes models. Waist circumference remained a significant predictor of prediabetes or IR. Subjects with a large waist circumference were nearly twice as likely to develop prediabetes or IR in the higher education strata (some college and college+) and among the highest income group ($50,000+). The association with multiple risk factors remained nonsignificant. The odds ratios for large waist circumference are in the same positive direction and of similar magnitude across all education levels with a range of 1.55 to 1.88, where a larger waist circumference nearly doubles the likelihood of prediabetes or IR. However the association in the <HS and HS or GED strata is nonsignificant. The same strength of association with waist circumference is seen between income strata, where the odds ratios range between

Table 3: Association (OR [95% CI]) between diet quality, sleep duration, and physical activity and prediabetes (versus unaffected), by socioeconomic status level.

	Education level Odds ratio [95% CI][A] $N = 2175$*				Income Odds ratio [95% CI][A] $N = 2175$*		
	<HS $N = 323$	HS or equiv. $N = 628$	College or equiv. $N = 472$	Postgrad $N = 752$	<$20,000 $N = 782$	$20,000–$49,999 $N = 654$	$50,000+ $N = 738$
Individual factor							
Model 1[C]: Total diet quality (lower tertile versus upper and middle tertiles)	1.57 [0.29, 8.38]	0.98 [0.40, 2.42]	1.40 [0.63, 3.12]	0.81 [0.46, 1.42]	1.06 [0.45, 2.50]	1.07 [0.54, 2.14]	0.94 [0.55, 1.61]
Model 2: BMI (≥25 versus <25)	2.08 [0.61, 7.06]	1.17 [0.53, 2.62]	1.31 [0.60, 2.87]	1.17 [0.73, 1.88]	1.08 [0.55, 2.13]	1.46 [0.75, 2.83]	1.23 [0.73, 2.07]
Model 3: Waist circumference, cm mean SE (Larger: ≥88 for women, ≥102 for men, versus smaller)	1.31 [0.47, 3.70]	1.74 [0.79, 3.86]	1.90 [0.99, 3.65]	**1.68 [1.07, 2.62]**[B]	0.97 [0.45, 2.10]	**2.13 [1.10, 4.10]**	**1.88 [1.22, 2.92]**
Model 4: Physical activity (low versus med/high)	2.37 [0.70, 8.01]	1.29 [0.62, 2.66]	0.93 [0.44, 1.98]	1.09 [0.62, 1.91]	1.41 [0.67, 2.97]	1.20 [0.57, 2.53]	1.01 [0.59, 1.73]
Model 5: Sleep length/quality (low versus med/high)	2.36 [0.87, 6.40]	1.00 [0.50, 2.00]	1.20 [0.59, 2.43]	1.19 [0.74, 1.91]	1.12 [0.60, 2.10]	0.93 [0.49, 1.77]	1.45 [0.89, 2.35]
Two factors							
Low diet and sleep scores	3.43 [0.49, 24.18]	0.60 [0.19, 1.96]	1.13 [0.36, 3.50]	0.94 [0.36, 2.47]	0.81 [0.23, 2.87]	1.47 [0.48, 4.47]	0.92 [0.37, 2.28]
Three factors							
Low diet and sleep scores, plus low activity	2.83 [0.24, 33.06]	1.64 [0.37, 7.21]	1.06 [0.17, 6.48]	2.80 [0.34, 22.91]	1.43 [0.35, 5.77]	2.75 [0.28, 27.34]	2.19 [0.36, 13.38]
Four factors							
Low diet, sleep, activity, plus high BMI or waist (select the relevant one)	3.33 [0.29, 38.89]	1.58 [0.32, 7.74]	0.89 [0.09, 8.43]	2.02 [0.22, 18.66]	1.33 [0.33, 5.43]	2.33 [0.21, 25.44]	2.33 [0.25, 21.90]

*These analyses exclude 980 subjects with diabetes.

[A]From multivariable models adjusting for age, gender, race/ethnicity, smoking status, and family history of diabetes.

[B]In bold, $P \leq 0.05$.

[C]Dietary models are adjusted for age, gender, race/ethnicity, total caloric intake, and family history of diabetes.

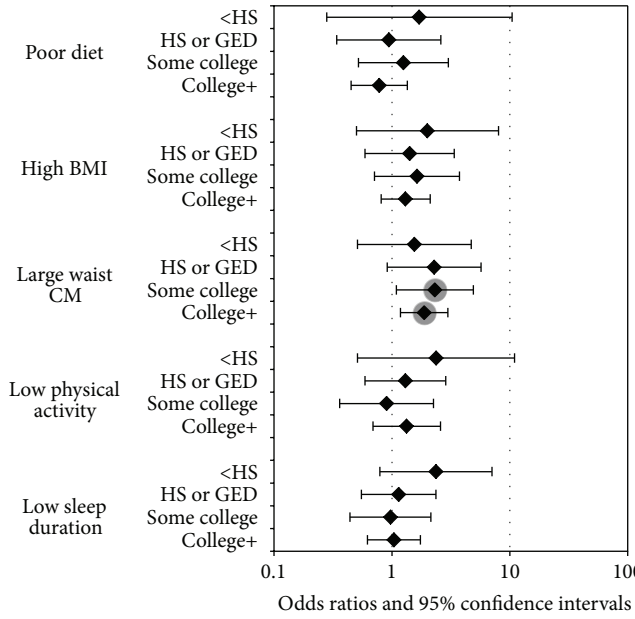

Figure 2: Odds ratio estimates for prediabetes or insulin resistance by education strata.

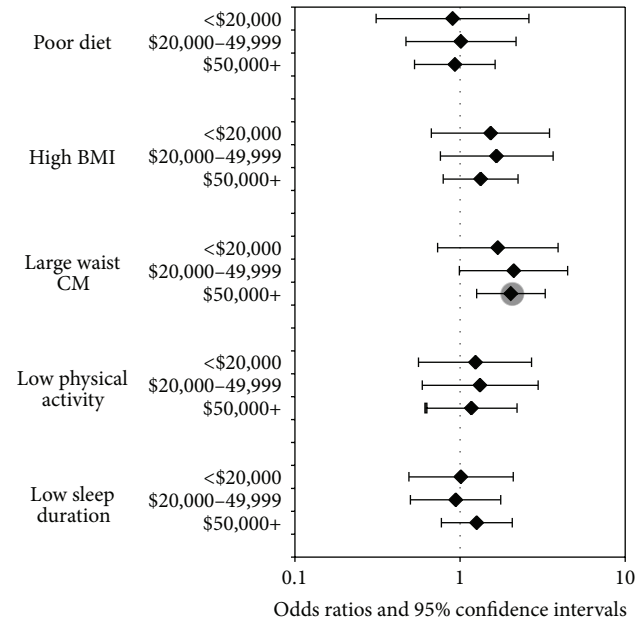

Figure 3: Odds ratio estimates for prediabetes or insulin resistance by income strata.

1.69 and 2.11 and reached significance only within the highest income strata.

4. Conclusions

Our analysis shows that, after adjusting for SES indicators (education and income), commonly known diabetes behavioral risk factors (namely, diet quality, sleep duration, and physical activity) are poor predictors of prediabetes and insulin resistance. Larger waist circumference was a consistent predictor of prediabetes and insulin resistance with large waist circumference increasing the probability of prediabetes or IR nearly twofold. While this magnitude of effect is seen across all levels of SES, it reaches significance primarily within higher SES levels. The examination of multiple risk factors revealed that the presence of two or more risk factors is not associated with prediabetes or IR. While many studies have shown significant associations between diet and diabetes risk [17, 27, 43–45], these results have been mixed. For example, other studies have shown no association between diet quality, specifically total fat intake and red meat are not predictive of diabetes risk [46, 47]. Other studies have shown that BMI

adjustment attenuates the association between diet quality and type 2 diabetes [48–50] which supports our findings of BMI being a significant predictor of insulin resistance across all levels of SES.

The significant findings between BMI and waist circumference indicate that, independent of education and income, increased BMI and waist circumference are important predictors of prediabetes and insulin resistance. The strength of our waist circumference results is corroborated by a study which examined the interrelationships between demographic (age, income, marital, race, and education) and physical activity and poor diet on prediabetes in path models, concluding that large waist circumference had the strongest direct effect on prediabetes [51]. In addition, findings from the NHANES 2009-2010 survey indicated that the significance of sleep disorders on diabetes is attenuated when BMI is added to the model, where the odds ratio for diabetes drops from 2.04 (1.40, 2.95) to 1.38 (0.95, 2.00). They conclude that the effect of sleep disorders on diabetes may be explained through a subject's obesity status [52]. While they controlled for various factors such as age, gender, ethnicity, education, and income, they did not examine other modifiable risk factors (e.g., diet and sleep).

The significant association of waist circumference with both prediabetes and insulin resistance may help guide future primary and secondary prevention programs where, in addition to socioeconomic factors, subjects with high BMI and large waist circumference are likely to produce the most beneficial outcomes. The finding that waist circumference is not predictive across all education levels in prediabetes suggests that there may be different predictors of prediabetes among the lower SES groups providing new opportunities for more targeted intervention programs. On a broader level, our analyses showed no association between known diabetes risk factors and prediabetes. This may be explained by the fact that prediabetes is considered an early precursor state indicating likely eventual development of diabetes, and not the eventual established diabetes state itself, by which time the associated risk factors are more evident and strongly associated. Moreover, while the majority of prediabetic cases may end up being diagnosed with diabetes, not all cases will be; therefore the expected relationship of known diabetes risk factors with prediabetes is diluted.

There are some limitations to this epidemiologic investigation. First, because it is cross-sectional; the temporality of the relationships uncovered remains uncertain. Fasting insulin was also only measured at a single time point and maybe subject to measurement error. The fact that measures are obtained at a single time point may reduce the likelihood of finding significant differences as research has shown that repeated measures of health behaviors may increase the significance and the effect size of such modifiable behaviors [53]. Physical, direct measures such as BMI and waist circumference may serve as a lifetime proxy for diet quality and level of physical activity compared to self-reported measures of health behaviors, taken at a single time point. While the reliability of self-reports of health conditions may be questioned, there is evidence that they are generally well-correlated with medical record review [54–57]. Fortunately, medication data were available and over 80% of those self-reporting diabetes were taking a diabetes-related medication.

Countervailing strengths of this study include use of a community-based random sample and the composition of the sample, which covers a broad age range, inclusion of both genders, and a racial/ethnic diversity, with roughly equal numbers of Blacks, Whites, and Hispanic participants. Inclusion of a broad range of recognized behavioral risk factors permitted assessment of their independent and joint influences on prediabetic states. Finally, we examined a variety and combination of multiple risk factors, sleep duration, diet, and physical activity to test whether targeting multiple behaviors were predictive of prediabetes or IR. We found that, among the behavioral risk factors considered, BMI and waist circumference were consistent predictors of prediabetes outcomes independent of SES. Our results have both clinical and public health significance: many different risk factors (including BMI and waist circumference) are variably associated with diabetes, prediabetes, and IR and offer variably effective opportunities for primary and secondary prevention. By identifying, among the broad range of risk factors, the most promising influences, future primary and secondary prevention initiatives can be more precisely targeted, resulting in more effective and cost efficient outcomes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] Centers for Disease Control and Prevention, Ed., *National Diabetes Fact Sheet: National Estimates and General Information on Diabetes and Prediabetes in the United States*, Centers for Disease Control and Prevention, Atlanta, Ga, USA, 2001.

[2] J. E. Shaw, R. A. Sicree, and P. Z. Zimmet, "Global estimates of the prevalence of diabetes for 2010 and 2030," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 4–14, 2010.

[3] "Awareness of prediabetes—United States, 2005–2010," *Morbidity and Mortality Weekly Report*, vol. 62, no. 11, pp. 209–212, 2013.

[4] J. P. Boyle, T. J. Thompson, E. W. Gregg, L. E. Barker, and D. F. Williamson, "Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence," *Population Health Metrics*, vol. 8, article 29, 2010.

[5] G. N. Ioannou, C. L. Bryson, and E. J. Boyko, "Prevalence and trends of insulin resistance, impaired fasting glucose, and diabetes," *Journal of Diabetes and its Complications*, vol. 21, no. 6, pp. 363–370, 2007.

[6] A. J. Garber, Y. Handelsman, D. Einhorn et al., "Diagnosis and management of prediabetes in the continuum of hyperglycemia—when do the risks of diabetes begin? A consensus statement from the American College of Endocrinology and the American Association of Clinical Endocrinologists," *Endocrine Practice*, vol. 14, no. 7, pp. 933–946, 2008.

[7] D. M. Nathan, M. B. Davidson, R. A. DeFronzo et al., "Impaired fasting glucose and impaired glucose tolerance: implications for care," *Diabetes Care*, vol. 30, no. 3, pp. 753–759, 2007.

[8] L. S. Geiss, C. James, E. W. Gregg, A. Albright, D. F. Williamson, and C. C. Cowie, "Diabetes risk reduction behaviors among U.S. adults with prediabetes," *The American Journal of Preventive Medicine*, vol. 38, no. 4, pp. 403–409, 2010.

[9] "Symptoms," http://www.diabetes.org/diabetes-basics/symptoms/.

[10] National Cholesterol Education Program Expert Panel on Detection Evaluation—Treatment of High Blood Cholesterol in Adults, "Third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report," *Circulation*, vol. 106, pp. 3143–3421, 2002.

[11] R. A. Miech and R. M. Hauser, "Socioeconomic status and health at midlife: a comparison of educational attainment with occupation-based Indicators," *Annals of Epidemiology*, vol. 11, no. 2, pp. 75–84, 2001.

[12] J. Morgan and C. Lininger, "Education and income: comment," *The Quarterly Journal of Economics*, vol. 78, no. 2, pp. 346–347, 1964.

[13] Centers for Disease Control and Prevention, *Physical Activity and Health: Report of the Surgeon General*, Centers for Disease Control and Prevention, Atlanta, Ga, USA, 1996.

[14] American Diabetes Association, J. P. Bantle, J. Wylie-Rosett et al., "Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association," *Diabetes Care*, vol. 31, pp. S61–S78, 2008.

[15] K. Kempf, W. Rathmann, and C. Herder, "Impaired glucose regulation and type 2 diabetes in children and adolescents," *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 6, pp. 427–437, 2008.

[16] S. H. Ley, O. Hamdy, V. Mohan, and F. B. Hu, "Prevention and management of type 2 diabetes: dietary components and nutritional strategies," *Lancet*, vol. 383, no. 9933, pp. 1999–2007, 2014.

[17] K. Esposito and D. Giugliano, "Increased consumption of green leafy vegetables, but not fruit, vegetables or fruit and vegetables combined, is associated with reduced incidence of type 2 diabetes," *Evidence-Based Medicine*, vol. 16, no. 1, pp. 27–28, 2011.

[18] N. T. Ayas, D. P. White, W. K. Al-Delaimy et al., "A prospective study of self-reported sleep duration and incident diabetes in women," *Diabetes Care*, vol. 26, no. 2, pp. 380–384, 2003.

[19] F. P. Cappuccio, L. D'Elia, P. Strazzullo, and M. A. Miller, "Quantity and quality of sleep and incidence of type 2 diabetes: a systematic review and meta-analysis," *Diabetes Care*, vol. 33, no. 2, pp. 414–420, 2010.

[20] J. E. Gangwisch, S. B. Heymsfield, B. Boden-Albala et al., "Sleep duration as a risk factor for diabetes incidence in a large U.S. sample," *Sleep*, vol. 30, no. 12, pp. 1667–1673, 2007.

[21] H. K. Yaggi, A. B. Araujo, and J. B. McKinlay, "Sleep duration as a risk factor for the development of type 2 diabetes," *Diabetes Care*, vol. 29, no. 3, pp. 657–661, 2006.

[22] J. Engeda, B. Mezuk, S. Ratliff, and Y. Ning, "Association between duration and quality of sleep and the risk of prediabetes: evidence from NHANES," *Diabetic Medicine*, vol. 30, no. 6, pp. 676–680, 2013.

[23] C. L. Jackson, S. Redline, I. Kawachi, and F. B. Hu, "Association between sleep duration and diabetes in black and white adults," *Diabetes Care*, vol. 36, pp. 3557–3565, 2013.

[24] Y. Ma, J. R. Hebert, J. E. Manson et al., "Determinants of racial/ethnic disparities in incidence of diabetes in postmenopausal women in the U.S.: the women's health initiative 1993–2009," *Diabetes Care*, vol. 35, pp. 2226–2234, 2012.

[25] Y. Qiao, L. Tinker, B. C. Olendzki et al., "Racial/ethnic disparities in association between dietary quality and incident diabetes in postmenopausal women in the United States: the Women's Health Initiative 1993–2005," *Ethnicity and Health*, vol. 19, pp. 328–347, 2014.

[26] C. C. Cowie and M. S. Eberhardt, "Sociodemographic characteristics of persons with diabetes," in *Diabetes in America*, pp. 85–116, National Institutes of Health, Bethesda, Md, USA, 1995.

[27] A. F. Brown, A. G. Gross, P. R. Gutierrez, L. Jiang, M. F. Shapiro, and C. M. Mangione, "Income-related differences in the use of evidence-based therapies in older persons with diabetes mellitus in for-profit managed care," *Journal of the American Geriatrics Society*, vol. 51, pp. 665–670, 2003.

[28] A. J. Karter, A. Ferrara, J. A. Darbinian, L. M. Ackerson, and J. V. Selby, "Self-monitoring of blood glucose: language and financial barriers in a managed care population with diabetes," *Diabetes Care*, vol. 23, pp. 477–483, 2000.

[29] J. B. McKinlay and C. L. Link, "Measuring the urologic iceberg: design and implementation of the Boston Area Community Health (BACH) Survey," *European Urology*, vol. 52, pp. 389–396, 2007.

[30] R. S. Piccolo, A. B. Araujo, N. Pearce, and J. B. McKinlay, "Cohort profile: the Boston Area Community Health (BACH) survey," *International Journal of Epidemioloy*, vol. 43, pp. 42–51, 2014.

[31] R. A. Washburn, K. W. Smith, A. M. Jette, and C. A. Janney, "The Physical Activity Scale for the Elderly (PASE): development and evaluation," *Journal of Clinical Epidemiology*, vol. 46, pp. 153–162, 1993.

[32] "According to waist circumference," in *Guidelines on Overweight and Obesity: Electronic Textbook*, NHLBI, 1998.

[33] K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy et al., "Harmonizing the metabolic syndrome: a joint interim statement of the international diabetes federation task force on epidemiology and prevention; National heart, lung, and blood institute; American heart association; World heart federation; International atherosclerosis society; And international association for the study of obesity," *Circulation*, vol. 120, no. 16, pp. 1640–1645, 2009.

[34] G. Block, A. M. Hartman, and C. M. Dresser, "A data-based approach to diet questionnaire design and testing," *The American Journal of Epidemiology*, vol. 124, no. 3, pp. 453–469, 1986.

[35] *Dietary Guidelines for Americans 2010*, Government Printing Office, Washington, DC, USA, 2010.

[36] American Heart Association Nutrition Committee, A. H. Lichtenstein, L. J. Appel, and M. Brands, "Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee," *Circulation*, vol. 114, pp. 82–96, 2006.

[37] S. Javaheri, A. Storfer-Isser, C. L. Rosen, and S. Redline, "Association of short and long sleep durations with insulin

sensitivity in adolescents," *Journal of Pediatrics*, vol. 158, no. 4, pp. 617–623, 2011.

[38] K. M. Bullard, S. H. Saydah, G. Imperatore et al., "Secular changes in U.S. prediabetes prevalence defined by hemoglobin A 1c and fasting plasma glucose: National Health and Nutrition Examination Surveys, 1999–2010," *Diabetes Care*, vol. 36, no. 8, pp. 2286–2293, 2013.

[39] A. E. Sumner and C. C. Cowie, "Ethnic differences in the ability of triglyceride levels to identify insulin resistance," *Atherosclerosis*, vol. 196, no. 2, pp. 696–703, 2008.

[40] W. C. Willett, L. Sampson, and M. J. Stampfer, "Reproducibility and validity of a semiquantitative food frequency questionnaire," *The American Journal of Epidemiology*, vol. 122, no. 1, pp. 51–65, 1985.

[41] *SUDAAN User's Manual, Release 11.0 [Package Insert]*, Research Triangle Institute, Research Triangle Park, NC, USA, 2012.

[42] S. van Buuren and K. Groothuis-Oudshoorn, "Mice: multivariate imputation by chained equations in R," *Journal of Statistical Software*, vol. 45, no. 3, 2011.

[43] S. A. McNaughton, D. W. Dunstan, K. Ball, J. Shaw, and D. Crawford, "Dietary quality is associated with diabetes and cardio-metabolic risk factors," *Journal of Nutrition*, vol. 139, no. 4, pp. 734–742, 2009.

[44] P. Carter, L. J. Gray, J. Troughton, K. Khunti, and M. J. Davies, "Fruit and vegetable intake and incidence of type 2 diabetes mellitus: Systematic review and meta-analysis," *British Medical Journal*, vol. 341, Article ID c4229, 2010.

[45] A. M. Hodge, D. R. English, K. O'Dea, and G. G. Giles, "Dietary patterns and diabetes incidence in the Melbourne collaborative cohort study," *The American Journal of Epidemiology*, vol. 165, no. 6, pp. 603–610, 2007.

[46] J. Salmeron, F. B. Hu, J. E. Manson et al., "Dietary fat intake and risk of type 2 diabetes in women," *American Journal of Clinical Nutrition*, vol. 73, pp. 1019–1026, 2001.

[47] D. Aune, G. Ursin, and M. B. Veierød, "Meat consumption and the risk of type 2 diabetes: a systematic review and meta-analysis of cohort studies," *Diabetologia*, vol. 52, no. 11, pp. 2277–2287, 2009.

[48] R. M. van Dam, W. C. Willett, E. B. Rimm, M. J. Stampfer, and F. B. Hu, "Dietary fat and meat intake in relation to risk of type 2 diabetes in men," *Diabetes Care*, vol. 25, no. 3, pp. 417–424, 2002.

[49] R. Villegas, X. O. Shu, Y.-T. Gao et al., "The association of meat intake and the risk of type 2 diabetes may be modified by body weight," *International Journal of Medical Sciences*, vol. 3, no. 4, pp. 152–159, 2006.

[50] A.-H. Harding, N. E. Day, K.-T. Khaw et al., "Dietary fat and the risk of clinical type 2 diabetes: the European prospective investigation of Cancer-Norfolk study," *American Journal of Epidemiology*, vol. 159, no. 1, pp. 73–82, 2004.

[51] B. H. Bardenheier, K. M. Bullard, C. J. Caspersen, Y. J. Cheng, E. W. Gregg, and L. S. Geiss, "Novel use of structural equation models to examine factors associated with prediabetes among adults aged 50 years and older: National Health and Nutrition Examination Survey 2001–2006," *Diabetes Care*, vol. 36, no. 9, pp. 2655–2662, 2013.

[52] J. Liu, J. Hay, and B. E. Faught, "The Association of Sleep Disorder, Obesity Status, and Diabetes Mellitus among US Adults-The NHANES, 2009-2010 survey results," *International Journal of Endocrinology*, vol. 2013, Article ID 234129, 6 pages, 2013.

[53] S. Stringhini, S. Sabia, M. Shipley et al., "Association of socioeconomic position with health behaviors and mortality," *JAMA*, vol. 303, pp. 1159–1166, 2010.

[54] M. M. Bergmann, T. Byers, D. S. Freedman, and A. Mokdad, "Validity of self-reported diagnoses leading to hospitalization: a comparison of self-reports with hospital records in a prospective study of American adults," *American Journal of Epidemiology*, vol. 147, pp. 969–977, 1998.

[55] Y. Okura, L. H. Urban, D. W. Mahoney, S. J. Jacobsen, and R. J. Rodeheffer, "Agreement between self-report questionnaires and medical record data was substantial for diabetes, hypertension, myocardial infarction and stroke but not for heart failure," *Journal of Clinical Epidemiology*, vol. 57, pp. 1096–1103, 2004.

[56] J. L. St Sauver, P. T. Hagen, S. S. Cha et al., "Agreement between patient reports of cardiovascular disease and patient medical records," *Mayo Clinic Proceedings*, vol. 80, pp. 203–210, 2005.

[57] L. M. Martin, M. Leff, N. Calonge, C. Garrett, and D. E. Nelson, "Validation of self-reported chronic conditions and health services in a managed care population," *American Journal of Preventive Medicine*, vol. 18, pp. 215–218, 2000.

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Research Article

Thyroid Autoimmunity in the Context of Type 2 Diabetes Mellitus: Implications for Vitamin D

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Vitamin D deficiency has been associated with both type 2 diabetes mellitus (T2DM) and autoimmune disorders. The association of vitamin D with T2DM and thyroid autoimmunity (TAI) has not been investigated. Thus, we aimed to explore the putative association between T2DM and thyroid autoimmunity (TAI) focusing on the role of 25-hydroxy-vitamin D (25(OH)D). Study population included 264 T2DM patients and 234 controls. To explore the potential association between 25(OH)D and thyroid autoimmunity while controlling for potential confounders—namely, age, gender, body mass index, and presence of T2DM—multivariate logistic regression analyses were undertaken. Patients with T2DM were younger ($P < 0.001$) and had significantly lower 25(OH)D levels ($P < 0.001$) and higher anti-TPO titers ($P = 0.005$). Multivariable logistic regression analyses suggested that T2DM and 25(OH)D levels were significantly associated with the presence of thyroid autoimmunity. In an elderly population of diabetic patients and controls with a high prevalence of vitamin D deficiency/insufficiency, a patient with T2DM was found to be 2.5 times more likely to have thyroid autoimmunity compared to a nondiabetic individual and the higher the serum 25(OH)D levels were, the higher this chance was.

1. Introduction

The association between type 2 diabetes mellitus (T2DM) and the presence of thyroid autoimmunity (TAI) has been a matter of debate. Although earlier reports dismissed any link between them [1–3], there is evidence to suggest an increased prevalence of TAI in patients with T2DM [4, 5]. Considering the discordant results, the direct association between TAI and newly identified hypothyroidism in the context of T2DM [6], and evidence implying a detrimental role for hypothyroidism in insulin sensitivity [7], it could be advocated that the association between T2DM and TAI requires further clarification from both the clinical and research perspectives.

Vitamin D, beyond its pivotal role in the regulation of calcium and phosphate homeostasis, emerges as a potentially important coregulator of both autoimmunity [8, 9] and insulin sensitivity [10, 11]. Vitamin D is considered to promote Th2 over Th1 immune response phenotype [12] acting through the vitamin D receptor- (VDR-) expressing immune cells [12, 13], while influencing both insulin secretion and sensitivity acting at the level of pancreatic beta-cells and muscle cells [10, 14].

In general, vitamin D deficiency or insufficiency is thought to be a predisposing factor to both autoimmune disorders and glucose intolerance. This preliminary evidence might provide the biological background to suggest that vitamin D could be involved in the putative association

between T2DM and TAI; however, such hypothesis has not been addressed in the literature as yet.

To this end, we aimed to explore the association between T2DM and TAI focusing on the role of 25(OH)D in an ethnically homogenous, elderly population, using a cross-sectional, nested within case-control, study design.

2. Materials and Methods

Subjects with an established diagnosis of T2DM were consecutively recruited from the outpatient diabetes clinic of a tertiary reference hospital from December 2011 to March 2012. Community-dwelling individuals from the same region, in whom normal glycaemia was documented, both by fasting glucose (FPG) and glycated haemoglobin (HbA1c), were also recruited as controls during the same period. Controls were recruited from community centres providing social services to senior citizens (KAPI, Open Centres for the Protection of the Elderly). Current use of corticosteroids served as an exclusion criterion, since this could act as a confounder both at the levels of autoimmunity and glucose homeostasis. Subjects under vitamin D supplementation were also excluded from the study. At the day of the recruitment, a structured medical interview and a physical examination were performed in each subject, medical records were retrieved, and blood samples were drawn and stored at −80°C. Informed consent was provided and the study was conducted in accordance with the Declaration of Helsinki. Descriptive characteristics of the study population are summarized in Table 1.

2.1. Measurements. 25-Hydroxy-vitamin D (25(OH)D), glycated haemoglobin (HbA1c), autoantibodies against thyroid peroxidase (TPOab) and thyroglobulin (TGab), and thyroid-stimulating hormone (TSH) were determined for each subject. Radioimmunoassays (DiaSorin, RIA) were performed according to the manufacturer's instructions for the measurements of serum 25(OH)D (intra-assay coefficient of variation (CV): 5.19%, interassay CV: 7.90%, and detection limit: 4 ng/mL) and thyroid parameters (TPOab intra-assay CV: 4.1%, interassay CV: 9.1%, and detection limit: 0.8 U/mL and TGab intra-assay CV: 2.9%, interassay CV: 11.6%, and detection limit: 6 U/mL). HbA1c measurements were performed by a standardized high performance liquid chromatography (HPLC) assay.

2.2. Definitions. A subject was included in the control group (subjects without diabetes) if FPG was less than 7.0 mmol/L (126 mg/dL) and HbA1c less than 48 mmol/mol (6.5%) and no use of diabetes medications was reported. A subject was designated as TAI positive if either TPOab or TGab was higher than 100 U/mL and as vitamin D deficient/insufficient if 25(OH)D was below 75 nmol/L (30 ng/mL). Presence of hypothyroidism was documented by a TSH value greater than 4.0 mU/L or thyroxine treatment.

2.3. Statistical Analysis. Continuous and dichotomous variables were described as mean (standard deviation) or n (%), respectively. Normality assumption was assessed by visual inspection of the distribution as well as the Kolmogorov-Smirnov test. Differences in means were explored using Mann-Whitney and chi-squared tests for continuous and dichotomous variables, respectively. To explore the potential association between 25(OH)D and thyroid autoimmunity while controlling for potential confounders, namely, age, gender, body mass index (BMI), and presence of T2DM, multivariate logistic regression analyses were undertaken in all study populations. Standardized values (z-scores) of the natural logarithms for all continuous variables were used. All analyses were undertaken within Stata 10.0.

3. Results

A total of 498 participants (264 patients with T2DM and 234 healthy controls) constituted the study population. Patients with T2DM were younger and more obese and had significantly lower 25(OH)D and higher TPOab titres compared to controls (Table 1). Prevalence of hypothyroidism was comparable between groups; however the prevalence of thyroid autoimmunity was approximately twofold greater in patients with T2DM compared to controls. Interestingly, the great majority of the study population (78%) was found to be 25(OH)D deficient or insufficient and the prevalence of vitamin D deficiency/insufficiency was significantly higher in patients with T2DM compared to controls. This finding was robust when a lower threshold for 25(OH)D (50 nmol/L (20 ng/mL), 65.5% versus 47% in patients with T2DM and controls, resp., and chi2 P value < 0.001) was applied. 25(OH)D levels by study group and TAI status are presented in Figure 1.

Multivariable logistic regression analyses adjusting for age, gender, and body mass index suggested that both the presence of T2DM (odds ratio (OR): 3.31, 95% confidence interval (CI): 1.58–6.90) and 25(OH)D levels (OR: 1.32, 95% CI: 1.01–1.72) were significantly associated with the presence of TAI (Table 2). Interpreting this finding in clinical terms, each unit increase in 25(OH)D levels (in standard deviations, corresponding to approximately 30 nmol/L (12 ng/mL) in the study population) is associated with a 30% increase (in odds) for TAI. 25(OH)D is depicted in ng/mL.

4. Discussion

In an elderly population of patients with T2DM and controls with a high prevalence of vitamin D deficiency/insufficiency, T2DM and 25(OH)D levels were significantly associated with TAI. The association between 25(OH)D and TAI was noted only in the presence of T2DM and, interestingly, it was found to be positive. Conflicting results have been reported in the literature regarding the association between T2DM and TAI [1–7]. These contradictory findings might either reflect T2DM phenotypic heterogeneity or result from the limited statistical power, multiple confounders, uncontrolled design, and ethnic heterogeneity in individual studies. We hypothesized that this inconsistency might imply a role for vitamin D in light of evidence suggesting its potential link to both thyroid autoimmunity and glucose intolerance

Table 1: Descriptive characteristics of the study population.

	Normal	Type 2 diabetes mellitus	*P*
N	234	264	
Male gender (female)	89 (38)	109 (41)	NS
Age (years)	72.2 (6.5)	67.6 (9.7)	0.0001
Body mass index (kg/m^2)	30.6 (4.9)	31.6 (5.7)	0.032
Type 2 diabetes mellitus duration (years)	N/A	10.0 (8.4)	
Glycated haemoglobin (%)	4.7 (0.5)	7.1 (1.5)	0.0001
25-Hydroxy-vitamin D (ng/mL)	22.6 (12.6)	16.5 (10.4)	0.0001
Presence of vitamin D deficiency/insufficiency	172 (73.5%)	215 (81.4)	0.04
Thyroid peroxidase Ab (IU/mL)	60 (156)	90 (200)	0.005
Thyroglobulin Ab (IU/mL)	44 (131)	54 (136)	NS
Thyroid autoimmunity	18 (7.7)	38 (14.4)	0.018
Thyroid stimulating hormone (μIU/mL)	1.95 (1.60)	2.25 (3.64)	NS
Hypothyroidism	8 (3.4)	11 (4.2)	NS

Data presented as mean (standard deviation) or *N* (%). *P* values refer to Mann-Whitney test or Pearson chi-square. NS: nonsignificant at the level of 0.05. Abs: autoantibodies.

Table 2: Multivariable logistic regression using thyroid autoimmunity as the dependent variable.

Covariates	Odds ratio	95% CI	*P*
Type 2 diabetes mellitus	3.31	1.58–6.90	0.001
Gender	0.64	0.35–1.18	NS
Age	0.97	0.26–3.55	NS
Body mass index	1.24	0.89–1.72	NS
25-Hydroxy-vitamin D	1.32	1.01–1.72	0.047

CI: confidence interval.

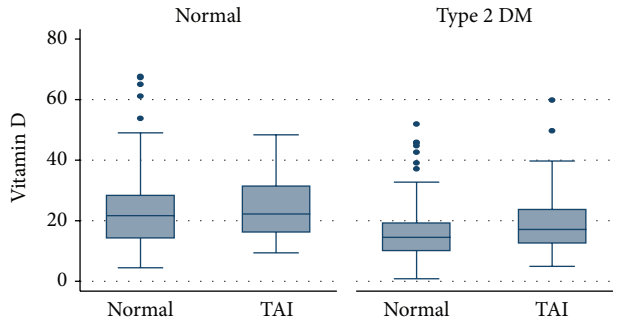

Figure 1: Box-plot representing serum 25-hydroxy-vitamin D levels by study group and thyroid autoimmunity status.

individually. In specific, it has been reported that allelic variations within the VDR gene may mediate susceptibility to thyroid autoimmunity [15, 16]. Vitamin D insufficiency has been associated with thyroid autoimmunity [17], serum 25(OH)D levels were found to be significantly lower in patients with thyroid autoimmunity compared to controls [18, 19], and the severity of 25(OH)D deficiency correlated with thyroid antibody levels in both adult [18, 19] and children populations [20]. However, VDR knockout mice did not differ in parameters of thyrocyte function and morphology compared to wild-type controls [21], the prevalence of thyroid autoimmunity did not differ between subjects stratified on the basis of 25(OH)D levels [22], and vitamin D status was not associated with thyroid autoimmunity after controlling for sex and age [23]. Furthermore, euthyroid subjects with documented genetic susceptibility for developing thyroid autoimmunity did not differ in 25(OH)D levels compared to controls at baseline, which was also the case in those who finally developed de novo TPOAb compared to controls, questioning the role of vitamin D at least during the early stages of thyroid autoimmunity [24].

Similarly, a higher prevalence of vitamin D deficiency has been reported in the presence of T2DM [25] and an inverse association between circulating 25(OH)D levels and risk of incident T2DM was documented (each 10 ng/mL increment in 25(OH)D levels was associated with a 4% lower risk of T2DM) [26]. It should be noted though that this association has been recently questioned when taking into account genetic determinants [27] and that vitamin D3 supplementation had no beneficial effect on glycaemic indices in healthy overweight or obese women [28]. Collectively, it could be extrapolated that these data suggest that any role vitamin D might have in the putative association between thyroid autoimmunity and T2DM would be complex and certainly not self-evident. In fact, our findings seem to corroborate this notion. First, the association between thyroid autoimmunity and T2DM was documented adjusting for the major confounders; a subject with T2DM is 2.5 more likely (in odds) to have thyroid autoimmunity compared to a normal individual. Second, vitamin D levels are significantly associated with the presence of thyroid autoimmunity in subjects with T2DM, but not in controls. Third, it was shown that the higher the serum 25(OH)D levels are in these patients, the higher the chance was for thyroid autoimmunity. The latter finding is counterintuitive and of borderline statistical significance; therefore it requires further confirmation in subsequent studies. Of note, when 25(OH)D levels were modeled as a categorical variable, this association was not robust and no threshold effect was documented. Patients

with T2DM have statistically significant higher percentage of thyroid autoimmunity compared to controls (Table 1). Of note, both groups have a high prevalence of vitamin D deficiency/insufficiency and the difference between the two groups is marginally statistically significant. On the other hand, it was found that TAI is more prevalent when the levels of vitamin D are higher. Vitamin D is one among many factors that could potentially modify thyroid autoimmunity. This discrepant finding should be attributed to the confounding effect of age, BMI, and gender, namely, all variables used in the multivariable regression analysis. This is why we undertook a regression analysis, adjusting for potential confounders. Any inferences were based on the findings of the latter analysis. In this model of regression analysis, both vitamin D levels and T2DM were included as covariates although associated. However, the degree of correlation between T2DM and 25(OH)D levels in our sample was not as high as to undermine the analysis. In any case, the theoretical risk of multicollinearity overinflates standard errors. Thus, there would be a risk of an insignificant finding. This was not the case in our study with regard to vitamin D.

Since the complex interplay between thyroid autoimmunity and vitamin D in the context of T2DM has not been explored to date, it might be premature to suggest an alternative hypothesis before further confirmation nor could it be substantiated on the basis of our data or study design. However, it might be intriguing to hypothesize that the effects noted in our study might be compatible with a tissue-specific model in the action of vitamin D on autoimmunity. Possible immunomodulatory effects of vitamin D include inhibition of proinflammatory activity of CD4+ Th1 cells and their production of cytokines (interleukin 2 (IL-2), interferon-(IFN-) γ, and tumor necrosis factor-α [29]).

In addition to its anti-inflammatory effects, vitamin D also promotes Th2 responses by enhancing IL-4, IL-5, and IL-10 production, thus promoting a more anti-inflammatory phenotype (Th2 state) of the T cell compartment over the inflammatory Th1 state [12]. On the other hand, the formation of thyroid autoantibodies by thyroid-infiltrating lymphocytes and blood lymphocytes has been found to be a distinct procedure. Although the intrathyroidal process is characterized by a shift towards Th1, the stimulus inducing the formation of thyroid autoantibodies by blood lymphocytes commonly shifts the balance towards Th2. Therefore, immune deviation towards Th2 has long been questioned as appropriate therapy for autoimmune thyroid disorders and has even been suggested that it "could project the patient from the frying pan into the fire" [30]. It is obvious that the above hypothesis requires further experimental confirmation.

Considering the observational nature of the evidence and, thus, the inappropriateness for causality inference, caution is advisable in the interpretation of the findings. Notably, the study findings should not be extrapolated to different populations with different baseline characteristics. Our sample demonstrated significant prevalence of vitamin D deficiency and a higher prevalence of thyroid autoimmunity in patients with T2DM compared to controls. Both of these sample characteristics are consistent with previous reports [31, 32], thus reassuring external validity concerns. It should also be noted that oral glucose tolerance test was not performed for the diagnosis of diabetes mellitus and, thus, a marginal misclassification of patients with diabetes as controls could not be excluded.

However, the impact of limitation on the study findings is probably minimal, since the discrimination between the diabetic and control groups was performed on the basis of two glycemic indices (fasting glucose and HbA1c measurements), which secured a clear distinction between groups. Similarly, the potential confounding effect of seasonal variation in serum 25(OH)D levels [33] is also expected to be minimal, since the collection of blood samples was performed during the same season (winter).

5. Conclusions

In summary, in an elderly population of patients with T2DM and controls with a high prevalence of vitamin D deficiency/insufficiency, it was shown that T2DM and vitamin D were associated with TAI. A patient with T2DM was 2.5 times more likely (in odds) to have thyroid autoimmunity compared to a normal individual and the higher the serum 25(OH)D levels were, the higher the chance for thyroid autoimmunity was.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] C. Cardoso, A. E. Ohwovoriole, and S. F. KuKu, "A study of thyroid function and prevalence of thyroid autoantibodies in an African diabetic population," *Journal of Diabetes and its Complications*, vol. 9, no. 1, pp. 37–41, 1995.

[2] S. Kasim and A. Bessman, "Thyroid autoimmunity in Type 2 (non-insulin-dependent) diabetic patients of Caucasoid, black and Mexican origin," *Diabetologia*, vol. 27, no. 1, pp. 59–61, 1984.

[3] A.-R. M. Radaideh, M. K. Nusier, F. L. Amari et al., "Thyroid dysfunction in patients with type 2 diabetes mellitus in Jordan," *Saudi Medical Journal*, vol. 25, no. 8, pp. 1046–1050, 2004.

[4] Z. Schroner, I. Lazurova, and J. Petrovicova, "Autoimmune thyroid diseases in patients with diabetes mellitus," *Bratislavské Lekárske Listy*, vol. 109, no. 3, pp. 125–129, 2008.

[5] T. Yasmin, F. Ghafoor, T. Malik, R. Naz S, and A. U. Khan, "Pattern of thyroid autoimmunity in type 1 and type 2 diabetics," *Journal of the College of Physicians and Surgeons Pakistan*, vol. 16, no. 12, pp. 751–754, 2006.

[6] J. J. Díez and P. Iglesias, "An analysis of the relative risk for hypothyroidism in patients with Type2 diabetes," *Diabetic Medicine*, vol. 29, no. 12, pp. 1510–1514, 2012.

[7] L. H. Duntas, J. Orgiazzi, and G. Brabant, "The interface between thyroid and diabetes mellitus," *Clinical Endocrinology*, vol. 75, no. 1, pp. 1–9, 2011.

[8] N. Agmon-Levin, M. Blank, G. Zandman-Goddard et al., "Vitamin D: an instrumental factor in the anti-phospholipid syndrome by inhibition of tissue factor expression," *Annals of the Rheumatic Diseases*, vol. 70, no. 1, pp. 145–150, 2011.

[9] Y. Arnson, H. Amital, N. Agmon-Levin et al., "Serum 25-OH vitamin D concentrations are linked with various clinical aspects in patients with systemic sclerosis: a retrospective cohort study and review of the literature," *Autoimmunity Reviews*, vol. 10, no. 8, pp. 490–494, 2011.

[10] C. M. Girgis, R. J. Clifton-Bligh, M. W. Hamrick, M. F. Holick, and J. E. Gunton, "The roles of vitamin D in skeletal muscle: form, function, and metabolism," *Endocrine Reviews*, vol. 34, no. 1, pp. 33–83, 2013.

[11] J.-C. Souberbielle, J.-J. Body, J. M. Lappe et al., "Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendations for clinical practice," *Autoimmunity Reviews*, vol. 9, no. 11, pp. 709–715, 2010.

[12] A. Boonstra, F. J. Barrat, C. Crain, V. L. Heath, H. F. J. Savelkoul, and A. O'Garra, "1α,25-Dihydroxyvitamin D3 has a direct effect on naive $CD4^+$T cells to enhance the development of Th2 cells," *The Journal of Immunology*, vol. 167, no. 9, pp. 4974–4980, 2001.

[13] G. Penna and L. Adorini, "1α,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation," *Journal of Immunology*, vol. 164, no. 5, pp. 2405–2411, 2000.

[14] T. Takiishi, C. Gysemans, R. Bouillon, and C. Mathieu, "Vitamin D and diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 2, pp. 419–446, 2010.

[15] W. Horst-Sikorska, M. Ignaszak-Szczepaniak, M. Marcinkowska, M. Kaczmarek, M. Stajgis, and R. Slomski, "Association analysis of vitamin D receptor gene polymorphisms with bone mineral density in young women with Graves' disease," *Acta Biochimica Polonica*, vol. 55, no. 2, pp. 371–380, 2008.

[16] M. Stefanic, S. Papic, M. Suver, L. Glavas-Obrovac, and I. Karner, "Association of vitamin D receptor gene 3′-variants with Hashimoto's thyroiditis in the Croatian population," *International Journal of Immunogenetics*, vol. 35, no. 2, pp. 125–131, 2008.

[17] G. Tamer, S. Arik, I. Tamer, and D. Coksert, "Relative vitamin D insufficiency in Hashimoto's thyroiditis," *Thyroid*, vol. 21, no. 8, pp. 891–896, 2011.

[18] N. C. Bozkurt, B. Karbek, B. Ucan et al., "The association between severity of vitamin D deficiency and Hashimoto's thyroiditis," *Endocrine Practice*, vol. 19, no. 3, pp. 479–484, 2013.

[19] S. Kivity, N. Agmon-Levin, M. Zisappl et al., "Vitamin D and autoimmune thyroid diseases," *Cellular & Molecular Immunology*, vol. 8, no. 3, pp. 243–247, 2011.

[20] O. M. Çamurdan, E. Döğer, A. Bideci, N. Çelik, and P. Cinaz, "Vitamin D status in children with Hashimoto thyroiditis," *Journal of Pediatric Endocrinology and Metabolism*, vol. 25, no. 5-6, pp. 467–470, 2012.

[21] I. Clinckspoor, A. C. Gérard, J. van Sande et al., "The vitamin D receptor in thyroid development and function," *European Thyroid Journal*, vol. 1, no. 3, pp. 168–175, 2012.

[22] R. Goswami, R. K. Marwaha, N. Gupta et al., "Prevalence of vitamin D deficiency and its relationship with thyroid autoimmunity in Asian Indians: a community-based survey," *British Journal of Nutrition*, vol. 102, no. 3, pp. 382–386, 2009.

[23] L. O. Chailurkit, W. Aekplakorn, and B. Ongphiphadhanakul, "High vitamin D status in younger individuals is associated with low circulatingthyrotropin," *Thyroid*, vol. 23, no. 1, pp. 25–30, 2013.

[24] G. Effraimidis, K. Badenhoop, J. G. P. Tijssen, and W. M. Wiersinga, "Vitamin D deficiency is not associated with early stages of thyroid autoimmunity," *European Journal of Endocrinology*, vol. 167, no. 1, pp. 43–48, 2012.

[25] T. L. van Belle, C. Gysemans, and C. Mathieu, "Vitamin D and diabetes: the odd couple," *Trends in Endocrinology and Metabolism*, vol. 24, no. 11, pp. 561–568, 2013.

[26] Y. Song, L. Wang, A. G. Pittas et al., "Blood 25-hydroxy vitamin D levels and incident type 2 diabetes: a meta-analysis of prospective studies," *Diabetes Care*, vol. 36, no. 5, pp. 1422–1428, 2013.

[27] B. Buijsse, H. Boeing, F. Hirche et al., "Plasma 25-hydroxyvitamin D and its genetic determinants in relation to incident type 2 diabetes: a prospective case-cohort study," *European Journal of Epidemiology*, vol. 28, no. 9, pp. 743–752, 2013.

[28] A. Salehpour, F. Shidfar, F. Hosseinpanah, M. Vafa, M. Razaghi, and F. Amiri, "Does vitamin D3 supplementation improve glucose homeostasis in overweight or obese women? A double-blind, randomized, placebo-controlled clinical trial," *Diabetic Medicine*, vol. 30, no. 12, pp. 1477–1481, 2013.

[29] M. Cippitelli and A. Santoni, "Vitamin D3: a transcriptional modulator of the interferon-gamma gene," *European Journal of Immunology*, vol. 28, no. 10, pp. 3017–3030, 1998.

[30] J. Guo, B. Rapoport, and S. M. McLachlan, "Balance of Th1/Th2 cytokines in thyroid autoantibody synthesis *in vitro*," *Autoimmunity*, vol. 30, no. 1, pp. 1–9, 1999.

[31] A. Tsagari, K. A. Toulis, P. Makras, K. Skagias, A. Galanos, and G. Lyritis, "Performance of the mini nutritional assessment score in the detection of vitamin D status in an elderly Greek population," *Hormone and Metabolic Research*, vol. 44, no. 12, pp. 896–899, 2012.

[32] K. Vondra, J. Vrbikova, and K. Dvorakova, "Thyroid gland diseases in adult patients with diabetes mellitus," *Minerva Endocrinologica*, vol. 30, no. 4, pp. 217–236, 2005.

[33] V. Carnevale, S. Modoni, M. Pileri et al., "Longitudinal evaluation of vitamin D status in healthy subjects from southern Italy: seasonal and gender differences," *Osteoporosis International*, vol. 12, no. 12, pp. 1026–1030, 2001.

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Research Article

Evidence of Insulin Resistance and Other Metabolic Alterations in Boys with Duchenne or Becker Muscular Dystrophy

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Aim. Our aim was (1) to determine the frequency of insulin resistance (IR) in patients with Duchenne/Becker muscular dystrophy (DMD/BMD), (2) to identify deleted exons of DMD gene associated with obesity and IR, and (3) to explore some likely molecular mechanisms leading to IR. *Materials and Methods*. In 66 patients with DMD/BMD without corticosteroids treatment, IR, obesity, and body fat mass were evaluated. Molecules involved in glucose metabolism were analyzed in muscle biopsies. Results show that 18.3%, 22.7%, and 68% were underweight, overweight, or obese, and with high adiposity, respectively; 48.5% and 36.4% presented hyperinsulinemia and IR, respectively. Underweight patients (27.3%) exhibited hyperinsulinemia and IR. Carriers of deletions in exons 45 (OR = 9.32; 95% CI = 1.16–74.69) and 50 (OR = 8.73; 95% CI = 1.17–65.10) from DMD gene presented higher risk for IR than noncarriers. We observed a greater staining of cytoplasmic aggregates for GLUT4 in muscle biopsies than healthy muscle tissue. *Conclusion*. Obesity, hyperinsulinemia, and IR were observed in DMD/BMD patients and are independent of corticosteroids treatment. Carriers of deletion in exons 45 or 50 from DMD gene are at risk for developing IR. It is suggested that alteration in GLUT4 in muscle fibers from DMD patients could be involved in IR.

1. Introduction

Duchenne muscular dystrophy (DMD) is a recessive X-chromosome-linked disease that affects ~1/3600–6000 live-born males. DMD usually presents in early childhood with generalized motor delays and gait difficulties. The muscular weakness is progressive, causing loss of ambulation by early adolescence (between 9 and 12 years of age) [1]. DMD is caused by mutations in the *DMD* gene that code for dystrophin, a sarcolemmal cytoskeletal protein [2–4]. *DMD* gene mutations that result in complete loss of dystrophin interrupt their translation, giving rise to DMD. Mutations that conserve the open reading frame produce reduced quantities of dystrophin or a dysfunctional or truncated form of dystrophy, resulting in Becker muscular dystrophy (BMD), a less severe phenotype [5].

Dystrophin is an element of the dystrophin-glycoprotein complex (DGC) that provides a mechanical link between the

extracellular matrix and cytoskeleton of muscle cells, allowing the plasma membrane of the muscle fiber to resist the mechanical process of the muscle during muscle contraction [6, 7]. It has been suggested that the DGC also participates in important cell-signalling processes, functioning as a binding platform for certain ligands [8] such as neuronal nitric acid synthase, which stimulates glucose transport [9]. Skeletal muscle is responsible for >80% of insulin-stimulated glucose uptake in the body [10]. Therefore, destabilization in DGC or their components may generate abnormal signalling of insulin in muscle fibers and lead to alterations in functionality. In fact, it was reported that the disturbance within the surface DGC may contribute to insulin resistance (IR) and abnormalities characteristic of the skeletal muscle of diabetic Goto-Kakizaki rats, because an abnormal subcellular location of glucose transporter 4 (GLUT4) vesicles in muscle fiber was observed in these rats [11]. However, information on this regard from human studies is lacking. This information led us to propose that DGC alterations may generate changes in glucose metabolism such as IR in skeletal muscle of patients with DMD or BMD.

DGC modifications provoke an imbalance in plasma membranes permeability, inducing myofibers through deterioration-regeneration cycles until exhausting their repair capacity [12]. This poses muscle fibers susceptible to necrotic development and to be replaced by both fibrous connective tissue and adipose tissue [1, 13], increasing adiposity. Previous body composition studies have showed that DMD patients present greater body fat mass than similarly aged healthy subjects [13, 14]. In addition, other investigators have observed that patients with DMD develop overweight or obesity from the age of 7, reaching a frequency of >50% at 13 years of age [15].

In addition to the alteration of DGC, overweight and obesity are also risk for IR, which increases the risk for other severe morbidities such as cardiovascular disease and type 2 diabetes in DMD patients [16]. However, the concomitant effect of alterations in DGC and obesity on the risk for insulin resistance has not been properly addressed. Thus, the purposes of this investigation were to determine the frequency of IR in patients with DMD/BMD and to evaluate the association of deletions in specific regions of the *DMD* gene with obesity and IR, as well as exploring molecular mechanisms likely leading to IR, by evaluating molecules involved in glucose metabolism such as insulin receptor, insulin receptor substrate, and GLUT4 localization in muscle biopsies of DMD/BMD patients.

2. Subjects and Methods

2.1. Patients. The Institutional (Instituto Mexicano del Seguro Social) Ethics Committee approved the study prior to the start of patient recruitment. All DMD/BMD patients seen at the outpatient Electrodiagnostic Muscular Dystrophy Service at the National Institute of Rehabilitation were recruited for the cross-sectional study between January 2011 and December 2013; 117 patients (aged 4 years to <18 years) were included. Subjects with a clinical diagnosis of DMD/BMD were candidates to participate in the study, and confirmatory molecular diagnosis of dystrophy was carried out. Patients were eligible for inclusion in this study if they had a deletion in the *DMD* gene analyzed by multiplex polymerase chain reaction (MPCR). Children were not included if they received corticosteroids. None of the patients was taking medications during the study.

Parents and patients received an explanation of study fundamentals, procedures, benefits, right to confidentiality, and the right to withdraw from the study if they wished. All parents provided written informed consent in adherence with the human subjects' guidelines of the Institutional Ethics Committee.

On the day of the study, a peripheral blood sample was collected in vacutainer with and without anticoagulant in a fasting state for genomic DNA extraction from leukocytes to determine glucose and insulin, respectively. Serum samples were kept at −70°C until analysis. A medical history was obtained, and weight and height were measured. Body composition to measure adiposity was evaluated by dual-energy X-ray absorptiometry (DEXA).

2.2. Anthropometric Measurements. Trained personnel carried out measurements of body weight (kg) and height (mts). For subjects who were able to stand erect, height was measured with a wall-mounted stadiometer (Model 208, Seca). For subjects unable to stand erect, length was measured on a flat table with the subject supine. Subjects' weight was measured (Model BWB-700, Tanita, for ambulatory patients and model 954 Seca for wheelchair-bound patients) wearing light clothing and without shoes.

Diagnosis of overweight and obesity was obtained using body mass index (BMI), expressed as percentiles. Children with BMI ≤ 5th percentile were classified as underweight, those with BMI > 5th but <85th percentile as normal weight, those with BMI ≥ 85th but <95th percentile as overweight, and those with BMI ≥ 95th percentile as obese, in accordance with criteria established by the Centers for Disease Control and Prevention (CDC) in 2009 about BMI for children and teens (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html).

2.3. Body Composition. Body composition measurements were carried out by DEXA (Lunar Prodigy, GE Medical Systems, Madison, WI) and enCore software, v. 2004 (Lunar Corporation), was used to analyze whole-body DEXA scans. Body fat mass was considered high according to the classification as described previously [17] that used DEXA to predict % body fat mass corresponding to the BMI cutoff in male children and adolescents (3–18 years) as overweight (range: 18%–23%) and obesity (range: 24–36%).

2.4. Molecular Diagnosis of DMD. DNA was extracted from peripheral blood samples according to standard procedures [18]. One hundred seventeen patients were screened for deletions and duplications by MPCR with the primer sets of Chamberlain et al. [19] and Beggs et al. [20] using the MPCR kit for Human DMD/BMD set I + II (Maxim Biotech, San

Francisco, CA) according to the protocol recommended by the manufacturer.

2.5. Biochemical Assays. Serum insulin (μU/mL) was quantified utilizing a commercial kit (Linco Research, St. Louis, MO) based on radioimmunoanalysis. *A* value >12 μU/mL was considered as hyperinsulinemia as was previously reported [21]. Serum glucose (mg/dL) concentration was measured by the glucose-oxidase method (Glucose-LQ, Spin-React, S.A., Girona, Spain). IR was calculated from insulin and glucose data using the homeostasis model assessment-insulin resistance (HOMA-IR) method (HOMA-IR = [fasting insulin, μU/mL] $*$ [fasting glucose, mmol/L])/22.5 [22]. Values of HOMA-IR > 3.16 [23] were considered as IR.

2.6. Cellular Localization of Dystrophin, Insulin Receptor, Insulin Receptor Substrate, and GLUT4 Using Immunofluorescence Analysis

2.6.1. Source of Human Muscle. Muscle biopsies were obtained surgically from deltoid or biceps muscle for cytological diagnosis. A portion was used for immunofluorescence analysis. In this study, we had availability for tissues from only five patients with DMD/BMD of the entire population studied. Patient ages ranged from 8 to 11 years. We also used five biopsies of gastrocnemius muscle from five healthy individuals as controls (age 44–55 years) to compare cellular localization of dystrophin, insulin receptor, insulin receptor substrate, and GLUT4. We analyzed skeletal muscle biopsy from older controls because we have not access to biopsies of muscle from healthy subjects of the same age [13]. Nevertheless, older controls allowed the main purpose to evaluate the cellular localization of these molecules in human healthy muscle.

Specific mouse monoclonal primary antibodies used were GLUT4, insulin receptor (IRe) (Santa Cruz Biotechnology, Santa Cruz, CA), and dystrophin N-terminus (Dys-N) and C-terminus (Dys-C) (Vector Laboratories, Burlingame, CA). Polyclonal antibody against insulin receptor substrate (IRS) (Santa Cruz Biotechnology) was also used.

2.6.2. Immunofluorescence Assay. Skeletal muscle biopsy was isolated and rapidly frozen in liquid nitrogen-cooled isopentane. Afterwards, 7 μm cryosections were prepared and added to the coverslip covered with poly-L-lysine.

Next, nonspecific binding was blocked by incubations of the cryosections with 5% bovine serum albumin in phosphate-buffered solution (PBS) for 60 min at 25°C. Tissues were then washed with PBS. Sections were incubated overnight at 4°C with the primary antibodies. Sections were then washed with PBS. Samples were incubated with Cy3-secondary antibody (goat anti-mouse, Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min at 25°C in the dark. Subsequently, samples were washed with PBS and mounted with DAPI (labeling of nuclei) and Vectashield (Vector Laboratories). Negative controls omitting the primary antibody were included. Tissues were observed under an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan). Five areas of 1,443,520 mm^3 were analyzed in each section. A subjective value was assigned (normal + + +, decreased + + −, considerably decreased + − −, and absent − − −) to describe staining, indicating the presence of dystrophin.

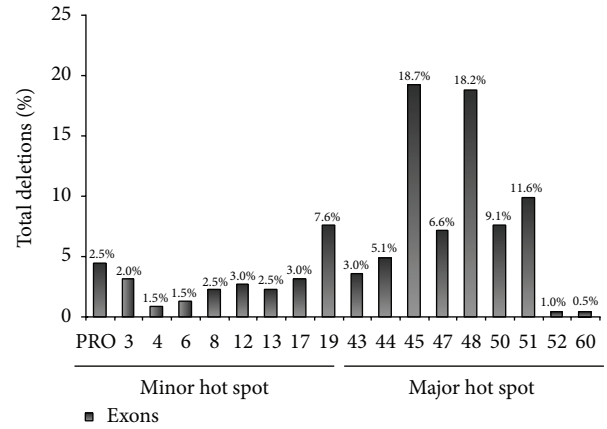

Figure 1: Distribution of the mutations detected in the *DMD* gene in patients with Duchenne muscular dystrophy/Becker muscular dystrophy (DMD/BMD).

2.7. Statistical Analysis. Statistical analysis was performed using the Minitab statistical software (Minitab 14, State College, PA). Results are presented as median (minimum, maximum); *P* value ≤0.05 was considered significant.

Pearson correlation analyses were used to evaluate associations between the most frequently deleted exons and fasting insulin, HOMA-IR, BMI, and body composition. Comparisons among nutritional status groups were conducted with one-way ANOVA and Dunnett's method as post hoc test, considering the normal nutritional status group as control. Associations between IR and nutritional status were analyzed with χ^2 analyses. Logistic regression models were carried out to identify risk factors for IR, introducing each exon deletion as a predictor and taking into account adiposity.

3. Results

3.1. Study Population. We are reporting the results of 66 subjects identified as carriers in at least one exon of the two deletion-prone regions in the *DMD* gene. Most alterations (73.7%) were clustered in exons 43–60 in the major *hot spot*. Distribution of the incriminated mutations is presented in Figure 1. Deletions of exons 19, 45, 47, 48, 50, and 51 were more frequently detected.

3.2. Anthropometric and Metabolic Parameters. Patient ages were 8.96 (4.61, 17.75) years, median (minimum, maximum), and 53% of the population were between 7 and 10 years of age. A low percentage of patients (9.1%) were between 15 and 18 years of age. Fasting insulin concentration ranged from 5.2 to 59.9 μU/mL, but ~50% present hyperinsulinemia. HOMA-IR values range from 1.04 to 12.9, and 36% of the patients had IR (Table 1). Most boys presented normal nutritional

Table 1: Anthropometric and metabolic parameters in the study sample of DMD/BMD patients ($n = 66$).

	Median	Minimum, maximum
Age (years)	8.96	4.61, 17.75
Body weight (kg)	25.8	12.40, 79.35
Height (mts)	1.23	0.97, 1.75
BMI (kg/m^2)	16.05	10.40, 29.50
Percentile	47.5	0.0, 99.74
Body fat (%)	26.2	7.62, 60.0
Body fat mass (kg)	6.37	0.88, 46.08
Fat-free mass (kg)	16.89	10.69, 34.99
Glucose (mg/dL)	91.8	72, 135
Insulin (μU/mL)	11.75	5.2, 59.9
>12 (μU/mL)	48.5%	
HOMA-IR	2.6	1.04, 12.91
IR > 3.16	36.4%	
Loss of ambulation	18	

DMD/BMD: Duchene/Becker muscular dystrophy; BMI: body mass index; IR: insulin resistance; HOMA-IR: homeostasis model assessment-insulin resistance.

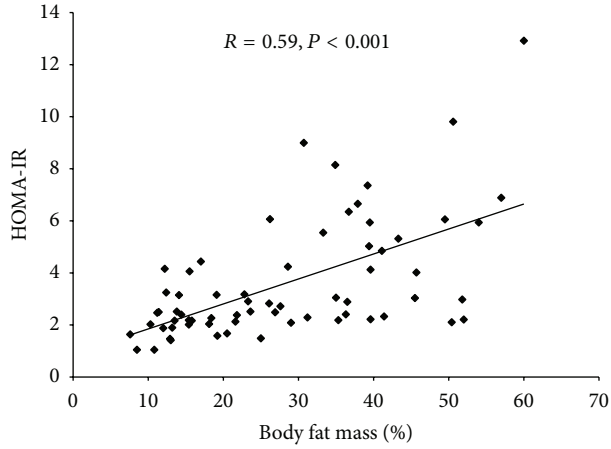

Figure 2: Scatter plot of body fat mass and HOMA-IR in DMD/BMD patients. HOMA-IR, homeostasis model assessment-insulin resistance.

status (59%), but 22.7% were overweight/obese and 18.2% were underweight. According to the classification of Taylor et al. (see Subjects and Methods), a higher (68%) prevalence of overweight/obesity was observed in contrast to using BMI (22.7%). Thus, we show group data according to nutritional status from DMD/BMD patients as underweight, normal, and overweight/obese (Table 2).

3.3. Associations among IR and BMI, Body Composition, and Deletions in DMD Gene. We observed significant differences in insulin concentrations and HOMA-IR values among nutritional status groups, but no differences in age were detected. Overweight/obese boys presented higher glucose, insulin, and HOMA-IR values as compared to normal BMI group. That group exhibited also a higher proportion (80%) of subjects with IR determined by HOMA-IR > 3.16 (χ^2: 11.48, $P = 0.004$) compared to normal nutritional status. Interestingly, an important percentage of underweight patients presented hyperinsulinemia and IR (27.3%). Body fat mass was higher in overweight/obese and lower in underweight than in patients with normal nutritional status (Table 2). Both fasting insulin and HOMA-IR were positively correlated with all anthropometric and body composition parameters. However, the highest correlation was found between HOMA-IR and body fat mass (Figure 2).

Carriers of deletions in exon 45 (OR = 9.32; 95% CI 1.16–74.69; $P = 0.036$) or exon 50 (OR = 8.73; $CI_{95} = 1.17$–65.10; $P = 0.035$) were associated with the risk for IR, even adjusting for adiposity (Figure 3).

3.4. Cellular Localization of Molecules Involved in Glucose Metabolism. The staining of GLUT4 shows its subcellular localization in myofiber sections from muscle biopsies. Staining of GLUT4 vesicles was observed as cytoplasmic aggregates in biopsies from patients 3, 5, and 7 (Figure 4), although in patients 3 and 7 there are a smaller number of these aggregates; the three patients presented hyperinsulinemia and IR (Table 3) and patient 5 showed hyperglycemia. Aggregates were not observed in control tissue. Biopsies of remaining patients showed staining for GLUT4 similar to control tissues. Staining for IRe and IRS was similar to control tissue muscle in myofiber sections from five patients. Dystrophin staining intensities in myofibers from five patients (Figure 3) were lower than those observed in normal biopsy specimens.

4. Discussion

Hyperinsulinemia and IR observed in our patients are important risk factors for developing pathologies such as cardiovascular disease or type 2 diabetes. The frequency of these metabolic alterations increases (80%) importantly when DMD/BMD patients are overweight/obese, with a rate higher than in nondystrophic obese boys reported in our country (~50% in children between 3 and 18 years) [24]. The increased intramuscular body fat mass deposition [25] in DMD patients and hyperinsulinemia and IR are strong indicators of muscle metabolic defects [26]; this deposition is present in DGC-related muscular dystrophy [27]. But it is important to consider that almost a third (27.3%) of underweight patients presented hyperinsulinemia and IR which indicates that there is not an intramuscular body fat mass deposition in those patients. Therefore, IR in DMD/BMD may be an independent result of fat mass content where alterations of components of DGC such as dystrophin may be involved.

To our knowledge, there are no studies that clearly demonstrate whether patients with DMD or BMD present IR or alterations in glucose metabolism. Freidenberg and Olefsky reported that serum glucose and insulin concentrations after oral glucose administration showed an abnormal increase in the area under their respective curves, suggesting alterations in glucose metabolism from DMD patients [28].

Table 2: Characteristics and metabolic variables according to nutritional status in DMD/BMD patients from 4 to 18 years of age[a].

	Underweight ($n = 12$)	Normal ($n = 39$)	Overweight/obese ($n = 15$)
Age (years)	9.2 (4.9, 17.8)	8.9 (4.6, 17.1)	8.5 (6.2, 15.0)
Height (mts)	1.21 (0.97, 1.64)	1.26 (1.0, 1.75)	1.23 (1.15, 1.75)
Percentile of BMI	0.63 (0.0, 5.34)*	46.37 (6.54, 81.38)	93.2 (86.35, 99.74)*
Body weight (kg)	19.3 (12.4, 28.1)*	24.3 (14.8, 59.3)	34.7 (24.7, 79.4)*
BMI (kg/m^2)	13.3 (10.4, 14.1)*	16.0 (13.9, 22.9)	21.3 (18.5, 29.5)*
Lean body mass (kg)	15.44 (10.69, 22.7)	16.97 (12.22, 34.07)	19.24 (13.76, 34.99)*
Body fat mass (%)	13.8 (7.6, 26.2)*	23.6 (10.3, 57.0)	39.6 (27.6, 60.0)*
Body fat mass (kg)	2.6 (0.88, 5.42)*	6.16 (1.58, 30.74)	13.14 (8.85, 46.08)*
Glucose (mg/dL)	90.08 (77.43, 104.4)	91.88 (75.67, 104.5)	97.29 (75.67, 135.12)*
Insulin (μU/mL)	9.4 (5.2, 23.9)	11.0 (5.9, 43.5)	23.7 (9.4, 59.9)*
HOMA-IR	2.08 (1.04, 6.06)	2.49 (1.04, 8.99)	5.54 (2.21, 12.91)*
Wheelchair bound (number of boys)	2	11	5

DMD/BMD: Duchene/Becker muscular dystrophy; BMI: body mass index; HOMA-IR: homeostasis model assessment-insulin resistance.
*$P < 0.01$ compared to normal group (ANOVA, Dunnett's method).
[a]Values are median (minimum, maximum).

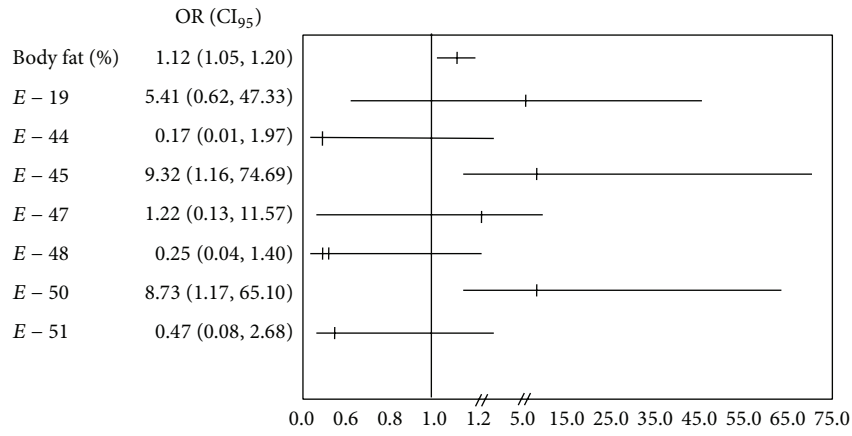

Figure 3: Risk of developing insulin resistance (homeostasis model assessment-insulin resistance > 3.16). P values and odds ratio calculated by logistic regression model. CI, confidence interval. Data adjusted by % body fat mass. E = Exon.

Abnormal subcellular accumulation of GLUT4 vesicles was observed in nonobese, type 2 diabetic skeletal muscle fibers of rats [11]. Accordingly, we also observed abnormal cytoplasmic aggregates of GLUT4 in myofibers from DMD/BMD patients, suggesting a possible alteration of glucose incorporation into the muscle, leading to hyperglycemia. Interestingly, these patients also presented hyperinsulinemia and IR. It is possible that these GLUT4 abnormalities could be secondary due to IR.

Furthermore, we observed that deletions of exons 45 or 50 increase the risk (~9.0 times) for developing IR. In this sense, underweight patients who present hyperinsulinemia and IR had deletion of exons 45 or 50. These exons encode an acting-binding domain and maybe the alteration between F-actin and dystrophin link may disturb some cell-signalling process as binding platform ligands arising in metabolic alteration [8]. Anyway, our data suggest that the result of the mutation in the *DMD* gene may possibly be associated with a metabolic alteration in DMD/BMD patients. The presence of these metabolic alterations in underweight subjects suggests a possible relationship with DMD/BMD.

Because we included a low number of muscle biopsies from DMD/BMD patients with different grades of damage in the muscle fiber, we detected abnormal cytoplasmic aggregates of GLUT4 in myofibers only in three patients. At any rate, this information opens an interesting field of study to explore, in muscle biopsies from DMD/BMD patients, cellular signalling pathways involved in glucose metabolism and possibly related to muscle morphology. Another limitation of this study is the lack of distinction between DMD and BMD and the lack of appropriate control subjects of the same age. Anyway, the main strength of this study is that we demonstrated that those patients develop metabolic alterations, which is essential information for caregivers and physicians in order to prevent other pathologies.

We identified differences in nutritional status of DMD/BMD patients according to BMI (percentiles). Prevalence of overweight or obesity in these patients was lower (22.7%)

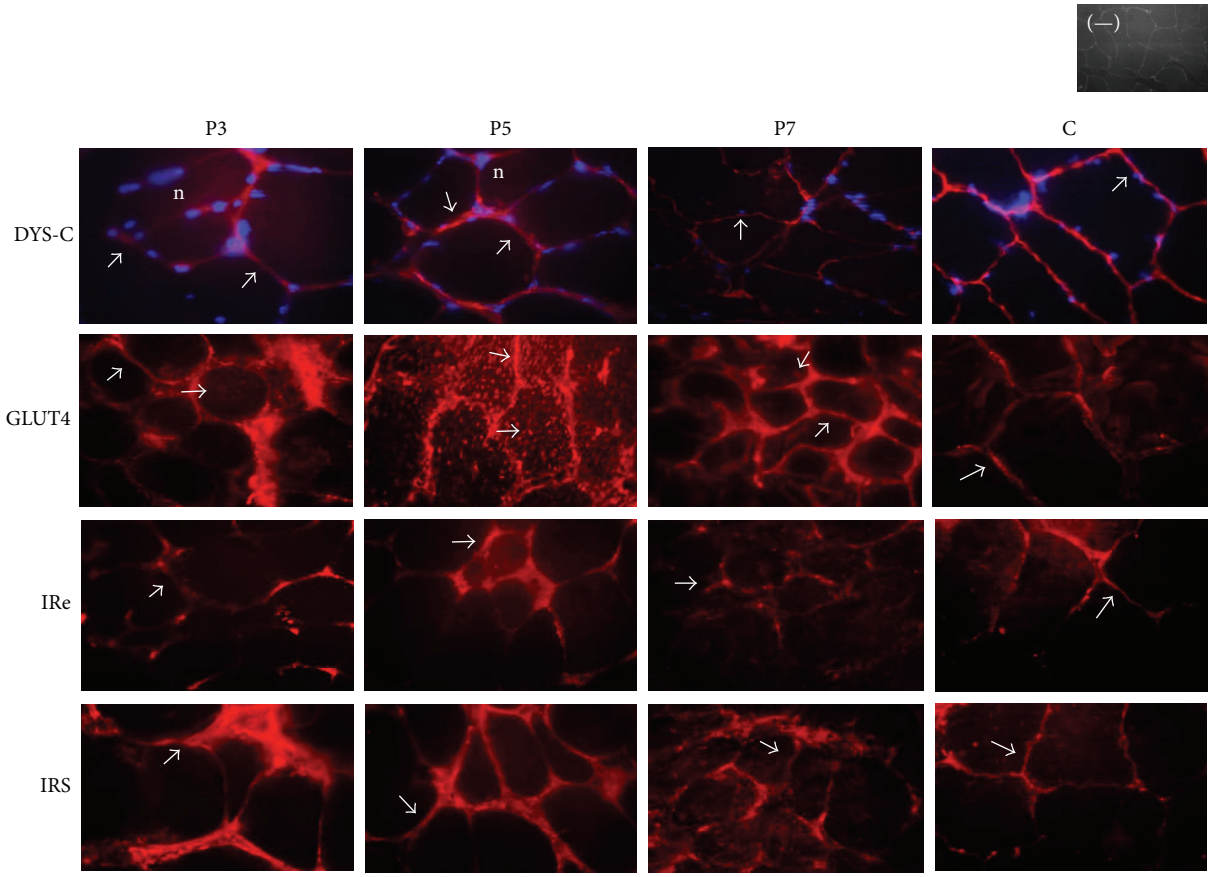

Figure 4: Immunofluorescence analysis of dystrophin (DYS), insulin receptor (IRe), insulin receptor substrate (IRS), and glucose transporter 4 (GLUT4) of healthy individuals and DMD/BMD patients. Immunostaining demonstrated semiabsence of dystrophin on the muscle fibers of the patients. DYS-C, GLUT4, IRe, and IRS are stained in red (arrows) and nuclei are stained in blue (n). Negative control omitted the primary antibody.

Table 3: Characteristics of patients and cellular localization of molecules involved in glucose metabolism.

	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Variable					
Age (years)	11.2	8.07	6.2	6.48	11.2
Age of onset (years)	1.5	2	5	1.5	3
Wheelchair dependency	No	No	No	No	No
Exon deleted	48	45	45, 47, 48	44	45, 48
Dys C	+ − −	− − −	+ + +	+ − −	+ + −
Dys N	+ − −	+ − −	+ + −	+ − −	+ − −
Insulin (μU/mL)	32.1	6.4	27.5	10.09	38.9
Glucose (mg/dL)	90.6	88.4	102.3	100.5	84.5
HOMA-IR	9.8	1.41	7.4	2.51	8.1

A subjective value was assigned (normal + + +, decreased + + −, considerably decreased + − −, and absent − − −) to describe staining indicating the presence of dystrophin. ND: not detected by multiplex PCR; GLUT4: glucose transporter 4; Dys-C: dystrophin C-terminus; Dys-N: dystrophin N-terminus; HOMA-IR: homeostasis model assessment-insulin resistance.

than that reported by other authors where ~50% of boys with DMD/BMD were obese by 13 years of age according to BMI [13, 15]. Nevertheless, in these studies the interaction between weight and steroid treatment is noteworthy. For the first time, we present information about the prevalence of obesity in DMD without effect of steroid treatment. The higher total body fat mass observed in boys with DMD is mostly due to increased intramuscular body fat mass deposition in both the central and peripheral regions [25]. In agreement with the classification of Taylor et al., 2002, body fat mass was higher (~68%) than in healthy boys and in boys with DMD [13–15, 25].

In conclusion, to our knowledge, these novel results present evidence regarding the presence of metabolic alterations in DMD/BMD patients such as obesity, hyperinsulinemia, and IR, without the effects of steroid treatment. IR had a high frequency and increased significantly when DMD/BMD patients are overweight/obese. Deletion of exons 45 or 50 increases the risk (~9 times) for developing IR. Abnormal cytoplasmic aggregates of GLUT4 in myofibers from DMD/BMD patients suggest a possible alteration of glucose incorporation into the muscle.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] K. Bushby, R. Finkel, D. J. Birnkrant et al., "Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management," *The Lancet Neurology*, vol. 9, no. 1, pp. 77–93, 2010.

[2] B. T. Darras, P. Blattner, J. F. Harper, A. J. Spiro, S. Alter, and U. Francke, "Intragenic deletions in 21 Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy (BMD) families studied with the dystrophin cDNA: location of breakpoints on Hind III and BglII exon-containing fragment maps, meiotic and mitotic origin of the mutations," *American Journal of Human Genetics*, vol. 43, pp. 620–629, 1988.

[3] E. F. Gillard, J. S. Chamberlain, E. G. Murphy et al., "Molecular and phenotypic analysis of patients with deletions within the deletion-rich region of the Duchenne muscular dystrophy (DMD) gene," *The American Journal of Human Genetics*, vol. 45, no. 4, pp. 507–520, 1989.

[4] R. Coral-Vazquez, D. Arenas, D. Cisneros et al., "Pattern of deletions of the dystrophin gene in Mexican Duchenne/Becker muscular dystrophy patients: the use of new designed primers for the analysis of the major deletion "hot spot" region," *The American Journal of Medical Genetics*, vol. 70, pp. 240–246, 1997.

[5] A. P. Monaco, C. J. Bertelson, S. Liechti-Gallati, H. Moser, and L. M. Kunkel, "An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus," *Genomics*, vol. 2, no. 1, pp. 90–95, 1988.

[6] J. M. Ervasti, K. Ohlendieck, S. D. Kahl, M. G. Gaver, and K. P. Campbell, "Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle," *Nature*, vol. 345, no. 6273, pp. 315–319, 1990.

[7] K. A. Lapidos, R. Kakkar, and E. M. McNally, "The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma," *Circulation Research*, vol. 94, no. 8, pp. 1023–1031, 2004.

[8] A. A. Hack, M. Y. J. Lam, L. Cordier et al., "Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoprotein complex," *Journal of Cell Science*, vol. 113, no. 14, pp. 2535–2544, 2000.

[9] J. E. Brenman, D. S. Chao, H. Xia, K. Aldape, and D. S. Bredt, "Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy," *Cell*, vol. 82, no. 5, pp. 743–752, 1995.

[10] J. B. Boord, A. L. Graber, J. W. Christman, and A. C. Powers, "Practical management of diabetes in critically ill patients," *The American Journal of Respiratory and Critical Care Medicine*, vol. 64, pp. 1763–1767, 2001.

[11] C. Mulvey, E. Harno, A. Keenan, and K. Ohlendieck, "Expression of the skeletal muscle dystrophin-dystroglycan complex and syntrophin-nitric oxide synthase complex is severely affected in the type 2 diabetic Goto-Kakizaki rat," *European Journal of Cell Biology*, vol. 84, no. 11, pp. 867–883, 2005.

[12] D. G. Allen and N. P. Whitehead, "Duchenne muscular dystrophy—what causes the increased membrane permeability in skeletal muscle?" *The International Journal of Biochemistry and Cell Biology*, vol. 43, no. 3, pp. 290–294, 2011.

[13] C. M. McDonald, G. T. Carter, R. T. Abresch et al., "Body composition and water compartment measurements in boys with Duchenne muscular dystrophy," *The American Journal of Physical Medicine and Rehabilitation*, vol. 84, no. 7, pp. 483–491, 2005.

[14] M. C. Zanardi, A. Tagliabue, S. Orcesi, A. Berardinelli, C. Uggetti, and A. Pichiecchio, "Body composition and energy expenditure in Duchenne muscular dystrophy," *European Journal of Clinical Nutrition*, vol. 57, no. 2, pp. 273–278, 2003.

[15] T.-N. Willig, L. Carlier, M. Legrand, H. Riviere, and J. Navarro, "Nutritional assessment in Duchenne muscular dystrophy," *Developmental Medicine and Child Neurology*, vol. 35, no. 12, pp. 1074–1082, 1993.

[16] S. Z. Yanovski and J. A. Yanovski, "Obesity," *The New England Journal of Medicine*, vol. 346, no. 8, pp. 591–602, 2002.

[17] R. W. Taylor, I. E. Jones, S. M. Williams, and A. Goulding, "Body fat percentages measured by dual-energy x-ray absorptiometry corresponding to recently recommended body mass index cutoffs for overweight and obesity in children and adolescents aged 3–18 y," *The American Journal of Clinical Nutrition*, vol. 76, no. 6, pp. 1416–1421, 2002.

[18] J. Sambrook and D. Russell, "Preparation and analysis of eukaryotic genomic DNA," in *Molecular Cloning: A Laboratory Manual*, C. Nolan, Ed., pp. 6.1–6.31, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2001.

[19] J. S. Chamberlain, R. A. Gibbs, J. E. Ranier, P. N. Nguyen, and C. T. Caskey, "Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification," *Nucleic Acids Research*, vol. 16, no. 23, pp. 11141–11156, 1988.

[20] A. H. Beggs, M. Koenig, F. M. Boyce, and L. M. Kunkel, "Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction," *Human Genetics*, vol. 86, no. 1, pp. 45–48, 1990.

[21] A. C. B. Rizzo, T. B. L. Goldberg, C. C. Silva, C. S. Kurokawa, H. R. C. Nunes, and J. E. Corrente, "Metabolic syndrome risk factors in overweight, obese, and extremely obese brazilian adolescents," *Nutrition Journal*, vol. 12, no. 1, article no. 19, 2013.

[22] D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner, "Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[23] M. Keskin, S. Kurtoglu, M. Kendirci, M. E. Atabek, and C. Yazici, "Homeostasis model assessment is more reliable than the fasting glucose/insulin ratio and quantitative insulin sensitivity check index for assessing insulin resistance among obese children and adolescents," *Pediatrics*, vol. 115, no. 4, pp. e500–e503, 2005.

[24] J. J. García, G. G. Rojas, and N. M. León, "Prevalencia de resistencia a la insulina y síndrome metabólico en niños obesos

que acuden a la Clínica de Obesidad del Hospital Pediátrico de Sinaloa," *Pediatría de México*, vol. 12, pp. 18–22, 2010.

[25] A. Leroy-Willig, T. N. Willig, M. C. Henry-Feugeas et al., "Body composition determined with MR in patients with Duchenne muscular dystrophy, spinal muscular atrophy, and normal subjects," *Magnetic Resonance Imaging*, vol. 15, no. 7, pp. 737–744, 1997.

[26] T. Ploug, B. van Deurs, H. Ai, S. W. Cushman, and E. Ralston, "Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions," *The Journal of Cell Biology*, vol. 142, no. 6, pp. 1429–1446, 1998.

[27] S. Groh, H. Zong, M. M. Goddeeris et al., "Sarcoglycan complex. Implications for metabolic defects in muscular dystrophies," *Journal of Biological Chemistry*, vol. 284, no. 29, pp. 19178–19182, 2009.

[28] G. R. Freidenberg and J. M. Olefsky, "Dissociation of insulin resistance and decreased insulin receptor binding in Duchenne muscular dystrophy," *Journal of Clinical Endocrinology and Metabolism*, vol. 60, no. 2, pp. 320–327, 1985.

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Research Article

Decreased Insulin Sensitivity and Impaired Fibrinolytic Activity in Type 2 Diabetes Patients and Nondiabetics with Ischemic Stroke

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We analyzed (a) insulin sensitivity (IS), (b) plasma insulin (PI), and (c) plasminogen activator inhibitor-1 (PAI-1) in type 2 diabetes (T2D) patients with (group A) and without (group B) atherothrombotic ischemic stroke (ATIS), nondiabetics with ATIS (group C), and healthy controls (group D). IS was determined by minimal model (Si). Si was lower in A versus B (1.18 ± 0.67 versus 2.82 ± 0.61 min−1/mU/L × 104; $P < 0.001$) and in C versus D (3.18 ± 0.93 versus 6.13 ± 1.69 min−1/mU/L × 104; $P < 0.001$). PI and PAI-1 were higher in A versus B (PI: 19.61 ± 4.08 versus 14.91 ± 1.66 mU/L; $P < 0.001$, PAI-1: 7.75 ± 1.04 versus 4.57 ± 0.72 mU/L; $P < 0.001$) and in C versus D (PI: 15.14 ± 2.20 versus 7.58 ± 2.05 mU/L; $P < 0.001$, PAI-1: 4.78 ± 0.98 versus 3.49 ± 1.04 mU/L; $P < 0.001$). Si correlated with PAI-1 in T2D patients and nondiabetics, albeit stronger in T2D. Binary logistic regression identified insulin, PAI-1, and Si as independent predictors for ATIS in T2D patients and nondiabetics. The results imply that insulin resistance and fasting hyperinsulinemia might exert their atherogenic impact through the impaired fibrinolysis.

1. Introduction

Impaired insulin sensitivity (IS) plays a crucial role in the development of type 2 diabetes (T2D) [1], but its relevance for the occurrence of ischemic stroke still remains unclear. In that context, some studies suggested that decreased IS, for example, insulin resistance, is an established risk factor for ischemic stroke, [2–4] while there are studies which could not demonstrate this relationship [5].

Moreover, it has been reported that decreased IS is directly related to different subtypes of ischemic stroke in T2D patients and nondiabetics, measured by different metabolic tests, short insulin tolerance test, homeostasis model assessment for insulin resistance, and immunoreactive insulin after glucose loading in 2 h OGTT [2, 3]. Simultaneously, it has been shown that higher levels of insulin resistance were found in nondiabetics with coexistence of intra- and extracranial atherosclerosis in contrast to those with only intra- or extracranial atherosclerosis [6].

Also, it has been suggested that hyperinsulinemia might be a risk factor for ischemic stroke [7, 8]. Hyperinsulinemia represents a surrogate measure for insulin resistance in nondiabetics, as well as in T2D patients with significant residual insulin secretion capacity [9].

Simultaneously, higher levels of PAI-1 have been found in blood from patients with T2D, in obese subjects [10],

and in other conditions associated with insulin resistance [11–13]. Moreover, it has been shown that hypofibrinolysis due to higher PAI-1 levels has been related to the insulin resistance [14–17] and might be involved together with insulin resistance, in the pathogenesis of ischemic stroke [18].

Therefore, in this study we tried to determine the role of impaired IS, together with the relevant changes in insulin and PAI-1 levels in T2D patients as well as nondiabetics with ischemic stroke.

2. Materials and Methods

2.1. Patients. We divided 62 T2D patients into two groups, with ($N = 33$) and without ($N = 29$) atherothrombotic ischemic stroke (ATIS), and 64 nondiabetics were assigned into group with ATIS ($N = 34$) and healthy subjects ($N = 30$), paired with the T2D patients with respect to sex and age. The diagnosis of T2D was based on the World Health Organization criteria [19]. Diagnosis of ATIS was done by a neurologist according to clinical signs and visualization methods, cranial computerized scan, and/or magnetic resonance imaging of the brain, repeated after the first 7 days from initial findings of ischemic stroke [20]. Only the patients with ATIS were included in the study, while the exclusion criteria involved patients with previously documented lacunar, cardioembolic, hemorrhagic cerebral infarction or coronary heart disease (history of myocardial infarction or coronary angiography). T2D patients were treated only with oral antihyperglycemic agents, while we excluded patients treated with insulin therapy or with other endocrine diseases, renal or hepatic insufficiency, previous and current infections, hematological or rheumatic diseases, uncontrolled hypertension, or severe alcohol consumption, during the last 4 weeks. At the time of metabolic evaluation, all the patients, irrespective of occurrence of the stroke, showed a uniform level of their physical activity.

The patients were completely informed about the study, before they gave an informed consent to participate.

The study was done at the Clinic for Endocrinology, Diabetes and Metabolic Diseases and at the Clinic for Neurology, Clinical Centre of Serbia, Faculty of Medicine, University of Belgrade, and was approved by the Institutional Ethics Committee.

2.2. Study Design. The obtaining of the medical history and physical examination, metabolic tests, and fibrinolytic activity evaluation were conducted in all the patients included the study within one-day visit.

Body mass index was calculated as weight in kilograms divided by the square of height in meters.

Arterial blood pressure was measured by sphygmomanometry and hypertension was diagnosed according to World Health Organisation criteria (systolic/diastolic blood pressure ≥140/≥90 mm Hg) or by the use of antihypertensive agents.

The metabolic test was performed after 6 months from occurrence of ATIS and following overnight fasting. IS was tested by using IVGTT with frequently sampled plasma glucose (PG) and PI levels, followed by the minimal model analysis [21]. The subjects were injected with glucose 0.3 g/kg body weight and the blood samples were taken immediately before intravenous glucose loading and sequentially every minute during the first 10 minutes and then 12, 14, 16, 20, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, and 180 minutes after intravenous glucose loading. Insulin was added during the test intravenously (4 mU/kg/min), between minutes 20 and 25, in order to substitute potentially diminished insulin response. The insulin sensitivity index (Si) was obtained from the data of PG and PI levels by computerized minimal model analysis (MINMOD program, kindly provided by Dr. Richard Bergman from the University of Southern California, Los Angeles).

2.3. Laboratory Analyses. PG was obtained by glucose oxidase method using a Beckman Glucose Analyser (Beckman Instruments). PI was measured by radioimmunoassay (INEP-Zemun) double antibody kits. Plasma PAI-1 activity was evaluated by plasminogen chromogenic plasmin substrate assay (Boehringer).

2.4. Statistical Analyses. Data are presented as means ± SD. Data were tested for normal distribution using Kolmogorov-Smirnov test. The continuous variables within each group of patients were analyzed with analysis of variance (ANOVA) with post hoc Tamhane test. Binary logistical regression analysis was performed. Correlation was estimated by Pearson's (r) correlation coefficient. The significance of the differences between correlation coefficients was analyzed by using Fisher r-to-z transformation. The differences were considered to be statistically significant at $P < 0.05$. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (Advanced Statistics, version 17.0), Chicago, IL.

3. Results

3.1. Clinical Characteristics. The clinical characteristics of study subject are shown in Table 1. All three investigated groups of patients were matched according to the age, duration of T2D, and period of time from the onset of ischemic stroke. Simultaneously, all patients were overweight and T2D patients had satisfactory metabolic control expressed as almost equal HbA_1c values, before metabolic investigation. The hypertension in patients with the stroke was significantly higher than in healthy controls, both in T2D patients and in nondiabetics. We did not find a significant difference in BMI between T2D patients and ATIS compared to T2D without ATIS and nondiabetics with ATIS compared to healthy subjects (Table 1).

3.2. Insulin Sensitivity. When we analysed IS, expressed as Si index, the lowest Si values were present in patients with T2D and ATIS and they were significantly lower in T2D patients with ATIS compared to T2D patients without ATIS (1.18 ± 0.67 versus 2.82 ± 0.61 $\text{min}^{-1}/\text{mU/L} \times 10^4$; $P < 0.001$). Moreover, Si values were significantly lower in nondiabetics with ATIS in comparison to healthy subjects (3.18 ± 0.93 versus 6.13 ± 1.69 $\text{min}^{-1}/\text{mU/L} \times 10^4$; $P < 0.001$) (Figure 1).

Table 1: Clinical characteristics of type 2 diabetes (T2D) patients and nondiabetics with and without atherothrombotic ischemic stroke (ATIS).

	$T2D^+$ $ATIS^+$ A	$T2D^+$ $ATIS^-$ B	Nondiabetics $ATIS^+$ C	Healthy subjects D
n (M/F)	33 (15/18)	29 (15/14)	34 (17/17)	30 (14/16)
Age (years)	56.97 ± 2.17	58.28 ± 2.43	57.76 ± 2.75	57.46 ± 2.19
Duration of diabetes (years)	4.81 ± 1.81	5.77 ± 2.44	—	—
Period of time from onset of ischaemic stroke (years)	1.14 ± 0.39	—	1.03 ± 0.23	—
HbA1c (%)	7.36 ± 0.24	7.22 ± 0.24	5.76 ± 0.57	4.97 ± 0.45*
Hypertension	21 (63.6%)	18 (62.1%)	20 (58.8%)	0 (0%)*
BMI (kg/m^2)	27.62 ± 3.14	27.62 ± 3.77	26.20 ± 4.09	26.39 ± 2.41

Data are *n*, means ± SD.
* $P < 0.001$, A versus B; C versus D; A versus C, D.

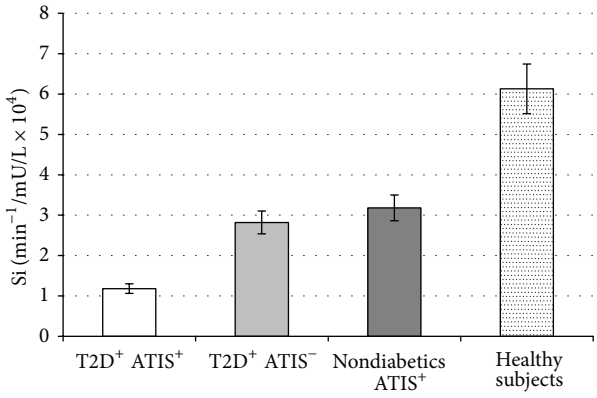

Figure 1: Values are means ± SE. Bar graphs show the values of Si determined by minimal model analysis. Si levels were significantly lower in type 2 diabetes (T2D) patients with atherothrombotic ischemic stroke (ATIS) compared to T2D patients without ATIS ($P < 0.001$) and in nondiabetics with ATIS compared to healthy subjects ($P < 0.001$). There is no difference in Si levels between T2D patients without ATIS and nondiabetics with ATIS (P = NS) (analysis of variance (ANOVA) with post hoc Tamhane test).

There is no difference in Si between T2D patients without ATIS and nondiabetics with ATIS (P = NS).

3.3. Insulin Levels. Simultaneously, we found that PI levels were higher in T2D patients and ATIS compared to T2D patients without ATIS (19.61 ± 4.08 versus 14.91 ± 1.66 mU/L; $P < 0.001$). In addition, PI levels were higher in nondiabetics with ATIS in comparison to healthy subjects (15.14 ± 2.20 versus 7.58 ± 2.05; $P < 0.001$). Moreover, T2D patients with ATIS showed significantly higher PI levels than nondiabetics with stroke ($P < 0.001$), but we did not document difference in PI levels between T2D patients without ATIS and nondiabetics with ATIS (Figure 2).

3.4. Fibrinolysis. Simultaneously, we found that PAI-1 levels were significantly higher in T2D patients with ATIS compared to T2D patients without ATIS (7.75 ± 1.04 versus 4.57 ± 0.72 mU/L; $P < 0.001$) and in nondiabetics with ATIS compared to healthy subjects (4.78 ± 0.98 versus 3.49 ± 1.04 mU/L; $P < 0.001$) (Figure 3). In addition, PAI-1 levels

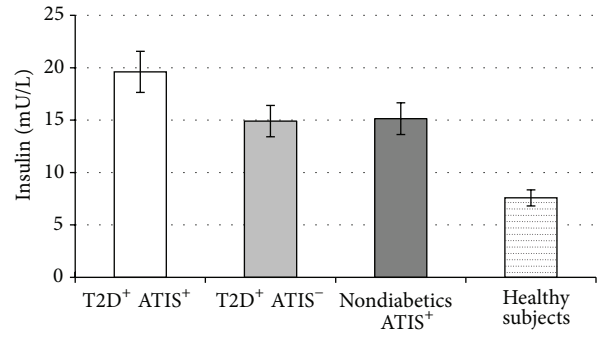

Figure 2: Values are means ± SE. Bar graphs show the values of basal PI level. PI levels were higher in type 2 diabetes (T2D) patients with atherothrombotic ischemic stroke (ATIS) compared to T2D patients without ATIS ($P < 0.001$) and in nondiabetics with ATIS in comparison to healthy subjects ($P < 0.001$). There is no difference in PI levels between T2D patients without ATIS and nondiabetics with ATIS (P = NS) (analysis of variance (ANOVA) with post hoc Tamhane test).

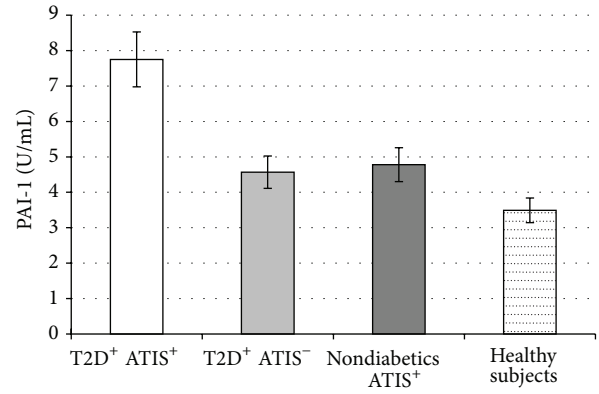

Figure 3: Values are means ± SE. Bar graphs show the values of PAI-1 levels. PAI-1 levels were significantly higher in type 2 diabetes (T2D) patients with atherothrombotic ischemic stroke (ATIS) compared to T2D patients without ATIS ($P < 0.001$) and in nondiabetics with ATIS compared to healthy subjects ($P < 0.001$). PAI-1 levels were not different among T2D patients without ATIS and nondiabetics with ATIS (P = NS) (analysis of variance (ANOVA) with post hoc Tamhane test).

Table 2: Correlation between sensitivity index (Si) and plasminogen activator inhibitor-1 (PAI-1) levels in patients with type 2 diabetes (T2D) and nondiabetics.

T2D	r	P	Nondiabetics	r	P
	PAI-1			PAI-1	
Si	−0.690	0.001	Si	−0.456	0.0001

Table 3: Independent factors related to T2D patients for development of ATIS in binary logistic regression analysis.

	OR (95%)	P
PAI-1	3.797–129.180	0.01
Insulin	3.203–5.848	0.01
Si	5.025–71.428	0.01

Table 4: Independent factors related to nondiabetics for development of ATIS in binary logistic regression analysis.

	OR (95%)	P
PAI-1	2.300–12.809	0.01
Insulin	1.443–25.526	0.014
Si	2.652–19.608	0.01

were not different among T2D patients without ATIS and nondiabetics with ATIS.

3.5. Correlations and Binary Logistic Regression Analysis. Moreover, we found that Si levels correlated with PAI-1 levels both in T2D ($r = -0.690$, $P < 0.001$) and in nondiabetic subjects ($r = -0.456$, $P < 0.001$) (Table 2). By using Fisher r-to-z transformation, we evaluated the difference between two correlations coefficients. The correlations of Si and PAI-1 in the T2D patients were stronger than Si and PAI-1 correlation in nondiabetics ($P < 0.05$).

The use of binary logistic regression analysis has identified levels of PAI-1, insulin, and Si as independent predictors for ATIS both in T2D patients and in nondiabetics (Tables 3 and 4).

4. Discussion

Our study results revealed decreased IS in patients with ATIS, whether they were T2D patients or nondiabetics, suggesting that insulin resistance may play a significant role in occurrence of the stroke. Simultaneously, T2D patients with ATIS showed the lowest level of IS, together with the fasting hyperinsulinemia. The documented hyperinsulinemia in these patients, in the settings of diminished IS levels, suggests the existence of a significant residual insulin secretion capacity. Moreover, both metabolic abnormalities, decreased IS or insulin resistance and fasting hyperinsulinemia in nondiabetics with ATIS, have confirmed the importance of decreased IS level in pathogenesis of ischemic stroke, which is consistent with the previous data [22]. Both experimental and human studies revealed the importance of insulin resistance for the occurrence of acute ischemic stroke [23–26]. However, our results signify the persistence of compromised IS and increased PI levels even 6 months after acute phase in T2D patients with ischemic stroke.

We decided to evaluate IS level in this study by using IVGTT with frequently sampled PG and PI levels with minimal model analysis, which correlates with hyperinsulinemic euglycemic clamp, previously implemented in the IS studies [27, 28].

The existence of impaired IS in different subtypes of ischemic stroke in patients with T2D has also been proposed [2]. Also, the novel study confirmed the highest values of two other parameters of insulin resistance, homeostasis model assessment for insulin resistance and insulin after glucose challenge in 2 h OGTT in patients with atherothrombotic cerebral infarction without previously documented abnormal glucose tolerance [3].

The results from extensive Atherosclerosis Risk in Communities (ARIC) Study showed that fasting insulin levels, among the other investigated risk factors, are positively associated with occurrence of ischemic stroke in the general population, which highlights the influence of insulin resistance [29], consistent with results from the Finnish cohort study that included elderly T2D patients and nondiabetics [30].

Novel data pointed out the important role of augmented insulin resistance and related metabolic abnormalities in the development of intracranial stenosis from its early stages even in nondiabetics [31].

On the other hand, lots of data suggested that PAI-1 plays a significant role in occurrence of coronary artery and cerebrovascular disease in T2D [32]. Recent studies shed new light on PAI-1 as an important pathway for cardiovascular events, including ischemic stroke [33].

Despite those facts and the background of occurrence and progression of atherosclerosis, the link between insulin resistance, diminished fibrinolytic activity, and ischemic stroke has not yet been clarified.

Our results showed higher PAI-1 levels in T2D and ATIS, in parallel with the absence of difference in PAI-1 levels between T2D patients without ATIS and nondiabetics with ATIS. However, there are some conflicting results suggesting higher PAI-1 levels in T2D without ischemic stroke compared to nondiabetics, implying the absence of further deterioration of fibrinolytic activity in ischemic stroke in T2D patients [34]. Moreover, there are indications of decreased IS, hyperinsulinemia, and increased PAI-1 in first-degree relatives of patients with ischemic stroke which is related to ethnicity, together with the findings supporting the hypothesis that diminished fibrinolytic activity may exist prior to ischemic stroke [35–37]. A previous study demonstrated that the higher PAI-1 activity in young adults with a first ischemic stroke was a consequence of acquired hypofibrinolysis [38], together with other investigations supporting the genetic control of decreased fibrinolysis [39].

Also, obese diabetics had higher PAI-1 levels compared to nondiabetics, implying permanent impaired fibrinolytic activity, which potentiates thrombosis, based on effect of combination of hormonal (hyperinsulinemia) and metabolic (hyperglycemia) changes characteristic for T2D [13, 40]. In general, increased PAI-1 level in patients with ATIS or T2D

or nondiabetics could be present even 4 years after acute cerebral ischemic event [41]. Therefore, we speculate that disturbances in fibrinolysis may precede the occurrence of ATIS, having in mind higher PAI-1 level in T2D patients without ATIS and almost equal PAI-1 levels in nondiabetics with ATIS [14, 42].

We found that Si levels correlated with PAI-1 levels both in T2D and nondiabetic subjects, with stronger correlation in T2D than in nondiabetics. IS has been independently related to PAI-1 levels in our study and previously in patients with T2D and obese diabetic and nondiabetic subjects [43, 44]. In BARI 2D trial it has been shown that diminishing of insulin resistance favorably changes the balance between fibrinolysis and thrombosis potentiating fibrinolysis during long term followup in T2D patients [13]. In addition, it was suggested that patients who experience progression of symptomatic intracranial atherosclerosis are characterized by impaired endogenous fibrinolysis. Moreover, insulin resistance might be associated with recurrence of ischemic stroke [45].

PAI-1 levels may influence stroke mechanisms in multiple ways, and they might differentiate between responders and nonresponders to reperfusion therapies, and they might represent potential target for stroke prevention [46].

In order to minimize the previously confirmed harmful effect of "glucose toxicity" to the IS level and fibrinolytic activity [13, 47], we selected T2D patients with or without ischemic stroke matched with respect to duration of disease, showing optimal metabolic control before the evaluation of insulin sensitivity level. Previously described association of obesity, insulin resistance, and higher PAI-1 levels, hypersecreted from adipocytes, was the reason to include overweight subjects in our investigation [48]. Since age is known to be strongly and independently correlated with the occurrence of ischemic stroke, investigated patients were younger than 65 years. Measurements of IS were not made until at least 6 months after the stroke, providing enough time for the patients to approach maximum recovery, showing similar level of the physical activity. In order to diminish the heterogeneity of stroke, patients were matched according to the duration of ischemic brain disease.

The binary logistic regression analysis applied to our data has demonstrated that insulin, PAI-1, and Si levels are independent predictors of the ischemic stroke occurrence, in T2D patients as well as in nondiabetics.

5. Conclusions

In conclusion, the results demonstrated that decreased IS levels together with fasting hyperinsulinemia are strongly associated with the onset of the ATIS, while this atherogenic effect might be strongly potentiated by increased level of PAI-1. In this context, insulin resistance and impaired fibrinolytic activity might be important targets for secondary stroke prevention.

Conflict of Interests

The authors have no financial or any conflict of interests to declare.

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References

[1] R. A. Defronzo, R. C. Bonadonna, and E. Ferrannini, "Pathogenesis of NIDDM: a balanced overview," *Diabetes Care*, vol. 15, no. 3, pp. 318–368, 1992.

[2] K. Matsumoto, S. Miyake, M. Yano et al., "Insulin resistance and classic risk factors in type 2 diabetic patients with different subtypes of ischemic stroke," *Diabetes Care*, vol. 22, no. 7, pp. 1191–1195, 1999.

[3] T. Urabe, H. Watada, Y. Okuma et al., "Prevalence of abnormal glucose metabolism and insulin resistance among subtypes of ischemic stroke in Japanese patients," *Stroke*, vol. 40, no. 4, pp. 1289–1295, 2009.

[4] T. Rundek, H. Gardener, Q. Xu et al., "Insulin resistance and risk of ischemic stroke among nondiabetic individuals from the Northern Manhattan study," *Archives of Neurology*, vol. 67, no. 10, pp. 1195–1200, 2010.

[5] R. G. Wieberdink, P. J. Koudstaal, A. Hofman, J. C. M. Witteman, M. M. B. Breteler, and M. A. Ikram, "Insulin resistance and the risk of stroke and stroke subtypes in the nondiabetic elderly," *American Journal of Epidemiology*, vol. 176, no. 8, pp. 699–707, 2012.

[6] H.-Y. Park, D.-S. Park, H.-S. Lee, H. Chang, Y.-S. Kim, and K.-H. Cho, "Correlation Between insulin resistance and intracranial atherosclerosis in patients with ischemic stroke without diabetes," *Journal of Stroke and Cerebrovascular Diseases*, vol. 17, no. 6, pp. 401–405, 2008.

[7] M. Pyörälä, H. Miettinen, M. Laakso, and K. Pyörälä, "Hyperinsulinemia and the risk of stroke in healthy middle-aged men: the 22-year follow-up results of the Helsinki Policemen Study," *Stroke*, vol. 29, no. 9, pp. 1860–1866, 1998.

[8] A. R. Folsom, M. L. Rasmussen, L. E. Chambless et al., "Prospective associations of fasting insulin, body fat distribution, and diabetes with risk of ischemic stroke," *Diabetes Care*, vol. 22, no. 7, pp. 1077–1083, 1999.

[9] R. E. Pratley and C. Weyer, "The role of impaired early insulin secretion in the pathogenesis of Type II diabetes mellitus," *Diabetologia*, vol. 44, no. 8, pp. 929–945, 2001.

[10] P. Vague, I. Juhan-Vague, M. F. Aillaud et al., "Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects," *Metabolism*, vol. 35, no. 3, pp. 250–253, 1986.

[11] J. Auwerx, R. Bouillon, D. Collen, and J. Geboers, "Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus," *Arteriosclerosis*, vol. 8, no. 1, pp. 68–72, 1988.

[12] J.-H. Jansson, B. Johansson, K. Boman, and T. K. Nilsson, "Hypofibrinolysis in patients with hypertension and elevated cholesterol," *Journal of Internal Medicine*, vol. 229, no. 4, pp. 309–316, 1991.

[13] D. J. Schneider and B. E. Sobel, "PAI-1 and diabetes: a journey from the bench to the bedside," *Diabetes Care*, vol. 35, no. 10, pp. 1961–1967, 2012.

[14] A. Festa, R. D'Agostino Jr., R. P. Tracy, and S. M. Haffner, "Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study," *Diabetes*, vol. 51, no. 4, pp. 1131–1137, 2002.

[15] C. J. Lyon and W. A. Hsueh, "Effect of plasminogen activator inhibitor-1 in diabetes mellitus and cardiovascular disease," *The American Journal of Medicine*, vol. 115, no. 8, pp. 62–68, 2003.

[16] J. B. Meigs, C. J. O'Donnell, G. H. Tofler et al., "Hemostatic markers of endothelial dysfunction and risk of incident type 2 diabetes: the Framingham Offspring Study," *Diabetes*, vol. 55, no. 2, pp. 530–537, 2006.

[17] A. Natali, E. Toschi, S. Baldeweg et al., "Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes," *Diabetes*, vol. 55, no. 4, pp. 1133–1140, 2006.

[18] E. L. Air and B. M. Kissela, "Diabetes, the metabolic syndrome, and ischemic stroke: epidemiology and possible mechanisms," *Diabetes Care*, vol. 30, no. 12, pp. 3131–3140, 2007.

[19] World Health Organization, "Definition, diagnosis, and classification: diabetes mellitus: report of a WHO study group," Tech. Rep. 727, World Health Organization, Geneva, Switzerland, 1985.

[20] "Special report from the National Institute of Neurological Disorders and Stroke. Classification of cerebrovascular diseases III," *Stroke*, vol. 21, no. 4, pp. 637–676, 1990.

[21] G. Pacini and R. N. Bergman, "MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test," *Computer Methods and Programs in Biomedicine*, vol. 23, no. 2, pp. 113–122, 1986.

[22] K. Shinozaki, H. Naritomi, T. Shimizu et al., "Role of insulin resistance associated with compensatory hyperinsulinemia in ischemic stroke," *Stroke*, vol. 27, no. 1, pp. 37–43, 1996.

[23] Y.-Y. Wang, S.-Y. Lin, Y.-H. Chuang, C.-J. Chen, K.-C. Tung, and W. H.-H. Sheu, "Adipose proinflammatory cytokine expression through sympathetic system is associated with hyperglycemia and insulin resistance in a rat ischemic stroke model," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 300, no. 1, pp. E155–E163, 2011.

[24] Y.-Y. Wang, C.-J. Chen, S.-Y. Lin, Y.-H. Chuang, W. H.-H. Sheu, and K.-C. Tung, "Hyperglycemia is associated with enhanced gluconeogenesis in a rat model of permanent cerebral ischemia," *Molecular and Cellular Endocrinology*, vol. 367, no. 1-2, pp. 50–56, 2013.

[25] Y.-Y. Wang, S.-Y. Lin, Y.-H. Chuang, W. H.-H. Sheu, K.-C. Tung, and C.-J. Chen, "Activation of hepatic inflammatory pathways by catecholamines is associated with hepatic insulin resistance in male ischemic stroke rats," *Endocrinology*, vol. 155, no. 4, pp. 1235–1246, 2014.

[26] A. I. Calleja, E. Cortijo, P. García-Bermejo et al., "Blood biomarkers of insulin resistance in acute stroke patients treated with intravenous thrombolysis: temporal profile and prognostic value," *Journal of Diabetes Research & Clinical Metabolism*, vol. 2, article 2, 2013.

[27] M. F. Saad, R. L. Anderson, A. Laws et al., "A comparison between the minimal model and the glucose clamp in the assessment of insulin sensitivity across the spectrum of glucose tolerance," *Diabetes*, vol. 43, no. 9, pp. 1114–1121, 1994.

[28] G. Howard, R. Bergman, L. E. Wagenknecht et al., "Ability of alternative indices of insulin sensitivity to predict cardiovascular risk: comparison with the 'minimal model'," *Annals of Epidemiology*, vol. 8, no. 6, pp. 358–369, 1998.

[29] A. R. Folsom, M. L. Rasmussen, L. E. Chambless et al., "Prospective associations of fasting insulin, body fat distribution, and diabetes with risk of ischemic stroke," *Diabetes Care*, vol. 22, no. 7, pp. 1077–1083, 1999.

[30] J. Kuusisto, L. Mykkänen, K. Pyörälä, and M. Laakso, "Non-insulin-dependent diabetes and its metabolic control are important predictors of stroke in elderly subjects," *Stroke*, vol. 25, no. 6, pp. 1157–1164, 1994.

[31] E. López-Cancio, A. Galán, L. Dorado et al., "Biological signatures of asymptomatic extra- and intracranial atherosclerosis: the Barcelona-AsIA (Asymptomatic Intracranial Atherosclerosis) Study," *Stroke*, vol. 43, pp. 2712–2719, 2012.

[32] X. Liang, T. Kanjanabuch, S.-L. Mao et al., "Plasminogen activator inhibitor-1 modulates adipocyte differentiation," *The American Journal of Physiology: Endocrinology and Metabolism*, vol. 290, no. 1, pp. E103–E113, 2006.

[33] L. Iacoviello, C. Agnoli, A. de Curtis et al., "Type 1 plasminogen activator inhibitor as a common risk factor for cancer and ischaemic vascular disease: the EPICOR study," *British Medical Journal*, vol. 3, no. 11, article 12, 2013.

[34] M. W. Mansfield, A. J. Catto, A. M. Carter, and P. J. Grant, "Fibrinolytic measurements in type 2 diabetic patients with acute cerebral infarction," *Diabetic Medicine*, vol. 15, no. 11, pp. 953–957, 1998.

[35] L. Johansson, J.-H. Jansson, K. Boman, T. K. Nilsson, B. Stegmayr, and G. Hallmans, "Tissue plasminogen activator, plasminogen activator inhibitor-1, and tissue plasminogen activator/plasminogen activator inhibitor-1 complex as risk factors for the development of a first stroke," *Stroke*, vol. 31, no. 1, pp. 26–32, 2000.

[36] K. Kain, A. J. Catto, J. Young, J. Bamford, J. Bavington, and P. J. Grant, "Insulin resistance and elevated levels of tissue plasminogen activator in first-degree relatives of South Asian patients with ischemic cerebrovascular disease," *Stroke*, vol. 32, no. 5, pp. 1069–1073, 2001.

[37] A. Mavri, M.-C. Alessi, and I. Juhan-Vague, "Hypofibrinolysis in the insulin resistance syndrome: implication in cardiovascular diseases," *Journal of Internal Medicine*, vol. 255, no. 4, pp. 448–456, 2004.

[38] B. Kristensen, J. Malm, T. K. Nilsson, J. Hultdin, B. Carlberg, and T. Olsson, "Increased fibrinogen levels and acquired hypofibrinolysis in young adults with ischemic stroke," *Stroke*, vol. 29, no. 11, pp. 2261–2267, 1998.

[39] P.-G. Wiklund, L. Nilsson, S. N. Ardnor et al., "Plasminogen activator inhibitor-1 4G/5G polymorphism and risk of stroke: replicated findings in two nested case-control studies based on independent cohorts," *Stroke*, vol. 36, no. 8, pp. 1661–1665, 2005.

[40] J. B. Mcgill, D. J. Schneider, C. L. Arfken, C. L. Lucore, and B. E. Sobel, "Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients," *Diabetes*, vol. 43, no. 1, pp. 104–109, 1994.

[41] A. Lindgren, C. Lindoff, B. Norrving, B. Åstedt, and B. B. Johansson, "Tissue plasminogen activator and plasminogen activator inhibitor-1 in stroke patients," *Stroke*, vol. 27, no. 6, pp. 1066–1071, 1996.

[42] L. Mykkanen, T. Ronnemaa, J. Marniemi, S. M. Haffner, R. Bergman, and M. Laakso, "Insulin sensitivity is not an independent determinant of plasma plasminogen activator inhibitor-1 activity," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 14, no. 8, pp. 1264–1271, 1994.

[43] A. Rydzewski, K. Sakata, A. Kobayashi et al., "Changes in plasminogen activator inhibitor 1 and tissue-type plasminogen activator during exercise in patients with coronary artery disease," *Haemostasis*, vol. 20, no. 5, pp. 305–312, 1990.

[44] A. Panahloo, V. Mohamed-Ali, A. Lane, F. Green, S. E. Humphries, and J. S. Yudkin, "Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene," *Diabetes*, vol. 44, no. 1, pp. 37–42, 1995.

[45] J. F. Arenillas, J. Alvarez-Sabín, C. A. Molina et al., "C-reactive protein predicts further ischemic events in first-ever transient ischemic attack or stroke patients with intracranial large-artery occlusive disease," *Stroke*, vol. 3, pp. 2463–2470, 2003.

[46] A. Tjärnlund-Wolf, H. Brogren, E. H. Lo, and X. Wang, "Plasminogen activator inhibitor-1 and thrombotic cerebrovascular diseases," *Stroke*, vol. 43, no. 10, pp. 2833–2839, 2012.

[47] A. H. Kissebah, S. Alfarsi, D. J. Evans, and P. W. Adams, "Plasma low density lipoprotein transport kinetics in noninsulin-dependent diabetes mellitus," *Journal of Clinical Investigation*, vol. 71, no. 3, pp. 655–667, 1983.

[48] M. C. Alessi, F. Peiretti, P. Morange, M. Henry, G. Nalbone, and I. Juhan-Vague, "Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link between visceral fat accumulation and vascular disease," *Diabetes*, vol. 46, no. 5, pp. 860–867, 1997.

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Review Article

Diabetes, Endothelial Dysfunction, and Vascular Repair: What Should a Diabetologist Keep His Eye on?

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Cardiovascular complications are the most common complications of diabetes mellitus. A prominent attribute of diabetic cardiovascular complications is accelerated atherosclerosis, considered as a still incurable disease, at least at more advanced stages. The discovery of endothelial progenitor cells (EPCs), able to replace old and injured mature endothelial cells and capable of differentiating into healthy and functional endothelial cells, has offered the prospect of merging the traditional theories on the pathogenesis of atherosclerosis with evolving concepts of vascular biology. The literature supports the notion that EPC alterations are involved in the pathogenesis of vascular diseases in diabetics, but at present many questions remain unanswered. In this review the aspects linking endothelial progenitor cells to the altered vascular biology in diabetes mellitus are discussed.

1. Introduction

Cardiovascular complications are the most common and devastating complications of diabetes mellitus; they are a major cause of hospital admissions and leading cause of death among diabetic patients [1, 2]. A prominent attribute of diabetic cardiovascular complications is accelerated atherosclerosis, which is associated with oxidative stress, insulin resistance, and the metabolic syndrome [3, 4]. Current knowledge suggests that endothelial injury and dysfunction occur as the initial event in the pathogenesis of atherosclerosis, followed by platelet adhesion and aggregation [5]. Overproduction of cytokines and other inflammation mediators stimulates migration and proliferation of smooth muscle cells in the vascular intima and deposition of extracellular matrix molecules like collagen and elastin, leading to plaque expansion and fibrous cap formation [6, 7]. Fibrous caps may weaken and rupture eventually, exposing the underlying extremely thrombogenic tissues. Plaque rupture induces further thrombus formation and release of more inflammatory mediators, causing continued progression of the atherosclerotic plaque, finally resulting in luminal narrowing and/or occlusion. Dramatic events like myocardial infarction, ischemic stroke, or critical ischemia of peripheral tissues may appear, depending of the anatomic site of the injured vessel [8, 9]. With increasing knowledge about the pathogenesis of atherosclerosis, hope that human atheromata can regress has evolved, but over time this idea met considerable skepticism. Resistance to concepts of lesion regression was enhanced by the fact that advanced atheromata in humans and in animals contain necrosis, calcification, and fibrosis, giving an impression of still irreparable events [10–13].

The discovery of a cell subgroup of myeloid origin, able to replace old and injured mature endothelial cells and capable of differentiating into healthy and functional endothelial cells, challenged skeptics. Those cells, named endothelial progenitor cells (EPC), have offered the prospect of merging the traditional theories on the pathogenesis of atherosclerosis with evolving concepts of atherosclerosis regression. Indeed, it seems that these progenitor cells are able to repair the injured vessel wall and to enhance neovascularization of ischemic tissues [14–16].

On the contrary, reduced EPC levels are associated with more serious endothelial dysfunction and elevated risk of adverse cardiovascular events, compatible with the concept that impaired EPC-mediated vascular repair allows further progression of vascular disease [17].

This applies in particular to endangered patients with metabolic alterations such as compensatory hyperinsulinemia, impaired fasting glucose, impaired glucose tolerance, and diabetes, who have an impaired EPC number and function, and this could be a further challenge to future investigations [14, 18].

However, available data suggest that metabolic interventions by either lifestyle change, better glucose and lipid control, or certain other agents are able to improve EPC number and function [17].

This review will focus on the role of EPC in vascular repair and available therapeutic options in diabetic patients.

2. Endothelium Biology

Despite being originally considered to be just a simple mechanic barrier between the blood and vascular wall, the endothelium is now recognized as the most important component of healthy vascular function. It maintains the anticoagulant, antiplatelet, and fibrinolytic properties of vascular cells. The healthy endothelium in response to physical and chemical signals produces a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. In few words, the endothelium is regarded as a very complex endocrine and paracrine organ [19, 20].

Effects on the vascular tone were the first discovery unveiling the importance of the endothelium. The endothelium produces several vasoactive molecules that relax or constrict the vessels, interplaying with circulating vasoactive mediators like bradykinin or thrombin. This vasomotion is of crucial importance for tissue oxygen supply and metabolic demand and is also involved in remodeling of vascular structures and regulating long-term organ perfusion. Maintaining the functional integrity of the endothelium, therefore, is critical for the preservation of blood flow and the prevention of thrombosis [21, 22].

Nitric oxide (NO) is the most important mediator released by endothelial cells, historically named and originally identified as endothelium-derived relaxing factor. NO is produced from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of several cofactors. This gas activates guanylate cyclase in vascular smooth muscle cells, leading to cGMP-mediated vasodilatation. In addition, this enzyme may be activated under certain circumstances by other signaling molecules like bradykinin, adenosine, vascular endothelial growth factor (during hypoxia), and serotonin (during platelet aggregation) [23, 24].

The endothelium also mediates vasodilatation through other mechanisms, like the endothelium-derived hyperpolarizing factor via accumulation of potassium ions in the intercellular space and/or due to tissue electrical conductivity, allowing propagation of electrical signals along the axis of blood vessels by means of homocellular gap junctions and throughout the vascular wall itself by means of myoendothelial gap junctions [25, 26].

In normal vascular physiology, NO plays a key role in preserving the vessel wall in a quiescent state by inhibition of inflammation, thrombosis, and cellular proliferation through limiting oxidative phosphorylation in mitochondria and s-nitrosylation of cysteine residues in a wide range of proteins, including transcription factors like NFκB, cell cycle-controlling proteins, and proteins involved in generation of tissue factors [22, 27].

Another endothelium-derived vasodilator that acts independently of NO is prostacyclin, derived by the action of the cyclooxygenase system. Prostacyclin is an eicosanoid which chiefly prevents formation of the platelet plug involved in primary hemostasis. In humans, it appears to have a more limited role in the maintenance of vasodilator tone, although it may contribute to some of the other regulatory roles of the endothelium [22, 26].

Other substances important for vasomotion are constrictors like endothelin and vasoconstrictor prostanoids generated in the endothelium, as well as angiotensin I converted to angiotensin II at the endothelial surface. These vasoconstrictor agents predominantly act locally but may also exert some systemic effects and have a role in the regulation of arterial structure and remodeling. Because of these properties, vasoconstrictor substances are believed to be involved in the pathogenesis of vascular diseases of several organ systems, including the heart, general circulation, and brain [22, 28, 29].

3. Diabetes, Endothelial Injury, and Dysfunction

Chronic hyperglycemia leads to vascular disease, and multiple studies in patients and animal models and in vitro have revealed that hyperglycemia alters endothelial metabolism and function, causing vascular injury. Vascular injury contributes to all diabetic complications, whether micro- or macrovascular, in all forms of diabetes mellitus. Prolonged and/or repeated exposure to other cardiovascular risk factors can additionally seriously damage the endogenous protective mechanisms within endothelial cells. As a consequence, the endothelium may become dysfunctional, and lose is vasomotor properties [30, 31].

An important biochemical abnormality accompanying diabetes mellitus and also important for vascular injury is the formation of advanced glycation end products (AGEs). Driven by hyperglycemia and oxidant stress, the effects of AGEs on vessel wall homeostasis may account for the rapidly progressive atherosclerosis associated with diabetes [32, 33].

Although the mechanisms underlying this phenomenon are probably multifactorial, the role of the diacylglycerol-protein kinase C (PKC) pathway has recentyl been recognized as very important in in-vivo and in-vitro studies. PKC may have several adverse effects on endothelial function, such as activation of superoxide-producing enzymes like the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and increased expression of a dysfunctional, superoxide-producing, uncoupled endothelial nitric oxide synthase (NOS III). PKC-mediated superoxide production may inactivate NO derived from endothelial NOS III and may inhibit the activity and/or expression of the NO downstream target,

the soluble guanylyl cyclase, resulting in impaired vasomotion properties of the endothelium. Furthermore, within the vessel wall, collagen-linked AGEs may "trap" plasma proteins, interact with specific receptors, and modulate a large number of cellular properties. On plasma low density lipoproteins (LDL), AGEs initiate oxidative reactions that promote the formation of oxidized LDL [32–35].

However, in patients with diabetes, endothelial dysfunction is a coherent finding. There is a general consensus that hyperglycemia and diabetes lead to impaired NO production and damaged vasodilatatory activity [33, 35].

Even more importantly, endothelial cells exposed to chronic hyperglycemia can also lose integrity, progress to senescence, and undergo apoptosis [36, 37].

The outcome of this detrimental process is detachment of endothelial cells, which are released into the circulation. In the bloodstream they can be detected as circulating mature endothelial cells or as their apoptotic microparticles, if endothelial cells did not detach as entire cells [38].

McClung et al. have shown that circulating mature endothelial cell levels are higher in type 2 diabetic patients, irrespective of glucose control represented by HbA_{1c} levels. Elevated endothelial cell-derived endothelial microparticle levels are predictive of the presence of coronary heart disease, and it is an even more significant independent risk factor than presence of diabetes, lipid levels, or hypertension [38–40].

The result of this apoptotic process is arterial denudation, which triggers a cascade of proatherosclerotic processes like platelet adhesion and aggregation, inflammation, smooth muscle cell proliferation, migration, and matrix secretion [38, 41].

4. Endothelial Repair

As already mentioned, structural or functional damage of the endothelium is a result of cumulative exposure to cardiovascular risk factors. These factors have the ability to induce biochemical cellular toxicity and/or promote endothelial cell loss by apoptosis.

The resulting extent of endothelial dysfunction depends not only on the extent of injury, but also on the biologic capacity for repair. For this reason, endothelial reparatory mechanisms are crucial in reestablishing vessel integrity.

Two mechanisms involved in this process have been recently identified.

The endothelium itself has a relatively weak capacity for self-repair, because it is built from mostly terminally differentiated cells with low proliferative capacity. However, mature endothelial cells surrounding the injured locus in the endothelium can replicate in situ and replace lost and damaged cells [38, 42].

Recently, it has become evident that some forms of cells, recruited from the bone marrow and from some other tissues, circulate in the peripheral blood and have the ability to be embedded in the injured endothelium and differentiate into mature cells with endothelial characteristics. These cells were called EPCs, and they seemed to be an important mechanism for maintenance and repair of the endothelium.

Several studies have shown that EPCs may be derived from different sources such as the bone marrow and non-bone marrow organs like the spleen. EPCs are a heterogeneous group of cells presenting in different stages of differentiation in the blood stream. There are at least two different known subtypes of EPCs: the early and late EPCs. Early EPCs occur as colony forming units (CFU) and have some endothelial characteristics, like harboring markers of CD31, TIE2, and VEGFR2. Late EPCs, or outgrowth EPCs, have different growth patterns. Outgrowth EPCs express additional endothelial characteristics, such as VE-cadherin and von Willebrand factor, in addition to CD31, CD133, CD34, and VEGFR2 [4]. These outgrowth EPCs will further differentiate into mature endothelial cells for angiogenesis and vasculogenesis [14, 38, 43]. The ability of EPCs for functional vascular repair varies with the maturation state of the cell [14, 38].

There are some difficulties in precise characterization of EPCs, because some of these cell surface markers are shared with other cell types like hematopoietic stem cells and mature endothelial cells. Currently, EPCs are defined as cells positive for both a hematopoietic stem cell marker such as CD34 and an endothelial marker protein such as VEGFR2 [14, 38, 44].

Mobilization of these cells into the circulation is in a certain magnitude NO-dependent and may be impaired in patients with present cardiovascular risk factors, like diabetes and smoking [45]. There are also several factors responsible for mobilizing EPCs from the bone marrow or other organs into the blood stream, like growth factors and cytokines, including VEGF (vascular endothelial growth factor), SDF-1α (stromal-cell-derived factor-1α), erythropoietin, and estrogens. Interestingly, the number of EPCs seems to be inversely correlated with the severity and degree of atherosclerosis [46, 47].

EPCs migrate toward injured endothelial regions, after they have been mobilized into the circulation. At these places they home or adhere and start to proliferate beginning vascular repair. An important factor in directing circulating progenitor cells to sites of vascular injury is chemokine signaling, like tissue hypoxia induced upregulation of SDF-1α in ischemic tissues. Homing to the injured sites is facilitated through interactions between SDF-1α and CXCR4 (CXC chemokine receptor 4) [48–50].

Once embedded in the injured site, EPCs are involved in endothelial repair either by proliferation and forming new endothelial cells or by releasing provasculogenic cytokines and growth factors important for the proliferation of local mature endothelial cells or other EPCs [48].

Recent evidence has suggested that cardiovascular risk factors interfere not only with the differentiation and function of endothelial progenitor cells, but also with the recruitment of these cells [14, 38, 39, 44, 45].

However, blood flow in ischemic tissues could be enhanced by the increase in the number of endothelial circulating progenitor cells, augmented whether by cell transfusion or induced mobilization, involving mechanisms like enhanced restoration and integrity of the endothelial lining, and neointimal formation [46, 51, 52].

5. Endothelial Repair in Diabetic Patients

Diabetes, hypercholesterolemia, hypertension, and smoking, leading to atherosclerosis, and several other forms of vascular disease are associated with a reduced number and impaired functional activity of circulating EPCs [14, 18, 31, 32, 38].

Apparently, there is a glucometabolic continuum in EPC biology. In particular, all forms of glucose disorders are associated with abnormalities in EPC biology, including impaired fasting glucose and impaired glucose tolerance [47].

Individuals with disorders of glucose metabolism have reduced levels of circulating EPCs. Damaged mobilization, decreased proliferation, and shortened survival in the circulation may contribute to a reduced number of circulating EPCs in diabetic patients. Several mechanisms could be responsible for defects in EPC mobilization, migration and homing of EPC in diabetic patients, including decreased NO bioavailability, defects in intracellular signaling, inflammation and adipokines, reactive oxygen species, and direct effects of insulin and IGF-1 [38, 53, 54].

NO bioavailability and PI3K/Akt signaling are crucial in EPC mobilization from bone marrow. In insulin resistant states both mechanism are damaged [53, 55].

A consistent finding in insulin resistant humans and animals with impaired EPC mobilization and function in experimental models is decreased NO bioavailability. NO is important for the normal function of EPCs after mobilization. A substantial requirement for NO in migration, homing, and neovascularization was shown in in vitro and in vivo studies [53, 55, 56].

Another important mechanism damaged in insulin resistant humans and animals is the PI3K/Akt pathway. This pathway mediates metabolic effects like insulin-stimulated glucose uptake in metabolically active tissues and insulin- or shear-stress induced NO production in endothelial cells. In EPCs, inhibition of this pathway abolishes their mobilization in response to several stimuli [47].

After mobilization, there are more issues limiting the regenerative ability of EPCs in diabetes.

The capacity for effective homing to the injured blood vessel, adhesion and integration into the endothelium, proliferation, and differentiation is crucial for EPCs to promote vascular repair. EPCs from diabetic animals and humans show impaired response to chemotactic stimuli, reduced proliferative potential, and diminished ability to form vascular-like structures in vitro. Hyperglycemia is associated with reduced expression of SDF-1α, a chemokine occurring in injured tissues, and decreased expression of CXCR4 in peripheral mononuclear cells and EPCs. Both may inhibit homing of EPCs from the circulation to the injured endothelium, since adhesion of EPCs to sites of vascular injury is dependent on an interaction between locally produced chemokines and the CXCR4 receptor [48, 49].

Damaged PI3K/Akt signalling has been implicated to impair EPC differentiation and inhibition of EPC apoptosis, like in inflammatory states [57]. Systemic inflammation is known to contribute to atherosclerotic vascular disease by stimulation of proatherogenic adhesion molecules in mature endothelial cells, but it affects also EPC-mediated vascular regeneration. Inflammatory factors impair EPC survival, differentiation, and function. On the contrary, inflammatory mediators released from the injured endothelium stimulate the production of growth factors and cytokines necessary to facilitate EPC release and homing. It is likely that persistent inflammation (even a low level inflammation) has harmful effects, but transient inflammatory response following endothelial injury is associated with EPC mobilization and may be beneficial [47, 58].

Adipocytes are another factor involved in damaged vascular repair in diabetic patients. These metabolic active cells produce several cytokines and contribute to a chronic inflammatory state by secreting the proinflammatory cytokine TNF-α which reduces the proliferation of EPCs [59].

Leptin, another adipokine, increased in vitro tubule formation in EPC cultures, but at higher concentration EPC migration was inhibited. Leptin was also shown to be able to enhance the capacity of EPC to adhere to matured endothelial cells or the extracellular matrix by increasing the expression of specific integrins. These effects led to enhanced reendothelialization when leptin-stimulated human EPCs were given to mice. However, obese insulin resistant humans have increased circulating leptin concentrations and impaired leptin effects and have been assumed to suffer from "leptin resistance" [60, 61].

Reactive oxygen species (ROS) produced in diabetic patients also contribute to endothelial injury and impair endothelial repair. ROS are directly cytotoxic for endothelial cells, react with NO, decrease NO bioavailability, and form peroxynitrite anions which act as powerful oxidants. Although it seems that EPCs from healthy individuals are relatively resistant to oxidant stress, in diabetic patients ROS may lead to EPC impairment. Mechanisms may include increased ROS production and impaired endogenous antioxidant defence [62, 63].

Among other substances, insulin and IGF-1 influence the mobilization and differentiation of EPCs. In a small study of patients with poorly controlled type 2 diabetes, insulin therapy led to an increase in circulating EPCs. Insulin-mediated EPC mobilization was significantly dependent on SDF-1 polymorphism; mobilization was significantly enhanced in subjects with the SDF-1 3′-A/G allele. Insulin stimulates the clonogenic and angiogenic potential of EPCs; the effect is more likely mediated through the IGF-1 receptor than the insulin receptor itself. Humpert and coworkers have demonstrated that insulin stimulates the outgrowth in vitro of EPCs from patients with type 2 diabetes. This effect was completely abrogated by IGF-1 receptor blockade but unaltered after blocking the insulin receptor itself. The IGF-1 receptor-dependent effect of insulin on EPC growth was largely mediated by the MAPKs ERK1/2 (extracellular-signal-regulated kinase) and p38 [64, 65].

6. Current Available Therapeutic Options

New concepts of endothelial injury and repair have offered exciting perspectives for new researches in preventing and/or treating cardiovascular disease. Despite the fact that EPCs are recently discovered, lifestyle interventions and several

drugs successfully used for treatment of diabetes and/or cardiovascular diseases have proven beneficial effects to EPC biology.

Many of those lifestyle and pharmacological interventions already have established favourably cardiovascular benefits [14, 17].

Exercise as a lifestyle modification has a potential to increase the number of endothelial progenitor cells and improve their migratory capacity, helping to repair the damaged endothelium [66–68]. In healthy volunteers, exercise increased the number of EPCs in the circulation [69]. In patients with stable coronary artery disease, an increased number of circulating EPCs and reduced EPC apoptosis were found after 28 days of moderate exercise training [70].

Vascular repair could be enhanced also with nutrition. There is some evidence for favourable effects of polyunsaturated fatty acids from different sources on vascular biology [71–73]. A hypocaloric Mediterranean diet has also been proved as enhancing endothelial repair [74, 75].

Considering current available medical treatment for diabetes and its comorbidities, there are several established classes of drugs with beneficial effects on endothelial repair.

Among these drugs are statins, angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor blockers, some sulphonylureas like gliclazide, metformin, PPAR-gamma agonists, GLP-1, DPP-4 inhibitors, and insulin, which are able to increase the number and/or functional activity of EPCs, affecting different mechanisms, as some experimental and clinical studies have suggested.

Statins are widely used among diabetic and nondiabetic patients as antilipemic drugs, with a profound effect on the incidence of cardiovascular events and mortality. They may also modulate vascular repair through increasing EPC numbers and inducing EPC differentiation by activation of the PI3K pathway and stimulation of NO production, in addition to their primary effects on lipoprotein metabolism [57, 76].

In general, the effect of statins on EPC count was a significant increase occurring shortly after treatment initiation. It seems that it could be a class effect of all statins, since there are many of them studied with similar results, including simvastatin, atorvastatin, pravastatin, and rosuvastatin. Some secondary outcomes like left ventricular (LV) volume, ejection fraction (EF), and flow mediated dilation (FMD) could also be improved in some studies. Furthermore, even herbal products containing lovastatin have the capacity to improve EPC counts. Only one study showed a negative correlation between statin therapy and EPC count, but this study was cross-sectional, on a group of patients receiving various statins. It is still unclear whether the effect of statins is dose dependent, and larger randomized trials are necessary for firmer proof.

An overview of clinical data involving statins treatment and their effects on EPCs is shown in Table 1.

Similarly, beneficial outcomes on EPC biology were found for some antihypertensive drugs with favourable metabolic effects, like ACEIs (angiotensin-converting enzyme inhibitors) and ARBs (angiotensin II type 1 receptor blockers). For both classes of drugs cardiovascular benefits are well established, beyond their antihypertensive effect [94]. It was shown that ACEi and ARB treatment increased numbers and function of EPCs in patients with a variety of cardiovascular diseases, including arterial hypertension, stable coronary artery disease, and acute coronary syndromes. Treatment with these classes of antihypertensive drugs could contribute to their cardioprotective effect in type 2 diabetes, even independent of their action on blood pressure [95] like in a study where both olmesartan and irbesartan increased the numbers of EPCs in a group of patients with type 2 diabetes.

Calcium channel blockers (CCBs) decrease blood pressure by inhibiting L-type voltage-gated calcium channels, leading to a decreased level of intracellular calcium. They act on vascular smooth muscle, inducing vasodilation followed by decreased blood pressure. Preliminary results from two studies reported affirmative outcomes on EPC numbers and function with CCBs in patients with essential hypertension [96, 97].

In addition to data on EPC, in some trials there are also data about improved flow mediated vessel dilatation, markers of inflammation, and in one trial there are also favorable mortality data. Most trials presented affirmative results in regard to EPCs count and/or function, with few exceptions. However, larger trials are needed to get more reliable data.

A summary of currently available clinical data on antihypertensives and their effect on EPC biology is shown in Table 2.

Several classes of agents used for treatment of diabetes are also shown to enhance EPC biology. Among them is metformin, commonly used as first-line treatment in patients with type 2 diabetes and as supplementary treatment in patients with type 1 diabetes. It has also established vasculoprotective effects. These effects could be at least in part explained by increased circulating EPCs after initiating treatment with metformin [107, 108]. The effect was even augmented with addition of gliclazide [108]. Otherwise, positive effects of gliclazide as monotherapy could be also proven; it improved also flow mediated vessel dilatation and some markers of oxidative stress [109].

A further class of antidiabetic medications found to have beneficial effects on vascular repair are thiazolidinediones. Thiazolidinediones are peroxisome proliferator-activated receptors (PPAR) γ agonists and are in clinical use for glucoregulation, but these insulin-sensitizing drugs could also improve some other cardiovascular risk factors. At the time, pioglitazone and rosiglitazone are approved for use by the FDA. Considering EPC biology, rosiglitazone was able to normalize impaired EPC migratory activity and to increase EPC numbers in culture [117]. In patients with type 2 diabetes rosiglitazone was effective in reducing NADPH oxidase activity and thus improving the reendothelialization by EPCs [63]. Pioglitazone was also shown to increase the number and function of EPCs and to decrease EPC apoptosis in animal models [118]. Human studies with pioglitazone were also mainly affirmative [110–113].

Other mechanisms in glucose control affect glucagon like peptide 1 (GLP-1) and its analogues and inhibitors of degradation.

Table 1

Reference	Study drug	Study population and design	Study duration	Findings
Vasa et al. [77]	Atorvastatin 40 mg/day	15 patients with coronary artery disease, no control group	4 weeks	1.5-fold ↑ in EPC count after 1 week 3-fold ↑ in EPC count after 4 weeks ↑ EPC functional activity
del Papa et al. [78]	Simvastatin 20 mg/day	40 patients (20 hypercholesterolemic versus 20 normocholesterolemic patients with systemic sclerosis)	4 weeks	Simvastatin ↑ EPC count in patients without systemic sclerosis In patients with systemic sclerosis there was an attenuated response, mainly in patients with late disease
Westerweel et al. [79]	Simvastatin 80 mg/day versus simvastatin 10 mg/ezetimibe 10 mg/day	20 obese patients with metabolic syndrome, randomized trial, crossover design	6 + 6 weeks	↑ EPC counts in both groups
Pesaro et al. [80]	Simvastatin 80 mg/day versus simvastatin 20 mg/ezetimibe 10 mg/day	68 patients with LDL levels >70 mg/dL pretreated with simvastatin 20 mg, randomized trial	6 weeks	No effect on EPC count in either group Similar reduction in LDL levels in both groups
Hibbert et al. [81]	Atorvastatin 80 mg/day versus no statin	20 male patients undergoing angiography for stent placement randomized to atorvastatin or no statin treatment	4 days	3.5-fold ↑ in EPC count in the statin group
Baran et al. [82]	Atorvastatin 40 mg/day versus placebo	60 patients undergoing first-time CABG, placebo controlled, randomized double-blind study	14 days	↑ EPC count in atorvastatin group ↓ incidence of postoperative atrial fibrillation in the statin group
Sobrino et al. [83]	Atorvastatin 20 mg/day	48 patients with first ever nonlacunar ischaemic Stroke 16 patients receiving atorvastatin during the first 4 days	7 days	↑ EPC count in atorvastatin group Effect probably due to NO related mechanisms
Huang et al. [84]	Atorvastatin 40 mg/day versus atorvastatin 10 mg/day	100 patients with ischemic cardiomyopathy randomized to 10 mg or 40 mg of atorvastatin Control group: 100 healthy volunteers	1 year	40 mg of atorvastatin had a more profound ↑ in EPC count Higher dose of atorvastatin was associated with a more marked ↓ in total and LDL cholesterol, hsCRP, oxLDL, and circulating endothelial microparticles
Spadaccio et al. [85]	Atorvastatin 20 mg/day versus placebo	50 patients undergoing elective coronary surgery, randomized crossover trial	3 weeks	↑ EPC count in atorvastatin group SDF-1α, CSF, and VEGF unaffected
Leone et al. [86]	Atorvastatin 80 mg/day immediately versus 20 mg/day atorvastatin after hospital discharge	40 patients with AMI undergoing PCI, randomized trial	4 months	Larger dose of atorvastatin related to larger ↑ EPC count LV volume, EF, and wall motion were similar in both groups after study completion
Lu et al. [87]	Pravastatin versus placebo versus Xuezhikang	88 patients with essential hypertension, randomized trial	8 weeks	↑ EPC count and proliferative ability in pravastatin and Xuezhikang (contains lovastatin) group

Table 1: Continued.

Reference	Study drug	Study population and design	Study duration	Findings
Paradisi et al. [88]	Pravastatin 40 mg/day	20 patients, healthy postmenopausal women, randomized, double-blind trial	8 weeks	↑ EPC colony forming units ↓ count of senescent cells
Tousoulis et al. [89]	Rosuvastatin 10 mg/day	60 patients with systolic heart failure, randomized trial	1 month	↑ EPC count improved No change in inflammatory and oxidative markers
Erbs et al. [90]	Rosuvastatin 40 mg/day versus placebo	42 patients with chronic heart failure, randomized trial	12 weeks	↑ EPC count ↑ homing of EPC ↑ FMD, ↑VEGF
Yoshida et al. [91]	Pitavastatin 2 mg/day versus placebo	30 male smokers, randomized trial	4 weeks	No effect on EPC count ↑ FMD, ↓ markers of oxidative stress in pitavastatin group
Spiel et al. [92]	Simvastatin 80 mg/day versus rosuvastatin 10 mg/day versus placebo	6 healthy volunteers, randomized, double-blind, placebo controlled, crossover study	5 days	3-fold ↑ EPC count in statin groups Class effect?
Hristov et al. [93]	Low dose of statins (10/20 mg/day) versus high dose (40 mg/day) versus untreated	209 CAD patients (without statin: 65, low dose statin: 101, and higher dose statin: 43 patients) cross-sectional study	None	40 mg/d of statin treatment has significantly ↓ EPC count Lower doses had no impact on EPC count Continuous statin therapy inversely correlated with EPC numbers

GLP-1 is a hormone, released from enteroendocrine cells in the intestine, and has been shown to exert cardiovascular protective effects. Results indicated that GLP-1 improves VEGF generation, which contributed to improvement of EPCs biological function. VEGF is a necessary mediator of the effects of GLP-1 on EPCs [119]. Interestingly, there are no data about GLP injectables and EPCs number and function in diabetic patients.

Dipeptidyl peptidase 4 (DPP 4) inhibitors are a recently introduced class of oral hypoglycemic agents. There is one study that showed an increase in EPC number in patients treated with sitagliptin after 4 weeks of treatment in comparison to metformin. In addition, plasma stromal-derived factor-1α (SDF-1α) levels also increased in sitagliptin treated patients, leading to enhanced EPC release from bone marrow [114]. In another study sitagliptin improved both SDF-1α levels and flow mediated dilatation. In this study voglibose was used as active comparator but with no effect on EPC biology despite its positive effect on blood vessels dilation [115].

In subjects with type 1 diabetes and in patients with type 2 diabetes who fail to respond adequately to oral therapies, insulin is used to achieve glycaemic control. Although there is some evidence that short-term insulin treatment and tight blood glucose control decrease adverse cardiovascular events after myocardial infarction, studies have failed to show superiority of insulin in comparison to other drugs used for glucose control on the long term [120]. However, as discussed previously, insulin and certain insulin analogues have been shown to mobilize EPCs and improve EPC parameters in vitro [47]. In patients with type 2 diabetes mellitus, long acting insulin analogues glargine and detemir were able to raise the EPC count, with no significant difference between both drugs. Differences were noticed in the number of hypoglycemic events and weight gain, in favour of insulin detemir [116].

Studies on effects of antidiabetic treatment on EPCs are listed in Table 3.

Some other hormones occasionally used in the treatment of specific subgroups of diabetic patient may also improve vascular repair, like estrogens and erythropoietin.

Estrogens are shown to be effective in mobilizing EPCs and reducing neointima formation after arterial injury in animals [121]. These effects are NOS-mediated and depend on FGF-2 (fibroblast growth factor-2) activity. Furthermore, in healthy fertile women, EPCs are mobilized cyclically in response to raising estrogens during the menstrual cycle, providing an interesting explanation for gender differences in cardiovascular risk [47, 122].

Erythropoietin, a kidney hormone that controls erythropoiesis, was also expected to be beneficial in improving vascular repair in humans but without definitive proof yet. In a trial on patients with ST-elevation acute myocardial infarction there was only a nonsignificant improvement in EPC count after a single dose of erythropoietin in the acute phase, with no impact on infarct size [123].

Table 2

Reference	Study drugs	Study population and design	Study duration	Findings
Cacciatore et al. [98]	Enalapril 20 mg/day versus zofenopril 30 mg/day,	36 patients with newly diagnosed mild hypertension, randomized trial	5 years	↑ EPC count No difference between groups ↓ intima media thickness
Sun et al. [99]	Perindopril 4 mg/day versus placebo	68 patients with acute myocardial infarction and T2DM	28 days after PCI	↑ EPC count ↑ VEGF ↑ SDF-1α ↑ LVF ↓CV mortality in the perindopril group
Min et al. [100]	Ramipril 5 mg/day	36 nondiabetic patients with acute myocardial infarction	4 weeks	↑ EPC count 1.5-fold after 1 week, 2.5-fold after 4 weeks ↑ EPC proliferation, migration, and adhesion
Cangiano et al. [101]	Perindopril 10 mg/day versus valsartan 320 m/day	Patients with acute coronary syndromes 16 receiving perindopril 17 receiving valsartan 20 healthy controls	30 days	↑ EPC mobilization, ↑ VEGF in the perindopril group No effects found for valsartan
Porto et al. [102]	Ramipril 5 mg/day versus telmisartan 80 mg/day	42 patients with acute coronary syndrome, randomized trial	20 days after PCI	↑ EPC count in both groups Telmisartan had a more profound anti-inflammatory effect
Pelliccia et al. [103]	Telmisartan 40 mg/day versus placebo	40 normotensive patients with CAD, randomized trial	4 weeks	↑ EPC count ↑ FMD
Bahlmann et al. [95]	Olmesartan 40 mg/day versus placebo, double-blind RCT Irbesartan 300 mg/day, open trial	18 patients with T2DM randomized to olmesartan or placebo 20 patients with T2DM receiving irbesartan	12 weeks	↑ EPC count with both olmesartan and irbesartan
Tan et al. [104]	Losartan 100 mg/day			↑ EPC count ↑ FMD
Suzuki et al. [105]	Losartan 50 mg/day versus trichlormethiazide 4 mg/day	36 patients with hypertension randomized to losartan or trichlormethiazide Control group: 18 normotensive patients	4 weeks	↑ EPC count with losartan Hypertensive patients had a lower EPC count in comparison to normotensive patients
Kampoli et al. [106]	Pioglitazone (15 m/day) versus perindopril (4 mg/day)	50 patients with T2DM, randomized trial	1 month	No effect on EPC
Sugiura et al. [96]	Nifedipine SR 20 mg/day versus placebo	37 hypertensive patients with stage I hypertension, randomized trial	4 weeks	↑ EPC count ↑ EPC differentiation migration, resistance to oxidative stress ↑ FMD
de Ciuceis et al. [97]	Barnidipine 20 mg/day versus hydrochlorothiazide 25 mg/day	29 hypertensive patients with mild essential hypertension, randomized trial	6 months	↑ EPC count with barnidipine No difference in RR reduction observed between drugs

7. Current Limitations and Future Perspectives

The discovery of circulating endothelial progenitor cells has challenged current concepts about the genesis and treatment of atherosclerosis and has unveiled very wide experimental and clinical perspectives. With respect to prominent medical, social, and economic impact of diabetic cardiovascular complications, researchers and clinicians involved in diabetology should not remain indifferent to these novel findings.

Since their discovery in 1997, accumulating findings suggest that EPCs promote postnatal vasculogenesis in adults, important for vascular repair of the injured endothelium, opening the way for new therapies of cardiovascular diseases focused on EPCs.

Despite these encouraging prospects, there are still issues and limitations that need to be addressed [124].

A special challenge is that patient groups who would gain the greatest benefit from new EPC based clinical concepts,

Table 3

Reference	Study drug	Study population and design	Study duration	Study findings
Chen et al. [109]	Gliclazide 30–90 g/day	33 patients with newly diagnosed T2DM versus 25 nondiabetic patients in the control group	12 weeks	↑ EPC count ↑ flow mediated dilatation ↓ some markers of oxidative stress in study group
Chen et al. [108]	Gliclazide (30–60 g/day) and metformin (250–1000 mg/day) versus metformin (500–2500 mg/day)	47 patients with newly diagnosed T2DM, randomized trial	16 weeks	more profound ↑ EPC count and function with combination treatment
Liao et al. [107]	Metformin (1700–2550 mg/day)	46 patients with newly diagnosed T2DM versus 51 healthy controls	16 weeks	↑ EPC count in both groups T2DM patients had a lower EPC count throughout the study ↑ FMD changed in both groups
Werner et al. [110]	Pioglitazone 45 mg/day versus placebo	54 patients without T2DM, with stable CAD, randomized trial	30 days	↑ EPC count ↑ migratory activity of EPCs ↑ clonogenic potential of EPCs after pioglitazone treatment
Wang et al. [111]	Pioglitazone 30 mg/day	24 patients with T2DM receiving pioglitazone versus 12 patients with T2DM receiving metformin, randomized trial	8 weeks	↑ EPC count and homing and decreased ↓ EPC apoptosis, ↓ hsCRP, ↓ triglycerides, ↓ LDL, ↑ HDL cholesterol, and ↑ insulin sensitivity after pioglitazone treatment No change in FMD
Makino et al. [112]	Pioglitazone (15–30 mg/day)	34 patients with T2DM	24 weeks	↑ EPC count ↑adiponectin ↓ hsCRP
Esposito et al. [113]	Pioglitazone (15–45 mg/day) versus metformin (1000–2000 mg/day)	110 patients with newly diagnosed T2DM, randomized trial	24 weeks	More profound ↑ EPC count ↑ weight ↑ HDL ↑ adiponectin ↓ CRP ↓ triglycerides in patients receiving pioglitazone
Kampoli et al. [106]	Pioglitazone (15 m/day) versus perindopril (4 mg/day)	50 patients with T2DM, randomized trial	1 month	No effect on EPC count Improved markers of inflammation and oxidative stress
Fadini et al. [114]	Sitagliptin 100 mg/day versus no additional treatment	16 patients with T2DM receiving sitagliptin, 16 patients with T2DM with no additional treatment, controlled, nonrandomized trial	4 weeks	↑ EPC count ↑ SDF-1α in the study group
Nakamura et al. [115]	Sitagliptin (50 m/day) versus voglibose (0,6 mg/day)	66 patients with T2DM, 31 patients with T2DM, receiving sitagliptin, 35 patients with T2DM receiving voglibose	12 weeks	↑ EPC count with sitagliptin ↑ FMD in both groups, no difference between groups
Fadini et al. [116]	Insulin detemir versus insulin glargine	42 patients with T2DM and macroangiopathy, randomized crossover study	6 months	↑ EPC count increased between month 3 and month 6 in both groups ↑ weight gain and ↑ hypoglycemic events with glargine

like diabetic patients, are the same in whom different problems with decreased EPC number and their dysfunction have been clearly demonstrated [124, 125]. This problem limits approaches based on EPCs isolation, cultivation, and autologous transplantation in diabetic patients. In fact, new insights on reduced EPC counts and EPC dysfunction in diabetic patients will maybe allow us to introduce diabetic osteomyelopathy as a new, but very important, diabetic chronic complication.

Therefore, it is necessary to put more efforts into understanding mechanisms of EPC dysfunction and then to design new strategies to improve EPC function ex vivo before therapeutic transplantation.

Similar problems face other groups of patients, like elderly people. EPCs derived from aged individuals also show a reduced capacity to proliferate, home into existing capillary networks, and enhance perfusion like in diabetic patients [124–126].

Another problem is how to obtain adequate numbers of those cells. Furthermore, every autologous delivery of cells inevitably involves a considerable time delay in treatment, due to the time needed for collection, identification, isolation, and then propagation of progenitors ex vivo [124].

Adverse effects of endothelial progenitor cell delivery may include microvascular embolism and unintended acceleration of pathological neovascularization in malignancies. Undirected growth and possible undesired pathological differentiation after transplantation of those cells may generate risks of late complication, like teratoma. In fact, vigorous differentiation and purification protocols are needed, and studies proving the long-term safety profile of progenitor cells are required before widespread use in humans [124].

It seems to be more likely that a greater impact on general health could have concepts focused on prophylactic measures like lifestyle changes and/or pharmacological interventions with drugs specially designed to address EPC proliferation, migration, and homing to injured vessels.

8. Conclusion

Studies have proven the prognostic significance of endothelial function, which is most often clinically demonstrated as the vasodilator response to various pharmacological or mechanical stimulations. Endothelial dysfunction may occur over time, progressing to atherosclerotic plaques and clinical apparent vascular disease.

Since endothelial injury and dysfunction precede clinically significant atherosclerotic vascular disease and play a role in its pathogenesis, the discovery of EPCs has provided a new concept of vascular disease as potentially preventable and curable, offering new strategies for medical intervention.

Since EPCs are of extreme importance for reendothelialization of the injured endothelium, promoting vascular repair may be an attractive therapuetic approach. Maintaining normal EPC numbers and function seems to be crucial in preventing cardiovascular diseases.

There are still unresolved questions and many challenges to face before EPC based therapies will be widely used, but even with contemporary medical interventions the number and function of EPC may be improved. This is especially important for patients with metabolic alterations such as compensatory hyperinsulinemia, impaired fasting glucose, and IGT, whose EPC number is decreased and function is impaired.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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References

[1] M. C. Riddle, "Glycemic control and cardiovascular mortality," *Current Opinion in Endocrinology, Diabetes & Obesity*, vol. 18, no. 2, pp. 104–109, 2011.

[2] L. G. Mellbin, M. Anselmino, and L. Rydén, "Diabetes, prediabetes and cardiovascular risk," *European Journal of Cardiovascular Prevention and Rehabilitation*, vol. 17, supplement 1, pp. S9–S14, 2010.

[3] I. Martín-Timón, C. Sevillano-Collantes, A. Segura-Galindo, and F. J. del Cañizo-Gómez, "Type 2 diabetes and cardiovascular disease: have all risk factors the same strength?" *World Journal of Diabetes*, vol. 5, no. 4, pp. 444–470, 2014.

[4] R. Stöhr and M. Federici, "Insulin resistance and atherosclerosis: convergence between metabolic pathways and inflammatory nodes," *Biochemical Journal*, vol. 454, no. 1, pp. 1–11, 2013.

[5] M. M. Polovina and T. S. Potpara, "Endothelial dysfunction in metabolic and vascular disorders," *Postgraduate Medicine*, vol. 126, no. 2, pp. 38–53, 2014.

[6] Y. Imai, A. D. Dobrian, J. R. Weaver et al., "Interaction between cytokines and inflammatory cells in islet dysfunction, insulin resistance and vascular disease," *Diabetes, Obesity and Metabolism*, vol. 15, supplement 3, pp. 117–129, 2013.

[7] J. L. Johnson, "Emerging regulators of vascular smooth muscle cell function in the development and progression of atherosclerosis," *Cardiovascular Research*, vol. 103, no. 4, pp. 452–460, 2014.

[8] J. F. Bentzon, F. Otsuka, R. Virmani, and E. Falk, "Mechanisms of plaque formation and rupture," *Circulation Research*, vol. 114, no. 12, pp. 1852–1866, 2014.

[9] Z. Sun, "Atherosclerosis and atheroma plaque rupture: normal anatomy of vasa vasorum and their role associated with atherosclerosis," *The Scientific World Journal*, vol. 2014, Article ID 285058, 6 pages, 2014.

[10] A. Sannino, L. Brevetti, G. Giugliano et al., "Non-invasive vulnerable plaque imaging: how do we know that treatment works?" *European Heart Journal—Cardiovascular Imaging*, vol. 15, no. 11, pp. 1194–1202, 2014.

[11] J. E. Feig, "Regression of atherosclerosis: insights from animal and clinical studies," *Annals of Global Health*, vol. 80, no. 1, pp. 13–23, 2014.

[12] R. W. Wissler and D. Vesselinovitch, "Brief overview of the mounting evidence that atherosclerosis is both preventable and

reversible," *Journal of Clinical Apheresis*, vol. 4, no. 2-3, pp. 52–58, 1988.

[13] P. Constantinides, "Overview of studies on regression of atherosclerosis," *Artery*, vol. 9, no. 1, pp. 30–43, 1981.

[14] P. S. S. Lee and K. K. Poh, "Endothelial progenitor cells in cardiovascular diseases," *World Journal of Stem Cells*, vol. 6, no. 3, pp. 355–366, 2014.

[15] L. Zhang and Q. Xu, "Stem/progenitor cells in vascular regeneration," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 6, pp. 1114–1119, 2014.

[16] E. Mannarino and M. Pirro, "Endothelial injury and repair: a novel theory for atherosclerosis," *Angiology*, vol. 59, no. 2, supplement, pp. 69S–72S, 2008.

[17] C. Besler, C. Doerries, G. Giannotti, T. F. Lüscher, and U. Landmesser, "Pharmacological approaches to improve endothelial repair mechanisms," *Expert Review of Cardiovascular Therapy*, vol. 6, no. 8, pp. 1071–1082, 2008.

[18] K.-H. Yiu and H.-F. Tse, "Specific role of impaired glucose metabolism and diabetes mellitus in endothelial progenitor cell characteristics and function," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 6, pp. 1136–1143, 2014.

[19] P. Rajendran, T. Rengarajan, J. Thangavel et al., "The vascular endothelium and human diseases," *International Journal of Biological Sciences*, vol. 9, no. 10, pp. 1057–1069, 2013.

[20] J. Xu and M. H. Zou, "Molecular insights and therapeutic targets for diabetic endothelial dysfunction," *Circulation*, vol. 120, no. 13, pp. 1266–1286, 2009.

[21] C. Aalkjær, D. Boedtkjer, and V. Matchkov, "Vasomotion—what is currently thought?" *Acta Physiologica*, vol. 202, no. 3, pp. 253–269, 2011.

[22] J. E. Deanfield, J. P. Halcox, and T. J. Rabelink, "Endothelial function and dysfunction: testing and clinical relevance," *Circulation*, vol. 115, no. 10, pp. 1285–1295, 2007.

[23] R. H. Böger, "The pharmacodynamics of L-arginine," *Alternative Therapies in Health and Medicine*, vol. 20, no. 3, pp. 48–54, 2014.

[24] J. Lei, Y. Vodovotz, E. Tzeng, and T. R. Billiar, "Nitric oxide, a protective molecule in the cardiovascular system," *Nitric Oxide*, vol. 35, pp. 175–185, 2013.

[25] M. Félétou and P. M. Vanhoutte, "Endothelium-derived hyperpolarizing factor: where are we now?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 6, pp. 1215–1225, 2006.

[26] L. Stoner, M. L. Erickson, J. M. Young et al., "There's more to flow-mediated dilation than nitric oxide," *Journal of Atherosclerosis and Thrombosis*, vol. 19, no. 7, pp. 589–600, 2012.

[27] A. J. Donato, G. L. Pierce, L. A. Lesniewski, and D. R. Seals, "Role of NFkappaB in age-related vascular endothelial dysfunction in humans," *Aging*, vol. 1, no. 8, pp. 678–680, 2009.

[28] I. Sudano, S. Roas, and G. Noll, "Vascular abnormalities in essential hypertension," *Current Pharmaceutical Design*, vol. 17, no. 28, pp. 3039–3044, 2011.

[29] M. Pacurari, R. Kafoury, P. B. Tchounwou, and K. Ndebele, "The renin-angiotensin-aldosterone system in vascular inflammation and remodeling," *International Journal of Inflammation*, vol. 2014, Article ID 689360, 13 pages, 2014.

[30] R. Madonna and R. de Caterina, "Cellular and molecular mechanisms of vascular injury in diabetes—part II: cellular mechanisms and therapeutic targets," *Vascular Pharmacology*, vol. 54, no. 3–6, pp. 75–79, 2011.

[31] A. C. Roberts and K. E. Porter, "Cellular and molecular mechanisms of endothelial dysfunction in diabetes," *Diabetes & Vascular Disease Research*, vol. 10, no. 6, pp. 472–482, 2013.

[32] M. M. Hartge, T. Unger, and U. Kintscher, "The endothelium and vascular inflammation in diabetes," *Diabetes and Vascular Disease Research*, vol. 4, no. 2, pp. 84–88, 2007.

[33] H. A. R. Hadi and J. A. Al Suwaidi, "Endothelial dysfunction in diabetes mellitus," *Vascular Health and Risk Management*, vol. 3, no. 6, pp. 853–876, 2007.

[34] P. Geraldes and G. L. King, "Activation of protein kinase C isoforms and its impact on diabetic complications," *Circulation Research*, vol. 106, no. 8, pp. 1319–1331, 2010.

[35] C. Manrique, G. Lastra, and J. R. Sowers, "New insights into insulin action and resistance in the vasculature," *Annals of the New York Academy of Sciences*, vol. 1311, no. 1, pp. 138–150, 2014.

[36] X. F. Liu, J. Q. Yu, R. Dalan, A. Q. Liu, and K. Q. Luo, "Biological factors in plasma from diabetes mellitus patients enhance hyperglycaemia and pulsatile shear stress-induced endothelial cell apoptosis," *Integrative Biology*, vol. 6, no. 5, pp. 511–522, 2014.

[37] L. Piconi, L. Quagliaro, R. Assaloni et al., "Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction," *Diabetes/Metabolism Research and Reviews*, vol. 22, no. 3, pp. 198–203, 2006.

[38] A. Avogaro, M. Albiero, L. Menegazzo, S. de Kreutzenberg, and G. P. Fadini, "Endothelial dysfunction in diabetes: the role of reparatory mechanisms," *Diabetes Care*, vol. 34, supplement 2, pp. S285–S290, 2011.

[39] J. A. McClung, N. Naseer, M. Saleem et al., "Circulating endothelial cells are elevated in patients with type 2 diabetes mellitus independently of HbA1c," *Diabetologia*, vol. 48, no. 2, pp. 345–350, 2005.

[40] S. Nomura, "Dynamic role of microparticles in type 2 diabetes mellitus," *Current Diabetes Reviews*, vol. 5, no. 4, pp. 245–251, 2009.

[41] A. A. Alsheikh-Ali, G. D. Kitsios, E. M. Balk, J. Lau, and S. Ip, "The vulnerable atherosclerotic plaque: scope of the literature," *Annals of Internal Medicine*, vol. 153, no. 6, pp. 387–395, 2010.

[42] V. J. Dzau, M. Gnecchi, A. S. Pachori, F. Morello, and L. G. Melo, "Therapeutic potential of endothelial progenitor cells in cardiovascular diseases," *Hypertension*, vol. 46, no. 1, pp. 7–18, 2005.

[43] T. Asahara, A. Kawamoto, and H. Masuda, "Concise review: circulating endothelial progenitor cells for vascular medicine," *Stem Cells*, vol. 29, no. 11, pp. 1650–1655, 2011.

[44] T. Resch, A. Pircher, C. M. Kähler, J. Pratschke, and W. Hilbe, "Endothelial progenitor cells: current issues on characterization and challenging clinical applications," *Stem Cell Reviews and Reports*, vol. 8, no. 3, pp. 926–939, 2012.

[45] B. Özüyaman, P. Ebner, U. Niesler et al., "Nitric oxide differentially regulates proliferation and mobilization of endothelial progenitor cells but not of hematopoietic stem cells," *Thrombosis and Haemostasis*, vol. 94, no. 4, pp. 770–772, 2005.

[46] A. Zampetaki, J. P. Kirton, and Q. Xu, "Vascular repair by endothelial progenitor cells," *Cardiovascular Research*, vol. 78, no. 3, pp. 413–421, 2008.

[47] R. M. Cubbon, M. B. Kahn, and S. B. Wheatcroft, "Effects of insulin resistance on endothelial progenitor cells and vascular repair," *Clinical Science*, vol. 117, no. 5, pp. 173–190, 2009.

[48] R. C. Rennert, M. Sorkin, R. K. Garg, and G. C. Gurtner, "Stem cell recruitment after injury: lessons for regenerative medicine," *Regenerative Medicine*, vol. 7, no. 6, pp. 833–850, 2012.

[49] S.-W. Youn, S.-W. Lee, J. Lee et al., "COMP-Ang1 stimulates HIF-1α-mediated SDF-1 overexpression and recovers ischemic

injury through BM-derived progenitor cell recruitment," *Blood*, vol. 117, no. 16, pp. 4376–4386, 2011.

[50] X. Liu, B. Duan, Z. Cheng et al., "SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion," *Protein and Cell*, vol. 2, no. 10, pp. 845–854, 2011.

[51] S. Wassmann, N. Werner, T. Czech, and G. Nickenig, "Improvement of endothelial function by systemic transfusion of vascular progenitor cells," *Circulation Research*, vol. 99, no. 8, pp. e74–e83, 2006.

[52] J. I. Rotmans, J. M. M. Heyligers, H. J. M. Verhagen et al., "In vivo cell seeding with anti-CD34 antibodies successfully accelerates endothelialization but stimulates intimal hyperplasia in porcine arteriovenous expanded polytetrafluoroethylene grafts," *Circulation*, vol. 112, no. 1, pp. 12–18, 2005.

[53] G. P. Fadini, S. Sartore, C. Agostini, and A. Avogaro, "Significance of endothelial progenitor cells in subjects with diabetes," *Diabetes Care*, vol. 30, no. 5, pp. 1305–1313, 2007.

[54] C. G. Egan, R. Lavery, F. Caporali et al., "Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes," *Diabetologia*, vol. 51, no. 7, pp. 1296–1305, 2008.

[55] G. P. Fadini, S. Sartore, M. Schiavon et al., "Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats," *Diabetologia*, vol. 49, no. 12, pp. 3075–3084, 2006.

[56] A. Aicher, C. Heeschen, and S. Dimmeler, "The role of NOS3 in stem cell mobilization," *Trends in Molecular Medicine*, vol. 10, no. 9, pp. 421–425, 2004.

[57] C. V. Desouza, F. G. Hamel, K. Bidasee, and K. O'Connell, "Role of inflammation and insulin resistance in endothelial progenitor cell dysfunction," *Diabetes*, vol. 60, no. 4, pp. 1286–1294, 2011.

[58] T. J. Rabelink, H. C. de Boer, E. J. P. de Koning, and A.-J. van Zonneveld, "Endothelial progenitor cells: more than an inflammatory response?" *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 5, pp. 834–838, 2004.

[59] H. Tilg and A. R. Moschen, "Inflammatory mechanisms in the regulation of insulin resistance," *Molecular Medicine*, vol. 14, no. 3-4, pp. 222–231, 2008.

[60] R. Wolk, A. Deb, N. M. Caplice, and V. K. Somers, "Leptin receptor and functional effects of leptin in human endothelial progenitor cells," *Atherosclerosis*, vol. 183, no. 1, pp. 131–139, 2005.

[61] M. R. Schroeter, M. Leifheit, P. Sudholt et al., "Leptin enhances the recruitment of endothelial progenitor cells into neointimal lesions after vascular injury by promoting integrin-mediated adhesion," *Circulation Research*, vol. 103, no. 5, pp. 536–544, 2008.

[62] T. Thum, D. Fraccarollo, S. Thum et al., "Differential effects of organic nitrates on endothelial progenitor cells are determined by oxidative stress," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 4, pp. 748–754, 2007.

[63] S. A. Sorrentino, F. H. Bahlmann, C. Besler et al., "Oxidant stress impairs in vivo reendothelialization capacity of endothelial progenitor cells from patients with type 2 diabetes mellitus: restoration by the peroxisome proliferator-activated receptor-γ agonist rosiglitazone," *Circulation*, vol. 116, no. 2, pp. 163–173, 2007.

[64] P. M. Humpert, R. Neuwirth, M. J. Battista et al., "SDF-1 genotype influences insulin-dependent mobilization of adult progenitor cells in type 2 diabetes," *Diabetes Care*, vol. 28, no. 4, pp. 934–936, 2005.

[65] P. M. Humpert, Z. Djuric, U. Zeuge et al., "Insulin stimulates the clonogenic potential of angiogenic endothelial progenitor cells by IGF-1 receptor-dependent signaling," *Molecular Medicine*, vol. 14, no. 5-6, pp. 301–308, 2008.

[66] S. Möbius-Winkler, A. Linke, V. Adams, G. Schuler, and S. Erbs, "How to improve endothelial repair mechanisms: the lifestyle approach," *Expert Review of Cardiovascular Therapy*, vol. 8, no. 4, pp. 573–580, 2010.

[67] K. A. Volaklis, S. P. Tokmakidis, and M. Halle, "Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and diseased patients," *Clinical Research in Cardiology*, vol. 102, no. 4, pp. 249–257, 2013.

[68] J.-S. Wang, M.-Y. Lee, H.-Y. Lien, and T.-P. Weng, "Hypoxic exercise training improves cardiac/muscular hemodynamics and is associated with modulated circulating progenitor cells in sedentary men," *International Journal of Cardiology*, vol. 170, no. 3, pp. 315–323, 2014.

[69] U. Laufs, A. Urhausen, N. Werner et al., "Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects," *European Journal of Cardiovascular Prevention and Rehabilitation*, vol. 12, no. 4, pp. 407–414, 2005.

[70] M. Sandri, V. Adams, S. Gielen et al., "Effects of exercise and ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes: results of 3 randomized studies," *Circulation*, vol. 111, no. 25, pp. 3391–3399, 2005.

[71] M. Keith, M. A. Kuliszewski, C. Liao et al., "A modified portfolio diet complements medical management to reduce cardiovascular risk factors in diabetic patients with coronary artery disease," *Clinical Nutrition*, 2014.

[72] V. Spigoni, C. Lombardi, M. Cito et al., "N-3 PUFA increase bioavailability and function of endothelial progenitor cells," *Food & Function*, vol. 5, no. 8, pp. 1881–1890, 2014.

[73] J. Turgeon, S. Dussault, F. Maingrette et al., "Fish oil-enriched diet protects against ischemia by improving angiogenesis, endothelial progenitor cell function and postnatal neovascularization," *Atherosclerosis*, vol. 229, no. 2, pp. 295–303, 2013.

[74] J. M. Fernández, D. Rosado-Álvarez, M. E. Da Silva Grigoletto et al., "Moderate-to-high-intensity training and a hypocaloric Mediterranean diet enhance endothelial progenitor cells and fitness in subjects with the metabolic syndrome," *Clinical Science*, vol. 123, no. 6, pp. 361–373, 2012.

[75] C. Marin, R. Ramirez, J. Delgado-Lista et al., "Mediterranean diet reduces endothelial damage and improves the regenerative capacity of endothelium," *The American Journal of Clinical Nutrition*, vol. 93, no. 2, pp. 267–274, 2011.

[76] B. Hibbert, T. Simard, F. D. Ramirez et al., "The effect of statins on circulating endothelial progenitor cells in humans: a systematic review," *Journal of Cardiovascular Pharmacology*, vol. 62, no. 5, pp. 491–496, 2013.

[77] M. Vasa, S. Fichtlscherer, K. Adler et al., "Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease," *Circulation*, vol. 103, no. 24, pp. 2885–2890, 2001.

[78] N. del Papa, M. Cortiana, C. Vitali et al., "Simvastatin reduces endothelial activation and damage but is partially ineffective in inducing endothelial repair in systemic sclerosis," *The Journal of Rheumatology*, vol. 35, no. 7, pp. 1323–1328, 2008.

[79] P. E. Westerweel, F. L. J. Visseren, G. R. Hajer et al., "Endothelial progenitor cell levels in obese men with the metabolic syndrome and the effect of simvastatin monotherapy vs. simvastatin/ezetimibe combination therapy," *European Heart Journal*, vol. 29, no. 22, pp. 2808–2817, 2008.

[80] A. E. P. Pesaro, C. V. Serrano Jr., M. Katz et al., "Increasing doses of simvastatin versus combined ezetimibe/simvastatin: effect on circulating endothelial progenitor cells," *Journal of Cardiovascular Pharmacology and Therapeutics*, vol. 18, no. 5, pp. 447–452, 2013.

[81] B. Hibbert, X. Ma, A. Pourdjabbar et al., "Pre-procedural Atorvastatin mobilizes endothelial progenitor cells: clues to the salutary effects of statins on healing of stented human arteries," *PLoS ONE*, vol. 6, no. 1, Article ID e16413, 2011.

[82] Ç. Baran, S. Durdu, K. Dalva et al., "Effects of preoperative short term use of atorvastatin on endothelial progenitor cells after coronary surgery: a randomized, controlled trial," *Stem Cell Reviews and Reports*, vol. 8, no. 3, pp. 963–971, 2012.

[83] T. Sobrino, M. Blanco, M. Pérez-Mato, M. Rodríguez-Yáñez, and J. Castillo, "Increased levels of circulating endothelial progenitor cells in patients with ischaemic stroke treated with statins during acute phase," *European Journal of Neurology*, vol. 19, no. 12, pp. 1539–1546, 2012.

[84] B. Huang, Y. Cheng, Q. Xie et al., "Effect of 40 mg versus 10 mg of atorvastatin on oxidized low-density lipoprotein, high-sensitivity c-reactive protein, circulating endothelial-derived microparticles, and endothelial progenitor cells in patients with ischemic cardiomyopathy," *Clinical Cardiology*, vol. 35, no. 2, pp. 125–130, 2012.

[85] C. Spadaccio, F. Pollari, A. Casacalenda et al., "Atorvastatin increases the number of endothelial progenitor cells after cardiac surgery: a randomized control study," *Journal of Cardiovascular Pharmacology*, vol. 55, no. 1, pp. 30–38, 2010.

[86] A. M. Leone, S. Rutella, M. B. Giannico et al., "Effect of intensive vs standard statin therapy on endothelial progenitor cells and left ventricular function in patients with acute myocardial infarction: statins for regeneration after acute myocardial infarction and PCI (STRAP) trial," *International Journal of Cardiology*, vol. 130, no. 3, pp. 457–462, 2008.

[87] L. Lu, J.-Z. Zhou, L. Wang, and T.-X. Zhang, "Effects of Xuezhikang and Pravastatin on circulating endothelial progenitor cells in patients with essential hypertension," *Chinese Journal of Integrative Medicine*, vol. 15, no. 4, pp. 266–271, 2009.

[88] G. Paradisi, M. Bracaglia, F. Basile et al., "Effect of pravastatin on endothelial function and endothelial progenitor cells in healthy postmenopausal women," *Clinical and Experimental Obstetrics and Gynecology*, vol. 39, no. 2, pp. 153–159, 2012.

[89] D. Tousoulis, I. Andreou, M. Tsiatas et al., "Effects of rosuvastatin and allopurinol on circulating endothelial progenitor cells in patients with congestive heart failure: the impact of inflammatory process and oxidative stress," *Atherosclerosis*, vol. 214, no. 1, pp. 151–157, 2011.

[90] S. Erbs, E. B. Beck, A. Linke et al., "High-dose rosuvastatin in chronic heart failure promotes vasculogenesis, corrects endothelial function, and improves cardiac remodeling—results from a randomized, double-blind, and placebo-controlled study," *International Journal of Cardiology*, vol. 146, no. 1, pp. 56–63, 2011.

[91] O. Yoshida, T. Kondo, Y. Kureishi-Bando et al., "Pitavastatin, an HMG-CoA reductase inhibitor, ameliorates endothelial function in chronic smokers," *Circulation Journal*, vol. 74, no. 1, pp. 195–202, 2010.

[92] A. O. Spiel, F. B. Mayr, J. M. Leitner, C. Firbas, W. Sieghart, and B. Jilma, "Simvastatin and rosuvastatin mobilize Endothelial Progenitor Cells but do not prevent their acute decrease during systemic inflammation," *Thrombosis Research*, vol. 123, no. 1, pp. 108–113, 2008.

[93] M. Hristov, C. Fach, C. Becker et al., "Reduced numbers of circulating endothelial progenitor cells in patients with coronary artery disease associated with long-term statin treatment," *Atherosclerosis*, vol. 192, no. 2, pp. 413–420, 2007.

[94] C. Werner, M. Baumhäkel, K. K. Teo et al., "RAS blockade with ARB and ACE inhibitors: current perspective on rationale and patient selection," *Clinical Research in Cardiology*, vol. 97, no. 7, pp. 418–431, 2008.

[95] F. H. Bahlmann, K. De Groot, O. Mueller, B. Hertel, H. Haller, and D. Fliser, "Stimulation of endothelial progenitor cells: a new putative therapeutic effect of angiotensin II receptor antagonists," *Hypertension*, vol. 45, no. 4, pp. 526–529, 2005.

[96] T. Sugiura, T. Kondo, Y. Kureishi-Bando et al., "Nifedipine improves endothelial function: role of endothelial progenitor cells," *Hypertension*, vol. 52, no. 3, pp. 491–498, 2008.

[97] C. de Ciuceis, A. Pilu, D. Rizzoni et al., "Effect of antihypertensive treatment on circulating endothelial progenitor cells in patients with mild essential hypertension," *Blood Pressure*, vol. 20, no. 2, pp. 77–83, 2011.

[98] F. Cacciatore, G. Bruzzese, D. F. Vitale et al., "Effects of ACE inhibition on circulating endothelial progenitor cells, vascular damage, and oxidative stress in hypertensive patients," *European Journal of Clinical Pharmacology*, vol. 67, no. 9, pp. 877–883, 2011.

[99] J. Y. Sun, L. Zhai, Q. L. Li et al., "Effects of ACE inhibition on endothelial progenitor cell mobilization and prognosis after acute myocardial infarction in type 2 diabetic patients," *Clinics*, vol. 68, no. 5, pp. 665–673, 2013.

[100] T. Q. Min, C. J. Zhu, W. X. Xiang, Z. J. Hui, and S. Y. Peng, "Improvement in endothelial progenitor cells from peripheral blood by ramipril therapy in patients with stable coronary artery disease," *Cardiovascular Drugs and Therapy*, vol. 18, no. 3, pp. 203–209, 2004.

[101] E. Cangiano, J. Marchesini, G. Campo et al., "ACE inhibition modulates endothelial apoptosis and renewal via endothelial progenitor cells in patients with acute coronary syndromes," *The American Journal of Cardiovascular Drugs*, vol. 11, no. 3, pp. 189–198, 2011.

[102] I. Porto, L. di Vito, G. L. de Maria et al., "Comparison of the effects of ramipril versus telmisartan on high-sensitivity C-reactive protein and endothelial progenitor cells after acute coronary syndrome," *The American Journal of Cardiology*, vol. 103, no. 11, pp. 1500–1505, 2009.

[103] F. Pelliccia, V. Pasceri, C. Cianfrocca et al., "Angiotensin II receptor antagonism with telmisartan increases number of endothelial progenitor cells in normotensive patients with coronary artery disease: a randomized, double-blind, placebo-controlled study," *Atherosclerosis*, vol. 210, no. 2, pp. 510–515, 2010.

[104] H. Tan, X. Li, W. Chen et al., "Effects of losartan on the mobilization of endothelial progenitor cells and improvement of endothelial function," *Annals of Clinical and Laboratory Science*, vol. 43, no. 4, pp. 402–406, 2013.

[105] R. Suzuki, N. Fukuda, M. Katakawa et al., "Effects of an angiotensin II receptor blocker on the impaired function of

endothelial progenitor cells in patients with essential hypertension," *The American Journal of Hypertension*, vol. 27, no. 5, pp. 695–701, 2014.

[106] A.-M. Kampoli, D. Tousoulis, Z. Pallantza et al., "Comparable effects of pioglitazone and perindopril on circulating endothelial progenitor cells, inflammatory process and oxidative stress in patients with diabetes mellitus," *International Journal of Cardiology*, vol. 157, no. 3, pp. 413–415, 2012.

[107] Y.-F. Liao, L.-L. Chen, T.-S. Zeng et al., "Number of circulating endothelial progenitor cells as a marker of vascular endothelial function for type 2 diabetes," *Vascular Medicine*, vol. 15, no. 4, pp. 279–285, 2010.

[108] L.-L. Chen, Y.-F. Liao, T.-S. Zeng, F. Yu, H.-Q. Li, and Y. Feng, "Effects of metformin plus gliclazide compared with metformin alone on circulating endothelial progenitor cell in type 2 diabetic patients," *Endocrine*, vol. 38, no. 2, pp. 266–275, 2010.

[109] L.-L. Chen, F. Yu, T.-S. Zeng, Y.-F. Liao, Y.-M. Li, and H.-C. Ding, "Effects of gliclazide on endothelial function in patients with newly diagnosed type 2 diabetes," *European Journal of Pharmacology*, vol. 659, no. 2-3, pp. 296–301, 2011.

[110] C. Werner, C. H. Kamani, C. Gensch, M. Böhm, and U. Laufs, "The peroxisome proliferator-activated receptor-γ agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance," *Diabetes*, vol. 56, no. 10, pp. 2609–2615, 2007.

[111] C.-H. Wang, M.-K. Ting, S. Verma et al., "Pioglitazone increases the numbers and improves the functional capacity of endothelial progenitor cells in patients with diabetes mellitus," *The American Heart Journal*, vol. 152, no. 6, pp. 1051.e1–1051.e8, 2006.

[112] H. Makino, S. Okada, A. Nagumo et al., "Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients," *Diabetes Research and Clinical Practice*, vol. 81, no. 3, pp. 327–330, 2008.

[113] K. Esposito, M. I. Maiorino, C. Di Palo et al., "Effects of pioglitazone versus metformin on circulating endothelial microparticles and progenitor cells in patients with newly diagnosed type 2 diabetes—a randomized controlled trial," *Diabetes, Obesity and Metabolism*, vol. 13, no. 5, pp. 439–445, 2011.

[114] G. P. Fadini, E. Boscaro, M. Albiero et al., "The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1α," *Diabetes Care*, vol. 33, no. 7, pp. 1607–1609, 2010.

[115] K. Nakamura, H. Oe, H. Kihara et al., "DPP-4 inhibitor and alpha-glucosidase inhibitor equally improve endothelial function in patients with type 2 diabetes: EDGE study," *Cardiovascular Diabetology*, vol. 13, no. 1, p. 110, 2014.

[116] G. P. Fadini, S. V. de Kreutzenberg, V. Mariano et al., "Optimized glycaemic control achieved with add-on basal insulin therapy improves indexes of endothelial damage and regeneration in type 2 diabetic patients with macroangiopathy: a randomized crossover trial comparing detemir versus glargine," *Diabetes, Obesity and Metabolism*, vol. 13, no. 8, pp. 718–725, 2011.

[117] F. Pistrosch, K. Herbrig, U. Oelschlaegel et al., "PPARγ-agonist rosiglitazone increases number and migratory activity of cultured endothelial progenitor cells," *Atherosclerosis*, vol. 183, no. 1, pp. 163–167, 2005.

[118] H.-F. Zhang, L. Wang, H.-J. Yuan et al., "Ppar-γ agonist pioglitazone prevents apoptosis of endothelial progenitor cells from rat bone marrow," *Cell Biology International*, vol. 37, no. 5, pp. 430–435, 2013.

[119] X. Xiao-Yun, M. Zhao-Hui, C. Ke, H. Hong-Hui, and X. Yan-Hong, "Glucagon-like peptide-1 improves proliferation and differentiation of endothelial progenitor cells via upregulating VEGF generation," *Medical Science Monitor*, vol. 17, no. 2, pp. BR35–BR41, 2011.

[120] J. Papak and D. Kansagara, "Management of hyperglycemia in a hospitalized patient with diabetes mellitus and cardiovascular disease," *The American Journal of Cardiology*, vol. 110, no. 9, supplement, pp. 24B–31B, 2012.

[121] K. Strehlow, N. Werner, J. Berweiler et al., "Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation," *Circulation*, vol. 107, no. 24, pp. 3059–3065, 2003.

[122] A. Iwakura, C. Luedemann, S. Shastry et al., "Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury," *Circulation*, vol. 108, no. 25, pp. 3115–3121, 2003.

[123] T. J. Povsic, S. S. Najjar, K. Prather et al., "EPC mobilization after erythropoietin treatment in acute ST-elevation myocardial infarction: the REVEAL EPC substudy," *Journal of Thrombosis and Thrombolysis*, vol. 36, no. 4, pp. 375–383, 2013.

[124] N. J. Leeper, A. L. Hunter, and J. P. Cooke, "Stem cell therapy for vascular regeneration: adult, embryonic, and induced pluripotent stem cells," *Circulation*, vol. 122, no. 5, pp. 517–526, 2010.

[125] G. Spinetti, N. Kraenkel, C. Emanueli, and P. Madeddu, "Diabetes and vessel wall remodelling: from mechanistic insights to regenerative therapies," *Cardiovascular Research*, vol. 78, no. 2, pp. 265–273, 2008.

[126] T. Asahara and A. Kawamoto, "Endothelial progenitor cells for postnatal vasculogenesis," *The American Journal of Physiology—Cell Physiology*, vol. 287, no. 3, pp. C572–C579, 2004.

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Review Article

Vascular Effects of Dietary Advanced Glycation End Products

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Evidence has accumulated lately demonstrating that advanced glycation end products (AGEs) play an important role in the development of diabetic and cardiovascular complications as well as the development of other chronic diseases. AGEs originating from diet have a significant contribution to the AGEs body pool and therefore dietary interventions aiming at reducing AGEs load are believed to exert health promoting effects. This review summarizes the evidence from clinical studies regarding effects of dietary AGEs on the vascular system, highlighting also the different aspects of vascular tests. It also advocates an extension of dietary recommendations towards the promotion of cooking methods that reduce dietary AGEs in consumed foods.

1. Introduction

Cardiovascular disease (CVD) belongs to the leading causes of increased mortality and morbidity in diabetes and many attempts have been made to lower its incidence [1, 2]. Endothelial dysfunction (ED) represents an early, reversible stage of atherosclerosis and is exacerbated in subjects with diabetes mellitus [3]. Several studies suggested, for example, the assessment of endothelial function by ultrasound (e.g., the measurement of flow-mediated dilatation, FMD) to be a prognostic factor for cardiovascular (CV) events [4–6]. Indeed, FMD improves with therapies that decrease cardiovascular risk in people with diabetes, suggesting that restoration of endothelial function might promote CV health, while its impairment promotes atherosclerosis [7, 8].

2. Measurement of Endothelial Function

The measurement of endothelial function has become an important tool for both clinicians and researcher for at least 3 reasons: it allows the identification of patients at risk; it can be used to measure positive vascular effects of different interventions (e.g., medicaments); and it enables the quantification of detrimental vascular effects, for example, during the postprandial state.

Early identification of patients at high risk is clinically important enabling the implementation of more intensive prevention strategies. Therefore, several methods have been developed and proved their efficacy in predicting CV risk, with the ultrasound measurement of FMD of the brachial artery being one of the widest used [9, 10]. Indeed, several studies have shown that the measurement of FMD predicts cardiovascular risk and improves risk stratification especially in patients with known CVD [11–13]. FMD has the advantage that it is noninvasive and has a good repeatability (when conditions are well standardized), but it requires special equipment and highly skilled investigators. Moreover, this technique investigates the endothelial function of conductance vessels. Endothelial function of conductance vessels underlies a different regulation than that of the microcirculation and a growing body of evidence suggests that the microcirculation might be the initial site where endothelial damage occurs [14]. Therefore, techniques investigating microvascular function have been developed too. They add to the early prediction of CV risk in the general population and the risk for the development of diabetes complications in subjects with diabetes mellitus. At least, three comprehensive reviews on available methods for the evaluation of peripheral

neurovascular function have been made available lately by Vinik et al. [15], Cracowski et al. [16], and Stirban [17]. One of the most used methods assesses with a single-point laser-Doppler the skin reactive hyperemia following transient ischemia of the forearm [18]. The method has several advantages: it is investigator independent and it can be performed in parallel to the FMD, thus enabling the assessment of both macrovascular and microvascular endothelial functions.

Beyond the above-mentioned functional tests of micro- and macrocirculation, biomarkers of endothelial dysfunction are available like E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [19].

3. Postprandial State and Postprandial Endothelial Dysfunction

Zilversmit [20] called already in 1979 the atherogenesis to be a "postprandial phenomenon." Indeed, there is compelling data linking postprandial or postload hyperglycemia [21] and hypertriglyceridemia [22] to cardiovascular events suggesting that the postprandial state plays an important role in the development of CVD [23, 24]. The pathogenic importance of the postprandial state becomes even more understandable if we consider that it covers around 2/3 of our daytime.

The mechanisms that link postprandial dysmetabolism to CVD might be the exacerbation of oxidative stress and the occurrence of endothelial dysfunction [25–27].

At least 4 factors deteriorate postprandial endothelial function: hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and food toxins like the so-called advanced glycation end products (AGEs). Postprandial hyperglycemia and hypertriglyceridemia have not only independent but also cumulative effects on postprandial endothelial dysfunction both in healthy subjects and in diabetic patients [28]. Campia and coworkers demonstrated that acute hyperinsulinemia impairs conduit vessel endothelial function independent of insulin sensitivity and lipid profile concluding that hyperinsulinemia may trigger ED and promote atherosclerosis too [29]. Several study groups, including ours, have extensively investigated the effects of food AGEs in humans.

4. AGEs: Definition, Pathogenic Effects, and Sources

AGEs are a heterogeneous group of compounds formed by the nonenzymatic glycation of proteins, lipids, or nucleic acids [30, 31] within the so-called "Maillard reaction." The reaction was named in honor of the French scientist Louis Camille Maillard (1878–1936). It consists of several steps; the first, reversible step takes place between the carbonyl group of a reducing sugar such as glucose and an aminoterminal group of a protein, lipid, or nucleic acid generating a so-called "Schiff base." By structural irreversible rearrangements, more stable ketoamines are formed, called Amadori products (such as the $HbA1_c$) [32]. The Amadori products undergo further structural changes through a series of reactions such as oxidation, dehydration, and degradation to finally yield highly stable AGE compounds [32, 33].

We have recently reviewed the pathogenic mechanisms of AGEs on the vascular system [34]. Briefly, some of the mechanisms are related to inflammation and oxidative stress [35], increased glycation of low-density and high-density lipoproteins (LDL and HDL) [36], activation of the proinflammatory inducible nitric oxide- (NO-) synthase (iNOS) [37], and inhibition of NO availability [38]. Further mechanisms comprise the increased production of cytokines, for example, insulin-like growth factor-1 (IGF-1) or the platelet derived growth factor (PDGF), which modify the migration of monocytes and macrophages as well as the proliferation of vascular smooth muscle cells (VSMC) [39, 40]. Overall, AGEs exert their deleterious effects by receptor-dependent mechanisms and receptor-independent mechanisms. Receptor-independent effects comprise glycation of proteins and lipoproteins (thus altering their normal function [41]), glycation of LDL particles on the apolipoprotein B (ApoB) and phospholipid components [42, 43], glycation of matrix proteins such as collagen VI, laminin, and vitronectin [44], and so forth. AGEs receptors are present on the surface of different cell types such as macrophages, adipocytes, endothelial cells, and vascular smooth muscle cells (VSMC) and several types of receptors including scavenger receptors (macrophage scavenger receptor-AI, macrophage scavenger receptor-AII, CD68, and CD36), RAGE, AGE-R1, AGE-R2, and AGE-R3 have been described, [45, 46]. Although scavenger receptors are responsible for the removal of AGEs, RAGE likely mediates most biological effects of AGEs [47]. The AGEs-RAGE interaction triggers oxidative stress, inflammation, and apoptosis [48–50].

AGEs can be formed within the organism (endogenous source) or can originate from exogenous sources [34]. Although AGEs are better known as by-products of hyperglycemia, they also form within food during heat-enhanced cooking [51]. Evidence has accumulated that dietary AGEs are partially absorbed [52, 53] and either retained in the body or excreted in the urine [53–55]. These dietary AGEs represent an important source for circulating AGEs under in vivo conditions [56–58]. Moreover, smoking also serves as an additional exogenous source of AGEs [59].

The amount of AGEs in food is dependent on the nutrients used, but the AGEs concentration can be greatly influenced by the cooking method. The AGEs generation during cooking increases with temperature, decreasing moisture, cooking time, and increased pH [51]. An excellent article by Uribarri and colleagues provides information on the AGEs amount in several hundreds of meals; this database provides a valuable instrument for estimating food AGEs and contains recommendations of how to reduce dietary AGEs intake [51].

5. In Vivo Effects of Food AGEs

We have recently reviewed [34] the vascular effects of food AGEs in animal models, highlighting that higher circulating concentrations of AGEs, particularly food-derived AGEs, can induce cross-linking of arterial wall connective tissue

protein [60], aortic atherosclerotic lesions [61], and neointimal formation after arterial injury [62], increase vascular permeability, and markedly impair vascular vasodilatory response [63]. Moreover, dietary AGEs restriction improves insulin sensitivity, prevents from the development of diabetic nephropathy, and increases life span [64, 65].

Chilelli et al. [66] reviewed the importance of AGEs for the development of microvascular complications in diabetes advocating that their prevention and treatment must focus not only on early glycemic control, but also on reducing oxidative stress, and especially the dietary intake of exogenous AGEs.

Indeed, several studies have investigated the effects of food AGEs in humans. These studies will be briefly presented and discussed especially from the perspective of vascular effects. For other aspects of the effects of dietary AGEs in humans, two excellent reviews on AGEs in food and their effects on health have been recently made available by Poulsen et al. [67] and Kellow and Savige [68].

Most of the studies investigating effects of dietary AGEs on health dealt with at least 3 important questions. First, at what amount can a dietary modulation of AGEs influence circulating AGEs concentration in populations with different AGEs load? Second, can dietary AGEs modulation influence endothelial function? Third, are these effects due to AGEs or rather due to other dietary toxins generated during cooking?

These questions were addressed mainly in 3 populations: healthy subjects (having a low endogenous AGEs production and an unaltered renal AGEs excretion), subjects with diabetes mellitus (high endogenous AGEs production), and subjects with renal failure (exacerbated endogenous AGEs production and reduced renal excretion).

6. Modulation of Circulating AGEs by Changing the Dietary AGEs Load

From clinical point of view, it makes sense to reduce dietary AGEs only if this has a substantial impact on circulating AGEs. Several kinetic studies have suggested that approximately 10–30% of dietary AGEs are absorbed and around one-third of ingested AGEs are excreted into the urine and feces [69]. An important contributor to circulating AGEs seems to be also the capacity of the body to eliminate AGEs [55, 70].

An acute intervention demonstrated in patients with type 2 diabetes mellitus that the ingestion of a single meal with a high AGEs (H-AGEs) content increased carboxymethyllysine (CML) by 15.6%* and methylglyoxal (MG) by 20.7%*‡ compared to fasting, while following a meal with a low AGEs (L-AGEs) content CML decreased by 5.4% and MG decreased by 10% ($^*P < 0.05$ versus low AGEs, $^‡P < 0.05$ versus fasting) [18]. In healthy subjects, the intake of a beverage containing AGEs increased serum AGEs by 29% [71].

Chronic interventions influence circulating AGEs at an even higher degree. In nondiabetic, renal failure patients on peritoneal dialysis, 4 weeks of low dietary L-AGEs intake decreased serum CML by 34%* and serum MG by 35%*, while a H-AGEs intake of similar length increased serum CML by 29%* and serum MG by 26%* ($^*P < 0.05$ versus baseline) [57]. In patients with diabetes, following a dietary intervention over 6 weeks, serum AGEs were increased by 28.2% on H-AGEs ($P = 0.06$ versus baseline) and reduced by 40% on L-AGEs ($P < 0.02$ versus baseline) [56].

In healthy subjects exposed in a cross-over manner to a dietary intervention of 1 month each, plasma CML was significantly higher (7%, $P = 0.002$) after the H-AGEs diet than after the L-AGEs diet [72]. In another study, in healthy adults, a 6-week L-AGEs diet reduced serum CML from 763 ± 24 to 679 ± 29 ng/mL (−11%, $P = 0.03$) and urine CML from 1.37 ± 1.47 to 0.77 ± 2.01 μg/mL creatinine (−43%, $P = 0.02$) [73].

Overall, it seems that dietary interventions significantly influence circulating AGEs. It is important to note that the absorption of different AGEs varies greatly and also the fact that usually for the assessment of the AGEs load measurements of CML, MG, or pentosidine are used. It is still a matter of debate whether these AGEs are representative for the dietary AGEs class and whether they can be mainly made responsible for the deleterious effects of AGEs.

7. Can Dietary AGEs Modulation Influence Endothelial Function?

In a proof of principle study [18], we tested the hypothesis that a single "real-life" H-AGEs-meal acutely induces more pronounced vascular dysfunction than does a low-AGEs (L-AGEs) meal matched for caloric as well as micronutrient and macronutrient content. We performed a randomized, crossover study, investigating in 20 in-patients with type 2 diabetes the effects of L-AGEs and H-AGEs meal on macrovascular (assessed by flow-mediated dilatation (FMD)) and microvascular (assessed by laser-Doppler flowmetry) function, serum markers of endothelial dysfunction (E-selectin, intracellular adhesion molecule 1, and vascular cell adhesion molecule 1), oxidative stress, and serum AGEs. The meals had identical ingredients but different AGE amounts (15.100 compared with 2.750 kU AGE for the H-AGEs and L-AGEs meals, resp.), which were obtained by varying the cooking temperature and time. Vascular measurements were performed at baseline and 2, 4, and 6 h after each meal. Following the H-AGEs meal, FMD decreased by 36.2%, from 5.77 ± 0.65% (baseline) to 3.93 ± 0.48 (2 h), 3.70 ± 0.42 (4 h), and 4.42 ± 0.54% (6 h) ($P < 0.01$ for all compared with baseline). After the L-AGEs meal, FMD decreased by 20.9%, from 6.04 ± 0.68% (baseline) to 4.75 ± 0.48% (2 h), 4.69 ± 0.51% (4 h), and 5.62 ± 0.63% (6 h), respectively ($P < 0.01$ for all compared with baseline; $P < 0.001$ for all compared with the HAGEs meal). This impairment of macrovascular function after the HAGE meal was paralleled by an impairment of microvascular function (−67.2%) and increased concentrations of serum AGE and markers of endothelial dysfunction and oxidative stress. Following both meals, glucose, triglycerides, and insulin excursions were comparable. We concluded that, in patients with T2DM, HAGEs meal induces a more pronounced acute impairment of vascular function than does an otherwise identical LAGEs meal. Therefore, chemical modifications of food by means

of cooking play a major role in influencing the extent of postprandial vascular dysfunction.

In contrast, in 19 healthy individuals completing a crossover trial, Poulsen et al. showed that a single high-AGEs meal compared to a low-AGEs meal did not show effects on appetite and markers of inflammation or endothelial activation but affected postprandial ghrelin, oxidative stress (urinary F2-isoprostanes), and glucose responses [74]. FMD and functional microvascular tests were not applied in this study; therefore, the comparison to our study is limited.

Several studies investigated the effects of chronic dietary AGEs modulation on endothelial function in patients with type 2 diabetes mellitus.

In one study performed in 24 diabetic subjects, Vlassara et al. [56] investigated the effects of two equivalent diets, one regular (H-AGEs) and the other with 5-fold lower AGEs (L-AGEs) content on inflammatory mediators and markers of ED. Eleven of the subjects participated in a 2-week crossover and 13 subjects in a 6-week parallel study. The authors demonstrated that, after 2 weeks on H-AGEs, serum AGEs increased by 64.5% ($P = 0.02$) and on L-AGEs decreased by 30% ($P = 0.02$) and serum vascular adhesion molecule-1 was 1,108 ± 429 and 698 ± 347 ngml ($P = 0.01$) on H- and L-AGEs, respectively. After 6 weeks, vascular adhesion molecule-1 declined by 20% on L-AGEs ($P < 0.01$) and increased by 4% on H-AGEs. This study shows that, lowering dietary AGEs, a significant reduction in circulating AGEs levels can be achieved, along with a decrease in soluble factors that mirror ED.

In another study, Cai and coworkers [75] demonstrated that, in 24 diabetic subjects randomized to either a standard diet (H-AGEs) or a diet 5-fold lower in AGEs (L-AGEs) for 6 weeks, LDL pooled from patients on H-AGEs diet was more glycated than LDL pooled from the L-AGEs diet group (192 versus 92 AGE U/mg apolipoprotein B) and more oxidized (5.7 versus 1.5 nmol malondialdehyde/mg lipoprotein). They noted that the LDL pooled from patients on H-AGEs added to human endothelial cells promoted marked ERK1/2 phosphorylation (pERK1/2) (5.5- to 10-fold of control) and stimulated NF-kappaB activity compared to the LDL pooled from patients on L-AGEs. Since glycated and oxidized LDL might promote atherosclerosis, this study shows that dietary AGEs might have atherogenic effects by enhancing LDL-induced vascular toxicity via redox-sensitive mitogen-activated protein kinase activation, an effect that can be reduced by dietary AGEs restriction.

Peppa et al. [76] demonstrated in a group of 18 patients with chronic renal failure randomly assigned to a 4-week diet with either L-AGEs or H-AGEs that dietary AGEs modulation resulted in a significant decrease in levels of serum AGEs, CRP, and PAI-1 in the L-AGEs group (approximately 35%, 44%, and 17%, resp.; $P < 0.03$), whereas only serum AGEs levels increased significantly in the H-AGEs group. VCAM-1 and TNF-alpha levels, although similar at baseline, became significantly lower in patients on L-AGEs compared with H-AGEs diet ($P < 0.05$) at the end of the study.

Patients with diabetes mellitus and/or renal failure show increased AGEs production and reduced AGEs renal clearance. But healthy subjects have presumably intact protective mechanisms that might compensate an increased dietary AGEs load. Therefore, the question arises whether dietary AGEs restriction has also quantifiable effects in healthy subjects.

The study by Birlouez-Aragon dealt with this question and investigated the effects of dietary AGEs in healthy subjects [72]. The study performed in 62 volunteers was a randomized, crossover, diet-controlled intervention trial with the duration of 4 weeks, designed to compare the potential metabolic effects of 2 diets: one based on mild steam cooking (and thus with a low AGEs content: L-AGEs) and another based on high-temperature cooking (H-AGEs). The 2 diets differed mainly in their AGEs content. Authors assessed AGEs in the diet and in subjects' feces, blood, and urine samples, using CML as an indicator of AGEs. In comparison with the L-AGEs diet, 1 month of H-AGEs induced significantly lower insulin sensitivity and plasma concentrations of long-chain n-3 (omega-3) fatty acids and vitamins C and E (−17% ($P < 0.002$), −13% ($P < 0.0001$), and −8% ($P < 0.01$), resp.). However, concentrations of plasma cholesterol and triglycerides increased (+5% ($P < 0.01$) and +9% ($P < 0.01$), resp.). The authors' conclusion was that, in healthy people, a diet based on high-heat-treated foods increases markers associated with increased risk of type 2 diabetes and cardiovascular diseases. These effects can be counteracted by changing the cooking method, that is, by replacing high-heat-treatment techniques by mild cooking techniques.

In contrast to these data, Semba et al. [73] recently published an article showing in 24 healthy subjects that a 6-week H-AGEs diet compared to an isocaloric L-AGEs diet had no impact on endothelial function (measured as peripheral arterial tonometry) and inflammatory mediators. Nevertheless, the conclusion regarding the impact on endothelial function has to be regarded with caution as should be the comparison to our study [18]. The endothelium is the main place where vasoactive humoral factorsare produced accounting for vasodilatation (e.g., nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor) or vasoconstriction (e.g., thromboxane A2 and endothelin-1) [77]. Of note, macrovascular function (mirrored by FMD) and microvascular function (mirrored by laser-Doppler measurements) underlies different regulatory mechanisms. While FMD is predominantly mediated by NO [78], the microcirculation is largely independent of NO-mediated regulation and is subject to prostaglandin [79] and non-endothelium-dependent pathways [80]. This explains why a poor correlation exists between the vascular reactivity of the microcirculation and macrocirculation [81]. Moreover, peripheral arterial tonometry and other functional vascular tests are not interchangeable and seem to reflect at least slightly different aspects of endothelial function [82].

8. Are the Above-Mentioned Effects due to AGEs?

Dietary AGEs interventions have several drawbacks: (1) blinding of subjects is not possible and therefore studies were

performed in the best case investigator-blinded and (2) the findings might have been confounded by the fact that the methods to increase food AGEs content, namely, application of heat, might also affect vitamin activity and the formation of other non-AGEs substances with toxic potential, that is, oxidized lipids.

We therefore performed a first study investigating the effects of a beverage containing small molecules of AGEs.

An AGEs-rich beverage free of carbohydrates or lipids or other known vasoactive substances was administered to diabetic ($n = 44$) as well as healthy subjects ($n = 10$), and its acute effects on arterial endothelial function were assessed by means of FMD measurements as well as measurements of VCAM-1 and plasminogen activator inhibitor-1 (PAI-1) levels. The oral AGEs challenge beverage (300 mL) contained 1.8×106 AGE units, but neither carbohydrates nor lipids [71]. The beverage was prepared from glucose and caffeine-free Coca-Cola light, which was concentrated 10 times by rotary evaporation at room temperature. The diabetic subjects had higher baseline levels of serum AGEs ($P < 0.020$), PAI-1 (NS), and VCAM-1 ($P < 0.033$) and lower baseline values of FMD ($P < 0.032$) compared with nondiabetic subjects. Ninety minutes after a single oral AGE challenge, serum AGEs and PAI-1 levels increased and FMD decreased significantly in both healthy subjects (AGEs: 7.2 ± 0.5 to 9.3 ± 1.0 units/mL, $P < 0.014$; PAI-1: 5.4 ± 0.4 to 6.8 ± 0.4 ng/mL, $P < 0.007$; and FMD: 9.9 ± 0.7 to 7.4 ± 0.9%, $P < 0.019$) and diabetic subjects (AGEs: 10.5 ± 0.7 to 14.2 ± 1.0 units/mL, $P < 0.020$; PAI-1: 6.5 ± 1.0 to 10 ± 2 ng/mL, $P < 0.030$; and FMD: 5.4 ± 0.4 to 4.0 ± 0.3%, $P < 0.032$). Serum glucose and VCAM-1 levels remained unchanged. We therefore concluded that significant increases in serum AGEs can occur together with altered clinical measures of endothelial function in diabetic and nondiabetic subjects after a single AGEs-rich beverage.

However, the results have been criticized as potentially not being representative for dietary AGEs derived from common foods, as cola-derived AGEs are only present in low amounts and as small molecules, whereas large AGE modified proteins are missing [83]. It also could not be excluded that substances other than AGEs contained in Coca-Cola might have influenced vascular function. Moreover, the study was not subject-blinded.

We therefore performed a randomized, double-blind, controlled, cross-over study that aimed at investigating the acute effects of dietary AGEs resulting from nonenzymatic glycation during heating of beta-lactoglobulins (a protein class frequently encountered in food) and compared these effects to nonglycated, but heated beta-lactoglobulins (BLG) [84]. Thus, the sole difference between the 2 protein preparations was the advanced protein glycation. Nineteen patients with type 2 diabetes mellitus received on 2 different occasions beverages containing either glycated, heat-treated BLG (AGEs-BLG) or nonglycated, heat-treated BLG (C-BLG). We measured macrovascular (FMD) and microvascular (laser-Doppler measurements of reactive hyperemia in the hand, RH) functions at baseline (T_0), as well as 90 (T_{90}) and 180 (T_{180}) minutes after each beverage. Following the AGEs-BLG, FMD decreased at T_{90} by $80\%^{*\ddagger}$ from baseline and remained decreased by $42\%^{*}$ at T_{180} ($^{*}P < 0.05$ versus baseline, $^{\ddagger}P < 0.05$ versus C-BLG). In comparison, FMD decrease following C-BLG was lower, with a maximum decrease of 51% at T_{180}. A significant decrease in nitrite (T_{180}) and nitrate (T_{90} and T_{180}) as well as a significant increase in CML accompanied the changes following the AGEs-BLG. No change in microvascular function followed any of the 2 beverages.

We concluded that in patients with type 2 diabetes mellitus, an acute oral administration of AGEs from heat-treated, glycated BLG transiently, but significantly impair macrovascular function. These effects are more pronounced than following administration of heat-treated, nonglycated BLG and are accompanied by an increase in circulating CML and a decrease in nitrate. We therefore suggested that the mechanisms leading to vascular dysfunction are related to a decrease in NO bioavailability.

Thus, we demonstrated for the first time in humans that dietary AGEs transiently impair endothelial function in patients with type 2 diabetes mellitus. The relevance for the development of cardiovascular disease still has to be established.

9. Conclusions

AGEs play a major role in the development of cardiovascular and diabetes complications as well as other chronic diseases. Dietary AGEs contribute significantly to circulating AGEs and probably to the AGEs pool of the body. Dietary interventions are effective in reducing circulating AGEs in healthy volunteers, patients with diabetes mellitus, and patients with renal failure.

There is compelling data showing that dietary AGEs significantly contribute to the development of postprandial endothelial dysfunction, thus increasing atherosclerotic risk. Accordingly, reducing the dietary AGEs load might represent a cost-effective and easy to implement method showing protective cardiovascular effects. Moreover, a large body of evidence shows that dietary AGEs restriction improves insulin sensitivity and reduces inflammation and oxidative stress. Therefore, beyond recommendations aiming at reducing, for example, dietary fat and carbohydrates, changing the cooking method might also promote health. Uribarri and coworkers [51] published in 2010 a seminal work that reveals the AGEs content of over 540 foods along with recommendations for low-AGEs cooking. They highlighted that dry heat, for example, enhances AGEs formation by 10–100 times compared to the uncooked state across food categories, thus recommending cooking with moist heat, using shorter cooking times, cooking at lower temperatures, and the use of acidic ingredients such as lemon juice or vinegar.

We therefore advocate extending dietary recommendations to healthy cooking. Nevertheless, further studies are needed to better understand the pathogenic effects of food AGEs and to develop strategies for dietary AGEs reduction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] F. D. R. Hobbs, "Type-2 diabetes mellitus related cardiovascular risk: new options for interventions to reduce risk and treatment goals," *Atherosclerosis Supplements*, vol. 7, no. 4, pp. 29–32, 2006.

[2] D. M. Nathan, P. A. Cleary, J.-Y. C. Backlund et al., "Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes," *The New England Journal of Medicine*, vol. 353, no. 25, pp. 2643–2653, 2005.

[3] H. A. R. Hadi, C. S. Carr, and J. Al Suwaidi, "Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome," *Vascular Health and Risk Management*, vol. 1, no. 3, pp. 183–198, 2005.

[4] T. Neunteufl, S. Heher, R. Katzenschlager et al., "Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain," *American Journal of Cardiology*, vol. 86, no. 2, pp. 207–210, 2000.

[5] T. Neunteufl, R. Katzenschlager, A. Hassan et al., "Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease," *Atherosclerosis*, vol. 129, no. 1, pp. 111–118, 1997.

[6] B. Takase, Y. Matsushima, A. Uehata, M. Ishihara, and A. Kurita, "Endothelial dysfunction, carotid artery plaque burden, and conventional exercise-induced myocardial ischemia as predictors of coronary artery disease prognosis," *Cardiovascular Ultrasound*, vol. 6, article 61, 2008.

[7] B. Guerci, A. Kearney-Schwartz, P. Böhme, F. Zannad, and P. Drouin, "Endothelial dysfunction and type 2 diabetes: part 1: physiology and methods for exploring the endothelial function," *Diabetes and Metabolism*, vol. 27, no. 4, pp. 425–434, 2001.

[8] B. Guerci, P. Bohme, A. Kearney-Schwartz, F. Zannad, and P. Drouin, "Endothelial dysfunction and type 2 diabetes. Part 2: altered endothelial function and the effects of treatments in type 2 diabetes mellitus," *Diabetes and Metabolism*, vol. 27, no. 4 I, pp. 436–447, 2001.

[9] D. S. Celermajer, K. E. Sorensen, V. M. Gooch et al., "Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis," *The Lancet*, vol. 340, no. 8828, pp. 1111–1115, 1992.

[10] C. T. Minson, "Thermal provocation to evaluate microvascular reactivity in human skin," *Journal of Applied Physiology*, vol. 109, no. 4, pp. 1239–1246, 2010.

[11] N. Gokce, J. F. Keaney Jr., L. M. Hunter, M. T. Watkins, J. O. Menzoian, and J. A. Vita, "Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study," *Circulation*, vol. 105, no. 13, pp. 1567–1572, 2002.

[12] N. Gokce, J. F. Keaney Jr., L. M. Hunter et al., "Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease," *Journal of the American College of Cardiology*, vol. 41, no. 10, pp. 1769–1775, 2003.

[13] M. Guazzi, G. Reina, P. Gripari, G. Tumminello, M. Vicenzi, and R. Arena, "Prognostic value of flow-mediated dilatation following myocardial infarction," *International Journal of Cardiology*, vol. 132, no. 1, pp. 45–50, 2009.

[14] S. V. Brodsky, O. Gealekman, J. Chen et al., "Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen," *Circulation Research*, vol. 94, no. 3, pp. 377–384, 2004.

[15] A. I. Vinik, T. Erbas, K. K. Pierce, and K. B. Stansberry, "Methods for evaluation of peripheral neurovascular dysfunction," *Diabetes Technology and Therapeutics*, vol. 3, no. 1, pp. 29–50, 2001.

[16] J.-L. Cracowski, C. T. Minson, M. Salvat-Melis, and J. R. Halliwill, "Methodological issues in the assessment of skin microvascular endothelial function in humans," *Trends in Pharmacological Sciences*, vol. 27, no. 9, pp. 503–508, 2006.

[17] A. Stirban, "Microvascular dysfunction in the context of diabetic neuropathy," *Current Diabetes Reports*, vol. 14, no. 11, article 541, 2014.

[18] M. Negrean, A. Stirban, B. Stratmann et al., "Effects of low- and high-advanced glycation endproduct meals on macro- and microvascular endothelial function and oxidative stress in patients with type 2 diabetes mellitus," *The American Journal of Clinical Nutrition*, vol. 85, no. 5, pp. 1236–1243, 2007.

[19] J. B. Meigs, F. B. Hu, N. Rifai, and J. E. Manson, "Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus," *Journal of the American Medical Association*, vol. 291, no. 16, pp. 1978–1986, 2004.

[20] D. B. Zilversmit, "Atherogenesis: a postprandial phenomenon," *Circulation*, vol. 60, no. 3, pp. 473–485, 1979.

[21] M. Coutinho, H. C. Gerstein, Y. Wang, and S. Yusuf, "The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years," *Diabetes Care*, vol. 22, no. 2, pp. 233–240, 1999.

[22] M. J. Stampfer, R. M. Krauss, J. Ma et al., "A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction," *Journal of the American Medical Association*, vol. 276, no. 11, pp. 882–888, 1996.

[23] M. E. Tushuizen, M. Diamant, and R. J. Heine, "Postprandial dysmetabolism and cardiovascular disease in type 2 diabetes," *Postgraduate Medical Journal*, vol. 81, no. 951, pp. 1–6, 2005.

[24] J. H. O'Keefe and D. S. H. Bell, "Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor," *American Journal of Cardiology*, vol. 100, no. 5, pp. 899–904, 2007.

[25] A. Ceriello, M. Hanefeld, L. Leiter et al., "Postprandial glucose regulation and diabetic complications," *Archives of Internal Medicine*, vol. 164, no. 19, pp. 2090–2095, 2004.

[26] A. Ceriello, "Postprandial hyperglycemia and diabetes complications: is it time to treat?" *Diabetes*, vol. 54, no. 1, pp. 1–7, 2005.

[27] A. J. Garber, "Postprandial dysmetabolism and the heart," *Heart Failure Clinics*, vol. 8, no. 4, pp. 563–573, 2012.

[28] A. Ceriello, C. Taboga, L. Tonutti et al., "Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment," *Circulation*, vol. 106, no. 10, pp. 1211–1218, 2002.

[29] U. Campia, G. Sullivan, M. B. Bryant, M. A. Waclawiw, M. J. Quon, and J. A. Panza, "Insulin impairs endothelium-dependent vasodilation independent of insulin sensitivity or lipid profile," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 286, no. 1, pp. H76–H82, 2004.

[30] H. Vlassara, "Recent progress in advanced glycation end products and diabetic complications," *Diabetes*, vol. 46, supplement 2, pp. S19–S25, 1997.

[31] H. Vlassara and M. R. Palace, "Glycoxidation: the menace of diabetes and aging," *Mount Sinai Journal of Medicine*, vol. 70, no. 4, pp. 232–241, 2003.

[32] N. Ahmed, "Advanced glycation endproducts—role in pathology of diabetic complications," *Diabetes Research and Clinical Practice*, vol. 67, no. 1, pp. 3–21, 2005.

[33] J. D. Méndez, J. Xie, M. Aguilar-Hernández, and V. Méndez-Valenzuela, "Trends in advanced glycation end products research in diabetes mellitus and its complications," *Molecular and Cellular Biochemistry*, vol. 341, no. 1-2, pp. 33–41, 2010.

[34] A. Stirban, T. Gawlowski, and M. Roden, "Vascular effects of advanced glycation endproducts: clinical effects and molecular mechanisms," *Molecular Metabolism*, vol. 3, no. 2, pp. 94–108, 2014.

[35] W. Cai, Q.-D. Gao, L. Zhu, M. Peppa, C. He, and H. Vlassara, "Oxidative stress-inducing carbonyl compounds from common foods: novel mediators of cellular dysfunction," *Molecular Medicine*, vol. 8, no. 7, pp. 337–346, 2002.

[36] P. B. Duell, J. F. Oram, and E. L. Bierman, "Nonenzymatic glycosylation of HDL and impaired HDL-receptor-mediated cholesterol efflux," *Diabetes*, vol. 40, no. 3, pp. 377–384, 1991.

[37] R. M. F. Wever, T. F. Lüscher, F. Cosentino, and T. J. Rabelink, "Atherosclerosis and the two faces of endothelial nitric oxide synthase," *Circulation*, vol. 97, no. 1, pp. 108–112, 1998.

[38] B. Xu, Y. Ji, K. Yao, Y.-X. Cao, and A. Ferro, "Inhibition of human endothelial cell nitric oxide synthesis by advanced glycation end-products but not glucose: relevance to diabetes," *Clinical Science*, vol. 109, no. 5, pp. 439–446, 2005.

[39] H. Vlassara, "Advanced glycation end-products and atherosclerosis," *Annals of Medicine*, vol. 28, no. 5, pp. 419–426, 1996.

[40] A. Stirban, "The role of AGEs and ROS in atherosclerosis," *Herz*, vol. 35, no. 3, pp. 170–180, 2010.

[41] D. Aronson and E. J. Rayfield, "How hyperglycemia promotes atherosclerosis: molecular mechanisms," *Cardiovascular Diabetology*, vol. 1, article 1, 2002.

[42] R. Bucala, R. Mitchell, K. Arnold, T. Innerarity, H. Vlassara, and A. Cerami, "Identification of the major site of apolipoprotein B modification by advanced glycosylation end products blocking uptake by the low density lipoprotein receptor," *The Journal of Biological Chemistry*, vol. 270, no. 18, pp. 10828–10832, 1995.

[43] R. Bucala, Z. Makita, T. Koschinsky, A. Cerami, and H. Vlassara, "Lipid advanced glycosylation: pathway for lipid oxidation in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 14, pp. 6434–6438, 1993.

[44] M. Brownlee, A. Cerami, and H. Vlassara, "Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications," *The New England Journal of Medicine*, vol. 318, no. 20, pp. 1315–1321, 1988.

[45] A. W. Stitt, C. He, and H. Vlassara, "Characterization of the advanced glycation end-product receptor complex in human vascular endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 256, no. 3, pp. 549–556, 1999.

[46] S. McFarlane, J. V. Glenn, A. M. Lichanska, D. A. C. Simpson, and A. W. Stitt, "Characterisation of the advanced glycation endproduct receptor complex in the retinal pigment epithelium," *British Journal of Ophthalmology*, vol. 89, no. 1, pp. 107–112, 2005.

[47] L. G. Bucciarelli, T. Wendt, L. Rong et al., "RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease," *Cellular and Molecular Life Sciences*, vol. 59, no. 7, pp. 1117–1128, 2002.

[48] S. F. Yan, R. Ramasamy, and A. M. Schmidt, "The receptor for advanced glycation endproducts (RAGE) and cardiovascular disease," *Expert Reviews in Molecular Medicine*, vol. 11, article e9, 2009.

[49] A. Bierhaus, P. M. Humpert, M. Morcos et al., "Understanding RAGE, the receptor for advanced glycation end products," *Journal of Molecular Medicine*, vol. 83, no. 11, pp. 876–886, 2005.

[50] S. F. Yan, R. Ramasamy, and A. M. Schmidt, "The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature," *Circulation Research*, vol. 106, pp. 842–885, 2010.

[51] J. Uribarri, S. Woodruff, S. Goodman et al., "Advanced glycation end products in foods and a practical guide to their reduction in the diet," *Journal of the American Dietetic Association*, vol. 110, no. 6, pp. 911–916, 2010.

[52] A. Förster, Y. Kühne, and T. Henle, "Studies on absorption and elimination of dietary Maillard reaction products," *Annals of the New York Academy of Sciences*, vol. 1043, pp. 474–481, 2005.

[53] A. Foerster and T. Henle, "Glycation in food and metabolic transit of dietary AGEs (advanced glycation end-products): studies on the urinary excretion of pyrraline," *Biochemical Society Transactions*, vol. 31, part 6, pp. 1383–1385, 2003.

[54] H. F. Erbersdobler and V. Faist, "Metabolic transit of *Amadori* products," *Die Nahrung*, vol. 45, no. 3, pp. 177–181, 2001.

[55] T. Koschinsky, C.-J. He, T. Mitsuhashi et al., "Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 12, pp. 6474–6479, 1997.

[56] H. Vlassara, W. Cai, J. Crandall et al., "Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15596–15601, 2002.

[57] J. Uribarri, M. Peppa, W. Cai et al., "Restriction of dietary glycotoxins reduces excessive advanced glycation end products in renal failure patients," *Journal of the American Society of Nephrology*, vol. 14, no. 3, pp. 728–731, 2003.

[58] J. Uribarri, M. Peppa, W. Cai et al., "Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients," *The American Journal of Kidney Diseases*, vol. 42, no. 3, pp. 532–538, 2003.

[59] C. Cerami, H. Founds, I. Nicholl et al., "Tobacco smoke is a source of toxic reactive glycation products," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13915–13920, 1997.

[60] M. Brownlee, H. Vlassara, A. Kooney, P. Ulrich, and A. Cerami, "Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking," *Science*, vol. 232, no. 4758, pp. 1629–1632, 1986.

[61] R.-Y. Lin, R. P. Choudhury, W. Cai et al., "Dietary glycotoxins promote diabetic atherosclerosis in apolipoprotein E-deficient mice," *Atherosclerosis*, vol. 168, no. 2, pp. 213–220, 2003.

[62] R. Y. Lin, E. D. Reis, A. T. Dore et al., "Lowering of dietary advanced glycation endproducts (AGE) reduces neointimal formation after arterial injury in genetically hypercholesterolemic mice," *Atherosclerosis*, vol. 163, no. 2, pp. 303–311, 2002.

[63] H. Vlassara, H. Fuh, Z. Makita, S. Krungkrai, A. Cerami, and R. Bucala, "Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 24, pp. 12043–12047, 1992.

[64] S. M. Hofmann, H.-J. Dong, Z. Li et al., "Improved insulin sensitivity is associated with restricted intake of dietary glycoxidation products in the db/db mouse," *Diabetes*, vol. 51, no. 7, pp. 2082–2089, 2002.

[65] F. Zheng, C. He, W. Cai, M. Hattori, M. Steffes, and H. Vlassara, "Prevention of diabetic nephropathy in mice by a diet low in glycoxidation products," *Diabetes/Metabolism Research and Reviews*, vol. 18, no. 3, pp. 224–237, 2002.

[66] N. C. Chilelli, S. Burlina, and A. Lapolla, "AGEs, rather than hyperglycemia, are responsible formicrovascular complications in diabetes: a "glycoxidation-centric" point of view," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 23, no. 10, pp. 913–919, 2013.

[67] M. W. Poulsen, R. V. Hedegaard, J. M. Andersen et al., "Advanced glycation endproducts in food and their effects on health," *Food and Chemical Toxicology*, vol. 60, pp. 10–37, 2013.

[68] N. J. Kellow and G. S. Savige, "Dietary advanced glycation end-product restriction for the attenuation of insulin resistance, oxidative stress and endothelial dysfunction: a systematic review," *European Journal of Clinical Nutrition*, vol. 67, no. 3, pp. 239–248, 2013.

[69] V. Faist and H. F. Erbersdobler, "Metabolic transit and in vivo effects of melanoidins and precursor compounds deriving from the Maillard reaction," *Annals of Nutrition and Metabolism*, vol. 45, no. 1, pp. 1–12, 2001.

[70] C. Delgado-Andrade, F. É. J. Tessier, C. Niquet-Leridon, I. Seiquer, and M. P. Navarro, "Study of the urinary and faecal excretion of Ne-carboxymethyllysine in young human volunteers," *Amino Acids*, vol. 43, no. 2, pp. 595–602, 2012.

[71] J. Uribarri, A. Stirban, D. Sander et al., "Single oral challenge by advanced glycation end products acutely impairs endothelial function in diabetic and nondiabetic subjects," *Diabetes Care*, vol. 30, no. 10, pp. 2579–2582, 2007.

[72] I. Birlouez-Aragon, G. Saavedra, F. J. Tessier et al., "A diet based on high-heat-treated foods promotes risk factors for diabetes mellitus and cardiovascular diseases," *The American Journal of Clinical Nutrition*, vol. 91, no. 5, pp. 1220–1226, 2010.

[73] R. D. Semba, S. K. Gebauer, D. J. Baer et al., "Dietary intake of advanced glycation end products did not affect endothelial function and inflammation in healthy adults in a randomized controlled trial," *Journal of Nutrition*, vol. 144, no. 7, pp. 1037–1042, 2014.

[74] M. W. Poulsen, M. J. Bak, J. M. Andersen et al., "Effect of dietary advanced glycation end products on postprandial appetite, inflammation, and endothelial activation in healthy overweight individuals," *European Journal of Nutrition*, vol. 53, no. 2, pp. 661–672, 2014.

[75] W. Cai, J. C. He, L. Zhu et al., "High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients," *Circulation*, vol. 110, no. 3, pp. 285–291, 2004.

[76] M. Peppa, J. Uribarri, W. Cai, M. Lu, and H. Vlassara, "Glycoxidation and inflammation in renal failure patients," *The American Journal of Kidney Diseases*, vol. 43, no. 4, pp. 690–695, 2004.

[77] J. R. Vane, E. E. Anggard, and R. M. Botting, "Regulatory functions of the vascular endothelium," *The New England Journal of Medicine*, vol. 323, no. 1, pp. 27–36, 1990.

[78] S. N. Doshi, K. K. Naka, N. Payne et al., "Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide," *Clinical Science*, vol. 101, no. 6, pp. 629–635, 2001.

[79] I. Carlsson, A. Sollevi, and A. Wennmalm, "The role of myogenic relaxation, adenosine and prostaglandins in human forearm reactive hyperaemia," *The Journal of Physiology*, vol. 389, pp. 147–161, 1987.

[80] J. Bjornberg, U. Albert, and S. Mellander, "Resistance responses in proximal arterial vessels, arterioles and veins during reactive hyperaemia in skeletal muscle and their underlying regulatory mechanisms," *Acta Physiologica Scandinavica*, vol. 139, no. 4, pp. 535–550, 1990.

[81] M. Dhindsa, S. M. Sommerlad, A. E. de van et al., "Interrelationships among noninvasive measures of postischemic macro- and microvascular reactivity," *Journal of Applied Physiology*, vol. 105, no. 2, pp. 427–432, 2008.

[82] M. Hedetoft and N. V. Olsen, "Evaluation of endothelial function by peripheral arterial tonometry and relation with the nitric oxide pathway," *Nitric Oxide C*, vol. 42, pp. 1–8, 2014.

[83] N. Ahmed, B. Mirshekar-Syahkal, L. Kennish, N. Karachalias, R. Babaei-Jadidi, and P. J. Thornalley, "Assay of advanced glycation endproducts in selected beverages and food by liquid chromatography with tandem mass spectrometric detection," *Molecular Nutrition and Food Research*, vol. 49, no. 7, pp. 691–699, 2005.

[84] A. Stirban, P. Kotsi, K. Franke et al., "Acute macrovascular dysfunction in patients with type 2 diabetes induced by ingestion of advanced glycated β-lactoglobulins," *Diabetes Care*, vol. 36, no. 5, pp. 1278–1282, 2013.

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Research Article

Trends in the Levels of Serum Lipids and Lipoproteins and the Prevalence of Dyslipidemia in Adults with Newly Diagnosed Type 2 Diabetes in the Southwest Chinese Han Population during 2003–2012

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Objective. To determine the trends of serum lipid levels and dyslipidemia in adults newly diagnosed with type 2 diabetes mellitus during 2003–2012 in Southwest China. *Methods*. Serum lipid measurements of 994 adults were obtained from 5 independent, cross-sectional studies (2003-2004, 2005-2006, 2007-2008, 2009-2010, and 2011-2012). The main outcome measures were mean serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride levels; body mass index; hemoglobin A1C level; and the percentages of patients with dyslipidemia, hypertension, coronary heart disease, and cerebrovascular disease. *Results*. The mean total cholesterol and low-density lipoprotein cholesterol levels increased from 4.92 ± 1.15 to 5.30 ± 1.17 mmol/L ($P = 0.039$) and 2.72 ± 0.83 to 3.11 ± 1.09 mmol/L ($P = 0.004$), respectively, and the mean HDL cholesterol level declined from 1.22 ± 0.30 to 1.06 ± 0.24 mmol/L ($P < 0.001$). The percentages of patients with dyslipidemia increased gradually. The incidence of coronary heart and cerebrovascular diseases increased from 8.2% to 19.1% and 6.6% to 15.3%, respectively ($P < 0.05$). *Conclusion*. Unfavorable upward trends were observed in serum lipid levels and the prevalence of dyslipidemia, coronary heart disease, and cerebrovascular disease in adults newly diagnosed with type 2 diabetes mellitus in Southwest China during 2003–2012.

1. Introduction

In recent years, owing to the rapid economic development and changes in lifestyle, the number of patients with diabetes mellitus in China has increased dramatically. The age-standardized prevalence rates of total diabetes and prediabetes in China are reportedly 9.7% and 15.5% [1]. Among the patients with type 2 diabetes mellitus, cardiovascular disease is the most important cause of mortality and morbidity. Epidemiologic studies have demonstrated that dyslipidemia increases the risk of cardiovascular diseases [2–4]. Because dyslipidemia is asymptomatic, it is difficult to diagnose and manage, and many clinicians ignore the importance of dyslipidemia in patients with diabetes.

A previous study based on the Chinese national nutrition and health survey in 2002 investigated the prevalence of dyslipidemia in 14252 Chinese adults and found that the overall prevalence of dyslipidemia was 18.6% (22.2% in men and 15.9% in women) [5]. In addition, although numerous studies have shown that serum lipid levels are high in diabetes patients, data on serum lipid and lipoprotein levels in patients newly diagnosed with type 2 diabetes mellitus are scarce, especially in Chongqing, a midlevel developed city in Southwest China with living conditions representative of the majority of Chinese cities and thus a fitting representative of the general Chinese population.

The aim of the study was to analyze the trends in serum lipid levels and the prevalence of patients with dyslipidemia, coronary heart disease (CHD), hypertension, and cerebrovascular disease in adults with newly diagnosed type 2 diabetes mellitus in the Southwest Chinese Han population during 2003–2012.

2. Materials and Methods

2.1. Patients and Data Collection. Overall, 994 inpatients newly diagnosed with type 2 diabetes mellitus between January 2003 and December 2012 were collected from 3 different hospitals including The Second Affiliated Hospital of Chongqing Medical University, Bishan District People's Hospital of Chongqing, China, and Fuling District People's Hospital of Chongqing, China. All participants were Han ethnic. None of these patients received prescribed lipid or hormone replacement therapy, and those taking lipid-lowering medication were excluded. All subjects had complete data regarding total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and serum triglyceride (TG) levels from each study year. This project was approved by the Human Research Ethics Committee of Chongqing Medical University. Because our study is retrospective, patient information was anonymized and deidentified when data were collected and analyzed, thus exempting the requirement for informed consent.

2.2. Diagnostic Criteria. According to National Cholesterol Education Program Adult Treatment Panel (NCEP ATP) III [6], the diagnosis of dyslipidemia for patients with diabetes is based on the presence of one or more of the following criteria: high TC (≥5.18 mmol/L), high LDL cholesterol (≥2.60 mmol/L), low HDL cholesterol (≤1.04 mmol/L for men and ≤1.30 mmol/L for women), and high TG (≥1.70 mmol/L) levels. Hypertension was defined as systolic and diastolic blood pressure ≥140 and ≥90 mmHg, respectively, and/or current treatment with antihypertensive medications. CHD was diagnosed on the basis of a history of acute coronary syndrome or was confirmed by computed tomography angiography, or coronary angiography. Cerebrovascular disease, including ischemic stroke and hemorrhagic stroke, was diagnosed using brain computed tomography or brain magnetic resonance imaging.

2.3. Design and Measurements. The trends of the mean serum lipid levels were analyzed by comparing the data of 30–89-year-old adults obtained from 5 studies: 2003-2004, 2005-2006, 2007-2008, 2009-2010, and 2011-2012. These 5 sets of data were obtained independent of each other.

At the time of diagnosis, blood was drawn after an 8 h fast for measuring TG, TC, HDL-C, LDL-C, and hemoglobin A1C (HbA1C) levels. The same methods of lipid level measurements were used in all 5 examinations. The GPO-PAP method and COD-CE-PAP assay were used to determine triglyceride and total cholesterol levels, respectively. HDL and LDL cholesterol levels were measured using the CAT method (Maker Biotechnology Co., Ltd., Sichuan, China). The HbA1C level was measured using a high performance liquid chromatography method. The body mass index (BMI) was calculated by dividing the weight (kg) by the square of the height (m^2). Information about the diagnosis of CHD, hypertension, and cerebrovascular disease was collected from the patients' medical records.

2.4. Statistical Analyses. The results are expressed as mean ± standard deviation for TC, HDL-C, and LDL-C. Geometric means and standard errors of geometric means are presented for serum triglyceride levels because the distribution is highly skewed. The prevalence of dyslipidemia and the morbidity rates of CHD, hypertension, and cerebrovascular disease are presented as percentages. We calculated the differences between the time periods using analysis of variance (ANOVA) for continuous variables; Kruskal-Wallis test was used for nonnormally distributed variables; to test whether the percentage of patients with dyslipidemia, hypertension, CHD, and cerebrovascular disease changed over the years, the chi-square test was used. All data were analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA), and P values less than 0.05 were considered statistically significant.

3. Results

The patient characteristics are shown in Table 1. The medical data of 994 subjects (550 men and 444 women; age range: 30–89 years) were analyzed. The time trends in the mean age ($P = 0.723$), BMI level ($P = 0.052$), and haemoglobin A1c (HbA1C) level ($P = 0.391$) of the patients with newly diagnosed type 2 diabetes were not of statistical significance during the study period (Table 1).

3.1. Trends in Serum Total Cholesterol and LDL Cholesterol Levels. The mean TC level of 30–89-year-old adults increased from 4.92 ± 1.15 mmol/L in 2003-2004 to 5.30 ± 1.17 mmol/L in 2011-2012 ($P = 0.039$; Figure 1). No significant differences were observed when men and women were analyzed separately. The mean serum TC level was higher in women than in men in each period (Table 2). The mean LDL-C levels gradually increased between 2003-2004 and 2011-2012 ($P = 0.004$; Figure 1). A significant increase was observed in the LDL-C levels in men (from 2.64 ± 0.80 mmol/L in 2003-2004 to 3.09 ± 1.08 mmol/L in 2011-2012; $P = 0.001$; Table 2). In women, the LDL-C level increased from 2.81 ± 0.86 mmol/L in 2003-2004 to 3.18 ± 1.26 mmol/L in 2011-2012, but this increase was not statistically significant ($P = 0.280$; Table 2). In each period, the mean LDL-C level was higher in women than in men (Table 2).

3.2. Trends in Triglycerides and HDL Cholesterol Levels. The mean serum triglyceride levels of all patients increased till 2005-2006 and then decreased till 2011-2012 ($P = 0.148$; Figure 1). The mean HDL-C level decreased continuously from 1.22 ± 0.30 mmol/L in 2003-2004 to 1.06 ± 0.24 mmol/L in 2011-2012 ($P < 0.001$; Figure 1). In men, the HDL cholesterol level showed a significant ($P < 0.001$) downward linear trend from 1.19 ± 0.36 mmol/L in 2003-2004 to 1.02 ± 0.22 mmol/L in 2011-2012 (Table 2), whereas, in women, the HDL-C levels showed no significant change. The mean serum HDL-C level was lower in men than in women in each period (Table 2).

3.3. Prevalence of Dyslipidemia, Hypertension, CHD, and Cerebrovascular Disease. The percentage of patients with a

Table 1: Patient characteristics.

	2003-2004	2005-2006	2007-2008	2009-2010	2011-2012
Sample sizes	122	212	184	240	236
Men	61	128	108	132	121
Women	61	84	76	108	115
Mean age (years)	56.89 ± 12.87	57.35 ± 13.00	56.24 ± 12.42	56.00 ± 12.13	55.90 ± 11.93
BMI (kg/m^2)	25.12 ± 2.14	25.18 ± 2.64	25.19 ± 2.96	25.20 ± 2.08	24.58 ± 2.94
HbA1C (%)	10.25 ± 1.22	10.16 ± 2.37	10.64 ± 2.32	10.26 ± 3.10	10.27 ± 2.60

Values are expressed as means ± SD. BMI: body mass index; HbA1c: haemoglobin A1c.

Table 2: Serum lipids and lipoproteins levels in patients with newly diagnosed type 2 diabetes in Chongqing, 2003–2012.

		03~04	05~06	07~08	09~10	11~12	P
TC (mmol/L)	Total	4.92 ± 1.15	5.04 ± 1.37	5.15 ± 1.46	5.25 ± 1.19	5.30 ± 1.17	0.039
	Men	4.81 ± 1.20	4.90 ± 1.29	5.13 ± 1.51	5.12 ± 1.15	5.26 ± 1.19	0.056
	Women	5.03 ± 1.09	5.40 ± 1.83	5.19 ± 1.38	5.50 ± 1.28	5.37 ± 1.40	0.247
TG (mmol/L)	Total	2.48 ± 2.02	2.93 ± 2.63	2.75 ± 2.50	2.67 ± 1.91	2.41 ± 2.08	0.148
	Men	2.72 ± 2.32	3.10 ± 2.82	3.19 ± 2.96	2.95 ± 2.30	2.76 ± 2.45	0.659
	Women	2.24 ± 1.67	2.64 ± 2.28	2.14 ± 1.48	2.34 ± 1.22	2.03 ± 1.50	0.127
HDL (mmol/L)	Total	1.22 ± 0.30	1.18 ± 0.45	1.09 ± 0.45	1.06 ± 0.29	1.06 ± 0.24	<0.001
	Men	1.19 ± 0.36	1.14 ± 0.32	1.00 ± 0.35	1.01 ± 0.29	1.02 ± 0.22	<0.001
	Women	1.26 ± 0.24	1.24 ± 0.59	1.18 ± 0.54	1.11 ± 0.28	1.12 ± 0.26	0.091
LDL (mmol/L)	Total	2.72 ± 0.83	2.91 ± 1.04	2.98 ± 1.02	3.07 ± 0.74	3.11 ± 1.09	0.004
	Men	2.64 ± 0.80	2.77 ± 1.00	2.95 ± 0.98	2.96 ± 0.78	3.09 ± 1.08	0.001
	Women	2.81 ± 0.86	3.13 ± 1.08	3.03 ± 1.08	3.12 ± 0.84	3.18 ± 1.26	0.280

Values are expressed as means ± SD. TC: total cholesterol; TG: triglyceride; HDL: high- density lipoprotein; LDL: low-density lipoprotein.

serum total cholesterol level of at least 5.18 mmol/L increased gradually from 40.7% in 2003-2004 to 55.6% in 2011-2012 ($P = 0.001$; Figure 2). The prevalence of hypo-HDL-cholesterolemia for men increased greatly from 32.8% in 2003-2004 to 71.2% in 2009-2010 and then decreased to 53.7% in 2011-2012 ($P < 0.001$; Figure 2). In women the percentage of hypo-HDL-cholesterolemia increased from 54.8% to 72.1% during the study period ($P = 0.008$; Figure 2). No significant differences were observed in the prevalence of hyper-LDL-cholesterolemia and hypertriglyceridemia between 2003 and 2012. But we observed that majority of patients newly diagnosed with type 2 diabetes mellitus have high LDL cholesterol levels.

The prevalence rates of hypertension, CHD, and cerebrovascular disease at the time of diagnosis of type 2 diabetes mellitus are shown in Figure 3.

4. Discussion

In this study, we found that both the TC levels and the percentage of patients with hypercholesterolemia increased gradually, and the same trend was observed for LDL cholesterol. The NCEP ATP III constantly monitors the elevated levels of LDL cholesterol, which is a major risk factor contributing to CHD [7]. The recommended LDL cholesterol levels for individuals at high risk for cardiovascular disease and for those without overt cardiovascular disease are <1.8 mmol/L and <2.6 mmol/L, respectively [8]. In our study, the lowest mean LDL cholesterol level for all participants was 2.72 ± 0.83 mmol/L observed in 2003-2004 and not only was higher than the recommended levels, but was also found to have increased since 2003-2004, reaching a high of 3.11 ± 1.09 mmol/L in 2011-2012. Hence, the management of high LDL cholesterol level in diabetes patients is vital for reducing the risk of cardiovascular events.

Although lowering LDL cholesterol level is the major focus in this field of study, benefits associated with targeting other lipids have also been previously demonstrated. For example, HDL cholesterol level is a strong negative indicator for cardiovascular events, with high levels of HDL cholesterol having been suggested to protect against atherosclerotic manifestations. On the basis of several epidemiological studies, when HDL cholesterol levels are increased by 1.0 mg/dL, the occurrence of CHD simultaneously decreases by 2-3% [9]. In our study, unfavorable upward trends were observed in HDL cholesterol levels and the prevalence of low HDL cholesterol for men and women. Accordingly, several studies have shown that HDL cholesterol levels were markedly reduced in both men and women with diabetes mellitus compared with those in nondiabetic individuals [10]. Therefore, management of HDL cholesterol levels should be essential in patients with newly diagnosed type 2 diabetes mellitus.

Although no significant changes were observed in the serum triglycerides levels during 2003–2012, we speculate that this trend may have been influenced by differences in the diets of the patients. According to the values of NCEP

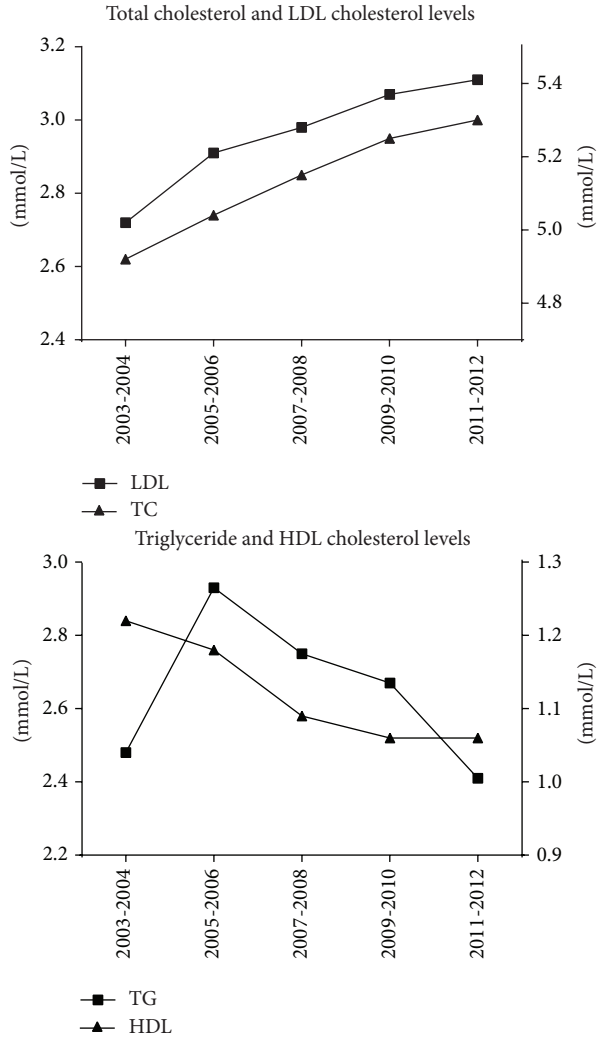

Figure 1: Trends in serum total cholesterol, LDL cholesterol, triglyceride, and HDL cholesterol levels between 2003 and 2012.

ATP III classification, both men and women had elevated triglyceride levels in each time period. Being overweight, sedentary, or a cigarette smoker is associated with elevated triglyceride levels; thus, positive lifestyle changes can help lower triglyceride levels [11].

In our study, we found that, at the time of diagnosis of diabetes, elevated triglyceride levels, high LDL cholesterol levels, and low HDL cholesterol levels were commonly noted, and these are all characteristic features of diabetic dyslipidemia [12]. Moreover, we found that the mean total and LDL cholesterol levels were higher in women than in men. One possible reason for this observation is that hormonal changes after menopause cause a rapid increase in lipid levels, which become higher than those in men [13].

In the past several years, total serum cholesterol levels have declined in most Western populations, such as in Australia, North America, and Europe; however, they have increased in the East, Southeast Asia, and the Pacific regions [14, 15]. In China, the mean levels of serum lipids have been reported to be much higher in areas with rapid economic growth, such as Shanghai and Beijing, compared to those in rural areas [16]. Remarkable socioeconomic development along with a rise in the living standards of Chinese Han population in the past few decades may explain the unfavorable trends in the lipid profiles observed herein. Numerous studies have indicated that lifestyle factors such as high calorie intake, population-wide sedentary lifestyle, decreased physical activities, and increased obesity are related to increased serum lipids levels [17, 18]. Although we did not investigate changes in the eating habits and physical activities of individuals in this study, other studies have reported that the eating habits of the Chinese population have changed dramatically from the traditional dietary pattern (plant-based, low-fat diet) to the Western dietary pattern (high-fat, low-carbohydrate diet), which is characterized by excess calorie intake [19]. Another study reported that China is experiencing rapidly escalating rates of overweightness and obesity in recent years [20]. In China, the rate of overweightness has doubled from 13.5% in

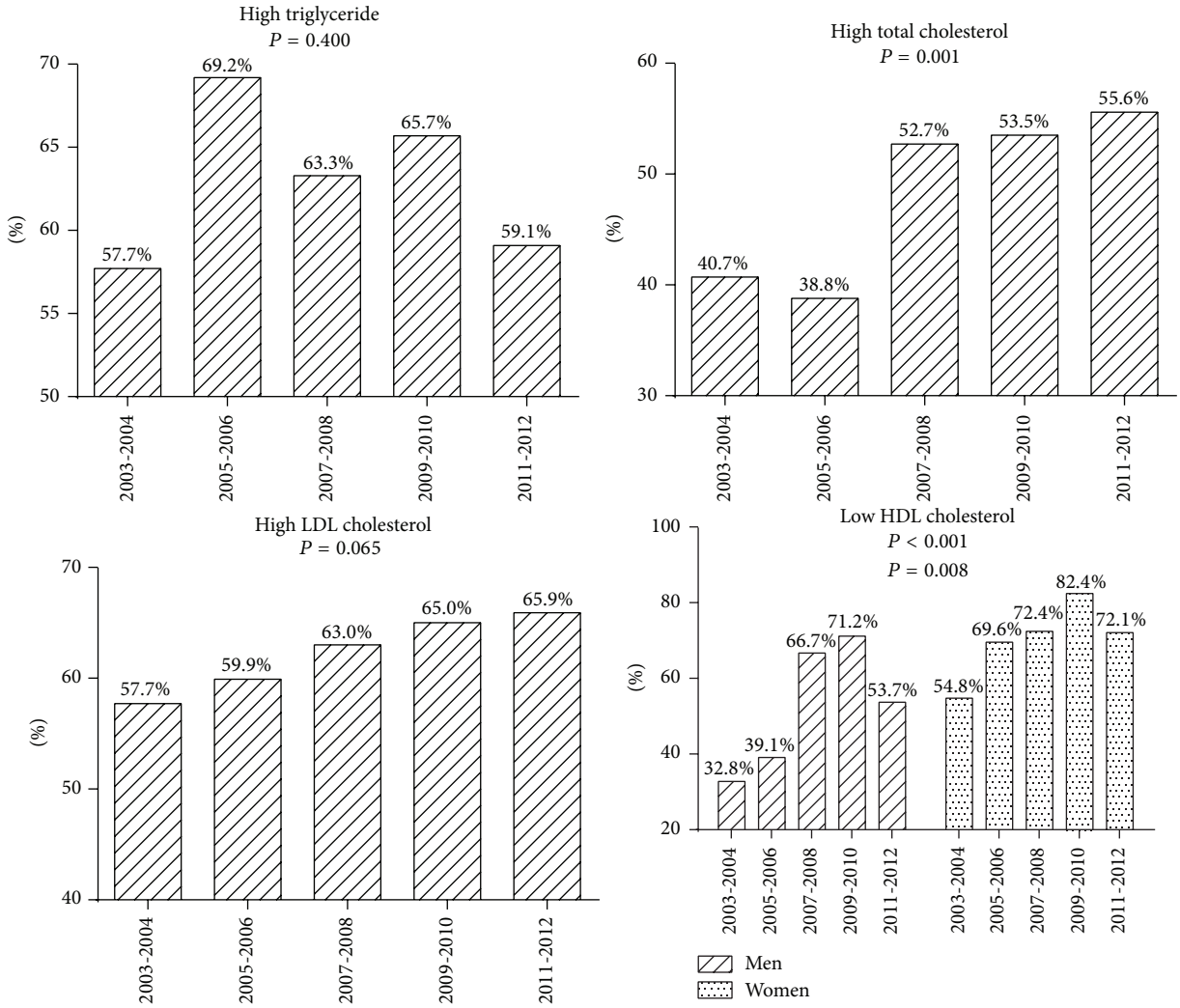

Figure 2: The proportions of patients with dyslipidemia during 2003–2012.

1991 to 26.7% in 2006, and the number of obese individuals has tripled [21]. The classifications of overweightness and obesity in Chinese individuals are based on the BMI cutoffs of 24 kg/m^2 and 28 kg/m^2, respectively [22], and in our study, the average BMI was higher than 24 kg/m^2 in each period, indicating that most of the patients with newly diagnosed type 2 diabetes mellitus were overweight.

Another possible factor responsible for the changes in serum lipid levels is a decrease in physical activity. A sedentary lifestyle is now becoming widespread, especially among urban residents, with the average weekly physical activity among adults having decreased by more than a third between 1991 and 2006 [23]. Increasing reliance on automobiles and increasing availability of buses have replaced the traditional ways of transportation such as biking or walking, and the increased use of television and computers has replaced the traditional means of recreation such as swimming and running [24, 25]. Thus, lifestyle and behavioral changes need to be addressed in order to reduce the incidence of dyslipidemia.

The United Kingdom Prospective Diabetes Study provided conclusive evidence that, with effective control of hyperglycemia in diabetes patients, morbidity due to microvascular complications can be significantly reduced. However, intensive treatment of hyperglycemia did not significantly reduce macrovascular disease [26]. In our study, in accordance with the increase in serum lipid levels, the trends of CHD and cerebrovascular disease in people newly diagnosed with diabetes also continuously increased during the study period. Hence, immediate measures should be taken to prevent macrovascular disease in this population.

The results of this study should be interpreted in light of the following limitations. First, when we classified the study participants into different ages, no statistical significance was observed, and this could be because we did not use a very large enough sample size. Second, this was a cross-sectional analysis and we could investigate factors affecting the prevalence of dyslipidemia, such as dietary intake and physical activities. Hence, further research is needed to assess

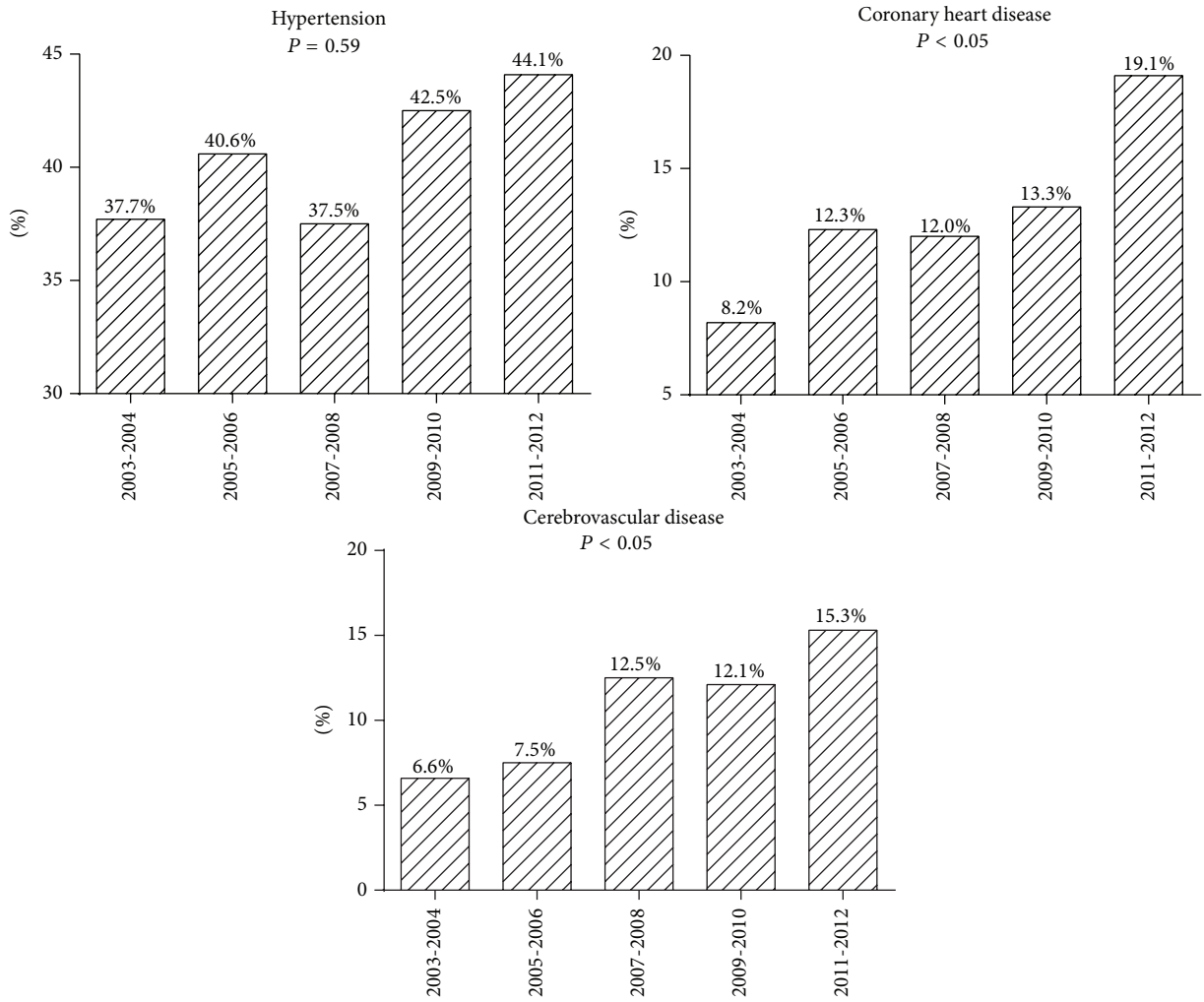

Figure 3: Percentages of patients with hypertension, coronary heart disease, and cerebrovascular disease between 2003 and 2012.

the effects of lifestyle factors on serum levels of lipids. Third, the patients taking lipid-lowering medication were excluded; the percentages of people with dyslipidemia did not include these patients.

5. Conclusion

The results of this study indicated unfavorable upward trends in serum lipid levels and in the prevalence of dyslipidemia, CHD, and cerebrovascular disease among patients with newly diagnosed type 2 diabetes mellitus in the Southwest Chinese Han population. Widespread promotions of ways to lower lipid levels and of treatment strategies should be intensified to reduce cardiovascular morbidity and mortality. In conclusion, dyslipidemia is preventable and now is the ideal time for implementing appropriate strategies to battle this epidemic.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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References

[1] W. Yang, J. Lu, J. Weng et al., "Prevalence of diabetes among men and women in China," *The New England Journal of Medicine*, vol. 362, no. 12, pp. 1090–1101, 2010.

[2] W. P. Castelli, "The triglyceride issue: a view from Framingham," *American Heart Journal*, vol. 112, no. 2, pp. 432–437, 1986.

[3] P. P. Toth, "Subclinical atherosclerosis: what it is, what it means and what we can do about it," *International Journal of Clinical Practice*, vol. 62, no. 8, pp. 1246–1254, 2008.

[4] P. W. F. Wilson, R. B. D'Agostino, D. Levy, A. M. Belanger, H. Silbershatz, and W. B. Kannel, "Prediction of coronary heart disease using risk factor categories," *Circulation*, vol. 97, no. 18, pp. 1837–1847, 1998.

[5] W.-H. Zhao, J. Zhang, Y. Zhai et al., "Blood lipid profile and prevalence of dyslipidemia in chinese adults," *Biomedical and Environmental Sciences*, vol. 20, no. 4, pp. 329–335, 2007.

[6] National Cholesterol Education Program, "Detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel III)," *Circulation*, vol. 106, pp. 3143–3421, 2002.

[7] S. M. Grundy, "United States cholesterol guidelines 2001: Expanded scope of intensive low-density lipoprotein-lowering therapy," *American Journal of Cardiology*, vol. 88, no. 7, pp. 23J–27J, 2001.

[8] American Diabetes Association, "Standards of medical care in diabetes—2013," *Diabetes Care*, vol. 36, supplement 1, pp. S11–S66, 2013.

[9] S. M. Grundy, D. Bilheimer, A. Chait et al., "Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II)," *Journal of the American Medical Association*, vol. 269, no. 23, pp. 3015–3023, 1993.

[10] W. B. Kannel, "Lipids, diabetes, and coronary heart disease: insights from the Framingham study," *American Heart Journal*, vol. 110, no. 5, pp. 1100–1107, 1985.

[11] National Institutes of Health, "Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults," The Evidence Report NIH 98-4083, National Heart, Lung and Blood Institute, Bethesda, Md, USA, 1998.

[12] A. D. Mooradian, "Dyslipidemia in type 2 diabetes mellitus," *Nature Clinical Practice Endocrinology and Metabolism*, vol. 5, no. 3, pp. 150–159, 2009.

[13] D. de Aloysio, M. Gambacciani, M. Meschia et al., "The effect of menopause on blood lipid and lipoprotein levels," *Atherosclerosis*, vol. 147, no. 1, pp. 147–153, 1999.

[14] F. Farzadfar, M. M. Finucane, G. Danaei et al., "National, regional, and global trends in serum total cholesterol since 1980: systematic analysis of health examination surveys and epidemiological studies with 321 country-years and 3.0 million participants," *The Lancet*, vol. 377, no. 9765, pp. 578–586, 2011.

[15] E. V. Kuklina, P. W. Yoon, and N. L. Keenan, "Trends in high levels of low-density lipoprotein cholesterol in the United States, 1999-2006," *Journal of the American Medical Association*, vol. 302, no. 19, pp. 2104–2110, 2009.

[16] S. Wang, L. Xu, J. B. Jonas, Q. S. You, Y. X. Wang, and H. Yang, "Prevalence and associated factors of dyslipidemia in the adult Chinese population," *PLoS ONE*, vol. 6, no. 2, Article ID e17061, 2011.

[17] T. O. Cheng, "Prevention of cardiovascular disease in modern China: utile or futile?" *CVD Prevention and Control*, vol. 4, no. 1, pp. 1–7, 2009.

[18] T. O. Cheng, "Effects of fast foods, rising blood pressure and increasing serum cholesterol on cardiovascular disease in China," *American Journal of Cardiology*, vol. 97, no. 11, pp. 1676–1678, 2006.

[19] B. M. Popkin, "Will China's nutrition transition overwhelm its health care system and slow economic growth?" *Health Affairs*, vol. 27, no. 4, pp. 1064–1076, 2008.

[20] T. O. Cheng, "Obesity epidemic in modern China," *International Journal of Cardiology*, vol. 118, no. 3, p. 397, 2007.

[21] Y. Lu and D. Goldman, "The effects of relative food prices on obesity—evidence from China: 1991–2006," NBER Working Paper no. 15720. JEL no. D01, I1, J88, 1991.

[22] C. Chen and F. C. Lu, "The guidelines for prevention and control of overweight and obesity in Chinese adults," *Biomedical and Environmental Sciences*, vol. 17, pp. 1–36, 2004.

[23] B. M. Popkin, "Global nutrition dynamics: the world is shifting rapidly toward a diet linked with noncommunicable diseases," *The American Journal of Clinical Nutrition*, vol. 84, no. 2, pp. 289–298, 2006.

[24] T. O. Cheng, "Fast food, automobiles, television and obesity epidemic in Chinese children," *International Journal of Cardiology*, vol. 98, no. 1, pp. 173–174, 2005.

[25] B. M. Popkin, "The nutrition transition and obesity in the developing world," *Journal of Nutrition*, vol. 131, no. 3, pp. 871S–873S, 2001.

[26] R. Turner, "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

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Research Article

Inverse Levels of Adiponectin in Type 1 and Type 2 Diabetes Are in Accordance with the State of Albuminuria

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Aims. To investigate the behaviour of adiponectin (ApN) in patients with type 1 and type 2 diabetic nephropathy. *Methods.* ApN and inflammatory and other markers of the metabolic syndrome were compared across diabetes types, albumin excretion rate (AER), and creatinine clearance (CrCl) categories in 219 type 1 and type 2 diabetic patients. *Results.* Significant differences among ApN levels according to AER were found in both types of diabetes ($F = 8.45$, df = 2, $P < 0.001$). With the progression of albuminuria, ApN increased in type 1 and decreased in type 2 diabetes. Patients with decreased CrCl had higher ApN levels than those with normal CrCl in either type of diabetes ($F = 12.7$, df = 1, $P < 0.001$). The best model for ApN ($R^2 = 0.9002$) obtained from stepwise regression in type 1 diabetes included CrCl, BMI, WBC, CRP, and age, while in type 2 diabetes ($R^2 = 0.2882$) it included ppPG, LDL, and UA. *Conclusion.* ApN behaved differently in relation to albuminuria, increasing with its progression in type 1 diabetes and decreasing in type 2 diabetes. It was however increased in the subgroups with decreased CrCl in both types of diabetes. Albuminuria seems to be more important than renal insufficiency in the definition of ApN levels in type 1 and type 2 diabetes.

1. Introduction

Adiponectin (ApN) has an impact on endothelial cell function by its anti-inflammatory properties and stimulation of nitric oxide production [1, 2]. On the other hand, dyslipidemia is characterized by increased serum triglycerides and decreased high-density lipoprotein cholesterol (HDL-C), which correlates with low ApN levels [3]. Dyslipidemia as part of the metabolic syndrome is a risk factor for endothelial dysfunction and atherosclerosis as well. Its levels are significantly higher in women than in men, probably due to variations in sex hormones during lifetime [3, 4]. Although serum ApN in healthy people might be associated with vascular function independently of insulin resistance, increased inflammatory markers are connected with an increase in insulin resistance in patients with impaired glucose tolerance, type 2 diabetes, and obesity, who also have low circulating ApN concentrations [2, 5–7]. Nevertheless, due to its insulin sensitizing action, ApN seems to have a role in both insulin resistance and vascular protection [8]. Furthermore,

because low ApN affects dyslipidemia, inflammation, insulin sensitivity, and vascular protection, it is important in the onset of cardiovascular events [9].

As an anti-inflammatory mediator, ApN might also be responsible for the prevention of diabetic microangiopathy. In type 1 diabetic patients nephropathy correlates with increased ApN levels [3, 10]. Recent prospective studies have found a link between hyperadiponectinemia and mortality in chronic kidney disease. ApN has been reported to play a protective role in male wild-type mice by reducing albuminuria through an effect on podocytes through the AMP-activated protein kinase (AMPK) pathway [7, 11].

A family history of diabetes could be associated with hypoadiponectinemia. Adiponectin gene polymorphisms have been determined to be associated with a risk of diabetic nephropathy [12]. Our previous study has demonstrated significantly higher adiponectin levels in type 1 diabetes as compared with type 2 diabetes and also identified C-peptide as a significant determinant of this difference [13].

The aim of the present study was to investigate the relationship of adiponectin and other markers of the metabolic syndrome with nephropathy in patients with type 1 and type 2 diabetes.

2. Materials and Methods

The study protocol was approved by the hospital's ethics committee. The patients received both written and oral information about the study and signed a written informed consent.

2.1. Patients. A total of 219 patients treated at our outpatient department were included in the study: 87 with type 1 diabetes and 132 with type 2 diabetes. Blood samples were taken after 12 hr fast. Patients with type 2 diabetes were on oral hypoglycemic agents and/or diet, and patients with type 1 diabetes were on either intensive insulin treatment or 2 to 3 doses of premixed insulin. Diabetes mellitus was defined according to the American Diabetes Association classification [14]. Patients with malignancies and immunologic and infectious inflammatory diseases, pregnant women, and patients receiving corticosteroids or cytostatics were not included in the study. In all patients, fundoscopy was performed to determine the presence of retinopathy and to decide on further diagnostic procedures in patients with albuminuria and without retinopathy.

Clinical and laboratory markers of diabetes, obesity, and metabolic syndrome included age, diabetes duration, body mass index (BMI), ApN, C-reactive protein (CRP), fibrinogen (FIB), homocysteine (HCY), creatinine clearance (CrCl), creatinine, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting (fPG) and postprandial plasma glucose (ppPG), glycated hemoglobin (A1c), liver function tests (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and gamma-glutamyl transpeptidase [GGT]), lipids (high density lipoprotein [HDL-C], low density lipoprotein [LDL-C], and triglycerides [TG]), ferritin, uric acid (UA), creatine phosphokinase (CPK), and leucocyte count (WBC) were determined. Patients were assigned to subgroups based on albumin excretion rate (AER) (<30 [normoalbuminuria], 30–300 [microalbuminuria], >300 mg/24 h [macroalbuminuria]), and CrCl (normal >0.83 mL/sec for women and >1.17 mL/sec for men). AER and CrCl were calculated from three consecutive urine sample collections. Blood pressure was measured after five minutes of supine rest and mean values of three measurements were used in statistical analysis. Previous myocardial infraction and stroke were also determined.

2.2. Laboratory Tests. Serum ApN was measured by sandwich ELISA (DRG, Marburg, Germany), plasma FIB by the Clauss method, and hs-CRP by an immunoturbidimetric assay on an Olympus AU600 analyzer (Beckman-Coulter, Brea, CA, USA). Hemoglobin A1c was measured by an automated immunoturbidimetric procedure on a dedicated analyzer (Integra, TinaQuant, Roche Diagnostics, Hoffmann-LaRoche, Basel, Switzerland) with results traceable to the NGSP-standard.

HCY in EDTA plasma was measured by an automated chemiluminescence assay (Advia Centaur, Siemens Diagnostic Solutions, Tarrytown, NY, USA). Cholesterol, TG, UA, and glucose were analyzed using standard enzymatic procedures and HDL-C using a homogeneous assay on an automated analyzer (Olympus AU600, Beckman-Coulter, Brea, CA, USA).

2.3. Data Analysis. All variables, age, diabetes duration, BMI, ApN, CRP, FIB, HCY, CrCl, creatinine, SBP, DBP, fPG, ppPG, A1c, AST, ALT, GGT, HDL-C, LDL-C, and TG, ferritin, UA, CPK, and WBC, were analyzed using descriptive statistics. Type error $I(\alpha)$ of 0.05 was considered statistically significant.

Differences between type 1 and type 2 diabetes were tested using Student's t-test or Mann-Whitney U test if assumption of homogeneity of variance was not satisfied. Difference in ApN between the groups according to AER (<30, 30–300, >300) and CrCl (normal >0.83 mL/sec for women and >1.17 mL/sec for men) and their interactions were tested using analysis of variance (ANOVA). If a significant difference was observed, Tukey's HSD post hoc test was used to determine which groups were significantly different from each other. Stepwise regression was used to detect main predictors of ApN in DM groups. Student's t-test, Mann-Whitney U test, and ANOVA were performed using STATISTICA 8 and stepwise regression using SAS 9.1 [15]. The graphs were created using STATISTICA [16].

3. Results

ApN and HDL were significantly increased in type 1 diabetes in comparison with type 2 diabetes, whereas CRP, FIB, HCY, and GGT were significantly increased in DM2 (Table 1).

Statistically significant differences among ApN levels according to AER were found ($F = 8.45$, df = 2, $P < 0.001$) between type 1 diabetes (<30 = 12.37 ± 6.62, 30–300 = 21.38 ± 7.98, and >300 = 31.85 ± 18.05) and type 2 diabetes (<30 = 9.05 ± 5.63, 30–300 = 7.46 ± 4.58, and >300 = 5.26 ± 3.3) (Figure 1). The difference in duration of disease between

Table 1: Differences between biochemical data of the study groups according to type of diabetes.

Variable	Group 1: DM1 *t*-test			Group 2: DM2 *t*-test			*t*-value	df	*P*
	Mean 1	Std. Dev.	*N* 1	Mean 2	Std. Dev.	*N* 2			
Hs-CRP (mg/L)	2.06	3.45	87	3.66	4.17	131	−1.98	160	0.049
FIB (g/L)	3.76	1.43	87	4.73	1.22	132	−3.86	161	<0.001
Ferritin (μg/mL)	135.2	136.9	84	155.9	122.75	89	−0.72	111	0.474
	Mann-Whitney *U* test			Mann-Whitney *U* test			*U*	*Z*	*P*
	Mean 1	Std. Dev.	*N* 1	Mean 2	Std. Dev.	*N* 2			
ApN (μg/mL)	15.37	9.27	87	8.07	5.15	131	886.5	4.87	<0.001
HCY (μmol/mL)	11.1	2.92	87	15.6	6.92	132	1105.5	−3.98	<0.001
Lp(a) (mg/dL)	23.52	21.65	87	39.9	52.31	119	1172	−1.38	0.167
HDL-C (mmol/L)	1.6	0.5	87	1.34	0.32	132	1314	3.09	0.002
AST (U/L)	25.67	10.9	85	22.86	7.09	131	1641	1.40	0.160
ALT (U/L)	31.12	17.5	87	29.05	13.4	131	1956	0.32	0.751
GGT (U/L)	23.03	13.6	85	37.37	30.1	131	1171	−3.45	0.001
UA (μmol/L)	283.13	83.8	85	383.3	350.43	132	1127	−3.68	<0.001

DM1, type 1 diabetes; DM2, type 2 diabetes.

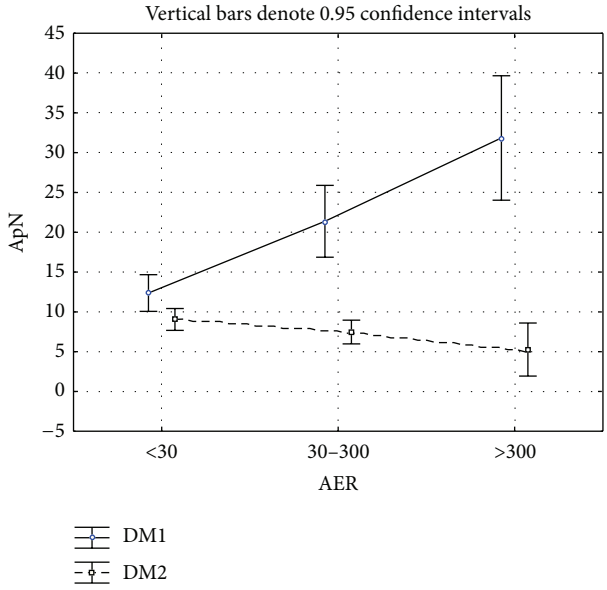

Figure 1: ApN mean values and 95% confidence intervals in patients with type 1 and type 2 diabetes according to AER.

type 1 and type 2 diabetic patients was not observed (6.02 ± 5.25 yrs and 7.14±7.51 yrs) ($P = 0.97$). There were also significant differences in ApN between the types of diabetes ($F = 73.402$, df = 1, $P < 0.001$) and in the interaction between the type of diabetes and AER ($F = 18.12$, df = 2, $P < 0.001$), indicating that ApN did not behave comparably across AER categories in both types of diabetes. In type 1 diabetes ApN levels increased with an increase in AER, whereas in type 2 they were found to decrease (Table 2, Figure 1). Patients with type 1 diabetes had significantly higher ApN values than those with type 2 diabetes. Tukey's post hoc test pointed to a significant difference in ApN between type 1 diabetic subgroups with normoalbuminuria and microalbuminuria and those with normoalbuminuria and macroalbuminuria, as well as between type 1 and type 2 subgroups with normoalbuminuria and macroalbuminuria (Table 3). Patients with type 1 and type 2 diabetes differ (Student's *t*-test) in the occurrence of myocardial infarction (type 1: 3.4% and type 2: 4.5%) and stroke (type 1: 2.3% and type 2: 5.3%).

In a model for ApN as a dependent variable and the type of diabetes, CrCl, and the interaction between the type of DM and CrCl as factors, a statistically significant difference was found for all analyzed factors (Figure 2). ApN levels according to CrCl ($F = 12.7$, df = 1, $P < 0.001$) were higher in patients with decreased CrCl (1) than in those with normal CrCl (2). In type 1 diabetic patients with normal CrCL (2) ApN levels were 13.9 ± 7.93, and in patients with decreased CrCL (1) they were 23 ± 12.8. In type 2 diabetic patients with normal CrCl (2), those levels were 7.63 ± 4.76 and in patients with decreased CrCl (1) they were 9.86 ± 6.25 (Table 2). Although ApN was not significantly increased in the group with decreased CrCl, post hoc results pointed to a significant increase in ApN in type 1 diabetes subgroups with normal and decreased CrCL as compared with type 2 diabetes (Table 3).

The best model for ApN ($R^2 = 0.9002$) obtained from stepwise regression in type 1 diabetes included CrCl, BMI, WBC, CRP, and age, while in type 2 diabetes the best model ($R^2 = 0.2882$) included ppPG, LDL, and UA (Table 4).

In type 1 diabetes ApN correlated significantly ($P < 0.05$) with HCY ($r = 0.57$), CrCL ($r = -0.61$), AER ($r = 0.61$), and creatinine ($r = 0.40$), and in type 2 diabetes it correlated with HCY ($r = 0.25$), CrCl ($r = -0.22$), creatinine ($r = 0.20$), and diastolic BP ($r = -0.19$).

4. Discussion

ApN and inflammatory factors are inversely correlated [6], which was confirmed by the observed significant differences in ApN, CRP, FIB, and HCY according to the type of diabetes. Levels of ApN and HDL were significantly increased in

Table 2: Results of ANOVA for ApN as dependent variables according to AER, type of diabetes, CrCl, and their interactions.

Dependent variable	Factors	Sums of squares	Degree of freedom	Mean squares	F	P
ApN	Type of DM	2299.98	1	2299.98	73.4	<0.0001
	AER	529.55	2	264.78	8.45	0.0003
	DM ∗ AER	1135.66	2	567.83	18.12	<0.0001
ApN	Type DM	1321.04	1	1321.04	37.32	<0.0001
	CrCl	449.71	1	449.71	12.7	0.0005
	DM ∗ CrCl	165.76	1	165.76	4.68	0.032

Table 3: Interaction between the type of diabetes and AER and the type of diabetes and CrCl for ApN as dependent variable using Tukey's HSD post hoc test.

(a)

Cell number	Type of DM	AER mg/24 h	{1}	{2}	{3}	{4}	{5}
{1}	1	<30					
{2}	1	30–300	0.0060				
{3}	1	>300	0.0001	0.1972			
{4}	2	<30	0.1422	<0.0001	<0.0001		
{5}	2	30–300	0.0056	<0.0001	<0.0001	0.6316	
{6}	2	>300	0.0071	<0.0001	<0.0001	0.2991	0.8415

(b)

Cell number	Type of DM	CrCl mL/s	{1}	{2}	{3}
{1}	1	$F \leq 0.83, M \leq 1.17$			
{2}	1	$F > 0.83, M > 1.17$	0.0094		
{3}	2	$F \leq 0.83, M \leq 1.17$	<0.0001	0.0642	
{4}	2	$F > 0.83, M > 1.17$	<0.0001	<0.0001	0.3083

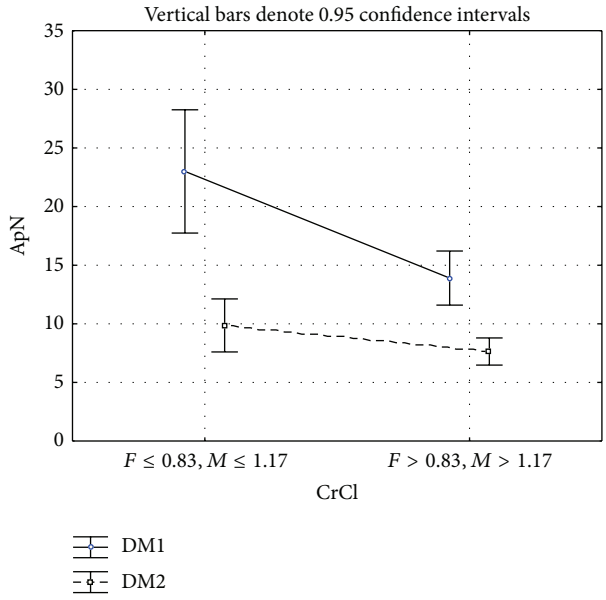

Figure 2: ApN mean values and 95% confidence intervals in patients with type 1 and type 2 diabetes according to CrCl.

type 1 diabetes in comparison with type 2 diabetes, whereas CRP, FIB, HCY, and GGT were significantly increased in type 2 diabetes, indicating that this type of diabetes is an inflammatory state. This is in agreement with the reports on decreased ApN levels in patients with prediabetes and type 2 diabetes [17] and a negative association between plasma ApN and the levels of CRP and WBC [18].

GGT, a marker of the metabolic syndrome and fatty liver, was significantly increased in type 2 diabetes in comparison with type 1 diabetes. A significant association between GGT and insulin resistance has previously been demonstrated in diabetic patients, introducing GGT as one of the markers for metabolic syndrome [19]. Serum ApN and tumor necrosis factor-α are independent predictors of liver steatosis, and ApN has additionally been found to be an independent predictor of response to chronic hepatitis C therapy [20].

Recent reports have pointed to an interrelation between C-peptide levels and ApN production in adipocytes [13]. In nonobese subjects ApN has been shown to correlate better with β-cell function than with insulin sensitivity [21]. The relationship between intensive insulin treatment and ApN is

Table 4: Results of stepwise regression analysis for ApN as a dependent variable in DM1 and DM2.

	Variable	Parameter estimate	Standard error	F	P	Partial R^2	R^2
DM1	Age	0.265	0.064	17.08	0.0020	0.0608	0.9063
	BMI	−2.073	0.294	49.86	<0.0001	0.3454	
	Hs-CRP	0.727	0.251	8.41	0.0158	0.0441	
	CrCl	−10.975	2.035	29.06	0.0003	0.3197	
	WBC	1.830	0.503	13.24	0.0046	0.0593	
DM2	ppPG	−0.603	0.284	4.53	0.0426	0.1418	0.2882
	LDL	2.035	1.039	3.83	0.0606	0.0862	
	UA	0.018	0.012	2.28	0.1423	0.0602	

All variables left in the model are significant at the 0.15 level.

still not clear. The DIGAMI study has demonstrated that diabetic patients with acute myocardial infarction on intensive insulin treatment have a better prognosis and an absolute reduction in mortality [22]. The effect of intensive insulin treatment on ApN levels has been discussed as a possible explanation [23]. Several authors have observed that intensive insulin treatment normalized elevated serum C-peptide and increased circulating ApN levels, thus improving insulin sensitivity [24, 25]. This is in agreement with our results which showed that diabetic patients on intensive insulin treatment had increased ApN and decreased CRP levels.

In this study, diastolic BP correlated significantly with ApN in type 2 diabetes. Low ApN level could be considered to be a marker that predicts arterial hypertension and stiffness as a result of impaired vasodilation caused by decreased nitric oxide (NO) production in insulin resistant patients [26]. A correlation between epicardial adipose tissue expression, low ApN level, and hypertension has already been reported [2]. Although HCY is also a risk factor for cardiovascular diseases, its association with nephropathy seems even more important. The present study showed a correlation between HCY and ApN levels in both types of diabetes and an increased HCY level in type 2 diabetes. Seshardi and coworkers have shown that HCY and CRP are associated with the onset and progression of albuminuria as measured by the albumin/creatinine ratio and with the promotion of inflammatory state but through different mediators of inflammation [27].

Our results did not reveal a correlation between ApN and BMI either in type 1 diabetic patients or in type 2 patients, which corresponds to the lack of such a correlation reported in Japanese men [10, 28]. A study of the association of ApN with insulin resistance and dyslipidemia has demonstrated that ApN does not correlate with overall obesity but with subcutaneous abdominal fat. Furthermore, the impact of overall obesity on ApN production has also been found to be less significant than that of epicardial fat tissue [2].

As reported in the literature, ApN is associated with endothelial protection [8]. Its presumed protective role, however, is not in agreement with the finding of increased ApN levels in type 1 diabetic patients and especially in those with advanced stages of nephropathy. Higher ApN levels in type 1 diabetes have been reported to be associated with the onset and progression of microalbuminuria [10]. A possible explanation could be that ApN can change the integrity of endothelial junctions and induce NO production, thus affecting hyperfiltration [29]. Increased ApN levels in patients with renal failure and proteinuria could be attributed to increased ApN production and reduced clearance in renal failure [30]. Increased ApN has been hypothesized to be a marker of cachexia and catabolism in subjects with renal failure, type 1 diabetes, or weight loss [31]. Another reason why ApN is increased in nephropathy could be that, belonging to the soluble collagen family, it accumulates in the subintimal space of the arterial wall through its interaction with collagens in the vascular intima and consequently attenuates TNF-α–induced expression of adhesion molecules in endothelial cells [2]. Studies in male wild-type mice have demonstrated the protective role of ApN in affecting podocytes through the AMPK pathway, thus reducing albuminuria [7, 11].

In this study, statistically significant differences in ApN levels among different stages of albuminuria were found between type 1 diabetes and type 2 diabetes (Figure 1). There were also significant differences in ApN between the types of diabetes and in the interaction between the type of diabetes and AER. Patients with type 1 diabetes had significantly higher ApN than those with type 2 diabetes at any AER level. Significant differences in ApN were also found between type 1 diabetic subgroups with normoalbuminuria and microalbuminuria and with normoalbuminuria and macroalbuminuria. Such differences were also observed in the subgroups with normoalbuminuria and macroalbuminuria between type 1 and type 2 diabetic patients (Table 3). ApN increases with the progression of albuminuria in type 1 diabetic patients, which is concordant with the observed hyperadiponectinemia in patients with chronic kidney disease [32]. High plasma ApN concentrations decrease after renal transplantation, suggesting that renal insufficiency may either have an effect on ApN clearance and/or stimulate ApN production. The metabolic state improves after transplantation [33]. Our patients with type 2 diabetes showed a decrease in ApN levels with the progression of albuminuria and a rise in other inflammatory markers, which pointed to an increase in inflammation accompanying albuminuria. The decrease in ApN in type 2 diabetes could also be explained by obesity, as suggested by Guebre-Egziabher and coworkers [34].

In a study of 50 patients with type 2 diabetes, ApN was increased in subjects with macroalbuminuria and inversely correlated with creatinine levels. The authors have concluded that macroalbuminuria is superior to impaired renal function in determining the level of ApN [35]. The decrease in ApN relative to the progression of albuminuria in patients with type 2 diabetes observed in our study confirmed results of previous studies which have demonstrated that low circulating ApN that accompanies proteinuria regardless of the degree of renal function impairment is an important predictor of endothelial dysfunction [36].

ApN levels were higher in patients with decreased CrCl than in those with increased CrCl in both types of diabetes (Table 2). A model for ApN as a dependent variable and the type of diabetes mellitus, CrCl, and the interaction between the type of diabetes and CrCl as factors revealed statistically significant differences for all analyzed factors (Figure 2). ApN was significantly higher in type 1 diabetes than in type 2 diabetes in both normal and decreased CrCl subgroups (Table 3). This increase could also be associated with a possible role of C-peptide as one of the determinants responsible for the difference in ApN levels between type 1 and type 2 diabetes [13, 21–24]. A high plasma ApN level observed in a mice renal failure model has been explained by a low clearance rate [7]. The decrease in CrCl in our study population could also be responsible for changes in ApN levels. CrCl-related ApN in the present study behaved the same in both types of diabetes, in contrast to the behavior it exhibited in relation to albuminuria. This points to the importance of albuminuria in comparison with CrCl in the determination of ApN level, as concluded by Ran and coworkers [35].

In patients with type 1 diabetes ApN was best predicted by CrCl, BMI, WBC, CRP, and age. In patients with type 2 diabetes, the main predictors were ppPG, LDL-cholesterol, and UA (Table 4). Besides other known predictors such as CRP, LDL-cholesterol, and UA, WBC could be held responsible for the development of atherosclerosis [18]. Higher UA levels have been proven to be a protective antioxidant mechanism in hyperglycemic states and obesity. They have also been connected to microvascular complications in diabetes [37]. There is evidence that plasma ApN level is associated with hyperlipidemia and a consequently increased cardiovascular risk in patients with renal insufficiency [38]. Although there was a difference in occurrence of myocardial infarction and stroke between groups of patients with type 1 and type 2 diabetes, the number of patients limited the comparison between groups according to albuminuria levels. As it is known, albuminuria and ApN are among markers of cardiovascular disease. In this study, ApN behaved differently in different type of diabetes which could be among the reasons for different cardiovascular risk [39].

Our finding of decreased HDL-C and ApN in type 2 diabetes in comparison with type 1 diabetes corresponds to the reports hypothesizing that low ApN might be a trigger for dyslipidemia [25], the finding that LDL was among the main predictors of ApN in type 2 diabetes further corroborating the hypothesis.

5. Conclusion

ApN was increased in type 1 and type 2 diabetic subgroups with decreased CrCl but behaved differently in relation to albuminuria, showing an increase with the progression of albuminuria in type 1 diabetes and a decrease in type 2 diabetes. It was higher in the subgroups with normoalbuminuria and macroalbuminuria in type 1 diabetes than in comparable type 2 subgroups. It seems that the interaction between various degrees of renal insufficiency and albumin loss could be important on ApN levels. Increased levels of ApN in type 1 diabetes could be explained by a positive feedback loop, where loss of ApN due to albuminuria causes increased synthesis of ApN. This in turn suggests a protective anti-inflammatory action of ApN. Other inflammatory markers investigated in this study were found to be decreased in type 1 diabetes. The interaction between inflammation as part of the metabolic syndrome and ApN level is important in the development of nephropathy, possibly being responsible for different courses of nephropathy in the two types of diabetes. We can expect that patients with different types of diabetes might be under different risk for cardiovascular disease, in part because of a period of undiagnosed type 2 diabetes but mostly because of the difference in inflammation, adiponectin level, and presentation of metabolic syndrome in each type of diabetes. ApN behavior opens many questions which will be answered in futures studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] G. K. Shetty, P. A. Economides, E. S. Horton, C. S. Mantzoros, and A. Veves, "Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes," *Diabetes Care*, vol. 27, no. 10, pp. 2450–2457, 2004.

[2] E. Teijeira-Fernandez, S. Eiras, L. Grigorian-Shamagian, A. Fernandez, B. Adrio, and J. R. Gonzalez-Juanatey, "Epicardial adipose tissue expression of adiponectin is lower in patients with hypertension," *Journal of Human Hypertension*, vol. 22, no. 12, pp. 856–863, 2008.

[3] N. L. Brooks, K. S. Moore, R. D. Clark, M. T. Perfetti, C. M. Trent, and T. P. Combs, "Do low levels of circulating adiponectin represent a biomarker or just another risk factor for the metabolic syndrome?" *Diabetes, Obesity and Metabolism*, vol. 9, no. 3, pp. 246–258, 2007.

[4] H. Nishizawa, L. Shimomura, K. Kishida et al., "Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein," *Diabetes*, vol. 51, no. 9, pp. 2734–2741, 2002.

[5] P. M. Ridker, N. Rifai, L. Rose, J. E. Buring, and N. R. Cook, "Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events," *The New England Journal of Medicine*, vol. 347, no. 20, pp. 1557–1565, 2002.

[6] N. Ouchi, S. Kihara, T. Funahashi et al., "Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue," *Circulation*, vol. 107, no. 5, pp. 671–674, 2003.

[7] N. Komura, S. Kihara, M. Sonoda et al., "Increment and impairment of adiponectin in renal failure," *Cardiovascular Research*, vol. 86, no. 3, pp. 471–477, 2010.

[8] J.-M. Fernández-Real, A. Castro, G. Vázquez et al., "Adiponectin is associated with vascular funiction independent of insulin sensitivity," *Diabetes Care*, vol. 27, no. 3, pp. 739–745, 2004.

[9] T. Pischon, C. J. Girman, G. S. Hotamisligil, N. Rifai, F. B. Hu, and E. B. Rimm, "Plasma adiponectin levels and risk of myocardial infarction in men," *Journal of the American Medical Association*, vol. 291, no. 14, pp. 1730–1737, 2004.

[10] S. Hadjadj, R. Aubert, F. Fumeron et al., "Increased plasma adiponectin concentrations are associated with microangiopathy in type 1 diabetic subjects," *Diabetologia*, vol. 48, no. 6, pp. 1088–1092, 2005.

[11] K. Sharma, S. RamachandraRao, G. Qiu et al., "Adiponectin regulates albuminuria and podocyte function in mice," *The Journal of Clinical Investigation*, vol. 118, no. 5, pp. 1645–1656, 2008.

[12] Y. Cai, T. Zeng, and L. Chen, "Association of adiponectin polymorphisms with the risk of diabetic nephropathy in type 2 diabetes: a meta-analysis," *Journal of Diabetes*, vol. 7, no. 1, pp. 31–40, 2015.

[13] S. Ljubic, J. Boras, A. Jazbec et al., "Adiponectin has different mechanisms in type 1 and type 2 diabetes with C-peptide link," *Clinical & Investigative Medicine*, vol. 32, no. 4, pp. E271–E279, 2009.

[14] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 32, supplement 1, pp. S62–S67, 2009.

[15] SAS Institute, Cary, NC, USA: SAS Online Doc, 2008, http://support.sas.com/documentation/onlinedoc/91pdf/index_913.html#stat.

[16] StatSoft, *Electronic Statistics Textbook*, StatSoft, Tulsa, Okla, USA, 2011, http://www.statsoft.com/textbook/.

[17] S. Yaturu, J. Bridges, and D. R. R. S. Reddy, "Decreased levels of plasma adiponectin in prediabetes, type 2 diabetes and coronary artery disease," *Medical Science Monitor*, vol. 12, no. 1, pp. CR17–CR20, 2006.

[18] M. Matsubara, K. Namioka, and S. Katayose, "Decreased plasma adiponectin concentrations in women with low-grade C-reactive protein elevation," *European Journal of Endocrinology*, vol. 148, no. 6, pp. 657–662, 2003.

[19] H. Yokoyama, S. Moriya, Y. Homma, and T. Ogawa, "Association between γ-glutamyl transpeptidase activity and status of disorders constituting insulin resistance syndrome," *Alcoholism: Clinical and Experimental Research*, vol. 27, no. 8, pp. 22S–25S, 2003.

[20] T. A. Zografos, C. Liaskos, E. I. Rigopoulou et al., "Adiponectin: a new independent predictor of liver steatosis and response to IFN-α treatment in chronic hepatitis C," *The American Journal of Gastroenterology*, vol. 103, no. 3, pp. 605–614, 2008.

[21] M. Furuta, M. Tamai, T. Hanabusa, Y. Yamamoto, K. Nanjo, and T. Sanke, "Serum adiponectin is associated with fasting serum C-peptide in non-obese diabetic patients," *Diabetes Research and Clinical Practice*, vol. 72, no. 3, pp. 302–307, 2006.

[22] K. Malmberg, "Prospective randomised study of intensive insulin treatment on long term survival after acute myocardial infarction in patients with diabetes mellitus," *British Medical Journal*, vol. 314, no. 7093, pp. 1512–1515, 1997.

[23] P. Dandona, A. Aljada, P. Mohanty et al., "Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect?" *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 7, pp. 3257–3265, 2001.

[24] L. Langouche, S. Vander Perre, P. J. Wouters, A. D'Hoore, T. K. Hansen, and G. van den Berghe, "Effect of intensive insulin therapy on insulin sensitivity in the critically ill," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 10, pp. 3890–3897, 2007.

[25] M. K. Heliövaara, A.-M. Teppo, S.-L. Karonen, J. A. Tuominen, and P. Ebeling, "Improved glycaemia in type 1 diabetes results in decreased levels of soluble adhesion molecules with no change in serum adiponectin or most acute phase proteins," *Experimental and Clinical Endocrinology and Diabetes*, vol. 114, no. 6, pp. 295–300, 2006.

[26] Y. Iwashima, T. Katsuya, K. Ishikawa et al., "Hypoadiponectinemia is an independent risk factor for hypertension," *Hypertension*, vol. 43, no. 6, pp. 1318–1323, 2004.

[27] N. Seshadri, A. W. Messerli, N. Acharya et al., "Relation of homocysteine and C-reactive protein to urinary albumin loss," *The American Journal of Cardiology*, vol. 93, no. 7, pp. 926–928, 2004.

[28] M. Komatsu, H. Ohfusa, T. Aizawa, and K. Hashizume, "Adiponectin inversely correlates with high sensitive C-reactive protein and triglycerides, but not with insulin sensitivity, in apparently healthy Japanese men," *Endocrine Journal*, vol. 54, no. 4, pp. 553–558, 2007.

[29] J. Malyszko, J. S. Malyszko, S. Brzosko, S. Wolczynski, and M. Mysliwiec, "Adiponectin is related to CD146, a novel marker of endothelial cell activation/injury in chronic renal failure and peritoneally dialyzed patients," *The Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 9, pp. 4620–4627, 2004.

[30] C. G. Schalkwijk, N. Chaturvedi, M. T. Schram, J. H. Fuller, and C. D. A. Stehouwer, "Adiponectin is inversely associated with renal function in type 1 diabetic patients," *The Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 1, pp. 129–135, 2006.

[31] C. J. Behre, "Adiponectin: saving the starved and the overfed," *Medical Hypotheses*, vol. 69, no. 6, pp. 1290–1292, 2007.

[32] V. Menon, L. Li, X. Wang et al., "Adiponectin and mortality in patients with chronic kidney disease," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2599–2606, 2006.

[33] M. Saraheimo, C. Forsblom, L. Thorn et al., "Serum adiponectin and progression of diabetic nephropathy in patients with type 1 diabetes," *Diabetes Care*, vol. 31, no. 6, pp. 1165–1169, 2008.

[34] F. Guebre-Egziabher, J. Bernhard, T. Funahashi, A. Hadj-Aissa, and D. Fouque, "Adiponectin in chronic kidney disease is related more to metabolic disturbances than to decline in renal function," *Nephrology Dialysis Transplantation*, vol. 20, no. 1, pp. 129–134, 2005.

[35] J. Ran, X. Xiong, W. Liu et al., "Increased plasma adiponectin closely associates with vascular endothelial dysfunction in type 2 diabetic patients with diabetic nephropathy," *Diabetes Research and Clinical Practice*, vol. 88, no. 2, pp. 177–183, 2010.

[36] M. I. Yilmaz, M. Saglam, A. R. Qureshi et al., "Endothelial dysfunction in type-2 diabetics with early diabetic nephropathy is associated with low circulating adiponectin," *Nephrology Dialysis Transplantation*, vol. 23, no. 5, pp. 1621–1627, 2008.

[37] M. R. Hayden and S. C. Tyagi, "Uric acid: a new look at an old risk marker for cardiovascular disease, metabolic syndrome, and type 2 diabetes mellitus: the urate redox shuttle," *Nutrition and Metabolism*, vol. 1, no. 1, article 10, 2004.

[38] C. Zoccali, F. Mallamaci, G. Tripepi et al., "Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease," *Journal of the American Society of Nephrology*, vol. 13, no. 1, pp. 134–141, 2002.

[39] N. Sattar, G. Wannamethee, N. Sarwar et al., "Adiponectin and coronary heart disease: a prospective study and meta-analysis," *Circulation*, vol. 114, no. 7, pp. 623–629, 2006.

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Clinical Study

A Randomised Controlled Trial to Delay or Prevent Type 2 Diabetes after Gestational Diabetes: Walking for Exercise and Nutrition to Prevent Diabetes for You

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Aims. To develop a program to support behaviour changes for women with a history of Gestational Diabetes Mellitus (GDM) and a Body Mass Index (BMI) > 25 kg/m^2 to delay or prevent Type 2 Diabetes Mellitus. *Methods*. Women diagnosed with GDM in the previous 6 to 24 months and BMI > 25 kg/m^2 were randomized to an intervention (I) ($n = 16$) or a control (C) ($n = 15$) group. The intervention was a pedometer program combined with nutrition coaching, with the primary outcome increased weight loss in the intervention group. Secondary outcomes included decreased waist and hip measurements, improved insulin sensitivity and body composition, increased physical activity, and improved self-efficacy in eating behaviours. *Results*. Median (IQR) results were as follows: weight: I −2.5 (2.3) kg versus C +0.2 (1.6) kg ($P = 0.009$), waist: I −3.6 (4.5) cm versus C −0.1 (3.6) cm ($P = 0.07$), and hip: I −5.0 (3.3) cm versus C −0.2 (2.6) cm ($P = 0.002$). There was clinical improvement in physical activity and eating behaviours and no significant changes in glucose metabolism or body composition. *Conclusion*. A pedometer program and nutrition coaching proved effective in supporting weight loss, waist circumference, physical activity, and eating behaviours in women with previous GDM.

1. Introduction

Gestational diabetes mellitus (GDM) is a well-established predictor for the development of type 2 diabetes (T2DM) [1]. The incidence of GDM has been increasing over the last fifteen years [2], and, with the introduction of updated clinical guidelines for the diagnosis and management of GDM, the prevalence in Australia could be as high as 13% [3]. Worldwide, the prevalence of T2DM following GDM may be as high as 70% [4–9].

In 2007, the economic burden of T2DM was estimated at approximately $US218 billion [10]. The global burden of T2DM is immense [11] with one potential solution being a targeted delay or prevention of progression to T2DM in high risk populations [12–15]. However, programs designed to target women following GDM have met with varied levels of success [16]. Lifestyle intervention trials incorporating dietary modification and promoting increased physical activity to support weight loss have been successful in preventing T2DM [16–19], demonstrating a reduced risk of progression to T2DM in high risk groups by up to 58% [20, 21], with a continuing influence up to eight years after the intervention [22].

In a secondary analysis of the US Diabetes Prevention Program study, women with documented prior GDM had a 71% greater chance of progressing to T2DM three years later, a

risk which was reduced by 50% through lifestyle intervention [18]. However, women were over a decade from their delivery, and it was not known whether this last delivery was in fact their GDM delivery. Therefore, although interventions successfully reduced the incidence of diabetes, the onset of diabetes likely occurred after subsequent pregnancies. Surveys of women with GDM suggest that six months–two years is an optimal time to offer a lifestyle modification intervention as women felt they would be more able to include changes in their life after the birth of their baby [23], and earlier intervention would also offer the chance to reduce the risk of glucose intolerance during subsequent pregnancies. Targeting these reproductive-aged women with recognised risk factors with programs that both engage and provide education for long-term healthy behaviour may provide the optimal prevention strategy for both maternal and fetal outcomes.

A recent systematic review examined types of physical activity and found the most successful exercise programs in postpartum women were those with objectively set goals usually incorporating devices such as pedometers [24]. Previous studies that specifically used pedometers in the postpartum population report an increase in physical activity [25, 26]. Both studies relied on self-reporting of step counts from the pedometer, with no indication as to whether the women would have preferred web-based storage of the step data. Kim et al. suggested the combination of internet based support with a more traditional approach may be more successful than the internet support alone [27].

2. Objectives

This study aimed to develop, implement, and evaluate a low intensity exercise and diet program for women who were diagnosed with GDM during a prior pregnancy and had a body mass index (BMI) > 25 kg/m^2 in the postpartum period. Our primary hypothesis was that the women in the intervention group would achieve significantly more weight loss than the control group. Our secondary hypotheses were that, compared with women in the control group, women in the intervention group would have significantly (1) better diet quality and self-efficacy, (2) more minutes of physical activity/week, (3) lower fasting glucose and insulin levels, and (4) lower body fat mass (FM) and significantly higher fat free mass (FFM). The trial was named "walking for exercise and nutrition to prevent diabetes for you" (WENDY).

3. Method

The intervention took place at a tertiary maternity hospital in Brisbane, Australia, from June 2011 to December 2012. The study was approved by Mater Health Services Human Research Ethics Committee and The University of Queensland Medical Research Ethics Committee.

We evaluated the intervention using a randomised controlled trial. Women were eligible if they were 18 years of age or over and had been diagnosed and treated for GDM, six months to two years postpartum, had a self-reported BMI > 25 kg/m^2, had routine access to a computer, computer skills to navigate websites, and e-mail, and understood that the primary physical activity would be walking. Women were ineligible if they were currently pregnant, had T2DM, were not fluent in English, used hypoglycaemic medications, or had any mental or physical disabilities which would have hindered participation in study activities. Randomisation was stratified according to BMI (25–30 kg/m^2; >30 kg/m^2).

Women were recruited through several venues, including telephone contact obtained from the hospital database of women with GDM diagnoses, hospital-based electronic resources, advertisements placed through the Australian National Diabetes Services Scheme (NDSS) [28] dedicated website to GDM (You2), and television advertisements.

Participants were contacted by the research team, with three attempts at contact (fixed and mobile phones). Women not contactable after three attempts were classified as "unable to contact." Those who were contacted and refused had their reasons for refusal noted. For those who agreed to participate, an e-mail address and basic data such as height and weight to allow calculation of current BMI and updated contact details were collected, and an oral glucose tolerance test (OGTT) was performed to exclude T2DM.

4. Randomisation

An independent service generated a stratified, variable block, computer generated randomisation schedule and sealed the individual allocations in opaque envelopes. The envelopes were stored in a locked, secured container until eligibility was established. Once eligibility was established through baseline measurements (BMI, no T2DM on OGTT), the next envelope for the appropriate stratum was opened.

Women allocated to the intervention group received a pedometer linked to a tailored web-based program "step up to health" and a four-week nutrition coaching workshop. The women in the control group formed a wait-list group and were offered the nutrition workshop following the three-month assessment.

The pedometer had an opaque sticker that covered the digital display and was worn continuously for the first week, without providing feedback to record baseline steps. Once the baseline steps were uploaded via USB, the sticker was removed and the step count was visible. The web-based program generated weekly goals based on the previous weeks steps. As the steps were uploaded each week, the goals were gradually increased, until the maximum of 10,000 steps/day was reached [27]. The user was encouraged to log on weekly to receive updated weekly goals, feedback on their walking progress, messages, and "tips" regarding diet and exercise targeted at diabetes prevention.

The nutrition coaching workshop was delivered by accredited practising dietitians. The workshop consisted of four one-hour group sessions incorporating evidence-based strategies to facilitate behaviour change aimed at healthy sustainable weight loss [29] and to build self-efficacy such as goal setting and self-monitoring and use of group activities to model recommended behaviour and engender peer support.

Resources provided to all women included tools designed to encourage portion control [30, 31].

5. Data Collection and Outcome Measures

Data were collected at baseline and three months. Baseline observations included survey-based assessments of dietary and physical activity, mental health assessments, assessments of anthropometrics, body composition, serum insulin, and OGTT performance. Weight was measured to the nearest 0.1 kg using a spring balance scale, and height was measured with a wall mounted stadiometer to the nearest 0.5 cm. Hip and waist measurements were taken with a standard tape measure, and estimation of body composition (fat mass and lean body mass) was assessed using using a multifrequency bioelectrical impedance analyser (BodyStat 1500MDD, Bodystat, United Kingdom), with a measured resistance at a fixed frequency of 50 Hz.

Dietary quality was assessed using the Fat, Fibre Index [32], eating behaviour self-efficacy was assessed using The Health and Wellbeing Self Efficacy Survey (WEL) [33], physical activity was assessed using Australian Women's Activity Survey (AWAS) [34], and mental health was assessed using the Kessler Psychological Distress scale (K10) [35]. Any results indicative of anxiety or depression were discussed with the participant and referred to relevant health care providers if necessary [36]. The homeostasis model assessment of insulin resistance (HOMA-IR), a widely used estimate of insulin resistance in the fasting state, was calculated as fasting plasma insulin (FPI)-[mU/L] × fasting plasma glucose (FPG) [mmol/L]/22.5 [37].

Table 1: Demographic characteristics of women in intervention and control groups.

Characteristic	Group	Total $N = 31$
Age at OGTT* (years)		36.0 (4.5) Range 28–44
Ethnicity	Caucasian	28 (90%)
	Other	3 (10%)
Health insurance status	Public (NHS)	17 (55%)
	Privately funded	14 (45%)
Gravidity	1	6 (19%)
	2+	25 (81%)
Parity	0	6 (19%)
	1+	25 (81%)
Diabetic control with GDM	Insulin	19 (61%)
	Metformin	4 (13%)
	Diet	8 (26%)
Weight* (kg)		85.7 (17.5)
BMI^ (kg/m^2)		30.3 (8.2)
Waist* (cm)		100.7 (11.8)
Hip* (cm)		116.6 (14.1)
Body fat %*		37.4 (7.1)
Lean mass %*		52.5 (6.2)
Fasting glucose^		4.8 (0.8)
Fasting insulin^&		8.7 (6.0)
2 hr glucose^		5.5 (2.8)

*Mean (standard deviation), independent samples t-test.
^Median (interquartile range), Mann-Whitney U test.
&4 cases missing (1 intervention; 3 control).

6. Outcome Measures

The primary outcome was weight loss from baseline to three months, reported as absolute weight loss for each participant.

Secondary outcomes were change in measurements from baseline to three months for (1) hip and waist measurements, (2) diet quality measured by a self-reported survey, (3) WEL overall and domain scores, (4) minutes of physical activity/week (as health enhancing physical activity, HEPA), (5) glucose and HOMA-IR, and (6) body FM and FFM.

7. Statistical Methods

Analysis was by intention-to-treat with all analyses comparing the control and intervention groups. Analysis was undertaken with blinding to study assignment.

Data were checked for normality of the distributions of continuous variables. Normally distributed variables underwent parametric analyses; continuous non-normally distributed data were analyzed using nonparametric methods and categorical data were analyzed using chi-squared or Fisher's exact test. Analysis of the primary outcome used independent samples t-test, examining percentage of weight loss between the control and intervention groups. Analyses were performed in SPSS version 15 [38]. Results are reported as mean (standard deviation [SD]) or median (interquartile range [IQR]).

8. Results

Demographic and anthropometric characteristics of the study participants were similar in each group (Table 1). There were more multigravidas in the intervention group, and an equal proportion of women had public and private health insurance. Ethnicity was predominately Caucasian women, with three women of Asian descent. The majority of women had required insulin therapy (control $n = 10$ [67%], intervention $n = 9$ [56%]) to control their glucose levels during pregnancy, followed by diet (control $n = 4$ [27%], intervention $n = 4$ [25%]) and then metformin (control $n = 1$ [7%], intervention $n = 3$ [19%]).

We attempted to contact two hundred and forty-six women (Figure 1). Thirty-one women were randomised, with twenty-three women completing the three-month primary outcome measurements.

Five participants in the intervention group discontinued over the course of the 3-month period for differing reasons (Figure 1). One control participant (who was randomised in

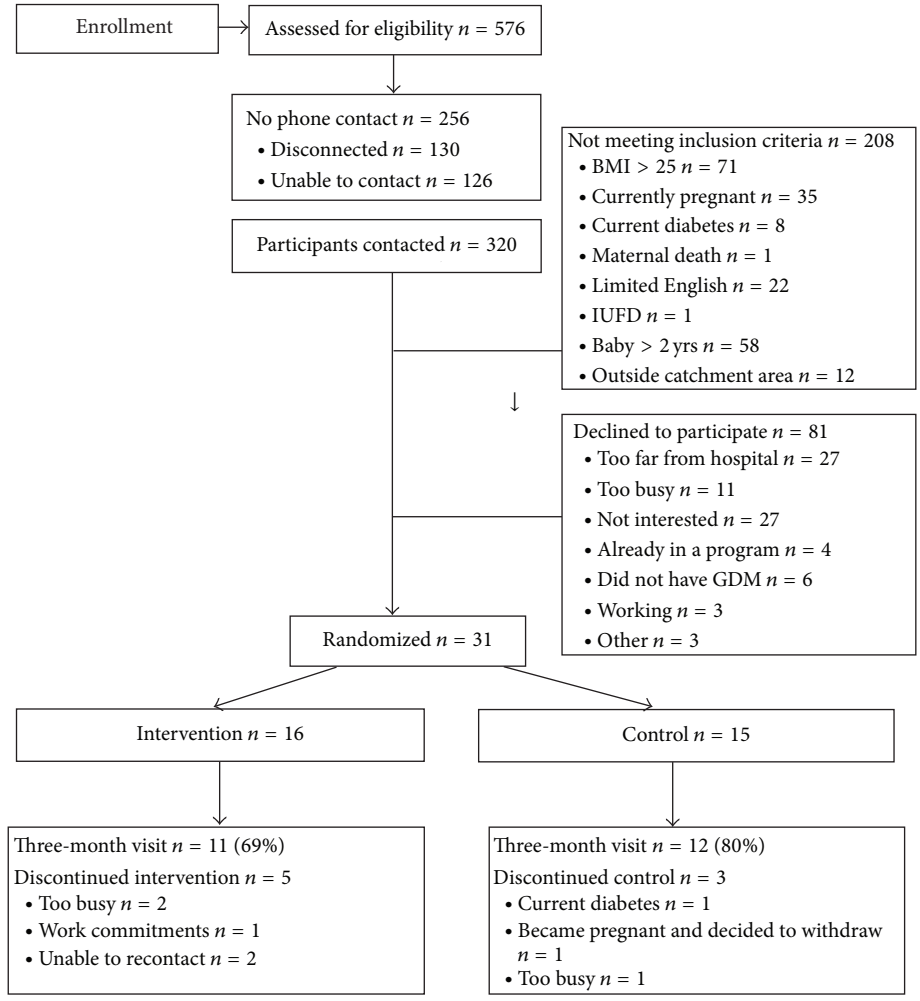

Figure 1: Consort diagram of the study.

error prior to OGTT results) was diagnosed as T2DM following baseline OGTT and two other participants withdrew for unspecified reasons. Eleven participants in the intervention group (69%) and 12 participants in the control group (80%) completed both baseline and three-month assessments.

Weight loss was greater in the intervention group, with a median loss of 2.5 kg (1.4) compared with a static weight in the control group ($P = 0.002$), leading to a reduction in BMI of 0.9 kg/m^2 (IQR 0.7) ($P = 0.002$) in the intervention group (Table 2).

9. Secondary Outcomes

Changes in hip circumference were also significant with a median loss of 3 cm (5.0) in the intervention group compared with 0 cm (4.8) ($P = 0.006$). Intervention group waist circumference decreased by a median of 3 cm (4.0) compared with 0.5 cm (4.8) ($P = 0.037$).

There was a slight decrease in body fat and increase in lean body mass in the intervention group, but this was not statistically significant. Fasting glucose taken at both data collection points showed a small difference between the two groups that had borderline statistical significance ($P = 0.052$); however, there was no change in HOMA-IR.

The intervention group increased their daily activity by one hundred and thirty-five minutes/week at the three-month time point compared to the control group, although this difference was not statistically significant. The WEL results showed participants in the intervention group at three months feeling empowered when presented with opportunity for poor food choices ($P = 0.036$). Despite being not statistically significant, trends towards improvements in the domains of negative emotions, social pressure, physical discomfort, and positive activities were noted, all related to the participants' feelings regarding food and food choices (Table 2). There were no differences in factors related to depression or mood changes between groups.

10. Website "Stepping up to Health"

All women randomised to the intervention group accessed the website during the three-month intervention. The mean

Table 2: Change between 3-month and baseline measurements.

Characteristic	Intervention $N = 11$	Control $N = 12$	P-value
Weight^ (kg)	−2.5 (1.4)	0.0 (2.3)	0.002
BMI^ (kg/m^2)	−0.9 (0.7)	0.0 (0.8)	0.002
Waist^ (cm)	−3.0 (4.0)	0.5 (4.8)	0.037
Hip^ (cm)	−3.0 (5.0)	0.0 (4.8)	0.006
Body fat %^&	−1.1 (4.5)	−0.3 (1.3)	0.393
Lean mass %^&	0.9 (3.3)	0.0 (2.6)	0.436
Fasting glucose^&	0.3 (0.5)	−0.1 (0.6)	0.052
Fasting insulin^#	−0.5 (2.4)	0.173	0.830
K 10 total score^ (measure of distress and anxiety over the previous month)	0.0 (4.0)	1.0 (4.0)	0.193
WEL total score (measure of attitudes, feelings, and efficacy related to food and eating behaviours)	27.8 (20.1)	13.9 (37.4)	0.290
Negative emotions	5.5 (2.9)	3.5 (8.5)	0.472
Availability	7.1 (5.5)	1.0 (7.0)	0.036
Social pressure	6.1 (5.4)	4.1 (9.4)	0.545
Physical discomfort	4.5 (6.7)	4.5 (8.4)	0.978
Positive activities	4.6 (4.9)	0.9 (7.6)	0.188
HEPA	135 (225)	0 (418)	0.190
Fat	0.2 (0.4)	0.2 (0.5)	0.824
Fibre	−0.04 (0.8)	0.1 (0.4)	0.576
Total	0.1 (0.5)	0.2 (0.4)	0.682

All results are mean (standard deviation) unless otherwise stated.
^Median (interquartile range), Mann-Whitney U test.
&3 cases missing (1 intervention; 2 control).
#5 cases missing (1 intervention; 4 control).

Table 3: Pedometer and nutrition workshop data.

Characteristic	Mean (SD)	Range
Number of times pedometer data uploaded	90 (31)	39–145
Number of days where steps were recorded	71 (31)	30–109
Number of steps per day	4,687 (3,510)	0–16,645
Number of times website messages accessed	28 (26)	3–74
Number of nutrition workshops attended	3 (1)	0–4

SD: standard deviation.

number of participant pedometer uploads was 90 (SD 31). The mean recorded steps/day were 5,916 (SD 2,878, range 5–16,645) in the three-month period (Table 3).

11. Discussion

Although women with GDM are at increased risk for diabetes and a significant proportion will develop T2DM within the decade after their GDM delivery, interventions successfully targeting women during this time are few. In this study, we demonstrate that a simple, brief intervention consisting of only 4 sessions of counseling and a web-based activity component could successfully reduce weight, increase physical activity, and improve constructs associated with improved lifestyle behaviours. Such a program has the potential to be delivered in multiple care settings for limited cost. However, our study also demonstrates the challenges of engaging women with young children in an intervention aimed at changing lifestyle behaviours, as willingness to participate in the relatively "simple" intervention was low.

Obesity is a primary risk factor for the development of T2DM [1]. At least two systematic reviews [39, 40] have suggested that a combination of diet and exercise rather than diet alone may be more efficacious for postpartum weight loss [24, 39, 40]. A previous report using only the web-based pedometer component targeting physical activity did not demonstrate significant weight loss [27], suggesting that both diet and exercise components are necessary, even though we did not note significant changes in dietary quality. Of note, the pattern of clinically significant changes in physical activity with smaller, nonsignificant diet quality changes was also observed in recent dietary and physical intervention underpinned by similar behaviour-change strategies for high BMI women in the postpartum period [41]. The results for the secondary outcomes in our study such as the trend of increased incidental activity and improved self-efficacy in food behaviour in the intervention group may be a collateral effect of goal setting behaviour. The value of increased physical activity in all domains is an important factor in overall lifestyle change.

Our study also suggests that an in-person counseling component may be more effective for behaviour change in this specific at-risk group of women than the web-based program alone. The mean attendance in the four counseling sessions was three (range 0–4 sessions) (Table 3) with the majority of participants attending all four group sessions. These results suggest that the primary impact of the intervention was mediated through the in-person counseling session. Other interventions targeting obesity and risk reduction of T2DM have noted that behaviour may be successfully modified by counseling sessions only [17], but participant populations in those studies were older and had different motivators and enablers of behaviour change.

Recruitment of participants in the early postpartum phase has been proven to be difficult. Although we demonstrated promising weight and behaviour changes amongst participants, it is also notable that the participation was low and needed extensive advertising and outreach to obtain the small numbers enrolled in this study. Common themes encountered by other intervention studies in this population such as lack of time, no childcare, and difficulties "fitting the changes" into the family were also a factor in this study and affected all stages of the project from recruitment of

possible participants and attrition during the trial to poor followup attendance [27, 42, 43]. While the intervention was designed to reduce barriers to behaviour change, this experience suggests that additional motivators will need to be explored in order to successfully change behaviour in this group of young mothers.

The strength of our study lies in the physical and lifestyle changes achieved in the intervention group of our sample. The feedback from the participants on the combination of the pedometer and website was positive and the delivery and content of the nutrition workshop were well received. The ability to provide the intervention in a central location was also a strength as most women found the hospital a familiar environment.

There were limitations in this project. Despite our efforts to recruit a larger number of participants, actual recruitment was low; therefore, the statistical power to detect significant differences between intervention and control arms was limited. Moreover, the women in this study were predominantly Caucasian and in their mid-thirties, and thus our results may not apply to women of other age or racial/ethnic groups. Younger women may have lower perceptions of risk and less motivation to alter behaviour [44, 45], and women of other races/ethnicities may have different perceptions and understanding of lifestyle changes required to decrease their risk of developing T2DM [46].

12. Conclusion

Despite encountering similar barriers to recruitment and retention of participants as in other intervention trials, results from this study demonstrate that the combination of a web-based pedometer intervention with nutrition coaching underpinned by behaviour change theory based on long-term behaviour change can lead to overall weight loss and increased physical activity (known risk factors for the development of T2DM) over a three-month period. The availability of a program that combines these features in a suitably delivered format to engage women previously diagnosed with GDM in a larger scale trial may delay or prevent T2DM in this high risk group.

This trial is registered with Australia and New Zealand Clinical Trials registry ACTRN12611000075987.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] L. Bellamy, J.-P. Casas, A. D. Hingorani, and D. Williams, "Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis," *The Lancet*, vol. 373, no. 9677, pp. 1773–1779, 2009.

[2] D. Dabelea, J. K. Snell-Bergeon, C. L. Hartsfield, K. J. Bischoff, R. F. Hamman, and R. S. McDuffie, "Increasing prevalence of gestational diabetes mellitus (GDM) over time and by birth cohort: kaiser Permanente of Colorado GDM screening program," *Diabetes Care*, vol. 28, no. 3, pp. 579–584, 2005.

[3] R. G. Moses, G. J. Morris, P. Petocz, F. Sangil, and D. Garg, "The impact of potential new diagnostic criteria on the prevalence of gestational diabetes mellitus in Australia," *The Medical Journal of Australia*, vol. 194, no. 7, pp. 338–340, 2011.

[4] C. Kim, K. M. Newton, and R. H. Knopp, "Gestational diabetes and the incidence of type 2 diabetes: a systematic review.," *Diabetes Care*, vol. 25, no. 10, pp. 1862–1868, 2002.

[5] A. J. Lee, R. J. Hiscock, P. Wein, S. P. Walker, and M. Permezel, "Gestational diabetes mellitus: clinical predictors and long-term risk of developing type 2 Diabetes. A retrospective cohort study using survival analysis," *Diabetes Care*, vol. 30, no. 4, pp. 878–883, 2007.

[6] H. Lee, H. C. Jang, H. K. Park, B. E. Metzger, and N. H. Cho, "Prevalence of type 2 diabetes among women with a previous history of gestational diabetes mellitus," *Diabetes Research and Clinical Practice*, vol. 81, no. 1, pp. 124–129, 2008.

[7] Y. Linné, B. Barkeling, and S. Rössner, "Natural course of gestational diabetes mellitus: long term follow up of women in the SPAWN study," *BJOG*, vol. 109, no. 11, pp. 1227–1231, 2002.

[8] D. S. Feig, B. Zinman, X. Wang, and J. E. Hux, "Risk of development of diabetes mellitus after diagnosis of gestational diabetes," *Canadian Medical Association Journal*, vol. 179, no. 3, pp. 229–234, 2008.

[9] D. S. Feig, B. Zinman, X. Wang, and J. E. Hux, "Correction to "Risk of development of diabetes mellitus after diagnosis of gestational diabetes"," *Canadian Medical Association Journal*, vol. 179, no. 4, p. 344, 2008.

[10] T. M. Dall, Y. Zhang, Y. J. Chen, W. W. Quick, W. G. Yang, and J. Fogli, "The economic burden of diabetes," *Health Affairs*, vol. 29, no. 2, pp. 297–303, 2010.

[11] S. S. Lim, T. Vos, A. D. Flaxman et al., "A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study," *The Lancet*, vol. 380, no. 9859, pp. 2224–2260, 2010.

[12] M. Y. Bertram, S. S. Lim, J. J. Barendregt, and T. Vos, "Assessing the cost-effectiveness of drug and lifestyle intervention following opportunistic screening for pre-diabetes in primary care," *Diabetologia*, vol. 53, no. 5, pp. 875–881, 2010.

[13] N. Lohse, E. Marseille, and J. G. Kahn, "Development of a model to assess the cost-effectiveness of gestational diabetes mellitus screening and lifestyle change for the prevention of type 2 diabetes mellitus," *International Journal of Gynecology and Obstetrics*, vol. 115, no. 1, pp. S20–S25, 2011.

[14] G. M. Frierson, J. R. Morrow Jr., and A. Vidales, "Successful minority recruitment and adherence in physical activity internet-based research: the WIN study," *Journal of the National Medical Association*, vol. 104, no. 11-12, pp. 544–554, 2012.

[15] K. Baptiste-Roberts, B. B. Barone, T. L. Gary et al., "Risk factors for type 2 diabetes among women with gestational diabetes: a systematic review," *The American Journal of Medicine*, vol. 122, no. 3, pp. 207.4–214.e4, 2009.

[16] H. D. McIntyre, A. Peacock, Y. D. Miller, D. Koh, and A. L. Marshall, "Pilot study of an individualised early postpartum intervention to increase physical activity in women with previous gestational diabetes," *International Journal of Endocrinology*, vol. 2012, Article ID 892019, 5 pages, 2012.

[17] W. C. Knowler, E. Barrett-Connor, S. E. Fowler et al., "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin," *The New England Journal of Medicine*, vol. 346, no. 6, pp. 393–403, 2002.

[18] R. E. Ratner, C. A. Christophi, B. E. Metzger et al., "Prevention of diabetes in women with a history of gestational diabetes: effects of metformin and lifestyle interventions," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 12, pp. 4774–4779, 2008.

[19] N. W. Cheung, B. J. Smith, H. Henriksen, L. C. Tapsell, M. McLean, and A. Bauman, "A group-based healthy lifestyle program for women with previous gestational diabetes," *Diabetes Research and Clinical Practice*, vol. 77, no. 2, pp. 333–334, 2007.

[20] W. C. Knowler, S. E. Fowler, R. F. Hamman, C. A. Christophi, H. J. Hoffman, A. T. Brenneman et al., "10-year follow-up of diabetes incidence and weight loss in the Diabetes Prevention Program Outcomes Study," *The Lancet*, vol. 374, no. 9702, pp. 1677–1686, 2009.

[21] J. Tuomilehto, P. Schwarz, and J. Lindström, "Long-term benefits from lifestyle interventions for type 2 diabetes prevention: time to expand the efforts," *Diabetes Care*, vol. 34, no. 2, pp. S210–S214, 2011.

[22] V. Sterne, T. Logan, and M. A. Palmer, "Factors affecting attendance at postpartum diabetes screening in women with gestational diabetes mellitus," *Practical Diabetes International*, vol. 28, no. 2, pp. 64–68, 2011.

[23] H. D. McIntyre, A. Peacock, Y. Miller, D. Koh, and A. Marshall, "Pilot study of an individualised early postpartum intervention to increase physical activity in women with previous gestational diabetes," *International Journal of Endocrinology*, vol. 2012, Article ID 892019, 5 pages, 2012.

[24] S. L. Nascimento, J. Pudwell, F. G. Surita, K. B. Adamo, and G. N. Smith, "The effect of physical exercise strategies on weight loss in postpartum women: a systematic review and meta-analysis," *International Journal of Obesity*, 2013.

[25] K. K. Clarke, J. Freeland-Graves, D. M. Klohe-Lehman, T. J. Milani, H. J. Nuss, and S. Laffrey, "Promotion of physical activity in low-income mothers using pedometers," *Journal of the American Dietetic Association*, vol. 107, no. 6, pp. 962–967, 2007.

[26] M. S. Maturi, P. Afshary, and P. Abedi, "Effect of physical activity intervention based on a pedometer on physical activity level and anthropometric measures after childbirth: a randomized controlled trial," *BMC Pregnancy and Childbirth*, vol. 11, article 103, 2011.

[27] C. Kim, M. Draska, M. L. Hess, E. J. Wilson, and C. R. Richardson, "A web-based pedometer programme in women with a recent history of gestational diabetes," *Diabetic Medicine*, vol. 29, no. 2, pp. 278–283, 2012.

[28] "Gestational Diabetes Brisbane, Queensland, Australia: Diabetes Australia," 2014, http://www.diabetesqld.org.au/.

[29] NHMRC, Australian Dietary Guidelines, National Health and Research Council, 2013, https://www.nhmrc.gov.au/guidelines/publications/n55.

[30] T. Williams, *This=That: A Life Sized Photoguide to Food Serves*, Trudy Williams Nutrition and Dietetics Pty Ltd, Toowong, Australia, 2011.

[31] A. Clark, *Great Ideas in Nurition: Portion Perfection*, 2014, http://www.greatideas.net.au/about-portion-perfection.

[32] M. Reeves, E. Winkeler, A. Hodge, and E. Eakin, "Measuring dietary change: which is more responsive—a food frequency questionnaire or a dietary behaviour questionnaire?" *Nutrition & Dietetics*, vol. 67, supplement 1, pp. 57–58, 2010.

[33] M. M. Clark, D. B. Abrams, R. S. Niaura, C. A. Eaton, and J. S. Rossi, "Self-efficacy in weight management," *Journal of Consulting and Clinical Psychology*, vol. 59, no. 5, pp. 739–744, 1991.

[34] B. S. Fjeldsoe, A. L. Marshall, and Y. D. Miller, "Measurement properties of the Australian women's activity survey," *Medicine & Science in Sports & Exercise*, vol. 41, no. 5, pp. 1020–1033, 2009.

[35] R. C. Kessler, G. Andrews, L. J. Colpe et al., "Short screening scales to monitor population prevalences and trends in non-specific psychological distress," *Psychological Medicine*, vol. 32, no. 6, pp. 959–976, 2002.

[36] G. Andrews and T. Slade, "Interpreting scores on the kessler psychological distress Scale (K10)," *Australian and New Zealand Journal of Public Health*, vol. 25, no. 6, pp. 494–497, 2001.

[37] D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner, "Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[38] *IBM SPSS Statistics for Windows Version 21.0*, IBM Corporation, Armonk, NY, USA, 2012.

[39] A. R. Amorim Adegboye and Y. M. Linne, "Diet or exercise, or both, for weight reduction in women after childbirth," *Cochrane Database of Systematic Reviews*, no. 7, Article ID CD005627, 2013.

[40] P. van der Pligt, J. Willcox, K. D. Hesketh et al., "Systematic review of lifestyle interventions to limit postpartum weight retention: implications for future opportunities to prevent maternal overweight and obesity following childbirth," *Obesity Reviews*, vol. 14, no. 10, pp. 792–805, 2013.

[41] S. A. Wilkinson, P. van der Pligt, K. S. Gibbons, and H. D. Mcintyre, "Trial for reducing weight retention in new mums: a randomised controlled trial evaluating a low intensity, postpartum weight management programme," *Journal of Human Nutrition and Dietetics*, 2013.

[42] J. M. Nicklas, C. A. Zera, E. W. Seely, Z. S. Abdul-Rahim, N. D. Rudloff, and S. E. Levkoff, "Identifying postpartum intervention approaches to prevent type 2 diabetes in women with a history of gestational diabetes," *BMC Pregnancy and Childbirth*, vol. 11, no. 1, article 23, 2011.

[43] A. Ferrara, M. M. Hedderson, C. L. Albright et al., "A pregnancy and postpartum lifestyle intervention in women with gestational diabetes mellitus reduces diabetes risk factors: a feasibility randomized control trial," *Diabetes Care*, vol. 34, no. 7, pp. 1519–1525, 2011.

[44] C. Kim, L. N. McEwen, J. D. Piette, J. Goewey, A. Ferrara, and E. A. Walker, "Risk perception for diabetes among women with histories of gestational diabetes mellitus," *Diabetes Care*, vol. 30, no. 9, pp. 2281–2286, 2007.

[45] M. K. Morrison, J. M. Lowe, and C. E. Collins, "Perceived risk of type 2 diabetes in Australian women with a recent history of gestational diabetes mellitus," *Diabetic Medicine*, vol. 27, no. 8, pp. 882–886, 2010.

[46] H. Razee, H. P. van der Ploeg, I. Blignault et al., "Beliefs, barriers, social support, and environmental influences related to diabetes risk behaviours among women with a history of gestational diabetes," *Health Promotion Journal of Australia*, vol. 21, no. 2, pp. 130–137, 2010.

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Research Article

Sitagliptin: Is It Effective in Routine Clinical Practice?

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Aim. The present study was conducted to determine the glycaemic effects of sitagliptin in type 2 diabetes patients. *Methods*. Data was collected from patient medical records of a major teaching hospital in Malaysia, from 2009 to 2012. Glycated hemoglobin (HbA_{1c}) values prior to and up to 12 months after the initiation of sitagliptin were analysed. The change in HbA_{1c} values was accounted for based on a generalized linear model generated using the Generalized Estimating Equations (GEE) method. *Results and Discussion*. Of the 457 patients, 53.6% were elderly and 81.4% were overweight. The mean HbA_{1c} (standard deviation) before initiation of sitagliptin was 8.5 (1.4)%. This dropped to 7.7 (1.4)%, 3 to 6 months after initiation of sitagliptin, with a mean difference of 0.8% (95% confidence interval (CI): 0.7–1.0; $P < 0.001$). However, this value increased to 8.0 (1.7)% after 7 to 12 months on sitagliptin ($P = 0.002$) with a mean difference from baseline of 0.6% (95% CI: 0.4–0.7; $P < 0.001$). *Conclusion*. In routine clinical practice, sitagliptin produces a significant reduction in mean HbA_{1c} (0.8%) within the first 6 months of use which corresponds to efficacy data obtained in controlled clinical trials. However, this reduction was lesser, 7 to 12 month later.

1. Introduction

The prevalence of diabetes is increasing worldwide. According to the World Health Organization (WHO), approximately 171 million people have diabetes globally, with 82 million in the Association of South East Asian Nations (ASEAN) region [1]. The International Diabetes Federation reported that 366 million people have diabetes in year 2011 and this figure is expected to increase to 552 million in 2030 [2]. According to the Malaysian National Health Morbidity Survey, the prevalence of diabetes in Malaysia has almost doubled among those aged 30 and above within a 10-year period, increasing from 8.3% in 1996 to 14.9% in 2006 [3]. Subsequently, the overall prevalence of diabetes in Malaysia has escalated to 22.9% [4].

The glycemic goal recommended by the Malaysian Clinical Practice Guidelines (CPG) is a glycated hemoglobin (HbA_{1c}) of less than 6.5% (48 mmol/mol) but the American Diabetes Association (ADA) recommended a HbA_{1c} less than 7% (53 mmol/mol) [5, 6]. Most patients with type 2 diabetes do not achieve the HbA_{1c} target despite being on multiple medications. A study in Malaysia reported that only 17.4% (95% CI, 13.7 to 21.1%) of the patients achieved HbA_{1c} less than 6.5% (48 mmol/mol) [7]. Therefore, newer therapeutic agents such as dipeptidyl peptidase-4 (DPP-4) inhibitors have been introduced with the aim of achieving better glycemic control [8].

Sitagliptin (Januvia, Merck & Co. Inc., USA) is the first DPP-4 inhibitor marketed in the United States (USA) and was approved by the Food and Drug Administration of the United States (FDA, USA) in October 2006, for the treatment of type 2 diabetes [9]. The inhibition of DPP-4 leads to an increase in the active levels of incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) which are involved in glucoregulation [10, 11]. Sitagliptin not only reduces the HbA_{1c} levels but also improves the fasting and postprandial plasma glucose [12–14].

Studies showed that sitagliptin reduced HbA_{1c} by 0.5 to 0.7% [8, 12, 13, 15]. However it has been suggested that DPP-4 inhibitors should be used only for patients with type

2 diabetes who are unable to tolerate other oral antidiabetes medications or who have not managed to achieve the glycemic target with the standard first-line agents [8, 16]. Sitagliptin confers several advantages in comparison to other antidiabetes medications as it is well tolerated, weight neutral and does not cause hypoglycemia [14, 17–19].

Most of the studies on the effectiveness of DPP-4 inhibitors are controlled trials [12, 14, 18]. However, data on the effects of this group of medications in clinical practice is still lacking although the demand for DDP-4 inhibitors has been increasing since its introduction into the market. Therefore, the present study was conducted to determine the glycemic effects of sitagliptin.

2. Materials and Methods

2.1. Patients and Setting. A retrospective study was conducted in a major teaching hospital in Kuala Lumpur, Malaysia. All patients prescribed with sitagliptin from 2009 to 2012 were identified from the Pharmacy Information System (PIS) of the hospital and data related to the patients were extracted from the patient medical records. The study was approved by the Medical Ethics Committee of the hospital (MEC reference number 890.32) prior to initiation of the study. A pilot study was conducted to assess the feasibility and practicability of the methodology as well as to ensure that the data collection form was able to gather all the information required to meet the study objectives.

Patients included were those with type 2 diabetes who were prescribed with sitagliptin by the teaching hospital within the study period. These patients must have obtained their sitagliptin supply from the hospital more than once and their medical records must be available for data extraction. Patients excluded were those who were on other DPP-4 inhibitors prior to the initiation of sitagliptin and patients prescribed with sitagliptin as first-line drug or were not started on sitagliptin by the teaching hospital under study since patients' data prior to the initiation of sitagliptin would not be available for comparison.

To analyze the effectiveness of sitagliptin, patients must have HbA_{1c} readings before and 3 to 6 as well as 7 to 12 months after the initiation of sitagliptin. Insulin must not be added to or discontinued from patients medications during the 12-month period.

2.2. Data Collection. A list of sitagliptin transactions in the teaching hospital was retrieved from its PIS. Patients who were dispensed with sitagliptin only once were excluded from the list. Duplicated registration numbers were then deleted, leaving only the first transaction of sitagliptin for each patient. The list of remaining patients' names with registration number was submitted to the Patient Information Department of the teaching hospital to retrieve the patient medical records. The medical records were screened and relevant information was extracted and recorded in a preprepared data collection form.

The primary outcome of this study was a change in HbA_{1c} values prior to and after the initiation of sitagliptin. Patients' HbA_{1c} values were collected at three points: at baseline prior to initiation of sitagliptin, 3 to 6 months, and subsequently 7 to 12 months after initiation of sitagliptin. Other information analyzed included demographic data of patients, medical condition(s), medication(s) and other clinical data before and after initiation of sitagliptin, reason(s) for initiating sitagliptin, and reported side effects or hypoglycaemia episodes.

2.3. Statistical Analysis. All data were entered into and analyzed using the IBM SPSS Statistics for Windows, version 20 (IBM Corporation, Armonk, NY). All data were subjected to descriptive analysis which generated frequencies and percentages. For numeric data, the mean (standard deviation) and median were also obtained.

Repeated measures of HbA_{1c} values obtained at three points during the one-year period from the same cohort of patients were assumed to be "dependent." Therefore, a generalized linear model was generated to account for the change in HbA_{1c} values using the Generalized Estimating Equations (GEE) method. This model was used to assess any change in HbA_{1c} values with time (due to the initiation of sitagliptin) while controlling for patients' baseline characteristics. The equation is as follows:

$$\begin{aligned} Y_{ij} &= \alpha + \beta 1 * (\text{gender}) + \beta 2 * (\text{age}) + \beta 3 * (\text{race}) \\ &\quad + \beta 4 * (\text{marital status}) + \beta 5 * (\text{employment status}) \\ &\quad + \beta 6 * (\text{number of years of diagnosis with diabetes}) \\ &\quad + \beta 7 * (\text{baseline } HbA_{1c}) \\ &\quad + \beta 8 * (\text{number of antidiabetes agents before sitagliptin}) \\ &\quad + \beta 9 * (\text{change in regimen}) \\ &\quad + \beta 10 * (\text{initial sitagliptin dose}) \\ &\quad + \beta 11 * (\text{medication prior to sitagliptin}) \\ &\quad + \beta 12 * (\text{sitagliptin added or substituted}) + \varepsilon_{ij}, \end{aligned} \tag{1}$$

where Y_{ij} is the ith patient's HbA_{1c} value measured at the jth time point and ε_{ij} is the error term that cannot be explained by the model with $\varepsilon_{ij} \sim N(0, \sigma 2)$.

In addition, patients were divided into two groups: (1) patients with improvement in HbA_{1c} and (2) patients with no improvements in HbA_{1c} after using sitagliptin for 7 to 12 months. The same parameters as mentioned in the equation above were analysed using the GEE to test if there is any association between these parameters and the two groups of patients.

3. Results

3.1. Demographic and Baseline Characteristics of Patients. A total of 904 patients were prescribed with sitagliptin at the major teaching hospital in Malaysia from 2009 to 2012.

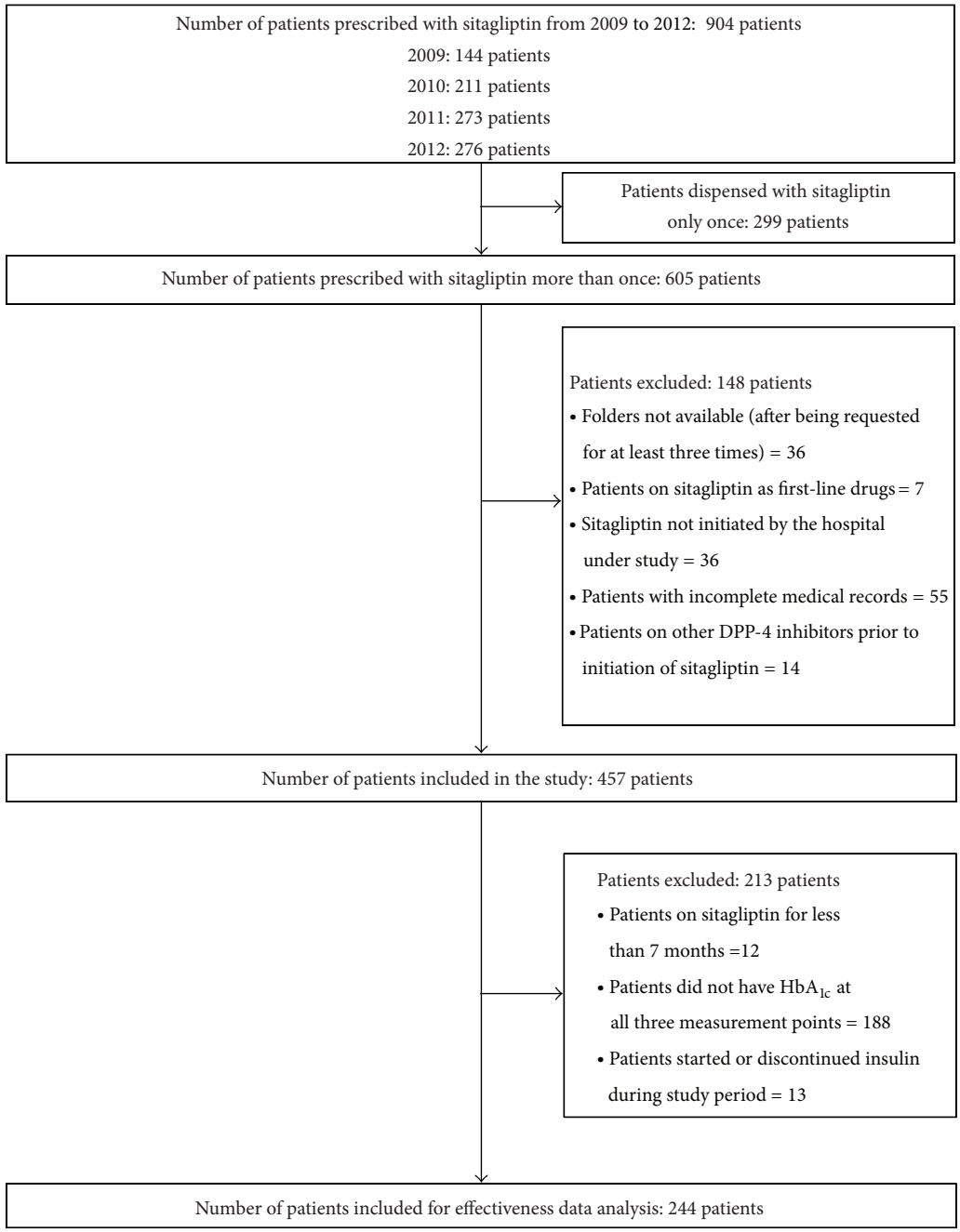

Figure 1: Inclusion of patients in the study.

The number of patients dispensed with sitagliptin showed an increasing trend during the four-year period. However, only data from 457 patients who met the inclusion criteria was analyzed in this study (Figure 1).

Table 1 shows the demographic and clinical characteristics of the patients prior to the initiation of sitagliptin. At least half of the patients on sitagliptin (53.6%) were 65 years old and above. Three foreigners (a Burmese, an Eurasian, and an Iranian) were classified as "Others" under the ethnic groups. Amongst the patients who were still working, only six had health-related jobs.

At the time when the patients were started on sitagliptin, 93.8% had uncontrolled diabetes (defined as HbA_{1c} 6.5% (48 mmol/mol) and above) [5]. However, if based on the more commonly used HbA_{1c} target of 7% (53 mmol/mol) and above, 88.3% of the patients had uncontrolled diabetes. Most of the patients (78.4%) possessed a glucose meter at home and were able to monitor their own blood glucose level if desired.

Based on the body mass index (BMI), 81.4% of the patients were overweight (defined as BMI 23 kg/m^2 and above) while 48.5% were obese (defined as BMI of 27.5 kg/m^2 and above) [20]. If based on waist circumference, then 83% of

Table 1: Baseline demographic and clinical characteristics of patients.

Patients characteristics	Number of patients (%)	Mean (SD) (median) [range]
Age (years) (N = 457)		
21–30	3 (0.7)	
31–40	9 (2.0)	
41–50	33 (7.2)	65 (11.7) (65) [23–100]
51–60	104 (22.7)	
61–70	152 (33.3)	
>70	156 (34.1)	
Gender (N = 457)		
Male	200 (43.8)	
Female	257 (56.2)	
Ethnic groups (N = 457)		
Malay	153 (33.5)	
Chinese	182 (39.8)	
Indian	119 (26.0)	
Others	3 (0.7)	
Marital status (N = 432)		
Single	21 (4.9)	
Married	392 (90.7)	
Divorced	2 (0.5)	
Widower	17 (3.9)	
Employment status (N = 329)		
Employed	115 (35.0)	
Unemployed	214 (65.0)	
Baseline HbA_{1c} (%) (mmol/mol)[a] (N = 402)		
<6.5 (<48)	25 (6.2)	
≥6.5 (≥48)	377 (93.8)	8.7 (1.7) (8.5) [4.9–17.1]
<7.0 (<53)	47 (11.7)	
≥7.0 (≥53)	355 (88.3)	
Fasting blood glucose (mmol/L) (mg/dL)[b] (N = 373)		
<4.4 (<79.2)	6 (1.6)	
4.4–6.1 (79.2–109.8)	50 (13.4)	8.8 (3.2) (8.3) [2.9–36.9]
>6.1 (>109.8)	317 (85.0)	
Presence of comorbidities[c] (N = 457)		
Hypertension	379 (82.9)	
Dyslipidemia	322 (70.5)	
Cardiovascular disease	143 (31.3)	
Kidney disease	103 (22.5)	
Stroke	23 (5.0)	
Duration of diabetes (number of years) (N = 406)		
1–5	21 (5.2)	
6–10	91 (22.2)	14.9 (7.6) (13) [0–41]
11–15	118 (29.1)	
16–20	81 (19.9)	
>20	95 (23.4)	
Body weight (kg) (N = 294)		
<50	21 (7.1)	
50–59	66 (22.5)	70.9 (19.1) (66.9) [36.8–195.0]
60–69	84 (28.6)	
70–79	43 (14.6)	
≥80	80 (27.2)	
Body mass index (BMI) (kg/m^2)[d] (N = 140)		
<18.5	1 (0.7)	
18.5–22.9	25 (17.9)	27.9 (5.4) (27.4) [17.2–45.4]
23–27.4	46 (32.9)	
≥27.5	68 (48.5)	
Waist circumference (cm)[b]		
Male (N = 74)		95.5 (11.8) (96.0) [65.0–132.5]
<90	20 (27.0)	
≥90	54 (73.0)	
Female (N = 85)		93.8 (18.0) (91.0) [67–204]
<80	7 (8.2)	
≥80	78 (91.8)	
Number of diabetes medications (N = 449)		
1	94 (20.9)	
2	224 (49.9)	2.12 (0.8) (2) [1–4]
3	112 (25.0)	
4	19 (4.2)	
Type of antidiabetes medications (N = 449)		
Biguanide only	41 (9.1)	
Sulphonylurea only	43 (9.6)	
Insulin only	16 (3.6)	
Biguanide and sulphonylurea	186 (41.4)	
Biguanide and insulin	20 (4.5)	
Sulphonylurea and insulin	8 (1.8)	
Biguanide, sulphonylurea, and insulin	24 (5.3)	
Others[e]	111 (24.7)	

Table 1: Continued.

Patients characteristics	Number of patients (%)	Mean (SD) (median) [range]
Initial dose of sitagliptin ($N = 457$)		
25 mg	34 (7.4)	
50 mg	158 (34.6)	
100 mg	265 (58.0)	

SD: standard deviation.
[a]HbA_{1c} is categorised based on the Clinical Practice Guidelines of Malaysia of <6.5% [5] and the American Diabetes Association of <7% [6].
[b]Fasting blood glucose and waist circumference are categorized based on the Clinical Practice Guidelines of Malaysia [5].
[c]Some patients may have more than one comorbidity.
[d]Body mass index (BMI) is categorized based on the Clinical Practice Guidelines on Management of Obesity [20].
[e]Others include combinations such as meglitinides, thiazolidinediones, and alpha-glucosidase inhibitors.

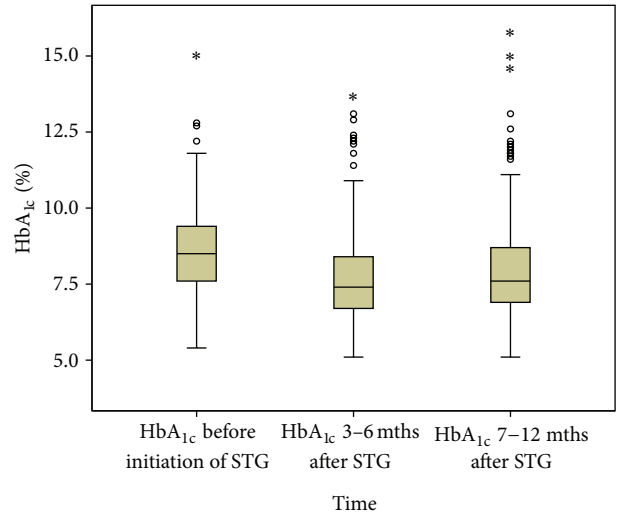

Figure 2: HbA_{1c} levels at all three measurement points ($N = 244$); o: outlier; ∗: extreme case; STG: sitagliptin; mths: months.

the patients were classified as overweight (defined as ≥80 cm for female and ≥90 cm for male) [5].

Almost all the patients initiated on sitagliptin had diabetes for more than 5 years (94.8%). Most of the patients have other comorbidities (Table 1). Some patients also presented with complications of diabetes: 27 patients had nephropathy; 45 had retinopathy; 33 had neuropathy; 9 had diabetes foot ulcer; and 4 had amputation done.

Sitagliptin was prescribed to substitute patients' current antidiabetes medication in 19.2% of the cases whereas, in most cases (80.8%), sitagliptin was added to the current antidiabetes therapy. Sitagliptin was often added as an adjunct to metformin and sulphonylurea (42.3%).

The most common reason for the initiation of sitagliptin was uncontrolled blood glucose even though patients were already on other antidiabetes medications (82.6%). Intolerance to the side effects of other antidiabetes medications (6.1%) such as gastrointestinal disturbances (19 cases) which were associated with the use of metformin and acarbose was also reported. Other reasons were the occurrence of hypoglycemia (5.4%), concern on possible adverse effects of rosiglitazone on the cardiovascular system (2.6%), patients' unwillingness to start insulin (1.6%), nonadherence to insulin (4 patients), difficulty in obtaining rosiglitazone (2 patients), and request by a patient who claimed that sitagliptin has better effect on his blood glucose.

3.2. Effectiveness of Sitagliptin. Effectiveness of sitagliptin was analyzed based on 244 patients whose HbA_{1c} levels could be obtained for all the three points (before initiation of sitagliptin, 3 to 6 months and 7 to 12 months after initiation of sitagliptin). Prior to the initiation of sitagliptin, the mean HbA_{1c} (standard deviation (SD)) was 8.5 (1.4)% (69 mmol/mol). After the initiation of sitagliptin, the mean HbA_{1c} value reduced significantly to 7.7 (1.4)% (61 mmol/mol) within the first 3 to 6 months ($P < 0.001$). Subsequently, after 7 to 12 months, the mean HbA_{1c} value was 8.0 (1.7)% (64 mmol/mol). This is significantly higher than that of the first 3 to 6 months ($P = 0.002$) but still significantly lower than before the initiation of sitagliptin ($P < 0.001$). The changes in HbA_{1c} before, 3 to 6 months and 7 to 12 months after initiation of sitagliptin are shown in Figure 2.

In the presence of missing data among the confounders, only 157 patients could be included in the generalized linear model. Due to the nature of the study design with repeated measures, there were 87 cases (35.7%) with incomplete covariates. These incomplete data were retained in the analysis using multiple imputation approach [21]. This means that all the 244 patients can be included using the imputed data as shown in Table 2. The model predicts that, on an average, a patient will experience a reduction in HbA_{1c} of 0.8% (95% confidence interval (CI): 0.7–1.0; $P < 0.001$) within the first 3 to 6 months after the initiation of sitagliptin and a reduction from baseline of 0.6% (95% CI: 0.4–0.7; $P < 0.001$) after being on sitagliptin for 7 to 12 months. This model also indicates that the patient's baseline HbA_{1c} value was the only factor which has a significant effect on the HbA_{1c} levels of patients after the initiation of sitagliptin.

Amongst the 244 patients included in determining the effectiveness of sitagliptin, 166 patients (68.0%) showed an overall reduction in HbA_{1c} after 7–12 months, whereas 69 patients (28.3%) showed an increase in HbA_{1c} and 9 patients (3.7%) had no change in HbA_{1c} after 7–12 months. The GEE analysis showed similar results when the patients were divided into two groups, that is, those with and without improvement in HbA_{1c} after being on sitagliptin for 7 to 12 months. Only patient's baseline HbA_{1c} value was associated with the two groups.

In addition, the proportion of patients who achieved the recommended glycemic target increased by twofold 7–12 months after the patient was on sitagliptin. After sitagliptin was initiated for 7–12 months, the number of patients who achieved HbA_{1c} below 6.5% increased from 16 patients (6.6%) at baseline to 29 patients (11.9%) ($\chi^2 = 41.886$; $P < 0.001$) whereas the number of patients with HbA_{1c} below 7%

Table 2: Parameter estimates using the Generalized Estimating Equations (GEE) model (imputed) ($N = 244$).

Parameter	B	Std. error	95% Wald confidence interval		Hypothesis test		
			Lower	Upper	Wald chi-square	df	Sig.
(Intercept)	1.990	.5912	.831	3.149	11.326	1	.001
Years diagnosed							
>20 years	.467	.2467	−.016	.951	3.590	1	.058
16–20 years	.319	.2447	−.161	.798	1.697	1	.193
11–15 years	.366	.2301	−.085	.817	2.528	1	.112
6–10 years	.292	.2186	−.137	.720	1.780	1	.182
1–5 years	0[a]	—	—	—	—	—	—
Employment status							
No	−.118	.1402	−.393	.157	.707	1	.401
Yes	0[a]	—	—	—	—	—	—
Race							
Indian	−.116	.1250	−.361	.129	.865	1	.352
Chinese	−.201	.1122	−.421	.019	3.218	1	.073
Malay	0[a]	—	—	—	—	—	—
Marital status							
Widower	.163	.3932	−.607	.934	.173	1	.678
Divorced	.274	.2920	−.299	.846	.878	1	.349
Married	.136	.2674	−.388	.660	.259	1	.611
Single	0[a]	—	—	—	—	—	—
Gender							
Female	.047	.985	−.147	.240	.223	1	.637
Male	0[a]	—	—	—	—	—	—
Time of HbA_{1c} level							
7–12 months after sitagliptin	−.562	.0941	−.746	−.377	35.586	1	.000
3–6 months after sitagliptin	−.831	.0801	−.988	−.674	107.550	1	.000
Before initiation of sitagliptin (Baseline)	0[a]	—	—	—	—	—	—
Number of antidiabetes	−.038	.1301	−.292	.217	.084	1	.773
Age	−.010	.0065	−.023	.003	2.345	1	.126
Changes in regimen							
During initiation of sitagliptin	.101	.2169	−.324	.526	.216	1	.642
After initiation of sitagliptin	.020	.0981	−.172	.213	.043	1	.837
No change	0[a]	—	—	—	—	—	—
Initiation dose of sitagliptin							
100 mg	.057	.3022	−.536	.649	.035	1	.852
50 mg	.273	.2901	−.295	.842	.887	1	.346
25 mg	0[a]						
Medications prior to sitagliptin initiation							
Others	.281	.2851	−.278	.840	.969	1	.325
Biguanide, sulphonylurea, and insulin	.384	.3392	−.281	1.049	1.281	1	.258
Sulphonylurea and insulin	.009	.3411	−.660	.678	.001	1	.979
Biguanide and insulin	.226	.3094	−.381	.832	.533	1	.465
Biguanide and sulphonylurea	.194	.1855	−.170	.558	1.092	1	.296
Insulin alone	.140	.4808	−.802	1.083	.085	1	.770
Sulphonylurea alone	.062	.1920	−.314	.438	.104	1	.747
Biguanide alone	0[a]						
Initiation of sitagliptin							
Added to current regime	−.159	.1385	−.431	.112	1.318	1	.251
Switched from current regime	0[a]						
Baseline HbA_{1c}	.790	.0407	.711	.870	377.159	1	000
(Scale)	1.114						

Dependent variable: HbA_{1c}.
[a]Set to zero because this parameter is redundant.

increased from 28 patients (11.5%) at baseline to 68 patients (27.9%) ($\chi^2 = 25.160$; $P < 0.001$).

3.3. Safety of Sitagliptin. The incidence of hypoglycemia reduced significantly from 61 patients (13.3%) to 40 patients (8.8%) ($\chi^2 = 7.591$; $P = 0.006$) after the initiation of sitagliptin. Prior to the initiation of sitagliptin, the mean weight (SD) of 176 patients was 70.9 (17.2) kg. However, 12 months after the initiation of sitagliptin, the mean weight (SD) decreased to 70.4 (17.3) kg, that is, a mean decrease of 0.5 kg (95% CI: −0.02 to 0.96; $P = 0.061$) but was not statistically significant.

Sitagliptin was associated with side effects in eleven patients. Three patients had hypoglycaemia, whereas each of the remaining patients had worsening of allergic reaction, cough, weight loss, drowsiness, increase in creatinine level, swelling of the leg, and bloating and one patient just could not tolerate sitagliptin with no specific reason given.

4. Discussion

Sitagliptin is a DPP-4 inhibitor which is a relatively new group of antidiabetes medications in the market. Despite the lack of data on its effectiveness in clinical practice, its use has escalated since its introduction over the past few years. The number of patients prescribed with sitagliptin in the present study has doubled from 2009 to 2012.

Most of the patients on sitagliptin were 65 years old and above. This could be due to its potential benefits for causing minimal or no hypoglycemia in comparison to other antidiabetes medications such as the sulphonylureas [18, 19]. In addition, it is also more convenient due to its once daily oral dosing [18, 22].

A majority of the patients prescribed with sitagliptin were overweight based on their BMI or waist circumference as defined by the clinical practice guidelines [20]. Sitagliptin is preferred for overweight patients as it is weight neutral [12, 13, 17, 18]. This is an advantage over other antidiabetes medications such as the thiazolidinediones and sulphonylureas which are often associated with weight gain [18, 23]. However, this study did not demonstrate any significant change in body weight after the initiation of sitagliptin.

The mean (SD) duration of diabetes of patients prescribed with sitagliptin was 14.9 (7.6) years. This is similar to a study conducted in Taiwan although other studies had shown a shorter duration of 2 to 6 years [14, 17, 18]. Patients with a shorter duration of diabetes showed greater reduction in HbA_{1c} with the addition of sitagliptin; hence sitagliptin should be started earlier for better effect [18].

Sitagliptin was added to the existing antidiabetes regimens of most patients due to uncontrolled diabetes. This is consistent with that of other studies [9, 14, 24, 25]. This also accounts for the high baseline HbA_{1c} values in a majority of the patients (88.3% of the patients with $HbA_{1c} > 7\%$) in the present study which was reported in another similar retrospective study [9]. Sitagliptin was usually prescribed for patients who were already on metformin and sulphonylurea. This means that sitagliptin was only initiated when the older groups of antidiabetes medications failed to produce adequate glycemic control. This is because sitagliptin is a relatively new antidiabetes agent and hence is reserved as an add-on therapy for patients who are unable to tolerate other antidiabetes medications or who have not reached the glycemic target with the standard first-line agents [8].

In the present study, 6.1% of the patients were started on sitagliptin when the other antidiabetes medications caused side effects. Sitagliptin is generally well tolerated with minimal adverse effects [14, 24]. This is a potential benefit of DPP-4 inhibitors as the occurrence of adverse effects often led to nonadherence to antidiabetes medications which in turn contributes to poor glycemic control [14]. It has been reported that patients on sitagliptin were less likely to discontinue their medications due to adverse reactions as compared to metformin monotherapy [26].

The Generalized Estimating Equations (GEE) model predicted that, on an average, sitagliptin resulted in a significant reduction of HbA_{1c} by 0.6% (95% CI: 0.4–0.7; $P < 0.001$) after 7 to 12 months. Studies have shown that sitagliptin reduced HbA_{1c} by 0.5% to 0.7% [8, 12, 13, 15]. However, the GEE model predicted a greater HbA_{1c} reduction 3 to 6 months after initiation of sitagliptin which is 0.8% (95% CI: 0.7–1.0; $P < 0.001$) compared to 7 to 12 months later. This indicates that sitagliptin produced the most glycemic effect during the first 6 months but this effect reduced significantly after that ($P = 0.002$) although still better than before the initiation of sitagliptin. This increment in HbA_{1c} may not be seen in studies which only had two-point measurements of HbA_{1c} (at baseline and at the end of the study). However, studies with more than two-point measurements showed similar effects with the use of sitagliptin [18, 27]. A study in Japan which reported similar outcomes attributed this increase to a reduction in compliance with diet and exercise therapy [28].

In addition, twice as many patients managed to attain glycemic control after the initiation of sitagliptin for 7 to 12 months. Other clinical studies showed similar results although they were carried out specifically to compare the efficacy of sitagliptin with placebo [12, 18]. One recent retrospective study which also assessed the effectiveness of sitagliptin in a clinical practice reported similar increase in the proportion of patients achieving glycemic control after using sitagliptin although the average reduction in HbA_{1c} is higher than that of the present study [29].

Mafauzy reported that 26.8% of patients with diabetes in public hospitals in Malaysia conducted self-monitoring of blood glucose [30]. On the contrary, 78.4% of the patients in the present study had a glucose meter at home. The difference may be attributed to the advance in technology from 2006 to 2012 and hence more patients have access to more convenient and cheaper glucose meters. The increase in the use of home glucose meters may also be due to an increase in awareness on the importance of self-monitoring of blood glucose.

There are several limitations in this study. Some data were not available as only information written in the patient medical records could be extracted. The dose of sitagliptin dispensed to the patients could not be standardized and this ranged from 25 to 100 mg, with most patients being on 100 mg. However, a study carried out in Japan showed

that patients on sitagliptin 50 mg also showed a reduction in HbA_{1c} of 0.6% which is similar to that achieved by patients on sitagliptin 100 mg in other studies [31]. In the present study, a change in medications during and after the initiation of sitagliptin may occur but this was taken into account during the GEE model analysis which showed that changes in patients' medications did not significantly affect the change in HbA_{1c} levels. Patients' adherence to their medications could not be ascertained as no adherence assessment was carried out. Dietary habit and exercise could not be controlled, which may have affected the change in HbA_{1c}.

5. Conclusion

In conclusion, the study provides evidence that sitagliptin produces a significant reduction of 0.8% in the mean HbA_{1c} value, 3 to 6 months after use. However, this reduction in HbA_{1c} was lesser 7 to 12 month later (0.6%) but still similar to that reported in clinical trials. Further investigations are required to determine if reduced adherence to sitagliptin is the reason for the increase in HbA_{1c} with prolonged usage of sitagliptin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] World Health Organization, "Diabetes programme: country and regional data," http://www.who.int/diabetes/facts/world_figures/en/.

[2] International Diabetes Federation, *One Adult in Ten will have Diabetes by 2030*, http://www.idf.org/media-events/press-releases/2011/diabetes-atlas-5th-edition.

[3] G. R. Letchuman, W. M. Wan Nazaimoon, W. B. Wan Mohamad et al., "Prevalence of diabetes in the Malaysian National Health Morbidity Survey III 2006," *The Medical Journal of Malaysia*, vol. 65, no. 3, pp. 173–179, 2010.

[4] W. M. Wan Nazaimoon, S. H. Md Isa, W. B. Wan Mohamad et al., "Prevalence of diabetes in Malaysia and usefulness of HbA_{1c} as a diagnostic criterion," *Diabetic Medicine*, vol. 30, no. 7, pp. 825–828, 2013.

[5] *Clinical Practice Guidelines: Management of Type 2 Diabetes Mellitus*, MOH/P/PAK/184.09(GU), Malaysian Endocrine and Metabolic Society, Ministry of Health Malaysia, Academy of Medicine Malaysia, Persatuan Diabetes Malaysia, Kuala Lumpur, Malaysia, 4th edition, 2009.

[6] American Diabetes Association (ADA), "Standards of medical care in diabetes—2014," *Diabetes Care*, vol. 37, pp. S14–S80, 2014.

[7] S. S. Chua and S. P. Chan, "Medication adherence and achievement of glycaemic targets in ambulatory type 2 diabetic patients," *Journal of Applied Pharmaceutical Science*, vol. 1, no. 4, pp. 55–59, 2011.

[8] S. VanDeKoppel, H. M. Choe, and B. V. Sweet, "Managed care perspective on three new agents for type 2 diabetes," *Journal of Managed Care Pharmacy*, vol. 14, no. 4, pp. 363–380, 2008.

[9] D. T. Eurich, S. Simpson, A. Senthilselvan, C. V. Asche, J. K. Sandhu-Minhas, and F. A. McAlister, "Comparative safety and effectiveness of sitagliptin in patients with type 2 diabetes: retrospective population based cohort study," *The British Medical Journal*, vol. 346, no. 7908, Article ID f2267, 2013.

[10] G. A. Herman, A. Bergman, C. Stevens et al., "Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 11, pp. 4612–4619, 2006.

[11] A. J. Bergman, C. Stevens, Y. Zhou et al., "Pharmacokinetic and pharmacodynamic properties of multiple oral doses of sitagliptin, a dipeptidyl peptidase-IV inhibitor: a double-blind, randomized, placebo-controlled study in healthy male volunteers," *Clinical Therapeutics*, vol. 28, no. 1, pp. 55–72, 2006.

[12] K. Nonaka, T. Kakikawa, A. Sato et al., "Efficacy and safety of sitagliptin monotherapy in Japanese patients with type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 79, no. 2, pp. 291–298, 2008.

[13] P. Aschner, M. S. Kipnes, J. K. Lunceford, M. Sanchez, C. Mickel, and D. E. Williams-Herman, "Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with type 2 diabetes," *Diabetes Care*, vol. 29, no. 12, pp. 2632–2637, 2006.

[14] M.-N. Chien, C.-C. Lee, W.-C. Chen, S.-C. Liu, C.-H. Leung, and C.-H. Wang, "Effect of sitagliptin as add-on therapy in elderly type 2 diabetes patients with inadequate glycemic control in Taiwan," *International Journal of Gerontology*, vol. 5, no. 2, pp. 103–106, 2011.

[15] B. J. Goldstein, M. N. Feinglos, J. K. Lunceford, J. Johnson, and D. E. Williams-Herman, "Effect of initial combination therapy with sitagliptin, a dipeptidyl peptidase-4 inhibitor, and metformin on glycemic control in patients with type 2 diabetes," *Diabetes Care*, vol. 30, no. 8, pp. 1979–1987, 2007.

[16] National Institute for Health and Clinical Excellence, *NICE Clinical Guideline: Type 2 Diabetes*, National Institute for Health and Clinical Excellence, London, UK, 2009.

[17] T. L. Seck, S. S. Engel, D. E. Williams-Herman et al., "Sitagliptin more effectively achieves a composite endpoint for A1C reduction, lack of hypoglycemia and no body weight gain compared with glipizide," *Diabetes Research and Clinical Practice*, vol. 93, no. 1, pp. e15–e17, 2011.

[18] I. Raz, M. Hanefeld, L. Xu, C. Caria, D. Williams-Herman, and H. Khatami, "Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy in patients with type 2 diabetes mellitus.," *Diabetologia*, vol. 49, no. 11, pp. 2564–2571, 2006.

[19] G. A. Herman, G. C. Mistry, B. Yi et al., "Evaluation of pharmacokinetic parameters and dipeptidyl peptidase-4 inhibition

following single doses of sitagliptin in healthy, young Japanese males," *British Journal of Clinical Pharmacology*, vol. 71, no. 3, pp. 429–436, 2011.

[20] *Clinical Practice Guidelines on Management of Obesity*, Ministry of Health Malaysia, Academy of Medicine Malaysia, Malaysian Association for the Study of Obesity, Malaysian Endocrine and Metabolic Society, Kuala Lumpur, Malysia, 2004.

[21] R. J. Little and D. B. Rubin, *Statistical Analysis with Missing Data*, John Wiley & Sons, New York, NY, USA, 1987.

[22] B. Ahrén, "DPP-4 inhibitors," *Best Practice and Research in Clinical Endocrinology and Metabolism*, vol. 21, no. 4, pp. 517–533, 2007.

[23] C. L. Boland, M. DeGeeter, D. S. Nuzum, and M. Tzefos, "Evaluating second-line treatment options for type 2 diabetes: focus on secondary effects of GLP-1 agonists and DPP-4 inhibitors," *Annals of Pharmacotherapy*, vol. 47, no. 4, pp. 490–505, 2013.

[24] M. A. Nauck, G. Meininger, D. Sheng et al., "Efficacy and safety of the dipeptidyl peptidase-4 inhibitor, sitagliptin, compared with the sulfonylurea, glipizide, in patients with type 2 diabetes inadequately controlled on metformin alone: a randomized, double-blind, non-inferiority trial," *Diabetes, Obesity and Metabolism*, vol. 9, no. 2, pp. 194–205, 2007.

[25] J. Rosenstock, R. Brazg, P. J. Andryuk, K. Lu, and P. Stein, "Efficacy and safety of the dipeptidyl peptidase-4 inhibitor sitagliptin added to ongoing pioglitazone therapy in patients with type 2 diabetes: a 24-week, multicenter, randomized, double-blind, placebo-controlled, parallel-group study," *Clinical Therapeutics*, vol. 28, no. 10, pp. 1556–1568, 2006.

[26] T. Karagiannis, P. Paschos, K. Paletas, D. R. Matthews, and A. Tsapas, "Dipeptidyl peptidase-4 inhibitors for treatment of type 2 diabetes mellitus in the clinical setting: systematic review and meta-analysis," *British Medical Journal*, vol. 344, no. 7850, Article ID e1369, 2012.

[27] O. Giampietro, C. Giampietro, L. D. Bartola, M. C. Masoni, and E. Matteucci, "Sitagliptin as add-on therapy in insulin deficiency: biomarkers of therapeutic efficacy respond differently in type 1 and type 2 diabetes," *Drug Design, Development and Therapy*, vol. 7, pp. 99–104, 2013.

[28] A. Kanamori and I. Matsuba, "Factors associated with reduced efficacy of sitagliptin therapy: analysis of 93 patients with type 2 diabetes treated for 1.5 years or longer," *Journal of Clinical Medicine Research*, vol. 5, no. 3, pp. 217–221, 2013.

[29] T. Y. Chen and C. J. Hsieh, "Real-world effectiveness of sitagliptin as add-on therapy in patients with Type 2 diabetes mellitus," *Postgraduate Medicine*, vol. 126, no. 3, pp. 205–215, 2014.

[30] M. Mafauzy, "Diabetes control and complications in public hospitals in Malaysia," *Medical Journal of Malaysia*, vol. 61, no. 4, pp. 477–483, 2006.

[31] T. Nomiyama, Y. Akehi, H. Takenoshita et al., "Contributing factors related to efficacy of the dipeptidyl peptidase-4 inhibitor sitagliptin in Japanese patients with type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 95, no. 2, pp. e27–e28, 2012.

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Research Article

Effects of Aerobic Exercise Based upon Heart Rate at Aerobic Threshold in Obese Elderly Subjects with Type 2 Diabetes

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In obese diabetic subjects, a correct life style, including diet and physical activity, is part of a correct intervention protocol. Thus, the aim of this study was to evaluate the effects of aerobic training intervention, based on heart rate at aerobic gas exchange threshold ($AerT_{ge}$), on clinical and physiological parameters in obese elderly subjects with type 2 diabetes (OT2DM). Thirty OT2DM subjects were randomly assigned to an intervention (IG) or control group (CG). The IG performed a supervised aerobic exercise training based on heart rate at $AerT_{ge}$ whereas CG maintained their usual lifestyle. Anthropometric measures, blood analysis, peak oxygen consumption ($\dot{V}O_{2peak}$), metabolic equivalent (MET_{peak}), work rate (WR_{peak}), and WR_{AerTge} were assessed at baseline and after intervention. After training, patients enrolled in the IG had significantly higher ($P < 0.001$) $\dot{V}O_{2peak}$, MET_{peak}, WR_{peak}, and WR_{AerTge} and significantly lower ($P < 0.005$) weight, BMI, %FM, and waist circumference than before intervention. Both IG and CG subjects had lower glycated haemoglobin levels after intervention period. No significant differences were found for all the other parameters between pre- and posttraining and between groups. Aerobic exercise prescription based upon HR at $AerT_{ge}$ could be a valuable physical intervention tool to improve the fitness level and metabolic equilibrium in OT2DM patients.

1. Introduction

Obesity is a leading risk factor for premature mortality and chronic health hazards such as type 2 diabetes, coronary heart diseases, and hypertension. According to the World Health Organization at least 2.8 million adults die each year as a result of being overweight or obese. In addition, 44% of the diabetes burden, 23% of the ischaemic heart disease burden, and 7 to 41% of certain cancers are also attributable to overweight and obesity. The prevalence of overweight and obesity has increased to epidemic proportions in the industrialized world and it is now dramatically rising in low- and middle-income countries, particularly in urban settings. It is well known that regular physical activity (PA) provides health benefits and it is considered an essential component of primary and secondary prevention for most of metabolic-syndrome related pathologies [1, 2]. Recent experimental data suggests that subjects who increased their level of PA over time have a decreased mortality rate compared to those who were consistently unfit [3, 4].

Despite such evidence, physical inactivity remains a global health problem and its negative effects on health were well documented [5] as well as negative economic consequences [6]. The role of exercise intensity on physical

training adherence is supported by several large studies [7–9]. Furthermore, Dishman and Buckworth [10] showed that activity-promotion efforts were more effective when the intensity was low rather than high. Accordingly, exercise intensity should be set in order to reach positive physiological effects, decrease the risk of injury, and increase adherence. It is well known that exercise can elicit different physiological responses in obese individuals when prescribed in absolute terms [11], since, usually, no adjustment is usually made for each individual's exercise capacity. Moreover, the use of relative terms, such as $\%VO_{2\,max}$, has been substantially criticized [12–14], since it seems that the relative parameters alone without considering the aerobic threshold (AerT) are not enough to individualize the exercise intensity [12]. AerT is the point after which ventilation begins to increase disproportionately relative to oxygen uptake and it is considered a useful parameter for optimal aerobic exercise intensity prescription in DM2 patients [12, 15–17]. To clarify this specific terminology, Meyer et al. [14] suggested using the terms $AerT_{ge}$ when aerobic threshold is evaluated by gas analysis.

Moreover, during the last years, $AerT_{ge}$ has been more frequently used to prescribe exercise intensity in obese and diabetic populations [12, 14, 15, 18, 19]. Interestingly, there are two different methods to exercise at a relative $AerT_{ge}$: the first aims at maintaining a work load, such as speed and power, corresponding to AerT, and the second one is keeping a constant HR corresponding to $AerT_{ge}$ [20]. However, since musculoskeletal complications commonly present in diabetic patients [21] could prevent obese elderly patients from performing 30 min exercise at constant average load, it could be useful to choose an approach based on constant HR at $AerT_{ge}$.

The effects of 30 min exercise at constant HR on physiological parameters were previously described by Kindermann et al. [20] who showed that it is possible to maintain the optimal individual workload intensity by regulating HR during exercise. The intensity of PA has been found to be negatively related to adherence in overweight or obese subjects [22]. Moreover, DaSilva et al. [22] showed that overweight individuals choose to exercise at intensities below or around the ventilatory threshold and that this intensity results in low perceived exertion and positive affective responses. Also, frequent walking of sufficient duration performed more than 3 days a week might improve glycaemic control and lipoprotein profiles of subjects with type 2 diabetes and, also, cardiorespiratory fitness [15, 23].

To the best of our knowledge, only one study [23] evaluated the effects of heart rate intensity prescribed walking training program on cardiorespiratory fitness and glycaemic control in individuals affected by type 2 diabetes mellitus. Subjects' peak heart rates, obtained from the Balke-Ware protocol, were used to set a training intensity at the 80% HR_{peak} and only walking exercise was performed for a training period of 7 weeks.

Therefore, the aim of our study was to evaluate the effects of three months of aerobic exercise, based upon heart rate at $AerT_{ge}$ on glycemic control, body weight, and fitness in obese elderly subjects with type 2 diabetes.

2. Methods

2.1. Participants. Thirty obese elderly subjects (age 66.8 ± 6.3 years), body mass index (BMI) of 34.6 ± 3.2 kg/m^2, and percent fat mass (%FM) of 36.5 ± 6.8% with type 2 diabetes, were recruited by a diabetes care unit hospital. Participants provided written informed consent and protocol was approved by the Local Scientific Committee. All subjects were sedentary and they had not been previously engaged in regular physical exercise program. Diagnosis of Type 2 diabetes mellitus (T2DM) was established according to the World Health Organization criteria [24]. Subjects underwent clinical examination to rule out any contraindications to PA such as neuropathy, autonomic dysfunction, cardiovascular diseases, and high blood pressure (≥140/90 mmHg). All subjects were on oral pharmacological treatments.

2.2. Experimental Design. Subjects were randomly assigned to an intervention group (IG) (15 subjects, age 66.7±4.9 years) or to a control group (CG) (15 subjects, age 66.9 ± 4.2 years). Pulmonary function tests and a resting electrocardiogram (ECG) recording were performed as initial screening in both groups. Prior to the first test session, participants took part in a familiarization session to become accustomed to PA tests. Anthropometric measurements, blood sampling, dietary and physiological evaluation, and a submaximal graded exercise test were performed at baseline and after 3 months in both groups. During the 3-month intervention period, IG group performed supervised aerobic exercise training while CG did not perform any organized exercise. Lifestyle and food behaviour of the subjects have not changed during the experimental procedure.

2.3. Body Composition. Participant's height was measured at the time of hospital referral using a stadiometer to the nearest 0.1 cm. Body composition was determined using a multifrequency bioimpedance analysis (InBody 720, Biospace Inc., Cerritos, CA, USA) [25]. All subjects were tested in the morning after 12-hour overnight fast. All body mass (BM) measurements were taken on a calibrated digital scale (InBody 720, Biospace Inc., Cerritos, CA, USA) when the subjects wore minimal clothing (i.e., underwear). Body mass index (BMI) was calculated as body mass divided by squared height (kg/m^2).

Waist circumferences were taken at 2.5 cm above the umbilicus [26] using an inextensible metallic tape placed directly on the skin, perpendicularly to the long axis of the body, and horizontally to the floor at the end of normal expiration. Average values from two measurements were considered.

2.4. Blood Analysis. All subjects had 5 mL blood drawn into a vacutainer from an antecubital vein in the morning after the overnight fast. Serum obtained after clotting and centrifugation at 1500 g for 20 min was used for glucose, triglyceride, and total/HDL cholesterol determination by a Kodak Autoanalyzer. Low density lipoprotein cholesterol (LDLC) was determined using Friedwald's equation for

triglycerides <400 mg/dL. Additional 2 mL blood samples were also drawn into EDTA-treated vacutainers for glycated hemoglobin (HbA1c) determination using high-performance liquid chromatography (BioRad Dia-STAT Analyzer 1).

2.5. Aerobic Power. $\dot{V}O_{2peak}$ was assessed in all participants by means of a continuous, maximal graded exercise test on a cycle ergometer or on a treadmill according to the individual abilities. In particular, subjects who were able to walk safely performed a modified Balke protocol [27] on a treadmill (Run Med Excite, Technogym, Gambettola (FC), Italy), while those that were not able to walk safely performed a bike-ramp 10w protocol [28] on a cycle ergometer (Bike Med Excite, Technogym, Gambettola (FC), Italy). Heart rate (beats·min^{-1}) was continuously recorded before and throughout the trial using a HR monitor (RS 400, Polar Electro, Kempele, Finland). $\dot{V}O_2$ and pulmonary ventilation ($\dot{V}E$) were measured by a semiportable gas analysis system (Fitmate Pro Cosmed, Rome, Italy) [29]. Prior to each test the Fitmate Pro underwent an automatic gas calibration cycle and the turbine flow meter was periodically calibrated using a 3 L syringe according to the manufacturer's recommendations. During the test, the highest VO_2 attained was chosen as the peak oxygen uptake ($\dot{V}O_{2peak}$). The individual aerobic gas exchange threshold ($AerT_{ge}$) was determined offline for each subject by plotting the ventilatory equivalent ($\dot{V}E/\dot{V}O_2$) as a function of $\dot{V}O_2$ to identify the point during exercise where this curve reached its lowest value [17, 30]. The level of $\dot{V}O_2$ at which we observed the lowest value of the $\dot{V}E/\dot{V}O_2$, in the individual plot, was the individual aerobic threshold [12, 31]. Moreover, work rate (WR) and metabolic equivalent (MET) were calculated at $AerT_{ge}$ (WR_{AerTge} and MET_{AerTge}, resp.) and at maximal effort (WR_{peak} and MET_{peak}, resp.).

2.6. Training Protocol. Subjects of the IG group performed a 3-month aerobic training (AT) twice a week based on HR corresponding to their individual $AerT_{ge}$. All training sessions, lasting 50 min, were supervised by a PA specialist. The AT was performed on a treadmill (Run Med Excite, Technogym, Gambettola (FC), Italy) or on a cycle ergometer (Bike Med Excite, Technogym, Gambettola (FC), Italy) in accordance with the device used for the evaluation of $\dot{V}O_{2peak}$. Subjects' HR, corresponding to $AerT_{ge}$ obtained from maximal exercise test, was used to set the intensity of training protocol which consisted of a 5 min warm-up, 30 min AT, and 5 min cooldown. All devices were programmed at the beginning of the training to change the external work load (inclination for the treadmill and wattage for the cycle ergometer) to maintain the subjects' HR below (warm-up and cool-down periods) or equal to (training period) the individual subject's HR at $AerT_{ge}$. Moreover, the maximal treadmill speed was set not higher than 5 km/h to perform all training sessions as safely as possible. Subjects training on a cycle ergometer maintained the pedaling rate at 40 RPM. This pedaling rate was the same used during the incremental graded test. Stretching (5 min) exercises, involving main lower limb muscle groups, were performed after warm-up and cool-down periods.

2.7. Dietary and Psychological Counselling. Dietary and psychological counselling was performed by a dietitian and a psychologist, respectively, to minimize these items' variability between groups. A low-calorie diet was set at approximately 400 Kcal less than total daily energy expenditure in both IG and CG groups. Total daily energy expenditure was set according to the following equation: resting metabolic rate + physical activity level. Resting metabolic rate was estimated by the Harris benedict equation [32] while physical activity level was estimated by the international physical activity questionnaire (IPAQ) [33]

2.8. Statistical Analysis. The similar baseline characteristics of the two groups were verified by unpaired t-test at T0. Due to the between subjects variability of glycated haemoglobin at baseline (T0), a 2×2 mixed analysis of covariance (ANCOVA) with group (IG versus CG) as between factor, time (before versus after) as within factor, and glycated haemoglobin baseline data as covariate was performed.

For each variable, mixed ANOVA with repeated measures on time was used to detect significant effects of two main factors: group (intervention versus control) and time (before versus after). Post hoc analysis of significant differences for group factor was performed using the unpaired Student's t-test, while for time factor it was performed using the paired t-test. All statistical analyses were performed with the SPSS statistical package (Version 20.0 for Windows; SPSS Inc., Chicago, IL, USA). All tests were two-tailed, with $\alpha \leq 0.05$ being taken as significant.

3. Results

The baseline characteristics were similar (no significant differences) between intervention and control groups for any studied variable as depicted in Table 1. Subjects in the IG reported a transitory, low muscle pain during training. A significant group × time interaction ($P < 0.05$) was found for body mass index (BMI), %FM, and abdominal circumference, indicating that the trend of these variables was different between groups after 3 months. In fact, the aerobic training based upon HR resulted in a significant decrease in BMI, %FM, and abdominal circumference, while there were not any differences in CG on anthropometric variables (Table 1). Moreover, significant group × time interaction ($P < 0.05$) was found for $\dot{V}O_{2peak}$, MET_{peak}, WR_{peak}, and WR_{AerTge}, indicating that only in IG group these variables increased ($P < 0.005$) in posttraining (Table 2). A significant main effect of time was found in glycated haemoglobin that significantly lowered after 3 months in both groups (Figure 1) but no significant effects were found in total cholesterol, high density lipoprotein cholesterol (HDLC), and low density lipoprotein cholesterol (LDLC).

4. Discussion

The results presented herein demonstrate that individually designed exercise, at a relative $AerT_{ge}$, improves fitness and metabolic parameters in untrained, sedentary, and obese

Table 1: Anthropometric measures, lipid profile, and glycated haemoglobin for the intervention group (IG) and the control group (CG) at baseline and after 3-month period.

	IG ($n = 15$)		CG ($n = 15$)	
	T0	T3	T0	T3
Weight (kg)	87.6 ± 19.5	85.0 ± 17.8*	87.0 ± 22.6	87.0 ± 22.1
BMI (kg/m^2)	33.6 ± 7.7	32.6 ± 7.1*	33.0 ± 5.4	32.9 ± 5.3
Fat mass (%)	31.5 ± 10.4	29.5 ± 9.5*	32.5 ± 4.2	31.1 ± 7.1
Abdominal circumference (cm)	117.8 ± 17.5	115.6 ± 15.9*	107.0 ± 12.2	105.4 ± 12.8
Glycated haemoglobin (mmol/mol)	49.9 ± 8.3	45.3 ± 7.3*	53.0 ± 12.2	45.0 ± 5.6*
Total cholesterol (mg/dL)	210.6 ± 57.9	217.1 ± 56.9	163.8 ± 33.1	162.0 ± 37.6
HDLC (mg/dL)	46.6 ± 16.2	48.7 ± 13.2	43.8 ± 12.9	46.7 ± 13.0
LDLC (mg/dL)	122.9 ± 51.6	136.3 ± 40.1	96.0 ± 51.2	99.3 ± 22.6

BMI: body mass index; HDLC: high density lipoprotein cholesterol; LDLC: low density lipoprotein cholesterol.
* $P < 0.05$ versus T0.

Table 2: Physiological parameters for the intervention group (IG) and the control group (CG) at baseline and after 3-month period.

	IG ($n = 15$)		CG ($n = 15$)	
	T0	T3	T0	T3
$\dot{V}O_{2peak}$ (ml·kg^{-1} min^{-1})	15.9 ± 3.0	18.5 ± 3.2*	18.6 ± 4.2	17.9 ± 5.7
MET_{peak}	4.5 ± 0.8	5.3 ± 1*	5.3 ± 1.2	5.1 ± 1.6
WR_{peak} (W)	65.7 ± 25.9	78.6 ± 26.1*	96.0 ± 23.0	88.3 ± 20.0
$\%HR_{max}$ (%)	77.1 ± 9.5	77.9 ± 7.9	79.0 ± 5.8	80.3 ± 7.5
$\%\dot{V}O_{2peak}$ at $AerT_{ge}$ (%)	57.7 ± 11.9	51.7 ± 11.6	58.1 ± 12.1	59.9 ± 16.5
$\%HR_{max}$ at $AerT_{ge}$ (%)	57.7 ± 8.3	56.1 ± 8.0	60.0 ± 7.6	60.0 ± 6.7
%HRR at $AerT_{ge}$ (%)	24.4 ± 12.2	21.6 ± 9.8	18.9 ± 4.7	18.4 ± 7.2
WR at $AerT_{ge}$ (w)	20.4 ± 4.7	28.0 ± 8.2*	20.8 ± 9.5	23.3 ± 0.3
ΔHR (bpm)	21.5 ± 11.6	18.9 ± 9.5	14.2 ± 3.9	14.3 ± 7.7
MET at $AerT_{ge}$	2.6 ± 0.6	2.6 ± 0.6	3.1 ± 0.6	2.9 ± 0.6

$\dot{V}O_{2peak}$: peak oxygen uptake; MET: metabolic equivalent; WR_{peak}: peak work rate.
HR_{max}: maximum heart rate; HRR: heart rate reserve; ΔHR: heart rate at $AerT_{ge}$-heart rate at rest.
* $P < 0.05$ versus T0.

diabetic subjects. These positive effects were controlled using baseline glycated haemoglobin as covariate.

Indeed, the first aim of this study was to determine the effects of three months of aerobic exercise training, based on heart rate at aerobic threshold, on long term glycaemic control, body composition, and exercise capacity in OT2DM. In our study, all type 2 diabetic subjects were obese and they did not practice any organized physical activity prior to this study. Moreover, subjects' fitness parameters, such as $\dot{V}O_{2peak}$, showed that they were very unfit. According to these observations, we chose not to use high-intensity exercise but a constant moderate intensity exercise as training method. American College of Sports Medicine PA guidelines for type 2 diabetic subjects recommend to perform low-to-moderate intensity physical activity (at 40–70% $\dot{V}O_{2\,max}$) to achieve cardiorespiratory and metabolic improvements. Most importantly, the lower intensity activity affords a more comfortable level of exertion and enhances the likelihood of adherence, while lessening the likelihood of musculoskeletal injury and foot trauma, particularly when weight-bearing activity is recommended [34]. At present, American College of Sports Medicine suggests using three variables to monitor exercise intensity: $\dot{V}O_{2\,max}$, HR, and rate of perceived exertion (RPE). Moreover, during the last years, the gas exchange threshold was identified as a valid tool to delineate the "training zone" for endurance training [14] and for unhealthy subjects [12].

In our exercise protocol, subjects performed 30 min aerobic exercise at an intensity corresponding to their $AerT_{ge}$. In detail, the heart rate determined at $AerT_{ge}$ was kept constant while the external work load, such as treadmill speed or cycle ergometer watt, decreased in accordance with HR. Kindermann et al. [20] studied the physiological responses of a constant exercise performed at a heart rate corresponding to the anaerobic threshold (4 mmol/L). In accordance with our results they showed that treadmill speed must be reduced continuously to maintain the HR constant. In our study, subjects who exercised on a cycle ergometer finished the aerobic program with a work load lower than that set at the beginning of training (decrement range 5–20 watts). In accordance, subjects who trained on a treadmill finished the aerobic program with a lower inclination than that at the beginning of training (decrement range 1–4°). The decrease in external work load was chosen to allow subjects to perform all

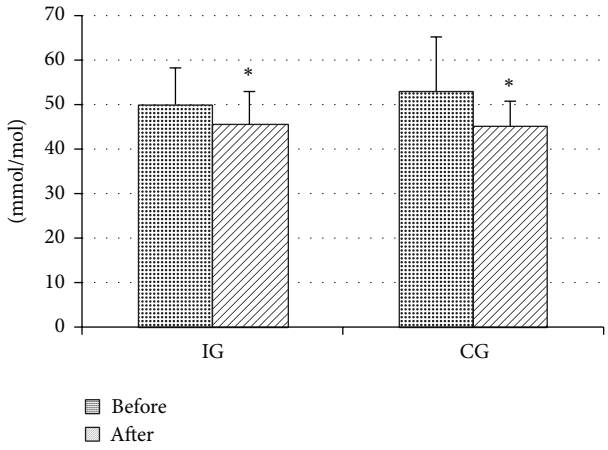

Figure 1: Glycated hemoglobin (HbA1c) before and after 3-month intervention period in control (CG) and intervention groups (IG).

30 min aerobic training because we originally noticed that our obese elderly type 2 diabetic patients were not able to exercise for 30 min at a constant external work load corresponding to $AerT_{ge}$.

It is well known that aerobic training associated with resistance exercise might improve glycemic control to an extent comparable to some oral hypoglycemic agents [35, 36]. The reduction of blood glucose in type 2 diabetic subjects could be reached by either exercising at constant moderate intensity or performing brief high-intensity exercise [15, 37]. Moreover, no data are available regarding the effects of aerobic training based upon constant heart rate at $AerT_{ge}$ on glycemic control and physiological parameters in diabetes subjects. The reduction in glycated haemoglobin observed in IG (−9.2%) was similar to that found in other studies. For example, Belli et al. [15] found that after a 12-week supervised walking training at $AerT_{ge}$, glycated haemoglobin decreased by 11,6%. Moreover, other two studies, Walker et al. [38] and Shenoy et al. [39], showed a decrease of glycated haemoglobin ranging from 7.6% to 9.7%, respectively. Contrary to our results, Morton et al. [23] showed no effects on glycated haemoglobin after 7 weeks of heart rate prescribed walking training. The difference between our results and those reported by Morton could be explained by the different duration of the two intervention periods. In fact, as suggested by Kilpatrick [40], glycation of haemoglobin is dependent on mean blood glucose concentrations over the 120-day lifespan of red blood cells. The lower glycated haemoglobin in CG at the end of the study than at baseline could be explained by the fact that all subjects were recruited from local health facilities and they were under pharmacological treatment since the beginning of the study. Therefore, our training protocol did not modify the positive effect on glycated haemoglobin of pharmacological treatment that was equal for both groups.

No significant differences were found in high density lipoprotein cholesterol (HDLC) and low density lipoprotein cholesterol (LDLC) levels in both groups after the training period. This was an unexpected result considering that all subjects received a low-calorie diet at baseline and that the positive effects of diet on lipid profile and weight are well known [41]. Clearly, dieting strategies provide benefits only in complying patients [42]. Indeed, a restricted diet was given at baseline to all subjects, but it was not possible to evaluate adherence to nutritional protocol throughout the study, which might lead to the hypothesis that subjects were not fully compliant to the diet intervention, as also suggested by the small, but significant decrease in body weight, fat mass, and abdominal circumference in IG group.

These results could be explained by the positive effects of physical exercise on energy expenditure. In fact, even if diet adherence was not controlled, IG performed a supervised physical training protocol leading to an energy expenditure increase during the study period.

To our knowledge the aerobic training of the subjects of our study could not be compared with any other study in the literature. In fact, to allow participants to perform a 30 min aerobic exercise we chose to maintain constant the heart rate corresponding to $AerT_{ge}$ while the external work load decreased automatically. This method was chosen for two main reasons: firstly, untrained obese older T2DM subjects were very unfit and therefore they could not perform 30 min exercise at the constant work load corresponding to $AerT_{ge}$; secondly, the right balance between exercise duration and intensity is a challenging task, since both factors have the potential to negatively impact adherence. PA intensity has been found to be negatively related to adherence in several studies involving overweight participants [43–45]. In fact, on average, overweight individuals choose an intensity below or around the $AerT_{ge}$ during a 20 min bout exercise [22]. Self-paced exercise was reported to fall within the zone of fat maximal oxidation [45] and, when performed over ground, resulted in lower perceived exertion and more positive affective responses than on treadmill [7].

Our results showed that 30 min aerobic exercise based upon HR corresponding to $AerT_{ge}$ could improve physical exercise capacity in obese T2DM subjects as demonstrated by $\dot{V}O_{2peak}$, MET_{peak}, and WR_{peak} increase after the training period in IG. These findings are in agreement with previous studies even if a different methodology of exercise prescription was applied due to the different characteristics of patients (obese and elderly). For instance, it is well known that when exercise is prescribed referring to absolute parameters (velocity or watt) corresponding to $AerT_{ge}$, exercise capacity can improve in obese adult T2DM [15, 38]. In addition, Morton et al. showed that walking at a heart rate of 80% HR_{peak}, type 2 diabetic subjects improved peak and submaximal cardiorespiratory responses. However, the $AerT_{ge}$ of our subjects was lower than the patients evaluated in other studies (80% HR_{peak}). Our positive results support the hypothesis that the lower exercise intensity was balanced by the longer training period used in our protocol.

Moreover the workload corresponding to $AerT_{ge}$ improved after training. In support of this, also Larose et al. [46] observed that workload at $AerT_{ge}$ increased while $\%\dot{V}O_{2peak}$ at $AerT_{ge}$ remained unchanged after 6 months of walking and cycling exercise at 60–75% HR_{max}

for 25–45 min, three times a week in adult type 2 diabetic subjects.

In conclusion, a 3-month aerobic exercise training based on HR, corresponding to subjects' $AerT_{ge}$, improved maximal exercise capacity and had positive effects on glycated haemoglobin levels. Thus, the prescription of exercise intensity PA, according to $AerT_{ge}$, should become more frequent in obese diabetic populations, since training at this intensity improves aerobic capacity, cost effectiveness of treatment and could also increase adherence to physical activity in obese subjects.

Conflict of Interests

The authors declare no conflict of interests.

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References

[1] J. E. Donnelly, S. N. Blair, J. M. Jakicic, M. M. Manore, J. W. Rankin, and B. K. Smith, "Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults," *Medicine and Science in Sports and Exercise*, vol. 41, no. 2, pp. 459–471, 2009.

[2] C. E. Garber, B. Blissmer, M. R. Deschenes et al., "Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: Guidance for prescribing exercise," *Medicine and Science in Sports and Exercise*, vol. 43, no. 7, pp. 1334–1359, 2011.

[3] L. M. León-Muñoz, D. Martínez-Gómez, T. Balboa-Castillo, E. López-García, P. Guallar-Castillón, and F. Rodríguez-Artalejo, "Continued sedentariness, change in sitting time, and mortality in older adults," *Medicine and Science in Sports and Exercise*, vol. 45, no. 8, pp. 1501–1507, 2013.

[4] Q.-L. Xue, K. Bandeen-Roche, T. J. Mielenz et al., "Patterns of 12-year change in physical activity levels in community-dwelling older women: can modest levels of physical activity help older women live longer?" *American Journal of Epidemiology*, vol. 176, no. 6, pp. 534–543, 2012.

[5] F. W. Booth and S. J. Lees, "Fundamental questions about genes, inactivity, and chronic diseases," *Physiological Genomics*, vol. 28, no. 2, pp. 146–157, 2007.

[6] G. A. Colditz, "Economic costs of obesity and inactivity," *Medicine and Science in Sports and Exercise*, vol. 31, no. 11, pp. S663–S667, 1999.

[7] S. G. Dasilva, L. Guidetti, C. F. Buzzachera et al., "Psychophysiological responses to self-paced treadmill and overground exercise," *Medicine and Science in Sports and Exercise*, vol. 43, no. 6, pp. 1114–1124, 2011.

[8] P. Ekkekakis, G. Parfitt, and S. J. Petruzzello, "The pleasure and displeasure people feel when they exercise at different intensities: decennial update and progress towards a tripartite rationale for exercise intensity prescription," *Sports Medicine*, vol. 41, no. 8, pp. 641–671, 2011.

[9] E. Lind, P. Ekkekakis, and S. Vazou, "The affective impact of exercise intensity that slightly exceeds the preferred level: 'Pain' for no additional 'gain'," *Journal of Health Psychology*, vol. 13, no. 4, pp. 464–468, 2008.

[10] R. K. Dishman and J. Buckworth, "Increasing physical activity: a quantitative synthesis," *Medicine and Science in Sports and Exercise*, vol. 28, no. 6, pp. 706–719, 1996.

[11] B. M. Pinet, D. Prud'homme, C. A. Gallant, and P. Boulay, "Exercise intensity prescription in obese individuals," *Obesity*, vol. 16, no. 9, pp. 2088–2095, 2008.

[12] G. P. Emerenziani, S. Migliaccio, M. C. Gallotta et al., "Physical exercise intensity prescription to improve health and fitness in overweight and obese subjects: a review of the literature," *Health*, vol. 5, no. 6, pp. 113–121, 2013.

[13] T. Meyer, H. H. W. Gabriel, and W. Kindermann, "Is determination of exercise intensities as percentages of V̇O2 max or HR(max) adequate?" *Medicine and Science in Sports and Exercise*, vol. 31, no. 9, pp. 1342–1345, 1999.

[14] T. Meyer, A. Lucía, C. P. Earnest, and W. Kindermann, "A conceptual framework for performance diagnosis and training prescription from submaximal gas exchange parameters—theory and application," *International Journal of Sports Medicine*, vol. 26, no. 1, pp. S38–S48, 2005.

[15] T. Belli, L. F. P. Ribeiro, M. A. Ackermann, V. Baldissera, C. A. Gobatto, and R. Galdino da Silva, "Effects of 12-week overground walking training at ventilatory threshold velocity in type 2 diabetic women," *Diabetes Research and Clinical Practice*, vol. 93, no. 3, pp. 337–343, 2011.

[16] J.-F. Brun, S. Bordenave, J. Mercier, A. Jaussent, M.-C. Picot, and C. Préfaut, "Cost-sparing effect of twice-weekly targeted endurance training in type 2 diabetics: a one-year controlled randomized trial," *Diabetes & Metabolism*, vol. 34, no. 3, pp. 258–265, 2008.

[17] W. Hollmann, "42 Years ago—development of the concepts of ventilatory and lactate threshold," *Sports Medicine*, vol. 31, no. 5, pp. 315–320, 2001.

[18] T. G. Babb, B. L. Wyrick, P. J. Chase et al., "Weight loss via diet and exercise improves exercise breathing mechanics in obese men," *Chest*, vol. 140, no. 2, pp. 454–460, 2011.

[19] N. Miyatake, H. Nishikawa, A. Morishita et al., "Evaluation of exercise prescription for hypertensive obese men by ventilatory threshold," *Journal of the Chinese Medical Association*, vol. 66, no. 10, pp. 572–578, 2003.

[20] W. Kindermann, G. Simon, and J. Keul, "The significance of the aerobic-anaerobic transition for the determination of work load intensities during endurance training," *European Journal*

of Applied Physiology and Occupational Physiology, vol. 42, no. 1, pp. 25–34, 1979.

[21] R. P. Kim, S. V. Edelman, and D. D. Kim, "Musculoskeletal complications of diabetes mellitus," *Clinical Diabetes*, vol. 19, no. 7, pp. 132–135, 2001.

[22] S. G. DaSilva, L. Guidetti, C. F. Buzzachera et al., "The influence of adiposity on physiological, perceptual, and affective responses during walking at a self-selected pace," *Perceptual and Motor Skills*, vol. 109, no. 1, pp. 41–60, 2009.

[23] R. D. Morton, D. J. West, J. W. Stephens, S. C. Bain, and R. M. Bracken, "Heart rate prescribed walking training improves cardiorespiratory fitness but not glycaemic control in people with type 2 diabetes," *Journal of Sports Sciences*, vol. 28, no. 1, pp. 93–99, 2010.

[24] WHO Document Production Services, *Report of a WHO/IDF Consultation Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia*, WHO Document Production Services, Geneva, Switzerland, 2006.

[25] T. Sofková, M. Přidalová, J. Mitáš, and J. Pelclová, "The level of neighborhood walkability in a place of residence and its effect on body composition in obese and overweight women," *Central European Journal of Public Health*, vol. 21, no. 4, pp. 184–189, 2013.

[26] R. S. Guerra, T. F. Amaral, E. A. Marques, J. Mota, and M. T. Restivo, "Anatomical location for waist circumference measurement in older adults: a preliminary study," *Nutricion Hospitalaria*, vol. 27, no. 5, pp. 1554–1561, 2012.

[27] B. Balke and R. W. Ware, "An experimental study of physical fitness of Air Force personnel," *United States Armed Forces Medical Journal*, vol. 10, no. 6, pp. 675–688, 1959.

[28] S. E. Selig, I. Levinger, A. D. Williams et al., "Exercise & Sports Science Australia Position Statement on exercise training and chronic heart failure," *Journal of Science and Medicine in Sport*, vol. 13, no. 3, pp. 288–294, 2010.

[29] J. M. Lee, D. R. Bassett Jr., D. L. Thompson, and E. C. Fitzugh, "Validation of the cosmed fitmate for prediction of maximal oxygen consumption," *Journal of Strength and Conditioning Research*, vol. 25, no. 9, pp. 2573–2579, 2011.

[30] R. D. Hagan and M. G. Smith, "Pulmonary ventilation in relation to oxygen uptake and carbon dioxide production during incremental load work," *International Journal of Sports Medicine*, vol. 5, no. 4, pp. 193–197, 1984.

[31] C. Baldari and L. Guidetti, "VO_{2max}, ventilatory and anaerobic thresholds in rhythmic gymnasts and young female dancers," *Journal of Sports Medicine and Physical Fitness*, vol. 41, no. 2, pp. 177–182, 2001.

[32] E. L. Rosado, R. S. de Brito, J. Bressan, and J. A. M. Hernández, "Effectiveness of prediction equations in estimating energy expenditure sample of Brazilian and Spanish women with excess body weight," *Nutricion Hospitalaria*, vol. 29, no. 3, pp. 513–518, 2014.

[33] B. E. Ainsworth, J. Bassett D.R., S. J. Strath et al., "Comparison of three methods for measuring the time spent in physical activity," *Medicine and Science in Sports and Exercise*, vol. 32, no. 9, pp. S457–S464, 2000.

[34] N. Gordon, "The exercise prescription," in *The Health Professional's Guide to Diabetes and Exercise*, pp. 71–82, American Diabetes Association, Alexandria, Va, USA, 1995.

[35] D. Umpierre, P. A. B. Ribeiro, C. K. Kramer et al., "Physical activity advice only or structured exercise training and association with HbA1c levels in type 2 diabetes: a systematic review and meta-analysis," *JAMA—Journal of the American Medical Association*, vol. 305, no. 17, pp. 1790–1799, 2011.

[36] N. G. Boulé, C. Robert, G. J. Bell et al., "Metformin and exercise in type 2 diabetes: examining treatment modality interactions," *Diabetes Care*, vol. 34, no. 7, pp. 1469–1474, 2011.

[37] O. P. Adams, "The impact of brief high-intensity exercise on blood glucose levels," *Diabetes, Metabolic Syndrome and Obesity*, vol. 6, no. 6, pp. 113–122, 2013.

[38] K. Z. Walker, L. S. Piers, R. S. Putt, J. A. Jones, and K. O'Dea, "Effects of regular walking on cardiovascular risk factors and body composition in normoglycemic women and women with type 2 diabetes," *Diabetes Care*, vol. 22, no. 4, pp. 555–561, 1999.

[39] S. Shenoy, R. Guglani, and J. S. Sandhu, "Effectiveness of an aerobic walking program using heart rate monitor and pedometer on the parameters of diabetes control in Asian Indians with type 2 diabetes," *Primary Care Diabetes*, vol. 4, no. 1, pp. 41–45, 2010.

[40] E. S. Kilpatrick, "Haemoglobin A_{1c} in the diagnosis and monitoring of diabetes mellitus," *Journal of Clinical Pathology*, vol. 61, no. 9, pp. 977–982, 2008.

[41] A. Makris and G. D. Foster, "Dietary approaches to the treatment of obesity," *Psychiatric Clinics of North America*, vol. 34, no. 4, pp. 813–827, 2011.

[42] M. L. Dansinger, J. A. Gleason, J. L. Griffith, H. P. Selker, and E. J. Schaefer, "Comparison of the Atkins, Ornish, Weight Watchers, and Zone Diets for weight loss and heart disease risk reduction: a randomized trial," *Journal of the American Medical Association*, vol. 293, no. 1, pp. 43–53, 2005.

[43] J. M. Jakicic, B. H. Marcus, K. I. Gallagher, M. Napolitano, and W. Lang, "Effect of exercise duration and intensity on weight loss in overweight, sedentary women: a randomized trial," *Journal of the American Medical Association*, vol. 290, no. 10, pp. 1323–1330, 2003.

[44] P. Ekkekakis and E. Lind, "Exercise does not feel the same when you are overweight: the impact of self-selected and imposed intensity on affect and exertion," *International Journal of Obesity*, vol. 30, no. 4, pp. 652–660, 2006.

[45] S. G. Dasilva, L. Guidetti, C. F. Buzzachera et al., "Gender-based differences in substrate use during exercise at a self-selected pace," *Journal of Strength and Conditioning Research*, vol. 25, no. 9, pp. 2544–2551, 2011.

[46] J. Larose, R. J. Sigal, N. G. Boulé et al., "Effect of exercise training on physical fitness in type II diabetes mellitus," *Medicine and Science in Sports and Exercise*, vol. 42, no. 8, pp. 1439–1447, 2010.

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Research Article

Resistance to the Beneficial Metabolic Effects and Hepatic Antioxidant Defense Actions of Fibroblast Growth Factor 21 Treatment in Growth Hormone-Overexpressing Transgenic Mice

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Fibroblast growth factor 21 (FGF21) modulates a diverse range of biological functions, including glucose and lipid metabolism, adaptive starvation response, and energy homeostasis, but with limited mechanistic insight. FGF21 treatment has been shown to inhibit hepatic growth hormone (GH) intracellular signaling. To evaluate GH axis involvement in FGF21 actions, transgenic mice overexpressing bovine GH were used. Expectedly, in response to FGF21 treatment control littermates showed metabolic improvements whereas GH transgenic mice resisted most of the beneficial effects of FGF21, except an attenuation of the innate hyperinsulinemia. Since FGF21 is believed to exert its effects mostly at the transcriptional level, we analyzed and observed significant upregulation in expression of various genes involved in carbohydrate and lipid metabolism, energy homeostasis, and antioxidant defense in FGF21-treated controls, but not in GH transgenics. The resistance of GH transgenic mice to FGF21-induced changes underlines the necessity of normal GH signaling for the beneficial effects of FGF21.

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) worldwide was estimated to be 170 million people in 2000, and this figure is expected to increase to more than 360 million by 2030 and impose a huge public health burden [1]. The recently discovered metabolic regulator fibroblast growth factor 21 (FGF21) has been shown to exert profound antidiabetic and triglyceride-lowering effects in rodent models of diabetes and obesity, as well as in diabetic rhesus monkeys [2–4].

FGF21, a member of the FGF19 subfamily that also includes FGF19 and FGF23, lacks the conventional FGF heparin-binding domain yet exerts systemic, hormone-like effects. There is evidence that FGF21 initiates its action by interacting with a dual receptor complex of β-klotho and fibroblast growth factor receptor (FGFR), activating the tyrosine-kinase activity of the FGFR. The expression of both FGF21 [5, 6] and its coreceptor, β-klotho [7], has been demonstrated in metabolically relevant tissues such as liver, pancreas, and white adipose tissue. In deciphering

Table 1: Body weight and plasma constituent parameters in control and GH transgenic mice.

Parameters	Normal		Transgenic	
	PBS	FGF21	PBS	FGF21
Body weight (g)	29.9 ± 2.3^{a}	29.8 ± 1.0^{a}	55.3 ± 0.8^{b}	51.2 ± 4.0^{b}
Fasting glucose (mg/dl)	143.25 ± 11.20^{a}	137.75 ± 11.44^{a}	117.00 ± 16.37^{b}	140.50 ± 4.79^{a}
Insulin (ng/ml)	0.86 ± 0.32^{a}	0.97 ± 0.16^{a}	2.56 ± 0.80^{b}	1.00 ± 0.25^{a}
β-Hydroxybutyrate (mmol/l)	0.43 ± 0.03	0.47 ± 0.02	0.37 ± 0.02	0.37 ± 0.02
NEFA (mmol/l)	0.51 ± 0.07^{a}	0.35 ± 0.04^{b}	$0.57 \pm 0.04^{a,c}$	0.69 ± 0.01^{c}
Triglycerides (mmol/l)	1.21 ± 0.09^{a}	0.79 ± 0.22^{b}	0.98 ± 0.13^{a}	1.14 ± 0.10^{a}
Total cholesterol (mg/dl)	54.47 ± 5.44^{a}	36.46 ± 2.79^{b}	119.15 ± 5.79^{c}	110.40 ± 3.63^{c}
IGF-1 (ng/ml)	238.33 ± 7.93^{a}	130.00 ± 21.55^{a}	1112.50 ± 77.67^{b}	933.33 ± 124.39^{c}

Data is expressed as mean ± SD for $n = 5$ in each group. Different superscripts denote significant difference at $P < 0.05$.

the mechanistic basis for the observed effects of FGF21 *in vivo*, several molecules and their corresponding pathways have been proposed as key players [8–10]. In fact, FGF21 has been shown to transduce its signal in a typical FGF manner by stimulating FGF receptor substrate (FRS2α) phosphorylation and activating ERK1/2 and Akt signaling pathways. Lately, it has been suggested that there is crosstalk between FGF21 and growth hormone (GH) signaling and that FGF21 can cause a state of GH resistance [11]. FGF21 overexpressing mice are reported to have elevated levels of GH and decreased levels of insulin-like growth factor-1 (IGF-1) in circulation [11]. The authors proposed that FGF21 causes GH resistance by reducing hepatic concentrations of the active form of signal transducer and activator of transcription 5 (STAT5), a major mediator of GH actions, and downregulating the expression of its target genes, including IGF-1.

GH is widely known to exert anti-insulin or diabetogenic effects on carbohydrate and lipid metabolism [12]. Hyperinsulinemia is a common feature associated with GH excess in GH overexpressing transgenic mice [13, 14] and in humans with acromegaly who often progress from GH-mediated insulin resistance to overt diabetes [15], but the cellular mechanisms underlying this form of insulin resistance remain enigmatic. While there is evidence from transgenic mouse models to show that GH excess leads to chronic activation of the IR/IRS-1/PI3K pathway, thereby reducing the extent of insulin-induced activation and resulting in decreased insulin-induced activation of key proteins including glycogen synthase [14, 16], we recently demonstrated that glucose tolerance and insulin sensitivity in transgenic mice that overexpress the bovine GH (bGH) gene are not impaired and are actually somewhat enhanced [17]. The aim of the present study was to investigate the effects of FGF21 treatment in GH overexpressing transgenic mice and to determine if FGF21 administration can rescue the hyperinsulinemic phenotype of these mice. Moreover, it has been reported that high continuous GH levels *in vivo* produce desensitization of the JAK2/STAT5 pathway of GH signaling in the liver of GH overexpressing mice [18]. We expected that as FGF21 and GH share similar signaling pathways, alterations in some of the GH signaling mediators that are involved in FGF21 signaling would hamper FGF21 action as a consequence of signaling crosstalk.

2. Results

2.1. Anatomical and Physiological Characteristics. Body weight and blood constituent parameters are summarized in Table 1. Expectedly, the body weights of GH overexpressing mice were considerably greater than their control littermates (29.9 ± 2.3 g versus 55.3 ± 0.78 g; $P < 0.001$). However, when comparing body weights before and after FGF21 treatment in chow-fed lean mice, we failed to observe a weight-lowering effect of FGF21 in mice of either phenotype. Previously, a dose dependent weight reduction effect of FGF21 has been reported in diet-induced obese mice and *ob/ob* mice [4, 19]. Moreover, FGF21 did not alter food consumption in mice of either genotype (data not shown). While a 3-fold higher concentration of insulin was observed in the GH transgenics relative to littermate controls ($P < 0.001$), fasted plasma glucose levels were only modestly ($P = 0.034$) lower in the GH transgenic mice relative to their control littermates. FGF21 treatment did not affect fasted blood glucose levels in either group of animals; however, it was able to alleviate the hyperinsulinemia in the GH transgenic mice (PBS 2.56 ± 0.80 ng/mL and FGF21 1.00 ± 0.25 ng/mL; $P < 0.005$) without causing significant change in insulin levels in normal mice (PBS 0.86 ± 0.32 ng/mL and FGF21 0.97 ± 0.16 ng/mL). Moreover, we determined the levels of β-hydroxybutyrate, a ketone body that is produced by the liver and serves as an alternative energy substrate peripherally when glucose is in short supply. However, as seen in Table 1, β-hydroxybutyrate in blood of overnight-fasted mice did not differ significantly as an effect of either genotype or treatment. In agreement with previous reports [4, 20], concentrations of plasma triglycerides and circulating NEFAs were lowered in FGF21-treated littermate control mice ($P = 0.007$ and $P = 0.044$, resp.); nonetheless, no effect was observed in the GH transgenics (Table 1).

The bovine GH overexpressing transgenics are hypercholesterolemic ($P < 0.001$) relative to control littermates, and while FGF21 treatment resulted in a modest but significant ($P < 0.01$) reduction in total cholesterol levels in control littermates, the lipid-lowering benefits of FGF21 seen in control mice did not extend to the bGH mice (Tg-PBS 119.15 ± 5.79 mg/dL and Tg-FGF21 110.40 ± 3.63 mg/dL). We also examined plasma IGF-1 levels in the mice under study and

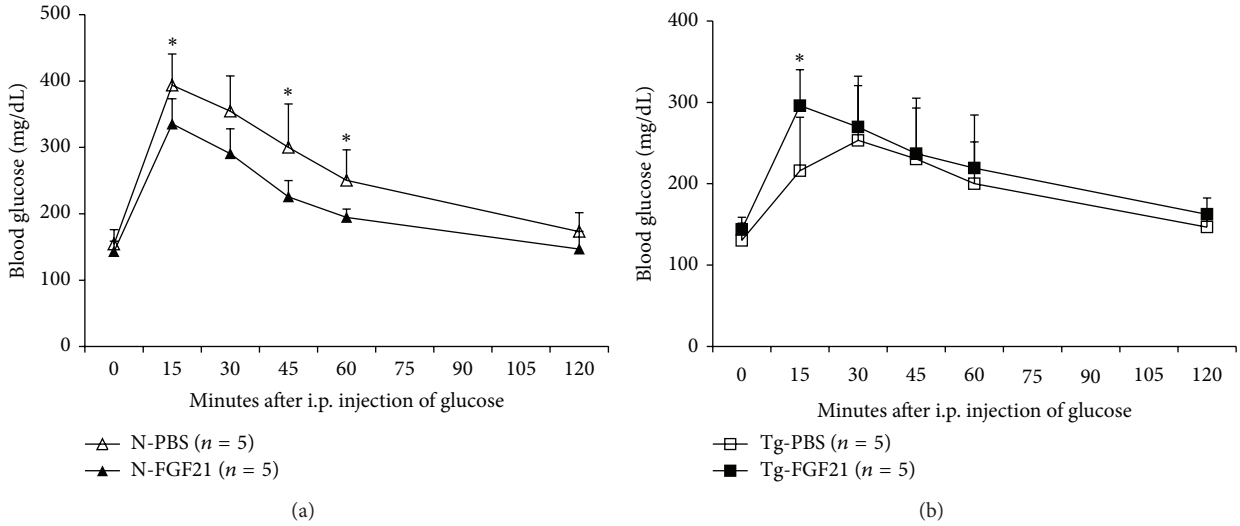

Figure 1: FGF21 improves glucose tolerance in normal mice. (a) Glucose tolerance testing in normal mice. (b) Glucose tolerance testing in bGH transgenic mice. Glucose levels were measured at indicated times after i.p. injection with a bolus of glucose (2 g/kg body weight). Data are mean ± SD. $P < 0.05$ versus vehicle-treated group (2-tailed, unpaired, homoscedastic Student's t-test).

(expectedly) found a 5-fold higher concentration of IGF-1 in circulation of mice with GH excess ($P < 0.001$). While FGF21 treatment tended to cause a numerical reduction in circulating IGF-1 levels in control littermates ($P = 0.061$), it resulted in a significant ($P = 0.032$) lowering of the elevated IGF-1 levels in the GH transgenic mice (Table 1). In addition, hepatic gene expression analysis showed that FGF21 treatment resulted in significant downregulation of IGF-1 expression in normal mice (N-PBS 1.00 ± 0.15 and N-FGF21 0.46 ± 0.02; Tg-PBS 3.73 ± 0.58 and Tg-FGF21 4.07 ± 1.12) while enhancing the expression of suppressor of cytokine signaling 2 (SOCS2) in control mice but not in GH transgenic mice (N-PBS 1.00 ± 0.36 and N-FGF21 1.44 ± 0.14; Tg-PBS 6.31 ± 0.55 and Tg-FGF21 4.67 ± 0.88).

2.2. Intraperitoneal Glucose Tolerance Test. Next, we investigated glucose disposal after a glucose challenge in normal and PEPCK-bGH transgenic mice treated with FGF21. As seen in Figure 1(a), FGF21-treated normal mice showed better glucose clearance compared to vehicle-treated animals (N-PBS versus N-FGF21; $P = 0.016$) in an intraperitoneal glucose tolerance test. At the 15-minute time-point, transgenic mice were able to clear glucose from their blood faster than control littermates and as a result showed improved glucose tolerance for the initial phase of response to the glucose challenge ($P = 0.014$). However, at later time points differences in glucose levels between PEPCK-bGH transgenics and their control littermates failed to reach significance. This seems to suggest that the elevated circulating insulin in the GH overexpressing vehicle-treated mice (Table 1) is initially able to clear the glucose load and hence only a modest increase (65%) is seen in the glucose levels of vehicle-treated transgenics at the fifteen-minute time-point in contrast to the more marked increase in blood glucose in the N-PBS group (155%). Further, as mentioned above, FGF21-treated bGH mice manifested a near normalization of the hyperinsulinemia (Table 1), which might explain the greater surge in blood glucose in the Tg-FGF21 group at 15 minutes (Figure 1(b), $P < 0.05$), relative to the Tg-vehicle-treated group, upon an exogenous glucose load. Nonetheless, exogenous FGF21 did not otherwise affect glucose disposal in mice with GH excess at later time points (Figure 1(b)).

2.3. Effects on Hepatic Carbohydrate Metabolism. Since FGF21 is believed to exert its effects through regulation of gene transcription [21] and the liver is the primary target for GH action, we analyzed changes in expression of genes involved in glucose metabolism in hepatic tissues of treated mice. As seen in Table 2, expression of the genes for insulin receptor (IR), insulin receptor substrate 1 (IRS1), and insulin receptor substrate 2 (IRS2) was found to be elevated in livers of littermate control mice treated with FGF21. Conversely, exogenous FGF21 failed to potentiate the expression of these constituents of the early steps of the insulin signaling pathway in livers of GH overexpressing mice (Table 2). In addition, FGF21 may have stimulated the hepatic gluconeogenic pathway, as surmised based upon observed increases in the expression of genes for the gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) in control littermates (Table 2). The expression of hepatocyte nuclear factor 4α (HNF4α), hepatocyte nuclear factor-1α (HNF1α), and peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1α), which are thought to be major mediators of the gluconeogenic process, was also induced by administration of FGF21 in normal mice without altering their expression in the GH transgenic mice (Table 2).

2.4. Effects on Lipid Metabolism. We examined the effects of FGF21 treatment on genes that control β-oxidation as well as fatty acid biosynthesis. The hepatic expression of genes involved in fatty acid oxidation was putatively promoted by

Table 2: FGF21-induced transcriptional changes in hepatic carbohydrate and lipid metabolism.

Gene of interest	N-PBS	N-FGF21	Tg-PBS	Tg-FGF21
		Insulin signaling		
IR	1 ± 0.17^a	4.68 ± 0.91^b	1.37 ± 0.44^a	1.01 ± 0.12^a
IRS-1	1 ± 0.18^a	3.01 ± 0.63^b	0.53 ± 0.09^a	0.82 ± 0.15^a
IRS-2	1 ± 0.14^a	2.03 ± 0.21^b	1.40 ± 0.34^a	1.19 ± 0.22^a
		Gluconeogenesis		
PEPCK	1 ± 0.07^a	1.82 ± 0.21^b	0.64 ± 0.19^a	$0.54 \pm .021^a$
G6Pase	1 ± 0.10^a	2.15 ± 0.33^b	0.59 ± 0.20^a	0.63 ± 0.14^a
HNF4α	1 ± 0.08^a	2.34 ± 0.45^b	0.79 ± 0.10^a	0.79 ± 0.13^a
HNF1α	1 ± 0.16^a	3.54 ± 0.78^b	1.14 ± 0.37^a	1.47 ± 0.44^a
PGC1α	1 ± 0.14^a	2.37 ± 0.65^b	0.68 ± 0.09^a	0.88 ± 0.12^a
		Lipogenesis		
ACC	1 ± 0.12^a	1.75 ± 0.30^b	2.14 ± 0.36^b	2.22 ± 0.40^b
FASN	1 ± 0.15^a	1.94 ± 0.32^b	1.69 ± 0.31^b	1.54 ± 0.26^b
		Fatty acid oxidation		
CPT1α	1 ± 0.19^a	2.62 ± 0.35^b	0.36 ± 0.11^c	0.51 ± 0.09^c
ACOX1	1 ± 0.11^a	2.48 ± 0.28^b	0.49 ± 0.10^c	0.46 ± 0.08^c
UCP2	1 ± 0.13^a	1.90 ± 0.37^b	4.15 ± 1.01^c	4.33 ± 0.88^c
AMPK	1 ± 0.09^a	2.25 ± 0.52^b	0.84 ± 0.12^a	0.95 ± 0.10^a

Shown is RT-PCR analysis of gene expression in normal and GH transgenic mice treated with FGF21 and vehicle (mean ± SEM, $n = 5$). Different superscripts denote significant difference at $P < 0.05$.

FGF21 administration, as indicated by the marked increases in expression of genes involved in β-oxidation (Table 2). Littermate controls treated with FGF21 showed significantly increased expression of acyl-CoA oxidase 1 (ACOX1) and carnitine palmitoyltransferase 1α (CPT1α) relative to N-PBS. While bGH mice showed significantly lower mRNA levels for both ACOX1 and CPT1α compared to littermate controls consistent with previously published data [22], FGF21-mediated induction of genes related to hepatic fatty acid oxidation was blunted in these mice as evidenced by a lack of response for both ACOX1 and CPT1α in FGF21-treated bGH transgenic mice (Table 2). FGF21 had significant effects on the levels of transcripts for enzymes and transcription factors involved in the regulation of lipid metabolism. Intriguingly, it also induced expression of the transcripts for key enzymes of *de novo* lipogenesis, namely, fatty acid synthase (FASN) and acetyl CoA carboxylase (ACC), in control mice but not in GH overexpressing mice which showed baseline upregulation of the transcripts for these lipogenic enzymes (Table 2). Although investigation of corresponding enzyme activities was beyond the scope of this study, the gene expression data from our study nonetheless suggests that, in keeping with the role of FGF21 in potentiating hepatic fatty acid metabolism, it mediates parallel induction of genes related to both the lipogenic and the lipolytic pathways and hence accelerates the turnover of lipids in hepatic tissue.

We also studied the evidence for FGF21-mediated effects on uncoupling of oxidative phosphorylation as measured by changes in the mRNA for uncoupling protein 2 (UCP2), one of the mitochondrial uncoupling proteins thought to play a role in nonshivering thermogenesis and the control of mitochondria-derived reactive oxygen species (ROS). Higher expression of UCP2 was observed in littermate controls upon treatment with FGF21. While mice with GH excess showed a dramatic induction in hepatic UCP2 expression with respect to controls, exogenous FGF21 failed to alter UCP2 expression in transgenic livers (Table 2). Since 5′ adenosine monophosphate- (AMP-) activated protein kinase (AMPK) is known to be a major regulator of cellular energy homeostasis, we were interested in whether any of the observed changes in response to FGF21 administration may be mediated by AMPK. As seen in Table 2, FGF21 treatment induced AMPK expression in hepatic tissue of control littermates relative to the PBS treated controls without any effect in GH overexpressing mice.

2.5. FGF21 Effects on Hypothalamic Gene Expression. In view of the published evidence about the possible stimulatory effects of FGF21 on food intake, we profiled changes in the gene expression of neuropeptides involved in the control of satiety and hunger in response to FGF21 treatment. While there are some reports about increased food intake when being normalized by body weight in FGF21-treated animals and in FGF21 transgenic mice [4, 11, 19, 21], as previously mentioned, in the present study we did not observe changes in food intake in response to FGF21 administration. The hypothalamus is the site where peripheral signals and neural pathways interact to centrally regulate appetite and body weight [23]. To determine FGF21-mediated effects on central control of feeding, we looked at the mRNA levels of key hypothalamic neuropeptides such as the orexigenic neuropeptide Y (NPY), agouti-gene-related peptide (AgRP), and the anorexigenic proopiomelanocortin (POMC) in FGF21-dosed mice. As seen in Table 3, we did not observe any changes in the transcripts for any of these neuropeptides in either the control littermates or the bGH mice as a result of

Table 3: FGF21-induced effects on genes for hypothalamic neuropeptides.

Gene of interest	N-PBS	N-FGF21	Tg-PBS	Tg-FGF21
AgRP	1 ± 0.11	1.17 ± 0.08	0.93 ± 0.21	0.86 ± 0.10
POMC	1 ± 0.17	1.22 ± 0.09	1.04 ± 0.09	1.32 ± 0.14
CART	1 ± 0.01	1.01 ± 0.08	0.92 ± 0.11	1.08 ± 0.03
NPY	1 ± 0.06	1.04 ± 0.02	1.14 ± 0.13	1.17 ± 0.09
MCH	1 ± 0.12	0.95 ± 0.08	1.12 ± 0.04	1.26 ± 0.09
Orexin	1 ± 0.10	1.17 ± 0.12	1.09 ± 0.05	1.21 ± 0.14
LEPR	1 ± 0.12	1.21 ± 0.07	1.49 ± 0.29	1.77 ± 0.33

Shown is RT-PCR analysis of gene expression in normal and GH transgenic mice treated with FGF21 and vehicle (mean ± SEM, $n = 5$).

FGF21 administration. We also determined transcriptional changes in additional neuromodulators of feeding behavior, namely, cocaine- and amphetamine-regulated transcript (CART), orexin, leptin receptor (LEPR), and melanin concentrating hormone (MCH) and failed to observe alterations in their transcripts in response to FGF21 administration.

2.6. FGF21 Effects on Antioxidant Defenses. Since superoxide dismutase 2 (SOD2) is a gene whose expression is regulated by insulin/IGF-1 signaling through the O family of Forkhead transcription factors (FoxO), we determined its expression in hepatic tissue of FGF21-treated mice. As seen in Figure 2, FGF21 treatment induced the expression of the gene for SOD2 in littermate control mice but not in bGH transgenic mice. Since FGF21 altered the expression of SOD2, we were interested in the supplementary effects of this hormone on antioxidant status and therefore we assessed the expression of the genes for catalase (CAT) and glutathione peroxidase (GPX1). As was the case for SOD2, FGF21 administration resulted in parallel induction for CAT and GPX1 in normal mice without significantly altering expression in transgenic mice (Figure 2). Since silent information regulator two 1 (SIRT1) and FoxO3 are believed to play a pivotal role in cellular oxidative stress resistance [24], we also examined their expression in FGF21-treated mice and found that FGF21 increased hepatic expression of both SIRT1 and FoxO3 in normal mice, yet not in bGH transgenics (Figure 2).

3. Discussion

One of the key novel findings of the research presented herein includes the resistance of GH overexpressing mice to the favorable effects of FGF21, pointing to the importance of normal GH signaling in mediating at least some of the wide-ranging metabolic effects of FGF21. Another important result from the present study pertains to the FGF21-mediated induction of antioxidant defenses, which might contribute to the metabolic benefits of FGF21.

FGF21 is a recently described member of the FGF19 subfamily that can act in a local and an endocrine manner to regulate glucose and lipid homeostasis. Its pharmacologic administration has been shown to improve the metabolic profile in obese and diabetic rodents and rhesus monkeys [2–4]. Although there is a preponderance of data on the broad beneficial metabolic effects of FGF21 administration, its innate physiological role and mechanism of action remain to be elucidated. In addition to being of fundamental interest to the basic biology knowledge base of endocrinology and metabolism, elucidation of FGF21's mechanism(s) of action has translational implications, insofar as drug design and drug contraindications.

Consistent with earlier publications, FGF21 administration improved glucose tolerance in response to a glucose challenge in normal mice. We also observed improvements in lipid profile, including lowering of triglycerides, free fatty acids, and total cholesterol in circulation. However, the beneficial effects of FGF21 did not extend to the bGH transgenic mice used in our study. Consistent with our previously published findings, the young-adult PEPCK-bGH transgenic mice used in our study showed somewhat better clearance, compared to control littermates, in response to an exogenous glucose load [17]. The increased musculature of the young-adult bGH mice would be expected to contribute to better glucose clearance relative to littermate controls. However, the blunted, more gradual slope of the curve in case of the bGH transgenics is noteworthy (Figure 1(b)) and could be attributed to the presence of higher circulating insulin and to defects in hepatic insulin signaling and hence impaired glucose disposal as reported previously [13]. In addition to elevated plasma insulin levels, these authors observed alterations in the early steps of the insulin signaling pathway in liver and skeletal muscle of female bGH transgenic mice.

It is of interest to note that FGF21 administration ameliorated the hyperinsulinemia in the bGH transgenic mice. The reduction in insulin levels could be attributed to enhanced insulin clearance in the liver and/or a decrease in insulin secretion. Although we do not provide direct evidence here as to which of these mechanisms is the chief contributor to lower circulating insulin, work done previously suggests that, in leptin-deficient *ob*/*ob* mice, FGF21 administration lowered plasma insulin levels by affecting insulin secretion; as indicated by reduced levels of amylin, a pancreatic hormone cosecreted with insulin [4]. Interestingly, expression of the FGF21 coreceptor β-klotho has been detected in the pancreas, which might be suggestive of local effects of FGF21 on modulating insulin secretion from mouse islets.

Since FGF21 has been purported to act by improving insulin sensitivity, the increased gene expression of molecules involved in the early steps of the insulin signaling pathway seen in the present study would concur with an insulin-sensitizing role for FGF21 (Table 2). Although we were

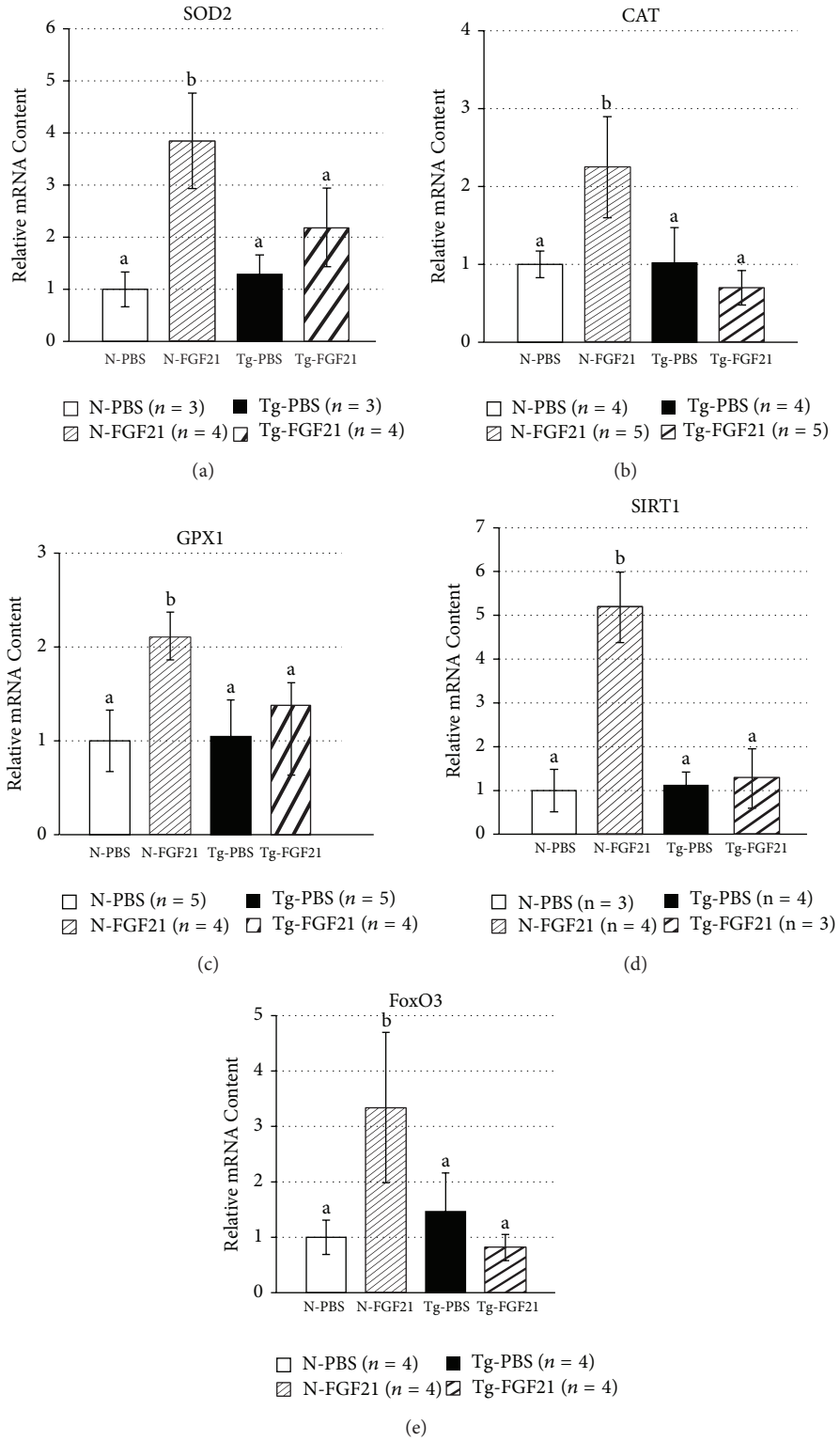

Figure 2: FGF21 stimulates mediators of oxidative stress resistance. RT-PCR analysis of gene expression in normal and GH transgenic mice treated with FGF21 (mean ± SEM) of the following genes: (a) superoxide dismutase 2, (b) catalase, (c) glutathione peroxidase, (d) sirtuin 1, and (e) Forkhead box class O, 3. Different superscripts denote significant difference at $P < 0.05$ (2-tailed, unpaired, homoscedastic Student's t-test).

not able to conduct direct insulin tolerance tests on these FGF21-treated mice, we assessed simple surrogate indices for insulin sensitivity/resistance such as the homeostasis model of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), which all rely on fasted insulin levels. The results from these calculations suggest that, compared to their littermate controls, bGH transgenic mice are insulin-resistant, which does not agree with the insulin signaling pathway gene expression data and is further startling considering concurrent studies in which we carried out insulin tolerance tests in GH overexpressing mice of both genders and different ages and revealed comparable, if not better, insulin sensitivity in the GH transgenic mice relative to their controls [17]. While such surrogates for insulin sensitivity do show modest correlations with more direct measures of insulin sensitivity in animal models [25], in this case the particular anatomic and physiologic peculiarities of the GH transgenic mice (i.e., innate hyperinsulinemia putatively due to increased β-cell-to-body weight/size, as well as different body composition) might explain the divergence between surrogate measures and actual physiologic responses.

Similarly, the decreased fasting blood glucose concentration in GH transgenic mice (Table 1) should logically result (primarily, if not exclusively) from some of the traits of GH overexpressing mice (i.e., higher insulin levels (Table 1) coupled with insulin sensitivity at the level of their littermate controls [17]).

Gluconeogenic effects of FGF21, although contrary to its role in glycemic regulation, have been previously reported [26] and are generally believed to be a part of the reputed role of FGF21 in the adaptive starvation response [11, 27]. Furthermore, since both insulin and GH are major regulators of cellular metabolism and can interact functionally by signaling crosstalk, the resistance of GH transgenic mice to FGF21 may be related to GH overexpression-induced alterations in the sensitivity of various insulin and GH signaling mediators that are needed for FGF21's actions [13, 28].

FGF21 is known to regulate lipolysis and lipid oxidation in adipose tissue [29]. β-oxidation of fatty acids occurs in both mitochondria and peroxisomes. The first step of peroxisomal β-oxidation is catalyzed by ACOX1, while CPT1α catalyzes the transfer of long-chain fatty acids into the mitochondria and is thought to be the rate-limiting enzyme in mitochondrial fatty acid oxidation [30]. In our study, FGF21 treatment for seven days increased expression of ACOX1 and CPT1α, as well as the lipogenic pathway genes FASN and ACC. In addition, it also increased the transcript for AMPK in the liver, which is known to stimulate hepatic fatty acid (FA) oxidation and inhibit lipogenesis [31]. ACC phosphorylation by activated AMPK would result in disinhibition of CPT1α and increased fatty acid oxidation. However, it is known that full phosphorylation of ACC by AMPK results in an inhibition of ACC activity by only 50–60% [20, 32]. While a partial inhibition of ACC would redirect acetyl-CoA and malonyl-CoA flux towards fatty acid oxidation, the substantial residual ACC activity would still allow a considerable rate of lipogenesis, consistent with the increased expression of lipogenic genes that we observed (Table 2). Thus, we propose that FGF21 could orchestrate energy-dissipating futile cycling between *de novo* lipogenesis and fatty acid oxidation.

Given the large size and weight of GH overexpressing mice (Table 1), it is possible that the herein documented FGF21 resistance is secondary to excess body fat [33]. Yet, GH overexpressing mice are actually much leaner than their littermate counterparts [34]. Therefore, insofar as the prospect of adiposity-induced FGF21 resistance, the littermate controls were more likely to exhibit this confound than the GH transgenic mic, making the resistance of the GH transgenics that much more remarkable.

Our observations on hypothalamic gene expression (Table 3) contrast with a previous report that showed increases in mRNA levels for the appetite-promoting AgRP and NPY in hypothalami of FGF21-treated mice on a high-fat diet. The variability in results pertaining to not only hypothalamic gene expression but also food intake and weight loss may be explained by differences in FGF21 dose levels, duration of dosing, and the animal models. It has been previously described that the dose of FGF21 required to exert weight-lowering effects is much higher than the dose required for improvements in glucose homeostasis and insulin sensitivity [4]. Therefore, it is likely that the 0.1 mg/kg/day dose of FGF21 used in our study is insufficient to cause changes in food consumption or the central regulation of feeding behavior. Increased oxidative stress is thought to be a deleterious factor leading to insulin resistance, β-cell dysfunction, and ultimately Type 2 diabetes [35]. In addition, there is suggestive evidence that increased generation of ROS may impair glucose-stimulated insulin secretion and affect the expression of key β-cell genes [36]. In our study, FGF21-treated mice showed increased capacity for resistance to oxidative stress, as measured by the increased hepatic expression of genes for key antioxidant enzymes, while there was a lack of an effect in mice with GH excess that was consistent with observations for other metabolic genes (Figure 2). We speculate that improving antioxidant defenses may be one possible mechanism by which FGF21 improves insulin sensitivity. Additionally, increased resistance to oxidative stress is also thought to be partially beneficial, yet not necessary, for longevity [37].

Previously, Chau and others (2010) have proposed a role for FGF21 in regulating energy metabolism based on the activation of the AMPK-SIRT1-PGC1α pathway in adipose tissue of FGF21 administered mice [38]. In our study, FGF21 induced hepatic UCP2 gene expression and, therefore, would be expected to stimulate UCP2-dependent uncoupled mitochondrial respiration. Since PGC1α is known to stimulate mitochondrial biogenesis through an induction of UCP2 [39], increased UCP2 expression in FGF21-treated mice may be attributable to the FGF21-mediated induction of PGC1α. Moreover, increased uncoupling of oxidative phosphorylation reduces ROS production and has been postulated to be a predictor of extended lifespan, as indicated by the short-lived $Ucp2^{-/-}$ mice [40]. Besides that, increased UCP2-dependent fatty acid oxidation appears to be another mechanism, in addition to reduced ROS generation, that can influence survival [41]. In addition, there is accumulating evidence that

FoxO and sirtuin proteins (such as SIRT1), which are thought to be lifespan modulators, influence diverse physiological functions including metabolism and ROS detoxification. In fact, there is convincing evidence to show that SIRT1 complexes with FoxO3 to enhance cellular stress resistance [42], while FoxO3 is implicated in the transcriptional activation of SOD2 [43]. Upregulation of both FoxO3 and SIRT1, with FGF21 treatment, together with the FGF21-mediated induction of UCP2 and antioxidant enzymes, might be suggestive of antiaging effects of FGF21. FGF21 may thus act to improve redox metabolism with possible beneficial effects on increasing healthspan (the period of life during which an organism is able to exist and function chiefly independently and free from substantial morbidity) and/or lifespan.

To the best of our knowledge, this is the first demonstration of a role for FGF21 in boosting antioxidant defenses. Since oxidative stress is associated with chronic hyperglycemia-induced insulin resistance [44] and a decline in insulin biosynthesis and secretion [45], we are tempted to speculate that the oxidative stress resistance putatively conferred by FGF21 contributes to the beneficial metabolic actions of FGF21. Because the ability to detoxify ROS and increased oxidative stress resistance are correlated with enhanced organismal longevity in many species [46], these particular functions of FGF21 may be relevant to its ability to engender longevity [47, 48].

Li and colleagues recently reported that, in the liver, SIRT1 is necessary for fasting-induced FGF21 gene expression [49]. As our results show that FGF21 treatment is sufficient to induce hepatic SIRT1 gene expression, this combination of results from these two studies suggests a paracrine positive feedback loop in which FGF21, produced in a SIRT1-containing hepatocyte, is exocytosed and stimulates (amongst other effects) the production of SIRT1 in neighboring hepatocytes; the resulting increase in circulating FGF21 might then travel to other parts of the body to engender salubrious effects on metabolism. Although we have no data on FGF-21's effects on isolated hepatocytes or *ex vivo* liver tissues, this hypothesis, if correct, would lend support to studies concluding that FGF21 acts directly through its receptors in the liver [50], partly via SIRT1 transactivation, while still allowing for conclusions of FGF21's salutary actions on other metabolic tissues.

Finally, the resilience of the GH transgenic mice to the metabolic benefits of FGF21 treatment seems to suggest that one of the mechanisms involved in mediating the beneficial effects of FGF21 on glucose and lipid metabolism may be through GH intracellular signaling. In ongoing studies, we are investigating the effects of FGF21 on GH-resistant, GH-signaling-suppressed Laron Dwarf ($Ghr/bp^{-/-}$) mice [51]. Specifically, we are assessing the effects of FGF21 on physiological (via tolerance tests) and macromolecular measures of carbohydrate metabolism, histological and macromolecular measures of lipid/cholesterol metabolism, and metabolism as ascertained by gas (O_2 and CO_2) exchange-based indirect calorimetry, as well as macromolecular analyses of insulin and/or lipid signal transduction in the blood, liver, white adipose tissue, and hypothalamus in these mice lacking growth hormone hormonal signaling. The results from those studies shall clarify the issue of whether the results from the present study are directly related to the GH signaling status or to some unrelated idiosyncrasy of PEPCK-bGH mice that makes them generally meek in response to any treatment, and might make the conclusion that GH intracellular signaling is antagonistic to the endocrinologically beneficial effects of FGF21 more cogent.

4. Methods

4.1. Animals. Transgenic mice that overexpress the bovine GH gene under the control of the rat phosphoenolpyruvate carboxykinase (PEPCK) promoter have been previously described [52]. These mice had markedly accelerated post-weaning growth, leading to a significant increase in body weight. Normal-sized siblings of transgenic mice were used as controls. The mice were housed three to five per cage in a room with controlled light (12 h light per day) and temperature (22 ± 2°C). The animals had free access to food (Lab Diet Formula 5001 containing a minimum of 23% protein, 4.5% fat, and a maximum of 6% crude fiber (Purina Mills Inc., St. Louis, MO)) and tap water. All experiments were performed using male mice in groups of five (3-4 months old) mice.

4.1.1. In Vivo Protocols. The protocols used in this study were approved by the Southern Illinois University Laboratory Animal Care and Use Committee. Mice were randomly assigned to treatment or vehicle groups, based on fed glucose levels and body weight. The mice were treated with vehicle (phosphate-buffered saline (PBS)) or with recombinant human FGF21 (Tany Technogene, Rehovot, Israel) at a dose of 0.1 mg/kg/d via continuous subcutaneous infusion with microosmotic pumps (Model 1007D, Alzet, Cupertino, CA) for one week {this minimal robustly effective dosing paradigm for FGF21 was determined based on previously published dose-response data [53]}. After one week, mice were euthanized between 0900 and 1100 h by cardiac puncture under Isoflurane (Phoenix Pharmaceuticals Inc., St Joseph, MO) anesthesia. Livers were removed quickly, snap-frozen, and stored at −80°C. Blood was centrifuged (4000 ×g for 10 min. at 4°C), and plasma was stored at −80°C.

4.2. Intraperitoneal Glucose Tolerance Test. Intraperitoneal (i.p.) glucose tolerance tests, on mice fasted for 16 h., were performed, at 0900 h. using One Touch Ultra 2 glucometers and blood glucose testing strips (Lifescan, Inc., Milpitas, CA) to measure glucose in blood sampled from the tail vein after an i.p. injection of glucose (2 g/kg body weight/10 mL).

4.3. Metabolite Analysis. Glucose and β-hydroxybutyrate were measured in blood using the One Touch Ultra 2 glucometer and blood glucose testing strips (Lifescan, Inc., Milpitas, CA) and the Precision Xtra β-ketone monitor and β-ketone test strips (Precision Xtra, Abbott Labs, Abbott Park, IL), respectively. Serum triglycerides (GPO; Pointe Scientific Inc, Canton, MI), cholesterol (Pointe Scientific Inc, Canton, MI), and nonesterified fatty acids (NEFA's) (Wako NEFA-HR; Wako Chemicals, Richmond, VA) were measured in

duplicate using enzymatic colorimetric assays. Insulin levels were determined by ultrasensitive mouse-specific enzyme-linked immunosorbent assay (ELISA) (Crystal Chem, Downers Grove, IL), while the immunoenzymometric rat/mouse insulin-like growth factor-1 (IGF-1) ELISA kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ) was used for determination of circulating IGF-1.

4.4. Real Time RT-PCR. Total hepatic RNA was extracted using the phenol-chloroform method [54]. cDNA was obtained using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), and the relative expression of the genes was analyzed by reverse transcriptase PCR (RT-PCR) as described previously [22]. Primer sequences are available upon request. Various genes with constitutive expression including β2-microglobulin, GAPDH, β-actin, and cyclophilin A were evaluated for use as internal control, and cyclophilin A was validated and used as a housekeeping gene for normalization of RNA expression in these animals. The relative expression levels were calculated according to the formula $2^{A-B}/2^{C-D}$ (A = threshold cycle (C_t) number of the gene of interest in the first control sample, $B = C_t$ number of the gene of interest in each sample, $C = C_t$ number of the housekeeping gene in the first control sample, and $D = C_t$ number of the housekeeping gene in each sample), as described previously [22]. The relative expression of the first normal sample was expressed as 1, and the relative expression of all other samples was calculated using this equation. The results from the normal group were averaged, and all the results were then divided by this average to get the fold change of expression of this gene compared with the appropriate control group (littermate control mice on PBS treatment (N-PBS)).

4.5. Statistical Analysis. Data were analyzed and assessed using SPSS software (SPSS Statistics 17.0, SPSS Institute Inc., Chicago, IL). Descriptive statistics of all variables were determined, including the mean and standard deviation (SD) or standard error of the mean (SEM) of each group. Data were analyzed by either Student's t-tests or one-way ANOVA's followed by Student Newman-Keuls *post hoc* test for pairwise comparisons, as appropriate. Data were considered significantly different when $P < 0.05$.

Ethical Approval

Animal Protocol #178-02-001, under which this study was conducted, was approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine.

Conflict of Interests

The authors declare that they have no conflict of interests related to this paper.

Authors' Contribution

Oge Arum, Michal M. Masternak, Andrzej Bartke, and Romesh K. Khardori acquired funding for this study; Ravneet K. Boparai, Romesh K. Khardori, and Andrzej Bartke conceived and designed this study; Ravneet K. Boparai, Oge Arum, and Johanna G. Miquet methodologically executed this study; Ravneet K. Boparai statistically analyzed the data from this study; and Ravneet K. Boparai, Oge Arum, and Romesh K. Khardori prepared the paper for this study. Andrzej Bartke and Romesh K. Khardori (senior authors) and Oge Arum and Ravneet K. Boparai (junior authors) contributed equally to this study. Oge Arum and Ravneet K. Boparai are regarded as colead authors.

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References

[1] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[2] A. Kharitonenkov, V. J. Wroblewski, A. Koester et al., "The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21," *Endocrinology*, vol. 148, no. 2, pp. 774–781, 2007.

[3] W. Wente, A. M. Efanov, M. Brenner et al., "Fibroblast growth factor-21 improves pancreatic β-cell function and survival by activation of extracellular signal-regulated kinase 1/2 and Akt signaling pathways," *Diabetes*, vol. 55, no. 9, pp. 2470–2478, 2006.

[4] J. Xu, D. J. Lloyd, C. Hale et al., "Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice," *Diabetes*, vol. 58, no. 1, pp. 250–259, 2009.

[5] E. S. Muise, B. Azzolina, D. W. Kuo et al., "Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor γ and altered metabolic states," *Molecular Pharmacology*, vol. 74, no. 2, pp. 403–412, 2008.

[6] T. Nishimura, Y. Nakatake, M. Konishi, and N. Itoh, "Identification of a novel FGF, FGF-21, preferentially expressed in the liver," *Biochimica et Biophysica Acta: Gene Structure and Expression*, vol. 1492, no. 1, pp. 203–206, 2000.

[7] S. Ito, S. Kinoshita, N. Shiraishi et al., "Molecular cloning and expression analyses of mouse *βklotho*, which encodes a novel Klotho family protein," *Mechanisms of Development*, vol. 98, no. 1-2, pp. 115–119, 2000.

[8] I. Dostálová, D. Haluzíková, and M. Haluzík, "Fibroblast growth gactor 21: a novel metabolic regulator with potential therapeutic properties in obesity/type 2 diabetes mellitus," *Physiological Research*, vol. 58, no. 1, pp. 1–7, 2009.

[9] A. Kharitonenkov, J. D. Dunbar, H. A. Bina et al., "FGF-21/FGF-21 receptor interaction and activation is determined by

βKlotho," *Journal of Cellular Physiology*, vol. 215, no. 1, pp. 1–7, 2008.

[10] J. S. Moyers, T. L. Shiyanova, F. Mehrbod et al., "Molecular determinants of FGF-21 activity-synergy and cross-talk with PPARγ signaling," *Journal of Cellular Physiology*, vol. 210, no. 1, pp. 1–6, 2007.

[11] T. Inagaki, V. Y. Lin, R. Goetz, M. Mohammadi, D. J. Mangelsdorf, and S. A. Kliewer, "Inhibition of growth hormone signaling by the fasting-induced hormone FGF21," *Cell Metabolism*, vol. 8, no. 1, pp. 77–83, 2008.

[12] M. B. Davidson, "Effect of growth hormone on carbohydrate and lipid metabolism," *Endocrine Reviews*, vol. 8, no. 2, pp. 115–131, 1987.

[13] F. P. Dominici, D. Cifone, A. Bartke, and D. Turyn, "Loss of sensitivity to insulin at early events of the insulin signaling pathway in the liver of growth hormone-transgenic mice," *Journal of Endocrinology*, vol. 161, no. 3, pp. 383–392, 1999.

[14] C. J. Quaife, L. S. Mathews, C. A. Pinkert, R. E. Hammer, R. L. Brinster, and R. D. Palmiter, "Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice," *Endocrinology*, vol. 124, no. 1, pp. 40–48, 1989.

[15] I. Hansen, E. Tsalikian, B. Beaufrere, J. Gerich, M. Haymond, and R. Rizza, "Insulin resistance in acromegaly: defects in both hepatic and extrahepatic insulin action," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 250, no. 3, part 1, pp. E269–E273, 1986.

[16] A. Valera, J. E. Rodriguez-Gil, J. S. Yun, M. M. Mcgrane, R. W. Hanson, and F. Bosch, "Glucose metabolism in transgenic mice containing a chimeric P-enolpyruvate carboxykinase/bovine growth hormone gene," *FASEB Journal*, vol. 7, no. 9, pp. 791–800, 1993.

[17] R. K. Boparai, O. Arum, R. Khardori, and A. Bartke, "Glucose homeostasis and insulin sensitivity in growth hormone-transgenic mice: a cross-sectional analysis," *Biological Chemistry*, vol. 391, no. 10, pp. 1149–1155, 2010.

[18] J. G. Miquet, A. I. Sotelo, A. Bartke, and D. Turyn, "Suppression of growth hormone (GH) Janus tyrosine kinase 2/signal transducer and activator of transcription 5 signaling pathway in transgenic mice overexpressing bovine GH," *Endocrinology*, vol. 145, no. 6, pp. 2824–2832, 2004.

[19] T. Coskun, H. A. Bina, M. A. Schneider et al., "Fibroblast growth factor 21 corrects obesity in mice," *Endocrinology*, vol. 149, no. 12, pp. 6018–6027, 2008.

[20] F. M. Fisher, P. C. Chui, P. J. Antonellis et al., "Obesity is a fibroblast growth factor 21 (FGF21)-resistant state," *Diabetes*, vol. 59, no. 11, pp. 2781–2789, 2010.

[21] A. Kharitonenkov, T. L. Shiyanova, A. Koester et al., "FGF-21 as a novel metabolic regulator," *Journal of Clinical Investigation*, vol. 115, no. 6, pp. 1627–1635, 2005.

[22] Z. Wang, M. M. Masternak, K. A. Al-Regaiey, and A. Bartke, "Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice," *Endocrinology*, vol. 148, no. 6, pp. 2845–2853, 2007.

[23] M. W. Schwartz, S. C. Woods, D. Porte Jr., R. J. Seeley, and D. G. Baskin, "Central nervous system control of food intake," *Nature*, vol. 404, no. 6778, pp. 661–671, 2000.

[24] Y. Kobayashi, Y. Furukawa-Hibi, C. Chen et al., "SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress," *International Journal of Molecular Medicine*, vol. 16, no. 2, pp. 237–243, 2005.

[25] T. T. Tran, N. Gupta, T. Goh et al., "Direct measure of insulin sensitivity with the hyperinsulinemic-euglycemic clamp and surrogate measures of insulin sensitivity with the oral glucose tolerance test: correlations with aberrant crypt foci promotion in rats," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 12, no. 1, pp. 47–56, 2003.

[26] M. J. Potthoff, T. Inagaki, S. Satapati et al., "FGF21 induces PGC-1α and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 26, pp. 10853–10858, 2009.

[27] M. K. Badman, P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier, and E. Maratos-Flier, "Hepatic fibroblast growth factor 21 is regulated by PPARα and is a key mediator of hepatic lipid metabolism in ketotic states," *Cell Metabolism*, vol. 5, no. 6, pp. 426–437, 2007.

[28] J. G. Miquet, L. González, M. N. Matos et al., "Transgenic mice overexpressing GH exhibit hepatic upregulation of GH-signaling mediators involved in cell proliferation," *Journal of Endocrinology*, vol. 198, no. 2, pp. 317–330, 2008.

[29] Y. Hotta, H. Nakamura, M. Konishi et al., "Fibroblast growth factor 21 regulates lipolysis in white adipose tissue but is not required for ketogenesis and triglyceride clearance in liver," *Endocrinology*, vol. 150, no. 10, pp. 4625–4633, 2009.

[30] J. D. McGarry and N. F. Brown, "The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis," *European Journal of Biochemistry*, vol. 244, no. 1, pp. 1–14, 1997.

[31] B. Viollet, F. Andreelli, S. B. Jørgensen et al., "Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models," *Biochemical Society Transactions*, vol. 31, no. 1, pp. 216–219, 2003.

[32] W. W. Winder and D. G. Hardie, "Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 270, no. 2, pp. E299–E304, 1996.

[33] F. M. Fisher, P. C. Chui, P. J. Antonellis et al., "Obesity is a fibroblast growth factor 21 (FGF21)-resistant state," *Diabetes*, vol. 59, no. 11, pp. 2781–2789, 2010.

[34] D. E. Berryman, E. O. List, K. T. Coschigano, K. Behar, J. K. Kim, and J. J. Kopchick, "Comparing adiposity profiles in three mouse models with altered GH signaling," *Growth Hormone & IGF Research*, vol. 14, no. 4, pp. 309–318, 2004.

[35] J. L. Evans, I. D. Goldfine, B. A. Maddux, and G. M. Grodsky, "Are oxidative stress—activated signaling pathways mediators of insulin resistance and β-cell dysfunction?" *Diabetes*, vol. 52, no. 1, pp. 1–8, 2003.

[36] R. A. Simmons, "Developmental origins of diabetes: the role of oxidative stress," *Free Radical Biology and Medicine*, vol. 40, no. 6, pp. 917–922, 2006.

[37] A. B. Salmon, A. Richardson, and V. I. Pérez, "Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging?" *Free Radical Biology & Medicine*, vol. 48, no. 5, pp. 642–655, 2010.

[38] M. D. L. Chau, J. Gao, Q. Yang, Z. Wu, and J. Gromada, "Fibroblast growth factor 21 regulates energy metabolism by

activating the AMPK-SIRT1-PGC-1α pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 28, pp. 12553–12558, 2010.

[39] Z. Wu, P. Puigserver, U. Andersson et al., "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1," *Cell*, vol. 98, no. 1, pp. 115–124, 1999.

[40] Z. B. Andrews and T. L. Horvath, "Uncoupling protein-2 regulates lifespan in mice," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 296, no. 4, pp. E621–E627, 2009.

[41] C. Pecqueur, T. Bui, C. Gelly et al., "Uncoupling protein-2 controls proliferation by promoting fatty acid oxidation and limiting glycolysis-derived pyruvate utilization," *The FASEB Journal*, vol. 22, no. 1, pp. 9–18, 2008.

[42] A. Brunet, L. B. Sweeney, J. F. Sturgill et al., "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase," *Science*, vol. 303, no. 5666, pp. 2011–2015, 2004.

[43] G. J. P. L. Kops, T. B. Dansen, P. E. Polderman et al., "Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress," *Nature*, vol. 419, no. 6904, pp. 316–321, 2002.

[44] J. W. Eriksson, "Metabolic stress in insulin's target cells leads to ROS accumulation—a hypothetical common pathway causing insulin resistance," *FEBS Letters*, vol. 581, no. 19, pp. 3734–3742, 2007.

[45] S. Kawahito, H. Kitahata, and S. Oshita, "Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress," *World Journal of Gastroenterology*, vol. 15, no. 33, pp. 4137–4142, 2009.

[46] R. Gredilla and G. Barja, "The role of oxidative stress in relation to caloric restriction and longevity," *Endocrinology*, vol. 146, no. 9, pp. 3713–3717, 2005.

[47] Y. Zhang, Y. Xie, E. D. Berglund et al., "The starvation hormone, fibroblast growth factor-21, extends lifespan in mice," *eLife*, vol. 2012, no. 1, Article ID e00065, 2012.

[48] C. Kenyon, "Longevity: could a hormone point the way to life extension?" *Elife*, vol. 1, Article ID e00286, 2012.

[49] Y. Li, K. Wong, A. Giles et al., "Hepatic SIRT1 attenuates hepatic steatosis and controls energy balance in mice by inducing fibroblast growth factor 21," *Gastroenterology*, vol. 146, no. 2, pp. 539.e7–549.e7, 2014.

[50] F. M. Fisher, J. L. Estall, A. C. Adams et al., "Integrated regulation of hepatic metabolism by fibroblast growth factor 21 (FGF21) *in vivo*," *Endocrinology*, vol. 152, no. 8, pp. 2996–3004, 2011.

[51] Y. Zhou, B. C. Xu, H. G. Maheshwari et al., "A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 24, pp. 13215–13220, 1997.

[52] M. M. McGrane, J. de Vente, J. Yun et al., "Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic mice," *Journal of Biological Chemistry*, vol. 263, no. 23, pp. 11443–11451, 1988.

[53] C. Hale, M. M. Chen, S. Stanislaus et al., "Lack of overt FGF21 resistance in two mouse models of obesity and insulin resistance," *Endocrinology*, vol. 153, no. 1, pp. 69–80, 2012.

[54] P. Chomczynski and N. Sacchi, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction," *Analytical Biochemistry*, vol. 162, no. 1, pp. 156–159, 1987.

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Research Article

Low Magnesium Levels and FGF-23 Dysregulation Predict Mitral Valve Calcification as well as Intima Media Thickness in Predialysis Diabetic Patients

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Background. Mitral valve calcification and intima media thickness (IMT) are common complications of chronic kidney disease (CKD) implicated with high cardiovascular mortality. *Objective*. To investigate the implication of magnesium and fibroblast growth factor-23 (FGF-23) levels with mitral valve calcification and IMT in CKD diabetic patients. *Methods*. Observational, prospective study involving 150 diabetic patients with mild to moderate CKD, divided according to Wilkins Score. Carotid-echodoppler and transthoracic echocardiography were used to assess calcification. Statistical tests used to establish comparisons between groups, to identify risk factors, and to establish cut-off points for prediction of mitral valve calcification. *Results*. FGF-23 values continually increased with higher values for both IMT and calcification whereas the opposite trend was observed for magnesium. FGF-23 and magnesium were found to independently predict mitral valve calcification and IMT ($P < 0.05$). Using Kaplan-Meier analysis, the number of deaths was higher in patients with lower magnesium levels and poorer Wilkins score. The mean cut-off value for FGF-23 was 117 RU/mL and for magnesium 1.7 mg/dL. *Conclusions*. Hypomagnesemia and high FGF-23 levels are independent predictors of mitral valve calcification and IMT and are risk factors for cardiovascular mortality in this population. They might be used as diagnostic/therapeutic targets in order to better manage the high cardiovascular risk in CKD patients.

1. Introduction

Patients with chronic kidney disease (CKD) are particularly susceptible to cardiovascular complications, and cardiovascular disease accounts for more than 50% of all deaths in this population [1]. Common complications like vascular (VC) and mitral valve calcification are clinically observed as changes in intima media thickness (IMT), coronary artery calcification, pulse pressure, or pulse wave velocity and have been implicated with the high cardiovascular mortality incidences observed [2, 3].

It is generally accepted that the interplay between kidney, bone, and vessels is important for maintaining mineral and bone homeostasis. It is during the early stages of renal disease that mechanisms responsible for keeping the balance start to get out of control, and an imbalance between inhibitory and inducing mediators has been shown to be the driving force for VC and mitral valve calcification [4]. Thus, especially the earlier stages of renal failure might be critical for the onset of the calcification process and for potential therapeutic interventions [5].

For many years calcification was believed to be an inert process, resulting exclusively from elevated concentrations of serum phosphate and calcium phosphate product [6–8]; however, it is currently being understood as a multifactorial, active, and dynamic process that shares similarities with

osteogenic differentiation [9]. Several factors are known to be involved in triggering the calcification process and inducing the phenotypical transformation of vascular smooth muscle to bone-forming cells [10].

Magnesium could be one potentially important factor in this process as several recent studies in the general population, in predialysis CKD patients, and in hemodialysis patients have shown a correlation of low magnesium levels with all-cause and cardiovascular mortality [11–14]. Low serum magnesium levels have been further associated with VC, both in animals [15–17] as well as in clinical observational [18–21] and interventional studies [22, 23] in hemodialysis patients. Last but not least low serum magnesium levels have also been associated with higher IMT values in several studies with dialysis or hypertensive patients [23–25].

Another important factor is fibroblast growth factor-23 (FGF-23). FGF-23 levels, known to rise early in patients with CKD [26], have recently been implicated with VC, left ventricular hypertrophy, endothelial dysfunction, and increased mortality in dialyzed patients [27, 28].

Therefore, both serum magnesium and FGF-23 seem to be potential markers of valvular calcification [29]. However, and to our knowledge, no study so far has assessed these two variables conjointly in a population of diabetics with impaired kidney function. Nonetheless, such analyses are of value as they provide insights into the early development of CKD and associated cardiovascular complications.

In the present study we investigated the association of serum levels of magnesium and FGF-23 with mitral valve calcification and IMT in diabetic patients with mild to moderate CKD to further elucidate the clinical developments for possible therapeutic approaches.

2. Material and Methods

This is a prospective, observational study in diabetic patients with mild to moderate CKD. Patients were screened and recruited in an outpatient diabetic nephropathy clinic and were followed from January 2008 to December 2013. Before its implementation, the study was submitted and approved by the local Ethics Committee. All principles of the Declaration of Helsinki of 1975, as revised in 2000, were followed and study procedures were only conducted after obtaining patients' written informed consent.

2.1. Subjects. In this study 150 patients with type 2 diabetes and with mild to moderate CKD ($15\,\text{mL/min/1.73}\,\text{m}^2 < \text{eGFR} \leq 89\,\text{mL/min/1.73}\,\text{m}^2$) were included. The classification of diabetes followed the guidelines established by the American Diabetes Association [30]. All included patients were, at the time of inclusion, undergoing several pharmacologic therapies, namely: antihypertensive drugs such as antagonist of receptor of angiotensin (ARA) and angiotensin converting enzyme inhibitor (ACEI), antidyslipidemic drugs, acetylsalicylic acid (ASA), and oral antidiabetic agents.

Patients were considered ineligible to participate in the study if they presented at least one of the following criteria: previous cardiovascular disease (defined as a history of one or more of the following: nonfatal myocardial infarction, angina pectoris (stable or unstable), stroke or transient ischemic attacks, and congestive heart failure), history of valvulopathies (including rheumatic fever), uncontrolled hypertension (BP ≥ 140/90 mmHg), albumin/creatinine ratio (UACR) > 500, estimated glomerular filtration rate (eGFR) ≤ 15 mL/min or ≥90 mL/min, parathyroid hormone (PTH) ≥ 350 pg/mL, phosphorus > 5.5 mg/dL, type 1 diabetes, renal disease other than diabetic nephropathy, and neoplastic or infectious diseases. Patients were not allowed to undergo therapy with thiazide or loop diuretics, spironolactone, magnesium supplements, or any laxative or chelate agent containing magnesium. Patients with any gastrointestinal pathology that could possibly interfere with magnesium absorption were also not included in this study.

All mortalities caused by other than cardiovascular events were also excluded.

2.2. Followup. Followup of patients was conducted 2-3 times a year during in-person visits on nephrology consultation. Patients with more severe conditions returned approximately every 3 months, with the other patients returning every 6 months. No patient was "lost to followup" since in the Algarve region all patients with renal disease are referred to Hospital de Faro, with the continuity of the followup being assured.

2.3. Blood Measurements. Serum samples were collected at baseline in fasting patients. Samples were centrifuged and plasma was frozen at −80°C. Several laboratory parameters were analyzed: glycated hemoglobin (HbA1c), lipid profile [total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides], mineral metabolism [calcium, phosphorus, magnesium, PTH], inflammation [interleukin-6 (IL-6)], active form of vitamin D [$1,25(OH)_2D3$], FGF-23, and serum creatinine.

FGF-23 serum levels were quantified using an enzyme-linked immunosorbent assay, *Human FGF-23 (C-Term)* ELISA kit (Cat. #60-6100 Immutopics Inc., San Clemente, CA, USA). Serum levels of $1,25(OH)_2D3$ were quantified with a radioimmunoassay (IDS, Boldon, UK). Total cholesterol, HDL, phosphorus, and magnesium were measured using the ARCHITECT c Systems and the AEROSET System (Abbott Diagnostics Division, Abbott Laboratories Abbott Park, IL, USA) and LDL cholesterol in human plasma was assessed using a MULTIGENT Direct LDL assay (Abbott Diagnostics Division, Abbott laboratories Abbott Park, IL, USA). Serum levels of IL-6 were measured using a sandwich enzyme-linked immunoassay (ELISA) kit (eBioscience, San Diego, CA, USA). HbA1c and PTH levels were measured using a spectrophotometry technique and electrochemiluminescent immunoassays (ECLIA), respectively.

2.4. Renal Function Assessment. Values of serum creatinine were obtained through an enzymatic method, using the ARCHITECT device (Abbott Diagnostics Division, Abbott Laboratories Abbott Park, IL, USA), while GFR was estimated using a formula derived by the Modification of Diet in Renal Disease study group [31].

Table 1: Grading of mitral valve characteristics according to Wilkins score.

Grade	Mobility	Thickening	Calcification	Subvalvular thickening
1	Highly mobile valve with only 1 leaflet tips restricted	Leaflets near normal in thickness (4-5 mm)	A single area of increased echo brightness	A single area of increased echo brightness
2	Leaflet midportions and base portions have normal mobility	Midleaflets normal, considerable thickening of margins (5–8 mm)	Scattered areas of brightness confined to leaflet margins	Scattered areas of brightness confined to leaflet margins
3	Valve continues to move forward in diastole, mainly from the base	Thickening extending through the entire leaflet (5–8 mm)	Brightness extending into the midportion of the leaflets	Thickening extending to the distal third of the chords
4	No or minimal forward movement of the leaflets in diastole	Considerable thickening of all leaflet tissue (>8–10 mm)	Extensive brightness throughout much of the leaflet tissue	Extensive thickening and shortening

2.5. Echocardiography. Transthoracic echocardiography was performed using a General Electrical Medical Systems echograph, model Vivid 7 with a probe (GE Healthcare, WI, USA). Data were recorded on computer and film and were always analyzed by the same technician.

2.6. Carotid Echodoppler. Carotid echodoppler was performed using a General Electrical Medical Systems echograph, model Vivid 4 with a linear probe of 10 MHz (GE Healthcare, WI, USA). For the assessment of the carotid artery intima-media thickness (IMT), the protocol of the American Society of Echocardiography was followed [32]. Data were recorded and analyzed by the same technician.

2.7. Outcomes. The primary outcome event studied was the presence of calcifications on the mitral valve annulus. The presence and extent of calcifications were assessed through echocardiographic examinations. Depending on the features of the echocardiographic findings, patients were stratified and divided into 4 groups according to the extent of mitral valve calcifications. This grading was performed following the Wilkins score, modified by Soliman and colleagues (Table 1) [33].

2.8. Statistical Analyses. Analyses were performed by using descriptive statistics, and for comparisons between groups ANOVA with Scheffé post hoc tests were used. Survival was estimated with the Kaplan-Meier method and the comparison between groups was made by using the log-rank test. Multivariate linear regressions were applied in order to identify risk factors. Receiver operating characteristic (ROC) curves were drawn in order to analyze sensitivity and specificity and to determine a cut-off point for serum FGF-23 and magnesium levels for predicting mitral valve calcification. In all analyses, $P < 0.05$ was considered significant. All analyses were performed using the SPSS program, v17.0.

3. Results

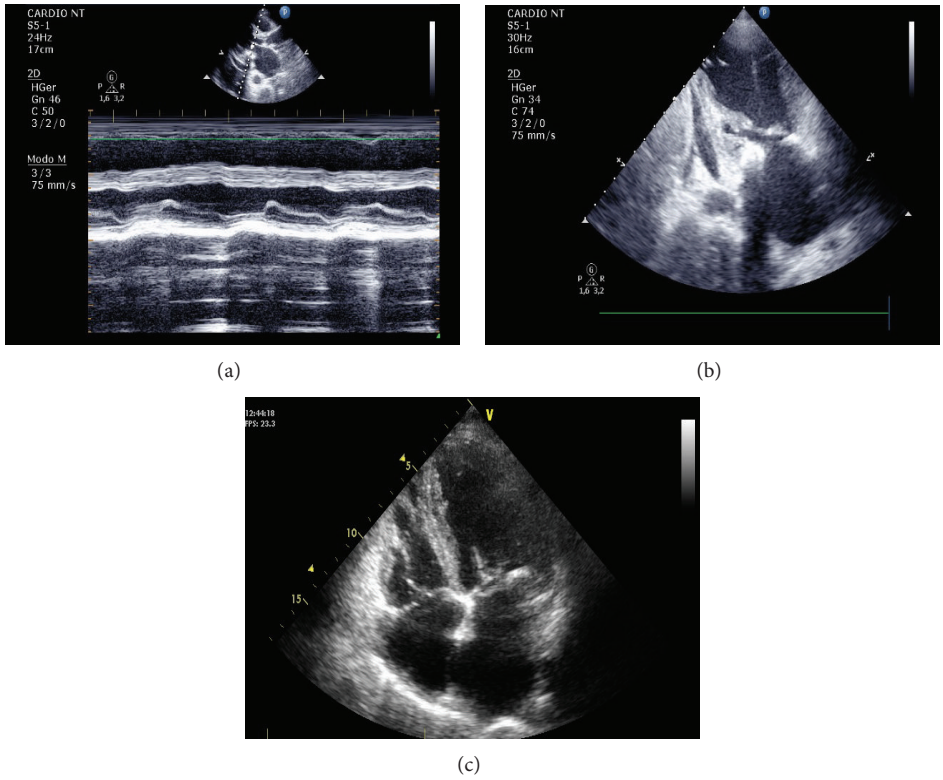
Patients' baseline characteristics are summarized in Table 2. The mean age of the patients was 66.6 ± 9.7 years (40–85) and 35.3% (53) were female. The study was conducted for 72 months, between January 2008 and December 2013.

Table 2: Patients' baseline characteristics.

Parameter	Values
Number of patients enrolled, *n*	150
Age (years)	66.6 ± 9.7
Gender, M/F (%)	97/53 (64.7/35.3)
Hb (g/dL)	13.0 ± 1.5
Blood Pressure (mmHg)	127.2/74.4 ± 8.5/8.1
HbA1c (%)	6.9 ± 0.8
Total cholesterol (mg/dL)	188.8 ± 40.8
HDL (mg/dL)	41.1 ± 10.1
LDL (mg/dL)	106.1 ± 34.4
Triglycerides (mg/dL)	141.6 ± 67.0
Creatinine (mg/dL)	1.7 ± 0.9
eGFR (mL/min)	49.7 ± 21.0
Albumin/creatinine ratio (μg/mg)	111.7 ± 78.7
Magnesium (mg/dL)	1.7 ± 0.7
Phosphorus (mg/dL)	3.7 ± 0.6
PTH (pg/mL)	145.7 ± 92.9
Calcium × phosphorus (mg/dL)	35.1 ± 5.8
FGF-23 (RU/mL)	112.5 ± 66.8
$1,25(OH)_2D3$ (pg/mL)	21.1 ± 8.6
IL-6 (pg/mL)	7.3 ± 3.7

After the echocardiographic assessments (Figure 1) patients were divided into 4 groups according to their Wilkins scores, 38 patients were allocated to Grade 1, 47 to Grade 2, 29 to Grade 3, and 36 patients to Grade 4. All the parameters assessed and depicted in Figure 2 present statistically significant differences between calcification groups.

Patients with poorer calcification features (Grade 4) presented higher levels of phosphorus, PTH, and FGF-23, as well as lower values of eGFR and magnesium. Continuous increase, accompanied by higher grading score, was seen for creatinine, PTH, and FGF-23 levels, whereas a continuous decrease in eGFR and magnesium levels was observed (Figure 2).



(a) (b)

(c)

Figure 1: Echocardiographic findings: (a) parasternal incidence long axis, mitral valve M-mode, fibrotic leaflets, with limited leaflet excursion mobility, and annular calcification; (b) apical 2-chamber view, left LV, LA, mitral valve with fibrotic leaflet, and subvalvular apparatus and annular calcification; (c) apical 4-chamber view, LV, LA, RV, RA, mitral valve with fibrotic leaflet, and subvalvular apparatus and annular calcification.

Table 3: Multivariate linear regression analysis of influencing factors of mitral valve calcification.

Variable	Coefficient	SE	P value
Age	0.011	0.006	0.101
Calcium	0.345	0.140	0.029
Phosphorus	0.066	0.158	0.675
Ca × P	0.999	0.018	0.591
PTH	<0.001	0.001	0.517
Creatinine	0.023	0.137	0.869
eGFR	−0.012	0.005	0.026
FGF-23	0.028	0.001	<0.001
Magnesium	−0.916	0.112	<0.001
$1,25(OH)_2D3$	−0.011	0.012	0.352
IL-6	<0.001	0.025	0.971

Variables such as age, calcium, phosphorus, Ca x P, PTH, creatinine, eGFR, FGF-23, magnesium, $1,25(OH)_2D3$, and IL-6 were analyzed using a multivariate linear regression to identify independent risk factors of mitral valve calcification. Calcium, eGFR, FGF-23, and magnesium were found to independently predict mitral valve calcification ($P < 0.05$) in opposition to the other variables (Table 3).

The trend of variables behavior was also assessed for IMT levels. Three groups were defined according to the IMT levels < 0.8, 0.8–1, and >1 mm and were analysed. Results demonstrated that variables such as FGF-23, PTH, and IL-6 continually increased with higher IMT levels, and magnesium and $1,25(OH)_2D3$ presented an opposite trend (Figure 3).

Furthermore, all variables were analyzed to identify independent risk factors of carotid intima-media thickness (IMT). IL-6, Wilkins score, FGF-23, and magnesium were found to independently predict IMT ($P < 0.05$) in contrast to the other variables (Table 4).

For detailed analyses all patients were grouped according to their magnesium levels resulting in 75 patients with levels lower than 1.85 mg/dL (= 0.76 mmol/L) (calcification score 1: $n = 4$; 2: $n = 10$; 3: $n = 25$; 4: $n = 36$) and 75 patients with levels of 1.85 mg/dL or higher (calcification score 1: $n = 34$; 2: $n = 37$; 3: $n = 4$; 4: $n = 0$). Using Kaplan-Meier analysis it was observed that the number of deaths was higher in patients with lower magnesium levels as well as in patients with poorer Wilkins score (Figure 4).

Finally, the cut-off levels of FGF-23 and magnesium were determined by the ROC curve analysis to differentiate between patients with and without mitral valve calcification. The area under the curve for FGF-23 and magnesium was 0.997 ± 0.003, $P < 0.001$ and 0.916 ± 0.024, $P < 0.001$, respectively (Figure 5). The mean cut-off value obtained for FGF-23 was 117 RU/mL and for magnesium the mean cut-off value was 1.7 mg/dL (= 0.7 mmol/L).

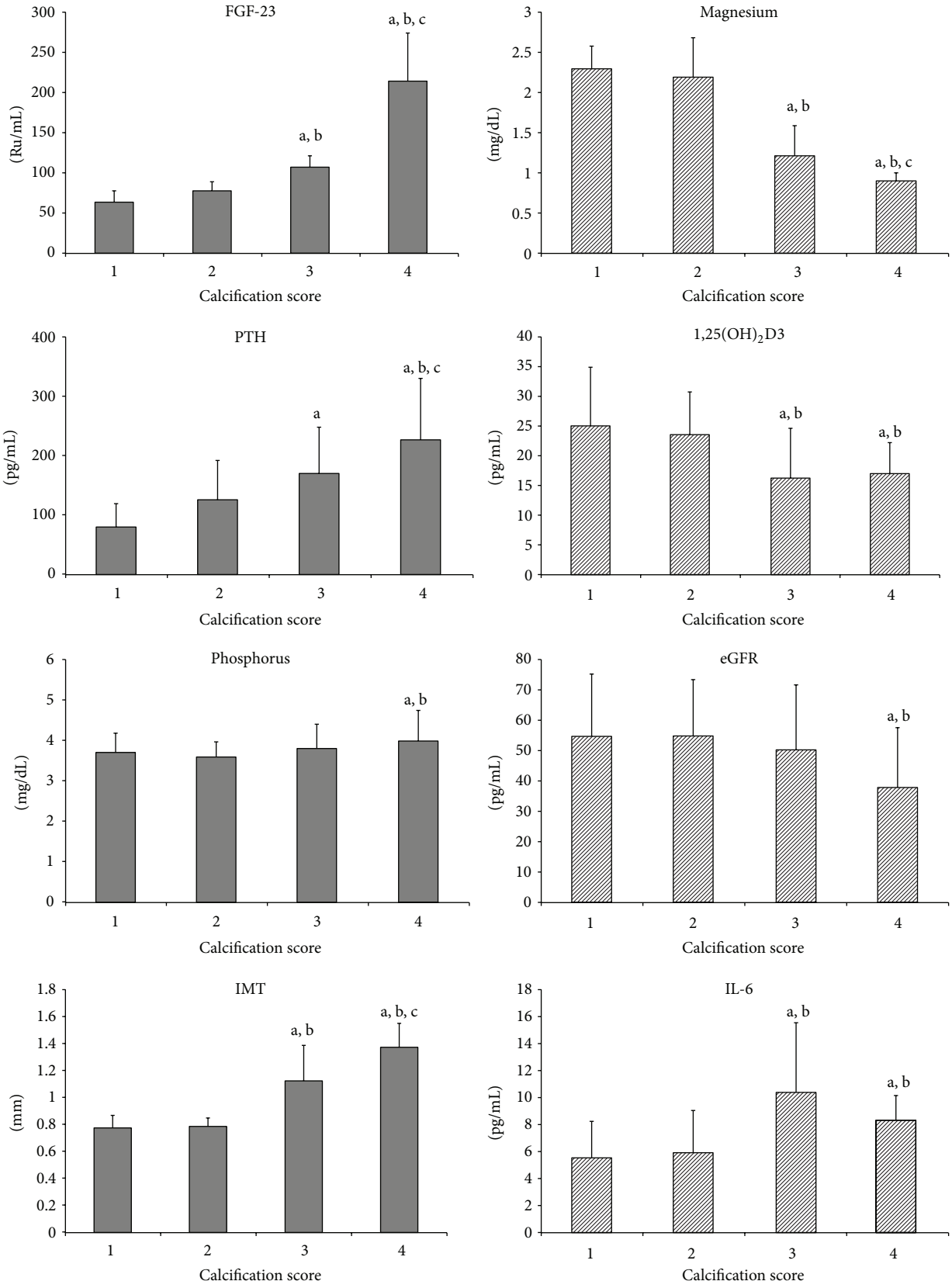


Figure 2: Parameters according to calcification score, 1 ($n = 38$), 2 ($n = 47$), 3 ($n = 29$), and 4 ($n = 36$). Results of post hoc analysis: a—$P < 0.05$ versus Group 1, b—$P < 0.05$ versus Group 2, and c—$P < 0.05$ versus Group 3.

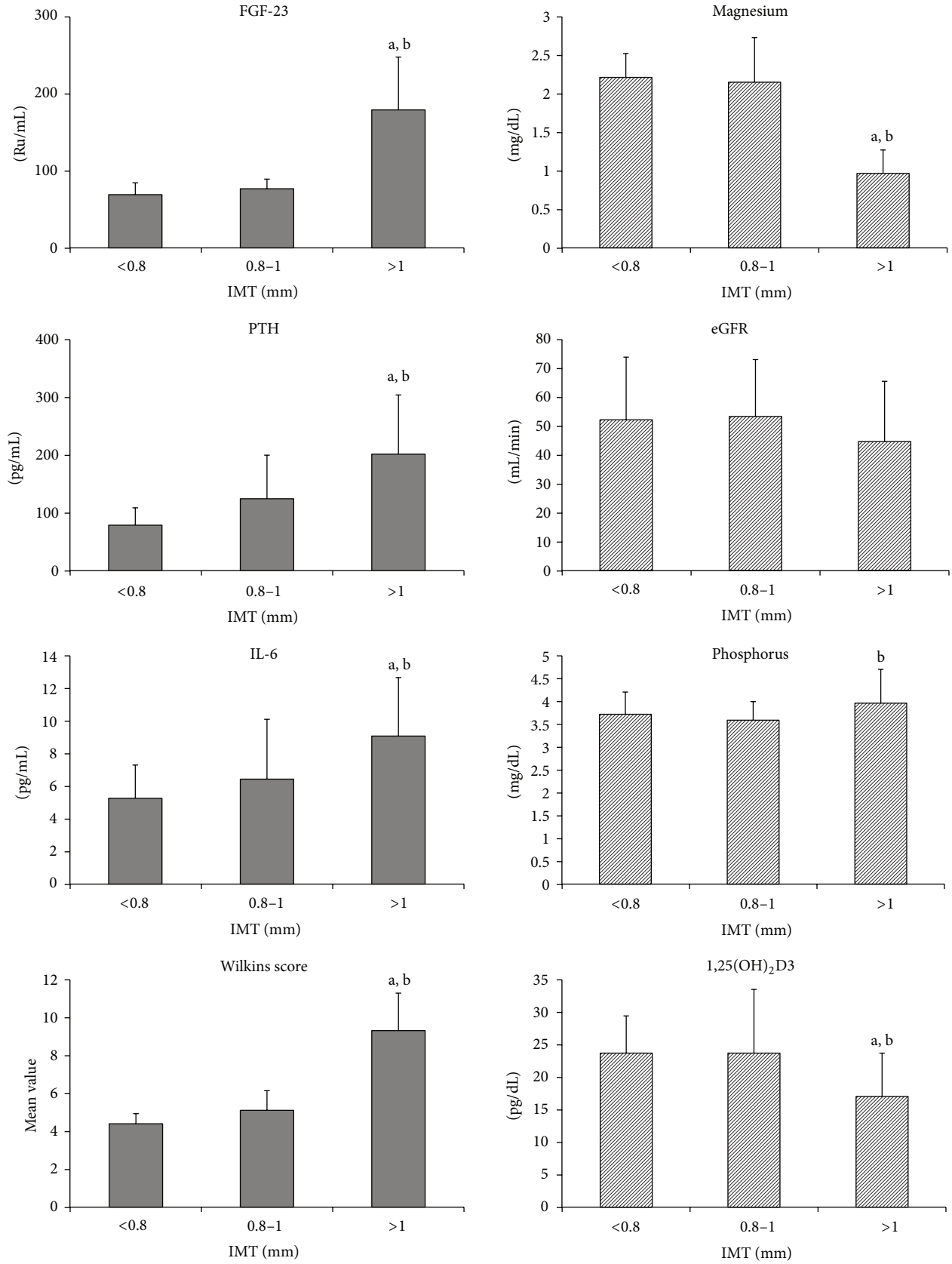


Figure 3: Parameters according to IMT values, <0.8 mm ($n = 25$), 0.8–1 mm ($n = 69$), and >1 mm ($n = 56$). Results of post hoc analysis: a—$P < 0.05$ versus Group 1 and b—$P < 0.05$ versus Group 2.

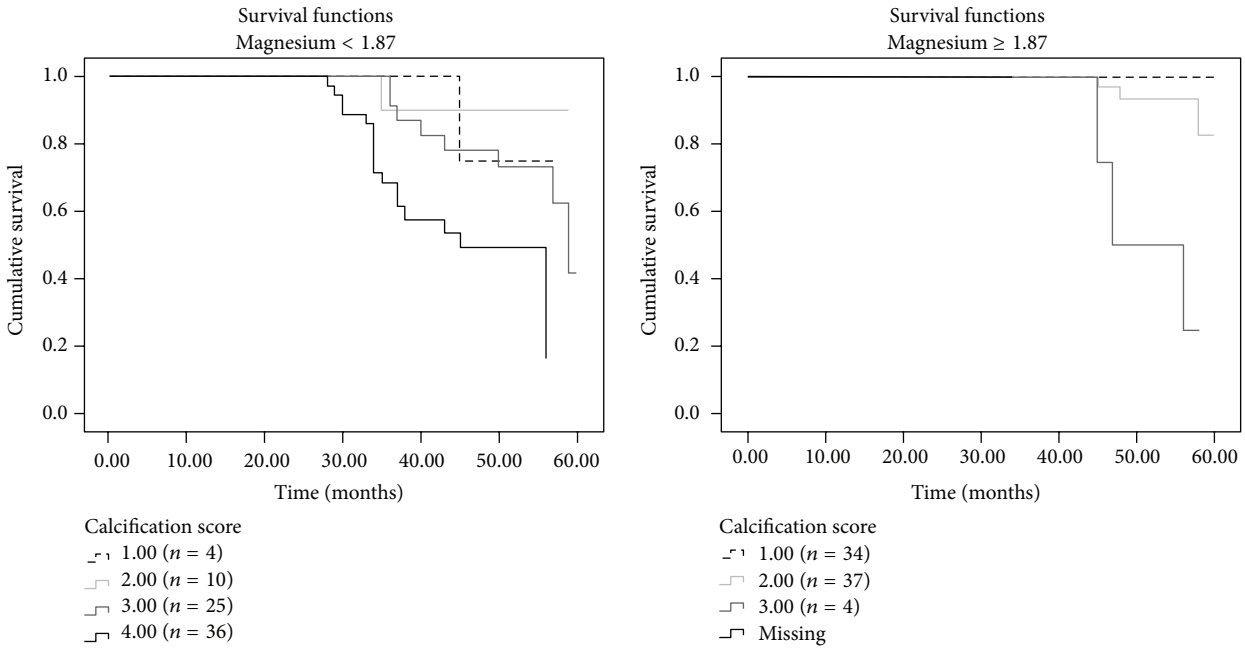


Figure 4: Survival analysis according to the serum magnesium level [mg/dL].

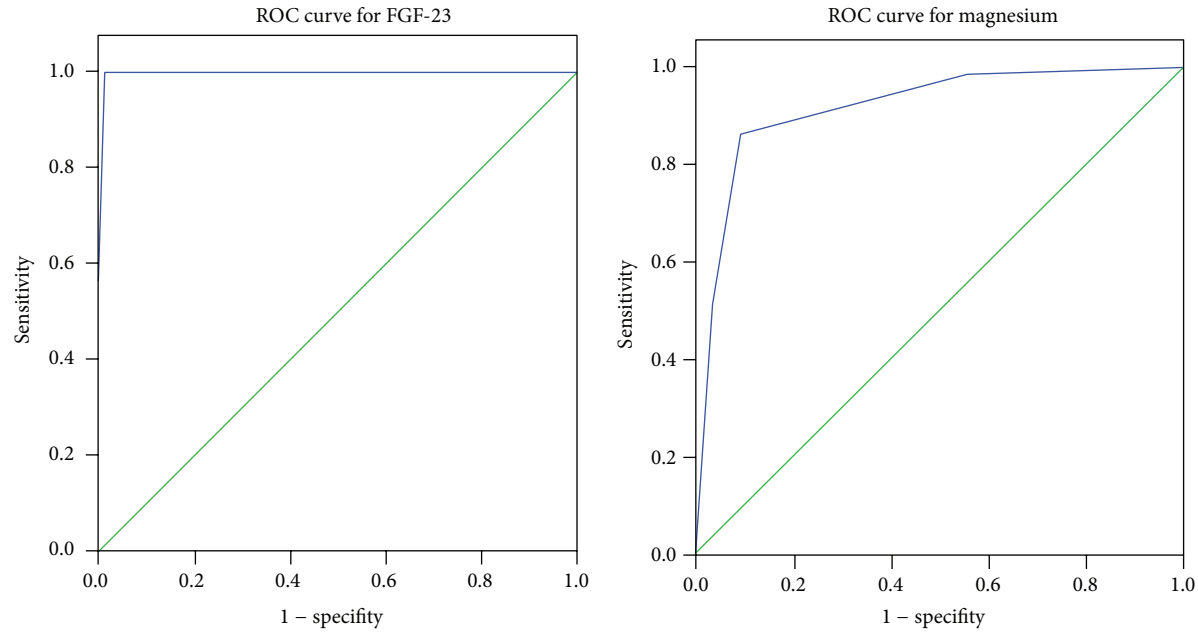


Figure 5: ROC curve analysis results for serum levels of FGF-23 (a) and magnesium (b).

4. Discussion

As a multifactorial process, many variables are thought to be responsible for vascular and valvular calcification in dialysis patients, including the duration of dialysis, diabetes and inflammation. Recently the role of mineral metabolism deregulation in the pathophysiology of calcification has been gaining relevance [34–36], with several studies associating serum magnesium and FGF-23 levels with increased vascular calcification in dialysis patients [4, 37, 38].

On the other hand the association between low serum magnesium levels and diabetes [39–41], particularly with hypomagnesemia being associated with a higher prevalence of diabetes [42] has also been demonstrated in several studies. However, as far as our knowledge goes, no other study before has assessed the relationship of magnesium and FGF-23 with mitral valve calcification and IMT in diabetic subjects with mild to moderate CKD. To elucidate this question we analyzed 150 diabetic patients with CKD 2–4. After patient

Table 4: Multivariate linear regression analysis of influencing factors of IMT.

Variable	Coefficient	SE	*P* value
Age	0.001	0.001	0.483
Calcium	0.004	0.031	0.908
Phosphorus	0.003	0.034	0.935
Ca × P	0.006	0.004	0.146
PTH	<0.001	<0.001	0.463
Creatinine	0.055	0.029	0.066
eGFR	0.001	0.001	0.471
FGF-23	0.001	0.001	0.041
Magnesium	−0.060	0.029	0.002
$1,25(OH)_2D3$	0.002	0.003	0.492
IL-6	0.016	0.005	0.005
Wilkins Score	0.073	0.018	<0.001

allocation to groups according to their Wilkins score almost every variable assessed had shown statistically significant differences between groups. However, in multivariate linear regression only magnesium, FGF-23, calcium, and eGFR were found to be independent predictors of mitral valve calcification. Furthermore, multivariate linear regression regarding IMT revealed once again magnesium and FGF-23 as independent predictors, this time together with IL-6 and the Wilkins score. Taken together the presented data are indicative that these two variables can be considered as predictors of mitral valve calcification and that this condition also alters vascular mechanical properties [4, 42, 43].

Magnesium exerts its protective effect on vascular calcification through multiple molecular mechanisms [4, 15, 23]. In particular it seems to negatively regulate vascular calcification and osteogenic differentiation through transient receptor potential melastatin (TRPM)7 activity and increased expression of anticalcification proteins [44]. Observational data suggest that magnesium may play an important role in the development and acceleration of arterial atherosclerosis and vascular calcification both in patients with CKD and in the general population [4, 42].

Levels of FGF-23 rise early in the course of CKD for that normal phosphorus levels can be maintained [45]. It is thought that these changes may be due to compensatory effects on phosphate retention caused by decreasing capacity of the damaged kidney to excrete dietary phosphorus loads, increased FGF-23 secretion into circulation, and decreased FGF-23 removal from circulation [46]. However, this rise has been associated with worse outcome [47]. Several studies with dialysis and predialysis CKD patients also suggest an association between high FGF-23 levels and vascular calcification [35, 37, 38, 40, 48].

More detailed analyses of our data regarding the role of magnesium on survival analyses suggested that mortality rates were higher both in patients with poorer grades of mitral valve calcification and in the subset of patients with lower magnesium levels. The association between hypomagnesaemia and mortality is further indicated by the strong inversed trend of patient numbers and calcification score in the two magnesium groups (magnesium < 1.85 mg/dL: calcification score 1: $n = 4$; 2: $n = 10$; 3: $n = 25$; 4: $n = 36$ versus ≥1.85 mg/dL: calcification score 1: $n = 34$; 2: $n = 37$; 3: $n = 4$; 4: $n = 0$) and is in accordance with previous studies [11, 13, 14, 49, 50]. Taken together, it seems plausible to assume that magnesium exerts a protective role and that FGF-23 might have a procalcification role in patients not yet undergoing a renal replacement therapy. The correct understanding of these risk factors (hypomagnesaemia and high FGF-23 levels) for mitral valve calcification in predialysis patients is extremely important as the presence and the extent of valvular calcifications impact patient survival.

Thus, magnesium levels might have a significant clinical relevance as a marker or predictor of mitral valve calcification as well as IMT as a measure of VC and last but not least, a therapeutic role for magnesium should be considered. In addition, magnesium and FGF-23 may potentially be used as targets for early interventions in predialysis patients in order to manage these risk factors for calcification, thereby possibly modulating its progression for that cardiovascular mortality might be reduced before dialysis as well as when these patients enter dialysis. In both ways the cut-off levels determined here might be of help for clinical practice.

There are several limitations in the current study such as the relatively small sample size and, consequently, the limited statistical power of the tests applied. Nevertheless, these are the first results of an ongoing long-time project and the main objective of this analysis was to establish primary associations and to put forward further studies to clarify and better understand the role of magnesium and FGF-23 in the pathophysiology of calcification in diabetic CKD patients. Thus, prospective studies with bigger sample size and robust statistical analysis are required in order to confirm these associations.

In conclusion, the present study shows that a deregulation of mineral metabolism, with particular attention directed to magnesium and FGF-23, impacts the extent and severity of mitral valve annulus calcification and IMT on type 2 diabetic patients with a diagnosis of mild to moderate CKD.

Conflict of Interests

Kristina Gündlach and Janine Büchel are current employees of Fresenius Medical Care Deutschland GmbH. The remaining authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors of this research paper have directly participated in the planning, execution, or analysis of this study.

References

[1] R. N. Foley, P. S. Parfrey, and M. J. Sarnak, "Clinical epidemiology of cardiovascular disease in chronic renal disease," *American Journal of Kidney Diseases*, vol. 32, no. 5, pp. S112–S119, 1998.

[2] G. M. London, A. P. Guérin, S. J. Marchais, F. Métivier, B. Pannier, and H. Adda, "Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality," *Nephrology Dialysis Transplantation*, vol. 18, no. 9, pp. 1731–1740, 2003.

[3] M. Noordzij, E. M. Cranenburg, L. F. Engelsman et al., "Progression of aortic calcification is associated with disorders of mineral metabolism and mortality in chronic dialysis patients," *Nephrology Dialysis Transplantation*, vol. 26, no. 5, pp. 1662–1669, 2011.

[4] Z. A. Massy and T. B. Drüeke, "Magnesium and outcomes in patients with chronic kidney disease: focus on vascular calcification, atherosclerosis and survival," *Clinical Kidney Journal*, vol. 5, no. 1, pp. i52–i61, 2012.

[5] Á. L. M. de Francisco and M. Rodríguez, "Magnesium—its role in CKD," *Nefrologia*, vol. 33, no. 3, pp. 389–399, 2013.

[6] G. A. Block, P. S. Klassen, J. M. Lazarus, N. Ofsthun, E. G. Lowrie, and G. M. Chertow, "Mineral metabolism, mortality, and morbidity in maintenance hemodialysis," *Journal of the American Society of Nephrology*, vol. 15, no. 8, pp. 2208–2218, 2004.

[7] M. Cozzolino, A. S. Dusso, and E. Slatopolsky, "Role of calcium-phosphate product and bone-associated proteins on vascular calcification in renal failure," *Journal of the American Society of Nephrology*, vol. 12, no. 11, pp. 2511–2516, 2001.

[8] S. K. Ganesh, A. G. Stack, N. W. Levin, T. Hulbert-Shearon, and F. K. Port, "Association of elevated serum PO4, Ca × PO4 product, and parathyroid hormone with cardiac mortality risk in chronic hemodialysis patients," *Journal of the American Society of Nephrology*, vol. 12, no. 10, pp. 2131–2138, 2001.

[9] S. Disthabanchong, "Vascular calcification in chronic kidney disease: pathogenesis and clinical implication," *World Journal of Gastroenterology*, vol. 1, pp. 43–53, 2012.

[10] S. M. Moe and N. X. Chen, "Pathophysiology of vascular calcification in chronic kidney disease," *Circulation Research*, vol. 95, no. 6, pp. 560–567, 2004.

[11] T. Reffelmann, T. Ittermann, M. Dörr et al., "Low serum magnesium concentrations predict cardiovascular and all-cause mortality," *Atherosclerosis*, vol. 219, no. 1, pp. 280–284, 2011.

[12] E. Ishimura, S. Okuno, T. Yamakawa, M. Inaba, and Y. Nishizawa, "Serum magnesium concentration is a significant predictor of mortality in maintenance hemodialysis patients," *Magnesium Research*, vol. 20, no. 4, pp. 237–244, 2007.

[13] Y. Sakaguchi, N. Fujii, T. Shoji, T. Hayashi, H. Rakugi, and Y. Isaka, "Hypomagnesemia is a significant predictor of cardiovascular and non-cardiovascular mortality in patients undergoing hemodialysis," *Kidney International*, vol. 85, no. 1, pp. 174–181, 2014.

[14] S. van Laecke, E. V. Nagler, F. Verbeke, W. van Biesen, and R. Vanholder, "Hypomagnesemia and the risk of death and GFR decline in chronic kidney disease," *The American Journal of Medicine*, vol. 126, no. 9, pp. 825–831, 2013.

[15] T. G. M. F. Gorgels, J. H. Waarsing, A. De Wolf, J. B. Ten Brink, W. J. P. Loves, and A. A. B. Bergen, "Dietary magnesium, not calcium, prevents vascular calcification in a mouse model for pseudoxanthoma elasticum," *Journal of Molecular Medicine*, vol. 88, no. 5, pp. 467–475, 2010.

[16] P. O. Schwille, A. Schmiedl, R. Schwille et al., "Media calcification, low erythrocyte magnesium, altered plasma magnesium, and calcium homeostasis following grafting of the thoracic aorta to the infrarenal aorta in the rat—differential preventive effects of long-term oral magnesium supplementation alone and in combination with alkali," *Biomedicine and Pharmacotherapy*, vol. 57, no. 2, pp. 88–97, 2003.

[17] F. A. R. van den Broek and A. C. Beynen, "The influence of dietary phosphorus and magnesium concentrations on the calcium content of heart and kidneys of DBA/2 and NMRI mice," *Laboratory Animals*, vol. 32, no. 4, pp. 483–491, 1998.

[18] E. Ishimura, S. Okuno, K. Kitatani et al., "Significant association between the presence of peripheral vascular calcification and lower serum magnesium in hemodialysis patients," *Clinical Nephrology*, vol. 68, no. 4, pp. 222–227, 2007.

[19] H. E. Meema, D. G. Oreopoulos, and A. Rapoport, "Serum magnesium level and arterial calcification in end-stage renal disease," *Kidney International*, vol. 32, no. 3, pp. 388–394, 1987.

[20] I. Tzanakis, A. Pras, D. Kounali et al., "Mitral annular calcifications in haemodialysis patients: a possible protective role of magnesium," *Nephrology Dialysis Transplantation*, vol. 12, no. 9, pp. 2036–2037, 1997.

[21] I. Tzanakis, K. Virvidakis, A. Tsomi et al., "Intra- and extracellular magnesium levels and atheromatosis in haemodialysis patients," *Magnesium Research*, vol. 17, no. 2, pp. 102–108, 2004.

[22] D. M. Spiegel and B. Farmer, "Long-term effects of magnesium carbonate on coronary artery calcification and bone mineral density in hemodialysis patients: a pilot study," *Hemodialysis International*, vol. 13, no. 4, pp. 453–459, 2009.

[23] F. Turgut, M. Kanbay, M. R. Metin, E. Uz, A. Akcay, and A. Covic, "Magnesium supplementation helps to improve carotid intima media thickness in patients on hemodialysis," *International Urology and Nephrology*, vol. 40, no. 4, pp. 1075–1082, 2008.

[24] F. Liu, X. Zhang, H. Qi et al., "Correlation of serum magnesium with cardiovascular risk factors in maintenance hemodialysis patients—a cross-sectional study," *Magnesium Research*, vol. 26, no. 3, pp. 100–108, 2013.

[25] A. R. Cunha, F. Medeiros, B. Umbelino, W. Oigman, R. M. Touyz, and M. F. Neves, "Altered vascular structure and wave reflection in hypertensive women with low magnesium levels," *Journal of the American Society of Hypertension*, vol. 7, no. 5, pp. 344–352, 2013.

[26] M. Fukagawa and J. J. Kazama, "With or without the kidney: the role of FGF23 in CKD," *Nephrology Dialysis Transplantation*, vol. 20, no. 7, pp. 1295–1298, 2005.

[27] D. Russo and Y. Battaglia, "Clinical significance of FGF-23 in patients with CKD," *International Journal of Nephrology*, vol. 2011, Article ID 364890, 5 pages, 2011.

[28] A. Fragoso, A. P. Silva, K. Gundlach, J. Büchel, and P. L. Neves, "Magnesium and FGF-23 are independent predictors of pulse pressure in pre-dialysis diabetic chronic kidney disease patients," *Clinical Kidney Journal*, vol. 7, no. 2, pp. 161–166, 2014.

[29] O. Gutierrez, T. Isakova, E. Rhee et al., "Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease," *Journal of the American Society of Nephrology*, vol. 16, no. 7, pp. 2205–2215, 2005.

[30] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 33, supplement 1, pp. S62–S69, 2010.

[31] A. S. Levey, J. P. Bosch, J. B. Lewis, T. Greene, N. Rogers, and D. Roth, "A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation," *Annals of Internal Medicine*, vol. 130, no. 6, pp. 461–470, 1999.

[32] J. H. Stein, C. E. Korcarz, R. T. Hurst et al., "Use of Carotid Ultrasound to Identify Subclinical Vascular Disease and Evaluate Cardiovascular Disease Risk: a Consensus Statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force Endorsed by the Society for Vascular Medicine," *Journal of the American Society of Echocardiography*, vol. 21, no. 2, pp. 93–111, 2008.

[33] O. I. I. Soliman, A. M. Anwar, A. K. Metawei, J. S. McGhie, M. L. Geleijnse, and F. J. Ten Cate, "New Scores for the assessment of mitral stenosis using real-time three-dimensional echocardiography," *Current Cardiovascular Imaging Reports*, vol. 4, no. 5, pp. 370–377, 2011.

[34] K. Nitta, "Relationship between fibroblast growth factor-23 and mineral metabolism in chronic kidney disease," *International Journal of Nephrology*, vol. 2010, Article ID 167984, 7 pages, 2010.

[35] N. D. Toussaint, E. Pedagogos, S.-J. Tan et al., "Phosphate in early chronic kidney disease: associations with clinical outcomes and a target to reduce cardiovascular risk," *Nephrology*, vol. 17, no. 5, pp. 433–444, 2012.

[36] C. M. McEniery, B. J. McDonnell, A. So et al., "Aortic calcification is associated with aortic stiffness and isolated systolic hypertension in healthy individuals," *Hypertension*, vol. 53, no. 3, pp. 524–531, 2009.

[37] J. Kendrick, A. K. Cheung, J. S. Kaufman et al., "FGF-23 associates with death, cardiovascular events, and initiation of chronic dialysis," *Journal of the American Society of Nephrology*, vol. 22, no. 10, pp. 1913–1922, 2011.

[38] J. Bernheim and S. Benchetrit, "The potential roles of FGF23 and Klotho in the prognosis of renal and cardiovascular diseases," *Nephrology Dialysis Transplantation*, vol. 26, no. 8, pp. 2433–2438, 2011.

[39] F. Corica, A. Corsonello, R. Ientile et al., "Serum ionized magnesium levels in relation to metabolic syndrome in type 2 diabetic patients," *Journal of the American College of Nutrition*, vol. 25, no. 3, pp. 210–215, 2006.

[40] M. M. Nasrallah, A. R. El-Shehaby, M. M. Salem, N. A. Osman, E. El Sheikh, and U. A. S. El Din, "Fibroblast growth factor-23 (FGF-23) is independently correlated to aortic calcification in haemodialysis patients," *Nephrology Dialysis Transplantation*, vol. 25, no. 8, pp. 2679–2685, 2010.

[41] P.-C. T. Pham, P.-M. T. Pham, S. V. Pham, J. M. Miller, and P.-T. T. Pham, "Hypomagnesemia in patients with type 2 diabetes," *Clinical Journal of the American Society of Nephrology*, vol. 2, no. 2, pp. 366–373, 2007.

[42] T. Hashimoto, A. Hara, T. Ohkubo et al., "Serum magnesium, ambulatory blood pressure, and carotid artery alteration: the ohasama study," *American Journal of Hypertension*, vol. 23, no. 12, pp. 1292–1298, 2010.

[43] A. R. Cunha, B. Umbelino, M. L. Correia, and M. F. Neves, "Magnesium and vascular changes in hypertension," *International Journal of Hypertension*, vol. 2012, Article ID 754250, 7 pages, 2012.

[44] A. C. Montezano, D. Zimmerman, H. Yusuf et al., "Vascular smooth muscle cell differentiation to an osteogenic phenotype involves TRPM7 modulation by magnesium," *Hypertension*, vol. 56, no. 3, pp. 453–462, 2010.

[45] J. J. Scialla and M. Wolf, "Roles of phosphate and fibroblast growth factor 23 in cardiovascular disease," *Nature Reviews Nephrology*, vol. 10, no. 5, pp. 268–278, 2014.

[46] R. Jimbo and T. Shimosawa, "Cardiovascular risk factors and chronic kidney disease—FGF23: a key molecule in the cardiovascular disease," *International Journal of Hypertension*, vol. 2014, Article ID 381082, 9 pages, 2014.

[47] S. Seiler, B. Reichart, D. Roth, E. Seibert, D. Fliser, and G. H. Heine, "FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment," *Nephrology Dialysis Transplantation*, vol. 25, no. 12, pp. 3983–3989, 2010.

[48] H. Jüppner, M. Wolf, and I. B. Salusky, "FGF-23: more than a regulator of renal phosphate handling?" *Journal of Bone and Mineral Research*, vol. 25, no. 10, pp. 2091–2097, 2010.

[49] M. Kanbay, M. I. Yilmaz, M. Apetrii et al., "Relationship between serum magnesium levels and cardiovascular events in chronic kidney disease patients," *The American Journal of Nephrology*, vol. 36, no. 3, pp. 228–237, 2012.

[50] A. Silva, A. Fragoso, C. Silva et al., "Magnesium and mortality in patients with diabetes and early chronic kidney disease," *Journal of Diabetes & Metabolism*, vol. 5, article 347, 2014.

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Research Article

Psychometric Properties of the Diabetes Management Self-Efficacy Scale in Korean Patients with Type 2 Diabetes

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Objectives. The aims of this study were to perform a cultural translation of the DMSES and evaluate the psychometric properties of the translated scale in a Korean population with type 2 diabetics. *Methods*. This study was conducted in patients with diabetes recruited from university hospitals. The first stage of this study involved translating the DMSES into Korean using a forward- and backward-translation technique. The content validity was assessed by an expert group. In the second stage, the psychometric properties of the Korean version of the DMSES (K-DMSES) were evaluated. *Results*. The content validity of the K-DMSES was satisfactory. Sixteen-items clustered into four-subscales were extracted by exploratory factor analysis, and supported by confirmatory factor analysis. The construct validity of the K-DMSES with the Summary of Diabetes Self-Care Activities scale was satisfactory ($r = 0.50$, $P < 0.001$). The Cronbach's alpha and intraclass correlation coefficient were 0.92 and 0.85 ($P < 0.001$; 95% CI = 0.75–0.91), respectively, which indicate excellent internal consistency reliability and test-retest reliability. *Conclusions*. The K-DMSES is a brief instrument that has demonstrated good psychometric properties. It is therefore feasible to use in practice, and is ready for use in clinical research involving Korean patients with type 2 diabetes.

1. Introduction

The prevalence of diabetes has reached an almost epidemic level. About 382 million people in the world have diabetes, and this number is expected to rise to 592 million by 2035 [1]. The prevalence of diagnosed diabetes in Korea has increased from 2% in the 1970s to 9.8% in 2011, and in 2012 the rate for patients with poor glycemic control was reportedly as high as 71.5% [2]. These findings suggest the presence of a substantial financial burden on the Korean health-care system.

Diabetes can be substantially improved by performing tasks such as taking prescribed medications, monitoring blood glucose levels, eating an appropriate diet, and exercising regularly. These are all day-to-day behaviors that patients must carry out to control their disease, a process that is termed self-management [3]. The traditional approach to diabetes self-management has been to educate patients about the disease and provide them with the skills necessary to control it [4]. According to one systematic review, although such self-management education appears to be successful, it exerts only small-to-moderate effects on the diabetes [5]. Diabetes researchers insist that providing patients with knowledge and skills is crucial, but these approaches appear to be insufficient for including the required behavioral changes

among patients with diabetes [6, 7]. Therefore, further factors that contribute to more effective diabetes self-management need to be considered.

Self-efficacy, a term that is derived from the social cognitive theory, refers to "belief in one's capability to organize and execute the course of action required to produce given levels of attainments" [8]. Self-efficacy influences the individual's choice of behaviors; people tend to engage in tasks when they feel competent to perform them and to avoid them when they feel that they exceed their capabilities. Self-efficacy also influences how people motivate themselves in the tasks that they undertake. That is, people with a strong sense of self-efficacy view their tasks or behaviors as challenges to be mastered, even if they are difficult. Efficacious people tend to set challenging goals and maintain commitment to them. In addition, self-efficacy beliefs influence emotional states; people with higher self-efficacy are likely to have reduced stress levels and lower risks of depression than those with low self-efficacy [9]. Thus, self-efficacy has emerged as a crucial factor in diabetes self-management behaviors [10–12].

Instruments that measure self-efficacy are broadly categorized into general and specific types of scales. Some researchers view self-efficacy as a more trait-like general construct, referring to one's overall competence to perform across a variety of different situations [13, 14]. Instruments developed based on this perspective are general self-efficacy scales. Others state that self-efficacy judgments are specific to behaviors and the situations in which those behaviors occur [15, 16]; that is, people perceive different levels of capability of performing in particular domains or situations of functioning. Instruments developed from this conceptualization are specific self-efficacy scales. Patients with diabetes must perform particular tasks to control their blood glucose in order to prevent complications. They may possess a high self-efficacy with respect to taking medication, but a low self-efficacy regarding physical exercise. Scales that are specifically designed for patients with diabetes are therefore more appropriate for measuring their self-efficacy [10, 17, 18].

There have been previous attempts in Korea to develop a specific scale measuring the perceived self-efficacy of diabetes self-management [19, 20], but they have produced only a primitive stage of scale development; the items were derived from the literature without verifying their psychometric properties. Applying such instruments in the studies for clinical interventions may threaten the reliability of their outcomes. The Diabetes Management Self-Efficacy Scale (DMSES) is a specific-type instrument that was developed by the members of the International Partnership in Self-Management and Empowerment [21]. Its psychometric properties were found to be acceptable for populations with type 2 diabetes in several countries: Netherlands [21], United Kingdom [22], Australia [23], Turkey [24], and Taiwan [25]. However, these psychometric studies had methodological and statistical problems related to factors such as sample size, item redundancy, and the underlying constructs. With these issues in mind, the aims of the present study were to perform a culture-sensitive translation of the DMSES and then evaluate the psychometric properties of the translated scale in a Korean population with type 2 diabetes.

2. Methods

2.1. Step I: Cultural Translation and Content Validity. The English-language version of the DMSES was translated into Korean using a forward and backward translation technique, based on the guidelines of Brislin [26]. A bilingual health professional and a layperson independently translated the English version into Korean using semantic equivalence. An expert panel of three bilinguals checked the two potential Korean versions and achieved a consensus on a Korean version. The Korean version was then independently translated back into English by another two bilinguals. The panel checked the back-translated versions against the original English version. Any discrepancies between the translated and original English versions were either confirmed by one of the original developers or else a consensus was reached by the panel. The preliminary Korean version was thus produced, and the Korean version was finalized after one professor majored in Korean literature had reviewed its grammar.

Five experts (one physician, one professor in nursing, and three diabetes educators) were involved in assessing the content validity of the final Korean version of the DMSES (K-DMSES). These experts were asked to rate each item of the preliminary K-DMSES whether they considered it "essential," "useful, but not essential," or "not essential" [27]. In addition, they were asked to answer open questions regarding whether or not there were any ambiguous words, jargon, or value-laden words and whether or not there were items that needed to be modified.

2.2. Step II: Psychometric Evaluation of the K-DMSES

2.2.1. Participants and Procedures. This was a methodological study to assess the psychometric properties of the K-DMSES. A convenience sample of 440 patients with type 2 diabetes was recruited from two university hospitals in South Korea. This sample size satisfied the requirement that at least 7 times the total number of items is needed for psychometric tests [28]. The inclusion criteria for the participants were being aged at least 20 years, being diagnosed with diabetes type 2, and being articulate in the Korean language. The participants were asked to sign a consent form and complete a package of questionnaires. Of these, 70 were given an envelope containing the K-DMSES questionnaire for the assessment of test-retest reliability. They were asked to take it home and complete it 10 days later; a time interval of 1-2 weeks between repeated measures is often recommended [28]. Each participant was asked to post the return envelope containing the completed questionnaire near home.

2.2.2. Ethical Consideration. Prior to data collection, this study was approved by the institutional review boards at the participating institutions. Participants were voluntary and those who agreed to participate signed a consent form. All participants were assured of their confidentiality.

2.2.3. Questionnaires. The DMSES [21] is a self-reported questionnaire that comprises 20 items with 4 subscales: nutrition specific and weight, nutrition general and medical

treatment, physical exercise, and blood sugar. Originally, each item was scored on a 5-point scale, but this was later revised to an 11-point scale on the UK English-language version [22]. Possible scores range from 0 to 200, with higher scores reflecting higher self-efficacy. The DMSES satisfied the content validity, factorial construct validity, internal consistency reliability, and test-retest reliability when it was developed. The English-language version of the DMSES, which was obtained from the developer, was translated into Korean and used in this study.

Based on previous studies [12, 25], it was hypothesized in this study that the DMSES was positively and moderately correlated with the Summary of Diabetes Self-Care Activities Scale (SDSCA) [29]. Therefore, the Korean version of the SDSCA was administered to test hypothesis testing construct validity. The SDSCA assesses the frequency of behavioral tasks in five aspects of the diabetes regimen: diet, exercise, self-monitoring of blood glucose, foot care, and smoking for the previous 7 days. The reliability and validity of the SDSCA, which comprises 11 items, were culturally adapted for Korean patients with type 2 diabetes [30, 31].

2.2.4. Statistical Analyses. Statistical analyses were completed using the PASW (version 18) statistical package. General characteristics and missing data were calculated using descriptive statistics. The zero-order correlation matrix among the K-DMSES items was computed using Pearson's analysis.

A cross-validation approach involving both exploratory factor analysis (EFA) and confirmatory factor analysis (CFA) was used for the factorial construct analysis, and for the cross-validation, 440 patients were split into 2 subsamples using a random-sampling function of the computer program (Table 1). The homogeneity of the subsamples with regard to general characteristics was computed using χ^2 or Fisher's exact test. With subsample 1, Bartlett's test of sphericity and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy were screened to justify undertaking EFA [32]. Then, EFA was performed using principal-axis factor analysis with Varimax rotation. Factors with an eigenvalue higher than 1 were retained, and the factor loading criterion was set at ≥0.4 [33]. For the CFA with subsample 2, a maximum-likelihood estimation procedure was performed. Multiple criteria were used to evaluate the model fit: the ratio of the χ^2 value to the degrees of freedom (CMIN/DF), goodness-of-fit index (GFI), standardized root-mean-square residual (SRMR), root-mean-square error of approximation (RMSEA), comparative fit index (CFI), and normed fit index (NFI). The following criteria were used to confirm that a model was an acceptable fit: relative CMIN/DF < 3, GFI > 0.9, SRMR < 0.08, RMSEA < 0.08, CFI > 0.9, and NFI > 0.9 [33–36].

Construct validity by means of the hypothesis testing approach was examined for the entire sample using Pearson's correlation analysis. Internal consistency reliability and test-retest reliability were evaluated using Cronbach's alpha and the intraclass correlation coefficient (ICC), respectively.

3. Results

3.1. Step 1: Cultural Translation and Content Validity. In Korean culture, workers often go out after work to socialize, either formally or informally, as a release from their job-related stresses, and this socializing often involves eating grilled meats or rice and drinking alcohol. It is difficult for a worker at a group dinner to refuse to eat or drink or to order other foods for only himself/herself. Thus, the term "company dinner" was added in the translation process to item 16: "... able to follow a healthy eating pattern when I am eating out, at a party, or at a company dinner." Clinicians in Korea usually recommend that patients with diabetes visit their physicians every 3 months, based on the guidelines of the Korean Diabetes Association [37]. Therefore, item 18 ("... able to visit my doctor once a year to monitor my diabetes") was changed to "... able to visit a clinic or a public health center four times a year to monitor my diabetes."

With respect to the content validity, all of the experts considered all of the items to be essential. However, item 11 ("... able to exercise more if the doctor advises me to") was refined by replacing the term "doctor" in this item with "health professional," since patients with diabetes in Korea receive advice not only from physicians but also from diabetes educators (e.g., nurses or nutritionists). Three experts commented that there were content similarities between items 4 and 5 and between items 13 and 14; however, no deletions were performed at this stage. The experts recommended additional quantitative analysis. All 20 items were retained for the next step of psychometric evaluation.

3.2. Step 2: Psychometric Evaluation of the K-DMSES

3.2.1. Missing Data. The rate of missing values was 0.23% for each of items 3, 11, and 15; these missing values were replaced by the mean value for each item. There were no missing values for any of the other items.

3.2.2. Zero-Order Correlation Matrix. In the 20 × 20 zero-order correlation matrix, items 4/5, 14/13, and 16/15 were strongly correlated (r = 0.80–0.90), as expected from the results for content validity. These strong correlations indicate the presence of redundancy [38], and hence only one item of each pair was retained. Items 4 and 14 were retained because their contents are more specific to diabetes than those of items 5 and 13. Furthermore, item 16 ("eat out, at a party, or at a company dinner") occurs more frequently in daily life than the content of item 15 ("eat on holiday"), and so item 16 was retained. Thus, items 5, 13, and 15 were deleted in order to remove content redundancy.

3.2.3. Factorial Construct Validity. The general characteristics did not differ between subsamples, as assessed by χ^2 or Fisher's exact test (Table 1). With the randomly split subsample 1, the KMO statistic (0.89) and Bartlett's sphericity (χ^2 = 2602.62, $P < 0.001$) indicated that the correlation matrix was suitable for factor analysis. The initial EFA extracted a four-factor solution (eigenvalue > 1, Table 2), which accounted

Table 1: General characteristics.

Variable	Subsample 1 (n = 220) n (%)	Subsample 2 (n = 220) n (%)	χ^2 or Fisher's exact test (P)
Gender			0.146 (0.703)
Male	111 (50.5)	115 (52.3)	
Female	109 (49.5)	105 (47.7)	
Age (years) (mean ± SD = 58.02 ± 0.88)			3.902 (0.561)
20–29	2 (0.9)	3 (1.4)	
30–39	7 (3.2)	10 (4.5)	
40–49	34 (15.5)	30 (13.6)	
50–59	87 (39.5)	71 (32.3)	
60–69	57 (25.9)	68 (30.9)	
≥70	33 (15.0)	38 (17.3)	
Marital status			0.705 (0.894)
Married/living together	178 (80.9)	173 (78.6)	
Divorced/widow(er)	32 (14.5)	37 (16.8)	
Unmarried	9 (4.1)	9 (4.1)	
Other	1 (0.5)	1 (0.5)	
Job			2.142 (0.295)
Employed	102 (46.4)	114 (51.8)	
None	117 (53.1)	106 (48.2)	
Data missing	1 (0.5)	0 (0.0)	
Education			1.230 (0.873)
Elementary school	34 (15.5)	35 (15.9)	
Middle school	30 (13.6)	32 (14.5)	
High school	88 (40.0)	88 (40.0)	
College and above	59 (26.8)	60 (27.3)	
Other	9 (4.1)	5 (2.3)	
Monthly income (KRW)			1.147 (0.766)
Less than 2,000,000	88 (40.0)	79 (35.9)	
2,000,000–2,999,999	40 (18.2)	39 (17.7)	
3,000,000–3,999,999	33 (15.0)	40 (18.2)	
4,000,000 and above	56 (25.4)	54 (24.5)	
Data missing	3 (1.4)	8 (3.6)	
Treatment regimen			1.164 (0.762)
Diet/exercise only	9 (4.1)	7 (3.2)	
Oral hypoglycemic agent	141 (64.1)	151 (68.6)	
Insulin	10 (4.5)	10 (4.5)	
Oral hypoglycemic agent + insulin	60 (27.3)	52 (23.6)	
HbA1c (mean ± SD = 7.70 ± 1.38)			0.011 (0.918)
Controlled (HbA1c < 7.0%)	69 (31.4)	70 (31.8)	
Uncontrolled (HbA1c ≥ 7.0%)	151 (68.6)	150 (68.2)	

HbA1c: hemoglobin A1c; KRW: South Korean won.

for 65.81% of the total variance. Item 7 was not significantly loaded on any factors at a criterion of > 0.40. EFA was conducted after deleting that item (Table 2), again yielding a four-factor solution that explained 67.28% of the total variance in all items. All items were significantly loaded onto one of four factors. There was no significant cross-loading of items on the factors. Factors 1–4 were labeled "nutrition" (items 4, 9, 10, 14, 16, and 17), "physical exercise/body weight" (items 6, 8, 11, and 12), "medical treatment" (items 18, 19, and 20), and "blood sugar" (items 1, 2, and 3).

To cross-validate the 16-item, 4-factor construct, CFA was conducted with the randomly split subsample 2. The

Table 2: Exploratory factor analyses.

Number	Abbreviated item description	First exploratory factor analysis[a]				Second exploratory factor analysis[b]			
		F1[c]	F2[d]	F3[e]	F4[f]	F1[c]	F2[d]	F3[e]	F4[f]
1	Checking blood sugar				0.46				0.46
2	Correcting high blood sugar				0.80				0.84
3	Correcting low blood sugar				0.83				0.81
4	Choosing foods	0.52				0.53			
6	Controlling body weight		0.59				0.58		
7	Examining feet for cuts					—	—	—	—
8	Taking physical exercise		0.74				0.74		
9	Adjusting eating plan during illness	0.53				0.54			
10	Following a healthy eating pattern	0.67				0.57			
11	Taking physical exercise on doctor's advice		0.84				0.81		
12	Balancing between exercise and eating plan		0.74				0.74		
14	Adjusting eating plan: when I am away from home	0.75				0.76			
16	Eating pattern: eating out, eating at a party or company dinner	0.80				0.79			
17	Eating plan related to stress or anxiety	0.76				0.74			
18	Visiting doctor four times a year			0.73				0.70	
19	Taking medication as prescribed			0.87				0.91	
20	Adjusting medication during illness			0.74				0.73	

[a]First exploratory factor analysis: Kaiser-Meyer-Olkin (KMO) statistic = 0.89, Bartlett's sphericity $\chi^2 = 2602.62$ ($P < 0.001$).
[b]Second exploratory factor analysis: KMO statistic = 0.89, Bartlett's sphericity $\chi^2 = 2461.10$ ($P < 0.001$).
[c]Factor 1: nutrition.
[d]Factor 2: physical exercise/body weight.
[e]Factor 3: medical treatment.
[f]Factor 4: blood sugar.

Table 3: Summary of fit indices from confirmatory factor analysis.

	χ^2	df	CMIN/DF	GFI	SRMR	RMSEA (90% CI)	CFI	NFI
Model 1	391.57*	98	3.99	0.81	0.07	0.12 (0.10–0.13)	0.87	0.84
Model 2	325.06*	97	3.35	0.85	0.06	0.10 (0.09–0.11)	0.90	0.97
Model 3	284.50*	96	2.96	0.87	0.06	0.09 (0.08–0.10)	0.92	0.88
Model 4	253.11*	95	2.66	0.88	0.06	0.08 (0.07–0.10)	0.93	0.90

df: degrees of freedom; CMIN/DF: ratio of χ^2 value to the degrees of freedom; GFI: goodness-of-fit index; SRMR: standardized root-mean-square residual; RMSEA (90% CI): root-mean-square error of approximation with 90% of confidence interval; CFI: comparative fit index; NFI: normed fit index.
*$P < 0.001$.

SRMR value indicated an acceptable model fit, where the values of the other indexes indicated a poor-fitting model (Model 1, Table 3). Thus, the possibility of model modification was explored using modification indices (MIs) [39], which revealed that the MI value of pairing of error terms between items 14 and 16 was the largest, at 57.38. After modifying the covariance between the error terms of items 14 and 16 (Model 2), the model fit was significantly improved ($\Delta\chi^2(1) = 66.51$, $P < 0.05$). However, the values of some model-fit indexes (CMIN/DF, GFI, and RMSEA) were unsatisfactory, and there was still a large MI value (36.63) between the error terms of items 16 and 17. With this modification, CFA produced a

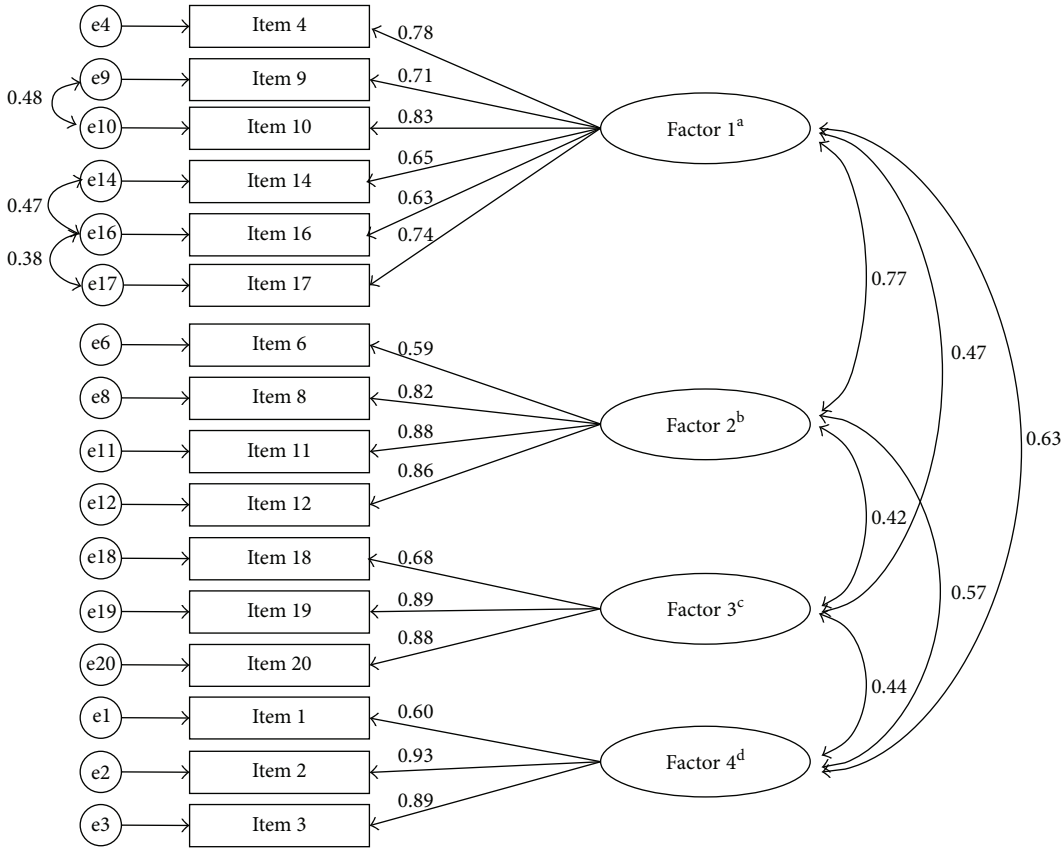

Figure 1: Confirmatory factor analysis for the Korean version of the Diabetes Management Self-Efficacy Scale. e: error term. [a]Factor 1: nutrition. [b]Factor 2: physical exercise/body weight. [c]Factor 3: medical treatment. [d]Factor 4: blood sugar.

significantly improved Model 3 ($\Delta\chi^2(1) = 40.56$, $P < 0.05$). After the final modification of the covariance between the error terms of items 9 and 10 (MI = 17.06), Model 4 was significantly improved compared with Model 3 ($\Delta\chi^2(1) = 31.39$, $P < 0.05$), and the values of all goodness-of-fit indexes, except GFI, were satisfactory. All items loaded meaningfully onto factors with standardized values ranged from 0.59 to 0.93 (Figure 1).

3.2.4. Hypothesis Testing Construct Validity with the Total Sample. The K-DMSES score was moderately correlated with the SDSCA score ($r = 0.50$, $P < 0.001$), as hypothesized for the construct validity.

3.2.5. Internal Consistency Reliability with the Total Sample. Overall Cronbach's alpha of the K-DMSES was 0.92, which indicates excellent internal consistency reliability. Cronbach's alpha values for the subscales of nutrition, physical exercise/body weight, medical treatment, and blood sugar were 0.89, 0.87, 0.86, and 0.84, respectively, which were all above the acceptability criterion of ≥0.70 [40].

3.2.6. Test-Retest Reliability. Of the 70 patients who were asked to complete the K-DMSES twice, 82.85% ($n = 58$) completed it twice. The ICC for the overall K-DMSES score was 0.85 ($P < 0.001$; 95% confidence interval = 0.75–0.91), reflecting a satisfactory test-retest reliability. ICCs for the nutrition, physical exercise/body weight, medical treatment, and blood sugar subscales were 0.87, 0.78, 0.62, and 0.88, respectively.

4. Discussion

This study translated the DMSES into Korean and evaluated its psychometric properties in Korean type 2 diabetes patients. The psychometric properties of the culturally adapted K-DMSES were satisfactory. The total number of items in the K-DMSES was 16, which is fewer than in all other language versions of the DMSES except for the UK-English version, which comprises 15 items [22]. A shorter K-DMSES may represent a smaller burden for patients with type 2 diabetes, rendering it more feasible to use in practice.

Translation and back-translation of a questionnaire requires not only literal translation but also social/cultural adaptation. In this study, item 18 of the K-DMSES was changed to "… four times a year to monitor my diabetes," based on the guidelines of the Korean Diabetes Association. A similar change was also made in the Taiwanese/Chinese version [25], in accordance with Taiwanese regulations of the Bureau of National Health Insurance. In the UK version, the item was deleted based on the National Health Service (such

as GP care system in the UK) [22]. The inclusion or wording of item 18 may depend upon the prevailing health system or health policy in the country in which the questionnaire will be used.

Item redundancy on the DMSES is constantly being discussed. McDowell et al. [23] reported strongly correlated items (items 2/3, 8/11, 13/14, 13/15, and 14/15) in the Australian-English version. Sturt et al. [22] also noted duplicated items (items 4/5, 5/10, 13/14, and 13/15) in the content validity of the UK-English version. Similarly, redundancy of items 4/5, 13/14, and 15/16 was found in the K-DMSES for the content validity and the zero-order correlation matrix of items. If items of a scale are strongly correlated, it is recommended that the redundant ones should be dropped. This prevents a methodological problem with multicollinearity [38].

Factorial construct validity in this study demonstrated that the K-DMSES comprises four subscales. The items clustered into each subscale were similar to those of the Taiwanese/Chinese version [25]. The Dutch version also comprises four subscales, wherein the clustered items on the physical exercise and blood sugar subscales were similar to those of the two aforementioned versions, but the items on the other two subscales ("nutrition specific and weight" and "nutrition general and medical treatment") were clustered differently [21]. This finding in the study of the Dutch version may be attributable to the use of an insufficient sample size ($N = 94$) for a principal component analysis. An inadequate sample size was also a weakness in the psychometric study of the Turkish version of the DMSES ($N = 101$), which revealed three subscales [24]. In addition, a single subscale was reported for the UK-English version [22], which accounted for only 41% of total variance of all items. This unidimensionality is not congruent with the assertion that diabetes management of self-efficacy is multifaceted [41]. Moreover, the total amount of variance accounted for by that unidimensionality did not meet the criterion of >50% [28].

Item 7 ("I am able to examine my feet for cuts") has been inconsistent in its loading on factor analyses: it loaded onto the general nutrition and medical treatment subscale of the Dutch version [21], the diet/feet control subscale of the Turkish version [24], and the blood sugar/feet check subscale of the Taiwanese/Chinese version [25]. Furthermore, the item was statistically deleted in the present study. This lack of consistency may be due to there being only one item related to the confidence of foot care in the DMSES, with this item possibly being treated as relatively heterogeneous, resulting in it being statistically clustered onto various subscales, or even deleted from the scale. If there were more items related to item 7, its own subscale might have been constructed. Given that at least three items are required for a latent construct [42], it is recommended that two items should be added in future studies, for example, "confident of protecting my feet from hot and cold" and "confident of putting on shoes and socks at all times."

Only EFA has been performed to evaluate the factorial construct validity of the DMSES—CFA has never been performed. This is the first study in which both EFA and CFA have been performed to validate the DMSES, applying a cross-validation approach. This approach has the merit of exploring the underlying construct of the items and simultaneously confirming the stability of those underlying constructs [43]. In the present study the four-subscale construct extracted from the EFA was empirically supported by CFA. However, the CFA revealed that there was covariance between the error terms of three pairs of items, items 9/10, 14/16, and 16/17, implying the presence of an unknown systematic error. Byrne [39] reported that a systematic error may occur due to an overlap in the content of items. The contents of the three pairs of items all related to "eating-related confidence." Therefore, further study is needed to remove the possibility of content overlap.

Construct validation by means of the hypothesis testing approach refers to the correlation with one or more well-established instruments, based on a prior hypothesis [44]. The present study has demonstrated the construct validity of the K-DMSES, with a moderate correlation with the SDSCA. The Taiwanese/Chinese version of the DMSES exhibited a similar correlation ($r = 0.58$) to the SDSCA [25].

A Cronbach's alpha value of between 0.70 and 0.95 indicates sufficient item correlations and a low redundancy of items [28]. Overall Cronbach's alpha was a little higher for the K-DMSES (0.92) than for the Dutch version (0.81) [21], the UK-English version (0.89) [22], and the Turkish version (0.88) [24] and was similar to that of the Australian-English version (0.91) [22] and the Chinese version (0.93) [25]. Together these findings suggest that the DMSES has a good internal consistency across languages.

Test-retest reliability refers to the temporal stability of a scale between two time points, and the most commonly used criteria for evaluating this parameter are Pearson's r or ICC > 0.70 [40]. Pearson's r for the test-retest reliability ranged from 0.76 to 0.86 for the Dutch [21], Australian-English [22], and Chinese [25] versions. However, Pearson's r is criticized for being insufficiently rigorous for assessing reliability. It does not consider systematic differences as a part of measurement error, so Pearson's r value is usually higher than the ICC. The ICC is considered a more reliable parameter for continuous variables [28] and so was calculated in the present study, yielding a value of 0.85, which is higher than that of the UK-English version (0.77) [22] and lower than that of the Turkish version (0.91) [24]. These findings suggest that the overall test-retest reliability of the DMSES is stable over time across languages. However, the medical treatment subscale in this study was characterized by a relatively low ICC (0.62). Similarly, the temporal stability of that subscale was unsatisfactory in the Taiwanese/Chinese version ($r = 0.69$) [25]. Other studies have determined only overall values, not values for the subscales, so it is currently difficult to determine why the medical treatment subscale lacks stability.

A limitation of this study is the lack of a responsiveness test to detect changes when patients improve or deteriorate [45]. A longitudinal study should therefore be conducted which assesses the K-DMSES scores of patients in whom changes are expected to occur.

Regarding test-retest reliability, the time interval between repeated measures should be justified. In general, it is preferable for the time interval to be sufficiently long to prevent recall, but short enough so as to ensure that a clinical

change has not occurred [28]. Diverse time intervals have been applied in reliability testing of the DMSES: 10 days (present study), 2 weeks [25], 3 weeks [23], 4 weeks [22, 24], and 5 weeks [21]. One empirical study found no significant differences in the test-retest reliability of health-status instruments when time intervals of 2 days and 2 weeks were applied [46]. More studies of the optimal time interval for the test-retest reliability of the DMSES are required.

5. Conclusion

The K-DMSES was subjected to culture-sensitive translation and its psychometric properties were validated in Korean type 2 diabetes patients. The underlying construct of the K-DMSES comprises four subscales: nutrition (items 4, 9, 10, 14, 16, and 17), physical exercise/body weight (items 6, 8, 11, and 12), medical treatment (items 18, 19, and 20), and blood sugar (items 1, 2, and 3). The K-DMSES demonstrated good content validity, factorial construct validity, hypothesis testing construct validity, internal consistency reliability, and test-retest reliability. This instrument is ready for use in both research and practice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] International Diabetes Federation, *Diabetes Atlas*, 6th edition, 2013, http://www.idf.org/diabetesatlas/introduction.

[2] Korea Centers for Disease Control and Prevention, *Korea Health Statistics 2011: Korea National Health and Nutrition Examination Survey (KNHANES V-2)*, Korea Center for Disease Control and Prevention & Ministry of Health and Welfare, Seoul, Republic of Korea, 2011.

[3] N. M. Clark, M. H. Becker, N. K. Janz, K. Lorig, W. Rakowski, and L. Anderson, "Self-management of chronic disease by older adults: a review and questions for research," *Journal of Aging and Health*, vol. 3, no. 1, pp. 3–27, 1991.

[4] K. Krichbaum, V. Aarestad, and M. Buethe, "Exploring the connection between self-efficacy and effective diabetes self-management," *Diabetes Educator*, vol. 29, no. 4, pp. 653–662, 2003.

[5] A. Warsi, P. S. Wang, M. P. LaValley, J. Avorn, and D. H. Solomon, "Self-management education programs in chronic disease: a systematic review and methodological critique of the literature," *Archives of Internal Medicine*, vol. 164, no. 15, pp. 1641–1649, 2004.

[6] R. E. Glasgow and V. L. Osteen, "Evaluating diabetes education: are we measuring the most important outcomes?" *Diabetes Care*, vol. 15, no. 10, pp. 1423–1432, 1992.

[7] K. M. Knight, T. Dornan, and C. Bundy, "The diabetes educator: trying hard, but must concentrate more on behaviour," *Diabetic Medicine*, vol. 23, no. 5, pp. 485–501, 2006.

[8] A. Bandura, "Health promotion from the perspective of social cognitive theory," *Psychology and Health*, vol. 13, no. 4, pp. 623–649, 1998.

[9] A. Bandura, "Health promotion by social cognitive means," *Health Education and Behavior*, vol. 31, no. 2, pp. 143–164, 2004.

[10] A. C. Hurley and C. A. Shea, "Self-efficacy: strategy for enhancing diabetes self-care," *The Diabetes Educator*, vol. 18, no. 2, pp. 146–150, 1992.

[11] K. M. Nelson, L. McFarland, and G. Reiber, "Factors influencing disease self-management among veterans with diabetes and poor glycemic control," *Journal of General Internal Medicine*, vol. 22, no. 4, pp. 442–447, 2007.

[12] K. E. Williams and M. J. Bond, "The roles of self-efficacy, outcome expectancies and social support in the self-care behaviours of diabetics," *Psychology, Health and Medicine*, vol. 7, no. 2, pp. 127–141, 2002.

[13] M. Sherer, J. E. Maddux, B. Mercandante, S. Prentice-Dunn, B. Jacobs, and R. W. Rogers, "The self-efficacy scale: construction and validation," *Psychological Reports*, vol. 51, no. 2, pp. 663–671, 1982.

[14] J. X. Zhang and R. Schwarzer, "Measuring optimistic self-beliefs: a Chinese adaptation of the general self-efficacy scale," *Psychologia*, vol. 38, pp. 174–181, 1995.

[15] A. Bandura, "Guide for constructing self-efficacy scales," in *Self-Efficacy Beliefs of Adolescent*, F. Pajares and T. Urban, Eds., pp. 307–337, Information Age Publishing, Charlotte, NC, USA, 2006.

[16] E. Maibach and D. A. Murphy, "Self-efficacy in health promotion research and practice: conceptualization and measurement," *Health Education Research*, vol. 10, no. 1, pp. 37–50, 1995.

[17] O. A. Al-Khawaldeh, M. A. Al-Hassan, and E. S. Froelicher, "Self-efficacy, self-management, and glycemic control in adults with type 2 diabetes mellitus," *Journal of Diabetes and its Complications*, vol. 26, no. 1, pp. 10–16, 2012.

[18] J. J. van der Bijl and L. M. Shortridge-Baggett, "The theory and measurement of the self-efficacy construct," *Research and Theory for Nursing Practice*, vol. 15, no. 3, pp. 189–207, 2001.

[19] J. H. Kim and S. A. Chang, "Effect of diabetes education program on glycemic control and self management for patients with type 2 diabetes mellitus," *Korean Diabetes Journal*, vol. 33, pp. 518–525, 2009.

[20] K. S. Paek and Y. H. Choi, "Sick-role behavioral compliance and influencing factors in patients with diabetes," *Korean Journal of Adult Nursing*, vol. 10, pp. 480–491, 1998.

[21] J. J. Van Der Bijl, A. Van Poelgeest-Eeltink, and L. Shortridge-Baggett, "The psychometric properties of the diabetes management self-efficacy scale for patients with type 2 diabetes mellitus," *Journal of Advanced Nursing*, vol. 30, no. 2, pp. 352–359, 1999.

[22] J. Sturt, H. Hearnshaw, and M. Wakelin, "Validation and reliability of the DMSES UK: a measure of self-efficacy for type 2 diabetes self-management," *Primary Health Care Research & Development*, vol. 11, pp. 374–381, 2010.

[23] J. McDowell, M. Courtney, H. Edwards, and L. Shortridge-Baggett, "Validation of the australian/english version of the

diabetes management self-efficacy scale," *International Journal of Nursing Practice*, vol. 11, no. 4, pp. 177–184, 2005.

[24] M. Kara, J. J. van der Bijl, L. M. Shortridge-Baggett, T. Asti, and S. Erguney, "Cross-cultural adaptation of the diabetes management self-efficacy scale for patients with type 2 diabetes mellitus: scale development," *International Journal of Nursing Studies*, vol. 43, no. 5, pp. 611–621, 2006.

[25] S.-F. Vivienne Wu, M. Courtney, H. Edwards, J. McDowell, L. M. Shortridge-Baggett, and P.-J. Chang, "Development and validation of the Chinese version of the diabetes management self-efficacy scale," *International Journal of Nursing Studies*, vol. 45, no. 4, pp. 534–542, 2008.

[26] R. W. Brislin, "Back-translation for cross-cultural research," *Journal of Cross-Cultural Psychology*, vol. 1, pp. 185–216, 1970.

[27] L. Veneziano and J. Hooper, "A method for quantifying content validity of health-related questionnaires," *American Journal of Health Behavior*, vol. 21, no. 1, pp. 67–70, 1997.

[28] C. B. Terwee, S. D. Bot, M. R. de Boer et al., "Quality criteria were proposed for measurement properties of health status questionnaires," *Journal of Clinical Epidemiology*, vol. 60, no. 1, pp. 34–42, 2007.

[29] D. J. Toobert, S. E. Hampson, and R. E. Glasgow, "The summary of diabetes self-care activities measure: results from 7 studies and a revised scale," *Diabetes Care*, vol. 23, no. 7, pp. 943–950, 2000.

[30] S. Chang and M. Song, "The validation and reliability of a Korean version of the Summary of Diabetes Self-Care Activities questionnaire for older patients with type 2 diabetes," *Korean Journal of Adult Nursing*, vol. 21, pp. 235–244, 2009.

[31] E. J. Choi, M. Nam, S. H. Kim et al., "Psychometric properties of a Korean version of the summary of diabetes self-care activities measure," *International Journal of Nursing Studies*, vol. 48, no. 3, pp. 333–337, 2011.

[32] H. F. Kaiser, "An index of factorial simplicity," *Psychometrika*, vol. 39, no. 1, pp. 31–36, 1974.

[33] B. G. Tabachnick and L. S. Fidell, *Using Multivariate Statistics*, Pearson Education, Upper Saddle River, NJ, USA, 6th edition, 2012.

[34] L.-T. Hu and P. M. Bentler, "Cutoff criteria for fit indexes in covariance structure analysis: conventional criteria versus new alternatives," *Structural Equation Modeling*, vol. 6, no. 1, pp. 1–55, 1999.

[35] R. B. Kline, *Principles and Practice of Structural Equation Modeling*, Guilford Press, New York, NY, USA, 2nd edition, 2005.

[36] R. C. MacCallum, M. W. Browne, and H. M. Sugawara, "Power analysis and determination of sample size for covariance structure modeling," *Psychological Methods*, vol. 1, no. 2, pp. 130–149, 1996.

[37] Korean Diabetes Association, *Clinical Practice Guideline: Type 2 Diabetes*, 2013, http://www.diabetes.or.kr/pro/publish/guide_2013.php?mode= list.

[38] M. A. Pett, N. R. Lackey, and J. J. Sullivan, *Make Sense of Factor Analysis*, Sage, Thousand Oaks, Calif, USA, 2003.

[39] B. M. Byrne, *Structural Equation Modeling with AMOS: Basic Concepts, Applications, and Programming*, Taylor & Francis, New York, NY, USA, 2nd edition, 2010.

[40] J. C. Nunnally and I. H. Bernstein, *Psychometric Theory*, McGraw-Hill, New York, NY, USA, 1994.

[41] P. Rapley, A. Passmore, and M. Phillips, "Review of the psychometric properties of the diabetes self-efficacy scale: Australian longitudinal study," *Nursing & Health Sciences*, vol. 5, no. 4, pp. 289–297, 2003.

[42] J. D. Cook, S. J. Hepworth, and P. B. Warr, *The Experience of Work*, Academic Press, San Diego, Calif, USA, 1981.

[43] H. C. W. De Vet, H. J. Adèr, C. B. Terwee, and F. Pouwer, "Are factor analytical techniques used appropriately in the validation of health status questionnaires? A systematic review on the quality of factor analysis of the SF-36," *Quality of Life Research*, vol. 14, no. 5, pp. 1203–1218, 2005.

[44] L. G. Portney and M. P. Watkins, *Foundations of Clinical Research. Applications to Practice*, Pearson/Prentice Hall, Upper Saddle River, NJ, USA, 2009.

[45] P. M. Fayers and D. Machin, *Quality of Life: The Assessment, Analysis and Interpretation of Patient-Related Outcomes*, John Wiley & Sons, West Sussex, UK, 2nd edition, 2007.

[46] R. G. Marx, A. Menezes, L. Horovitz, E. C. Jones, and R. F. Warren, "A comparison of two time intervals for test-retest reliability of health status instruments," *Journal of Clinical Epidemiology*, vol. 56, no. 8, pp. 730–735, 2003.

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Research Article

Altered Daytime Fluctuation Pattern of Plasminogen Activator Inhibitor 1 in Type 2 Diabetes Patients with Coronary Artery Disease: A Strong Association with Persistently Elevated Plasma Insulin, Increased Insulin Resistance, and Abdominal Obesity

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This study was aimed at investigating daily fluctuation of PAI-1 levels in relation to insulin resistance (IR) and daily profile of plasma insulin and glucose levels in 26 type 2 diabetic (T2D) patients with coronary artery disease (CAD) (group A), 10 T2D patients without CAD (group B), 12 nondiabetics with CAD (group C), and 12 healthy controls (group D). The percentage of PAI-1 decrease was lower in group A versus group B (4.4 ± 2.7 versus 35.0 ± 5.4%; $P < 0.05$) and in C versus D (14.0 ± 5.8 versus 44.7 ± 3.1%; $P < 0.001$). HOMA-IR was higher in group A versus group B ($P < 0.05$) and in C versus D ($P < 0.01$). Simultaneously, AUCs of PAI-1 and insulin were higher in group A versus group B ($P < 0.05$) and in C versus D ($P < 0.01$), while AUC of glucose did not differ between groups. In multiple regression analysis waist-to-hip ratio and AUC of insulin were independent determinants of decrease in PAI-1. The altered diurnal fluctuation of PAI-1, especially in T2D with CAD, might be strongly influenced by a prolonged exposure to hyperinsulinemia in the settings of increased IR and abdominal obesity, facilitating altogether an accelerated atherosclerosis.

1. Introduction

Fibrinolysis in blood is mediated by activation of tissue type plasminogen activator (t-PA) whose main role is to convert circulating plasminogen to plasmin resulting in lysis of clots and thrombi. The specific inhibitor of t-PA, plasminogen activator inhibitor 1 (PAI-1), a circulating protein, could attenuate the activity of the fibrinolytic system with consequent thrombus formation [1]. Moreover, it has been clearly shown that overexpression of PAI-1 in the vessel wall predisposes to the development of vulnerable plaques formation and acute coronary syndromes [2]. Previous studies found significantly increased levels of PAI-1 in patients with myocardial infarction (MI) [3], stable or unstable coronary artery diseases (CAD) [4], or even endothelial dysfunction [5]. In addition, increased concentration of PAI-1 in blood and arterial wall was also found in patients with obesity [6, 7], metabolic syndrome [8], and type 2 diabetes [9, 10]. Those results implied that increased expression of PAI-1, possibly induced by insulin resistance and hyperinsulinemia as major metabolic impairments underlying these diseases, could be a factor contributing to premature CAD frequently seen in patients with diabetes [2]. Furthermore, investigations in animal models have shown that infusion of insulin and proinsulin [11], as well as acute hyperglycemia and hyperinsulinemia [12], increased expression of PAI-1. In humans, localized intra-arterial infusions of insulin also induced increase in the concentration of PAI-1 in blood facilitating impaired fibrinolysis [13]. However, clamp studies performed in relatively small number of obese subjects (some of them with diabetes) do not

confirm that insulin acutely influenced fibrinolysis. It was speculated that hypofibrinolysis due to increased PAI-1 activity, detected in patients with obesity or T2D, may be linked rather to chronic hyperinsulinism, that is, insulin resistance [14].

It was known for many years that fibrinolytic activity in humans is significantly reduced in the morning mainly due to the highest value of PAI-1 activity at 06:00–08:00 and the nadir in the late afternoon (18:00) [15, 16]. Recently, it was shown that the morning peak of PAI-1 in healthy subjects is caused by internal circadian system independent of sleep/wake cycle and is not induced by behaviors that occur in the morning (altered posture or physical activity) [17]. Investigations done in patients with acute MI have shown that increased PAI-1 together with decreased fibrynolisis in the morning, found in these patients, could be associated with the morning peak of appearance of acute coronary syndrome which was frequently observed [18]. Although some rare previous studies have detected alteration in diurnal fluctuation of PAI-1 in patients with CAD [19], the impairments of PAI-1 circadian variation in T2D, especially in the presence of CAD, remain still unclarified. Therefore, this study was aimed at investigating the daily fluctuation of PAI-1 activity in T2D and nondiabetic patients with previously diagnosed CAD and to examine the relationship of these fluctuations to the changes in plasma insulin, insulin resistance, glucose, and lipid as well as anthropometric parameters, previously suggested to be possible determinants of PAI-1 daily profile in those patients.

2. Material and Methods

2.1. Study Populations. In this study, we included 60 T2D and nondiabetic patients divided into the groups according to the presence of CAD: 26 T2D patients with documented CAD (group A), 10 T2D without CAD (group B), and 12 nondiabetic patients with CAD (group C). Group D consisted of 12 healthy controls subjects. T2D was previously diagnosed according to the WHO criteria [20]. Patients with diabetes were treated by diet and/or oral agents (metformin and sulfonylurea) and none of them was using thiazolidinediones or insulin. The CAD was diagnosed by cardiologists, based on clinical feature, history of myocardial infarction, and stable angina pectoris or was angiographically verified. Patients with unstable angina pectoris, heart failure, acute myocardial infarction or coronary interventions within the last six months, acute or chronic infections, and malignant diseases were not included. None of the patients was on anticoagulants or corticosteroids at the time of the study. All the participants gave their informed consent for the study which was approved by the Institutional Ethics Committee.

2.2. Study Protocol. The investigations were performed in each subject included in the study within the same day. After an interview with questions regarding patient medical history, current medical condition, and medication use, anthropometric measurements were done. Body weight and height were measured with a digital scale and body mass index (BMI) was calculated (kg/m^2), while the relationship between waists and hip circumference (measured by soft tape) was expressed as waist-to-hip ratio (WHR) and served as marker of abdominal obesity. The blood samples for basal laboratory analysis were drawn in the morning, at 08:00, after 12-hour overnight fasting, in a supine position after 30 minutes of rest, from the antecubital vein. The participants were asked to refrain from any physical activity and drinking coffee or alcohol, while smokers were asked to refrain from smoking during the day when testing was done. In order to analyze daytime fluctuation of PAI-1 activity, blood was collected during the day, in hospital settings with standard hospital meals, before and 2 hours after the main meal (at 08:00, 10:00, 13:00, 15:00, and 18:00) from each subject included in the study. Simultaneously, at the same time points, blood samples were collected for determination of plasma glucose and plasma insulin levels. The differences between PAI-1 activity between morning (08:00) and evening values (18:00) were calculated and expressed as percentage of PAI-1 decrease during the day. Also, area under the curve (AUC) was calculated for PAI-1, insulin, and glucose values.

2.3. Laboratory Analysis. All assays were performed using commercially available kits on paired samples. Blood for determination of PAI-1 activity in plasma was collected into 5 mL tubes containing 0.5 mL of sodium citrate. The samples were immediately centrifuged for 10 minutes at 3000 g and 4°C, and then plasma was carefully separated, transferred to small vials, and stored at −80°C until analyzed. The level of PAI-1 activity in plasma was determined by using plasminogen/chromogenic plasmin substrate assay (kit Behring, Germany). The normal range for PAI-1 activity by using this test is 0.3–3.5 U/mL with an interassay CV of 7.7%. Plasma glucose was determined by using glucose oxidase method on Beckman glucose analyzer (Beckman Instruments, Fullerton, USA). Plasma insulin levels were measured by radioimmunoassay with double antibodies kits (INEP, Zemun, Serbia). Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) and was calculated from fasting plasma insulin and glucose levels according to the formula: (insulin (mU/L) × glucose (mmol/L))/22.5 [21]. Determination of lipid parameters, total cholesterol (chol), HDL-chol, and triglycerides concentrations were analyzed using commercial enzymatic kit (Boehringer Mannheim GmbH Diagnostics), while LDL-chol levels were calculated by using standard Friedewald formula.

2.4. Statistical Analysis. The statistical analyses were performed using SPSS software, version 17.0 (SPSS Inc., USA), and data are presented as mean ± SD. Kolmogorov-Smirnov test was used for testing normality of data distribution and variables not normally distributed were log-transformed. The presence of significant linear trends in variable distribution was assessed with analysis of variance (ANOVA) with a post hoc comparison using Bonferroni test. Categorical variables were tested with Kruskal-Wallis test. The total integrated response area under the curve (AUC) for PAI-1, glucose, and insulin was calculated by trapezoid method. The Pearson correlation coefficient or the Spearman's rank correlation was used to test the relationship between the variables. In order to

Table 1: Patient characteristics.

Variables	Group A T2D+ CAD+	Group B T2D+ CAD−	Group C T2D− CAD+	Group D Controls
n	26	10	12	12
Gender (m/f)	18/8	3/7	9/3	5/7
Age (years)	56.9 ± 9.2*	54.5 ± 7.2	55.9 ± 11.0	53.9 ± 11.0
Duration of diabetes (years)	9.8 ± 4.2	9.6 ± 3.6	/	/
Duration of CAD (years)	6.9 ± 4.5	/	6.2 ± 3.0	/
Hypertension (*n*, %)	18 (69.2)	7 (70.0)	9 (75.0)	4 (33.3)[a]
Smokers (*n*, %)	13 (50.0)	4 (40.0)	6 (50.0)	7 (58.0)

*Data are expressed as mean ± SD; T2D: type 2 diabetes; CAD: coronary artery disease.
[a]$P < 0.05$ versus groups A, B, and C.

Table 2: Metabolic and anthropometric parameters.

Variables	Group A T2D+ CAD+	Group B T2D+ CAD−	Group C T2D− CAD +	Group D Controls	*P*-trend ANOVA
Fasting glucose (mmol/L)	8.5 ± 2.5*	9.2 ± 2.3	5.2 ± 0.6	5.0 ± 0.9	<0.001
HbA1c (%)	7.6 ± 1.2	8.2 ± 1.5	5.3 ± 0.6	5.1 ± 0.6	<0.001
Fasting insulin (mU/L)	22.97 ± 2.99	13.29 ± 1.72[a]	13.19 ± 1.30	10.12 ± 3.88[b]	0.018
HOMA-IR	9.3 ± 1.9	5.6 ± 1.0[a]	3.1 ± 1.2	2.2 ± 0.8[b]	0.043
Total chol (mmol/L)	6.6 ± 1.1	6.6 ± 0.9	7.1 ± 1.2	6.2 ± 0.9	0.262
HDL-chol (mmol/L)	1.15 ± 0.38	1.39 ± 0.50	1.25 ± 0.37	1.56 ± 0.30[b]	0.023
LDL-chol (mmol/L)	4.3 ± 1.0	3.8 ± 1.0	4.8 ± 0.9	3.9 ± 0.9	0.127
Triglycerides (mmol/L)	2.26 ± 1.12	2.97 ± 1.20	1.91 ± 0.81	1.52 ± 0.52	0.010
PAI-1 (U/mL)	5.58 ± 1.48	3.83 ± 1.03[a]	4.34 ± 0.82	2.68 ± 0.63[b]	<0.001
BMI (kg/m^2)	27.5 ± 2.9	28.3 ± 4.7	27.2 ± 3.2	25.6 ± 3.6	0.116
WHR	0.97 ± 0.07	0.94 ± 0.08[a]	0.95 ± 0.06	0.83 ± 0.08[b]	<0.001

*Data are expressed as mean ± SD; T2D: type 2 diabetes; CAD: coronary artery disease; HOMA-IR: homeostasis model for insulin resistance; chol: cholesterol; PAI-1: plasminogen activator inhibitor 1; BMI: body mass index; WHR: waist-to-hip ratio.
[a]Group A versus group B: $P < 0.05$ for fasting insulin, HOMA-IR and WHR; $P < 0.01$ for PAI-1 (post hoc comparisons using Bonferroni test).
[b]Group C versus group D: $P < 0.05$ for fasting insulin, HOMA-IR, HDL-chol and PAI-1; $P < 0.001$ for WHR (post hoc comparisons using Bonferroni test).

analyze the determinants of PAI-1 fluctuations during the day, stepwise multiple regression analysis was performed with the percentage of PAI-1 decrease as a dependent variable. Differences were defined statistically significant if $P < 0.05$.

3. Results

3.1. Patients Characteristics. The characteristics of the T2D and nondiabetic patients included in the study are shown in Table 1. In both T2D groups the age and duration of diabetes were similar. Also, nondiabetics and control groups did not differ with respect to age and gender, while both groups with CAD (T2D and nondiabetics) were matched for duration of CAD. All three patients groups had similar prevalence of hypertension which was significantly higher in comparison to control group, while prevalence of smokers was similar in all investigated groups. The investigated T2D patients group (groups A and B) did not differ in HbA1c (Table 2).

3.2. Anthropometric Parameters. We did not find significant differences in BMI between the investigated groups, while WHR was significantly higher in group A in comparison to group B ($P < 0.05$), as well as in group C in comparison to group D ($P < 0.001$) (Table 2).

3.3. Lipid Parameters. The lipid levels in investigated groups are shown in Table 2. Groups A and B did not differ in fasting lipid levels (total chol, its subfractions, LDL-chol and HDL-chol, and triglycerides), although HDL-chol level was lower in group A, but without statistical significance. Also, we did not find any differences in the total chol, LDL-chol, and triglycerides levels between groups C and D, while HDL-chol levels were significantly lower in group C in comparison to group D ($P < 0.001$).

3.4. Insulin Resistance. HOMA-IR was significantly higher in group A in comparison to group B ($P < 0.05$), as well as in group C compared to group D ($P < 0.01$). Moreover, HOMA-IR was significantly higher in group A than in group C ($P < 0.01$) (Table 2).

3.5. Basal and Daytime Fluctuation of PAI-1 Activity Levels. We found that the basal PAI-1 activity levels were significantly higher in T2D patients with CAD (group A) compared to T2D patients without CAD (group B) ($P < 0.01$), as well as in nondiabetic patients with CAD (group C) compared to control group (group D) ($P < 0.05$). Moreover, the basal PAI-1 activity levels were significantly higher in group A than in group C ($P < 0.05$) (Table 2).

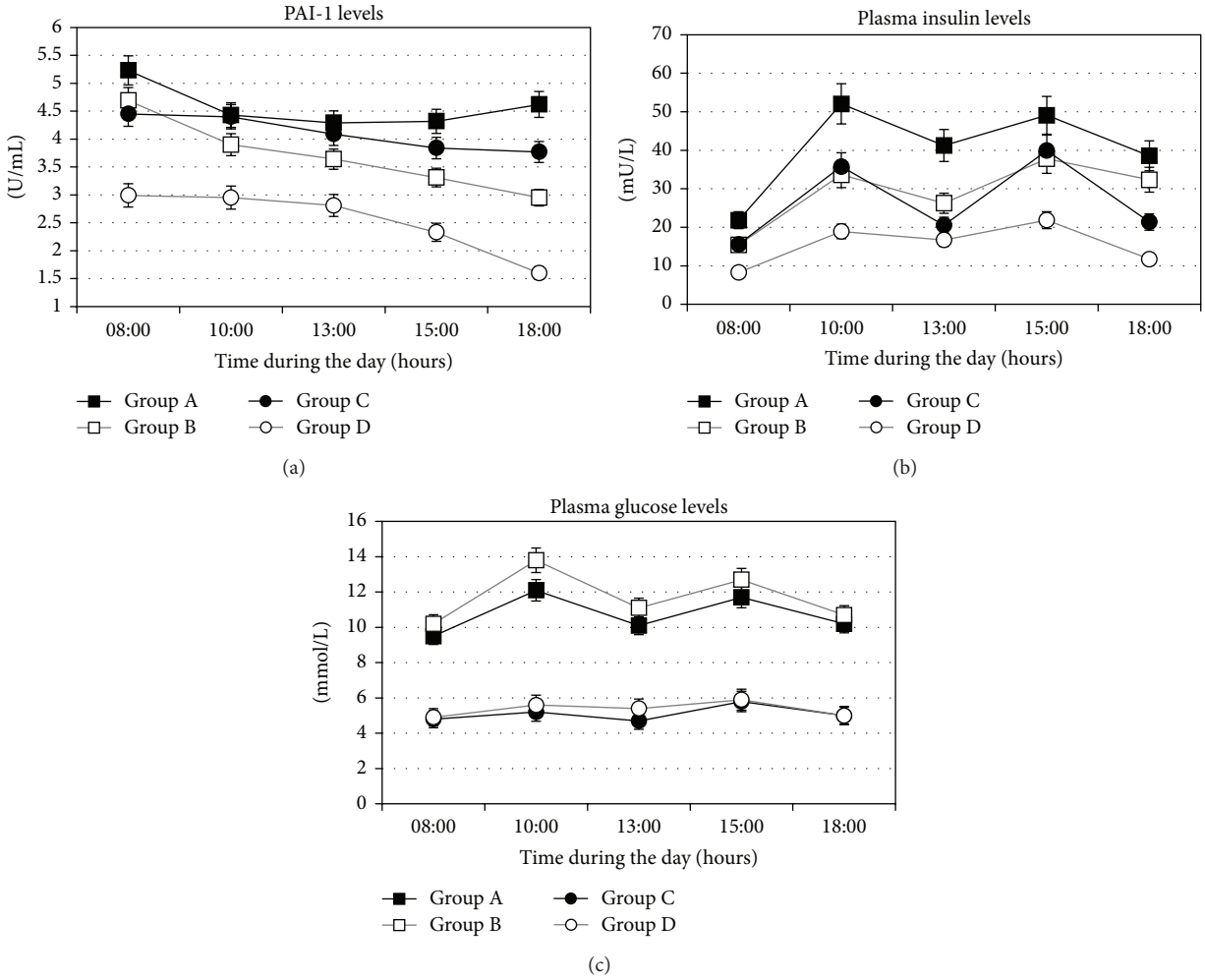

Figure 1: Daily profile of PAI-1 activity (a), plasma insulin (b), and plasma glucose (c) levels in T2D patients with CAD (group A), T2D without CAD (group B), nondiabetics with CAD (group C), and healthy controls (group D). Data are means ± SD.

The daily profile of PAI-1 levels in all investigated groups was shown in Figure 1(a). In group A, PAI-1 activity levels did not show a significant decrease between 08:00 and 18:00 (5.22 ± 0.27 versus 4.58 ± 0.33 U/mL; P = NS). In contrast, in group B, PAI-1 levels decreased during the same interval (4.69 ± 0.38 versus 2.95 ± 0.24 U/mL; $P < 0.05$). Similarly, in group C, we did not find the changes in PAI-1 activity levels during the day (4.45 ± 0.26 versus 3.77 ± 0.24 U/mL; P = NS), while they significantly decreased in group D (2.99 ± 0.21 versus 1.60 ± 0.08 U/mL; $P < 0.001$). Moreover, when we compared the percentage of reduction of PAI-1 during the day (08:00 versus 18:00) between the groups, we found that percentage of decrease in PAI-1 was significantly lower in group A than in group B (4.4 ± 2.7 versus 35.0 ± 5.4%; $P < 0.05$), as well as in group C versus group D (14.0 ± 5.8% versus 44.7 ± 3.1%; $P < 0.001$) (Figure 2). Simultaneously, AUC of the PAI-1 activity levels during the day was significantly higher in group A in comparison to group B (43.79 ± 8.16 versus 36.42 ± 7.05 U-hr/mL; $P < 0.05$), as well as in group C in comparison to group D (40.95 ± 7.52 versus 25.67 ± 5.53 U-hr/mL; $P < 0.001$) (Figure 3(a)).

3.6. Basal Plasma Insulin Levels and Daily Profile of Plasma Insulin. Basal plasma insulin levels were significantly higher in group A in comparison to group B ($P < 0.05$) and in group C compared to group D ($P < 0.05$), as well as in group A compared to group C ($P < 0.05$) (Table 2). The daily profile of plasma insulin levels in all investigated groups was shown in Figure 1(b). We found that at all investigated time points during the day, plasma insulin levels were higher in group A in comparison to group B, but being statistically significantly different only in the morning (at 8:00 h, $P < 0.05$). However, AUC of insulin levels during the day was significantly higher in group A than in group B (435.56 ± 270.14 versus 308.09 ± 99.71 mU-hr/L; $P < 0.05$). When we compared group C and group D, we also found that plasma insulin levels at all investigated time points were significantly higher in group C. Also, AUC of insulin levels during the day was significantly higher in group C than in group D (288.39 ± 89.01 versus 169.76 ± 17.50 mU-hr/L; $P < 0.01$) (Figure 3(b)).

3.7. Fasting Plasma Glucose Levels and Daily Profile of Plasma Glucose. The investigated T2D patients group (groups A and

Table 3: The variable independently related to altered daytime fluctuation pattern of PAI-1 in multiple stepwise linear regression analyses.

	B (95% CI)	β	R^2	Adjusted R^2	P value
(1) WHR	−89.05 (−162.54 to −15.56)	−0.357	0.167	0.145	0.019
(2) AUC of insulin	−0.04 (−0.07 to −0.02)	−0.310	0.261	0.220	0.040

Dependent variable in the model: percentage of PAI-1 decrease during the day.
Independent variable in the model: HOMA-IR, AUC of plasma insulin, AUC of plasma glucose, LDL-chol, HDL-chol, triglycerides, BMI, and WHR (adjusted for age, gender, and presence of diabetes).

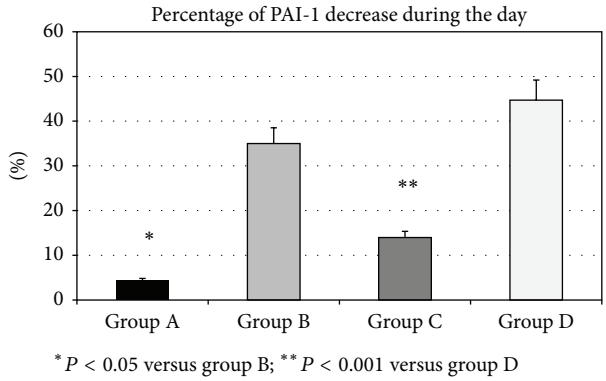

Figure 2: The percentage of PAI-1 decrease during the day, from 08:00 to 18:00, in T2D patients with CAD (group A), T2D without CAD (group B), nondiabetics with CAD (group C), and healthy controls (group D). P values indicate the statistical significance of the difference across the groups, as estimated by ANOVA with post hoc Bonferroni test. Data are means ± SD.

B) did not differ in fasting plasma glucose level. Similarly, we did not found any differences in the glucose level when we compared nondiabetic groups (groups C and D) (Table 2). The daily profile of plasma glucose levels in all investigated groups was shown in Figure 1(c). In contrast to plasma insulin levels, plasma glucose levels were similar at all-time points during the day when group A and group B were compared. Moreover, AUC of plasma glucose levels was similar in groups A and B (109.32 ± 34.66 versus 120.16 ± 31.83 mmol-hr/L; P = Ns). Similarly, in groups C and D plasma glucose levels at all investigated time points were without significant difference, as well as AUC of plasma glucose levels (51.38 ± 8.04 versus 54.86 ± 7.08 mmol-hr/L; P = NS) (Figure 3(c)).

3.8. Analysis of Correlations and Multiple Linear Regression Analysis. In T2D patients, basal PAI-1 activity levels significantly correlated with HOMA-IR ($r = 0.226$; $P < 0.05$), basal plasma insulin levels ($r = 0.225$; $P < 0.05$), and HDL-chol levels ($r = -0.384$; $P < 0.001$), while percentage of PAI-1 decrease during the day significantly correlated only with AUC of insulin levels ($r = -0.483$; $P < 0.05$). However, in nondiabetic patients, basal PAI-1 activity levels significantly correlated with basal plasma insulin levels ($r = 0.568$; $P < 0.01$) and WHR ($r = 0.228$; $P < 0.05$), while correlation with HOMA-IR was on the border of statistical significance ($r = 0.181$; $P = 0.06$). Similarly to T2D patients, in nondiabetics the percentage of PAI-1 decrease during the day significantly correlated only with AUC of insulin levels ($r = -0.512$; $P < 0.05$).

In order to investigate the independent determinants of altered daytime fluctuation pattern of PAI-1 activity levels we performed a stepwise multiple linear regression analysis with the percentage of PAI-1 decrease during the day as dependent variable (Table 3). After collinearity testing was used, in this analysis we included as independent variable HOMA-IR, AUC of plasma insulin, AUC of plasma glucose, LDL-chol, HDL-chol, triglycerides, BMI, and WHR (adjusted for age, gender, and presence of diabetes). We found that only WHR and AUC of plasma insulin were independent determinants of percentage in decrease of PAI-1 during the day, together explaining 26% ($R^2 = 0.261$) of the variability of degree of PAI-1 activity fluctuation during the day.

4. Discussion

The results from this study have shown that increased basal levels and impaired circadian variations of PAI-1, with protracted high PAI-1 levels during the day, are associated with CAD in patients with T2D as well as in nondiabetics. Moreover, the PAI-1 levels in T2D patients were higher compared to those in nondiabetics, both when basal levels or daytime variations were concerned.

In the past two decades, a number of studies have demonstrated elevated basal levels of PAI-1 in patients with CAD, reflecting impaired fibrinolysis [22–25]. However, the exact mechanisms by which a reduced fibrinolysis may lead to the appearance CAD in T2D are not yet fully understood [26, 27].

The results of testing of PAI-1 in this study are consistent with the above data. We found that the basal level of PAI-1 is significantly higher in patients with T2D and CAD compared with diabetic patients without CAD and in both groups of diabetic patients is significantly higher than in the group of healthy individuals. Also, in nondiabetics, our findings were similar with basal levels of PAI-1 in patients with CAD being significantly higher compared with the group of healthy subjects. Moreover, increased PAI-1 observed in T2D with CAD was significantly higher than in nondiabetics with CAD.

It has been previously shown that the secretion of PAI-1 in healthy individuals shows diurnal variation and that the level of PAI-1 secretion has a circadian rhythm with the highest values in the morning and the lowest values in the afternoon [28]. It has also been shown that in patients with elevated levels of PAI-1 (nondiabetics, persons in a state of infection, and pregnancy) there might be alterations in the daily variations in the level of PAI-1 [16]. On the other hand, some

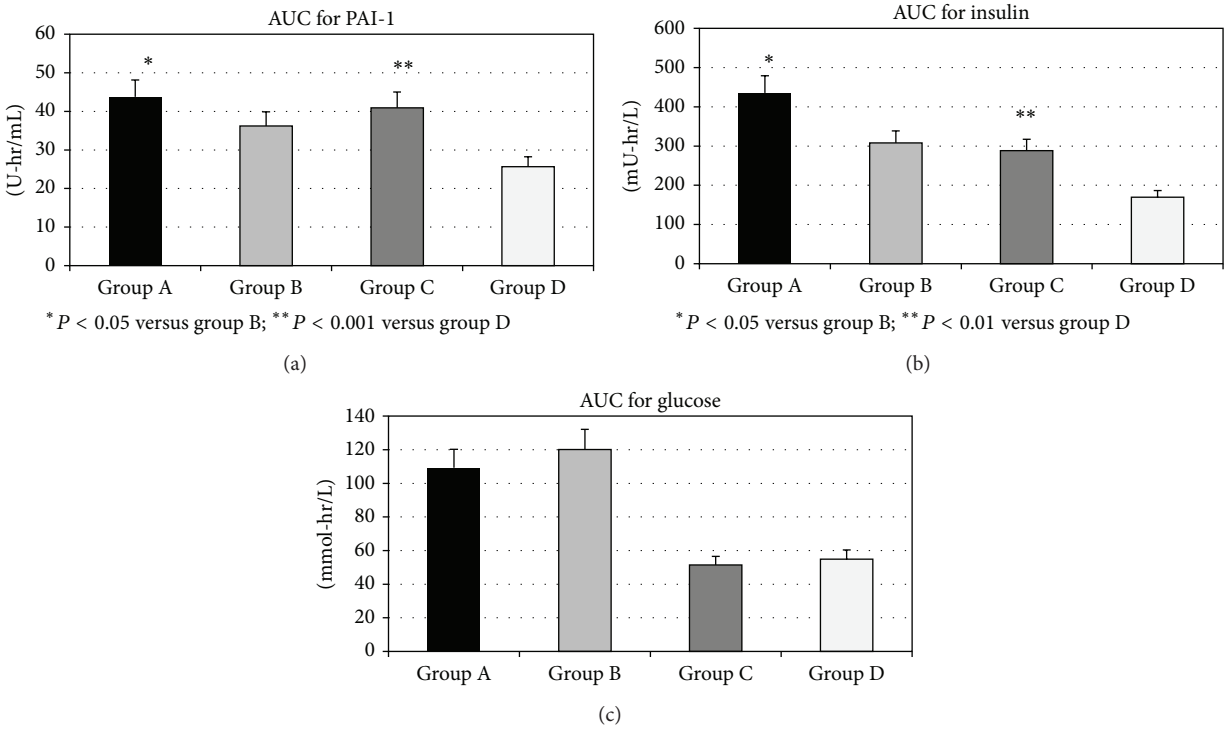

Figure 3: The value of area under the curve (AUC) for daily profile of PAI-1 activity (a), plasma insulin (b), and plasma glucose (c) levels in T2D patients with CAD (group A), T2D without CAD (group B), nondiabetics with CAD (group C), and healthy controls (group D). *P* values indicate the statistical significance of the difference across the groups, as estimated by ANOVA with post hoc Bonferroni test. Data are means ± SD.

previous data from large studies signify that the highest incidence of acute MI found in patients with CAD is in the early morning [29], which is also the period of the peak of PAI-1. Later, it was shown that under condition of elevated morning PAI-1, coronary atherosclerotic plaque could become vulnerable and prone to rupture, thereby precipitating acute coronary syndromes [2]. In contrast, recently it was shown that presence of diabetes completely abolished the morning predominance of MI and that a circadian pattern of MI onset could not be verified [30]. However, it is not known whether these influence of diabetes is solely due to the disappearance of daily variations of PAI-1 in patients with diabetes or that changes in insulin levels and insulin resistance might also be important contributors.

In our study, major new findings are that loss of circadian variation of PAI-1 was more significantly expressed in T2D patients with CAD compared to nondiabetics with CAD. To the best of our knowledge, this difference has not been demonstrated in the available literature. Some previous studies have shown minor impairment in diurnal variation of PAI-1, but only in male nondiabetic patients with CAD [19]. The authors found morning increase in PAI-1 in those patients, with 48% decrease in PAI-1 until evening. We also found increase in PAI-1 levels in the morning in nondiabetic patients (but both males and females) with CAD, although the evening decrease of PAI-1 in nondiabetics was remarkably lower (14%). In addition, in diabetic patients with CAD in our study, percentage of PAI-1 decrease during the day was the lowest (only 4.4%), showing an almost flat daytime curve of PAI-1 in these patients. This strongly suggested that significantly disturbed diurnal rhythm of PAI-1 secretion in these patients is related to appearance of CAD. The other similar investigation in patients with unstable and stable angina (some of them were diabetics) has shown significantly higher levels of PAI-1 during the whole day (measured at three time points: morning, early afternoon, and late evening) in both patients groups compared to healthy controls [31]. However, in our study, patients with diabetes were separated from nondiabetics. We also found higher whole daily levels of PAI-1 (expressed as AUC of PAI-1), but in both diabetic and nondiabetic groups with CAD (group A and group C) in comparison to corresponding groups without CAD, while in patients with CAD the daytime PAI-1 levels were higher in T2D than in controls.

Simultaneously, we have demonstrated significantly higher levels of insulin during the day in the patients with CAD, more expressed in T2D than in nondiabetics, which correlated with impairment in diurnal variation of PAI-1. These findings imply that exposure to hyperinsulinemia throughout the day could strongly contribute to the accelerated atherosclerosis seen in patient with diabetes. The relationship between insulin and PAI-1 has been investigated extensively in previous years. More than 20 years ago, the effects of insulin on PAI-1 have been studied in vitro, in cell cultures of endothelial cells [32] and the hepatocyte cell lines [33] that synthesize PAI-1. It has been shown that the addition

of insulin to the culture of hepatocytes, in concentration which exists in the portal vein after the meal, leads to a double increase in the production of PAI-1 in these cells, without modifying other hepatocyte products [34]. In addition, insulin, but also triglycerides and in some studies hyperglycemia, could increase the expression of PAI-1 by human arterial segments in vitro, both in normal vasculature and within atherosclerotic plaques [35]. These results from in vitro studies have demonstrated that this combination of metabolic impairments underlying T2D, usually associated with insulin resistance (see below), directly influence hypofibrinolysis and facilitate atherosclerosis in those patients [2]. In contrast, in vivo, these direct effects of insulin infusion on PAI-1 have not been observed so far, but investigations were done only in healthy subjects [36, 37], although studies in animal models suggested that infusion of insulin and proinsulin [11] increased expression of PAI-1. Results from our study are suggesting that higher insulin levels during the whole day could be related to observed impairments in fluctuation of PAI-1 during the day in both diabetic and nondiabetic patients with CAD, being more expressed in the T2D patients.

Previous study also suggested that insulin resistance, measured using different methodology, mostly HOMA-IR, is associated with CAD in patients with T2D [38]. One of the rare studies in which insulin resistance was directly measured using the clamp techniques has clearly shown that in diabetic patients insulin resistance is associated with vascular damage and impaired fibrinolysis, independent of obesity and poor glycemic control [39]. In line with these data are the findings in our study that T2D patients with CAD had more pronounced insulin resistance, measured by HOMA-IR, than diabetic patients without CAD. In addition, observed elevated basal PAI-1 level significantly correlated with HOMA-IR and plasma insulin levels, predominantly in the T2D patients.

Interestingly, it seems according to our results that hyperglycemia in the T2D patients is not related to diminished daily fluctuation in PAI-1, as it was already shown in clinical studies by other authors [40]. Also, we did not find differences in total LDL-chol and triglycerides between T2D patients with and without CAD, as well as when we compared nondiabetics with CAD and healthy controls, while HDL-chol levels were lower in both groups with CAD. Moreover, increased basal PAI-1 level significantly correlated with HDL-chol, but only in T2D patients. In accordance with our results, Mertens et al. demonstrated that PAI-1 activity was significantly related to HDL-chol in T2D patients free of CAD [41]. They also pointed out a strong relationship between PAI-1 and LDL peak particle diameter but not with LDL cholesterol levels.

Although the triglycerides did not differ in fasting conditions between groups with and without CAD in our study, the triglyceride levels during the day might be a factor potentially influencing the diurnal fluctuation of PAI-1, although we were not able to measure them in this study. In this context, some rare studies have previously shown that postprandial hypertriglyceridemia is associated with inflammatory and procoagulant state (including increase in PAI-1), but after high-fat meal and only in hypertensive patients [42].

In our study, we found that patients with CAD, both in T2D and nondiabetic, had higher WHR than those without CAD and healthy controls. Simultaneously, there were no differences in BMI between the groups, implying that fat distribution is more important than overall obesity as a risk for CAD. This is in accordance with previous studies showing that abdominal obesity is well-documented risk factors for cardiovascular disease and T2D [43]. Also, abdominal obesity has been found to be associated with insulin resistance and increased PAI-1 concentrations [7, 44, 45]. Moreover, it was previously clearly shown that abdominal visceral fat expressed 5-fold more PAI-1 than subcutaneous tissue [46]. In accordance with those data, the results from multiple regression analysis in our study have shown that impairments in daily fluctuation of PAI-1 were significantly and independently influenced only by WHR, as a marker of abdominal obesity, and AUC of insulin. These findings suggest that prolonged and chronic hyperinsulinemia, in the settings of abdominal obesity and insulin resistance, exhibits its atherogenic role through impairment of daily circadian rhythm of PAI-1.

5. Conclusion

In our results, we have demonstrated the alterations in the diurnal fluctuation pattern of PAI-1 activity, remaining persistently high instead of the afternoon decline, both in T2D patients and nondiabetics with CAD, which is more expressed in T2D. In addition, we have shown that these alterations are strongly influenced by a prolonged daily exposure to elevated plasma insulin levels, especially in T2D with CAD. The influence is suggested to be determined by coexistent insulin resistance and abdominal obesity, which altogether contribute to accelerated atherosclerosis in these patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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References

[1] E. D. Sprengers and C. Kluft, "Plasminogen activator inhibitors," *Blood*, vol. 69, no. 2, pp. 381–387, 1987.

[2] D. J. Schneider and B. E. Sobel, "PAI-1 and diabetes: a journey from the bench to the bedside," *Diabetes Care*, vol. 35, no. 10, pp. 1961–1967, 2012.

[3] A. Hamsten, G. Walldius, A. Szamosi et al., "Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction," *The Lancet*, vol. 2, no. 8549, pp. 3–9, 1987.

[4] P. Tretjakovs, A. Jurka, I. Bormane et al., "Circulating adhesion molecules, matrix metalloproteinase-9, plasminogen activator inhibitor-1, and myeloperoxidase in coronary artery disease patients with stable and unstable angina," *Clinica Chimica Acta*, vol. 413, no. 1-2, pp. 25–29, 2012.

[5] B. Eržen and M. Šabovič, "In young post-myocardial infarction male patients elevated plasminogen activator inhibitor-1 correlates with insulin resistance and endothelial dysfunction," *Heart and Vessels*, vol. 28, no. 5, pp. 570–577, 2013.

[6] I. Juhan-Vague, M.-C. Alessi, A. Mavri, and P. E. Morange, "Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk," *Journal of Thrombosis and Haemostasis*, vol. 1, no. 7, pp. 1575–1579, 2003.

[7] K. Landin, L. Stigendal, E. Eriksson et al., "Abdominal obesity is associated with an impaired fibrinolytic activity and elevated plasminogen activator inhibitor-1," *Metabolism*, vol. 39, no. 10, pp. 1044–1048, 1990.

[8] C. S. Coffey, F. W. Asselbergs, P. R. Hebert et al., "The association of the metabolic syndrome with PAI-1 and t-PA levels," *Cardiology Research and Practice*, vol. 2011, Article ID 541467, 8 pages, 2011.

[9] B. E. Sobel, J. Woodcock-Mitchell, D. J. Schneider, R. E. Holt, K. Marutsuka, and H. Gold, "Increased plasminogen activator inhibitor type 1 in coronary artery atherectomy specimens from type 2 diabetic compared with nondiabetic patients: a potential factor predisposing to thrombosis and its persistence," *Circulation*, vol. 97, no. 22, pp. 2213–2221, 1998.

[10] A. Pandolfi, D. Cetrullo, R. Polishuck et al., "Plasminogen activator inhibitor type 1 is increased in the arterial wall of type II diabetic subjects," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 21, no. 8, pp. 1378–1382, 2001.

[11] T. K. Nordt, H. Sawa, S. Fujii, and B. E. Sobel, "Induction of plasminogen activator inhibitor type-1 (PAI-1) by proinsulin and insulin *in vivo*," *Circulation*, vol. 91, no. 3, pp. 764–770, 1995.

[12] A. Pandolfi, A. Giaccari, C. Cilli et al., "Acute hyperglycemia and acute hyperinsulinemia decrease plasma fibrinolytic activity and increase plasminogen activator inhibitor type 1 in the rat," *Acta Diabetologica*, vol. 38, no. 2, pp. 71–76, 2001.

[13] F. Carmassi, M. Morale, L. Ferrini et al., "Local insulin infusion stimulates expression of plasminogen activator inhibitor-1 and tissue-type plasminogen activator in normal subjects," *The American Journal of Medicine*, vol. 107, no. 4, pp. 344–350, 1999.

[14] S. Fendri, B. Roussel, B. Lormeau, B. Tribout, and J.-D. Lalau, "Insulin sensitivity, insulin action, and fibrinolysis activity in nondiabetic and diabetic obese subjects," *Metabolism: Clinical and Experimental*, vol. 47, no. 11, pp. 1372–1375, 1998.

[15] V. Grimaudo, J. Hauert, F. Bachmann, and E. K. O. Kruithof, "Diurnal variation of the fibrinolytic system," *Thrombosis and Haemostasis*, vol. 59, no. 3, pp. 495–499, 1988.

[16] I. Juhan-Vague, M. C. Alessi, D. Raccah et al., "Daytime fluctuations of plasminogen activator inhibitor 1 (PAI-1) in populations with high PaI-1 levels," *Thrombosis and Haemostasis*, vol. 67, no. 1, pp. 76–82, 1992.

[17] F. A. J. L. Scheer and S. A. Shea, "Human circadian system causes a morning peak in prothrombotic plasminogen activator inhibitor-1 (PAI-1) independent of the sleep/wake cycle," *Blood*, vol. 123, no. 4, pp. 590–593, 2014.

[18] F. Andreotti, G. J. Davies, D. R. Hackett et al., "Major circadian fluctuations in fibrinolytic factors and possible relevance to time of onset of myocardial infarction, sudden cardiac death and stroke," *American Journal of Cardiology*, vol. 62, no. 9, pp. 635–637, 1988.

[19] P. Angleton, W. L. Chandler, and G. Schmer, "Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1)," *Circulation*, vol. 79, no. 1, pp. 101–106, 1989.

[20] World Health Organization, "Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation," Tech. Rep., World Health Organization, 2006.

[21] D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner, "Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[22] R. B. Francis Jr., D. Kawanishi, T. Baruch, P. Mahrer, S. Rahimtoola, and D. I. Feinstein, "Impaired fibrinolysis in coronary artery disease," *American Heart Journal*, vol. 115, no. 4, pp. 776–780, 1988.

[23] K. Huber, D. Rosc, I. Resch et al., "Circadian fluctuations of plasminogen activator inhibitor and tissue plasminogen activator levels in plasma of patients with unstable coronary artery disease and acute myocardial infarction," *Thrombosis and Haemostasis*, vol. 60, no. 3, pp. 372–376, 1988.

[24] I. Juhan-Vague, M. C. Alessi, P. Joly et al., "Plasma plasminogen activator inhibitor-1 in angina pectoris. Influence of plasma insulin and acute-phase response," *Arteriosclerosis*, vol. 9, no. 3, pp. 362–367, 1989.

[25] B. O. Olofsson, G. Dahlen, and T. K. Nilsson, "Evidence for increased levels of plasminogen activator inhibitor and tissue plasminogen activator in plasma of patients with angiographically verified coronary artery disease," *European Heart Journal*, vol. 10, no. 1, pp. 77–82, 1989.

[26] S. C. L. Gough, P. J. S. Rice, L. McCormack, C. Chapman, and P. J. Grant, "The relationship between plasminogen activator inhibitor-1 and insulin resistance in newly diagnosed Type 2 diabetes mellitus," *Diabetic Medicine*, vol. 10, no. 7, pp. 638–642, 1993.

[27] L. J. Garcia Frade, H. de la Calle, M. C. Torrado, J. I. Lara, L. Cuellar, and A. Garcia Avello, "Hypofibrinolysis associated with vasculopathy in non insulin dependent diabetes mellitus," *Thrombosis Research*, vol. 59, no. 1, pp. 51–59, 1990.

[28] C. Kluft, A. F. H. Jie, D. C. Rijken, and J. H. Verheijen, "Daytime fluctuations in blood of tissue-type plasminogen activator (t-PA) and its fast-acting inhibitor (PAI-1)," *Thrombosis and Haemostasis*, vol. 59, no. 2, pp. 329–332, 1988.

[29] J. E. Muller, P. H. Stone, Z. G. Turi et al., "Circadian variation in the frequency of onset of acute myocardial infarction," *The New England Journal of Medicine*, vol. 313, no. 21, pp. 1315–1322, 1985.

[30] R. Kanth, S. Ittaman, and S. Rezkalla, "Circadian patterns of ST elevation myocardial infarction in the new millennium," *Clinical Medicine & Research*, vol. 11, no. 2, pp. 66–72, 2013.

[31] T. Masuda, H. Ogawa, Y. Miyao et al., "Circadian variation in fibrinolytic activity in patients with variant angina," *British Heart Journal*, vol. 71, no. 2, pp. 156–161, 1994.

[32] D. J. Loskutoff, J. A. Van Mourik, L. A. Erickson, and D. Lawrence, "Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 80, no. 10, pp. 2956–2960, 1983.

[33] E. D. Sprengers, H. M. G. Princen, T. Kooistra, and V. W. M. van Hinsbergh, "Inhibition of plasminogen activators by conditioned medium of human hepatocytes and hepatoma cell line Hep G2," *The Journal of Laboratory and Clinical Medicine*, vol. 105, no. 6, pp. 751–758, 1985.

[34] M. C. Alessi, I. Juhan-Vague, T. Kooistra, P. J. Declerck, and D. Collen, "Insulin stimulates the synthesis of plasminogen activator inhibitor 1 by the human hepatocellular cell line Hep G2," *Thrombosis and Haemostasis*, vol. 60, no. 3, pp. 491–494, 1988.

[35] D. J. Schneider, P. M. Absher, and M. A. Ricci, "Dependence of augmentation of arterial endothelial cell expression of plasminogen activator inhibitor type 1 by insulin on soluble factors released from vascular smooth muscle cells," *Circulation*, vol. 96, no. 9, pp. 2868–2876, 1997.

[36] P. J. Grant, E. K. O. Kruithof, C. P. Felley, J. P. Felber, and F. Bachmann, "Short-term infusions of insulin, triacylglycerol and glucose do not cause acute increases in plasminogen activator inhibitor-1 concentrations in man," *Clinical Science*, vol. 79, no. 5, pp. 513–516, 1990.

[37] B. J. Potter Van Loon, A. C. W. De Bart, J. K. M. Radder Frolich, C. Kluft, and A. E. Meinders, "Acute exogenous hyperinsulinaemia does not result in elevation of plasma plasminogen activator inhibitor-1 (PAI-1) in humans," *Fibrinolysis*, vol. 4, no. 2, pp. 93–94, 1990.

[38] S. G. Wannamethee, G. D. O. Lowe, A. G. Shaper, A. Rumley, L. Lennon, and P. H. Whincup, "Insulin resistance, haemostatic and inflammatory markers and coronary heart disease risk factors in type 2 diabetic men with and without coronary heart disease," *Diabetologia*, vol. 47, no. 9, pp. 1557–1565, 2004.

[39] A. Natali, E. Toschi, S. Baldeweg et al., "Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes," *Diabetes*, vol. 55, no. 4, pp. 1133–1140, 2006.

[40] I. Juhan-Vague, C. Roul, M. C. Alessi, J. P. Ardissone, M. Heim, and P. Vague, "Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients—relationship with plasma insulin," *Thrombosis and Haemostasis*, vol. 61, no. 3, pp. 370–373, 1989.

[41] I. Mertens, I. Lemieux, A. Verrijken, J.-P. Després, and L. F. Van Gaal, "PAI-1 activity, but not fibrinogen or von Willebrand factor, is inversely related to LDL particle size in type 2 diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 2, pp. 141–147, 2008.

[42] L. Liu, S.-P. Zhao, T. Wen, H.-N. Zhou, M. Hu, and J.-X. Li, "Postprandial hypertriglyceridemia associated with inflammatory response and procoagulant state after a high-fat meal in hypertensive patients," *Coronary Artery Disease*, vol. 19, no. 3, pp. 145–151, 2008.

[43] M.-C. Alessi and I. Juhan-Vague, "PAI-1 and the metabolic syndrome: links, causes, and consequences," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 10, pp. 2200–2207, 2006.

[44] P. Vague, I. Juhan-Vague, M. F. Aillaud et al., "Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects," *Metabolism*, vol. 35, no. 3, pp. 250–253, 1986.

[45] L. M. Belalcazar, C. M. Ballantyne, W. Lang et al., "Metabolic factors, adipose tissue, and plasminogen activator inhibitor-1 levels in type 2 diabetes: findings from the look AHEAD study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 7, pp. 1689–1695, 2011.

[46] D. Bastelica, P. Morange, B. Berthet et al., "Stromal cells are the main plasminogen activator inhibitor-1—producing cells in human fat: evidence of differences between visceral and subcutaneous deposits," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 1, pp. 173–178, 2002.

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Research Article

The Chromosome 9p21 CVD- and T2D-Associated Regions in a Norwegian Population (The HUNT2 Survey)

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Background. Two adjacent regions upstream *CDKN2B* on chromosome 9p21 have been associated with type 2 diabetes (T2D) and progression of cardiovascular disease (CVD). The precise location and number of risk variants have not been completely delineated and a possible synergistic relationship between the adjacent regions is not fully addressed. By a population based cross-sectional case-control design, we genotyped 18 SNPs upstream of *CDKN2B* tagging 138 kb in and around two LD-blocks associated with CVD and T2D and investigated associations with T2D, angina pectoris (AP), myocardial infarction (MI), coronary heart disease (CHD; AP or AMI), and stroke using 5,564 subjects from HUNT2. *Results.* Single point and haplotype analysis showed evidence for only one common T2D risk haplotype (*rs10757282*|*rs10811661*: OR = 1.19, $P = 2.0 \times 10^{-3}$) in the region. We confirmed the strong association between SNPs in the 60 kb CVD region with AP, MI, and CHD ($P < 0.01$). Conditioning on the lead SNPs in the region, we observed two suggestive independent single SNP association signals for MI, *rs2065501* ($P = 0.03$) and *rs3217986* ($P = 0.04$). *Conclusions.* We confirmed the association of known variants within the 9p21 interval with T2D and CHD. Our results further suggest that additional CHD susceptibility variants exist in this region.

1. Introduction

One interesting region associated with type 2 diabetes (T2D) and cardiovascular disease (CVD) is on chromosome 9p21 in a gene desert ~130 kb upstream of *CDKN2B*. Several SNPs in the 9p21 interval are strongly associated with MI [1–4], vascular disease [5–7], and cancer [8], all highly correlated ($r^2 > 0.8$) and to be found in a ~60 kb region in high linkage disequilibrium (LD). The 9p21 region also contains two adjacent, but separate, T2D signals; a strong signal mapped to a 2 kb LD-block (represented by *rs10811661* and *rs10757282*) and a putatively independent second signal (*rs564398*) located ~100 kb from the T2D interval [9–11].

After the initial genome-wide association studies (GWASs), several investigations confirmed the association with the 9p21 candidate SNPs in T2D [12–17] and CVD [18–24] and extended the number of CVD phenotypes associated with the region [25–30]. A shared mechanistic link might

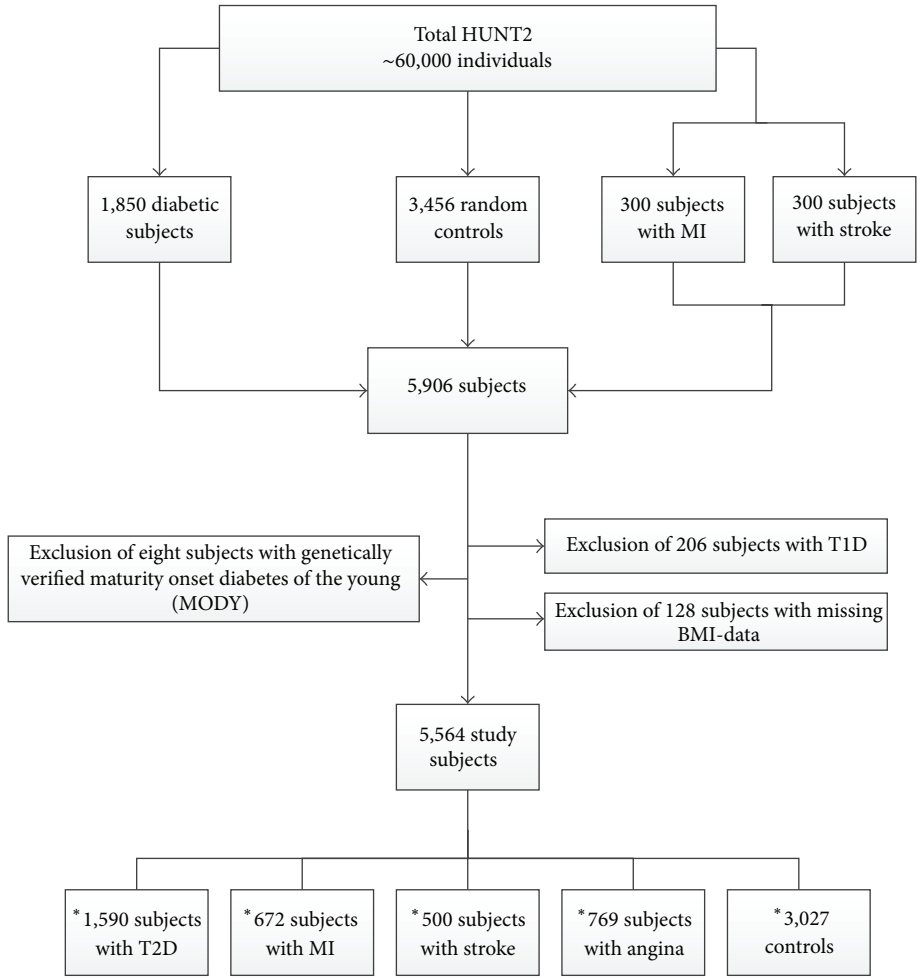

Figure 1: Flow chart presenting the selection of study subjects. Flow chart presenting the inclusion and exclusion criteria of the study subjects enrolled in the present study. A total of 5,564 subjects were eligible for analysis. *Some individuals have more than one outcome (e.g., myocardial infarction (MI) and type 2 diabetes (T2D)); hence, the sum of these counts does not match the total counts of study subjects. T1D denotes type 1 diabetes. The final set of controls was reduced as subjects with MI and stroke were incorporated after the initial controls.

therefore exist within this region increasing risk of both CVD and T2D through a common pathway. In patients with T2D, a variant within 9p21 showed significant interaction between poor glycemic control and risk of angiographically verified coronary artery disease (CAD) [31]. However, the effects of the disease susceptibility variants for the two major disease loci have shown to be independent, since T2D risk variants do not seem to confer increased risk of cardiovascular disease or the other way around [5, 32].

A multilocus analysis of the 9p21 region suggested a haplotype-effect on T2D risk rather than an effect from one single SNP [33], indicating that the *bona fide* locus could be situated somewhere in the vicinity of the test SNPs. However, a comprehensive sequencing study of the 9p21 locus that assessed rare variants and their association with T2D and MI did not discover any variants with stronger association than what was found in the initial GWASs [8]. Thus, we chose to evaluate the distribution of common tagSNPs within the region in individuals with overlapping T2D, angina pectoris (AP), previous MI, or stroke from the Norwegian population-based HUNT2 survey to assess the distribution of T2D and CVD risk alleles in HUNT 2.

2. Materials and Methods

2.1. Study Subjects and Ethics Statement. The second Nord-Trøndelag Health Study (HUNT2) is an extensive population-based health survey conducted in a Norwegian county with 127,000 inhabitants of which 60,000 participated [34]. HUNT2 is a subset of HUNT and was carried out in 1995–97. We had access to all subjects with diabetes (n = 1,850), in addition to 600 individuals selected for incident MI and/or stroke, but without diabetes, and 3,456 population-based random controls drawn from the same study population. After excluding 206 subjects with T1D, eight with genetically verified MODY [35], and 128 subjects with missing BMI data, 5,564 subjects were eligible for analysis (Figure 1). Diagnosis of diabetes, angina pectoris, previous MI, and stroke (ischemic or hemorrhagic strokes grouped as one phenotype) was self-reported. Written informed consent was obtained

Table 1: Clinical characteristic of the 5564 subjects included in the study and eligible for analysis.

	All	T2D	AP	MI	Stroke	No T2D and/or CVD
Individuals (*n*)	5,564	1,590[a]	769[a]	672[a]	500[a]	3,027[a]
Gender (male/female)	2,754/2,810	754/836	435/334	475/197	256/244	1,424/1,603
Age (years at examination)	60.4 ± 17.1	68.1 ± 12.0	72.4 ± 9.2	70.7 ± 10.3	70.8 ± 11.0	53.2 ± 17.6
BMI (kg/m^2)	27.3 ± 4.4	29.2 ± 4.8	28.0 ± 4.3	27.5 ± 3.9	27.4 ± 3.9	26.4 ± 4.1
Ever smoked (yes/no)	2,600/2,964	647/943	345/424	367/305	241/259	1,468/1,559
Nonfasting serum glucose[b] (mmol/L)	6.6 ± 3.1	9.6 ± 4.2	7.6 ± 3.6	7.2 ± 3.5	6.6 ± 2.7	5.4 ± 1.2
Serum triglyceride (mmol/L)	2.0 ± 1.3	2.5 ± 1.6	2.4 ± 1.6	2.3 ± 1.3	2.2 ± 1.5	1.8 ± 1.1
Serum cholesterol (mmol/L)	6.1 ± 1.3	6.2 ± 1.3	6.3 ± 1.3	6.2 ± 1.3	6.4 ± 1.3	6.0 ± 1.3
Serum HDL cholesterol (mmol/L)	1.3 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	1.3 ± 0.4	1.4 ± 0.4
Heart rate (bpm)	73.6 ± 13.6	75.5 ± 14.5	6.8 ± 13.6	67.6 ± 13.3	72.1 ± 13.4	74.1 ± 12.8
Type 2 diabetes (*n*, %)	1,590 (28.6%)	1,590 (100%)	326 (42.4%)	212 (31.5%)	110 (22%)	n/a
Myocardial infarction (*n*, %)	672 (12.1%)	212 (13.3%)	357 (46.9%)	672 (100%)	83 (16.6%)	n/a
Stroke (*n*, %)	500 (9.0%)	110 (6.9%)	115 (15.1%)	357 (53.1%)	500 (100%)	n/a
Angina pectoris (*n*, %)	769 (13.7%)	326 (20.5%)	769 (100%)	83 (12.4%)	115 (23%)	n/a

Values are presented as means ± SD or number (%). [a]Some individuals have more than one outcome (for example MI + diabetes); hence, the sum of these column counts does not match the total counts of individuals. [b]Only nonfasting glucose measures were available for participants in the HUNT2 cohort. MI denotes previous myocardial infarction. Abbreviations: T2D, Type 2 diabetes; AP, angina pectoris; MI, myocardial infarction; CVD, cardiovascular disease; bpm, beats per minute.

from all participants. This population based cross-sectional case-control study was approved by the Regional Committee for Research Ethics and the Norwegian Data Inspectorate, and was performed according to the latest version of the Helsinki Declaration.

2.2. SNP Selection, Genotyping, and Quality Control. We selected tagSNPs across 9p21 from the interval between Chr9:21,995,330 and 22,133,570 (NCBI Build 36). We selected 18 SNPs tagging a 138 kb region using the Haploview implementation of the Tagger algorithm [36] using the following criteria: minor allele frequency (MAF) of >5% and pairwise $r^2 > 0.80$. In addition, we added two previously GWAS-identified T2D susceptibility variants (*rs564398* and *rs10811661*) and three confirmed CVD susceptibility variants (*rs1333040*, *rs10757278,* and *rs1333049*). The genotyping was carried out by the multiplex MassARRAY *iPLEX* System (SEQUENOM Inc., San Diego, CA, USA) at CIGENE, Ås, Norway. Five variants (*rs1759417*, *rs1333049*, *rs7045889*, *rs4977761,* and *rs6475610*) did not pass quality control criteria (minimum call rate > 95% and Hardy-Weinberg equilibrium with $P > 0.01$) and were excluded from analyses. Thus, we assessed a total of 18 SNPs for association with T2D, angina pectoris, previous MI, and stroke.

2.3. Statistical Analysis. We used logistic regression to model single-point and haplotype association for the 18 SNPs with T2D, MI, angina pectoris, coronary heart disease, and stroke positive cases assuming additive effect of allele dosage. Gender, age, and BMI were used as covariates in the regression model in the analysis of T2D. Diabetes status and smoking were added to the list of covariates while analyzing AP, previous MI, CHD, and stroke. Individuals with a history of either AP, previous MI, or stroke were excluded as control subjects in the regression models when analyzing CVD traits. For T2D, AP, MI, and CHD, we carried out tests conditioning on the lead SNPs (MI, angina pectoris, CHD: *rs1333040* and *rs10757278*, T2D: *rs10811661*) to look for secondary signals of association. Multimarker haplotype analyses, haplotype frequency estimates, and haplotype comparisons for all phenotypes were performed using PLINK [37]. The sliding window approach used for multimarker haplotype analysis associates direct neighboring SNPs, generating 17 pairs of SNPs in the two-point analysis. All SNPs frequencies were consistent with Hardy-Weinberg equilibrium (HWE, $P > 0.01$). All analyses were carried out using PLINK version 1.07 software [37] and/or Stata SE v10.0 for Windows (Stata Corp LP, Brownsville, TX, USA). Figures displaying regional information such as the strength and extent of the association signals relative to genomic position, local linkage disequilibrium (LD), and recombination patterns and the positions of genes in the region were created using a combination of LocusZoom web interface [38], R package SNP Plotter [39], and Haploview [36]. We had >80% power to detect high-frequency alleles with ORs of 1.20 to 1.30 for both T2D and CVD phenotypes, but only around 50% and 30% power for T2D and CVD phenotypes, respectively, if the true ORs were 1.10. These estimates were performed using the Genetic Power Calculator [40]. All *P* values are presented without correction for multiple testing.

3. Results

Table 1 shows the clinical characteristics for the 5564 individuals enrolled in the present study.

3.1. Type 2 Diabetes. Regression analysis for association with T2D revealed only modest evidence for a single-point association for *rs10811661* ($P = 0.058$) after correction for age, gender, and BMI (Figure 2, Table 2). No SNP outside

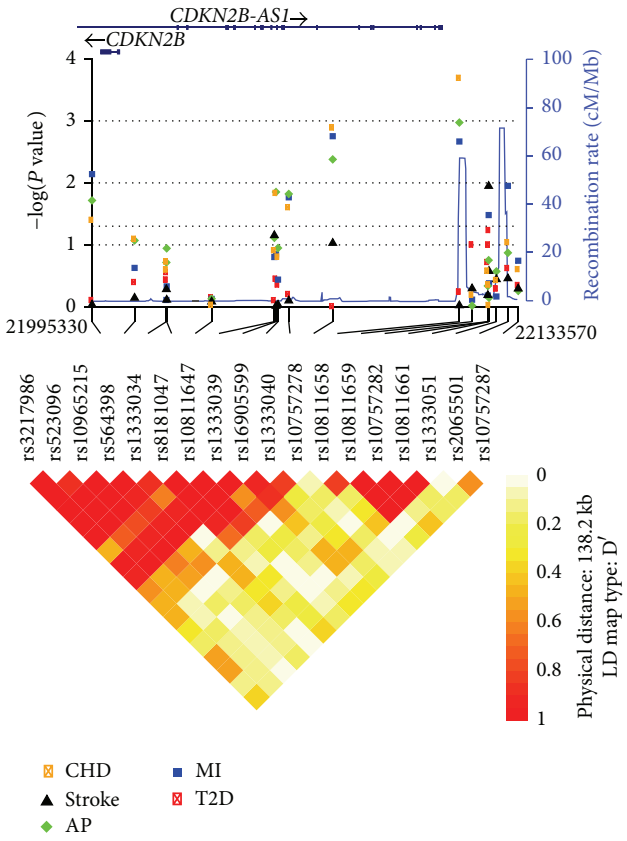

FIGURE 2: Plot summaries for single point association results. Plot summary of association results for 18 SNPs tagging the 138 kb CVD and T2D region on chromosome 9p21 for association with T2D, myocardial infarction, stroke, angina pectoris, or CHD (both MI and angina) using 5564 subjects from the HUNT2 study. The plot show local association results for all phenotypes together with the location and orientation of the genes it includes, local estimates of recombination rates and LD heat map with defined blocks (Gabriel et al.). The plots were created using the R-package SNP Plotter [39].

the previously implicated T2D block (LD-block 4 in Figure 2) showed evidence for an association with T2D.

Next, we performed a two-point sliding-window haplotype analysis and observed an increase in the association for this locus (*rs10757282*|*rs10811661*) with T2D ($P = 2.0 \times 10^{-3}$) (Table 2). The association seemed to be driven by the C-T risk haplotype (OR = 1.19, $P = 7.6 \times 10^{-4}$), compared to the two other common two-marker haplotypes (Table 3). Further haplotype analysis in this LD-block revealed that *rs10757282* and *rs10811661* completely tagged one distinct risk haplotype spanning four consecutive markers in a 2-kb region (LD block 4 in Figure 2). We observed a breakup of the haplotype at markers *rs10811658* and *rs2065501*, which confines a candidate region, located 117–128 kb upstream of *CDKN2B*. The risk haplotype had a frequency of 29 versus 26% in cases and controls (Table 3). HapMap data indicated similar boundaries and frequencies for the haplotype (not shown). An exploratory analysis of increasing haplotype window sizes were performed but did only produce less significant results; the strongest association was found for haplotypes incorporating both *rs10757282* and *rs10811661*.

3.2. Cardiovascular Diseases: Angina Pectoris, Myocardial Infarction, and Stroke. Figure 2 and Additional file 1 (in Supplementary Material available online at http://dx.doi.org/10.1155/2014/164652) show the association results for each of the 18 SNPs with AP, previous MI, CHD (AP or previous MI), and stroke positive cases after adjustment for age, gender, BMI, diabetes status, and smoking. We report replication of the strong association between SNPs in the 60 kb CVD region (defined by *rs8181047* to *rs10757278*, Figure 2) with AP (*rs10757278*: OR = 1.22; $P = 1.1 \times 10^{-3}$, Figure 2), MI (rs1333040: OR = 1.23, $P = 1.8 \times 10^{-3}$, Figure 2), and CHD (*rs10757278*: OR = 1.37; $P = 2.0 \times 10^{-4}$, Figure 2). Subanalyses showed that the effect of the CHD-associated SNPs was strongest in those having the most severe phenotype including both AP and previous MI. None of the SNPs in the CVD region demonstrated association with stroke, but one marker (*rs10757282*) in the previously implicated T2D region did show nominal evidence for association with stroke (OR = 1.2 (1.04–1.38), $P = 0.01$, Figure 2).

In exploratory analysis, we observed several nominally significant potentially novel single SNP associations for angina pectoris, previous MI, individuals with both AP and previous MI, and stroke in the 138 kb interval (Additional file 1). After conditioning upon the highly confirmed CVD susceptibil0ity SNPs *rs1333040* and *rs10757278*, only two remaining SNPs (*rs206550*, OR = 1.32, $P = 0.04$; and *rs3217986*, OR = 1.15, $P = 0.04$) showed nominal P values < 0.05 and only for MI (Table 4).

4. Discussion

Our findings highlight the genetic complexity of the chromosome 9p21 region. We found a weak but consistent single-point association between marker *rs10811661* and T2D, as previously found in several studies [9–11, 41]. This was in agreement with our former results obtained for this marker in a replication study performed in the same material from the HUNT2 population [13]. However, in the present study, we demonstrate a stronger association with a haplotype tagged by *rs10811661* and *rs10757282* and T2D. These results are in line with other studies [8]. Thus, these SNPs may tag a risk haplotype harboring an allele important for development of T2D. Alternatively, the 11 kb candidate region could harbor several variants associated with the disease.

Published data are conflicting regarding any additional T2D-associated signals in the 9p21 region [9–11]. Our data do not support the existence of additional signals. The role of *rs564398* as a T2D susceptibility variant is disputed [9, 12, 42]. Ethnicity may play a role, although our data are not supporting that this marker has a particularly strong effect in Caucasians [43].

The 9p21 risk variants are located in non-protein coding regions; their effects possibly influencing expression of nearby genes. The region contains two cyclin-dependent kinase inhibitors, *CDKN2A* ($p16^{INK4a}$) and *CDKN2B* ($p15^{INK4b}$), and

Table 2: Single point and two-point haplotype association results for T2D.

SNP	Minor allele	Single point		Two-point		Haplotype			
		OR (95% CI)	P	SNPs	Omnibus P	Haplotype	Frequency	OR	P
rs3217986	C	1.03 (0.86–1.22)	7.74×10^{-1}	*rs3217986/rs523096*	4.69×10^{-1}	AC/CT/AT	0.48/0.08/0.44	1.05/1.05/0.95	0.31/0.61/0.22
rs523096	C	1.04 (0.95–1.14)	3.97×10^{-1}	*rs523096/rs10965215*	4.70×10^{-1}	CA/TA/CG/TG	0.03/0.40/0.46/0.12	0.83/0.97/1.06/0.98	0.23/0.53/0.24/0.76
rs10965215	A	0.96 (0.88–1.05)	3.63×10^{-1}	*rs10965215/rs564398*	5.64×10^{-1}	GG/AA/GA	0.46/0.42/0.12	1.05/0.96/0.98	0.29/0.36/0.82
rs564398	G	1.05 (0.96–1.15)	2.75×10^{-1}	*rs564398/rs1333034*	5.69×10^{-1}	AG/GA/AA	0.12/0.46/0.43	0.97/1.05/0.96	0.71/0.29/0.41
rs1333034	G	0.97 (0.84–1.12)	6.96×10^{-1}	*rs1333034/rs8181047*	9.32×10^{-1}	AA/GG/AG	0.34/0.11/0.55	1.02/0.98/1.00	0.76/0.74/0.97
rs8181047	A	1.01 (0.92–1.12)	7.78×10^{-1}	*rs8181047/rs10811647*	6.02×10^{-1}	GG/AC/GC	0.40/0.34/0.26	0.96/1.01/1.04	0.35/0.78/0.44
rs10811647	G	0.96 (0.87–1.05)	3.51×10^{-1}	*rs10811647/rs1333039*	6.49×10^{-1}	CG/GC/CC	0.44/0.40/0.17	1.04/0.96/1.01	0.44/0.36/0.86
rs1333039	G	1.04 (0.95–1.14)	4.35×10^{-1}	*rs1333039/rs16905599*	5.47×10^{-1}	CA/GG/CG	0.06/0.44/0.50	1.05/1.04/0.95	0.62/0.40/0.28
rs16905599	A	1.05 (0.87–1.26)	6.20×10^{-1}	*rs16905599/rs1333040*	9.28×10^{-1}	AC/GC/GT	0.06/0.39/0.56	1.03/1.00/0.99	0.78/0.99/0.86
rs1333040	C	1.00 (0.91–1.09)	9.72×10^{-1}	*rs1333040/rs10757278*	5.95×10^{-1}	CG/TG/CA/TA	0.04/0.44/0.40/0.12	1.06/0.96/1.00/1.09	0.66/0.42/0.92/0.24
rs10757278	G	0.97 (0.89–1.07)	5.67×10^{-1}	*rs10757278/rs10811658*	3.46×10^{-1}	GA/AA/GG/AG	0.16/0.14/0.32/0.38	0.89/0.94/1.04/1.06	0.10/0.42/0.49/0.28
rs10811658	A	0.92 (0.83–1.02)	9.85×10^{-1}	*rs10811658/rs10811659*	4.60×10^{-1}	AC/GC/AT/GT	0.19/0.03/0.11/0.67	0.93/0.98/0.95/1.08	0.20/0.87/0.49/0.11
rs10811659	C	0.93 (0.83–1.04)	1.89×10^{-1}	*rs10811659/rs10757282*	1.98×10^{-1}	TC/CT/TT	0.44/0.21/0.35	1.07/0.92/0.98	0.14/0.14/0.72
rs10757282	C	1.08 (0.99–1.18)	9.88×10^{-2}	***rs10757282/rs10811661***	$\mathbf{2.05 \times 10^{-3}}$	**CT/TT/CC**	**0.16/0.28/0.56**	**1.19/0.93/0.89**	$\mathbf{7.63 \times 10^{-4}/0.11/5.71 \times 10^{-2}}$
rs10811661	C	0.89 (0.78–1.00)	5.76×10^{-2}	*rs10811661/rs1333051*	8.58×10^{-2}	CT/CA/TA	0.11/0.05/0.84	0.95/0.81/1.12	$0.45/0.04/6.48 \times 10^{-2}$
rs1333051	T	0.95 (0.82–1.10)	5.04×10^{-1}	*rs1333051/rs2065501*	5.05×10^{-1}	TA/AA/TC/AC	0.03/0.29/0.08/0.60	0.92/1.08/0.95/0.96	0.61/0.15/0.57/0.38
rs2065501	A	1.06 (0.96–1.17)	2.36×10^{-1}	*rs2065501/rs10757287*	6.15×10^{-1}	AT/CT/AA/CA	0.09/0.04/0.23/0.64	1.07/1.02/1.05/0.94	0.41/0.91/0.36/0.19
rs10757287	T	1.05 (0.92–1.21)	4.43×10^{-1}	n/a	n/a	n/a	n/a	n/a	n/a

Association results for T2D from single and two-point haplotype analysis after correction for gender, age, and BMI. Top associated haplotype *rs10757282* and *rs10811661* is outlined.

Table 3: T2D association results for haplotype *rs10757282*/*rs10811661*.

Haplotype	Frequency		OR	*P*
	Cases	Controls		
Overall evidence	—	—	—	2.05×10^{-3}
CT	0.29	0.26	1.19	7.63×10^{-4}
TT	0.56	0.57	0.93	1.06×10^{-1}
CC	0.15	0.17	0.87	5.71×10^{-2}

Association results for haplotypes defined by *rs10757282* and *rs10811661* in individuals with type 2 diabetes.

CDKN2BAS, a large antisense noncoding RNA gene. Expression of these genes is coregulated and most of the confirmed CVD risk variants correlate with decreased expression of *CDKN2BAS* and furthermore to atherosclerosis [44, 45]. Recent follow-up studies show correlation between the number of risk alleles and atherosclerotic CAD progression, but no predisposition to MI in patients with preexisting atherosclerotic CAD nor increased reoccurrence of MI [46–48]. This suggests 9p21 risk variants promote atherosclerosis rather than triggering MI [49]. Our associations with angina pectoris as well as MI and with the strongest associations in those having both AP and previous MI at the time of screening may thus likely be mediated through increased propensity for atherosclerosis.

The *rs10757278* SNP has been highlighted as a potential functional variant for the association with atherosclerotic disease based on effects on expression of the *INK4*/*ARF* locus ($p15^{INK4b}$, $p16^{INK4a}$, *ARF* and *CDKN2BAS*) [50–52]. In the present study, we confirmed the associations for SNPs in the CVD region with AP and MI. The associations were strongest among subjects having both AP and previous MI. This could be a marker for early progression of atherosclerotic CAD, supporting the aforementioned association between 9p21 risk variants and early progression. Moreover, the *rs10757278* SNP has been mapped to one of 33 identified enhancers in the 9p21 interval, in which the risk variant disrupts a transcription factor binding site, which could have functional relevance for an atherosclerosis-associated pathway in human endothelial cells [53].

We found no association between SNPs in the CVD region and stroke. Our results are in accordance with some studies [5, 54], but not with others [52, 55]. Several investigations aiming to address this discrepancy have confirmed 9p21 as a risk factor for stroke, but with evidence for heterogeneity of effect across stroke subtypes. The strongest association has been shown for large vessel stroke [56]. Thus, lacking stroke subtyping in our study may be the reason we did not find this association. Participants of the HUNT2 survey were identified having stroke through a self-administered questionnaire, hence details regarding type of stroke, hemorrhagic versus ischemic, or subtypes like atherothrombotic or cardioembolic were not available. One could anticipate that SNPs in the 9p21 region associated with ischemic, but not hemorrhagic stroke. Studies have indicated that sequence variation in 9p21 influences atherosclerosis development and progression; the strongest association being seen for large vessels [29]. On the other hand, *rs1333040* has recently been linked to sporadic brain arteriovenous malformations known to increase hemorrhagic stroke risk [7]. Moreover, the adjacent *rs10757278* has been linked to hemorrhagic stroke [52]. These results might suggest different pathways for ischemic and hemorrhagic stroke sharing common mechanisms linked to the same SNPs in the 9p21 region. Interestingly, when restricting the analysis to subjects with T2D, several SNPs in the 60 kb CVD region appeared associated with stroke, with the most significant being *rs1333040* (OR = 1.44; $P = 0.01$). This association was not seen in stroke subjects without T2D. Interaction between variants within the 9p21 region and poor glycemic control increasing risk of CVD in patients with T2D has been suggested [31]. If similar associations were to be found for stroke risk in diabetics, it would be interesting to see whether poor glycemic control also affects different types of stroke differently.

Our exploratory results also highlights two potential novel CVD susceptibility variants, *rs3217986* and *rs2065501*, which are located close to, but not in strong LD with the former and well-confirmed CVD region. The *rs3217986* is located in the 3′ UTR of *CDKN2B* as well as in intron 1 of the non-protein coding *CDKN2B* antisense RNA, *CDKN2BAS*. Although speculative, it could be hypothesized that the risk variant of *rs3217986* might exert an effect on atherosclerotic CAD susceptibility by influencing expression of one or both of these two genes. To our knowledge, there are no reports on whether the risk variant of *rs3217986* is correlated with expression of *CDKN2B* and/or *CDKN2BAS;* thus, this hypothesis needs to be further resolved.

The study must be viewed in light of its limitations. Although previous studies have confirmed highly significant associations between SNPs in the region and CVD and T2D, the many tests performed in this study could lead to a risk of false positive findings. Thus, while the primary single SNP associations and the T2D-risk haplotype are supported by previous studies, the more explorative findings of putative secondary signals need to be further investigated in much larger cohorts. The sparse risk increase associated with these common variants also renders our findings inadequate for clinical prediction. Fine-mapping studies of disease associated regions may still prove important to guide further investigation towards understanding the disease pathogenesis and possibly providing tools for cost-efficient risk stratification in the future.

Despite the close proximity between the CVD and T2D risk regions, our study is in line with previous studies and indicates that there is no apparent overlap between the two risk regions. Theories with reference to the concrete disease mechanism mediated by the risk variants of the 9p21 interval have increased in numbers the last years. However, since most of them still remain exploratory, the exact nature of the disease associated variants and their targets require further elucidation. They may possibly differ between CVD and T2D. It is possible that large-scale genome sequencing efforts may aid by identifying the underlying risk variants in the 9p21 region.

Table 4: Top five association results for CVD after conditioning upon lead SNPs.

SNP	Minor allele	AP		MI		Both MI and AP	
		OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
rs3217986	C	1.21 (0.95–1.53)	0.13	1.32 (1.01–1.71)	0.04	1.25 (0.89–1.75)	0.19
rs2065501	A	1.07 (0.94–1.21)	0.33	1.15 (1.01–1.32)	0.04	1.11 (0.94–1.33)	0.23
rs10757282	C	1.09 (0.96–1.24)	0.18	1.14 (1.00–1.31)	0.05	n/a	n/a
rs10811647	G	n/a		0.84 (0.69–1.03)	0.09	n/a	n/a
rs16905599	A	1.25 (0.95–1.63)	0.11	1.27 (0.95–1.71)	0.10	1.31 (0.89–1.94)	0.17
rs1333051	T	0.87 (0.71–1.07)	0.20	n/a	n/a	0.85 (0.64–1.13)	0.27
rs8181047	A	n/a	n/a	n/a	n/a	1.16 (0.89–1.53)	0.28

Association results for the top five associated SNPs after conditioning upon the lead CVD SNPs *rs1333040* and *rs10757278* for individuals with angina pectoris (AP), myocardial infarction (MI), and both MI and AP.

5. Conclusions

In conclusion, we confirm the association between variants in the 9p21 interval with T2D and CHD. Our results suggest that there exist additional CVD susceptibility variants in this region, highlighting the genetic complexity of the 9p21 region and human disease.

Abbreviations

AP: Angina pectoris
CAD: Coronary artery disease
CHD: Coronary heart disease
CVD: Cardiovascular disease
MI: Myocardial infarction
GWAS: Genome-wide association study
HUNT: Helseundersøkelsen Nord-Trøndelag
LD: Linkage disequilibrium
MODY: Maturity onset diabetes of the young
SNP: Single-nucleotide polymorphism
T2D: Type 2 diabetes
UTR: Untranslated region.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Øyvind Helgeland wrote the paper with assistance from Jens K. Hertel, Helge Ræder, Anders Molven, Pål R. Njølstad, and Stefan Johansson. Carl G. P. Platou, Kristian Midthjell, and Ottar Nygård reviewed and edited the paper. Øyvind Helgeland and Jens K. Hertel performed statistical analysis and interpreted the data with assistance from Anders Molven, Helge Ræder, Ottar Nygård, Pål R. Njølstad, and Stefan Johansson. Pål R. Njølstad and Stefan Johansson conceived the study design with contribution from Øyvind Helgeland, Jens K. Hertel, and Anders Molven. Kristian Midthjell collected background data. Stefan Johansson directed genotyping and statistical analysis. Øyvind Helgeland, Jens K. Hertel, Helge Ræder, Carl G. P. Platou, Kristian Midthjell, Ottar Nygård, Pål R. Njølstad, and Stefan Johansson contributed to discussion. All authors read and approved the final paper.

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References

[1] P. R. Burton, D. G. Clayton, L. R. Cardon et al., "Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls," *Nature*, vol. 447, no. 7145, pp. 661–678, 2007.

[2] A. Helgadottir, G. Thorleifsson, A. Manolescu et al., "A common variant on chromosome 9p21 affects the risk of myocardial infarction," *Science*, vol. 316, no. 5830, pp. 1491–1493, 2007.

[3] R. McPherson, A. Pertsemlidis, N. Kavaslar et al., "A common allele on chromosome 9 associated with coronary heart disease," *Science*, vol. 316, no. 5830, pp. 1488–1491, 2007.

[4] N. J. Samani, J. Erdmann, A. S. Hall et al., "Genomewide association analysis of coronary artery disease," *The New England Journal of Medicine*, vol. 357, no. 5, pp. 443–453, 2007.

[5] A. Helgadottir, G. Thorleifsson, K. P. Magnusson et al., "The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm," *Nature Genetics*, vol. 40, no. 2, pp. 217–224, 2008.

[6] C. Newton-Cheh, N. R. Cook, M. Vandenburgh, E. B. Rimm, P. M. Ridker, and C. M. Albert, "A common variant at 9p21 is associated with sudden and arrhythmic cardiac death," *Circulation*, vol. 120, no. 21, pp. 2062–2068, 2009.

[7] C. L. Sturiale, A. Puca, P. Sebastiani et al., "Single nucleotide polymorphisms associated with sporadic brain arteriovenous malformations: where do we stand?" *Brain*, vol. 136, no. 2, pp. 665–681, 2013.

[8] J. Shea, V. Agarwala, A. A. Philippakis et al., "Comparing strategies to fine-map the association of common SNPs at chromosome 9p21 with type 2 diabetes and myocardial infarction," *Nature Genetics*, vol. 43, no. 8, pp. 801–805, 2011.

[9] R. Saxena, B. F. Voight, V. Lyssenko et al., "Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels," *Science*, vol. 316, no. 5829, pp. 1331–1336, 2007.

[10] L. J. Scott, K. L. Mohlke, L. L. Bonnycastle et al., "A genome-wide association study of type 2 diabetes in finns detects multiple susceptibility variants," *Science*, vol. 316, no. 5829, pp. 1341–1345, 2007.

[11] E. Zeggini, M. N. Weedon, C. M. Lindgren et al., "Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes," *Science*, vol. 316, no. 5829, pp. 1336–1341, 2007.

[12] K. Duesing, G. Fatemifar, G. Charpentier et al., "Strong association of common variants in the *CDKN2A*/*CDKN2B* region with type 2 diabetes in French Europids," *Diabetologia*, vol. 51, no. 5, pp. 821–826, 2008.

[13] J. K. Hertel, S. Johansson, H. Ræder et al., "Genetic analysis of recently identified type 2 diabetes loci in 1,638 unselected patients with type 2 diabetes and 1,858 control participants from a Norwegian population-based cohort (the HUNT study)," *Diabetologia*, vol. 51, no. 6, pp. 971–977, 2008.

[14] Y. Horikawa, K. Miyake, K. Yasuda et al., "Replication of genome-wide association studies of type 2 diabetes susceptibility in Japan," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 8, pp. 3136–3141, 2008.

[15] M. C. Y. Ng, K. S. Park, B. Oh et al., "Implication of genetic variants near TCF7L2, SLC30A8, HHEX, CDKAL1, CDKN2A/B, IGF2BP2, and FTO in type 2 diabetes and obesity in 6,719 Asians," *Diabetes*, vol. 57, no. 8, pp. 2226–2233, 2008.

[16] Y. Wu, H. Li, R. J. F. Loos et al., "Common variants in CDKAL1, CDKN2A/B, IGF2BP2, SLC30A8, and HHEX/IDE genes are associated with type 2 diabetes and impaired fasting glucose in a Chinese Han population," *Diabetes*, vol. 57, no. 10, pp. 2834–2842, 2008.

[17] B. F. Voight, L. J. Scott, V. Steinthorsdottir et al., "Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis," *Nature Genetics*, vol. 42, no. 7, pp. 579–589, 2010.

[18] H. Schunkert, A. Götz, P. Braund et al., "Repeated replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease," *Circulation*, vol. 117, no. 13, pp. 1675–1684, 2008.

[19] T. L. Assimes, J. W. Knowles, A. Basu et al., "Susceptibility locus for clinical and subclinical coronary artery disease at chromosome 9p21 in the multi-ethnic ADVANCE study," *Human Molecular Genetics*, vol. 17, no. 15, pp. 2320–2328, 2008.

[20] Y. Hiura, Y. Fukushima, M. Yuno et al., "Validation of the association of genetic variants on chromosome 9p21 and 1q41 with myocardial infarction in a Japanese population," *Circulation Journal*, vol. 72, no. 8, pp. 1213–1217, 2008.

[21] M. G. Larson, L. D. Atwood, E. J. Benjamin et al., "Framingham Heart Study 100K project: genome-wide associations for cardiovascular disease outcomes," *BMC Medical Genetics*, vol. 8, no. 1, article S5, 2007.

[22] G.-Q. Shen, L. Li, S. Rao et al., "Four SNPs on chromosome 9p21 in a South Korean population implicate a genetic locus that confers high cross-race risk for development of coronary artery disease," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 2, pp. 360–365, 2008.

[23] G.-Q. Shen, S. Rao, N. Martinelli et al., "Association between four SNPs on chromosome 9p21 and myocardial infarction is replicated in an Italian population," *Journal of Human Genetics*, vol. 53, no. 2, pp. 144–150, 2008.

[24] L. Zhou, X. Zhang, M. He et al., "Associations between single nucleotide polymorphisms on chromosome 9p21 and risk of coronary heart disease in Chinese Han population," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 11, pp. 2085–2089, 2008.

[25] K. G. Abdullah, L. Li, G.-Q. Shen et al., "Four SNPS on chromosome 9p21 confer risk to premature, familial CAD and MI in an American Caucasian Population (GeneQuest)," *Annals of Human Genetics*, vol. 72, no. 5, pp. 654–657, 2008.

[26] Z. Chen, Q. Qian, G. Ma et al., "A common variant on chromosome 9p21 affects the risk of early-onset coronary artery disease," *Molecular Biology Reports*, vol. 36, no. 5, pp. 889–893, 2009.

[27] M. Matarin, W. M. Brown, A. Singleton, J. A. Hardy, and J. F. Meschia, "Whole genome analyses suggest ischemic stroke and heart disease share an association with polymorphisms on chromosome 9p21," *Stroke*, vol. 39, no. 5, pp. 1586–1589, 2008.

[28] B. Wahlstrand, M. Orho-Melander, L. Delling et al., "The myocardial infarction associated CDKN2A/CDKN2B locus on chromosome 9p21 is associated with stroke independently of coronary events in patients with hypertension," *Journal of Hypertension*, vol. 27, no. 4, pp. 769–773, 2009.

[29] S. Ye, J. Willeit, F. Kronenberg, Q. Xu, and S. Kiechl, "Association of genetic variation on chromosome 9p21 with susceptibility and progression of atherosclerosis: a population-based, prospective study," *Journal of the American College of Cardiology*, vol. 52, no. 5, pp. 378–384, 2008.

[30] R. Y. L. Zee and P. M. Ridker, "Two common gene variants on chromosome 9 and risk of atherothrombosis," *Stroke*, vol. 38, no. 10, article e111, 2007.

[31] A. Doria, J. Wojcik, R. Xu et al., "Interaction between poor glycemic control and 9p21 locus on risk of coronary artery disease in type 2 diabetes," *Journal of the American Medical Association*, vol. 300, no. 20, pp. 2389–2397, 2008.

[32] H. M. Broadbent, J. F. Peden, S. Lorkowski et al., "Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p," *Human Molecular Genetics*, vol. 17, no. 6, pp. 806–814, 2008.

[33] B. L. Browning and S. R. Browning, "Haplotypic analysis of wellcome trust case control consortium data," *Human Genetics*, vol. 123, no. 3, pp. 273–280, 2008.

[34] S. Romundstad, J. Holmen, H. Hallan, K. Kvenild, Ø. Krüger, and K. Midthjell, "Microalbuminuria, cardiovascular disease and risk factors in a nondiabetic/nonhypertensive population. The Nord-Trøndelag Health Study (HUNT, 1995–97), Norway," *Journal of Internal Medicine*, vol. 252, no. 2, pp. 164–172, 2002.

[35] S. Å. Eide, H. Ræder, S. Johansson et al., "Prevalence of *HNF1A* (MODY3) mutations in a Norwegian population (the HUNT2 Study)," *Diabetic Medicine*, vol. 25, no. 7, pp. 775–781, 2008.

[36] J. C. Barrett, B. Fry, J. Maller, and M. J. Daly, "Haploview: analysis and visualization of LD and haplotype maps," *Bioinformatics*, vol. 21, no. 2, pp. 263–265, 2005.

[37] S. Purcell, B. Neale, K. Todd-Brown et al., "PLINK: a tool set for whole-genome association and population-based linkage analyses," *American Journal of Human Genetics*, vol. 81, no. 3, pp. 559–575, 2007.

[38] R. J. Pruim, R. P. Welch, S. Sanna et al., "LocusZoom: regional visualization of genome-wide association scan results," *Bioinformatics*, vol. 26, no. 18, pp. 2336–2337, 2010.

[39] A. Luna and K. K. Nicodemus, "snp.plotter: an R-based SNP/haplotype association and linkage disequilibrium plotting package," *Bioinformatics*, vol. 23, no. 6, pp. 774–776, 2007.

[40] S. Purcell, S. S. Cherny, and P. C. Sham, "Genetic power calculator: design of linkage and association genetic mapping studies of complex traits," *Bioinformatics*, vol. 19, no. 1, pp. 149–150, 2003.

[41] D. Cugino, F. Gianfagna, I. Santimone et al., "Type 2 diabetes and polymorphisms on chromosome 9p21: a meta-analysis," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 22, no. 8, pp. 619–625, 2012.

[42] X. Y. Bao, C. Xie, and M. S. Yang, "Association between type 2 diabetes and CDKN2A/B: a meta-analysis study," *Molecular Biology Reports*, vol. 39, no. 2, pp. 1609–1616, 2012.

[43] F. Peng, D. Hu, C. Gu et al., "The relationship between five widely-evaluated variants in CDKN2A/B and CDKAL1 genes and the risk of type 2 diabetes: a meta-analysis," *Gene*, vol. 531, no. 2, pp. 435–443, 2013.

[44] M. S. Cunnington, M. S. Koref, B. M. Mayosi, J. Burn, and B. Keavney, "Chromosome 9p21 SNPs associated with multiple disease phenotypes correlate with ANRIL expression," *PLoS Genetics*, vol. 6, no. 4, Article ID e1000899, 2010.

[45] A. Congrains, K. Kamide, R. Oguro et al., "Genetic variants at the 9p21 locus contribute to atherosclerosis through modulation of ANRIL and CDKN2A/B," *Atherosclerosis*, vol. 220, no. 2, pp. 449–455, 2012.

[46] S. Dandona, A. F. R. Stewart, L. Chen et al., "Gene dosage of the common variant 9p21 predicts severity of coronary artery disease," *Journal of the American College of Cardiology*, vol. 56, no. 6, pp. 479–486, 2010.

[47] R. S. Patel, S. Su, I. J. Neeland et al., "The chromosome 9p21 risk locus is associated with angiographic severity and progression of coronary artery disease," *European Heart Journal*, vol. 31, no. 24, pp. 3017–3023, 2010.

[48] D. Ardissino, C. Berzuini, P. A. Merlini et al., "Influence of 9p21.3 genetic variants on clinical and angiographic outcomes in early-onset myocardial infarction," *Journal of the American College of Cardiology*, vol. 58, no. 4, pp. 426–434, 2011.

[49] J. L. Anderson and B. D. Horne, "The 9p21 locus and coronary heart disease: initiator, promoter, or precipitator?" *Journal of the American College of Cardiology*, vol. 56, no. 6, pp. 487–489, 2010.

[50] Y. Liu, H. K. Sanoff, H. Cho et al., "INK4/ARF transcript expression is associated with chromosome 9p21 variants linked to atherosclerosis," *PLoS ONE*, vol. 4, no. 4, Article ID e5027, 2009.

[51] L. M. Holdt, F. Beutner, M. Scholz et al., "ANRIL expression is associated with atherosclerosis risk at chromosome 9p21," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 3, pp. 620–627, 2010.

[52] W. Zhang, Y. Chen, P. Liu et al., "Variants on chromosome 9p21.3 correlated with ANRIL expression contribute to stroke risk and recurrence in a large prospective stroke population," *Stroke*, vol. 43, no. 1, pp. 14–21, 2012.

[53] O. Harismendy, D. Notani, X. Song et al., "9p21 DNA variants associated with coronary artery disease impair interferon-γ 3 signalling response," *Nature*, vol. 470, no. 7333, pp. 264–270, 2011.

[54] M. A. Ikram, S. Seshadri, J. C. Bis et al., "Genomewide association studies of stroke," *The New England Journal of Medicine*, vol. 360, no. 17, pp. 1718–1728, 2009.

[55] C. D. Anderson, A. Biffi, N. S. Rost, L. Cortellini, K. L. Furie, and J. Rosand, "Chromosome 9p21 in ischemic stroke: population structure and meta-analysis," *Stroke*, vol. 41, no. 6, pp. 1123–1131, 2010.

[56] M. Traylor, M. Farrall, E. G. Holliday et al., "Genetic risk factors for ischaemic stroke and its subtypes (the METASTROKE Collaboration): a meta-analysis of genome-wide association studies," *The Lancet Neurology*, vol. 11, no. 11, pp. 951–962, 2012.

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Research Article

The Difference Quantity of Urinary Peptides between Two Groups of Type 2 Diabetic Patients with or without Coronary Artery Disease

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Objectives. We aim to explore urinary biomarkers that could monitor CAD in type 2 diabetic patients. *Materials and Methods*. Urine samples from two groups, twenty-eight type 2 diabetic patients with coexisting CAD and thirty type 2 diabetic patients without CAD, were purified by MB-WCX and then analyzed by MALDI-TOF-MS. Subsequently, we compared the urinary peptide signatures of the two groups by use of ClinProTools2.1 and evaluated the potential ability of the differently expressed peptides to distinguish type 2 diabetic patients with coexisting CAD from type 2 diabetic patients without CAD by ROC analysis. Finally, the differently expressed peptides were identified by nanoliquid chromatography-tandem mass spectrometry. *Results*. There were six differently expressed peptides (*m/z* 1305.2, 1743.9, 2184.9, 2756.1, 3223.2, and 6196.1) between the two groups of subjects, and they were identified as fragments of isoform 1 of fibrinogen alpha chain precursor, prothrombin precursor, and interalpha-trypsin inhibitor heavy chain H4. The diagnostic efficacy of *m/z* 2756.1 and *m/z* 3223.2 was better than the other peptides. Area under ROC of the *m/z* 2756.1, and *m/z* 3223.2 was 0.98 and 0.93, respectively. *Conclusions*. These urinary peptides are potential urinary biomarkers for monitoring of type 2 diabetic patients with CAD.

1. Introduction

Coronary artery disease (CAD) is a frequently coexisting disorder for type 2 diabetic patients and presents as a major component of public health concerns and subsequent economic burdens worldwide. Diabetes mellitus is an established risk factor for coronary heart disease [1] and confers about a twofold excess risk for a wide range of vascular diseases, independently of other conventional risk factors [2]. Early diagnosis of CAD coexisting with type 2 diabetes is a key factor for successful treatment outcome, since early detection could offer the opportunity to initiate pharmacological treatments and even measures such as prophylactic stenting, to reduce the risk of developing cardiac ischemia and myocardial infarction [3, 4]. However, the diagnosis of CAD with or without diabetes in our current clinical practices must use a variety of invasive methods (angiography for CAD) or methods that deliver radiation (coronary perfusion testing, coronary angiography, and coronary artery calcification). Therefore, improved noninvasive methods to monitor diabetes and the coexisting CAD are needed.

To this end, we focus on the urinary proteomics for they contain extensive information [5–7], and the low molecular mass proteome in urine is quite stable [8, 9]. Furthermore, it is an excellent option for the discovery of biomarkers, which can be used for the early detection, diagnosis, and therapeutic evaluation of diseases in a clinical setting.

In this study, CLINPROT MALDI-TOF MS was used to analyze the urinary peptidome profiles that could offer insight into the potential biomarkers between type 2 diabetic patients with and without CAD. Then, nanoliquid chromatography-tandem mass spectrometry was used to identify the sequence of the differently expressed peptides.

Table 1: Demographics and clinical characteristics of two groups of type 2 diabetes mellitus.

	D ($n = 30$)	G ($n = 28$)	P value
Gender (M/F)	22/8	15/13	0.118
DM (year)	4.80 ± 4.90	7.81 ± 7.06	0.040
CAD (year)	/	5.25 ± 4.05	/
Mean age	53.1 ± 9.9	67.0 ± 9.9	0.000
FPG (mmol/L)	9.12 ± 1.82	8.89 ± 2.23	0.605
HbA1c%	7.66 ± 1.40	7.56 ± 1.01	0.716
HbA1c (mmol/mol)	60.2 ± 15.3	59.1 ± 11.1	0.716
Cholesterol (mmol/L)	5.46 ± 1.10	4.70 ± 1.06	0.004
HDL (mmol/L)	1.38 ± 0.56	1.32 ± 0.28	0.577
LDL (mmol/L)	3.41 ± 0.90	2.65 ± 0.92	0.001
TRIG (mmol/L)	1.51 ± 0.59	1.50 ± 1.05	0.960
A/Cr (mg/g)	11.86 ± 7.05	13.48 ± 6.69	0.371

Data is presented as mean ± SD unless otherwise indicated.
D: type 2 diabetes without coronary artery disease; G: type 2 diabetes coexisting with coronary artery disease; M/F: male/female; DM: the duration of type 2 diabetes; CAD: the history year of coronary artery disease; FPG: fasting plasma glucose; HDL: high density lipoprotein; LDL: low density lipoprotein; TRIG: triglyceride; A/Cr: albumin/creatine.

2. Materials and Methods

2.1. Study Population. This study was approved by the ethics committee of Beijing Shijitan Hospital, and the participants all gave informed consent, in accordance with the provisions of the Helsinki Declaration. The study analyzes two groups. Twenty-eight type 2 diabetic patients with coexisting clinically confirmed CAD (G, $n = 28$) and thirty type 2 diabetic patients without CAD (D, $n = 30$) from Beijing Shijitan Hospital were enrolled in this study from October 2012 to May 2013. All the type 2 diabetic patients had a fasting plasma glucose (FPG, fast for at least eight hours) ≥7.0 mmol/L and glycated hemoglobin (HbA1C) ≥6.0% (42 mmol/mol). Details of the clinical characteristics of selected subjects are shown in Table 1.

2.2. Urine Samples Collection and Preparation. Firstly, second void morning urine samples were collected from all the volunteers, discarding the first jet but not the final, and all volunteers were informed to refrain from unusual and heavy physical activity the day before urine collection. Moreover, the urine samples of all the selected subjects had no hematuresis, ketosis, and urinary albumin/creatinine ratio (A/Cr) less than 30 mg/g. Then, sterile polypropylene tubes were used to collect random urine samples. Immediately after collection, urine samples were centrifuged at 400 ×g for 5 minutes to remove cell debris and casts. Then we divided the supernatants into aliquots and froze them at −80°C.

The methods including fractionation of urinary peptides using weak cationic-exchange magnetic beads (Bruker Daltonics), MALDI-TOF MS AnchorChip spotting, and data acquisition were all performed as previously developed by Chu et al. [10].

2.3. Statistical Analyses. Descriptive patient characteristics are displayed as mean ± SD unless otherwise indicated and calculations were performed using SPSS 17.0. The peak area was used as quantitative standardization. The comparison of the peak area between two groups was performed by t-tests (normal distributed data) or Wilcoxon test (abnormal distributed data) using ClinProTools2.1 bioinformatics software. Two-tailed P values <0.05 were considered significant in all statistical comparisons. Receiver operating characteristic curve (ROC) analysis and area under the curve (AUC) calculations were constructed for determination of the diagnostic efficacy of each selected marker.

2.4. Peptide Sequence. A nanoliquid chromatography-tandem mass spectrometry, which consisted of an Aquity UPLC system (Waters) and a LTQ Obitrap XL mass spectrometer (Thermo Fisher) equipped with a nano-ESI source, was used to identify the sequences of differential expression peptides. Firstly the peptide solutions were loaded into a C18 trap column (symmetry 180 μm × 20 mm × 5 μm, nanoAcquity) with the flow rate of 15 μL/min for 3 minutes. Then the desalted peptides were analyzed by C18 analytical column (symmetry 75 μm × 150 mm × 3.5 μm, nanoAcquity) at a flow rate of 400 nl/min. The mobile phases A (5% acetonitrile, 0.1% formic acid, Sigma-Aldrich) and B (95% acetonitrile, 0.1% formic acid) were used for analytical columns. Gradient elution profile was as follows: 5%B-45%B-80%B-80%B-5%B-5%B in 60 minutes. The MS instrument was operated in a data-dependent model. The range of full scan was 400–2000 m/z with a mass resolution of 100,000 (m/z 400). The ten most intense monoisotope ions were the precursors for collision induced to two consecutive scans per precursor ion followed by 90s of dynamic exclusion.

2.5. Bioinformatics and Identification of Urine Biomarkers. The obtained spectra were analyzed with Bioworks-Browser3.3.1 SP1 (Thermo Fisher) and the resulting mass lists were matched against the IPI Human database (v3.45) using Sequest search. Parameters were set as follows: Delton ≥ 0.1; Charge2+, Xcorr2.0; charge3+, Xcorr2.5; peptide probability ≤ $1e - 003$; parent ion masses tolerance: 50 ppm; fragment ion masses tolerance: 1 Da; enzyme: no enzyme; variable modification: oxidation of methionine.

3. Results

3.1. Urinary Peptidome Profiling. Urine samples from fifty-eight volunteers purified by magnetic beads exhibited spectral peaks in the 1000–10,000 Da range. After analysis of MALDI-TOF MS, typical WCX spectra were shown in Figure 1.

3.2. Statistical Data Analysis between the Two Groups. Using ClinprotTools2.1, a total of 139 distinguishable peaks were detected within the 1,000 to 10,000 m/z range, with 90 peaks having differential expression and statistical significance $P < 0.05$. To avoid bias, we picked six relative higher peaks for further analysis and the mass-to-charge ratio of the six

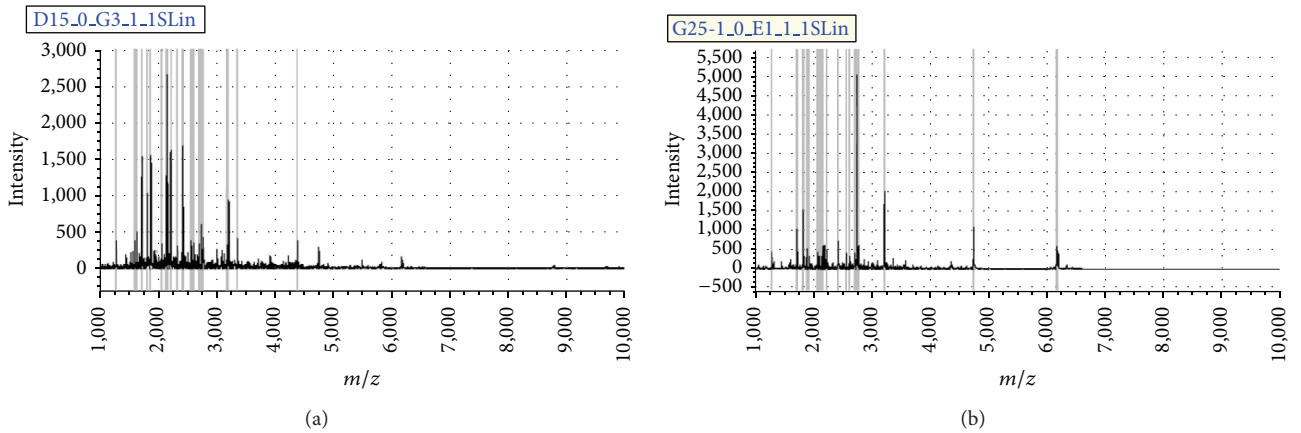


Figure 1: Typical urinary sample mass spectrum from MALDI-TOF MS after being purified by weak cation exchange magnetic beads. (a) From one sample of type 2 diabetic patients without coronary artery disease. (b) From one sample of type 2 diabetic patients with coronary artery disease.

Table 2: The statistics characteristic of the six selected peaks.

m/z	P value	D	G
1305.2	$3.66E-04$	639.7 ± 396.3	354.7 ± 231.3
1743.9	0.02	1219.3 ± 641.4	863.4 ± 474.1
2184.9	$2.93E-04$	1281.1 ± 667.2	708.7 ± 428.1
2756.1	$<1e-6$	531.8 ± 662.7	3516.8 ± 1363.3
3223.2	$<1e-6$	485.4 ± 499.1	2104.8 ± 1171.6
6196.1	0.002	269.4 ± 190.1	656.7 ± 645.6

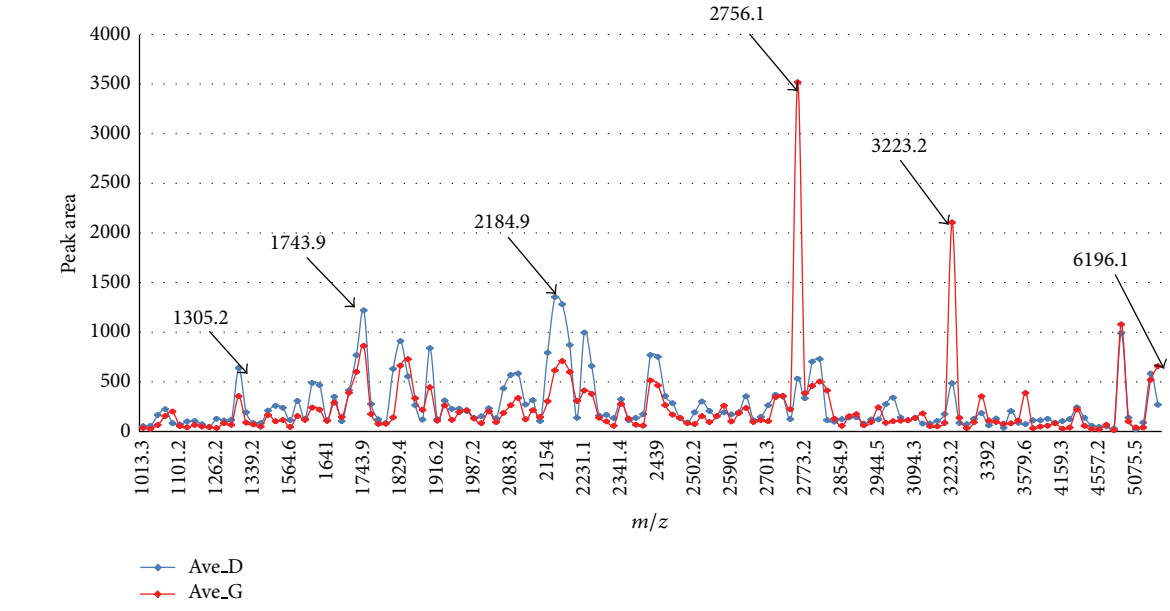
The peak area of every peak in two groups is presented as mean ± SD. P value was calculated by t-test (normally distributed continuous data) or Wilcoxon test (nonnormally distributed continuous data). $P < 0.05$ was accepted as statistically significant difference. D: type 2 diabetes mellitus without coronary artery disease; G: type 2 diabetes coexisting with coronary artery disease.

peaks was 1305.2, 1743.9, 2184.9, 2756.1, 3223.2, and 6196.1 (Figure 2(a)). Compared to D group, m/z 2756.1, 3223.2, and 6196.1 were upregulated (Figure 2(b)) and m/z 1305.2, 1743.9, and 2184.9 were downregulated in G group (Figure 2(c)). The statistical characteristics of the six selected peaks are shown in Table 2. To evaluate the diagnostic efficacy of these peptides, the ROC analysis was performed to calculate the sensitivities, specificities, and accuracies at different cut-off points for differentiating CAD type 2 diabetic patients from control subjects. In the ROC curves, m/z 2756.1 and 3223.2 had excellent area under the curve (AUC) values (0.98 and 0.93) which indicate a highly accurate diagnostic test, and m/z 6196.1, 1305.2, and 2184.9 had limited clinical utility AUC of 0.73, 0.773, and 0.755, respectively, while m/z 1743.9 had an AUC of 0.655 that suggests low diagnostic accuracy (Figure 3).

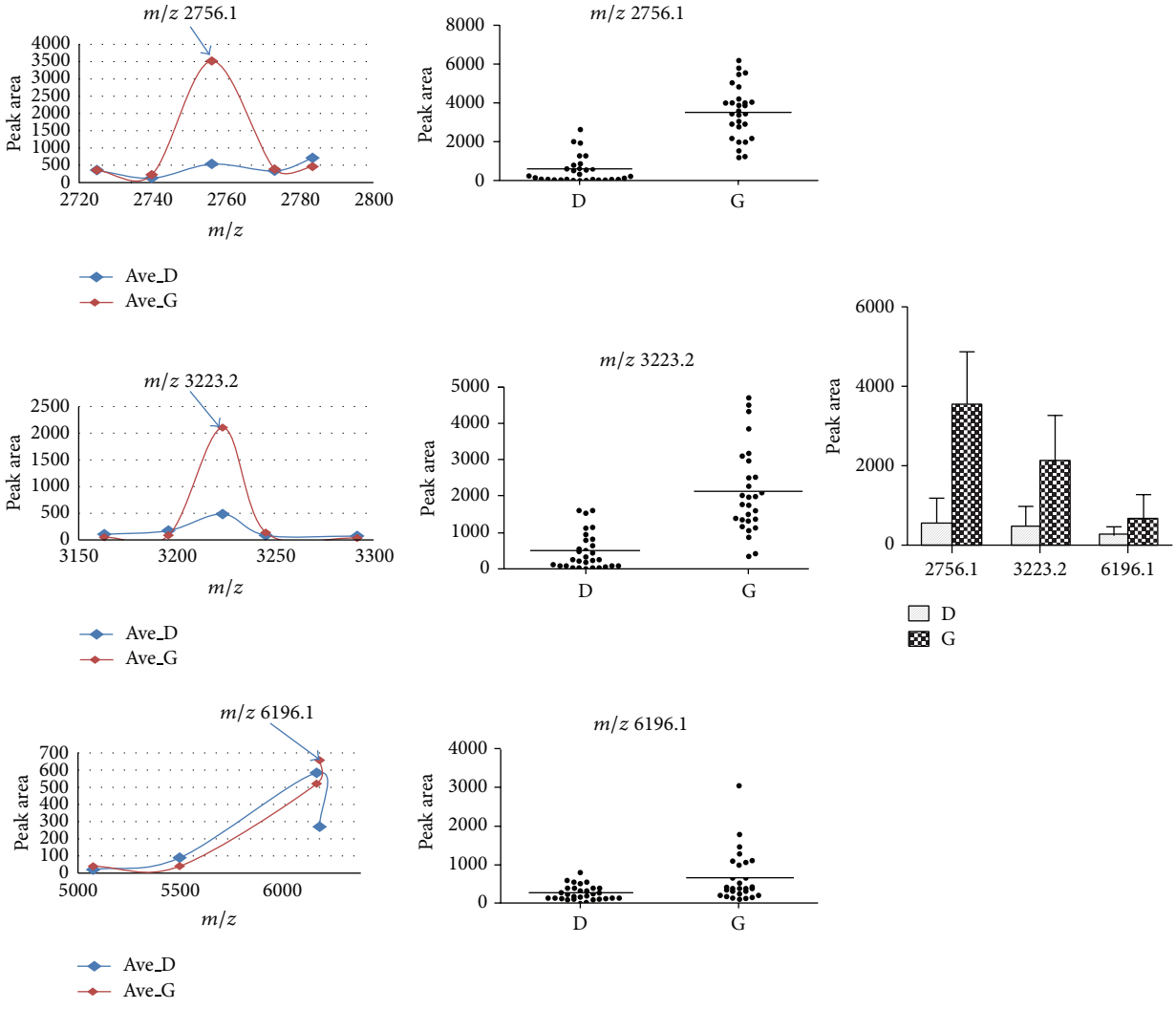
3.3. Identification of the Potential Urinary Biomarkers for CAD Patients with Coexisting Type 2 Diabetes. With this bead-based proteomic technology, we found six potential biomarkers for CAD patients with coexisting type 2 diabetes. The peptide sequence of the six differential peaks was identified by a nanoliquid chromatography-tandem mass spectrometry, and the Sequest search reported the protein name. Following MS/MS, the sequence of m/z 1305.2 was parsed as A.DSGEGDFLAEGGGV.R and it is a fragment of isoform 1 of fibrinogen alpha chain precursor. Similarly, m/z 1743.9 was parsed as K.MADEAGSEADHEGTHST.K and m/z 2756.1 was S.SYSKQFTSSTSYNRGDSTFESKSY.K and both of them are fragment of fibrinogen alpha chain precursor. The m/z 2184.9 comes from interalpha-trypsin inhibitor heavy chain H4 and its amino acid sequence is S.RQLGLPGPPDVPDHAAYHPF.R. The m/z 3223.2 is a fragment of prothrombin precursor and its amino acid sequence is C.GLRPLFEKKSLEDKTERELLESYIDGR.I. Unfortunately, the m/z 6196.1 peak sequence was not identified. The detailed results are shown in Table 3.

4. Discussion

Urine as a promising source of biomarkers identification associated with disease has recently been discussed and reviewed, especially when combined with proteomics [11], including an internationally harmonized urine collection protocol. Urinary proteomics, which would yield information pertinent to the function of both renal and extrarenal organs, was not only used in the urologic and genital diseases [6, 12], but also in other system diseases, like endocrine system and digestive system [10, 13]. Driven by the advancements in technology, mass spectrometry- (MS-) based proteomic studies aiming at defining clinically relevant biomarkers have been increasing in number. However, not all MS-analytical platforms used for biomarker discovery are suitable for clinical diagnostic applications. Common approaches of MS-based proteomics for clinical diagnosis include 2DE-MS, SELDI-MS, liquid chromatography- (LC-) MS, and capillary electrophoresis- (CE-) MS [14]. In view of urine containing low molecular mass proteome that does not undergo any significant change even when urine was stored for up to 3 days at 4°C or 6 h at room temperature [8, 9], so study of low mass protein/peptide may provide a new field for biomarker

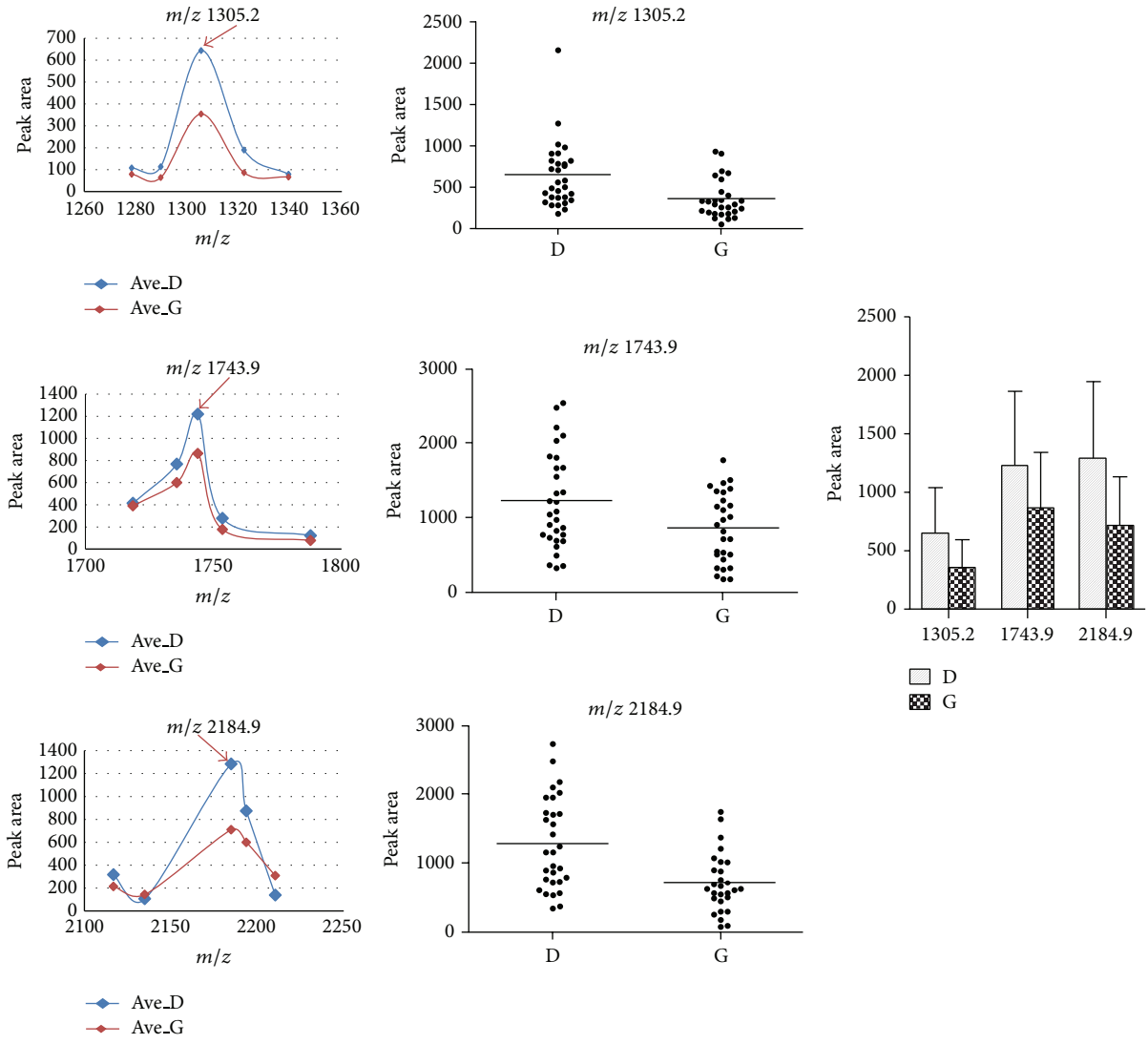


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: The feature of the 6 selected peaks in two groups. (a) The distribution of average peak area from two groups. (b) The average value of 3 elevated peaks in G group when compared with D group (left and right) and their distribution in all samples (middle). (c) The average value of 3 decreased peaks in G group when compared with D group (left and right) and their distribution in all samples (middle).

Table 3: Identified peptides sequence of the selected peaks.

m/z	Molecular weight	Amino sequences	Protein name
1305.2	1309.55	A.DSGEGDFLAEGGGV.R	Isoform 1 of fibrinogen alpha chain precursor
1743.9	1744.67	K.MADEAGSEADHEGTHST.K	Fibrinogen alpha chain precursor
2184.9	2181.92	S.RQLGLPGPPDVPDHAAYHPF.R	Interalpha-trypsin inhibitor heavy chain H4
2756.1	2756.22	S.SYSKQFTSSTSYNRGDSTFESKSY.K	Isoform 1 of fibrinogen alpha chain precursor
3223.2	3220.74	C.GLRPLFEKKSLEDKTERELLESYIDGR.I	Prothrombin precursor
6196.1		Identification failure	

discovery. On top of this, we focus our research on the bead-based MALDI-TOF mass spectrometry with its small sample sizes for analysis, high-throughput capability, exquisitely sensitive and high-resolution peptide detection, and monitoring of disease progression accurately [15–17]. We directly purified urinary protein/peptides using weak cationic-exchange magnetic beads without trypsinization and then develop a profile of urine proteome through MALDI-TOF-MS. By comparison with ClinProTools2.1 software, we determined several markers that differentiated type 2 diabetic patients with CAD

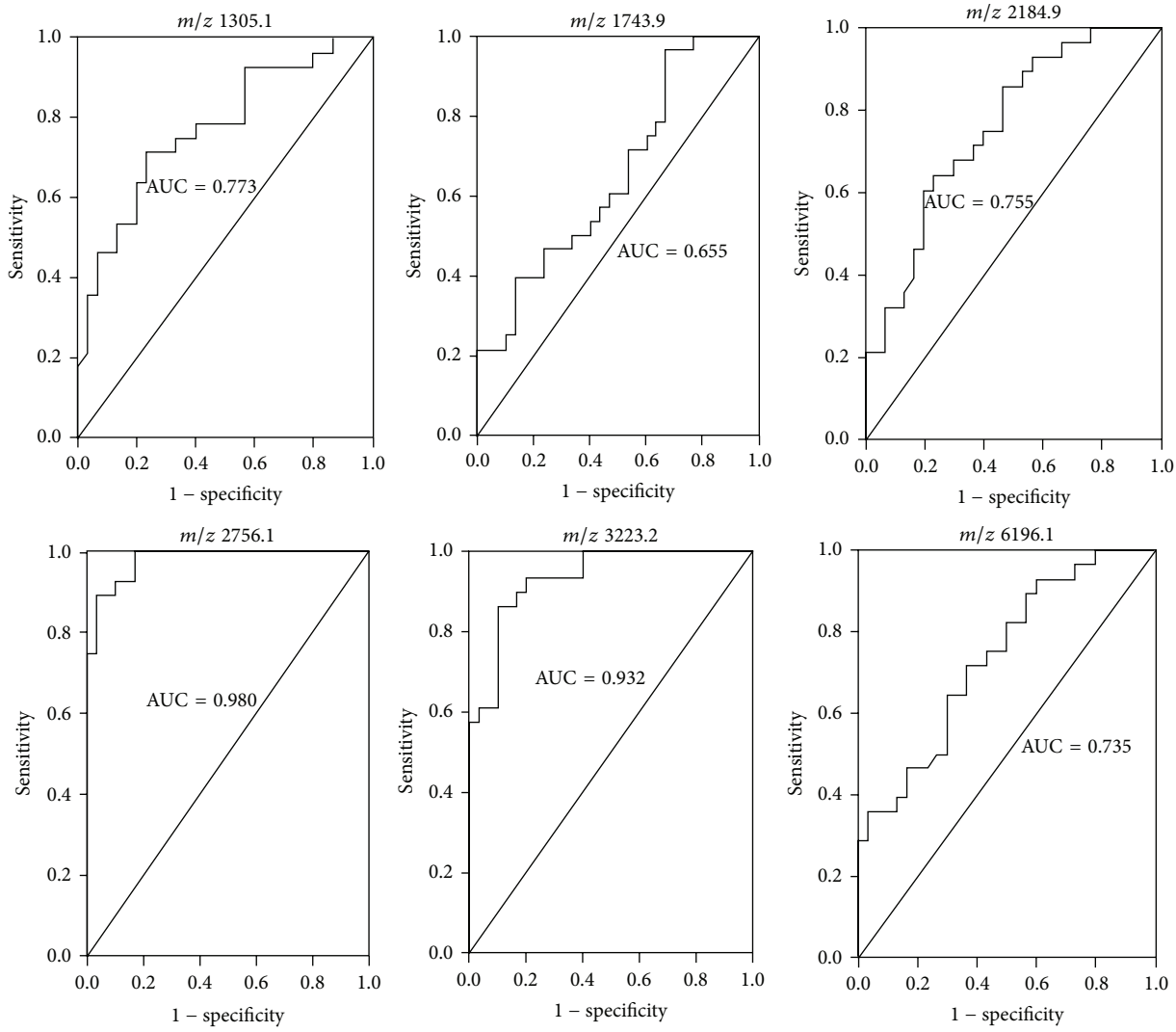


Figure 3: Receiver operator characteristics (ROC) curves generated with the *m/z* 1305.2, 1743.9, 2184.9, 2756.1, 3223.2, and 6196.1 used to distinguish type 2 diabetic patients coexisting with coronary artery disease from type 2 diabetic patients without coronary artery disease. The areas under the curve (AUC) were 0.773, 0.655, 0.755, 0.980, 0.932, and 0.735 for the above mentioned peaks, respectively.

from control samples. We selected several proteins/peptides biomarkers instead of single one because a single biomarker lacks specificity and perhaps does not exist.

Type 2 diabetes mellitus and CAD share several common characteristics in pathophysiology, including genetic and environmental factors, but type 2 diabetes with coexisting CAD takes on its own features. Insulin resistance and hyperglycemia are central features of type 2 diabetes mellitus, and they confer additional impairment for myocardial infection [18]. The underlying molecular mechanism of this kind of cardiac dysfunction is still largely unknown, but it is thought to involve causal alterations in gene and protein expression [19]. Proteomic technology now allows us to examine differential alterations in protein expression in the disease group and its controls.

In our bead-based urinary proteomic study, we found several urinary peptides patterns that were differently expressed in type 2 diabetic patients with CAD when compared with type 2 diabetic patients without CAD.

The downregulated peptides *m/z* 1305.1 and *m/z* 1743.9 and the upregulated peptide *m/z* 2756.1 were different fragments of fibrinogen alpha chain precursor. From our identified results we can see that different peptides can come from the same protein, but they play various roles in diagnosis of disease. Fibrinogen, synthesized by the liver, is a major plasma protein that consists of pairs of 3 different polypeptide chains α, β, and γ, joined by disulfide bonds to form a symmetric dimeric structure. It is directly involved in the clotting process as a clotting factor and releases two fibrinopeptides A and B from the NH_2 terminus of the α, β chains cleavage by thrombin. There are some other fragments [20] released from fibrin that degraded by plasmin. So the changes of the level of plasma fibrinogen or fragments from fibrinogen could represent some life state that associates with hypercoagulability such as cardiovascular and cerebrovascular thrombotic diseases [21, 22] and renal failure [23]. The peptide *m/z* 1305.1 is fibrinopeptide A (sites 20–35) and *m/z* 1743.9 is just a fragment (sites 602–620) released from

fibrinogen α chain. Alkjaerdsig and Fletcher had reported catabolism and excretion of fibrinopeptide A [24]. Plasma fibrinopeptide A had been a sensitive marker of in vivo fibrin formation and was significantly increased in type 2 diabetic patients with vascular complications [25]. In our study, the fragments *m/z* 1305.1 and 1743.9 of fibrinogen alpha chain in urine were significantly decreased and *m/z* 2756.1 was increased in type 2 diabetic patients with CAD. The former has longer duration of diabetes and thus is exposed longer in the hyperglycemia that could lead to protein metabolic disorder and dyslipidemia. The difference exhibited by the urinary proteomics may be able to explain the complex metabolism under the hyperglycemia.

Inter-trypsin inhibitor heavy chain H4 (ITIH4) from which the *m/z* 2184.9 peptide is derived is a plasma kallikrein-sensitive glycoprotein (120 kDa) [26] that is expressed mainly in liver and that acts as an acute-phase protein [27]. The urinary peptide we identified as a biomarker is a disease-associated fragment including cancer and inflammatory disease that had been discovered in the plasma [28]. There is increasing evidence that oxidative stress and inflammation play critical roles in the pathogenesis of type 2 diabetes mellitus and the development of its complications. Interestingly, several papers have suggested that ITIH4 may represent clinically surrogate markers for the detection and classification of different disease types [28, 29]. However, the exact biological function of ITIH4 in vivo remains unclear.

In total there were three elevated peptides in the complication group compared with the other group. Unfortunately, one of the three urinary peptides *m/z* 6196.1 was not identified. As mentioned above, the *m/z* 2756.1 peak sequence was identified as fibrinogen alpha chain. The other one peptide *m/z* 3223.2 was fragment of prothrombin precursor. In either diabetes mellitus patients or CAD patients, a hypercoagulable state is associated with the increase in thrombosis. Elevated level of coagulation factors is suggested to contribute to hypercoagulability and is considered one of the risk factors that play an important part in the development of stroke and myocardial infarction [30]. Its elevated expression in urine may be the result of cardiac injury by hyperglycemia and so it may be a risk signal for the diabetic patients accompanied with CAD. From the AUC of peptides *m/z* 2756.1 and *m/z* 3223.2, we can conclude that they had a higher diagnostic efficacy for these diabetic patients with CAD.

In conclusion, the urinary peptides hold important information that may have direct clinical utility for disease diagnosis and classification. We are very interested in these peaks and our next plan will go further to research for every urinary peptide. However, it is a long way to go to introduce this technology into clinical practice.

Abbreviations

HbA1C:	Hemoglobin A1c
MALDI-TOF MS:	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MB-WCX:	Weak cationic-exchange magnetic beads
m/z:	Mass-to-charge ratio
CAD:	Coronary artery disease
FGA:	Fibrinogen alpha chain
ITIH4:	Interalpha-trypsin inhibitor heavy chain H4
MS:	Mass spectrometry
2DE-MS:	Two-dimensional gel electrophoresis-mass spectrometry
SELDI-MS:	Surface enhanced laser desorption/ionization-mass spectrometry
ROC:	Receiver operating characteristic curve.

Disclosure

Guangzhen Fu and Mei Hu should be regarded as co-first authors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guangzhen Fu and Mei Hu contributed equally to this work.

Acknowledgments

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References

[1] E. A. Spencer, K. L. Pirie, R. J. Stevens et al., "Diabetes and modifiable risk factors for cardiovascular disease: the prospective Million Women Study," *European Journal of Epidemiology*, vol. 23, no. 12, pp. 793–799, 2008.

[2] N. Sarwar, P. Gao, S. R. Seshasai et al., "Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies," *The Lancet*, vol. 375, pp. 2215–2222, 2010.

[3] D. Grodos and R. Tonglet, "Scandinavian simvastatin study (4S)," *The Lancet*, vol. 344, pp. 1768–1769, 1994.

[4] W. B. Kannel and D. L. McGee, "Diabetes and cardiovascular disease. The Framingham study," *The Journal of the American Medical Association*, vol. 241, no. 19, pp. 2035–2038, 1979.

[5] J. Adachi, C. Kumar, Y. Zhang, J. V. Olsen, and M. Mann, "The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins," *Genome Biology*, vol. 7, no. 9, article R80, 2006.

[6] T. Lei, X. Zhao, S. Jin, Q. Meng, H. Zhou, and M. Zhang, "Discovery of potential bladder cancer biomarkers by comparative urine proteomics and analysis," *Clinical Genitourinary Cancer*, vol. 11, no. 1, pp. 56–62, 2013.

[7] T. Pisitkun, R. Johnstone, and M. A. Knepper, "Discovery of urinary biomarkers," *Molecular and Cellular Proteomics*, vol. 5, no. 10, pp. 1760–1771, 2006.

[8] S. Schaub, J. Wilkins, T. Weiler, K. Sangster, D. Rush, and P. Nickerson, "Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry," *Kidney International*, vol. 65, no. 1, pp. 323–332, 2004.

[9] D. Theodorescu, S. Wittke, M. M. Ross et al., "Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis," *The Lancet Oncology*, vol. 7, no. 3, pp. 230–240, 2006.

[10] L. Chu, G. Fu, Q. Meng, H. Zhou, and M. Zhang, "Identification of urinary biomarkers for type 2 diabetes using bead-based proteomic approach," *Diabetes Research and Clinical Practice*, vol. 101, no. 2, pp. 187–193, 2013.

[11] S. Decramer, A. G. de Peredo, B. Breuil et al., "Urine in clinical proteomics," *Molecular and Cellular Proteomics*, vol. 7, no. 10, pp. 1850–1862, 2008.

[12] J. Chen, L.-J. Chen, Y.-L. Xia et al., "Identification and verification of transthyretin as a potential biomarker for pancreatic ductal adenocarcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 7, pp. 1117–1127, 2013.

[13] D. Xiao, F. L. Meng, L. H. He, Y. X. Gu, and J. Z. Zhang, "Analysis of the urinary peptidome associated with *Helicobacter pylori* infection," *World Journal of Gastroenterology*, vol. 17, no. 5, pp. 618–624, 2011.

[14] D. Fliser, J. Novak, V. Thongboonkerd et al., "Advances in urinary proteome analysis and biomarker discovery," *Journal of the American Society of Nephrology*, vol. 18, no. 4, pp. 1057–1071, 2007.

[15] D. F. Ransohoff, "Rules of evidence for cancer molecular-marker discovery and validation," *Nature Reviews Cancer*, vol. 4, no. 4, pp. 309–314, 2004.

[16] F. Qiu, H.-Y. Liu, X.-J. Zhang, and Y.-P. Tian, "Optimization of magnetic beads for MALDI-TOF MS analysis," *Frontiers in Bioscience*, vol. 14, no. 10, pp. 3712–3723, 2009.

[17] F. Qiu, H. Y. Liu, Z. N. Dong, Y. J. Feng, X. J. Zhang, and Y. P. Tian, "Searching for potential ovarian cancer biomarkers with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry," *The American Journal of Biomedical Sciences*, vol. 1, pp. 80–90, 2009.

[18] D. P. Dutka, M. Pitt, D. Pagano et al., "Myocardial glucose transport and utilization in patients with type 2 diabetes mellitus, left ventricular dysfunction, and coronary artery disease," *Journal of the American College of Cardiology*, vol. 48, no. 11, pp. 2225–2231, 2006.

[19] E. McGregor and M. J. Dunn, "Proteomics of the heart: unraveling disease," *Circulation Research*, vol. 98, no. 3, pp. 309–321, 2006.

[20] C. A. Bootle-Wilbraham, S. Tazzyman, J. M. Marshall, and C. E. Lewis, "Fibrinogen E-fragment inhibits the migration and tubule formation of human dermal microvascular endothelial cells in vitro," *Cancer Research*, vol. 60, no. 17, pp. 4719–4724, 2000.

[21] M. G. Shlipak, J. H. Ix, K. Bibbins-Domingo, F. Lin, and M. A. Whooley, "Biomarkers to predict recurrent cardiovascular disease: the Heart and Soul Study," *The American Journal of Medicine*, vol. 121, no. 1, pp. 50–57, 2008.

[22] C. Zoccali, F. Mallamaci, G. Tripepi et al., "Fibrinogen, mortality and incident cardiovascular complications in end-stage renal failure," *Journal of Internal Medicine*, vol. 254, no. 2, pp. 132–139, 2003.

[23] P. Muntner, J. He, B. C. Astor, A. R. Folsom, and J. Coresh, "Traditional and nontraditional risk factors predict coronary heart disease in chronic kidney disease: results from the atherosclerosis risk in communities study," *Journal of the American Society of Nephrology*, vol. 16, no. 2, pp. 529–538, 2005.

[24] N. Alkjaerdsig and A. P. Fletcher, "Catabolism and excretion of fibrinopeptide-A," *Blood*, vol. 60, no. 1, pp. 148–156, 1982.

[25] M. C. Librenti, A. D'Angelo, P. Micossi, B. Garimberti, P. M. Mannucci, and G. Pozza, "β-Thromboglobulin and fibrinopeptide a in diabetes mellitus as markers of vascular damage," *Acta Diabetologica Latina*, vol. 22, no. 1, pp. 39–45, 1985.

[26] X. P. Pu, A. Iwamoto, H. Nishimura, and S. Nagasawa, "Purification and characterization of a novel substrate for plasma kallikrein (PK-120) in human plasma," *Biochimica et Biophysica Acta*, vol. 1208, no. 2, pp. 338–343, 1994.

[27] M. Piñeiro, M. A. Alava, N. González-Ramón et al., "ITIH4 serum concentration increases during acute-phase processes in human patients and is up-regulated by interleukin-6 in hepatocarcinoma HepG2 cells," *Biochemical and Biophysical Research Communications*, vol. 263, no. 1, pp. 224–229, 1999.

[28] J. Song, M. Patel, C. N. Rosenzweig et al., "Quantification of fragments of human serum inter-α-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay," *Clinical Chemistry*, vol. 52, no. 6, pp. 1045–1053, 2006.

[29] J. Villanueva, D. R. Shaffer, J. Philip et al., "Differential exoprotease activities confer tumor-specific serum peptidome patterns," *The Journal of Clinical Investigation*, vol. 116, no. 1, pp. 271–284, 2006.

[30] A. Ceriello, M. Pirisi, R. Giacomello et al., "Fibrinogen plasma levels as a marker of thrombin activation: new insights on the role of fibrinogen as a cardiovascular risk factor," *Thrombosis and Haemostasis*, vol. 71, no. 5, pp. 593–595, 1994.

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Research Article

Breaking Therapeutic Inertia in Type 2 Diabetes: Active Detection of In-Patient Cases Allows Improvement of Metabolic Control at Midterm

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Type 2 diabetes (T2D) exists in 25–40% of hospitalized patients. Therapeutic inertia is the delay in the intensification of a treatment and it is frequent in T2D. The objectives of this study were to detect patients admitted to surgical wards with hyperglycaemia (HH; fasting glycaemia > 140 mg/dL) as well as those with T2D and suboptimal chronic glycaemic control (SCGC) and to assess the midterm impact of treatment modifications indicated at discharge. A total of 412 HH patients were detected in a period of 18 months; 86.6% (357) had a diagnosed T2D. Their preadmittance HbA_{1c} was 7.7 ± 1.5%; 47% (189) had $HbA_{1c} \geq 7.4\%$ (SCGC) and were moved to the upper step in the therapeutic algorithm at discharge. Another 15 subjects (3.6% of the cohort) had T2D according to their current HbA_{1c}. Ninety-four of the 189 SCGC patients were evaluated 3–6 months later. Their HbA_{1c} before in-hospital-intervention was 8.6 ± 1.2% and 7.5 ± 1.2% at follow-up ($P < 0.004$). Active detection of hyperglycaemia in patients admitted in conventional surgical beds permits the identification of T2D patients with SCGC as well as previously unknown cases. A shift to the upper step in the therapeutic algorithm at discharge improves this control. Hospitalization is an opportunity to break therapeutic inertia.

1. Introduction

Maintaining good glycaemic control reduces the risk of microvascular and macrovascular complications associated with type 2 diabetes (T2D) [1, 2]. However, despite a broad armamentarium of effective glucose-lowering therapies, almost half of patients with T2D do not achieve globally recognized blood glucose targets [3, 4]. Between 25 and 40% of patients admitted in conventional hospitalization beds for other reasons have T2D. Hyperglycaemia in hospitalized patients, regardless of the cause, is associated with increased morbidity and mortality and it is known that early diagnosis and treatment improves the general outcomes [5–8].

Therapeutic inertia (TI) is the delay in the onset or intensification of a required treatment [9]. TI exists in a considerable percentage of patients with T2D, being reported in about 50% of cases [10, 11]. Hospitalization for other causes than diabetes could be a good opportunity to detect patients with T2D and poor glycaemic control and thus overcome TI, and, moreover, it may allow detecting unknown cases. Surgical departments of general hospitals usually have a very high prevalence of T2D patients.

The objectives of this prospective study were to set up a programme for active detection and treatment of hyperglycaemic patients during the admission in conventional surgical beds, to identify patients with previously known T2D that have a suboptimal chronic glycaemic control (SCGC) and to

evaluate the impact of treatment modifications indicated at the time of hospital discharge on the glycaemic control at midterm.

2. Material and Methods

This new intervention programme started in May 2012 in Hospital Germans Trias i Pujol, a tertiary referral hospital affiliated to the Universitat Autònoma of Barcelona, Catalonia, Spain. The Centre has 509 beds, with 353 of them in conventional hospitalization and 150 of those devoted to surgical services. The protocol has been progressively implemented in five surgical hospitalization departments (orthopaedics and traumatology, vascular surgery, general and digestive surgery, neurosurgery, and urology) in the last two years, and a pre- and postintervention assessment were planned in order to evaluate their efficacy. Admitted patients in these departments are noncritically ill ones and hyperglycaemia was defined as premeal blood glucose greater than 140 mg/dL, following the specific recommendations of ADA for management of hyperglycaemia in hospitalized patients [12–14]. Detection of hyperglycaemia in our centre uses an electronic warning message by which a glycaemic threshold of 140 mg/dL is automatically generated from admittance analyses for all the patients of surgical wards and then communicated to the Endocrinology Service by midday. The generated list of patients is then revised by a team of senior and junior residents and by a nurse and supervised by a senior staff member. The electronic clinical histories of these patients and their recent HbA_{1c} levels are evaluated; if there is no <3 months data regarding HbA_{1c}, a new measurement is performed with the recent blood sample from the present hospital stay, following the recommendations of the ADA [14]. HbA_{1c} was measured in blood samples with ethylenediaminetetraacetic acid (EDTA) by high-performance liquid chromatographic (HPLC) using a fully automated Adams Menarini HI-AUTO A1c 8160 analyzer manufactured by Arkray (Kyoto, Japan) with an interassay coefficient of variation of 1.8 and 1.5% at HbA_{1c} levels of 4.8 and 9.0%, respectively (reference range: 4–5.8%). This method is a cation exchange HPLC method certified by the NGSP (National Glycohemoglobin Standardization Program) of traceability to the Diabetes Control and Complications Trial Reference Method (DCCT).

Hyperglycaemic patients were categorized into one of the following four groups: (A) controlled T2D: T2D previously known and $HbA_{1c} < 7.4\%$; (B) T2D: previously known with SCGC as defined by a $HbA_{1c} \geq 7.4\%$; (C) T2D: not previously known as defined by hyperglycaemia > 140 mg/dL and $HbA_{1c} \geq 6.5\%$; (D) undetermined hyperglycaemic status, as defined by hyperglycaemia > 140 mg/dL and $HbA_{1c} < 6.5\%$.

During hospital stay, all patients were treated following the institutional protocol of control of hyperglycaemia consisting of a basal-bolus insulin therapy designed according to the level of hyperglycaemia and the fasting condition required for the perioperative period. When patients were discharged, those in group (B) ($HbA_{1c} \geq 7.4\%$) received a reassessment of their nutritional plan and were moved to an upper step of the therapeutic algorithm, following the recommendations by the Spanish societies of endocrinology and diabetes and the EASD/ADA [15, 16]. Patients in group (A) (well controlled diabetic patients) remained under the same treatment as that before their hospital admission. New diagnosis of T2D patients (group (C)) was initiated in diabetes treatment according to the HbA_{1c} level and clinical judgement by the consultant diabetologist team. All patients, regardless of the group, received the recommendation to make a follow-up appointment by their primary care team at 3–6 months of the hospital discharge. All treatments changes were specified in a highlighted manner in the discharge reports. In those patients with SCGC (group (B)) HbA_{1c} was determined again between three and six months after hospital discharge and evaluated according to the usual criteria by their primary care team. We compared these with previous results by consulting the electronic clinical history.

Continuous variables were expressed as mean standard deviations (SD) or median (interquartile range) and categorical variables as frequency and/or percentage. Differences between groups were assessed by the Student's t-test or the nonparametric Mann-Whitney U test, as appropriated. A P value less than 0.05 was considered statistically significant. Categorical variables were compared with χ^2 test. All statistical analyses were performed using the Statistical Package for Social Science (SPSS, Chicago, IL, USA) for personal computers, version 12.0 (SPSS).

3. Results

Four hundred and twelve hyperglycaemic patients were detected during the first 18 months after initiation of the programme. Of the 412 patients, 193 (47%) had an acute illness and the rest (219; 53%) had been planning program admission. The most common reasons for admission in patients with an acute process were bone fractures, mainly femur, arterial peripheral ischemia, cholecystitis, pancreatitis and other abdominal infectious processes, hemorrhagic stroke, and urological infections. The most frequent diagnoses of patients with scheduled hospital admission were chronic degenerative arthropathy, arterial stenosis, lesions that needed to be amputated, neoplasias of the digestive tract and the central nervous system, spinal disc herniation, and tumors of the kidney and prostate. Of the total 412 patients, 357 (86.6%) had previous known T2D (groups A and B) and 145 were women (40.6%), with a mean age of 69.7 ± 10.4 years, T2D evolution of 9 ± 9.5 years, and HbA_{1c} of 7.6% ± 1.4. From the 357 patients with known T2D, 168 (52.9%) were under good control (group (A), HbA_{1c} 6.5% ± 0.5). They had a mean age of 70.4 ± 11 years and a T2D evolution of 6.5 ± 6.1 years. The remaining 189 patients (47%, group (B), HbA_{1c} 8.6% ± 1.3) had a mean age of 69.2 ± 9.8 years and a T2D evolution of 10.9 ± 10.8 years. Patients in group (B) had a longer evolution of the T2D in comparison to those of group (A) and their HbA_{1c} was also higher ($P < 0.001$ for both). Fifteen patients had newly detected T2D (group (C)), corresponding to 3.6% of the total cohort, and in 40 patients the hyperglycaemic status was found in conjunction with an $HbA_{1c} < 6.5\%$ (group (D)). Mean age in group (C) was 68.8 ± 12.7 years and in group (D) 60.7 ± 18.3 years.

Table 1: Data of 94 out of 189 patients (group (B)) whose therapeutic changes were performed and were reassessed 3–6 months after discharge.

N	Women %	Age (years)	DM evolution (years)	HbA_{1c} PRE (%)	HbA_{1c} POST (%)
94	37 (39.4)	68.94 ± 9.89	12.44 ± 11.88	8.66 ± 1.27	7.50 ± 1.25*

*$P < 0.004$.
N: number of patients.
DM: diabetes mellitus.
HbA_{1c} PRE: preintervention.
HbA_{1c} POST: postintervention.

Patients in group (B) were moved to the upper step of the therapeutic algorithm [15, 16] at the time of hospital discharge. This action included the initiation of insulin therapy in 28 out of 357 patients with known T2D (14.8%). Ninety-four of the 189 patients from those whose therapeutic changes were performed have been assessed 3–6 months after discharge by consulting the primary care electronic clinical history. Their HbA_{1c} improved significantly (preadmission or in-hospital HbA_{1c} 8.6% ± 1.27 and after discharge 7.5% ± 1.25, $P < 0.004$). These data are shown in Table 1. In the remaining 95 patients with SCGC, 45 showed no HbA_{1c} in the clinical history due to a lack of scheduled follow-up visit after discharge and in the other 55 the follow-up visit was still not performed, as the time elapsed from discharge was less than 6 months.

4. Discussion

Recently, different diabetes medical societies have made specific recommendations for the care of diabetic patients regarding their glycaemic control when they are in hospital for any cause not specifically related to diabetes. Moreover, there is a general feeling that a certain delay exists in detecting diabetic patients and initiating the specific protocol for hyperglycaemia control while the patient is hospitalized, in particular in those admitted to surgical wards [17]. These scientific societies have proposed to take action against this situation by implementing both active detection and adequate treatment of diabetic patients when they are admitted to the hospital irrespective of the cause of admission [5]. Diabetes is present in a proportion as high as one-third to half of patients in community hospitals, a figure that will grow steadily in the near future in industrialized countries due to the increasing prevalence of diabetes, mostly related to ageing. The possibility to have automatic warnings that indicate the presence of the diabetic condition in a given patient provided from different hospital check points allows gaining time when classifying which patients require more prompt attention. Our tertiary hospital has an electronic clinical history shared with primary care physicians; thus current and past relevant information from a specific patient can travel across the health system on a real time basis.

Our protocol allowed us to detect 357 known T2D patients among those admitted to conventional beds of surgical services. Most of these would probably not have been considered as potential diabetologic consultations by their physicians in charge, as assessed by the comparison of the historical number of calls received from the specific surgical services included in the programme and the number of patients detected by the current programme. These patients are noncritically ill and a very heterogenic group. The challenge of defining the goals of antihyperglycaemic therapy arises from their heterogeneity mainly in relation to the aging process along with their diverse clinical characteristics. As a group, they should be treated as elderly diabetic patients. Therefore, it was considered that patients with $HbA_{1c} < 7.4\%$ were controlled [18]. Almost half of the patients detected (47%) had a previous SCGC at the time of hospital admission according to HbA_{1c} values. Some studies conducted in different geographical areas in Spain have confirmed a similar percentage of T2D patients with poor glycaemic control when primary care databases are evaluated [19–21]. These figures may be even higher when complex diabetic patients with active comorbidities and mostly followed at tertiary hospitals are concerned. Overall, the present study demonstrates that a substantial number of patients show a significant—either clinical or statistical—improvement in the glycaemic control at short- and midterm after active detection, evaluation, and modification of the therapeutic programme for every specific case. Consequently, we observed a 1% mean decrease of HbA_{1c}, thus reaching the recommended 7.5% HbA_{1c} value for this age group of patients. Additionally, 3.6% of the total cohort corresponded to new cases; thus our programme allowed an early treatment in these particular patients or at least did not further delay the diagnosis of T2D.

Therapeutic inertia (TI) is defined as the situation by which a given patient requiring a next step treatment modality usually with higher complexity does not receive the appropriate treatment. TI seems to be present approximately in one-third to 40% [22, 23] or even more [24, 25] of T2D patients with poor glycaemic control, especially those treated only with lifestyle changes or oral monotherapy, and also in older subjects. Assessing the true prevalence of TI is difficult and it should be noted that the methodology used to obtain these figures is heterogeneous. Moreover, TI is not the same as clinical inertia (CI) which includes not only the responsibility of the physician at the time of escalation in the therapeutic algorithm towards more complex treatment modalities but also the position of the patient, in which he/she voluntarily decides not to follow the therapy proposed by the diabetes team. In this regard, CI requires educational and emotional support, while TI requires medical training and support from expertise. Finally, the evaluation of HbA_{1c} as the indicator of TI should also be refined according to individual goal convenient for every patient, mostly related to concurrent diabetes complications and age. Therefore, a given patient may have

a convenient HbA_{1c} 8% value if he/she has major comorbidities and/or is very old and frail. However, this same value is inadequate for younger subjects with no apparent active comorbidities and relatively short duration of the disease.

In a recent multicentre, retrospective study of patients with poorly controlled diabetes and at least one hospitalization [26], less than a quarter received a change in their diabetes therapy upon discharge, and nearly one-third had no subsequent follow-up visit scheduled, suggesting widespread TI. In our cohort, a substantial number of subjects, around a quarter, did not have a primary care scheduled visit 6 months after discharge. This approach of controlling the whole process after discharge by temporal assessment of the shared electronic clinical histories also allows the implementation of rescue actions towards the reinclusion of patients lost in the follow-up by means of phone calls and other ways of contact. The overall approach could, therefore, increase the quality of care for T2D.

The implementation of an active detection programme and treatment of hyperglycaemia in patients admitted in conventional surgical beds, such as the one presented in this study, is, therefore, feasible in the habitual clinical practice and necessary for a substantial proportion of patients. We also demonstrated that the modification of the previous treatment to an upper step in the diabetes therapeutic algorithm together with the personalization of recommendations in patients with type 2 diabetes is able to obtain a significant improvement in the glycaemic control, at least at midterm.

5. Conclusions

Admission in a conventional surgical bed for any cause is a clear opportunity for overcoming therapeutic inertia and improving glycaemic control in patients with type 2 diabetes. We, therefore, propose the implementation of an active detection and treatment programme of hyperglycaemia, as we describe here, in all community and tertiary hospitals.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. Patel, S. MacMahon, J. Chalmers et al., "Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes," *The New England Journal of Medicine*, vol. 358, pp. 2560–2572, 2008.

[2] P. Gæde, H. Lund-Andersen, H.-H. Parving, and O. Pedersen, "Effect of a multifactorial intervention on mortality in type 2 diabetes," *The New England Journal of Medicine*, vol. 358, no. 6, pp. 580–591, 2008.

[3] T. J. Hoerger, J. E. Segel, E. W. Gregg, and J. B. Saaddine, "Is glycemic control improving in U.S. adults?" *Diabetes Care*, vol. 31, no. 1, pp. 81–86, 2008.

[4] M. F. B. Braga, A. Casanova, H. Teoh et al., "Treatment gaps in the management of cardiovascular risk factors in patients with type 2 diabetes in Canada," *Canadian Journal of Cardiology*, vol. 26, no. 6, pp. 297–302, 2010.

[5] G. E. Umpierrez, S. D. Isaacs, N. Bazargan, X. You, L. M. Thaler, and A. E. Kitabchi, "Hyperglycemia: an independent marker of in-hospital mortality in patients with undiagnosed diabetes," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 3, pp. 978–982, 2002.

[6] S. E. Inzucchi, "Management of hyperglycemia in the hospital setting," *The New England Journal of Medicine*, vol. 355, no. 18, pp. 1903–1911, 2006.

[7] A. Bruno, D. Gregori, A. Caropreso, F. Lazzarato, M. Petrinco, and E. Pagano, "Normal glucose values are associated with a lower risk of mortality in hospitalized patients," *Diabetes Care*, vol. 31, no. 11, pp. 2209–2210, 2008.

[8] A. P. Pérez, P. C. Gutiérrez, M. A. Diosdado et al., "Tratamiento de la hiperglucemia en el hospital," *Medicina Clínica*, vol. 132, no. 12, pp. 465–475, 2009.

[9] L. S. Phillips, J. Branch W.T., C. B. Cook et al., "Clinical inertia," *Annals of Internal Medicine*, vol. 135, no. 9, pp. 825–834, 2001.

[10] R. W. Grant, E. Cagliero, A. K. Dubey et al., "Clinical inertia in the management of type 2 diabetes metabolic risk factors," *Diabetic Medicine*, vol. 21, no. 2, pp. 150–155, 2004.

[11] D. C. Ziemer, C. D. Miller, M. K. Rhee et al., "Clinical inertia contributes to poor diabetes control in a primary care setting," *Diabetes Educator*, vol. 31, no. 4, pp. 564–571, 2005.

[12] S. E. Inzucchi and J. Rosenstock, "Counterpoint: inpatient glucose management: a premature call to arms?" *Diabetes care*, vol. 28, no. 4, pp. 976–979, 2005.

[13] G. E. Umpierrez, "Inpatient management of diabetes: an increasing challenge to the hospitalist physician," *Journal of Hospital Medicine*, vol. 2, no. 1, pp. 33–35, 2007.

[14] G. E. Umpierrez, R. Hellman, M. T. Korytkowski et al., "Management of hyperglycemia in hospitalized patients in noncritical care setting: an endocrine society clinical practice guideline," *Journal of Clinical Endocrinology and Metabolism*, vol. 97, no. 1, pp. 16–38, 2012.

[15] E. Menéndez Torre, F. J. Lafita Tejedor, S. Artola Menéndez et al., "Recommendations for the pharmacological treatment of hyperglycemia in type 2 diabetes," *Endocrinologia y Nutricion*, vol. 58, no. 3, pp. 112–120, 2011.

[16] S. E. Inzucchi, R. M. Bergenstal, J. B. Buse et al., "Management of hyperglycaemia in type 2 diabetes: a patient- centered approach. Position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD)," *Diabetologia*, vol. 56, pp. 303–316, 2012.

[17] M. Botella, J. A. Rubio, J. C. Percovich, E. Platero, C. Tasende, and J. Álvarez, "Control glucémico en pacientes hospitalizados no críticos," *Endocrinología y Nutrición*, vol. 58, no. 10, pp. 536–540, 2011.

[18] L. Kezerle, L. Shalev, and L. Barski, "Treating the elderly diabetic patient: special considerations," *Diabetes, Metabolic Syndrome and Obesity*, vol. 7, pp. 391–400, 2014.

[19] D. Orozco-Beltrán, V. F. Gil-Guillen, F. Quirce et al., "Control of diabetes and cardiovascular risk factors in patients with type 2

diabetes in primary care. The gap between guidelines and reality in Spain," *International Journal of Clinical Practice*, vol. 61, no. 6, pp. 909–915, 2007.

[20] P. Benito-López, R. García-Mayor, M. Puig-Domingo et al., "Perfil de los pacientes con Diabetes Mellitus tipo 2, en la Atención Primaria española," *Revista Clínica Española*, vol. 204, pp. 18–24, 2004.

[21] M. T. O. Tobarra, D. O. Beltrán, V. G. Guillén, and C. T. Moltó, "Frequency of attendance and degree of control of type-2 diabetic patients," *Atencion Primaria*, vol. 40, no. 3, pp. 139–144, 2008.

[22] M. Mata-Cases, B. Benito-Badorrey, P. Roura-Olmeda et al., "Clinical inertia in the treatment of hyperglycemia in type 2 diabetes patients in primary care," *Current Medical Research and Opinion*, vol. 29, no. 11, pp. 1495–1502, 2013.

[23] F. López-Simarro, C. Brotons, and I. Moral, "Inercia y cumplimiento terapéutico en pacientes con diabetes mellitus tipo 2 en atención primaria," *Medicina Clínica*, vol. 138, no. 9, pp. 377–384, 2012.

[24] R. W. Grant, E. Cagliero, A. K. Dubey et al., "Clinical inertia in the management of Type 2 diabetes metabolic risk factors," *Diabetic Medicine*, vol. 21, no. 2, pp. 150–155, 2004.

[25] R. van Bruggen, K. Gorter, R. Stolk, O. Klungel, and G. Rutten, "Clinical inertia in general practice: widespread and related to the outcome of diabetes care," *Family Practice*, vol. 26, no. 6, pp. 428–436, 2009.

[26] M. L. Griffith, J. B. Boord, S. K. Eden, and M. E. Matheny, "Clinical inertia of discharge planning among patients with poorly controlled diabetes mellitus," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 6, pp. 2019–2026, 2012.

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Research Article

The Blocking on the Cathepsin B and Fibronectin Accumulation in Kidney Glomeruli of Diabetic Rats

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Hyperglycemia results in the activation of tissue angiotensin II. Angiotensin II stimulates the synthesis of ECM proteins and causes a decrease activity of proteolytic enzymes. The aim of this study was to assess the impact of multilevel blocking of the RAAS, cathepsin B activity, and fibronectin accumulation in the glomerular in the rats diabetes model. Sixty male Wistar rats were initially included. Diabetes was induced by intravenous administration of streptozotocin. The animals were randomized to six groups of ten rats in group. Rats in the four groups were treated with inhibitors of the RAAS: enalapril (EN), losartan (LOS), enalapril plus losartan (EN + LOS), and spironolactone (SPIR); another group received dihydralazine (DIH) and the diabetic rats (DM) did not receive any drug. After six weeks, we evaluated blood pressure, 24 h urine collection, and blood for biochemical parameters and kidneys. In this study, fluorometric, ELISA, and immunohistochemical methods were used. Administration of EN + LOS increased activity of cathepsin B in homogenates of glomeruli compared to DM. Losartan treatment resulted in reduction of the ratio kidney weight/body weight compared to untreated diabetic rats. SPIR resulted in the increase activity of cathepsin B in the homogenate of glomeruli. The values of cathepsin B in the plasma of rats in all studied groups were similar and showed no tendency.

1. Introduction

Diabetes mellitus is a serious social problem. According to the World Health Organization (WHO), in 2030 the number of people with diabetes worldwide will increase to 360 million, representing 4.5% of the global population [1].

Diabetic nephropathy is the most frequent complication of diabetes that develops in up to 30–40% of patients. The main change of diabetic nephropathy is a thickening of the glomerular basement membrane and expansion of ECM proteins.

Hyperglycemia results in the activation of tissue angiotensin II, which plays an important role in the pathogenesis of kidney disease, through inflammation, fibrosis, vascular wall remodeling, and oxidative stress [2]. It was shown that blocking the AT1 receptor and angiotensin II-converting enzyme reduces the levels of inflammatory factors (NF-kB, IL-6, and TNF-β1) and is responsible for the processes of fibrosis (CTGF and TGF-β1) [3, 4].

Fibronectin is a glycoprotein present in the extracellular matrix, basal membranes, plasma, and other body fluids. FN and its receptors regulate many cellular functions. The gene expression of FN in tissues of healthy adults is generally low and increases in areas of wound healing and damaged tissue. Hyperglycemia causes increased expression of genes responsible for synthesis of FN [5, 6].

Cathepsin B (EC 3.4. 22.1) belongs to a class of cysteine proteinases and has optimum activity in acidic environment but can also be active in other pH ranges [7]. It participates in numerous physiological and pathological processes. It degrades structural proteins and enzymes in the cell, degrades the main elements of the basement membrane, and activates proenzymes, hormones, and growth factors involved in the induction phase and the executive apoptosis [8]. Glomerular

homogenates of healthy rats show high proteolytic activity of cathepsins. It has been shown that the decrease of the activity of proteolytic enzymes in the states of hyperglycemia is genetically determined as type 2 diabetes in rats and in isolated glomeruli of diabetic rats [9, 10].

The aim of this study was to assess the impact of multilevel blocking of the RAAS using an ACE inhibitor, AT1 receptor antagonist, administered separately and together, an inhibitor of aldosterone on the activity of cathepsin B, and fibronectin accumulation in the glomerular in the course of diabetic nephropathy in the diabetes model in rats.

2. Material and Methods

This study was performed in accordance with the Ethical Committee Affairs Experiments on Animals of the Medical University of Warsaw (Opinion number 5/2006). Sixty male Wistar rats weighing 180–200 g were initially included. Diabetes was induced by intravenous administration of streptozotocin [8]. After one week, blood glucose levels were evaluated. The animals were randomized to 6 groups of 10 rats in group. Rats in the 4 groups were treated with inhibitors of the RAA system, drugs were administered in drinking water in the morning: EN: enalapril 3.2 mg/kg/day; LOS: losartan 15 mg/kg/day; EN + LOS: enalapril and losartan, respectively, 3.2 and 7.5 mg/kg/day; SPIR: spironolactone 15 mg/kg/day. Another group received DIH, dihydralazine, 2.7 mg/kg/day and the DM group did not receive any drug.

The animals were followed up for six weeks (five weeks, the duration of action of drugs), and blood glucose was regularly analyzed. When blood glucose was above 700 mg/dL, insulin was administered at a dose of 0.25–1.0 IU/day. In the last week of the experiment, rats' blood pressure was measured using a pressure sensor APM MK-9301 (MK-Design, USA). A 24 hr urine collection was obtained at the end of the 6-week study using metabolic cages and urinary creatinine and microalbumin were measured. Then urine was frozen at −80°C and stored for future analysis.

The animals were euthanized and kidney and blood were collected. One of the kidneys was stored in saline in an ice bath until the isolation of glomeruli [11]. The second kidney was used for histological examination and placed in buffered formalin. The plasma biochemical tests performed are the following: glucose, total protein, albumin, creatinine, urea, and bicarbonate. Some of plasma destined for further research were frozen at −80°C.

Glomeruli were isolated according to the method developed and described by Spiro [12].

Isolated glomeruli were homogenized with a homogenizer Labsonic U (B Braun, USA).

DNA in homogenates of glomeruli was determined using the fluorometric reagent Bisbenzimide H 33258 (Hoechst, Germany) as previously described [11]. Protein in homogenates of glomeruli was determined spectrophotometrically using the BCA assay (bicinchoninic acid), protein assay reagent (Pierce, Beijerland, Netherlands) [13].

Cathepsin B activity in glomerular homogenates, urine, and plasma was measured fluorometrically using the synthetic substrate Z-Arg-Arg-AMC (N-CO_2-L-arginyl-arginine-7-amino-4-methylocoumarin salt) (Bachem, Biochemica GmbH, Heidelberg, Germany) as previously described [14].

FN concentration was determined by ELISA (enzyme-linked immunosorbent assay; immunoenzyme test) as previously described [15, 16].

Immunohistochemical staining was performed in paraffin sections of kidney with an antibody against fibronectin (Chemicon International, USA). Immunohistochemical analysis began with the assessment of the entire tissue section to determine representative areas. We evaluated 20 subsequent glomeruli within the renal cortex of representative areas. In relation to the glomerular vascular loops, each immunohistochemical reaction assessment was based on an analysis of two features: the intensity of the antibody reaction and the percentage of immunopositive vascular loops. Immunohistochemical expression was evaluated semiquantitatively using a 4-point scale:

(0) no reaction or very weak and focal (<1%) expression;

(1+) low intensity reaction, with moderate intensity expression of <50% of vascular loops and high intensity expression being segmental and in <25% of vascular loops;

(2+) moderate intensity expression involving >50% of vascular loops and high-intensity expression being segmental or continuous involving 25–75% of vascular loops;

(3+) high intensity expression that is continuous and includes >75% of vascular loops.

Immunohistochemical expression of all markers tested within the mesangium was evaluated in a similar fashion. The final evaluation for each specimen was the average (rounded to unity) from the analysis of 20 glomeruli.

The results obtained were analyzed using STATISTICA, version 9.0 which is available at the Medical University of Warsaw. Statistical nonparametric tests were used: ANOVA rank Kruskal-Wallis. Obtaining a result of analysis of the level of significance $P < 0.05$ was an indication for the use of post hoc test, Duncan. Statistical inference was performed at a significance level of $P \leq 0.05$.

3. Results

Biochemical characterization of the study groups is presented in Tables 1 and 2.

The value of kidney weight given as a percentage of final body weight in the group of untreated diabetic rats was 0.60 ± 0.06% and was significantly higher compared to the group of diabetic rats treated dihydralazine (0.54 ± 0.05%, $P = 0.01$). In addition, statistically significant differences were demonstrated between the group of diabetic rats treated dihydralazine and groups with diabetes treated enalapril ($P = 0.01$), losartan ($P = 0.0001$), enalapril and losartan in combination ($P = 0.01$), and spironolactone ($P = 0.04$), and between group of diabetic rats treated with spironolactone and losartan ($P = 0.03$) (Table 3, Figure 1).

Table 1: The characteristics of treatment groups.

Groups	Glucose mg/dL	Creatinine mg/dL	Urea mg/dL	HCO_3 mmol/L	Fibronectin μg/mL	Cathepsin B μIU/mL
DM						
Mean ± SD	746.6 ± 47.5	0.66 ± 0.11	62.6 ± 16.1	25.5 ± 1.1	20.91 ± 4.47	44.61 ± 11.88
Median (range)	763.0 (667.0–793.0)	0.7 (0.5–0.8)	64.0 (43.0–81.0)	25.8 (24.3–26.8)	20.23 (15.06–27.54)	41.03 (21.33–63.85)
EN						
Mean ± SD	706.8 ± 68.8	0.54 ± 0.17	61.6 ± 8.4	27.2 ± 1.8	25.08 ± 1.74	49.6 ± 6.60
Median (range)	703.5 (607.0–812.0)	0.5 (0.3–0.9)	61.5 (48.0–75.0)	27.1 (24.7–29.5)	25.59 (22.62–26.78)	46.57 (43.65–60.46)
LOS						
Mean ± SD	724.7 ± 99.0	0.66 ± 0.13	67.2 ± 15.8	24.7 ± 2.9	21.04 ± 2.52	49.08 ± 10.75
Median (range)	709.0 (577.0–856.0)	0.6 (0.5–0.9)	64.0 (51.0–105.0)	23.1 (21.7–28.3)	20.08 (18.70–24.94)	45.49 (39.07–66.40)
EN + LOS						
Mean ± SD	764.9 ± 61.0	0.57 ± 0.09	75.3 ± 17.7	25.8 ± 2.8	23.58 ± 3.74	50.54 ± 22.79
Median (range)	776.0 (657.0–833.0)	0.5 (0.5–0.7)	71.0 (53.0–116.0)	24.5 (21.9–29.8)	25.08 (16.62–27.06)	48.25 (25.63–80.59)
SPIR						
Mean ± SD	679.0 ± 82.0	0.61 ± 0.12	59.6 ± 10.9	26.1 ± 2.6	22.31 ± 3.02	44.7 ± 7.91
Median (range)	670.0 (570.0–834.0)	0.6 (0.4–0.8)	59.0 (47.0–80.0)	26.5 (22.2–30.0)	23.37 (17.28–25.55)	48.70 (26.88–51.10)
DIH						
Mean ± SD	680.6 ± 159.7	0.64 ± 0.09	59.8 ± 13.8	25.9 ± 2.3	23.87 ± 2.49	38.98 ± 12.90
Median (range)	699.0 (380.0–858.0)	0.6 (0.5–0.8)	63.5 (36.0–75.0)	26.2 (21.6–28.9)	23.95 (20.39–27.74)	37.07 (23.09–56.56)
ANOVA test	NS	NS	NS	NS	NS	NS

The results are presented as mean ± SD and median (range). Statistically significant differences between the groups have been shown, the levels of significance $P \leq 0.05$ in the 95% confidence interval.

Table 2: The values of blood pressure and biochemical parameters evaluated inurine collection.

Groups	Blood pressure mm Hg	Creatinine clearance μL/min/mc	Albuminuria/creatinine μg/mg	Fibronectin/creatinine ng/mg	Cathepsin B/creatinine mIU/mg
DM					
Mean ± SD	173.36 ± 26.7	2.83 ± 1.16	66.5 ± 44.9	46.73 ± 31.59	0.87 ± 0.27
Median (range)	165.00 (145.00–227.00)	2.68 (1.52–4.63)	39.3 (23.4–133.3)	31.94 (25.04–109.59)	0.81 (0.57–1.38)
EN					
Mean ± SD	160.50 ± 26.80	2.9 ± 0.80	40.3 ± 17.5	37.05 ± 11.88	0.81 ± 0.24
Median (range)	164.25 (122.00–202.00)	3.07 (1.57–3.94)	34.3 (20.7–70.3)	32.09 (24.77–61.43)	0.71 (0.58–1.32)
LOS					
Mean ± SD	164.90 ± 18.64	3.29 ± 0.85	46.1 ± 24.0	30.77 ± 8.29	0.75 ± 0.35
Median (range)	166.50 (135.00–192.00)	3.02 (2.37–4.46)	44.4 (13.2–98.2)	28.72 (18.82–44.97)	0.64 (0.41–1.48)
EN + LOS					
Mean ± SD	160.15 ± 21.81	3.98 ± 0.97	49.2 ± 29.6	29.45 ± 18.28	0.66 ± 0.25
Median (range)	164.50 (110.50–180.00)	4.26 (2.11–5.31)	35.8 (24.9–111.1)	24.00 (15.17–81.20)	0.56 (0.29–1.11)
SPIR					
Mean ± SD	168.95 ± 32.76	3.07 ± 0.73	41.2 ± 15.2	36.12 ± 7.67	0.71 ± 0.22
Median (range)	159.50 (120.00–222.00)	3.04 (1.52–4.07)	37.4 (17.6–74.1)	32.50 (29.70–49.70)	0.66 (0.40–1.04)
DIH					
Mean ± SD	159.00 ± 15.67	2.9 ± 0.39	39.3 ± 19.8*	37.57 ± 12.84	0.73 ± 0.29
Median (range)	159.75 (139.00–190.50)	2.82 (2.46–3.53)	32.8 (16.9–72.1)	37.06 (20.67–58.45)	0.70 (0.21–1.21)
ANOVA test	NS	NS	$P = 0.049$	NS	NS

The results are presented as mean ± SD and median (range). Statistically significant differences between the groups have been shown, the levels of significance $P \leq 0.05$ in the 95% confidence interval. *Statistical significance with the DM group.

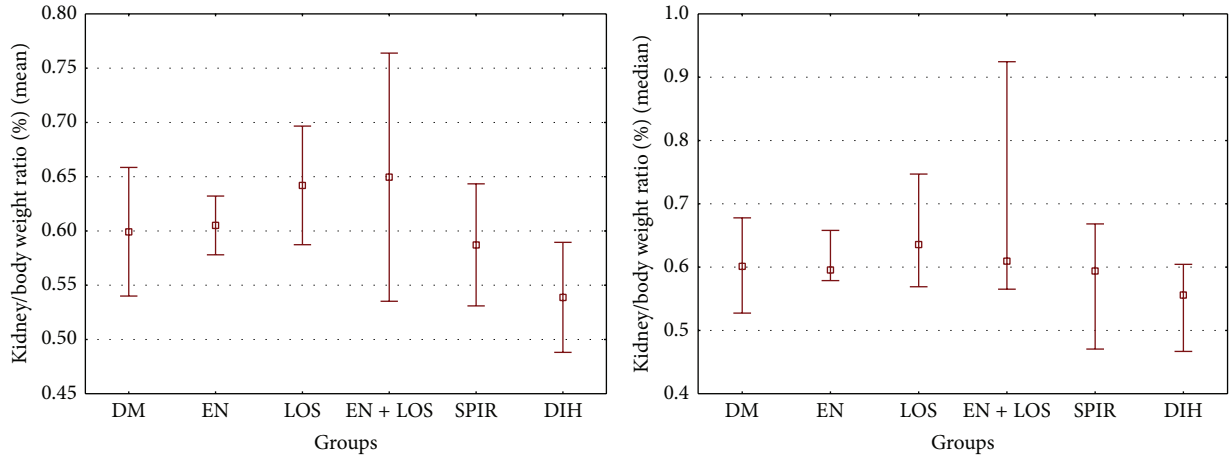

Figure 1: Kidney/body weight ratio in diabetic rats untreated and treated with enalapril, losartan, enalapril and losartan together, spironolactone, or dihydralazine. Results presented as mean ± SD and median (range). Statistically significant differences test of Kruskal-Wallis $P = 0.0099$ has been shown. P values calculated in the test and post hoc Duncan: DM versus DIH $P = 0.01$; DIH versus EN $P = 0.01$; DIH versus LOS $P = 0.0001$; DIH versus EN + LOS $P = 0.01$; DIH versus SPIR $P = 0.04$; SPIR versus LOS $P = 0.03$.

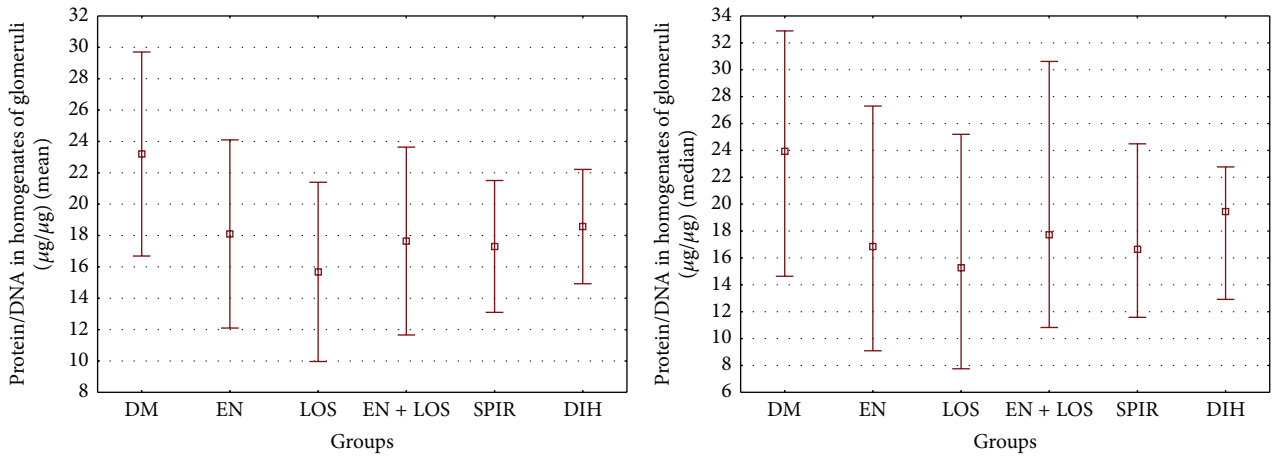

Figure 2: Comparison of the protein/DNA in homogenates of glomeruli in diabetic rats. Results presented as mean ± SD and median (range). P values calculated by post hoc test of Duncan were DM versus LOS $P = 0.01$ and DM versus SPIR. $P = 0.045$.

The coefficient protein/DNA in homogenates of glomeruli in the group of untreated diabetic rats was 23.3 ± 6.50 μg/μg and was significantly higher compared to a group of diabetic rats treated losartan 15.68 ± 5.71 μg/μg, P = 0.01, and a group of rats treated spironolactone 17.31 ± 4.2 μg/μg, $P = 0.045$ (Table 3, Figure 2).

Activity of cathepsin B in terms of microgram of DNA in homogenates of glomeruli in the group of untreated diabetic rats was 30.60 ± 9.65 μIU/μg and was lower compared to the group of diabetic rats treated with enalapril and losartan in combination ($P = 0.04$) and a group of rats diabetes treated with spironolactone (NS) and, respectively, was 51.79 ± 20.37 and 44.88 ± 32.18 μIU/μg. In the groups, diabetic rats treated with enalapril, losartan, and dihydralazine cathepsin B activity per μg of DNA were insignificantly lower compared with untreated diabetic rats and were, respectively, 25.99 ± 16.75, 21.71 ± 14.32, and 22.70 ± 14.97 μIU/μg. In addition, statistically significant differences were demonstrated between the group of diabetic rats treated with enalapril and losartan together, a group of diabetic rats treated with enalapril ($P = 0.01$), losartan ($P = 0.01$), and dihydralazine ($P = 0.01$), and groups of diabetic rats treated with spironolactone and enalapril ($P = 0.05$), losartan ($P = 0.02$), and dihydralazine ($P = 0.03$). Similar results were obtained converting activity of cathepsin B in homogenates of glomeruli in microgram of protein, Table 3, Figure 3.

Fibronectin concentration values per 1 μg of protein in homogenates of glomeruli in the untreated diabetic rats and the other treated diabetic rats showed no statistically significant differences. Similar results were obtained converting the concentration of fibronectin in glomerular homogenates for the presence of DNA (Table 3, Figure 4).

The content of fibronectin in kidney glomeruli evaluated immunohistochemical staining in the untreated diabetic rats was 2.33 ± 0.52 score and was higher compared to the other groups examined, including significantly higher ($P = 0.003$)

Table 3: The values of body weight and kidney weight and ratio of kidney weight/body weight and FN concentrations and cathepsin B activity in homogenates of glomeruli and the content of fibronectin within the glomerulus identified by immunohistochemistry.

Groups	Kidney weight/body weight %	Glomerular homogenates					Immunohistochemistry
		Protein/DNA μg/μg	FN/protein ng/μg	FN/DNA ng/μg	Cathepsin B/protein μIU/μg	Cathepsin B/DNA μIU/μg	FN score
DM							
Mean ± SD	0.60 ± 0.06**	23.2 ± 6.50	0.054 ± 0.020	0.95 ± 0.28	1.36 ± 0.37$^{\#\$}$	30.60 ± 9.65$^{\#}$	2.33 ± 0.52$^{\$}$
Median (range)	0.6 (0.53–0.68)	23.94 (14.64–32.89)	0.05 (0.029–0.088)	0.89 (0.68–1.48)	1.31 (0.92–1.79)	28.37 (21.09–50.96)	2.00 (2.00-3.00)
EN							
Mean ± SD	0.61 ± 0.03**	18.1 ± 6.00	0.034 ± 0.018	0.50 ± 0.28	1.5 ± 0.78$^{\#\$}$	25.99 ± 16.75$^{\#\$}$	2.0 ± 0.82$^{\$}$
Median (range)	0.6 (0.58–0.66)	16.85 (9.10–27.30)	0.031 (0.012–0.069)	0.42 (0.16–1.02)	1.43 (0.58–2.46)	19.14 (12.53–59.90)	2.00 (1.00–3.00)
LOS							
Mean ± SD	0.64 ± 0.05$^{\$**}$	15.68 ± 5.71*	0.056 ± 0.022	0.75 ± 0.30	1.39 ± 0.72$^{\#\$}$	21.71 ± 14.32$^{\#\$}$	1.60 ± 1.07
Median (range)	0.64 (0.57–0.75)	15.27 (7.75–25.19)	0.055 (0.016–0.09)	0.79 (0.24–1.10)	1.21 (0.49–2.97)	17.98 (10.71–49.64)	2.00 (0.00–3.00)
EN + LOS							
Mean ± SD	0.65 ± 0.11**	17.65 ± 5.99	0.058 ± 0.016	0.81 ± 0.33	3.11 ± 1.36*	51.79 ± 20.37*	2.30 ± 0.67$^{\$}$
Median (range)	0.61 (0.57–0.92)	17.73 (10.82–30.62)	0.058 (0.036–0.086)	0.80 (0.50–1.54)	2.78 (1.04–5.32)	53.96 (21.49–80.01)	2.00 (1.00–3.00)
SPIR							
Mean ± SD	0.59 ± 0.06**	17.31 ± 4.20*	0.058 ± 0.022	0.72 ± 0.27	2.58 ± 1.19*	44.88 ± 23.13**	0.90 ± 0.99$^{*\#}$
Median (range)	0.59 (0.47–0.67)	16.64 (11.58–24.49)	0.057 (0.021–0.091)	0.70 (0.33–1.13)	2.78 (1.04–4.09)	49.28 (16.59–83.64)	1.00 (0.00–3.00)
DIH							
Mean ± SD	0.54 ± 0.05*	18.57 ± 3.90	0.062 ± 0.022	0.81 ± 0.38	1.17 ± 0.64$^{\#\$}$	22.70 ± 14.97$^{\#\$}$	1.70 ± 0.95
Median (range)	0.56 (0.47–0.6)	19.45 (12.91–22.77)	0.054 (0.04–0.097)	0.62 (0.45–1.50)	0.96 (0.52–2.29)	16.02 (7.66–51.97)	1.50 (1.00–4.00)

Results are shown as mean ± SD and median (range). Statistically significant differences between the groups have been shown, the levels of significance $P \leq 0.05$ in the 95% confidence interval. *Statistical significance with the DM group; $^{\#}$statistical significance with the EN + LOS group; $^{\$}$statistical significance with the SPIR group; **statistical significance with the DIH group.

Kidney weight/body weight P value: DM versus DIH $P = 0.01$; DIH versus EN $P = 0.01$; DIH versus LOS $P = 0.0001$; DIH versus EN + LOS $P = 0.01$; DIH versus SPIR $P = 0.04$; SPIR versus LOS $P = 0.03$.

Protein/DNA P value: DM versus LOS $P = 0.01$; DM versus SPIR $P = 0.045$.

Cathepsin B/protein P value: DM versus EN + LOS $P = 0.001$; DM versus SPIR $P = 0.02$; EN + LOS versus EN $P = 0.002$; EN + LOS versus LOS $P = 0.001$; EN + LOS versus DIH $P = 0.0004$; SPIR versus EN $P = 0.03$; SPIR versus LOS $P = 0.02$; SPIR versus DIH $P = 0.009$.

Cathepsin B/DNA P value: DM versus EN + LOS $P = 0.04$; EN + LOS versus EN $P = 0.01$; EN + LOS versus LOS $P = 0.01$; EN + LOS versus DIH $P = 0.01$; SPIR versus EN $P = 0.05$; SPIR versus LOS $P = 0.02$; SPIR versus DIH $P = 0.03$.

FN by immunohistochemistry P-value: DM versus SPIR $P = 0.003$; SPIR versus EN $P = 0.02$; SPIR versus EN + LOS $P = 0.003$.

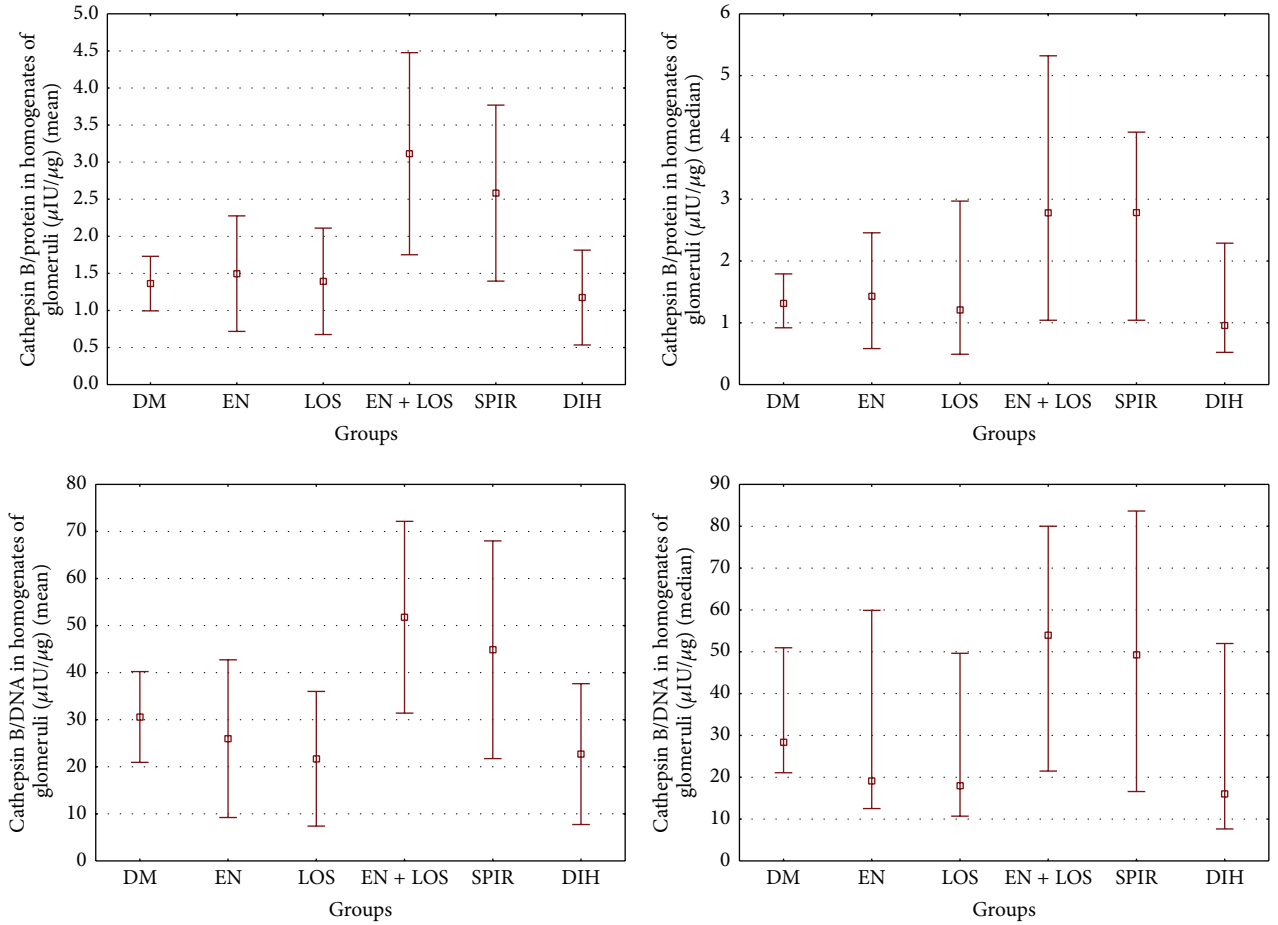

Figure 3: Comparison of cathepsin B activity in homogenates of glomeruli in study groups. Results were presented as mean ± SD and median (range). Activity of cathepsin B expressed per μg protein demonstrated statistically significant differences; ANOVA rang Kruskal-Wallis test P value 0.001 and Duncan's post hoc test DM versus EN + LOS $P = 0.001$; DM versus SPIR $P = 0.02$; EN versus EN + LOS $P = 0.002$; EN versus SPIR $P = 0.03$; LOS versus EN + LOS $P = 0.001$; LOS versus SPIR $P = 0.02$; EN + LOS versus DIH $P = 0.0004$; DIH versus SPIR $P = 0.009$. The activity of cathepsin B expressed per μg DNA. ANOVA rang Kruskal-Wallis test P value 0.0015 and post hoc Duncan test DM versus EN + LOS $P = 0.04$; EN versus EN + LOS $P = 0.01$; EN versus SPIR $P = 0.05$; LOS versus EN + LOS $P = 0.01$; LOS versus SPIR $P = 0.02$; EN + LOS versus DIH $P = 0.01$; SPIR versus DIH $P = 0.03$.

compared to the diabetic rats treated spironolactone 0.90 ± 0.99 score. Additionally, statistically significant differences have been shown between groups of diabetic rats treated with spironolactone and enalapril ($P = 0.02$) and diabetic rats treated with spironolactone and losartan and enalapril in combination ($P = 0.003$) (Table 3, Figures 5 and 6).

4. Discussion

Our study, performed on an animal model of diabetes mellitus induced by streptozotocin, aimed at assessing the impact of RAAS blocking on the activity of cathepsin B and the accumulation of FN. Diabetic rats were randomized into six treatment groups. Rats of groups 1–5 were treated with an ACE inhibitor, AT1 receptor antagonist, administered separately and together, and an inhibitor of aldosterone and dihydralazine. The rats in group number 6 were not treated and they constituted the control group. In the renal cortex of diabetic rats treated and untreated, performed immunohistochemical evaluation of fibronectin was observed. The activity of cathepsin B and the concentration of FN were assessed in homogenates of glomeruli, in 24 hour urine and plasma.

Fibronectin is a protein of the extracellular matrix, produced by the cells and endothelial mesangium [17, 18]. In mild and moderate diabetic nephropathy, accumulation of FN in the matrix mesangium has been shown and increased excretion in the urine does appear up earlier than microalbuminuria [17, 19].

In previous studies, the correlation between the accumulation FN and cathepsin B activity in the kidney glomerulus in diabetic rats has been shown [20].

The genetically determined type II diabetes in rats showed an increased accumulation of fibronectin (FN) in the kidney glomerulus. This phenomenon is accompanied by a decreased ability to degrade this protein [9]. Cumulative increase in ECM proteins in glomeruli depends on the increase of synthesis and/or reduces protein degradation.

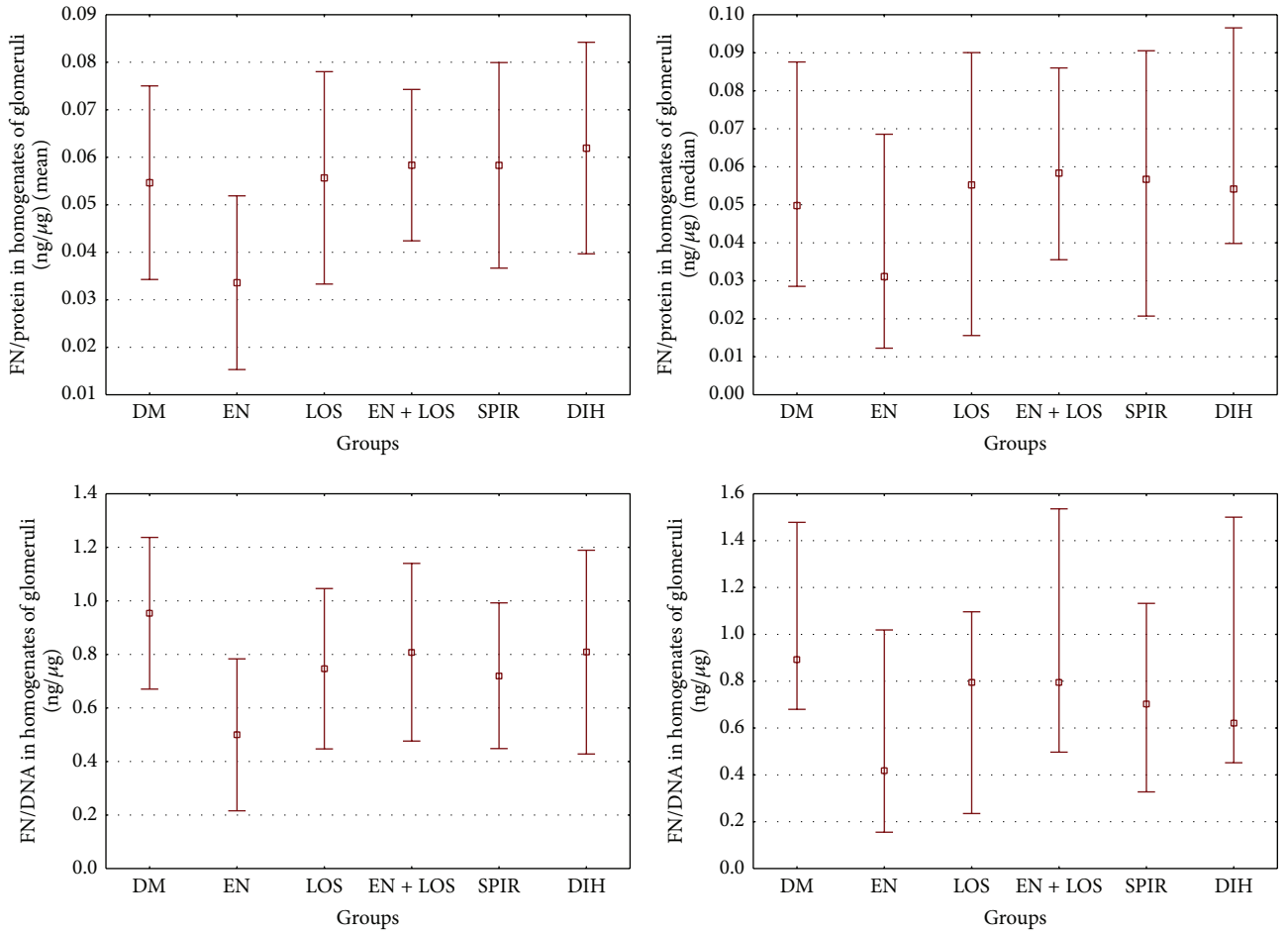

Figure 4: The comparison fibronectin concentration evaluated in glomerular homogenates. Results are presented as mean ± SD and median (range). Data are expressed per μg protein and per μg DNA. Differences between groups were not statistically significant.

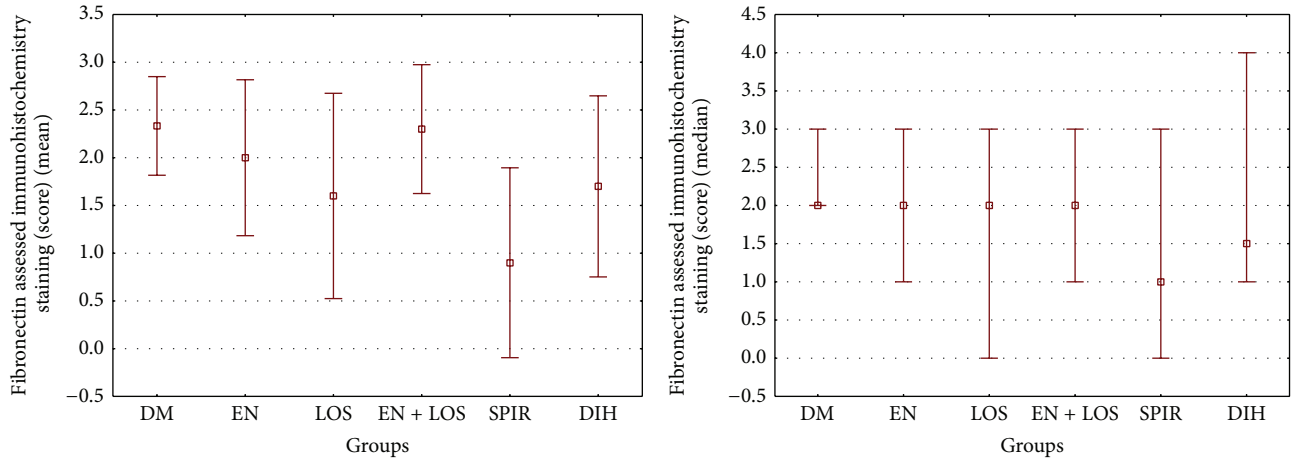

Figure 5: The content of fibronectin (FN) in glomeruli diabetic rats untreated (DM) and diabetic rats treated with enalapril (EN), losartan (LOS), enalapril and losartan together (EN + LOS), spironolactone (SPIR), or dihydralazine (DIH). Results were presented as mean ± SD and median (range). Differences between groups were statistically significant. ANOVA rang Kruskal-Wallis test P value = 0.02 and post hoc Duncan: DM versus SPIR $P = 0.003$, SPIR versus EN $P = 0.02$, and SPIR versus EN + LOS $P = 0.003$.

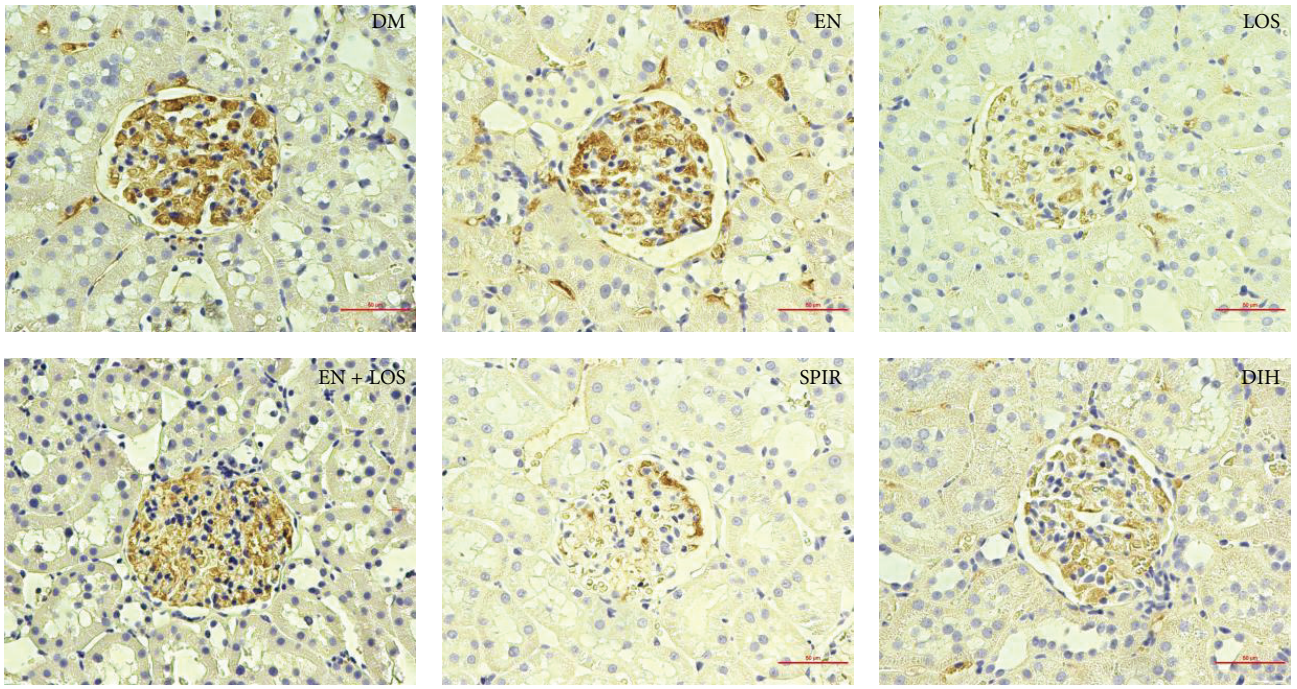

Figure 6: The contents of FN in glomeruli of STZ diabetic rats untreated (DM) and STZ diabetic treated enalapril (EN), losartan (LOS), including enalapril, and losartan (EN + LOS), spironolactone (SPIR), and dihydralazine (DIH), determined by immunohistochemistry staining, 40x magnification, scale 50 μm.

Proteolytic enzymes are responsible for maintaining the balance of synthesis and degradation of components of the extracellular matrix (ECM) [21].

Onozato et al. [22] showed a decrease of FN immunoreactivity in the kidney glomerulus in rats with hypertension that have blocked aldosterone and angiotensin II. In the present study the contents of the FN, determined by immunohistochemical staining in renal glomeruli of diabetic rats treated with enalapril, losartan, spironolactone, and dihydralazine were lower compared to the amount of FN in the renal glomeruli of rats with untreated diabetes. The largest decrease in the content FN was obtained in the group of diabetic rats receiving spironolactone ($P = 0.003$). No FN obtained reduction in kidney glomerulus of diabetic rats was treated with enalapril and losartan together.

The concentration of fibronectin in glomerular homogenates, based on DNA, in groups of diabetic rats treated has only a slight decrease compared to untreated diabetic rats. Similarly to the fibronectin excretion in the urine per mg creatinine, it has slightly decreased in the groups treated diabetic rats compared to untreated diabetic rats, while the concentration of FN in plasma of rats in all groups examined were in similar extent. Rao et al. [23] giving the rats streptozotocin diabetes ACEi and AT1 blocker received separately and together decrease the concentration of FN in the plasma.

The reduction ratio kidney weight/body weight, reflecting the development of kidney hypertrophy, was obtained only in the group of diabetic rats treated with dihydralazine. The coefficient of protein/DNA, reflecting glomerulosclerosis, was decreased in the group with diabetic rats treated with losartan ($P = 0.01$) and spironolactone ($P = 0.045$). In the other groups treated diabetic rats have shown a downward trend compared to the group of untreated diabetic rats.

A significant reduction of albuminuria has been shown in diabetic rats treated with dihydralazine compared to untreated diabetic rats. In other groups of diabetic rats receiving treatment, a decreasing trend of albuminuria has been shown in relation to untreated diabetic rats. Rao et al. [23] demonstrated reduction in daily urinary albumin excretion in treated diabetic rats compared to untreated diabetic rats.

Angiotensin II stimulates the synthesis of ECM proteins and causes a decrease activity of proteolytic enzymes. Cathepsin B (EC 3.4. 22.1) belongs to a class of cysteine proteinases and plays many functions in physiological and pathological processes. Glomerular homogenates of healthy rats show high proteolytic activity of cathepsins. Reduction of the activity of proteolytic enzymes in the states of hyperglycemia in the genetically determined type 2 diabetes in rats and in streptozotocin diabetic rats has been shown [9, 10, 24].

Multilevel blocking of the RAA system significantly increased activity of cathepsin B in homogenates of glomerular in diabetic rats treated with spironolactone ($P = 0.02$) and in combination with therapy with losartan and enalapril ($P = 0.001$) compared to the untreated diabetic rats. Dihydralazine administration, enalapril, or losartan in rats with diabetes did not lead to the expected results. Suzuki et al. [25] showed a beneficial effect of treatment ACEi to restore the activity of cathepsin B in the heart and Kim et al. [26] demonstrated that combination therapy ACEi and ARB (angiotensin-II receptor

blocker) can effectively prevent or reverse myocardial fibrosis. Maione et al. [27] showed that the development of an end-stage renal failure and change micro- to macroalbuminuria were significantly reduced after treatment of ACEi versus placebo and ARB versus placebo. This effect is not present in combined therapy (ACEi + ARB) versus monotherapy.

Assessing the activity of cathepsin B in 24-hour urine has shown a downward trend in the values of individual treatment groups compared to diabetic rats which are not treated. The values of cathepsin B in the plasma of rats in all groups studied were similar and showed no tendency.

Lysosomal enzymes, namely, cathepsins, are responsible for intracellular protein turnover [28]. Primarily its role is to maintain an intracellular pool of amino acids [29].

A part from controlling protein metabolism cathepsin is involved in the process of autophagy.

The summary of autophagy is provided by Deretic [30]. In summary autophagosomes remove irreversibly damaged mitochondria and toxic molecular macromolecules. Recently it is proved that the process of autophagy and activity of cathepsins are strictly linked to the process of inflammation.

Conflict of Interests

No conflict of interests was declared by the authors in regard to this work.

References

[1] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[2] C. R. Ban and S. M. Twigg, "Fibrosis in diabetes complications: pathogenic mechanisms and circulating and urinary markers," *Vascular Health and Risk Management*, vol. 4, no. 3, pp. 575–596, 2008.

[3] M. E. Cooper, "The role of the renin-angiotensin-aldosterone system in diabetes and its vascular complications," *The American Journal of Hypertension*, vol. 17, no. 11, pp. 16S–20S, 2004.

[4] G. Giacchetti, L. A. Sechi, S. Rilli, and R. M. Carey, "The renin-angiotensin-aldosterone system, glucose metabolism and diabetes," *Trends in Endocrinology and Metabolism*, vol. 16, no. 3, pp. 120–126, 2005.

[5] S. Roy, R. Sala, E. Cagliero, and M. Lorenzi, "Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 1, pp. 404–408, 1990.

[6] D. C. Dean, "Expression of the fibronectin gene," *American Journal of Respiratory Cell and Molecular Biology*, vol. 1, no. 1, pp. 5–10, 1989.

[7] B. Werle, B. Jülke, T. Lah, E. Spiess, and W. Ebert, "Cathepsin B fraction active at physiological pH of 7.5 is of prognostic significance in squamous cell carcinoma of human lung," *British Journal of Cancer*, vol. 75, no. 8, pp. 1137–1143, 1997.

[8] C. J. Olbricht and B. Geissinger, "Renal hypertrophy in streptozotocin diabetic rats: role of proteolytic lysosomal enzymes," *Kidney International*, vol. 41, no. 4, pp. 966–972, 1992.

[9] L. Paczek, M. Teschner, R. M. Schaefer, and A. Heidland, "Intraglomerular fibronectin accumulation and degradation in obese Zucker rats," *Diabetologia*, vol. 34, no. 11, pp. 786–789, 1991.

[10] L. Paczek, Z. Gaciong, I. Bartlomiejczyk, K. Sebekova, G. Birkenmeier, and A. Heidland, "Protease administration decreases enhanced transforming growth factor-β1 content in isolated glomeruli of diabetic rats," *Drugs under Experimental and Clinical Research*, vol. 27, no. 4, pp. 141–149, 2001.

[11] D. A. Harris, "Fluorometric assay for DNA with Hoechst 33258," in *Spectrophotometry and Spectrofluorimetry*, D. A. Harris and C. L. Bashford, Eds., pp. 64–65, IRL Press, Washington, DC, USA, 1987.

[12] R. G. Spiro, "Studies on the renal glomerular basement membrane. Preparation and chemical composition," *The Journal of Biological Chemistry*, vol. 242, no. 8, pp. 1915–1922, 1967.

[13] K. J. Wiechelman, R. D. Braun, and J. D. Fitzpatrick, "Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation," *Analytical Biochemistry*, vol. 175, no. 1, pp. 231–237, 1988.

[14] A. J. Barrett, "Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates," *Biochemical Journal*, vol. 187, no. 3, pp. 909–912, 1980.

[15] S. I. Rennard, R. Berg, G. R. Martin, J. M. Foidart, and P. G. Robey, "Enzyme-linked immunoassay (ELISA) for connective tissue components," *Analytical Biochemistry*, vol. 104, no. 1, pp. 205–214, 1980.

[16] I. Bartlomiejczyk, R. Krolak, J. Soin, L. Paczek, L. Gradowska, and Z. Gaciong, "Wydalanie z moczem białek macierzy zewnątrzkomórkowej w cukrzycy insulinozależnej," *Polskie Archiwum Medycyny Wewnętrznej*, vol. 98, pp. 493–500, 1997.

[17] A. J. Dixon, J. Burns, M. S. Dunnill, and J. McGee O'D., "Distribution of fibronectin in normal and diseased human kidneys," *Journal of Clinical Pathology*, vol. 33, no. 11, pp. 1021–1028, 1980.

[18] J. A. Madri, F. J. Roll, H. Furthmayr, and J. M. Foidart, "Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney," *The Journal of Cell Biology*, vol. 86, no. 2, pp. 682–687, 1980.

[19] R. J. Falk, J. I. Scheinman, S. M. Mauer, and A. F. Michael, "Polyantigenic expansion of basement membrane constituents in diabetic nephropathy," *Diabetes*, vol. 32, supplement 2, pp. 34–39, 1983.

[20] A. Wyczalkowska-Tomasik, I. Bartlomiejczyk, B. Gornicka, and L. Paczek, "Strong association between fibronectin accumulation and lowered cathepsin B activity in glomeruli of diabetic rats," *Journal of Physiology and Pharmacology*, vol. 63, no. 5, pp. 525–530, 2012.

[21] W. H. Baricos, "Decreased proteolysis of the glomerular extracellular matrix as a pathogenic mechanism in progressive renal disease," *Journal of Nephrology*, vol. 7, no. 6, pp. 322–328, 1994.

[22] M. L. Onozato, A. Tojo, N. Kobayashi, A. Goto, H. Matsuoka, and T. Fujita, "Dual blockade of aldosterone and angiotensin II additively suppresses TGF-β and NADPH oxidase in the hypertensive kidney," *Nephrology Dialysis Transplantation*, vol. 22, no. 5, pp. 1314–1322, 2007.

[23] R. P. Rao, A. K. Jain, and B. P. Srinivasan, "Dual therapy versus monotherapy of trandolapril and telmisartan on diabetic nephropathy in experimentally induced type 2 diabetes mellitus rats," *Journal of the Renin-Angiotensin-Aldosterone System*, vol. 12, no. 3, pp. 169–175, 2011.

[24] A. Tojo, M. L. Onozato, H. Kurihara, T. Sakai, A. Goto, and T. Fujita, "Angiotensin II blockade restores albumin reabsorption in the proximal tubules of diabetic rats," *Hypertension Research*, vol. 26, no. 5, pp. 413–419, 2003.

[25] H. Suzuki, L. Schaefer, H. Ling et al., "Prevention of cardiac hypertrophy in experimental chronic renal failure by long-term ACE inhibitor administration: potential role of lysosomal proteinases," *American Journal of Nephrology*, vol. 15, no. 2, pp. 129–136, 1995.

[26] S. Kim, M. Yoshiyama, Y. Izumi et al., "Effects of combination of ACE inhibitor and angiotensin receptor blocker on cardiac remodeling, cardiac function, and survival in rat heart failure," *Circulation*, vol. 103, no. 1, pp. 148–154, 2001.

[27] A. Maione, S. D. Navaneethan, G. Graziano et al., "Angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and combined therapy in patients with micro-and macroalbuminuria and other cardiovascular risk factors: a systematic review of randomized controlled trials," *Nephrology Dialysis Transplantation*, vol. 26, no. 9, pp. 2827–2847, 2011.

[28] A. Tojo, M. Onozato, H. Ha et al., "Reduced albumin reabsorption in the proximal tubule of early-stage diabetic rats," *Histochemistry and Cell Biology*, vol. 116, no. 3, pp. 269–276, 2001.

[29] Y. Uchiyama, "Autophagic cell death and its execution by lysosomal cathepsins," *Archives of Histology and Cytology*, vol. 64, no. 3, pp. 233–246, 2001.

[30] V. Deretic, "Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors," *Current Opinion in Immunology*, vol. 24, no. 1, pp. 21–31, 2012.

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Research Article

Role of the Insulin-Like Growth Factor Type 1 Receptor in the Pathogenesis of Diabetic Encephalopathy

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Defective cognitive function is common in patients with diabetes, suggesting that insulin normally exerts anabolic actions in neuron, namely, diabetic encephalopathy. However, because insulin can cross-activate the insulin-like growth factor type 1 receptor (IGF-1R), which also functions in most of tissues, such as muscle and bone, it has been difficult to establish the direct (IGF-1-independent) actions of insulin in the pathogenesis of diabetic encephalopathy. To overcome this problem, we examined insulin signaling and action in primary PC-12 cells engineered for conditional disruption of the IGF-1 receptor (ΔIGF-1R). The results showed that the lower glucose metabolism and high expression of IGF-1R occurred in the brain of the DE rat model. The results also showed the defect of IGF-1R could significantly improve the ability of glucose consumption and enhance sensitivity to insulin-induced IR and Akt phosphorylation in PC12 cells. And meanwhile, IGF-1R allele gene knockout (IGF-1R^{neo}) mice treated with HFD/STZ had better cognitive abilities than those of wild mice. Those results indicate that insulin exerts direct anabolic actions in neuron-like cells by activation of its cognate receptor and prove that IGF-1R plays an important role in the pathogenesis of diabetic encephalopathy.

1. Introduction

The case reports are presented in the order of increasing severity of the neuropathological changes [1, 2]. The severe damage found on histological examination of the brains from the patients justifies the term encephalopathy [3–5]. One point of interest was whether cerebral changes were the cause or a sequela of the disease [6, 7]. Diabetes and its treatment are associated with functional and structural disturbances in the brain [8–10]. Many existing publications focused on changes in cerebral function and structure that develop more insidiously [10]. These changes are referred to as diabetic encephalopathy (DE), a term that encompasses functional impairment of cognition, cerebral signal conduction, neurotransmission and synaptic plasticity, and underlying structural pathology associated with diabetes [11–14].

Insulin-like growth factor-1 (IGF-1) that is a single-chain polypeptide is widely expressed in central nervous system [15–17]. Overexpression and genetic ablation of components of the IGF system in animal models can lead to developmental anomalies and functional disturbances [18–20]. IGF-1 acts primarily through its receptor, IGF-1 receptor (IGF-1R), which is widely distributed in the brain [21]. Binding of IGF-1 to IGF-1R may activate two major signaling pathways, PI3K/Akt and MAPK pathways [22–24]. The activated form of Akt, phosphorylated Akt (p-Akt), may inhibit several proapoptotic factors including glycogen synthase kinase-3 beta (GSK-3 beta), fork-head homolog in rhabdomyosarcoma (FKHR), Bcl-2-associated death protein, and caspase-9; each of them may influence neuronal survival after stroke [25]. Autophosphorylation increases the kinase activity of IR-type receptors by 3 orders of magnitude, enabling them to phosphorylate a number of substrate proteins and engender growth or metabolic responses [26, 27]. In addition to forming homodimers, IR and IGF-1R can form heterodimers with each other [28–30].

To examine the direct actions of insulin in diabetic encephalopathy (DE) and elucidate signaling pathways downstream of the IR, we used a model for conditional removal of the IGF-1R in vitro by adenoviral introduction

of the Cre-recombinase to primary rat PC-12 cell derived from mice carrying floxed IGF-1R alleles. We show that PC-12 cells lacking the IGF-1R are two to three times more sensitive to insulin than are cells expressing both receptors. And in the model for downregulated IGF-1R in vivo, the knockdown (IGF-1R^{neo}) mice treated with HFD/STZ have better cognitive abilities than those of wild mice. It is concluded that insulin exerts direct anabolic actions in neuron-like cells by activation of its cognate receptor; the solid data provided in the study proves that IGF-1R plays an important role of in the pathogenesis of DE.

2. Materials and Methods

2.1. Experimental Animals and Creation of Animal Model. Wistar rats (male, weighing 180–200 g) were supplied by the Laboratory Animal Center of BeiJing. All animal experiments were conducted according to the guidelines of the local animal use and care committees and executed according to the National Animal Law. The animals were divided into three groups: normal controls (CON, $n = 25$), diabetic encephalopathy (D, $n = 25$), and diabetic encephalopathy (DE, $n = 25$). The rats were fed with HFD for 4 weeks; STZ was prepared before each use at 20 mg/mL in 0.1 M pH 4.4 citrate buffer and was injected at 150 mg/kg, i.p., into rats which had been fasted for 12 h prior to receiving the injection. Four days later, nonfasting blood glucose in a tail-vein sample was determined by a glucose analyzer; a value >15 mM/L was accepted as a successfully created diabetic model.

The IGF receptor null (IGF-1R^{-}) mice were not used in this study, because Epaud et al. reported that IGF-1R$^{-/-}$ embryos displayed severe lung hypoplasia and markedly underdeveloped diaphragms, leading to lethal neonatal respiratory distress [30]. IGF-1R allele gene knockout (IGF-1R^{neo}) mice described previously [6, 13, 21]. For studies in young adult mice, IGF-1R^{neo} mice were 9 to 12 weeks old. Mice lived under SPF conditions in individually ventilated filter-cages. All IGF-1R^{neo} mice were provided by Changchun ibiocc Co., Ltd. Preparation of HFD/STZ-induced diabetic mice refers to the publication [31].

2.2. Test of Abilities of Learning and Memory. Morris water maze tests were performed after training for 12 weeks. After the rats were familiar with the testing environment, normal training was performed from the second day. Orientation test: rats were trained twice per day, one time in the morning and one time in the afternoon. Each training lasted for 120 s and the gap time was 30 s. This training lasted for 4 days. Starting area was randomly selected and the number of times rats touch the platform in 120 s was recorded. The platform was removed, and the rats were put into water at the opposite side of the platform. The percent of residence time in the center area and number of times of passing the former platform in 120 s were recorded.

2.3. In Vivo PET Studies. PET studies were performed in the rats which were suffering from diabetes or diabetic encephalopathy ($n = 20$ per group). The PET protocol was the following report.

2.4. Biochemistry Markers Test. The brains of rats in each group after the test of abilities of learning and memory were collected on the ice and then the hippocampus was dissected. Tissues were crushed and centrifuged at the speed of 2000 r/min for 10 min. The supernate was collected and the activities of SOD, GSH-Px, CAT, and content of MDA in the rat hippocampal gyrus were detected. Coomassie brilliant blue staining was used to detect protein concentration.

2.5. HE Staining Test. Thirty μm brain coronal sections were collected from every 200 μm. The sections were deparaffinized, with two changes of xylene, 10 min each. They were rehydrated in 2 changes of absolute alcohol, for 5 min each, 95% alcohol for 2 min and 70% alcohol for 2 min, then washed briefly in distilled water, and stained in Harris hematoxylin solution for 8 min. They were washed in running tap water for 5 min and differentiated in 1% acid alcohol for 30 s. After this, they were washed in running tap water for 1 min and blued in 0.2% ammonia water or saturated lithium carbonate solution for 30 to 60 s. Again they were washed in running tap water for 5 min, rinsed in 95% alcohol, at 10 dips, and then counterstained in eosin-phloxine solution for 30 s. They were dehydrated in 95% alcohol, 2 changes of absolute alcohol, 5 min each. They were cleared in 2 changes of xylene, 5 min each, and mounted with xylene based mounting medium. The neurons in CA1 in hippocampus were observed using optical microscope.

2.6. IHC Staining Test. After dissecting tissues at 5 μm and fixing them in 4% paraformaldehyde for 10 m, slides were incubated 2 to 3 times in xylene for 10 m each and then incubated twice in 100% ethanol for 2 m each. They were hydrated by placing in 95, 70, 50, and 30% ethanol for 2 min each. Slides were placed into buffer containing 5% normal goat serum for 10 min. Slides were incubated in a humidified chamber overnight with primary antibody (rabbit anti-rat Akt/PKB 1 : 500, rabbit anti-rat GLUT4 1 : 1000). They were washed in 5 m in buffer for 3 times and incubated with secondary antibody in a humidified chamber for 30 min. DAB and hematoxylin staining, 5 discontinue brain sections were selected and 5 fields were selected randomly. The numbers of Akt/PKB and GLUT4 positive cells in CA1 were counted.

2.7. Western Blot. Run 20 μg protein per lane after heating at 100°C for 5 min. Run on an SDS-PAGE gel until the blue front is at the bottom of the gel. Transfer to a nitrocellulose membrane for 0.5 A-h. Block the membrane for 1 h in 5% nonfat dry milk in 1 × PBST, in a small Tupperware dish on a shaker. Incubate with primary antibody (rabbit anti-rat pAKT 1 : 500, rabbit anti-rat GLUT4 1 : 1000, and rabbit anti-rat β-actin 1 : 200) at 4°C overnight. Wash 3 times for 5 to 10 min in 50 mL 1 × PBS with 0.1% Tween 20 at RT. Incubate with goat anti-rabbit 1 : 200 for 1 h at RT in 1 × PBST, wash 3 × 10 min, and rinse with ddH_2O. Detect protein with ECL kit (2 mL/membrane). In a separate tube, mix black and white

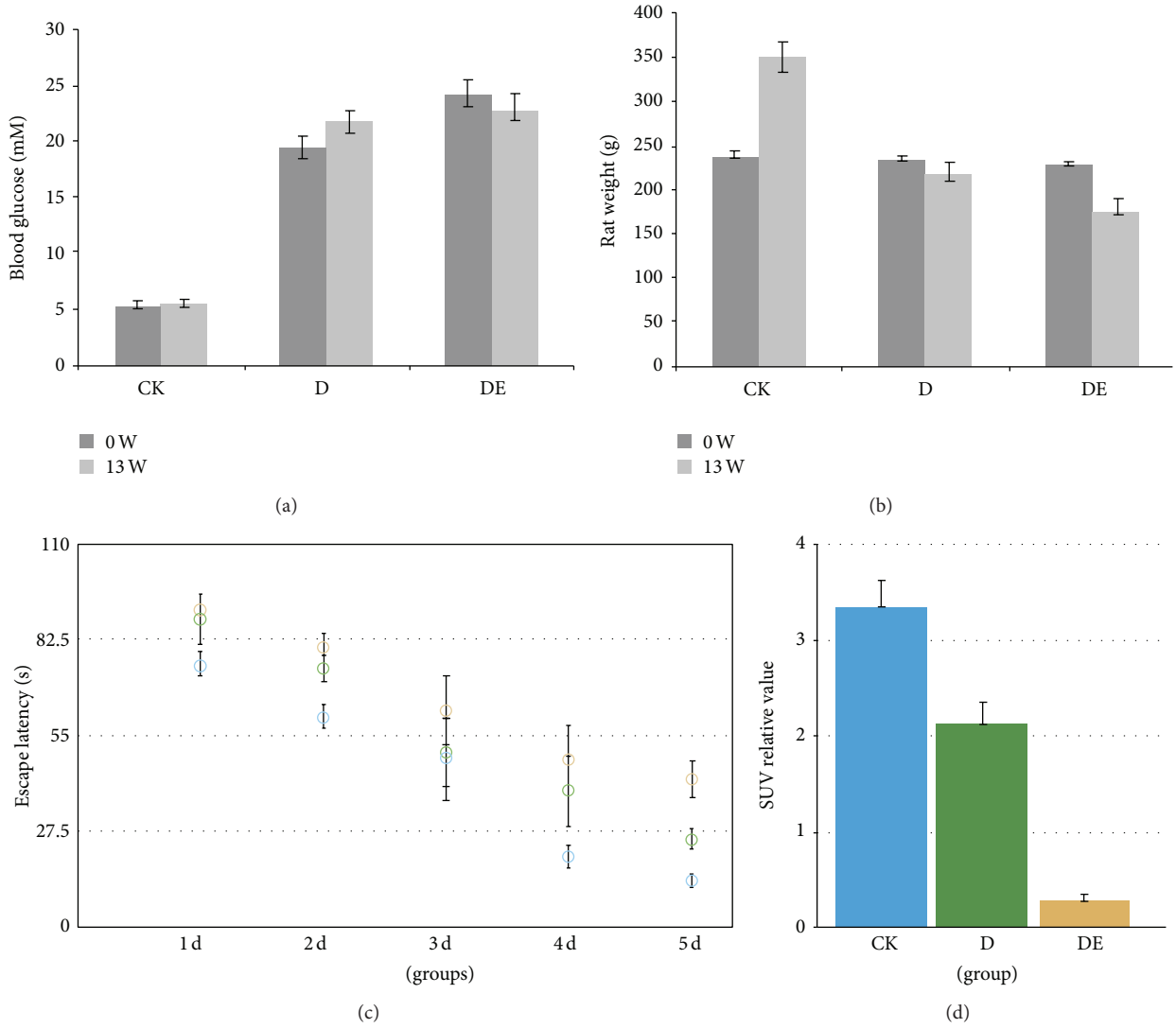

Figure 1: Blood glucose (a) and body weight (b) of mice in 3 groups. The ability analysis of learning and memory of mice in 3 groups (c). Evaluation of brain glycometabolism in DE animals by PET/CT (d).

ECL solutions in a 1 : 1 ratio. Then add aliquot solution onto membranes and wait for 1 min. Drain the ECL, wrap in plastic, and expose to film. The value of protein would be compared with β-actin, and the relative potency ratio would stand for the expression of protein.

2.8. Statistical Analysis. Data were expressed as mean ± standard deviation (M ± SD). Group differences in the swimming time in the Morris water maze test and the number of errors in the passageway water maze test were analyzed by SPSS 11.0 using Windows software to conduct two-way analysis of variance (ANOVA, equal variances assumed by S-N-K) on repeated measurements. Other data were analyzed by SPSS 11.0 using Windows software to conduct one-way ANOVA (equal variances assumed by S-N-K). A post hoc test was used to obtain the P values. $P < 0.05$ was considered significant.

3. Results

3.1. Comparation of Study and Memory Ability. The rats of D group and D + DE were with polydipsia, polyphagia, polyuria and weight loss, yellowish color, and poor spirit of the late, slow-moving symptoms. As shown in Figure 1, at the beginning of generating animal model, the values of blood glucose in D group and DE groups were much higher than Normal Control group in the 13th week ($P < 0.01$); the body weight of mice in 3 groups showed the same situation ($P < 0.01$).

During the training period, the escape latency in all rats decreased significantly as training days increased (F day = 1324.66, $P < 0.01$). To use the Morris water maze test, the rats in DE group had more swimming time than that in D group ($P < 0.05$) and made significantly more errors compared with that of Normal Control group ($P < 0.05$). The rats showed reversed behavioral alternation with levels returning close to that of rats in the control group (Figure 1(c)).

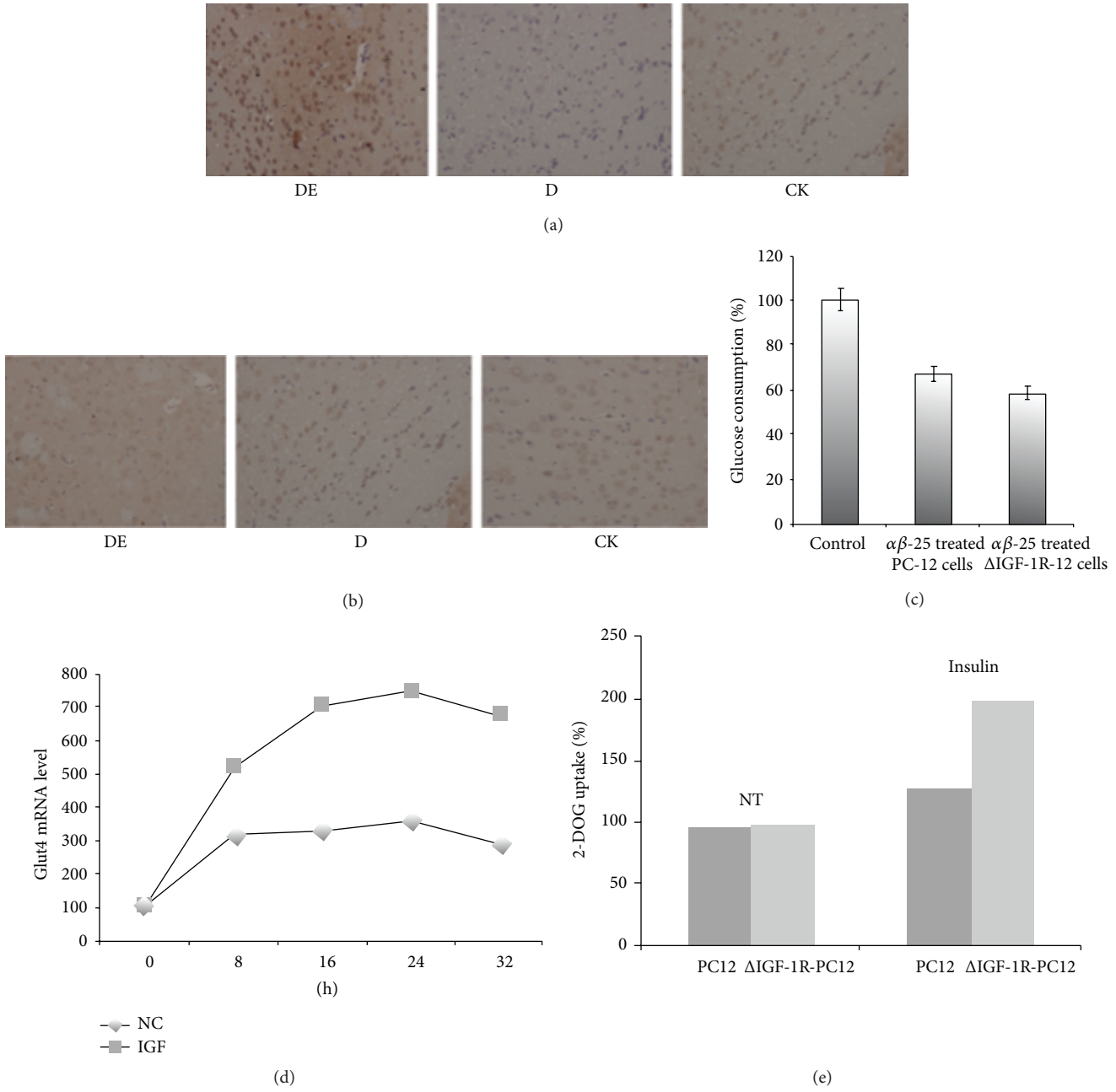

Figure 2: Expression of enhanced IGF-1R (a) and IR (b) in the hippocampal gyrus of DE rats. Enhanced insulin-induced glucose transport in ΔIGF-1R PC-12 cells (c, d, and e).

3.2. The Lower Glucose Metabolism in the Brain of the DE Rat Model. To investigate glucose metabolism in the DE rat model, the changes of [^{18}F]-FDG-PET images were recorded. A significant positive correlation was found between D group and DE group and the [^{18}F]-FDG uptake in the cortex and the hippocampus. Evaluation of glucose metabolism in animals revealed a decrease of cortical and hippocampal glucose uptake in the DE group compared with Normal Control group. In D group, more glucose was consumed, as compared with DE group. Based on the PET/CT data, low levels of glucose metabolism may be an important factor in the process of encephalopathy induced by diabetes (Figure 1(d)).

3.3. Abnormally High Expression of IGF-1R Occurs in the Brain Tissue of Rats Suffering from Diabetic Encephalopathy. IGF-1R was measured by immunohistochemistry assay in DE group and D group. As is shown in Figures 2(a) and 2(b), compared with Normal Control group, it was found a sharp increase of IGF-1R in DE group, but there was a decrease in the diabetic group. On the other hand, no difference of IGF-1R expression was seen among the Normal Control group, DE group and D group in Figures 2(a) and 2(b) using the same method. Abnormally high expression of IGF-1R has been found in the studies for the organizations rarely. This phenomenon was ever confusing to us, so we decided to

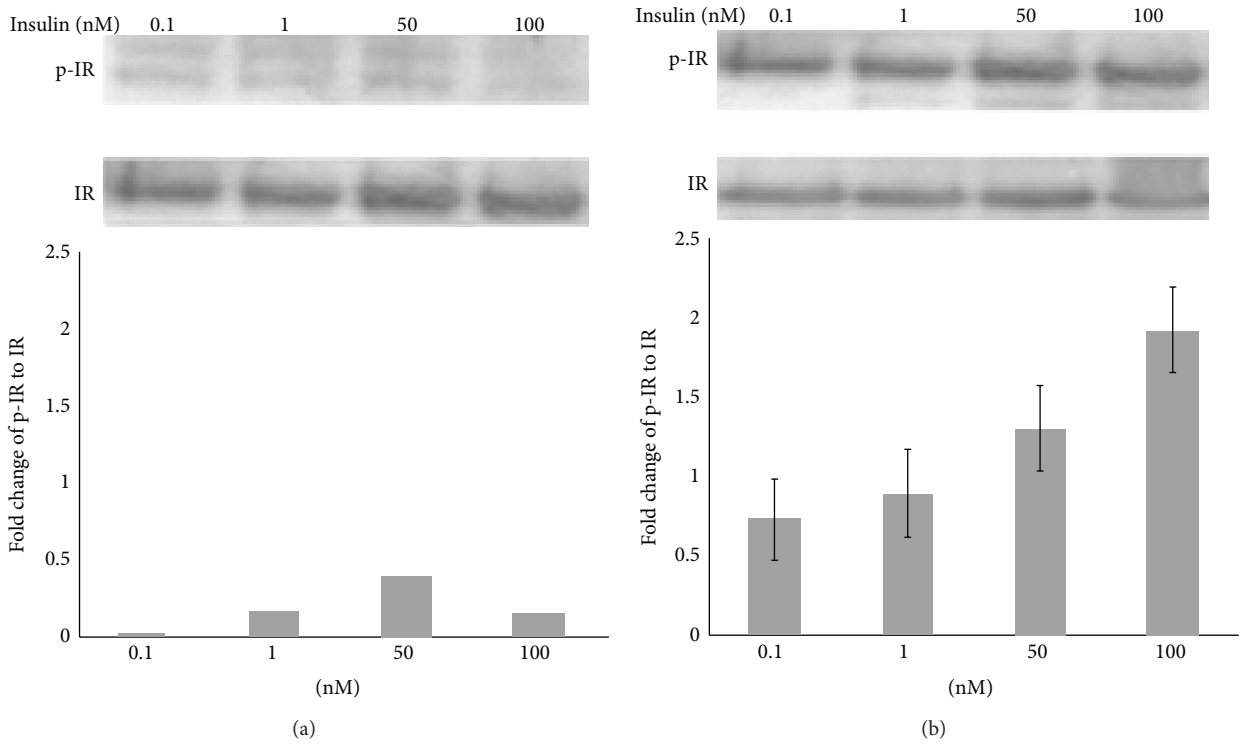

Figure 3: Selective insulin-induced phosphorylation of IRS-1 in ΔIGF-1R PC-12 cells (a, b).

construct the transformed PC12 cell that expressed IGF-1R lower than normal cells. Speculation confirmed earlier that low IGF-1R expression is good for the glucose metabolism of neurons cell.

3.4. The Defect of IGF-1R Significantly Improved the Ability of Glucose Consumption in PC12 Cells. It is known that Aβ25–35 peptide has toxicity to affect the ability of glucose consumption of PC12 cell. At the resting state (no insulin), glucose consumption of PC12 ΔIGF-1R cell and PC12 cell has no significant increase or decrease on the four time points ($P > 0.05$).

In Figure 4(a), compared with Normal Control group, PC12 cells and ΔIGF-1R-PC12 pretreated with Aβ25–35, the amount of glucose that was consumed by these two cells was decreased; in particular, PC12 cells treated with Aβ25–35 have shown the weakest glucose consumption capacity. Here it is important that ΔIGF-1R-PC12 cells consumed the glucose more than that of PC12 cell treated with Aβ25–35. The data suggest that the defect of IGF-1R significantly improved the ability of glucose consumption in PC12 cells but still did not completely reverse the damage caused by Aβ25–35.

At the resting state (no insulin) both of cell groups have no significant increase or decrease ($P > 0.05$) of glucose consumption on the four time points. As is shown in Figure 4(c), compared with PC12 cells, in insulin-pretreated PC12 cells and ΔIGF-1R-PC12 cells incubated by Aβ25–35, glucose uptake capacity was significantly improved by the defect of IGF-1R ($P < 0.05$), suggesting the defect of IGF-1R promotes mRNA level of Glut4 and the uptaking of glucose significantly in the insulin-stimulated PC12 cells (Figures 2(c), 2(d), and 2(e)).

3.5. The knock-Down (IGF-1R^{neo}) Mice Treated with HFD/STZ Have Better Cognitive Abilities Than Those of Wild Mice. Due to operational difficulties in molecular biology, we have not been able to use the rat model with lower expression of IGF-1R. The knock-down (IGF-1R^{neo}) mice were made applying some similar methods which were reported [30].

Considering respiratory failure and exhibiting a more severe growth deficiency in lung, null IGF-1R^{-} Mice have not been used as a model in the study. Using (PE) for 10 (for each treatment group) Histology of the overall digital analysis in hippocampus, compared to wild-type mice models, the knock-down (IGF-1R^{neo}) mice were significantly reduced in the control group that was approximately 33%. Due to insufficient accuracy of our PET-CT for animal, it cannot be used to detect levels of glucose metabolism in mice of the head; it is so regret in the study. Fortunately, the results of body weight, blood glucose, and cognitive abilities indicate the weight of knock-down IGF-1R^{neo} mice fed with high-fat high-sugar is lower than that of wild-type mice; this may be a positive tip that the IGF-1R^{neo} mice have a greater ability of glucose metabolism. More important is the knock-down (IGF-1R^{neo}) mice blood glucose levels were significantly lower than the wild type, with statistical significance ($P < 0.05$), but still higher than the normal diet of knock-down (IGF-1R^{neo}) mice ($P < 0.01$). As is shown in Tables 1 and 2, Morris water maze tests revealed the most important experimental result; only 2 knock-down (IGF-1R^{neo}) mice showed slight cognitive

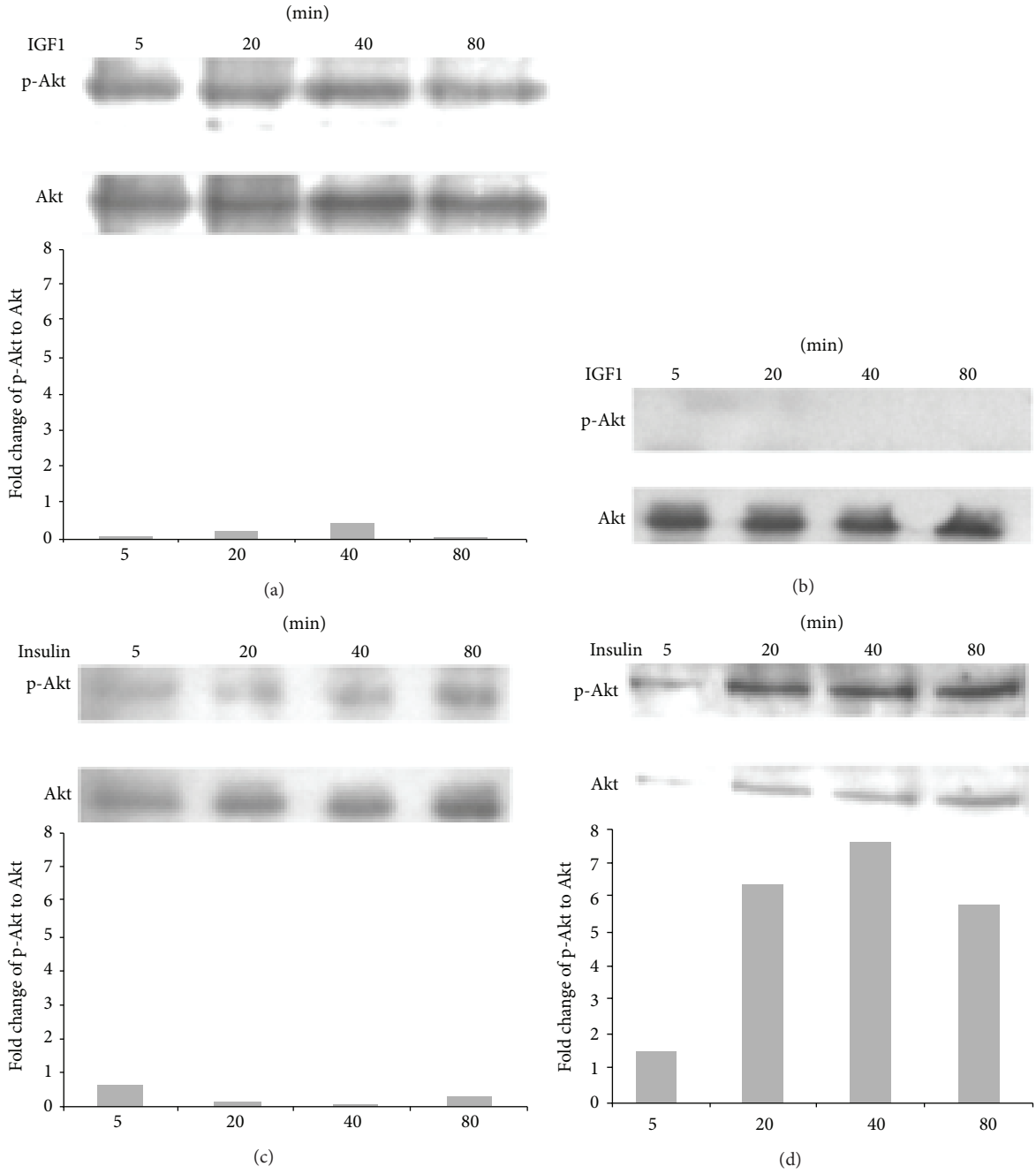

Figure 4: Loss of the IGF-1R enhances sensitivity to insulin-induced (a) and IGF1 (b) Akt phosphorylation.

barrier. However, 7 mice in wild-type mice fed with high-sugar high-fat treatment group had three in a serious cognitive disorder. Until testing the latter (25w), the living state of all wild mice was not suitable for water Morris maze test, while no knock-down (IGF-1R^{neo}) mice still have the athletic ability and cognitive ability.

In summary, the low expression of IGF-1R could be help to inhibit diabetic encephalopathy to some extent.

3.6. Loss of the IGF-1R Enhances Sensitivity to Insulin-Induced IR Phosphorylation. To investigate the effects of IGF-1R disruption on immediate IR signaling, we examined IR autophosphorylation in the cells. The ΔIGF-1R and control cells were serum-starved and treated with insulin, lysed, and immunoprecipitated with the anti-IR-β antibody. Western blot analysis with an anti-Tyr (P) antibody showed that insulin stimulated tyrosine phosphorylation of the insulin receptor in both ΔIGF-1R and control cells (Figures 3(a) and 3(b)). However, in the ΔIGF-1R cells, IR was more responsive to insulin, leading to IR autophosphorylation at 100-fold lower insulin concentrations (0.1 nM) when compared with equivalent activation in control cells at the concentration of 10 nM.

Table 1: Grade analysis of the pass-through time of mice in the water maze Automatic Control System test.

$T_p - T/T_p$	Wild-mice HFD ($n = 18$)	IGF-1R^{neo} mice HFD ($n = 18$)
0%~20%	11	16
20%~30%	3	2
30%~40%	3	0
>40%	1	0

T_p = the pass-through time; T = the middle time of negative control.

Table 2: The times of error in MS-2 water maze Automatic Control System test.

Groups	Times of error			
	Day 1	Day 2	Day 3	Day 4
NS control group	9.12 ± 8.01	7.50 ± 5.56	5.43 ± 4.42	3.65 ± 2.21
Wild-mice HFD	15.08 ± 5.13	15.71 ± 10.15	12.13 ± 5.70	7.30 ± 2.11
IGF-1R^{neo} mice HFD	11.12 ± 7.01	8.50 ± 4.11	7.24 ± 4.57*	5.88 ± 2.46*

*$P < 0.05$; $n = 18$ mice per group.

3.7. Loss of the IGF-1R Enhances Sensitivity to Insulin-Induced Akt Phosphorylation. Ligand binding to IR and IGF-1R activates Akt signaling pathways. As shown in Figure 4, IGF-1 acutely stimulated Akt (Figures 4(a) and 4(b), left panels) in control cells, whereas these effects were nearly abolished in ΔIGF-1R cells. Interestingly, insulin treatment resulted in significantly greater induction of Akt phosphorylation in PC12-ΔIGF-1R cells compared with control cells. The enhanced insulin sensitivity in ΔIGF-1R cells is opposite to that observed for growth hormone signaling where removal of the IGF-1R diminishes growth hormone induction of JAK/STAT phosphorylation.

4. Discussion

Diabetic encephalopathy is an unknown diabetes complication, characterized by electrophysiological, structural, neurochemical, and degenerative neuronal changes that lead to cognitive functioning limitations. Hence it is named as "type 3 diabetes"; the content of this title represents the most relevant risk factor for increased incidence of dementia, cognitive dysfunction, and consequently Alzheimer's disease.

As is known widely, insulin is an important anabolic hormone identified, since almost all of cell types are sensitive to this peptide [32]. More and More evidence proved that the hormone is widely located in the brain [32]. It plays a critical and central role in numerous actions in the brain, like neurotrophic, neuromodulatory, and neuroendocrine [33–35]. Additionally, insulin runs in the CNS through binding to the receptors on cell membrane—insulin receptor (IR) and insulin growth factor-1 receptor (IGF-1R); they are so abundant throughout the whole brain, such as hypothalamus, hippocampus [36, 37]. Once bound to the receptors, insulin triggers signaling cascades that include PI-3K and Akt pathways, which are the most relevant factors involved in learning and memory processes [38].

Insulin-like growth factor-1 receptor (IGF-1R) locates on the cell types in many human tissues [39]. Two peptide hormones called IGF-1 and IGF-2 both can activate it effectively. Their actions are mostly like insulin. Both of them have anabolic effects in adults—meaning that it can induce hypertrophy of brain and other target tissues. IGF-1R and other tyrosine kinase growth factor receptors signal through multiple pathways. In Figure 3, the results of Western blot showed that IR on ΔIGF-1R cells was more susceptible to insulin, so the pathways between IR and IGF-1R may cross and overlap; if one of them defects, the other will operate alternately. A key pathway is regulated by phosphatidylinositol-3 kinase (PI3K) and its downstream partner, the mammalian target of rapamycin. IGF-1 prosurvival action is mainly activated by the PI3K/Akt pathway [40, 41]. PI3K inhibitors or expression of an inactive Akt mutant can suppress the neuroprotective effects of IGF-1, supporting the hypothesis that the survival signal is mediated predominantly through this pathway. Furthermore, some inflammatory factors, such as tumor necrosis factor- (TNF-) alpha can also indirectly trigger the death of neurons by inhibiting essential components of the IGF-1 survival response, such as PI3K, further demonstrating the key role of the IGF-1/PI3K-Akt pathway in neuroprotection.

Activation of PI3K stimulates the phosphorylation of the survival kinase, Akt. Activated Akt can phosphorylate multiple downstream proteins related to cell survival. This is consistent with a recent study, which demonstrated that no regional or aging difference was observed in total Akt level, but activated Akt was significantly reduced in hippocampal CA1 region [42–45]. As shown in Figure 4, IGF-1 in ΔIGF-1R cell had no effects on ΔIGF-1R cells. On the other hand, more insulin-sensitivity was identified in ΔIGF-1R cells than the cells in control. These results suggest that the decrease of p-Akt signaling is related to the vulnerability of CA1 neurons to stressor such as ischemia.

5. Conclusion

It is concluded that insulin exerts direct anabolic actions in neuron-like cells by activation of its cognate receptor and proves that IGF-1R plays an important role of in the pathogenesis of diabetic encephalopathy.

Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work and there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the paper.

References

[1] M. Baldassarre, F. A. Giannone, L. Napoli et al., "The endocannabinoid system in advanced liver cirrhosis: pathophysiological implication and future perspectives," *Liver International*, vol. 33, no. 9, pp. 1298–1308, 2013.

[2] Y.-W. Chen, V. Boyartchuk, and B. C. Lewis, "Differential roles of insulin-like growth factor receptor- and insulin receptor-mediated signaling in the phenotypes of hepatocellular carcinoma cells," *Neoplasia*, vol. 11, no. 9, pp. 835–845, 2009.

[3] Q. L. Cui and G. Almazan, "IGF-I-induced oligodendrocyte progenitor proliferation requires PI3K/Akt, MEK/ERK, and Src-like tyrosine kinases," *Journal of Neurochemistry*, vol. 100, no. 6, pp. 1480–1493, 2007.

[4] M. A. Daulatzai, "Early stages of pathogenesis in memory impairment during normal senescence and alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 20, no. 2, pp. 355–367, 2010.

[5] M. L. De Paula, Q.-L. Cui, S. Hossain, J. Antel, and G. Almazan, "The PTEN inhibitor bisperoxovanadium enhances myelination by amplifying IGF-1 signaling in rat and human oligodendrocyte progenitors," *GLIA*, vol. 62, no. 1, pp. 64–77, 2014.

[6] A. M. Etgen, O. González-Flores, and B. J. Todd, "The role of insulin-like growth factor-I and growth factor-associated signal transduction pathways in estradiol and progesterone facilitation of female reproductive behaviors," *Frontiers in Neuroendocrinology*, vol. 27, no. 4, pp. 363–375, 2006.

[7] Y. F. Zhi, J. B. Prins, and T. H. Marwick, "Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications," *Endocrine Reviews*, vol. 25, no. 4, pp. 543–567, 2004.

[8] S. Fernandez, A. M. Fernandez, C. Lopez-Lopez, and I. Torres-Aleman, "Emerging roles of insulin-like growth factor-I in the adult brain," *Growth Hormone & IGF Research*, vol. 17, no. 2, pp. 89–95, 2007.

[9] A. Gozzi, V. Crestan, G. Turrini, M. Clemens, and A. Bifone, "Antagonism at serotonin 5-HT2A receptors modulates functional activity of frontohippocampal circuit," *Psychopharmacology*, vol. 209, no. 1, pp. 37–50, 2010.

[10] B. H. Harvey, "Is major depressive disorder a metabolic encephalopathy?," *Human Psychopharmacology: Clinical and Experimental*, vol. 23, no. 5, pp. 371–384, 2008.

[11] B. P. Head, J. N. Peart, M. Panneerselvam et al., "Loss of caveolin-1 accelerates neurodegeneration and aging," *PLoS ONE*, vol. 5, no. 12, Article ID e15697, 2010.

[12] A. Hematulin, D. Sagan, F. Eckardt-Schupp, and S. Moertl, "NBS1 is required for IGF-1 induced cellular proliferation through the Ras/Raf/MEK/ERK cascade," *Cellular Signalling*, vol. 20, no. 12, pp. 2276–2285, 2008.

[13] C. E. Hills, N. J. Brunskill, and P. E. Squires, "C-peptide as a therapeutic tool in diabetic nephropathy," *American Journal of Nephrology*, vol. 31, no. 5, pp. 389–397, 2010.

[14] S. Lim, J. Hong, C.-T. Liu et al., "Common variants in and near IRS1 and subclinical cardiovascular disease in the Framingham Heart Study," *Atherosclerosis*, vol. 229, no. 1, pp. 149–154, 2013.

[15] H. Meng, D. Zhang, and H. Yang, "Effects of amyloid precursor protein 17 peptide on the protection of diabetic encephalopathy and improvement of glycol metabolism in the diabetic rat," *Journal of Diabetes Research*, vol. 2013, Article ID 689841, 7 pages, 2013.

[16] M. A. Rogawski, "What is the rationale for new treatment strategies in Alzheimer's disease?" *CNS Spectrums*, vol. 9, no. 7, supplement 5, pp. 6–31, 2004.

[17] A. P. Ross, E. C. Bruggeman, A. W. Kasumu, J. G. Mielke, and M. B. Parent, "Non-alcoholic fatty liver disease impairs hippocampal-dependent memory in male rats," *Physiology and Behavior*, vol. 106, no. 2, pp. 133–141, 2012.

[18] B. A. Stoica, V. A. Movsesyan, P. M. Lea IV, and A. I. Faden, "Ceramide-induced neuronal apoptosis is associated with dephosphorylation of Akt, BAD, FKHR, GSK-3β, and induction of the mitochondrial-dependent intrinsic caspase pathway," *Molecular and Cellular Neuroscience*, vol. 22, no. 3, pp. 365–382, 2003.

[19] M. J. Strong, "The syndromes of frontotemporal dysfunction in amyotrophic lateral sclerosis," *Amyotrophic Lateral Sclerosis*, vol. 9, no. 6, pp. 323–338, 2008.

[20] H.-S. Suh, M.-L. Zhao, L. Derico, N. Choi, and S. C. Lee, "Insulin-like growth factor 1 and 2 (IGF1, IGF2) expression in human microglia: Differential regulation by inflammatory mediators," *Journal of Neuroinflammation*, vol. 10, article 37, 2013.

[21] K. Thind and M. N. Sabbagh, "Pathological correlates of cognitive decline in Alzheimer's disease," *Panminerva Medica*, vol. 49, no. 4, pp. 191–195, 2007.

[22] J. Trojan, J.-F. Cloix, M.-Y. Ardourel, M. Chatel, and D. D. Anthony, "Insulin-like growth factor type I biology and targeting in malignant gliomas," *Neuroscience*, vol. 145, no. 3, pp. 795–811, 2007.

[23] R. L. Tuttle, N. S. Gill, W. Pugh et al., "Regulation of pancreatic β-cell growth and survival by the serine/threonine protein kinase Akt1/PKBα," *Nature Medicine*, vol. 7, no. 10, pp. 1133–1137, 2001.

[24] H. U. Ung, A. R. Moehlig, R. A. Kudla et al., "Proton-bound dimers of 1-methylcytosine and its derivatives: vibrational and NMR spectroscopy," *Physical Chemistry Chemical Physics*, vol. 15, no. 43, pp. 19001–19012, 2013.

[25] X. Vafopoulou and C. G. H. Steel, "Insulin-like and testis ecdysiotropin neuropeptides are regulated by the circadian timing system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera)," *General and Comparative Endocrinology*, vol. 179, no. 2, pp. 277–288, 2012.

[26] A. I. Vinik, T. Erbas, K. S. Stansberry, and G. L. Pittenger, "Small fiber neuropathy and neurovascular disturbances in diabetes mellitus," *Experimental and Clinical Endocrinology and Diabetes*, vol. 109, no. 2, pp. S451–S473, 2001.

[27] A. Von Gunten, K. Ebbing, A. Imhof, P. Giannakopoulos, and E. Kövari, "Brain aging in the oldest-old," *Current Gerontology and Geriatrics Research*, vol. 2010, Article ID 358531, 10 pages, 2010.

[28] X. K. Wang, M. L. Michaelis, and E. K. Michaelis, "Functional genomics of brain aging and Alzheimer's disease: focus on selective neuronal vulnerability," *Current Genomics*, vol. 11, no. 8, pp. 618–633, 2010.

[29] B. K. Wicker, H. P. Hutchens, Q. Wu, A. T. Yeh, and J. D. Humphrey, "Normal basilar artery structure and biaxial mechanical behaviour," *Computer Methods in Biomechanics and Biomedical Engineering*, vol. 11, no. 5, pp. 539–551, 2008.

[30] R. Epaud, F. Aubey, J. Xu et al., "Knockout of insulin-like growth factor-1 receptor impairs distal lung morphogenesis," *PLoS ONE*, vol. 7, no. 11, Article ID e48071, 2012.

[31] S. Jiang, P. Du, L. An, G. Yuan, and Z. Sun, "Anti-diabetic effect of *Coptis Chinensis* polysaccharide in high-fat diet with STZ-induced diabetic mice," *International Journal of Biological Macromolecules*, vol. 55, pp. 118–122, 2013.

[32] A. A. F. Sima, W. Zhang, C. W. Kreipke, J. A. Rafols, and W. H. Hoffman, "Inflammation in diabetic encephalopathy is prevented by C-peptide," *Review of Diabetic Studies*, vol. 6, no. 1, pp. 37–42, 2009.

[33] S. M. de la Monte, "Insulin resistance and Alzheimer's disease," *BMB Reports*, vol. 42, no. 8, pp. 475–481, 2009.

[34] J. Havrankova, J. Roth, and M. Brownstein, "Insulin receptors are widely distributed in the central nervous system of the rat," *Nature*, vol. 272, no. 5656, pp. 827–829, 1978.

[35] L. Plum, M. Schubert, and J. C. Brüning, "The role of insulin receptor signaling in the brain," *Trends in Endocrinology and Metabolism*, vol. 16, no. 2, pp. 59–65, 2005.

[36] L. Gasparini and H. Xu, "Potential roles of insulin and IGF-1 in Alzheimer's disease," *Trends in Neurosciences*, vol. 26, no. 8, pp. 404–406, 2003.

[37] A. Wada, H. Yokoo, T. Yanagita, and H. Kobayashi, "New twist on neuronal insulin receptor signaling in health, disease, and therapeutics," *Journal of Pharmacological Sciences*, vol. 99, no. 2, pp. 128–143, 2005.

[38] C. C. Huang, C. C. Lee, and K. S. Hsu, "The role of insulin receptor signaling in synaptic plasticity and cognitive function," *Chang Gung Medical Journal*, vol. 33, no. 2, pp. 115–125, 2010.

[39] A. Gallardo, E. Lerma, D. Escuin et al., "Increased signalling of EGFR and IGF-1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas," *British Journal of Cancer*, vol. 106, no. 8, pp. 1367–1373, 2012.

[40] T. R. Kim, E. W. Cho, S. G. Paik, and I. G. Kim, "Hypoxia-induced SM22α in A549 cells activates the IGF-1R/PI3K/Akt pathway, conferring cellular resistance against chemo- and radiation therapy," *FEBS Letters*, vol. 586, no. 4, pp. 303–309, 2012.

[41] M. Goto, A. Iwase, T. Harata et al., "IGF1-induced AKT phosphorylation and cell proliferation are suppressed with the increase in PTEN during luteinization inhuman granulosa cells," *Reproduction*, vol. 137, no. 5, pp. 835–842, 2009.

[42] M. de Butte-Smith, R. S. Zukin, and A. M. Etgen, "Effects of global ischemia and estradiol pretreatment on phosphorylation of Akt, CREB and STAT3 in hippocampal CA1 of young and middle-aged female rats," *Brain Research*, vol. 1471, pp. 118–128, 2012.

[43] M. Racaniello, A. Cardinale, C. Mollinari et al., "Phosphorylation changes of CaMKII, ERK1/2, PKB/ Akt kinases and CREB activation during early long-term potentiation at Schaffer collateral-CA1 mouse hippocampal synapses," *Neurochemical Research*, vol. 35, no. 2, pp. 239–246, 2010.

[44] T. C. Jackson, A. Rani, A. Kumar, and T. C. Foster, "Regional hippocampal differences in AKT survival signaling across the lifespan: implications for CA1 vulnerability with aging," *Cell Death and Differentiation*, vol. 16, no. 3, pp. 439–448, 2009.

[45] V. Znamensky, K. T. Akama, B. S. McEwen, and T. A. Milner, "Estrogen levels regulate the subcellular distribution of phosphorylated Akt in hippocampal CA1 dendrites," *The Journal of Neuroscience*, vol. 23, no. 6, pp. 2340–2347, 2003.

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Research Article

Prevalence of Self-Reported Diabetes and Its Associated Factors: A Population-Based Study in Brazil

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Aim. The aim of this study was to estimate the prevalence of diabetes and its associated risk factors in adults from Brasilia, Brazil. *Methods*. The present cross-sectional population-based study consisted of interviews with individuals aged 18–65 years. Participants were selected through two-stage probability sampling by clusters and stratified by sex and age. Demographic and clinical data were collected directly with participants from February to May 2012. Self-reported diabetes prevalence was calculated at a 95% confidence interval (CI). Prevalence ratios (PR) were adjusted by Poisson regression with robust variance. *Results*. In all, 1,820 individuals were interviewed. Diabetes prevalence in the adult population of Brasilia was 10.1% (95% CI, 8.5%–11.6%). Variables associated with diabetes were an age between 35 and 49 years (PR = 1.83; 95% CI, 1.19–2.82) or 50 and 65 years (PR = 1.95; 95% CI, 1.17–3.23), hypertension (PR = 4.04; 95% CI, 2.66–6.13), respiratory disease (PR = 1.67; 95% CI, 1.11–2.50), cardiovascular disease (PR = 1.74; 95% CI, 1.15–2.63), and pain/discomfort (PR = 1.71; 95% CI, 1.21–2.41). *Conclusion*. Diabetes is a prevalent condition in adults living in Brasilia, and disease risk increases with age and comorbidities. Future health policies should focus on screening programs and prevention for the more vulnerable groups.

1. Introduction

Diabetes mellitus is a global health problem and an important cause of mortality and morbidity in many countries. Its prevalence in adults has been increasing worldwide over the last 30 years [1]. It is estimated that diabetes will affect 366 million individuals worldwide by 2030 [2]. The trend of increasing diabetes prevalence seems to prevail among developing countries. In Brazil, diabetes affected 11.3 million people in 2011, and this number is likely to triple by 2030 [3]. Estimates suggest that the diabetes rate in less developed countries will increase by 69% between 2010 and 2030 [4].

Diabetes imposes a burden for society such as high socioeconomic costs that have an impact on productivity as well as life and health quality [5]. This situation seems to be worse in developing countries, where the healthcare system often fails to meet demand [6]. Studies have concluded that a Western dietary pattern, sedentary lifestyle, and genetic factors play a central role in diabetes development [7].

The Brazilian Ministry of Health has followed the World Health Organization's recommendations and has taken some actions to monitor diabetes such as an annual telephone-based survey [8]. Socioeconomic disparities might contribute to some degree of heterogeneity in measures of prevalence between regions [9]. A study demonstrated that diabetes prevalence across the Brazilian states ranged from 11% to 25%, with an overall rate of 16% in 2001 [10].

Brasilia, the capital of Brazil, is located in the Central-West region of the country. The city has the highest Human Development Index in Brazil, but it has one of the highest levels of social inequality compared with other Brazilian regions [11, 12]. These characteristics of Brasilia warrant further investigation in many aspects, including the health status of its population.

Thus, the goal of this study was to estimate the prevalence of diabetes and its associated risk factors in adults of Brasilia, Brazil.

2. Materials and Methods

2.1. Study Design and Settings. The present cross-sectional population-based study was conducted in Brasilia, Brazil, from February to May 2012. The target population was 1,702,419 inhabitants aged 18–65 years [12].

2.2. Sample Size and Participants. The sample size was calculated based on an estimation of 16% of self-reported diabetes cases [10]. Considering a 95% confidence interval (CI), precision of 2.25%, and a design effect of 1.8, the estimated sample size was 1,835 individuals. We added 10% of the sample size to compensate for any eventual attrition, which resulted in a final sample of 2,019 individuals.

Participants were selected by a two-stage probability sampling process by cluster and were stratified by sex and age. A total of 220 census tracts were randomly selected from 3,886 urban tracts with more than 200 inhabitants [12]. Up to 10 households were selected from each census tract. In total, one adult per household was selected following the predefined quotas of sex and age to answer the interview. Trained professionals surveyed all of the participants in their homes using a semistructured questionnaire. To ensure reliability, 20% of the interviews were audited by telephone. To test the understanding and acceptability of the questionnaire, 150 pilot interviews were held prior to data collection.

2.3. Study Variables. The dependent variable was self-reported diabetes. Independent variables included demographic characteristics (age group, sex, marital status, living arrangements, and household location), socioeconomic characteristics (level of education, occupation, and social class), chronic health conditions (self-reported hypertension, depression, respiratory diseases, cardiovascular diseases, and other chronic diseases), access to healthcare (health insurance, medical consultation, and hospitalization), and perceived health status (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression) [13]. The stratification was based on the Brazilian criterion of economic classification, which defines five classes, with "A" being the wealthiest group and "E" being the poorest [14].

2.4. Statistical Analysis. In all of the analyses, the effects of complex sampling were considered. First, we described participant characteristics by weighted frequencies. Self-reported diabetes prevalence in the population was then calculated at a 95% CI. To identify factors related to diabetes prevalence, we calculated prevalence ratios (PR) using bivariate analysis and calculated the adjusted PR by a Poisson regression model with robust variance [15]. In this model, all of the variables were analyzed simultaneously. We preferred to use this more conservative model that included all of the variables to allow for better confounding adjustment. Other models that included only the most significant variables were tested and did not change the significance of the variables. Associations were considered to be statistically significant when $P < 0.05$. The STATA software version 10.1 was used for all of the calculations [16].

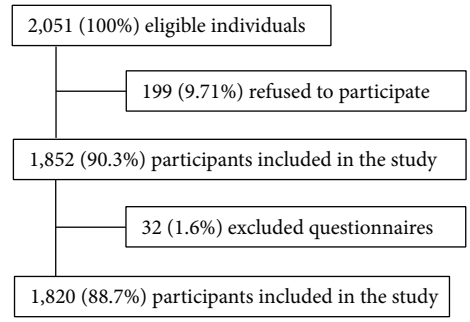

Figure 1: Sample selection.

2.5. Ethics Statement. This study was approved by the University of Brasilia Ethics Committee. All participants signed a term of free and informed consent.

3. Results

3.1. Participants and Sample Characteristics. In total, 1,820 individuals were included in the study (Figure 1). The main characteristics of the sample are shown in Table 1. Approximately 60% of the participants were women, and 57% were aged between 35 and 60 years. Most of the participants belonged to economic class "C," had completed high school, were married or cohabitating, lived with at least one more person in the household, and dwelled in a satellite town.

3.2. Diabetes and Correlates. Diabetes was self-reported by 10.1% (95% CI: 8.5%–11.6%) of the adult population in Brasilia. Table 1 depicts diabetes prevalence and prevalence ratios (PR) before and after adjustment by Poisson regression.

The age group of 35–65 years, hypertension, respiratory disease, cardiovascular disease, and pain/discomfort were significantly associated with diabetes. Sex, marital status, living arrangements, social class, education level, employability, living location, health insurance, medical consultation, hospitalization, physical mobility, self-care, usual activities, and anxiety/depression revealed no significant association.

Figure 2 illustrates differences in diabetes prevalence between all persons and the population with comorbidities. Diabetes prevalence in the age range 30–65 years is higher among individuals with cardiovascular disease, followed by those with hypertension and those with respiratory diseases. This result suggests that the likelihood of diabetes increases with age and is greater in persons with comorbidities.

4. Discussion

Diabetes was self-reported by one of every ten Brazilian adults. An age of 35 years and over, presence of pain or discomfort, cardiovascular disease, hypertension, and respiratory

Table 1: Sociodemographics of the sample population, diabetes prevalence, and unadjusted and adjusted prevalence ratios (PR) (N = 1,820).

Variables	Frequency distribution (%)	Diabetes prevalence (%)	Unadjusted PR	P value	Adjusted PR	95% CI	P value
Sex							
Male	40.7	9.6	1.00	—	1.00	—	—
Female	59.3	10.4	1.08	0.641	0.89	0.65–1.23	0.489
Age group (years)							
18–34	43.5	4.5	1.00	—	1.00	—	—
35–49	35.1	11.3	2.52	<0.001	1.83	1.19–2.82	0.006
50–65	21.4	19.0	4.24	<0.001	1.95	1.17–3.23	0.010
Marital status							
Single	47.8	7.9	1.00	—	1.00	—	—
Married/cohabitating	52.2	12.0	1.52	0.014	1.61	1.16–2.72	0.005
Living arrangements							
At least with one person	94.5	10.2	1.00	—	1.00	—	—
Alone	5.5	8.1	0.79	0.529	1.02	0.49–2.12	0.954
Social class							
Class A	8.5	7.6	1.00	—	1.00	—	—
Class B	34.4	10.1	1.33	0.373	1.72	0.83–3.65	0.145
Class C	47.5	10.8	1.42	0.257	1.47	0.67–3.22	0.331
Classes D-E	9.5	8.6	1.14	0.750	1.21	0.45–3.24	0.709
Level of education							
College or higher	17.4	8.5	1.00	—	1.00	—	—
High school	34.4	8.0	0.95	0.833	0.91	0.54–1.52	0.715
Primary school	21.6	9.3	1.10	0.738	1.19	0.65–2.16	0.567
Incomplete primary school	26.6	14.5	1.72	0.034	1.07	0.58–2.00	0.826
Occupation							
Employed	45.6	8.3	1.00	—	1.00	—	—
Unemployed or retired[a]	54.4	11.6	1.40	0.055	0.97	0.70–1.35	0.877
Location							
Downtown	17.2	8.0	1.00	—	1.00	—	—
Satellite towns	82.8	10.5	1.31	0.284	0.98	0.53–1.79	0.937
Self-reported chronic conditions							
Hypertension	21.5	29.9	6.43	<0.001	4.04	2.66–6.13	<0.001
Respiratory disease	7.3	20.9	2.37	<0.001	1.67	1.11–2.50	0.013
Cardiovascular disease	6.9	36.5	4.74	<0.001	1.74	1.15–2.63	0.009
Other chronic diseases	8.0	10.6	1.05	0.828	0.54	0.29–1.01	0.052
Healthcare services							
No health insurance	72.3	9.5	0.83	0.311	0.84	0.59–1.20	0.339
Medical consultation	42.5	12.8	1.66	<0.001	0.94	0.70–1.27	0.690
Hospitalization	9.9	16.6	1.80	0.005	1.43	0.98–2.10	0.062
Perceived health status							
Mobility	7.9	19.7	2.15	<0.001	1.29	0.84–2.00	0.242
Self-care	4.0	17.7	1.83	0.045	0.75	0.35–1.63	0.471
Usual activities	6.9	16.2	1.68	0.042	0.98	0.54–1.78	0.941
Pain/discomfort	37.0	15.7	2.30	<0.001	1.71	1.21–2.41	0.002
Anxiety/depression	23.0	14.8	1.70	0.001	1.06	0.70–1.63	0.777

Note: [a]included students not formally employed.
CI: confidence interval.

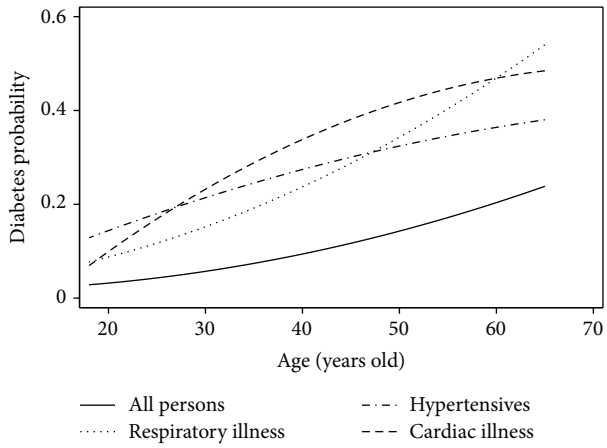

Figure 2: Diabetes prevalence by age in groups with different health conditions.

disease were positively associated with diabetes in the adult population of Brasilia.

The main limitations of our study were the self-reported assessments of the primary outcome and independent variables. Self-reported diabetes might be a source of bias because individuals need to be aware of the diagnosis prior to answering, which could result in disease underestimation [1]. However, performing a clinical test for diagnosing diabetes is not always possible in population-based studies. Thus, self-reported answers regarding diabetes have been a common practice according to the literature [17, 18]. Another shortcoming was the cross-sectional design of the study, which hampers a causal relationship between diabetes and the significantly associated factors identified herein.

A previous population-based study developed in Brazil in 2008 used telephone interviews to investigate self-reported diabetes prevalence and found low prevalence rates in Brasilia [19]. Another study found that Brasilia was the region with the highest diabetes prevalence compared with other Brazilian regions from 2002 to 2007 [20]. Research identified a significant increase in self-reported diabetes in the Brazilian population because it ascended from 3.3% in 1998 to 5.3% in 2008 [3]. In South and Central America, the estimated diabetes prevalence in 2013 was 8.0%; Brazil demonstrated the highest prevalence, followed by Colombia and Argentina [21]. The variability of diabetes prevalence may be due to a poorer diet and a lack of physical activity, or it could be related to better access to diagnostic testing [3].

As expected, our results demonstrated that the likelihood of having diabetes increases with age. From a healthcare policy perspective, diabetes prevention and management programs should target young people and not only the elderly population.

Diabetes prevalence was higher among individuals with cardiovascular disease, hypertension, and respiratory disease compared with the general population. There is convincing evidence of the association between diabetes and hypertension, which increases the risk of a cardiovascular event [22]. A 2003 study conducted in São Luis, a city located in one of the poorest areas of Brazil, observed a positive association between diabetes and hypertension [23]. A cross-sectional study conducted between 2004 and 2005 in São Jose do Rio Preto, a city in the Brazilian southeast region, revealed that the diabetes prevalence was almost threefold higher in a population of hypertensive individuals compared with the general population [9].

A cohort study performed in women between 1988 and 1996 throughout 11 states in the United States found that chronic obstructive pulmonary disease was a diabetes risk factor [24]. A retrospective cohort study conducted in northern California reported that individuals with diabetes are at a greater risk of developing asthma, chronic obstructive pulmonary disease, fibrosis, and pneumonia [25].

Socioeconomic factors were not associated with diabetes in our sample. In contrast, a systematic review of 10 studies suggests that growing up in a socioeconomically disadvantaged environment may contribute to diabetes in later life [26]. An Australian study also described a positive association between socioeconomic variables and diabetes in adults aged 45 years and over [27].

The perceived health dimensions physical mobility, self-care, usual activities, and anxiety/depression were not associated with diabetes in our sample. In 2012, a literature review found that diabetes was considered a potential risk factor for the poor performance of daily life activities among individuals aged 50 years and over [28]. A study conducted with older adult New York residents observed that self-reported diabetes was not associated with depression [29]. Other than depression, this finding might depict an association between diabetes and activities of daily living, which may be developed at older ages.

5. Conclusion

Diabetes is a common health condition in adults living in Brasilia and is positively associated with older age, cardiovascular disease, hypertension, respiratory disease, and presence of pain or discomfort. Preventive strategies should prioritize populations with at least one of the identified factors.

Conflict of Interests

The authors declare that they have no conflict of interests.

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References

[1] A. Rubinstein, L. Gutierrez, A. Beratarrechea, and V. E. Irazola, "Increased prevalence of diabetes in Argentina is due to easier health care access rather than to an actual increase in prevalence," *PLoS ONE*, vol. 9, no. 4, Article ID e92245, 2014.

[2] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[3] M. I. Schmidt, B. B. Duncan, G. A. E Silva et al., "Chronic non-communicable diseases in Brazil: burden and current challenges," *The Lancet*, vol. 377, no. 9781, pp. 1949–1961, 2011.

[4] J. E. Shaw, R. A. Sicree, and P. Z. Zimmet, "Global estimates of the prevalence of diabetes for 2010 and 2030," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 4–14, 2010.

[5] P. L. Bosi, A. M. Carvalho, D. Contrera et al., "Prevalência de diabetes melito e tolerância à glicose diminuída na população urbana de 30 a 79 anos da cidade de São Carlos, São Paulo," *Arquivos Brasileiros de Endocrinologia & Metabologia*, vol. 53, no. 6, pp. 726–732, 2009.

[6] N. Almeida-Filho, "Higher education and health care in Brazil," *The Lancet*, vol. 377, no. 9781, pp. 1898–1900, 2011.

[7] O. Garcia-Dominic, E. J. Lengerich, F. Camacho et al., "Prevalence of diabetes and associated obesity in Pennsylvania adults, 1995–2010," *Preventing Chronic Disease*, vol. 11, Article ID 130330, 2014.

[8] B. P. M. Iser, R. M. Claro, E. C. de Moura, D. C. Malta, and O. L. M. Neto, "Risk and protection factors for chronic non communicable diseases by telephone survey—VIGITEL Brazil—2009," *Revista Brasileira de Epidemiologia*, vol. 14, supplement 1, pp. 90–102, 2011.

[9] J. P. Cipullo, J. F. V. Martin, L. A. D. S. Ciorlia et al., "Hypertension prevalence and risk factors in a Brazilian urban population," *Arquivos Brasileiros de Cardiologia*, vol. 94, no. 4, pp. 519–526, 2010.

[10] L. B. Nucci, C. M. Toscano, A. L. M. Maia et al., "A nationwide population screening program for diabetes in Brazil," *Revista Panamericana de Salud Publica*, vol. 16, no. 5, pp. 320–327, 2004.

[11] "Programa das Nações Unidas para o Desenvolvimento. Atlas do Desenvolvimento Humano no Brasil 2013," 2014, http://atlasbrasil.org.br/2013/ranking.

[12] "IBGE- Instituto Brasileiro de Geografiae estatística. Censo Demográfico 2010," 2014, http://www.ibge.gov.br/home/estatistica/populacao/censo2010/default.shtm.

[13] R. Rabin and F. De Charro, "EQ-5D: a measure of health status from the EuroQol Group," *Annals of Medicine*, vol. 33, no. 5, pp. 337–343, 2001.

[14] CCEB, "Associação Brasileira de Empresas de Pesquisa. Critério de Classificação Econômica Brasil. 2012," August 2013, http://www.abep.org/novo/Content.aspx?ContentID=139.

[15] J. Lee, S. T. Chuen, and S. C. Kee, "A practical guide for multivariate analysis of dichotomous outcomes," *Annals of the Academy of Medicine Singapore*, vol. 38, no. 8, pp. 714–719, 2009.

[16] A. B. Siller and L. Tompkins, "The big four: analyzing complex sample survey data using SAS, SPSS, STATA, and SUDAAN," Tech. Rep. Paper 172-31, 2005.

[17] L. A. B. de Almeida, F. J. G. Pitanga, M. M. Freitas, C. P. S. Pitanga, E. H. M. Dantas, and C. C. Beck, "Caloric expenditure of different domains of physical activity as predictors of the absence of diabetes in adults," *Revista Brasileira de Medicina do Esporte*, vol. 18, no. 1, pp. 17–21, 2012.

[18] T. Borges, A. J. Rombaldi, L. Q. Corrêa, A. G. Knuth, and P. C. Hallal, "Prevalência de autorrelato da morbidade e conhecimento sobre diabetes: estudo populacional de uma cidade no sul do Brasil," *Revista Brasileira de Cineantropometria e Desempenho Humano*, vol. 14, no. 5, pp. 560–570, 2012.

[19] Brasil Ministério da Saúde, Secretaria de Vigilância em Saúde, and Secretaria de Gestão Estratégica e Participativa, *Vigitel Brasil 2008: vigilância de fatores de risco e proteção para doenças crônicas por inquérito telefônico*, Ministério da Saúde, 2009.

[20] J. C. R. Dias and J. A. D. B. Campos, "Diabetes mellitus: razão de prevalências nas diferentes regiões geográficas no Brasil, 2002–2007," *Ciência & Saúde Coletiva*, vol. 17, no. 1, pp. 239–244, 2012.

[21] International Diabetes Federation, *Diabetes Atlas*, IDF, 6th edition, 2013.

[22] S. M. Grundy, I. J. Benjamin, G. L. Burke et al., "Diabetes and cardiovascular disease: a statement for healthcare professionals from the american heart association," *Circulation*, vol. 100, no. 10, pp. 1134–1146, 1999.

[23] J. B. Barbosa, A. A. M. da Silva, A. M. dos Santos et al., "Prevalência da hipertensão arterial em adultos e fatores associados em São Luís—MA," *Arquivos Brasileiros de Cardiologia*, vol. 91, no. 4, pp. 260–266, 2008.

[24] J. S. Rana, M. A. Mittleman, J. Sheikh et al., "Chronic obstructive pulmonary disease, asthma, and risk of type 2 diabetes in women," *Diabetes Care*, vol. 27, no. 10, pp. 2478–2484, 2004.

[25] S. F. Ehrlich, C. P. Quesenberry Jr., S. K. van den Eeden, J. Shan, and A. Ferrara, "Patients diagnosed with diabetes are at increased risk for asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, and pneumonia but not lung cancer," *Diabetes Care*, vol. 33, no. 1, pp. 55–60, 2010.

[26] T. Tamayo, H. Christian, and W. Rathmann, "Impact of early psychosocial factors (childhood socioeconomic factors and adversities) on future risk of type 2 diabetes, metabolic disturbances and obesity: a systematic review," *BMC Public Health*, vol. 10, article 525, 2010.

[27] S. M. Shamshirgaran, L. Jorm, H. Bambrick, and A. Hennessy, "Independent roles of country of birth and socioeconomic status in the occurrence of type 2 diabetes," *BMC Public Health*, vol. 13, no. 1, article 1223, 2013.

[28] J. Petrofsky, L. Berk, and H. Al-Nakhli, "The influence of autonomic dysfunction associated with aging and type 2 diabetes on daily life activities," *Experimental Diabetes Research*, vol. 2012, Article ID 657103, 12 pages, 2012.

[29] P. Palta, S. H. Golden, J. A. Teresi et al., "Depression is not associated with diabetes control in minority elderly," *Journal of Diabetes and Its Complications*, vol. 28, no. 6, pp. 798–804, 2014.

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Research Article

Predictive Potential of Twenty-Two Biochemical Biomarkers for Coronary Artery Disease in Type 2 Diabetes Mellitus

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We investigated the potential of a panel of 22 biomarkers to predict the presence of coronary artery disease (CAD) in type 2 diabetes mellitus (DM2) patients. The study enrolled 96 DM2 patients with (n = 75) and without (n = 21) evidence of CAD. We assessed a biochemical profile that included 22 biomarkers: total cholesterol, LDL, HDL, LDL/HDL, triglycerides, glucose, glycated hemoglobin, fructosamine, homocysteine, cysteine, methionine, reduced glutathione, oxidized glutathione, reduced glutathione/oxidized glutathione, L-arginine, asymmetric dimethyl-L-arginine, symmetric dimethyl-L-arginine, asymmetric dimethyl-L-arginine/L-arginine, nitrate plus nitrite, S-nitrosothiols, nitrotyrosine, and n-acetyl-β-glucosaminidase. Prediction models were built using logistic regression models. We found that eight biomarkers (methionine, nitratate plus nitrite, n-acetyl-β-glucosaminidase, BMI, LDL, HDL, reduced glutathione, and L-arginine/asymmetric dimethyl-L-arginine) along with gender and BMI were significantly associated with the odds of CAD in DM2. These preliminary findings support the notion that emerging biochemical markers might be used for CAD prediction in patients with DM2. Our findings warrant further investigation with large, well-designed studies.

1. Introduction

Type 2 diabetes mellitus (DM2) is an important risk factor for atherosclerosis. Chronic hyperglycemia is related to pathophysiology of macrovascular and microvascular diseases. Even small changes in glucose metabolism may contribute to the onset of cardiovascular disease and endothelial dysfunction due to peripheral insulin resistance. Acute myocardial infarction affects a large proportion of patients with DM2; thus, the evidence of subclinical atherosclerosis would be helpful for preventive strategies in these individuals [1, 2].

Even knowing that hyperglycemia is one of the ways that can lead to the development of atherosclerosis, the mechanisms underlying coronary artery disease (CAD) in individuals with type II diabetes are not known for sure. Thus, besides serum glucose, other markers related to the pathophysiology of cardiovascular diseases might also be evaluated. Recent prominent candidates for prediction of CAD in DM2

patients include thiols (cysteine, homocysteine, methionine oxidized glutathione, and reduced glutathione), N-acetyl-β-D-glucosaminidase (NAGase), endogenous nitric oxide synthase inhibitors (ADMA), nitrate + nitrite (NOx), nitrotyrosine, and S-nitrosothiols (RSNO). Hence, the purpose of this study was to identify important biochemical alterations that could distinguish DM2 individuals with and without CAD.

2. Materials and Methods

2.1. Study Participants. In this study, 96 volunteers were enrolled and classified into the following groups: type 2 diabetes mellitus (DM2, $n = 21$) and type 2 diabetes mellitus with coronary artery disease (DM2 + CAD, $n = 75$) (Table 1). Volunteers of both genders (aged ≥ 21 years) were screened at the following Brazilian institutions: Instituto Dante Pazzanese de Cardiologia, Irmandade da Santa Casa de Misericórdia de São Paulo, and Faculdade de Ciências Médicas da Universidade Metodista de Santos. All subjects were fully informed about the details of the study and protocol and provided written informed consent. The present study was approved by the ethics committees of the three participating institutions. DM2 was defined according to the American Diabetes Society criteria, whereas participants with coronary artery disease were defined as patients who had acute myocardial infarctions confirmed by ECG, laboratory exams, and clinical symptoms [3]. Exclusion criteria used were pregnancy, psychiatric disorders, renal and/or hepatic diseases, smoking, alcoholism, cancer, or other pathological conditions that could interfere with the study. Venous blood was collected in tubes with and without EDTA after 12 hours of fasting. Serum and plasma were obtained by centrifugation and stored frozen at −70°C.

2.2. Biochemical Analysis. The concentrations of fructosamine, glycated hemoglobin (HbA_{1C}), glucose, total cholesterol, triglyceride, high-density lipoprotein- (HDL-) cholesterol, and very low density lipoprotein- (VLDL-) cholesterol were estimated by enzymatic methods using kits (Biosystems SA, Barcelona, Spain). The Friedewald equation was used to calculate the low density lipoprotein- (LDL-) cholesterol.

2.3. N-Acetyl-β-glucosaminidase (NAGase) Activity. N-Acetyl-β-glucosaminidase activity was determined based on the methodology described by Reglero and Cabezas (1976). 100 μL of plasma was added to 1.25 mL citrate buffer (0.1 M, pH 4.4) and incubated at 37°C with 0.25 mL 0.01 M p-nitrophenyl-N-acetyl-glucosaminide in citrate buffer for 15 minutes. The reaction was interrupted with 1.5 mL of sodium carbonate (0.2 M, pH 10.4) and the final product was measured by spectrophotometer at 405 nm. The calibration curve was made using standard solution of p-nitrophenol. The NAGase activity of the samples was calculated after subtraction of the blank sample. One unit of enzyme was defined as the amount released 1 μmol p-nitrophenol/min [4].

2.4. NOx (Nitrate + Nitrite). We used the nitric oxide analyzer (NOA^{280}, Sievers, USA) based on the chemiluminescence reaction between nitric oxide and ozone. The reduction of nitrate and nitrite (NOx) with vanadium chloride was used to convert NOx to oxide nitric. Reduction was done at 90°C in 0.1 M HCl. Calibration curves with multiple levels were performed with an external standard (sodium nitrate) using the Bag Program software (version 2.2, Sievers, USA). The samples were extracted with cold ethanol (0.5 mL sample and 1.0 mL of ethanol at 0°C). After vortexing, the solution was stored on ice during 30 minutes and then centrifuged at 9000 g for fifteen minutes. The supernatant was removed and analyzed in nitric oxide analyzer [5].

2.5. S-Nitrosothiols. The standard S-nitroso-albumin (SNO-Alb) was used to quantify total S-ntrosothiol serum. The synthesis was obtained by the reaction of nitrite with human albumin in 0.1 M HCl. This was incubated in the dark for 2 hours and then the absorbance was measured at 336 mm ($\varepsilon = 3874\ M^{-1}\ cm^{-1}$). The calibration curve was made with 10, 50, 100, 250, and 500 nM SNO-Alb. 1.0 mL of plasma was added to 10 μL of n-ethylmaleimide (500 mM). After homogenizing, 100 μuL of sulfanilamide (1%) was added and homogenized again. Then the samples were stored on ice until analysis. We used the equipment Nitric Oxide Analyzer (NOA^{280}, Sievers, USA) and injected 500 μL of sample and 500 μL of standard. The reaction solution was composed of 8 mL of glacial acetic acid, 2 mL of KI (50 mg/mL), 300 μL of decanol, and 200 μL of $CuSO_4$ (200 mM) at 70°C [6].

2.6. ADMA (Asymmetric Dimethyl-L-arginine), SDMA (Symmetric Dimethyl-L-Arginine), and L-arginine. Plasma concentrations of ADMA, SDMA, and L-arginine were determined by the technique of capillary electrophoresis (Bio-Focus 2000, Bio-Rad Laboratories, Inc.). The blood was collected with EDTA and centrifuged at 1,000 g for 10 minutes at 4°C to obtain plasma. It was added to the plasma L-homoarginine as an internal standard. The calibration curve was performed by adding the standard of ADMA, SDMA, and L-arginine in pooled plasma. Samples and standard were precipitated with ethanol, centrifuged at 9.000 g for 15 minutes at 4°C, and then derivatized with fluorescein 5-isothiocyanate. The injection was done under pressure (1 psi.sec) and the race was performed in a fused silica capillary (85 cm length and 50 internal diameter μm) at 20 kV. Running buffer consisted of 50 mM boric acid and 20 mM 3-cyclohexylamino-1-propanesulfonic adjusted to pH 10.8. The detector laser-induced fluorescence (BioFocus LIF^2, Bio-Rad Laboratories, Inc.) operated at 488 nm (excitation) and 520 nm (emission) (Caussé et al. 2000) [7].

2.7. Thiols (Homocysteine, Cysteine, Methionine, Oxidized Glutathione, and Reduced Glutathione). The blood was collected with EDTA and centrifuged at 1000 g for 10 minutes at 4°C to obtain plasma. In 200 μL in plasma 2 μL internal standard (1 mM n-(2-mercaptopropionyl) glycine) and 20 μL tri-n-butylphosphine 10% (v/v dimethylformamide) were added. The preparation was homogenized and incubated for 30 minutes at 4°C. Then, 200 μL 10% trichloroacetic acid containing 1 mM EDTA was added. After homogenizing samples, they were centrifuged at 13.000 g for 15 minutes at 4°C. In 100 μL of the supernatant was added to 100 μL

Table 1: The demographical clinical and biochemical characteristics of the 96 patients.

	DM2 + CAD ($n = 75$)	DM2 ($n = 21$)	P
Age (years)	59 (57–62)	59 (52–64)	0.66
BMI (kg/m^2)	29.1 ± 2.90	28.0 ± 2.96	0.11
Cholesterol (mg/dL)	216.7 ± 50.6	208.5 ± 41.1	0.53
Gender			
Male	10 (48%)	32 (43%)	0.80
Female	11 (52%)	43 (57%)	
LDL-cholesterol (mg/dL)	129 (111–146)	124 (98–164)	0.92
HDL-cholesterol (mg/dL)	35 (28–47)	45 (35–54)	0.01
Triglycerides (mg/dL)	185 (132–216)	184 (131–253)	0.60
LDL-cholesterol/HDL-cholesterol	3.23 (2.60–5.18)	2.88 (2.21–3.84)	0.08
Glucose (mg/dL)	164.1 ± 59.1	169.6 ± 88.3	0.48
Glycated hemoglobin (%)	8.4 (7.2–10.1)	8.2 (6.9–9.6)	0.69
Fructosamine (mg/dL)	2.96 (2.43–3.18)	3.83 (2.99–6.47)	<0.001
Homocysteine (μmol/L)	6.5 (3.5–10.6)	8.5 (5.6–12.3)	0.21
Cysteine (μmol/L)	168 (149–217)	198 (158–245)	0.17
Methionine (μmol/L)	21.2 (19.2–43.4)	26.0 (19.0–35.4)	0.81
Reduced glutathione (μmol/L)	3.28 (2.47–4.55)	4.05 (3.17–5.54)	0.03
Oxidized glutathione (μmol/L)	0.92 (0.68–1.16)	0.94 (0.66–1.34)	0.58
Reduced glutathione/oxidized glutathione	3.11 (2.69–4.47)	3.96 (3.10–6.56)	0.05
L-Arginine (μmol/L)	48.4 (35.8–57.3)	49.3 (35.6–68.0)	0.37
Asymmetric dimethyl-L-arginine (μmol/L)	0.96 (0.88–1.46)	0.89 (0.69–1.22)	0.08
Symmetric dimethyl-L-arginine (μmol/L)	0.70 (0.49–0.82)	0.79 (0.62–1.03)	0.04
L-Arginine/asymmetric dimethyl-L-arginine	39.4 (35.9–43.7)	51.9 (35.3–70.9)	0.02
Nitrate plus nitrite (μmol/L)	50.1 (36.9–57.0)	32.1 (25.5–41.1)	<0.001
S-Nitrosothiols (nM)	129 (93–164)	131 (95–170)	0.54
Nitrotyrosine (nM)	435 (375–453)	453 (315–615)	0.59
n-Acetyl-β-glucosaminidase (U/L)	33.2 (28.3–41.2)	26.3 (22.8–34.4)	0.02

DM2: type 2 diabetes mellitus. CAD: coronary artery disease. Results are given as means ± standard deviation, median (interquartile range), or counts (percentage). P values shown are not adjusted for multiple testing and refer to the univariate analysis.

0.5 M phosphate buffer and adjusted to pH 7.5 with 1 M Na_3PO_4. The derivatizer (5-bromomethylfluorescein) was added to the samples at a molar ratio of 5 to 10 times excess and the preparations are incubated for 15 minutes at 60°C. After derivatization, the sample was diluted at 1 : 10 with phosphate buffer 0.25 M pH 7.6 and injected with a pressure of 0.5 psi for 1.5 seconds on capillary (70 cm length and 50 μm internal diameter). The capillary electrophoresis instrument (BioFocus 2000, Bio-Rad Laboratories, Inc.) was performed at 25°C, 30 kV, and positive polarity on injection. The detector laser-induced fluorescence (BioFocus LIF^2, Bio-Rad Laboratories, Inc.) operated in 488 nm (excitation) and 520 nm (emission). For each run the capillary was washed with 1 M NaOH (Vecchione et al. 1999) [8].

2.8. Nitrotyrosine. The nitrotyrosine in proteins was determined by a competitive ELISA method. We used the polyclonal anti-nitrotyrosine antibody (Upstate, Catalog Number: 06-284). The standard used was nitrated bovine albumin (nitro-albumin) prepared by alkaline addition of 1 mM peroxynitrite and 1 mM albumin. The concentration of the nitro-albumin was determined using the molar extinction coefficient of 4300 $m^{-1}\,cm^{-1}$ at 438 nm and pH 9.0. The plate was sensitized with 0.05 ug nitro-albumin per well and then washed, blocked with milk protein, and rinsed again. The following was added to the plate anti-nitrotyrosine antibody and sample/standard. After incubation, the plate was washed and added to the peroxidase-conjugated antibody (Stressgen Biotechnologies Corp.). After incubation and washing, 2.3 mM luminol and 0.9 mM p-iodophenol (200 μL/well) and 3.9 mM hydrogen peroxide (50 μL/well) were added. The reading of the chemiluminescence produced was immediately performed (LumiCount, Packard, Meriden, USA). The present nitrotyrosine concentrations in the sample were estimated by using the calibration curve and nitrated albumin was expressed as equivalent nitro-albumin [9].

2.9. Statistical Analysis. There was no missing data in our study. Data were expressed as means ± standard deviation (SD), median (interquartile range), or counts (percentage) when appropriate. For univariate analyses, groups were compared by the t-test for approximately normally distributed

variables or by means of the Mann-Whitney U-test for variables with skewed distribution. Fisher's exact test was used to test differences in count data. In addition, we constructed multiple logistic regression models using the backward stepwise selection procedure to ascertain predictors for CAD in DM2 patients (coding scheme: DM2 + CAD = 1, DM2 = 0). Variables were sequentially removed from the full model (all regression terms included) when the correspondent P value was higher than 0.10. In order to assess the model performance, we did not employ k-fold partitioning due to the relatively low number of subjects. Instead, we used the resubstitution approach, in which the same data are used for both training and testing [10]. For these analyses, each participant has an estimated probability of being DM2 + CAD, which is calculated from the logistic model-derived equation based on the participant's own variables. If the calculated probability was ≥50%, the subject was assigned as DM2 + CAD or DM2 only, otherwise. Model predictions were then compared to the true (known) status. We quantified model performance by using two measures of classification accuracy that are not susceptible to class imbalance: balanced accuracy (BA) and normalized mutual information (NMI).

BA is an accuracy measure that takes into account both sensitivity and specificity of the models and is calculated as the average of sensitivity and specificity. This accuracy measure ranges from 0 to 100%. NMI is an information-theoretic measure of classifier performance. NMI ranges from 0 to 100% and is interpreted as the amount by which the examined model reduces one's uncertainty about the true state of the participants (e.g., 0% means that the status is independent of the studied explanatory variables, while 100% suggests that the model fully predicts the status for each individual) [11]. Permutation was used to construct empirical null distributions (2,000 shuffles) in order to compute the statistical significance of both BA and MNI measures. The hypothesis of a significantly better fit for the model with biochemical biomarkers plus classical variables for CAD (full model) compared to the simpler models (*nested* models) was tested by means of a likelihood-ratio test. This test assumes the form $\Delta = -2(l_0 - l_1)$, where l_1 and l_0 are the natural log-likelihoods under the full and nested models, respectively. We used permutation to test whether the full model fits the data significantly better than simpler models by comparing the observed test statistic Δ to those obtained in 2,000 randomly generated shuffles. P values were adjusted by the Holm-Bonferroni procedure in sensitivity analyses [11]. All data analyses were performed using the Stata package (version 11.0, STATA Corp., College Station, TX, USA). Two-sided P values < .05 were considered statistically significant.

3. Results

3.1. Analytical Validation. All methodologies presented in this study showed satisfactory accuracy and replication. All calibration curves were linear with a coefficient of determination (R^2) ≥ 0.98. The quantification limit (10 : 1; signal : noise) by capillary electrophoresis (ADMA, SDMA, and L-arginine: 25 nM; thiols: 50 nM) and nitric oxide analyzer (NOx and RSNO: 10 nM) were suitable for detection of the analytes in the samples. The ELISA also showed a quantification limit adequate for detection of nitrotyrosine (30 nM). All samples and standards were analyzed in duplicate or triplicate.

3.2. Participants' Biochemical and Demographic Characteristics. The main demographical, clinical, and biochemical characteristics of the studied participants are shown in Table 1. Twenty-one (22%) out of 96 participants with DM2 were classified as DM2 + CAD. Age, body mass index (BMI), and gender proportion were comparable between DM2 + CAD and DM2 groups. However, in an exploratory (unadjusted) analysis, levels of methionine, LDL/HDL ratio, N-acetyl-β-glucosaminidase, and nitrite plus nitrate (NOx) were significantly higher in DM2 + CAD. In addition, levels of L-arginine : ADMA ratio, fructosamine, and reduced glutathione were significantly lower in DM2 + CAD patients compared to their DM2 counterparts. After a Holm-Bonferroni correction for 25 tests, both NOx and fructosamine levels remained significantly higher in DM2 + CAD participants compared to the DM2 group ($P = 0.02$ and $P = 0.006$, resp.).

3.3. Predicting CAD in DM2. To determine putative predictor variables for CAD in DM2, we built a multiple logistic regression model with backward elimination. Of the 25 variables tested, eight remained in the final model: LDL/HDL ratio, methionine, reduced glutathione (GSH), L-arginine : ADMA, NOx (nitrate plus nitrite), N-acetyl-β-glucosaminidase (NAGase), gender, and BMI (Table 2). On the basis of this model (hereafter named *full model*), we constructed an equation that was applied to the complete dataset to predict the status of the 96 studied participants. The overall classification accuracy of this model (resubstitution method) is presented in Table 3, along with other two competing models including either only classical variables for CAD (i.e., LDL/HDL ratio, gender, and BMI; model 2) or emerging biochemical markers only (i.e., L-arginine : ADMA ratio, methionine, GSH, NAGase, and NOx; model 3).

All of the three models significantly predicted CAD more often than can be expected by chance, although it can be verified from Table 3 that the performance of the studied models ranges from small to moderate.

3.4. The Added Value of Emerging Biochemical Markers for CAD Prediction. We sought next to assess whether there is an increase in accuracy for prediction models when emerging biochemical markers are used in combination with classical risk factors for CAD. In other words, we tested whether simpler models (models 2 and 3) are equally capable of predicting CAD in DM2 patients compared to the full model. This is of paramount importance, since there is a potential of overfitting as the number of variables included in the model increases.

We observed that the full model fits significantly better the data compared to either model 2 ($P < 0.001$) or model 3 ($P = 0.004$), indicating that there is a statistically significant gain in accuracy when both emerging biochemical markers and classical risk factors are added to the model for CAD prediction.

Table 2: Final multiple logistic regression model for coronary artery disease in patients with type 2 diabetes ($n = 96$).

	Coefficient (β)	SE of β	Z	P	Odds ratio	95% CI
Intercept	−21.51	6.501	—	—	—	—
LDL-cholesterol/HDL-cholesterol	0.690	0.290	2.38	0.017	1.99	1.13 to 3.52
Methionine (μmol/L)	0.083	0.031	2.68	0.007	1.09	1.02 to 1.16
Reduced glutathione (μmol/L)	−0.512	0.261	−1.96	0.050	0.60	0.36 to 1.00
L-Arginine/asymmetric dimethyl-L-arginine	−0.052	0.022	−2.30	0.021	0.95	0.91 to 0.99
Nitrate plus nitrite (μmol/L)	0.095	0.028	3.40	0.001	1.10	1.04 to 1.16
n-Acetyl-β-glucosaminidase (U/L)	0.087	0.038	2.27	0.023	1.09	1.01 to 1.17
Gender (male = 1, female = 0)	−2.667	1.033	−2.58	0.010	0.07	0.01 to 0.52
BMI (kg/m^2)	0.588	0.189	3.11	0.002	1.80	1.24 to 2.61

(β): coefficient that reflects the expected increase in the log (odds ratio) for CAD per unit increase in the independent variable. SE: standard error. CI: confidence intervals.

Table 3: Performance of different models for CAD prediction in type 2 diabetes patients.

	BA (%)	P_{BA}*	NMI (%)	P_{NMI}*
Model 1 (full): classical factors + emerging biochemical factors	72.85%	0.002	20.52%	0.001
Model 2: classical factors only	57.14%	0.01	9.29%	0.003
Model 3: emerging biochemical factors only	66.38%	0.001	13.08%	0.003

BA: balanced accuracy. NMI: normalized mutual information.
Model 1: LDL : HDL ratio, methionine, reduced glutathione, L-arginine : asymmetric dimethyl-L-arginine, nitrate plus nitrite, n-acetyl-β-glucosaminidase, gender, and BMI.
Model 2: LDL : HDL ratio, gender, and BMI.
Model 3: methionine, reduced glutathione, L-arginine : asymmetric dimethyl-L-arginine, nitrate plus nitrite, and n-acetyl-β-glucosaminidase.
* P value based on 2,000 permutations. Age was not a significant predictor, yielding to virtually identical results after inclusion of this variable in the models (data not shown).

For comparison purposes, less conservative measures of diagnostic accuracy (e.g., classification error) also favor the full model over models 2 and 3 (data not shown).

4. Discussion

4.1. Main Findings. We found that individuals with DM2 + CAD have several significant metabolic changes related to endothelial dysfunction and oxidative stress compared to DM2 without CAD. In our sample, we observed significant alterations in the following variables: HDL-cholesterol, fructosamine, GSH, SDMA, L-arginine/ADMA, NOx, and NAGase.

In addition, we showed that the prediction accuracy for CAD in DM2 patients is significantly increased when six emerging biochemical markers are incorporated into the model along with classical risk factors for CAD.

4.2. Study Limitations. Our study has a number of important limitations. First, our investigation should be regarded as a "predictor finding study" only. Although we have developed a multivariable prediction model, we did not aim at the development of a model for use in clinical practice to guide patient management [12]. Instead, our study should be viewed as a hypothesis-generating study that warrants further confirmation in larger, well-powered investigations with external validation. Second, criticism might be directed at the fact that our definition of CAD might lack specificity. Because CAD was classified conservatively, silent CAD might be present among a few patients in the DM2 + CAD group. However, in our study, misclassification of the CAD status is likely to lead to downwardly biased estimates, ultimately leading to less impressive model predictions (i.e., more conservative models). Third, our sampling scheme was based on volunteers and was not designed to be representative of the population. With that said, our results are prone to a survival bias, since there is a reduced likelihood of enrolling patients who died acutely or who are experiencing severe consequences of myocardial infarction [13]. Again, these results are likely to lead to more conservative model predictions, since more prominent metabolic alterations are plausible in advanced CAD patients. On the other hand, even though we employed permutation to compute statistical significance, our small sample size is prone to overoptimistic results [14]. Indeed, there is a clear potential for overfitting and overestimation, because our models were constructed with a large number of biomarkers relative to the number of participants [15].

4.3. Usefulness of Novel Biochemical Markers for CAD Prediction in DM2. In another study conducted by our research group [16] we showed that hyperglycemia correlated with

increased production of nitric oxide based on the evaluation of concentrations of nitrate and nitrite. Endothelial dysfunction is generally defined as impaired endothelium-dependent vasodilation, related to lower $^{\bullet}NO$ production or bioavailability. Reactive oxygen and nitrogen species (RONS), such as H_2O_2, $O_2^{\bullet-}$, $^{\bullet}NO$, and $ONOO^{-}$, can be produced in blood vessels by certain drugs and pathological conditions and accentuate or induce endothelial dysfunction [17]. RONS may reduce $^{\bullet}NO$ bioactivity by formation of peroxynitrite or by decrease of enzyme or transporter activities by oxidation of their thiols groups [18]. Endothelial dysfunction can contribute to the initiation, progression, and clinical manifestations of CAD. The increase in S-nitrosothiols may be a consequence of glycation of proteins, which facilitates the nitrosation of protein thiols by nitric oxide [19]. The glycated proteins can act as a sink for nitric oxide because S-nitrosation reduces their competence to release nitric oxide which reflects directly on the pathogenesis of endothelial dysfunction [20]. Nitric oxide can react with superoxide anion, which leads to the formation of a potent oxidant, peroxynitrite, and in sequence generation of hydroxyl radicals and nitrogen dioxide [18, 21, 22]. The tyrosine, free or protein-bind, may react with nitrogen dioxide generating nitrotyrosine, which is considered a biomarker of the formation of peroxynitrite [23]. In this present study, only the increase of NOx was demonstrated with no differences for nitrotyrosine, even though reduced glutathione and reduced glutathione/oxidized glutathione ratio concentrations were observed.

The endogenous nitric oxide synthase inhibitors, represented by symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA), also play an important role in endothelial dysfunction. In the present study, reduced concentration of L-arginine/ADMA in DM2 + CAD group in comparison with DM2 without CAD reinforces the fact that endothelial nitric oxide synthase inhibition occurs along the atherosclerosis process. Accordingly, it has been shown that L-arginine/ADMA levels diminished parallel to the impairment of vasodilatation in DM2 patients after lipid ingestion [24]. Moreover, it was previously reported that low L-arginine/ADMA ratio was not related to either the presence of macroangiopathy or the time of disease manifestation in DM2 patients, but rather to the metabolic control of the diabetes [25]. Thus, in the present study, hypercholesterolemia observed in DM2 + CAD group could be related to ADMA increase, occasioning a lower production of $^{\bullet}NO$ and leading to endothelial dysfunction. However, nitric oxide low production could not be confirmed in this study based only on the increase of nitrite plus nitrate concentration generation. Studies have shown a reduction of endothelial dysfunction by rising L-arginine/ADMA ratio [26]. The source of ADMA in hypercholesterolemia is unknown. ADMA can be the result of the hydrolysis of methylated proteins [27]. In vivo lipid peroxidation causes peroxidative damage to tissue proteins and may raise the rate of proteolysis. Alternatively, there may be a dysfunction or downregulation of di-methylarginine dimethylaminohydrolase, the enzyme that converts ADMA to L-citrulline [28]. Hypercholesterolemia may alter the regulation or function of di-methylarginine dimethylaminohydrolase, thereby resulting in intracellular accumulation of ADMA. In fact, regenerating endothelial cells impairs vasodilation and produces more ADMA [29]. Hypercholesterolemia plus hyperglycemia is a factor that contributes to oxidative stress due to autoxidation of glucose and nonenzymatic protein glycation.

Thiols play an important role on the oxidative processes and therefore may directly or indirectly contribute in endothelial dysfunction. Glutathione is a major thiol used as a biomarker of oxidative stress and is found in the forms of GSH (reduced glutathione) and GSSG (oxidized glutathione). In the present study, the DM2 + CAD group showed lower GSH/GSSG and GSH concentration, compared with DM2 without CAD group. This decrease of reduced glutathione may be due to activation of the polyol pathway in which glucose is reduced to sorbitol by the NADPH-dependent aldose reductase [30, 31]. This enzyme presents a high Michaelis-Menten constant for glucose and therefore this metabolic via is only quantitatively significant in hyperglycemia. Thus, whenever the NADPH/NADP ratio is decreased in hyperglycemic state, it will result in prejudice in the regeneration of reduced glutathione. Also as a consequence, the synthesis of nitric oxide by nitric oxide synthase will be impaired due to the NADPH dependence [31, 32]. Apparently, the decrease in GSH concentration has a relation to the presence of atherosclerotic inflammation process in patients with CAD.

The activity of N-acetyl-β-D-glucosaminidase (NAGase) in plasma has been used as a biomarker of endothelial dysfunction [33]. In the present study, NAGase activity was significantly higher in DM2 + CAD group in comparison with the DM2 without CAD group. Previous studies have reported increased NAGase activity in individuals with CAD [34–36]. NAGase is a lysosomal enzyme produced by many cells, including not only endothelial cells, but also smooth muscle and kidney [37]. These enzymes can be thrown into the extracellular environment through stimulation of oxidative stress. Sánchez-Hueso et al. showed correlation between measurements of brachial artery diameter and blood flow via Doppler ultrasonography with NAGase activity in patients with DAC [35]. Additionally, NAGase activity showed positively correlated with insulin resistance in patients with CAD [38]. According to Komosińska-Vassev et al., the increase of NAGase activity can be considered an indicator of the intensity of endothelial cell dysfunction [39]. Several studies also demonstrated that NAGase is increased in individuals with DM2, when compared to healthy individuals and that NAGase activity is high in individuals with DM2 and previous history of cardiovascular disease (myocardial infarction) in comparison with individuals with DM2 without previous coronary events [34, 35, 37]. Thus, our data reinforce previous data showing that the increase of NAGase activity in individuals with DM2 can be associated with the presence of CAD.

Conflict of Interests

The authors report no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] J. H. Pinkney, C. D. A. Stehouwer, S. W. Coppack, and J. S. Yudkin, "Endothelial dysfunction: cause of the insulin resistance syndrome," *Diabetes*, vol. 46, no. 2, pp. S9–S13, 1997.

[2] F. Cosentino and T. F. Lüscher, "Endothelial dysfunction in diabetes mellitus," *Journal of Cardiovascular Pharmacology*, vol. 32, no. 3, pp. S54–S61, 1998.

[3] M. M. Gabir, R. L. Hanson, D. Dabelea et al., "The 1997 American Diabetes Association and 1999 World Health Organization criteria for hyperglycemia in the diagnosis and prediction of diabetes," *Diabetes Care*, vol. 23, no. 8, pp. 1108–1112, 2000.

[4] A. Reglero and J. A. Cabezas, "Glycosidases of molluscs. Purification and properties of α L fucosidase from Chamelea gallina L.," *European Journal of Biochemistry*, vol. 66, no. 2, pp. 379–387, 1976.

[5] J. F. Ewing and D. R. Janero, "Specific S-nitrosothiol (thionitrite) quantification as solution nitrite after vanadium(III) reduction and ozone-chemiluminescent detection," *Free Radical Biology and Medicine*, vol. 25, no. 4-5, pp. 621–628, 1998.

[6] R. Marley, M. Feelisch, S. Holt, and K. Moore, "A chemiluminescense-based assay for S-nitrosoalbumin and other plasma S- nitrosothiols," *Free Radical Research*, vol. 32, no. 1, pp. 1–9, 2000.

[7] E. Caussé, N. Siri, J. F. Arnal et al., "Determination of asymmetrical dimethylarginine by capillary electrophoresis-laser-induced fluorescence," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 741, no. 1, pp. 77–83, 2000.

[8] G. Vecchione, M. Margaglione, E. Grandone et al., "Determining sulfur-containing amino acids by capillary electrophoresis: a fast novel method for total homocyst(e)ine human plasma," *Electrophoresis*, vol. 20, no. 3, pp. 569–574, 1999.

[9] G. J. Chakmakjian, *Endocrinology*, Laboratory Medicine, Philadelphia, Pa, USA, 1982.

[10] A. H. Fielding and J. F. Bell, "A review of methods for the assessment of prediction errors in conservation presence/absence models," *Environmental Conservation*, vol. 24, no. 1, pp. 38–49, 1997.

[11] W. S. Bush, T. L. Edwards, S. M. Dudek, B. A. McKinney, and M. D. Ritchie, "Alternative contingency table measures improve the power and detection of multifactor dimensionality reduction," *BMC Bioinformatics*, vol. 9, article 238, 2008.

[12] W. Bouwmeester, N. P. A. Zuithoff, S. Mallett et al., "Reporting and methods in clinical prediction research: a systematic review," *PLoS Medicine*, vol. 9, no. 5, Article ID e1001221, 2012.

[13] T. V. Pereira, M. Rudnicki, R. F. Franco, A. C. Pereira, and J. E. Krieger, "Effect of the G-308A polymorphism of the tumor necrosis factor α gene on the risk of ischemic heart disease and ischemic stroke: a meta-analysis," *The American Heart Journal*, vol. 153, no. 5, pp. 821–830, 2007.

[14] T. V. Pereira, R. I. Horwitz, and J. P. A. Ioannidis, "Empirical evaluation of very large treatment effects of medical interventions," *The Journal of the American Medical Association*, vol. 308, no. 16, pp. 1676–1684, 2012.

[15] G. S. Collins, S. Mallett, O. Omar, and L. M. Yu, "Developing risk prediction models for type 2 diabetes: a systematic review of methodology and reporting," *BMC Medicine*, vol. 9, article 103, 2011.

[16] E. C. Pereira, S. Ferderbar, M. C. Bertolami et al., "Biomarkers of oxidative stress and endothelial dysfunction in glucose intolerance and diabetes mellitus," *Clinical Biochemistry*, vol. 41, no. 18, pp. 1454–1460, 2008.

[17] J. L. Witztum, "The oxidation hypothesis of atherosclerosis," *The Lancet*, vol. 344, no. 8925, pp. 793–795, 1994.

[18] N. Hogg, V. M. Darley-Usmar, A. Graham, and S. Moncada, "Peroxynitrite and atherosclerosis," *Biochemistry of Society Transplant*, vol. 21, no. 2, pp. 358–362, 1993.

[19] S. P. Wolff and R. T. Dean, "Glucose autoxidation and protein modification. The potential role of "autoxidative glycosylation" in diabetes," *Biochemical Journal*, vol. 245, no. 1, pp. 243–250, 1987.

[20] J. Farkas and E. J. Menzel, "Proteins lose their nitric oxide stabilizing function after advanced glycosylation," *Biochimica et Biophysica Acta—General Subjects*, vol. 1245, no. 3, pp. 305–310, 1995.

[21] J. S. Johansen, A. K. Harris, D. J. Rychly, and A. Ergul, "Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice," *Cardiovascular Diabetology*, vol. 4, article 5, pp. 1–11, 2005.

[22] N. Hogg, V. M. Darley-Usmar, M. T. Wilson, and S. Moncada, "The oxidation of α-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide," *FEBS Letters*, vol. 326, no. 1–3, pp. 199–203, 1993.

[23] O. Augusto, R. M. Gatti, and R. Radi, "Spin-trapping studies of peroxynitrite decomposition and of 3-morpholinosydnonimine N-ethylcarbamide autooxidation: direct evidence for metal-independent formation of free radical intermediates," *Archives of Biochemistry and Biophysics*, vol. 310, no. 1, pp. 118–125, 1994.

[24] B. Balint, S. A. Kharitonov, T. Hanazawa et al., "Increased nitrotyrosine in exhaled breath condensate in cystic fibrosis," *European Respiratory Journal*, vol. 17, no. 6, pp. 1201–1207, 2001.

[25] E. K. Hoogeveen, P. J. Kostense, C. Jakobs et al., "Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes: 5-year follow-up of the Hoorn study," *Circulation*, vol. 101, no. 13, pp. 1506–1511, 2000.

[26] Y. Xiong, M. Lei, S. Fu, and Y. Fu, "Effect of diabetic duration on serum concentrations of endogenous inhibitor of nitric oxide synthase in patients and rats with diabetes," *Life Sciences*, vol. 77, no. 2, pp. 149–159, 2005.

[27] R. H. Böger, S. M. Bode-Böger, A. Szuba et al., "Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia," *Circulation*, vol. 98, no. 18, pp. 1842–1847, 1998.

[28] J. R. McDermott, "Studies on the catabolism of Ng-methylarginine, Ng, Ng-dimethylarginine and Ng, Ng-dimethylarginine in the rabbit," *Biochemical Journal*, vol. 154, no. 1, pp. 179–184, 1976.

[29] S. A. Fickling, A. M. Leone, S. S. Nussey, P. Vallance, and G. J. Whitley St, "Synthesis of NG, NG dimethylarginine by human endothelial cells," *Endothelium: Journal of Endothelial Cell Research*, vol. 1, no. 3, pp. 137–140, 1993.

[30] H. Azuma, J. Sato, H. Hamaski, A. Sugimoto, E. Isotani, and S. Obayashi, "Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells," *British Journal of Pharmacology*, vol. 115, no. 6, pp. 1001–1004, 1995.

[31] A. N. Elias and S. Eng, "Homocysteine concentrations in patients with diabetes mellitus—relationship to microvascular and macrovascular disease," *Diabetes, Obesity and Metabolism*, vol. 7, no. 2, pp. 117–121, 2005.

[32] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

[33] N. E. Cameron, M. A. Cotter, M. Basso, and T. C. Hohman, "Comparison of the effects of inhibitors of aldose reductase and sorbitol dehydrogenase on neurovascular function, nerve conduction and tissue polyol pathway metabolites in streptozotocin-diabetic rats," *Diabetologia*, vol. 40, no. 3, pp. 271–281, 1997.

[34] J. Škrha, M. Prázný, J. Hilgertová, and H. Weiserová, "Serum α-tocopherol and ascorbic acid concentrations in Type 1 and Type 2 diabetic patients with and without angiopathy," *Clinica Chimica Acta*, vol. 329, no. 1-2, pp. 103–108, 2003.

[35] M. C. Sánchez-Hueso, J. Mateo-Caãs, and E. Zamora-Madaria, "Influence of glycemic blood glucose control and incipient diabetic nephropathy on the urinary excretion of N-acetyl-glucosaminidase (NAG) in diabetes mellitus," *Anales de Medicina Interna*, vol. 12, no. 5, pp. 216–220, 1995.

[36] J. Skrha, M. Prázný, J. Kvasnicka, and B. Kalvodová, "Changes in microcirculation and selected laboratory parameters in the early stages of diabetic microangiopathy," *Časopis Lékařů Českých*, vol. 140, no. 12, pp. 370–374, 2001.

[37] J. Škrha, M. Prázný, T. Haas, J. Kvasnička, and B. Kalvodová, "Comparison of laser-Doppler flowmetry with biochemical indicators of endothelial dysfunction related to early microangiopathy in Type 1 diabetic patients," *Journal of Diabetes and its Complications*, vol. 15, no. 5, pp. 234–240, 2001.

[38] T. Inoue, R. Matsunaga, S. Morooka, and Y. Uehara, "Serum N-acetyl-β-D-gulucosaminidase activity increases in association with insulin resistance in patients with coronary artery disease," *Atherosclerosis*, vol. 149, no. 1, pp. 117–122, 2000.

[39] K. Komosińska-Vassev, K. Olczyk, E. M. Koźma, P. Olczyk, G. Wisowski, and K. Winsz-Szczotka, "Alterations of glycosaminoglycan metabolism in the development of diabetic complications in relation to metabolic control," *Clinical Chemistry and Laboratory Medicine*, vol. 43, no. 9, pp. 924–929, 2005.

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Research Article

Treated Autoimmune Thyroid Disease Is Associated with a Decreased Quality of Life among Young Persons with Type 1 Diabetes

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Type 1 diabetes (T1D) in children and adolescents is relatively often accompanied by other immunopathological diseases, autoimmune thyroid disease (AITD) or celiac disease (CD). Our aim was to assess whether these conditions are associated with changes in the health-related quality of life (HRQOL) in pediatric patients with T1D. In a cross-sectional study we identified eligible 332 patients with T1D aged 8–18 years, of whom 248 (75%) together with their parents responded to the PedsQL Generic and Diabetes Modules. Compared to 143 patients without thyroid autoantibodies, 40 patients with a thyroxine-treated AITD scored lower in the overall generic HRQOL ($P = 0.014$), as well as in the overall diabetes-specific HRQOL ($P = 0.013$). After adjustment for age, gender, duration of diabetes, type of diabetes treatment, and diabetes control, this association remained statistically significant for the generic HRQOL ($P = 0.023$). Celiac disease was not associated with a change in the generic or diabetes-specific HRQOL ($P = 0.07$ and $P = 0.63$, resp.). Parental scores showed no association with AITD or celiac disease, except a marginally significant decrease in the overall generic HRQOL ($P = 0.039$) in the T1D + AITD compared to T1D group. Our study indicates that, in pediatric patients with T1D, concomitant thyroxine-treated AITD is associated with lower quality of life.

1. Introduction

The modern treatment of pediatric type 1 diabetes mellitus (T1D) relies not only on professional medical care but also on psychosocial support [1]. The impact of T1D on everyday life is much broader than a mere adaptation to the demanding treatment regime (balanced diet, blood glucose monitoring, insulin injections or infusion, and physical activity). Notably, the patients have been shown to experience worries of hypoglycemia or long-term complications, feelings of being different from peers, or conflicts with parents concerning limited autonomy [2–4]. The early recognition of a subgroup of children with diabetes with a decreased quality of life is of utmost importance, as it is frequently associated with an increased psychosocial distress potentially leading to a worse treatment adherence and T1D control [5, 6]. The ultimate goal of T1D control should not thus be limited to low HbA1c values and the absence of acute and late complications but must also include the subjective well-being of the children and their families.

The impact of a disease on the patients' lives is indirectly measurable by assessing their health-related quality of life

(HRQOL) which is defined as "patient's subjective perception of the impact of his disease and its treatment(s) on his daily life, physical, psychological and social functioning and well-being" [7]. The generic HRQOL questionnaires focus on general aspects of quality of life and are applicable in both healthy and diseased subjects whereas the diabetes-specific HRQOL tools refer to the disease-specific impacts on daily life and well-being [8–10]. The questionnaires have also a parental version which is often administered in parallel to the patients' questionnaires.

Children with T1D often suffer from concomitant autoimmune diseases. Of these, the most common are autoimmune thyroid disease (AITD) and celiac disease (CD) with a prevalence of 15% and 4–7%, respectively [11–14]. Both diseases are readily detectable by regular screening using autoantibodies, provable by imaging techniques (AITD) or biopsy (CD), and clinical recommendations have been developed on their management in young persons with T1D [15]. While there are many studies describing the influences of treatment regime, gender, HbA1c level, and age of patients with T1D on their quality of life [16, 17], up to our knowledge, no studies have assessed the HRQOL in children with T1D and AITD and only one study has published the impact of concomitant CD in children with T1D [18].

The aim of the present work was to assess whether two most prevalent comorbid conditions (AITD and CD) were associated with changes in the quality of life in young persons with T1D, by conducting a cross-sectional study at a large tertiary centre of reference for pediatric diabetes.

2. Materials and Methods

2.1. Participants and Their Screening for AITD and CD. The setting of this study was a tertiary referral centre for pediatric diabetes at the University Hospital in Motol in Prague, Czech Republic. The centre currently provides care for 513 children and young persons with diabetes, of whom overwhelming majority have T1D. It is accredited by the ISPAD/SWEET as one of its European Centres of Reference [19].

The eligibility criteria in the present study were (i) age 8 to 18 years (age constraints imposed by the questionnaires), (ii) the diagnosis of T1D made at least one year prior to the administration of the questionnaire, (iii) normal values of TSH (even if the patient was treated for AITD): hypothyroidism and hyperthyroidism were an exclusion criterion for their known effect on the psychological status, and (iv) absence of severe chronic concomitant diseases other than AITD or CD at the time of quality of life testing. In total, participation was offered to 332 eligible patients of the centre through their parents or guardians, of whom 248 (74.6%) participated. Demographic and clinical characteristics of the participants are shown in Table 1; the study group comprised 97 children aged 8–12 years (mean 10.6 years 48.4% of girls) and 151 adolescents aged 13–18 years (mean 15.8 years 47.0% of girls). For statistical analysis, the closest level of HbA1c to the date of the questionnaires administration as well as current type of T1D treatment was recorded.

All patients are regularly screened for complications, including an annual check-up for AITD and CD. The AITD is screened using the tests for TSH levels (thyroid-stimulating hormone, normal value for both genders 0.34–5.5 mIU/L in subjects aged 1–15 years, 0.35–4.8 mIU/L in subjects aged more than 15 years), anti-thyroglobulin autoantibodies (anti-Tg, normal values 0–60.9 kU/L for both genders and all ages), and thyroid peroxidase autoantibodies (anti-TPO, normal values 0–60.9 kU/L for both genders and all ages) as described previously [13]. The patients repeatedly positive for one or both autoantibodies and with elevated TSH levels were diagnosed as having AITD which was confirmed also by typical sonographical findings in all of them. Celiac disease was screened using anti-transglutaminase IgA and/or endomysial IgA antibodies and the diagnosis was subsequently confirmed by small bowel biopsy.

The annual thyroid autoantibody screening and testing of TSH levels discriminated the children into three groups: (i) 143 children without thyroid autoantibodies ("no thyroid disease"), (ii) 65 children having positive thyroid autoantibodies (against either thyroglobulin or thyroid peroxidase, "T1D+AB" group), and (iii) 40 children who had autoimmune thyroid disease with thyroid function compensated with L-thyroxine to the normal levels of TSH ("T1D+AITD" group). It is of note that four patients did not normalize TSH levels upon treatment at the time of the study and were thus excluded from the study.

CD was diagnosed in 26 of 248 (10.5%) patients. All of them were treated with gluten-free diet and 16 (61.5%) were ATGA negative at the time of HRQOL testing. Six patients had both CD and AITD and were therefore included in both AITD and CD related analyses. The work flow of the study is shown in Figure 1.

2.2. Quality of Life Questionnaires. The study was approved by the institutional ethics committee. The participation was offered through a personal letter signed by the attending physician and encouraged during the routine outpatient visits. The two anonymous questionnaires (generic and diabetes-specific HRQOL) were filled in either at home or at the hospital before the routine visit. Questionnaires were fully completed by 225 subjects whereas in the remaining 23 patients some of the questions were left unanswered and thus excluded from the particular subanalyses. We also collected 234 complete parent proxy reports. The characteristics of children responding to the questionnaires are summarized in Table 1.

The study was carried out using the PedsQL developed by Varni et al. [20]. The generic HRQOL was assessed by the PedsQL 4.0 Generic Core Scales (acute form) instrument which contains 23 items and encompasses four dimensions: physical, emotional, social, and school functioning. Diabetes-specific HRQOL was evaluated using the PedsQL 3.2 Diabetes Module (acute form) tool consisting of 32 or 33 items (depending on the age group) divided into five dimensions: diabetes symptoms, treatment barriers, treatment adherence, worry, and communication. Patients were requested to fill

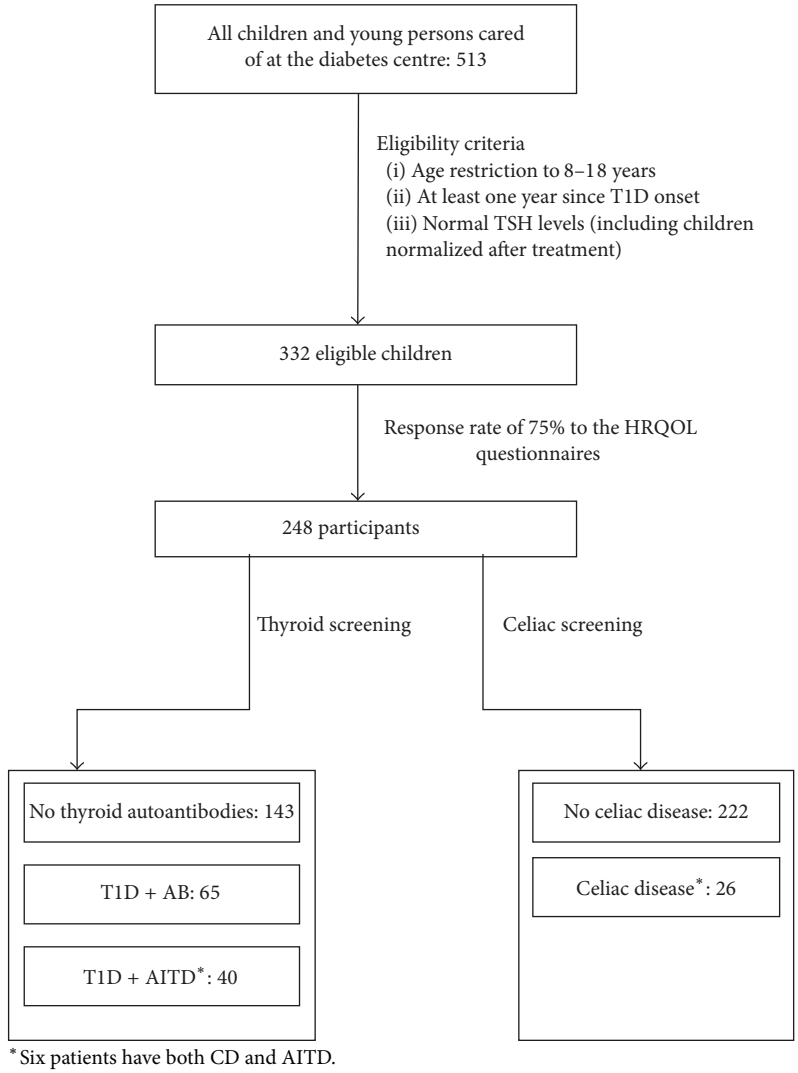

Figure 1: The flowchart of the study.

in self-report and parents were asked for a parallel proxy-report form. Respondents were asked how frequently each item had been problematic for them/their children during the past week and were supposed to record their attitudes on the five-point Likert scale. Summary scores may range from 0 to 100; higher scores indicate better HRQOL. Both Czech versions of instruments have linguistic validation certificates of the MAPI Research Trust (Lyon, France) proving that the translation process was supervised and included three steps: two translations into Czech by qualified translators, following a backward translation, and eventually cognitive debriefing with three healthy children and parents (linguistic validation certificate is available upon request from the authors). The instruments have appropriate psychometric properties [21, 22] and were endorsed for this study by Alena Spirkova (personal correspondence).

2.3. Statistical Analysis. Differences of HRQOL between two groups (children/adolescents, boys/girls, T1D+AITD/no thyroid disease, and T1D+CD/no celiac disease) were compared by two-sample t-test. Welch's correction for unequal variances was performed. Differences between three groups (T1D/T1D+AB/T1D+AITD) were evaluated by F-test analysis of variance. Dependence of HRQOL on continuous variables (age, diabetes duration, HbA1c, and treatment) was assessed by linear model. Finally, multiple linear regression analyses were used to explore the effects of different measures to HRQOL. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Autoimmune Thyroid Disease. Compared to patients with T1D only, the T1D+AITD patients scored in average lower in the generic HRQOL and in the diabetes-specific HRQOL (Table 2). Significantly lower scores were observed in many dimensions, including emotional functioning and school functioning (generic HRQOL) and diabetes symptoms and

Table 1: Characteristics of respondents.

	All patients	Thyroid disease status			Celiac disease status	
		No thyroid disease	Autoantibodies + normal TSH (T1D + AB, untreated)	Autoantibodies + high TSH upon diagnosis (T1D + AITD, treated)	No celiac disease	Celiac disease (T1D + CD)
n	248	143 (57.6%)	65 (26.2%)	40 (16.1%)	222 (89.5%)	26 (10.5%)
Females (*n*, %)	118 (47.6%)	63 (44.0%)	27 (41.5%)	28 (70.0%)[a]	104 (46.8%)	14 (53.8%)
Age (years)	13.7 (11.6–16.6)	13.3 (11.3–16.0)	14.0 (10.9–16.7)	15.2 (13.2–17.2)[b]	13.4 (11.7–13.9)	13.4 (11.7–13.9)
T1D treatment						
Insulin pen	156	90	39	27	142	14
Insulin pump	96 (38.7%)	53 (37.0%)	26 (40.0%)	13 (32.5%)	80 (36.0%)	12 (46.1%)
HbA1c (mmol/mol IFCC)	68 (60–77)	67 (60–75)	66 (60–76)	75 (63–82)[c]	68 (60–77)	72 (60–83)
Diabetes duration (years)	6.3 (3.0–9.3)	5.8 (2.8–9.3)	5.7 (2.8–8.8)	7.8 (5.2–10.6)	5.9 (2.8–9.3)	7.6 (4.9–9.1)

Data are described as median (interquartile range) or *n* (%).

[a] $P = 0.0065$ as compared to subjects with no thyroid disease; [b] $P = 0.0017$ as compared to subjects with no thyroid disease; [c] $P = 0.044$ as compared to subjects with no thyroid disease.

treatment adherence (diabetes-specific HRQOL). Further, a borderline nominal significance was noted in physical functioning ($P = 0.051$, generic HRQOL). The T1D+AB group (patients having autoantibodies but normal thyroid function) did not differ in either of the dimensions compared to patients with T1D only. There was, however, an apparent decreasing trend in many scores in the direction from no thyroid disease through T1D+AB to T1D+AITD group (Table 2).

The analysis was then adjusted for potential confounders and modifiers such as the level of HbA1c, age, diabetes duration, gender, and type of treatment. The adjusted model, specified in Table 3, included these five clinically most relevant variables. The association of AITD with a decrease in the generic HRQOL score persisted after this adjustment ($P = 0.023$) whereas no difference was appreciable for diabetes-specific HRQOL ($P = 0.11$).

The parental questionnaires did not show alterations of scores with the exception of a decrease in the overall generic score in the T1D+AITD group ($P = 0.039$) where also borderline-significant decrease in the physical functioning was noted ($P = 0.043$).

3.2. Celiac Disease. Neither overall generic nor overall diabetes-specific HRQOL of patients with T1D+CD differed from T1D patients without CD; this applied to both children's and parental scores (Table 4). The only differences were observed in single dimensions: the physical functioning, in which patients with T1D+CD had higher children's scores ($P = 0.025$), and the diabetes symptoms, where patients with T1D+CD had higher parental scores ($P = 0.020$).

4. Discussion

We observed an independent association between AITD and a decrease in several measures of quality of life in children and adolescents with T1D, whereas no such clear association was observable for CD.

The known factors as female gender, worse glycemic control, higher age, higher diabetes duration, and type of T1D treatment only partly explained the lower scores of HRQOL in the T1D+AITD group, as well as the apparent decreasing trend of HRQOL from diabetes-only group through patients with thyroid autoantibodies to children and adolescents with treated AITD (adjusted model, Table 3). All the confounders linked to AITD in the present study, with the exception of T1D treatment, had been described before as factors related to worse HRQOL in patients with T1D [23–29].

Upon adjustment, a significant independent association of AITD with decreased HRQOL was found for generic HRQOL but not for diabetes-specific HRQOL. It could be explained by the fact that the diabetes-specific questionnaire assessed the difficulties caused particularly by diabetes whereas the generic HRQOL covers a much broader concept of main life domains (physical, emotional, social, and school functioning). Therefore, the component of the association of AITD with HRQOL, which is independent of T1D, is more apparent in this measure.

There may be several reasons why thyroid disease is linked to a decreased HRQOL. Indeed, it is well conceivable that the diagnosis of AITD further increases the burden on the patient, having another disease requiring daily substitution treatment, although with tablets only. Two dimensions were impaired: the emotional and school functioning. We can only speculate whether there may be even a direct causative relation with AITD; for the emotional dimension, there could be a mechanism involving a direct impact of thyroid hormones on serotonin neurotransmission and subsequently on the mood [30]. The school functioning might be affected by an association of thyroid function with cognitive disturbances [31].

Table 2: Quality of life assessed using the HRQOL questionnaires, by thyroid disease.

	All patients	Thyroid disease status			*t*-test *P* value	
		No thyroid disease	T1D + AB	T1D + AITD	T1D + AB versus no thyroid disease	T1D + AITD versus no thyroid disease
Children's scores						
Generic HRQOL questionnaire						
Overall	82.51 (10.81)	83.57 (10.47)	82.64 (10.93)	78.55 (11.17)	0.567	**0.014***
Physical functioning	87.93 (10.58)	88.47 (10.35)	88.91 (10.21)	84.45 (11.48)	0.777	0.051
Emotional functioning	74.43 (18.57)	76.67 (18.21)	72.75 (18.59)	69.20 (18.95)	0.161	**0.030***
Social functioning	90.92 (12.53)	91.25 (11.19)	92.03 (13.24)	88.00 (15.43)	0.682	0.220
School functioning	73.77 (16.12)	75.39 (15.64)	73.28 (16.79)	68.88 (16.03)	0.396	**0.026***
Diabetes-specific HRQOL questionnaire						
Overall	76.90 (12.24)	78.46 (12.25)	75.86 (12.15)	73.05 (11.56)	0.158	**0.013***
Diabetes symptoms	73.29 (13.71)	74.76 (14.00)	72.77 (12.83)	68.87 (13.41)	0.318	**0.019***
Treatment barriers	77.60 (17.07)	78.71 (17.64)	75.85 (16.57)	76.49 (15.86)	0.261	0.452
Treatment adherence	84.82 (13.45)	86.96 (12.10)	83.15 (15.38)	79.92 (13.34)	0.082	**0.004****
Worry	73.77 (21.92)	75.03 (22.14)	73.65 (21.81)	69.35 (21.28)	0.681	0.159
Communication	81.07 (19.79)	82.66 (18.70)	79.83 (20.42)	77.39 (22.30)	0.352	0.189
Parental scores						
Generic HRQOL questionnaire						
Overall	78.47 (11.20)	79.57 (11.19)	77.97 (11.22)	75.30 (10.85)	0.350	**0.039**
Physical functioning	83.55 (11.79)	84.53 (11.37)	83.40 (12.84)	80.24 (11.11)	0.551	**0.043**
Emotional functioning	71.38 (17.07)	72.94 (17.60)	69.37 (16.18)	69.19 (16.39)	0.162	0.230
Social functioning	86.86 (14.22)	87.15 (13.63)	88.38 (13.70)	83.24 (16.76)	0.557	0.198
School functioning	69.14 (16.56)	70.74 (16.52)	67.78 (17.96)	65.68 (13.70)	0.193	0.061
Diabetes-specific HRQOL questionnaire						
Overall	74.13 (12.43)	75.09 (12.95)	72.91 (12.27)	72.70 (10.64)	0.251	0.253
Diabetes symptoms	71.70 (13.15)	73.20 (13.53)	69.94 (13.04)	69.19 (11.37)	0.105	0.072
Treatment barriers	74.48 (17.62)	75.04 (18.12)	73.69 (17.42)	73.81 (16.44)	0.615	0.695
Treatment adherence	79.62 (16.11)	81.02 (15.22)	77.31 (18.70)	78.46 (14.27)	0.167	0.343
Worry	72.12 (22.13)	72.43 (22.72)	72.36 (22.84)	70.53 (23.92)	0.985	0.630
Communication	76.25 (23.19)	75.91 (23.32)	76.16 (22.84)	77.67 (23.92)	0.944	0.695

Data are mean (SD).
Test for trend across the categories "no thyroid disease" > "T1D + AB" > "T1D + AITD" significant at $^{*}P < 0.05$ and $^{**}P < 0.005$.

4.1. Comparison to Previous Studies. We are not aware of a previous study on HRQOL in patients with AITD and diabetes, which is moreover pediatric. Several studies from adult patients without diabetes indicate that the burden of AITD is not as benign as it may seem to a healthcare professional knowledgeable about its relatively low overall health risks. It seems that the autoimmunity may affect the quality of life independent of thyroid function status: an impaired psychological well-being linked to altered quality of life was observed in treated patients with overt hypothyroidism with TSH in normal range [32–36] as well as in adults with untreated subclinical hypothyroidism [37]. Controversies exist regarding the prevalence of anxiety and depression in population with subclinical hypothyroidism: it was increased in some [38, 39] but not all studies [40]. Moreover, Ott et al. published a study where AITD had an impact on quality of life in adult women independently of their hormonal status [41]. The overall picture indicates that the AITD *per se* is a factor that may aggravate the psychological status of the patient which is in accord with the results of our multivariable model.

Interestingly, the other studied concomitant disease, CD, was not associated with worsening of HRQOL when compared to children and adolescents with T1D only. Apart from the considerably lower power to disclose such an association (only 10.5% of patients with T1D had CD), several

Table 3: The multivariable model describing the influence of AITD and other clinically relevant modifiers on the HRQOL.

Predictor	Change in HRQOL score per unit (95% conf. interval)	P value
Analysis of generic HRQOL score		
Presence of AITD	**−4.60 (−8.56, −0.64)**	**0.023**
Gender (male)	1.99 (−1.26, 5.25)	0.23
Age (per 1 year)	0.12 (−0.49, 0.74)	0.69
Diabetes duration (per 1 year)	0.21 (−0.24, 0.66)	0.36
HbA1c (per 1 mmol/mol)	−0.07 (−0.17, 0.02)	0.14
Treatment (insulin pump)	−1.68 (−5.08, 1.72)	0.33
Analysis of diabetes-specific HRQOL score		
Presence of AITD	−3.61 (−8.05, 0.83)	0.11
Gender (male)	**5.84 (2.23, 9.46)**	**0.002**
Age (per 1 year)	0.23 (−0.46, 0.92)	0.51
Diabetes duration (per 1 year)	0.13 (−0.38, 0.64)	0.61
HbA1c (per 1 mmol/mol)	**−0.11 (−0.22, −0.01)**	**0.041**
Treatment (insulin pump)	−2.38 (−6.15, 1.38)	0.21

The dependent variable was the total score in the respective questionnaire. The predictors were presence of autoimmune thyroiditis (1 = AITD; 0 = no thyroid disease), along with five major diabetes-related confounders identified from the literature as well as from our univariate analysis. The coefficients represent the change in the quality of life score per one unit of predictor (i.e., presence of AITD, sex, one year of age, one mmol/mol of HbA1c, or treatment by insulin pump). This model was selected for its biological plausibility; that is, no stepwise building was employed.

Table 4: Quality of life assessed using the HRQOL questionnaires, by presence of celiac disease.

	All patients	Celiac disease		t-test P value
		No	Yes	
Children's scores				
Generic HRQOL questionnaire				
Overall	82.51 (10.81)	82.19 (10.97)	85.19 (9.05)	0.128
Physical functioning	87.93 (10.58)	87.46 (10.68)	91.88 (8.91)	**0.025**
Emotional functioning	74.43 (18.57)	73.93 (18.71)	78.65 (17.18)	0.198
Social functioning	90.92 (12.53)	90.85 (12.47)	91.54 (13.25)	0.803
School functioning	73.77 (16.12)	73.67 (16.20)	74.62 (15.68)	0.774
Diabetes-specific HRQOL questionnaire				
Overall	76.90 (12.24)	76.74 (12.21)	78.30 (12.64)	0.562
Diabetes symptoms	73.29 (13.71)	72.84 (13.61)	77.00 (12.30)	0.169
Treatment barriers	77.60 (17.07)	77.83 (17.16)	75.58 (16.51)	0.516
Treatment adherence	84.82 (13.45)	84.73 (13.53)	85.54 (13.01)	0.767
Worry	73.77 (21.92)	73.64 (22.31)	74.84 (18.65)	0.769
Communication	81.07 (19.79)	81.58 (19.48)	76.72 (22.18)	0.303
Parental scores				
Generic HRQOL questionnaire				
Overall	78.47 (11.20)	78.16 (11.39)	80.88 (9.48)	0.187
Physical functioning	83.55 (11.79)	83.29 (11.97)	85.58 (10.30)	0.303
Emotional functioning	71.38 (17.07)	70.76 (17.23)	76.35 (15.14)	0.090
Social functioning	86.86 (14.22)	86.57 (14.32)	89.23 (13.39)	0.350
School functioning	69.14 (16.56)	69.06 (16.69)	69.81 (15.78)	0.822
Diabetes-specific HRQOL questionnaire				
Overall	74.13 (12.43)	73.70 (12.36)	77.62 (12.78)	0.149
Diabetes symptoms	71.70 (13.15)	70.98 (13.02)	77.57 (12.98)	**0.020**
Treatment barriers	74.48 (17.62)	74.47 (17.88)	74.54 (15.73)	0.985
Treatment adherence	79.62 (16.11)	79.27 (16.47)	82.46 (12.77)	0.253
Worry	72.12 (22.13)	71.72 (22.18)	75.44 (21.87)	0.428
Communication	76.25 (23.19)	76.33 (22.81)	75.56 (26.71)	0.891

Data are mean (SD).

other explanations for nonsignificant findings can be offered. First, gluten-free diet in patients with T1D+CD may have integrated into their diabetes treatment regime and therefore the awareness of dual diagnosis did not impair their HRQOL. Similar outcome was recently observed by Sud et al. [18], with a little difference, namely, that parents of children with dual diagnosis reported lower social dimension of generic HRQOL than parents of children with T1D.

Second, in children without diabetes, treated CD does not seem to decrease the quality of life total scores [42–45] although these results are not universal [46, 47]. The situation may be different in adults where a work on the dual diagnosis of T1D and CD showed a considerable negative impact on the diabetes-specific quality of life domains (diabetes related worry and social/vocational worry) as compared to adult patients with only T1D [48]. Conversely, we observed a mild, although not statistically significant, trend toward better HRQOL in T1D+CD group.

Finally, as 46% of patients with T1D+CD were using insulin pump compared to 36% of subjects with T1D only (Table 1), we adjusted the analyses for this factor: the type of treatment had been shown to affect the HRQOL in patients with T1D [49–51]. No net effect of the type of treatment was observed in our dataset, and the type of treatment did not modify the effects of the concomitant disorder on the HRQOL. Thus, further research of adequately sized longitudinal cohorts of pediatric patients with T1D+CD has to be conducted in order to clarify the herein observed subtle changes in HRQOL.

4.2. Parental Scores. Parental point of view did not show significant differences among studied groups with only few exceptions. The most apparent was the difference in generic HRQOL between T1D and T1D+AITD group, which well paralleled the children's questionnaires. Generally, parents assessed their child's HRQOL worse compared to their children themselves, which is in line with data from elsewhere [26].

4.3. The Strengths and Limitations of the Present Work. Our study, conducted at a single large pediatric centre, has the advantage of the homogeneity in language, in diabetes education, and in treatment targets and procedures. This may have rectified some of the difficulties which would inevitably arise with a bigger, yet more heterogeneous, population. Among the limitations, the most important one is the cross-sectional design which does not allow causal inference. Our findings warrant a follow-up in a longitudinal cohort to clarify the interesting contrast between the factual low disease severity of AITD and its problematic perception by the patients with T1D and parents. Secondly, the relatively low count of the individuals with a concomitant immunopathological disease limits the power to detect more subtle changes in the quality of life and does not allow meaningful investigation of the individual subdimensions of the scores.

5. Conclusions

Our study shows a decrease in quality of life measures in children and young persons with T1D, associated with the concomitant diagnosis of AITD. This association is partly independent of the known confounders as poor diabetes control, higher age, longer diabetes duration, and female sex and might draw attention to a specific group of patients with bigger need of (not only psychological) medical management by healthcare professionals caring for children and their families.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] A. M. Delamater, "Psychological care of children and adolescents with diabetes," *Pediatric Diabetes*, vol. 10, no. 12, pp. 175–184, 2009.

[2] J. H. Barlow and D. R. Ellard, "The psychosocial well-being of children with chronic disease, their parents and siblings: an overview of the research evidence base," *Child: Care, Health and Development*, vol. 32, no. 1, pp. 19–31, 2006.

[3] M. Marshall, B. Carter, K. Rose, and A. Brotherton, "Living with type 1 diabetes: perceptions of children and their parents," *Journal of Clinical Nursing*, vol. 18, no. 12, pp. 1703–1710, 2009.

[4] R. Whittemore, S. Jaser, A. Chao, M. Jang, and M. Grey, "Psychological experience of parents of children with type 1 diabetes: a systematic mixed-studies review," *Diabetes Educator*, vol. 38, no. 4, pp. 562–579, 2012.

[5] M. E. Hilliard, K. A. Mann, J. L. Peugh, and K. K. Hood, "How poorer quality of life in adolescence predicts subsequent type 1 diabetes management and control," *Patient Education and Counseling*, vol. 91, no. 1, pp. 120–125, 2013.

[6] H. Hoey, "Psychosocial factors are associated with metabolic control in adolescents: research from the Hvidoere Study Group on Childhood Diabetes," *Pediatric Diabetes*, vol. 10, supplement 13, pp. 9–14, 2009.

[7] Committee for Medicinal Products for Human Use, "Reflection Paper on the Regulatory Guidance for the Use of Health-Related Quality of Life (HRQL) Measures in the Evaluation of Medicinal Products," European Medicines Agency, London, UK, 2005, http://www.ispor.org/workpaper/emea-hrql-guidance.pdf.

[8] F. J. Cameron, "The impact of diabetes on health-related quality of life in children and adolescents," *Pediatric Diabetes*, vol. 4, no. 3, pp. 132–136, 2003.

[9] E. Davis, E. Waters, A. Mackinnon et al., "Paediatric quality of life instruments: a review of the impact of the conceptual framework on outcomes," *Developmental Medicine and Child Neurology*, vol. 48, no. 4, pp. 311–318, 2006.

[10] M. de Wit, H. A. Delemarre-van De Waal, F. Pouwer, R. J. B. J. Gemke, and F. J. Snoek, "Monitoring health related quality of life in adolescents with diabetes: a review of measures," *Archives of Disease in Childhood*, vol. 92, no. 5, pp. 434–439, 2007.

[11] F. Cerutti, G. Bruno, F. Chiarelli, R. Lorini, F. Meschi, and C. Sacchetti, "Younger age at onset and sex predict celiac disease in children and adolescents with type 1 diabetes: an Italian multicenter study," *Diabetes Care*, vol. 27, no. 6, pp. 1294–1298, 2004.

[12] E. Piątkowska and M. Szalecki, "Autoimmune thyroiditis in children and adolescents with type 1 diabetes," *Pediatric Endocrinology, Diabetes, and Metabolism*, vol. 17, no. 4, pp. 173–177, 2011.

[13] Z. Sumnik, P. Drevinek, M. Snajderova et al., "HLA-DQ polymorphisms modify the risk of thyroid autoimmunity in children with type 1 diabetes mellitus," *Journal of Pediatric Endocrinology and Metabolism*, vol. 16, no. 6, pp. 851–858, 2003.

[14] Z. Sumník, S. Kolousková, O. Cinek, R. Kotalová, J. Vavrinec, and M. Snajderová, "HLA-DQA1∗05-DQB1∗0201 positivity predisposes to coeliac disease in Czech diabetic children," *Acta Paediatrica*, vol. 89, no. 12, pp. 1426–1430, 2000.

[15] O. Kordonouri, A. M. Maguire, M. Knip et al., "Other complications and associated conditions," *Pediatric Diabetes*, vol. 8, no. 3, pp. 171–176, 2007.

[16] M. Abdul-Rasoul, F. Alotaibi, A. Abdulla, Z. Rahme, and F. Alshawaf, "Quality of life of children and adolescents with type 1 diabetes in Kuwait," *Medical Principles and Practice*, vol. 22, no. 4, pp. 379–384, 2013.

[17] M. E. Hilliard, J. M. Lawrence, A. C. Modi et al., "Identification of minimal clinically important difference scores of the PedsQL in children, adolescents, and young adults with type 1 and type 2 diabetes," *Diabetes Care*, vol. 36, no. 7, pp. 1891–1897, 2013.

[18] S. Sud, M. Marcon, E. Assor, D. Daneman, and F. H. Mahmud, "Quality of life in children with diabetes and celiac disease: minimal impact of the "double diagnosis"," *Pediatric Diabetes*, vol. 13, no. 2, pp. 163–169, 2012.

[19] T. Danne, B. Aschemeier, R. Perfetti et al., "SWEET—where are we heading with international type 1 diabetes registries?" *Pediatric Diabetes*, vol. 13, supplement 16, pp. 1–4, 2012.

[20] J. W. Varni, M. Seid, and C. A. Rode, "The PedsQL: measurement model for the pediatric quality of life inventory," *Medical Care*, vol. 37, no. 2, pp. 126–139, 1999.

[21] J. W. Varni, T. M. Burwinkle, J. R. Jacobs, M. Gottschalk, F. Kaufman, and K. L. Jones, "The PedsQL in type 1 and type 2 diabetes: reliability and validity of the pediatric quality of Life inventory generic core scales and type 1 diabetes module," *Diabetes Care*, vol. 26, no. 3, pp. 631–637, 2003.

[22] J. W. Varni, M. Seid, T. S. Knight, K. Uzark, and I. S. Szer, "The PedsQL 4.0 generic core scales: sensitivity, responsiveness, and impact on clinical decision-making," *Journal of Behavioral Medicine*, vol. 25, no. 2, pp. 175–193, 2002.

[23] M. Graue, T. Wentzel-Larsen, B. R. Hanestad, B. Båtsvik, and O. Søvik, "Measuring self-reported, health-related, quality of life in adolescents with type 1 diabetes using both generic and disease-specific instruments," *Acta Paediatrica*, vol. 92, no. 10, pp. 1190–1196, 2003.

[24] L. Hanberger, J. Ludvigsson, and S. Nordfeldt, "Health-related quality of life in intensively treated young patients with type 1 diabetes," *Pediatric Diabetes*, vol. 10, no. 6, pp. 374–381, 2009.

[25] J. E. Chaplin, R. Hanas, A. Lind, H. Tollig, N. Wramner, and B. Lindblad, "Assessment of childhood diabetes-related quality-of-life in West Sweden," *Acta Paediatrica*, vol. 98, no. 2, pp. 361–366, 2009.

[26] E. Kalyva, E. Malakonaki, C. Eiser, and D. Mamoulakis, "Health-related quality of life (HRQoL) of children with type 1 diabetes mellitus (T1DM): self and parental perceptions," *Pediatric Diabetes*, vol. 12, no. 1, pp. 34–40, 2011.

[27] L. Nardi, S. Zucchini, F. D'alberton et al., "Quality of life, psychological adjustment and metabolic control in youths with type 1 diabetes: a study with self- and parent-report questionnaires," *Pediatric Diabetes*, vol. 9, no. 5, pp. 496–503, 2008.

[28] M. J. Naughton, A. M. Ruggiero, J. M. Lawrence et al., "Health-related quality of life of children and adolescents with type 1 or type 2 diabetes mellitus: SEARCH for Diabetes in Youth Study," *Archives of Pediatrics and Adolescent Medicine*, vol. 162, no. 7, pp. 649–657, 2008.

[29] J. Wagner, A. Heapy, A. James, and G. Abbott, "Brief report: glycemic control, quality of life, and school experiences among students with diabetes," *Journal of Pediatric Psychology*, vol. 31, no. 8, pp. 764–769, 2006.

[30] M. Bauer, A. Heinz, and P. C. Whybrow, "Thyroid hormones, serotonin and mood: of synergy and significance in the adult brain," *Molecular Psychiatry*, vol. 7, no. 2, pp. 140–156, 2002.

[31] T. Leyhe and K. Müssig, "Cognitive and affective dysfunctions in autoimmune thyroiditis," *Brain, Behavior, and Immunity*, vol. 41, pp. 261–266, 2014.

[32] G. P. Bianchi, V. Zaccheroni, E. Solaroli et al., "Health-related quality of life in patients with thyroid disorders," *Quality of Life Research*, vol. 13, no. 1, pp. 45–54, 2004.

[33] R. Jaeschke, G. Guyatt, D. Cook, S. Harper, and H. C. Gerstein, "Spectrum of quality of life impairment in hypothyroidism," *Quality of Life Research*, vol. 3, no. 5, pp. 323–327, 1994.

[34] C. V. McMillan, C. Bradley, A. Woodcock, S. Razvi, and J. U. Weaver, "Design of new questionnaires to measure quality of life and treatment satisfaction in hypothyroidism," *Thyroid*, vol. 14, no. 11, pp. 916–925, 2004.

[35] P. Saravanan, W.-F. Chau, N. Roberts, K. Vedhara, R. Greenwood, and C. M. Dayan, "Psychological well-being in patients on "adequate" doses of l-thyroxine: results of a large, controlled community-based questionnaire study," *Clinical Endocrinology*, vol. 57, no. 5, pp. 577–585, 2002.

[36] E. M. Wekking, B. C. Appelhof, E. Fliers et al., "Cognitive functioning and well-being in euthyroid patients on thyroxine replacement therapy for primary hypothyroidism," *European Journal of Endocrinology*, vol. 153, no. 6, pp. 747–753, 2005.

[37] S. Razvi, L. E. Ingoe, C. V. McMillan, and J. U. Weaver, "Health status in patients with sub-clinical hypothyroidism," *European Journal of Endocrinology*, vol. 152, no. 5, pp. 713–717, 2005.

[38] C. Almeida, M. A. Brasil, A. J. Costa et al., "Subclinical hypothyroidism: psychiatric disorders and symptoms," *The Revista Brasileira de Psiquiatria*, vol. 29, no. 2, pp. 157–159, 2007.

[39] M. S. Gönen, G. Kisakol, A. S. Cilli et al., "Assessment of anxiety in subclinical thyroid disorders," *Endocrine Journal*, vol. 51, no. 3, pp. 311–315, 2004.

[40] R. Jorde, K. Waterloo, H. Storhaug, A. Nyrnes, J. Sundsfjord, and T. G. Jenssen, "Neuropsychological function and symptoms in subjects with subclinical hypothyroidism and the effect of thyroxine treatment," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 1, pp. 145–153, 2006.

[41] J. Ott, R. Promberger, F. Kober et al., "Hashimoto's thyroiditis affects symptom load and quality of life unrelated to hypothyroidism: a prospective case-control study in women undergoing thyroidectomy for benign goiter," *Thyroid*, vol. 21, no. 2, pp. 161–167, 2011.

[42] C. M. de Lorenzo, J. C. Xikota, M. C. Wayhs, S. M. Nassar, and M. M. D. S. Pires, "Evaluation of the quality of life of children with celiac disease and their parents: a case-control study," *Quality of Life Research*, vol. 21, no. 1, pp. 77–85, 2012.

[43] M. M. P. Kolsteren, H. M. Koopman, G. Schalekamp, and M. L. Mearin, "Health-related quality of life in children with celiac disease," *The Journal of Pediatrics*, vol. 138, no. 4, pp. 593–595, 2001.

[44] K. Nordyke, F. Norström, L. Lindholm, H. Stenlund, A. Rosén, and A. Ivarsson, "Health-related quality of life in adolescents with screening-detected celiac disease, before and one year after diagnosis and initiation of gluten-free diet, a prospective nested case-referent study," *BMC Public Health*, vol. 13, no. 1, article 142, 2013.

[45] E. J. van Koppen, J. J. Schweizer, C. G. D. S. Csizmadia et al., "Long-term health and quality-of-life consequences of mass screening for childhood celiac disease: a 10-year follow-up study," *Pediatrics*, vol. 123, no. 4, pp. e582–e588, 2009.

[46] T. Fidan, V. Ertekin, and K. Karabag, "Depression-anxiety levels and the quality of life among children and adolescents with coeliac disease," *Dusunen Adam*, vol. 26, no. 3, pp. 232–238, 2013.

[47] R. K. van Doorn, L. M. F. Winkler, K. H. Zwinderman, M. L. Mearin, and H. M. Koopman, "CDDUX: a disease-specific health-related quality-of-life questionnaire for children with celiac disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 47, no. 2, pp. 147–152, 2008.

[48] S. F. Bakker, F. Pouwer, M. E. Tushuizen, R. P. Hoogma, C. J. Mulder, and S. Simsek, "Compromised quality of life in patients with both Type 1 diabetes mellitus and coeliac disease," *Diabetic Medicine*, vol. 30, no. 7, pp. 835–839, 2013.

[49] A. Bayrakdar, S. Noureddine, L. Farhood, and M. P. Nasrallah, "Comparison of quality of life in a group of Lebanese type 1 diabetics on insulin pump and those on multiple daily injections," *Lebanese Medical Journal*, vol. 62, no. 1, pp. 22–26, 2014.

[50] E. Muller-Godeffroy, S. Treichel, and V. M. Wagner, "Investigation of quality of life and family burden issues during insulin pump therapy in children with Type 1 diabetes mellitus—a large-scale multicentre pilot study," *Diabetic Medicine*, vol. 26, no. 5, pp. 493–501, 2009.

[51] R. Nuboer, G. J. Borsboom, J. A. Zoethout, H. M. Koot, and J. Bruining, "Effects of insulin pump vs. injection treatment on quality of life and impact of disease in children with type 1 diabetes mellitus in a randomized, prospective comparison," *Pediatric Diabetes*, vol. 9, no. 4, pp. 291–296, 2008.

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Clinical Study

Effect of Omega-3 Fatty Acid on the Fatty Acid Content of the Erythrocyte Membrane and Proteinuria in Patients with Diabetic Nephropathy

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Diabetic nephropathy is the leading cause of end-stage renal disease and is associated with an increased risk of cardiovascular events. Dietary omega-3 fatty acid (FA) has cardioprotective effect and is associated with a slower deterioration of albumin excretion in patients with diabetic nephropathy. In this study, we evaluated the effect of omega-3 FA on proteinuria in diabetic nephropathy patients who are controlling blood pressure (BP) with angiotensin converting enzyme inhibitor (ACEi) or angiotensin II receptor blocker (ARB). In addition, we identified changes in erythrocyte membrane FA contents. A total of 19 patients who were treated with ACEi or ARB for at least 6 months were treated for 12 weeks with omega-3 FA (Omacor, 3 g/day) or a control treatment (olive oil, 3 g/day). Proteinuria levels were unchanged after 12 weeks compared with baseline values in both groups. The erythrocyte membrane contents of omega-3 FA and eicosapentaenoic acid (EPA) were significantly increased, and oleic acid, arachidonic acid : EPA ratio, and omega-6 : omega-3 FA ratio were significantly decreased after 12 weeks compared with the baseline values in the omega-3 FA group. Although omega-3 FA did not appear to alter proteinuria, erythrocyte membrane FA contents, including oleic acid, were altered by omega-3 FA supplementation.

1. Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD), and the incidence of diabetic nephropathy has been increasing rapidly [1]. Although diabetic nephropathy has been regarded as an irreversible and rapidly progressing disease, progression to kidney failure may be slowed by the use of angiotensin converting enzyme inhibitor (ACEi) or angiotensin II receptor blocker (ARB) [2]. Reducing proteinuria is very important and is the main target for the treatment of diabetic nephropathy [3]. However, there are few options for decreasing proteinuria in diabetic patients who are controlling blood pressure (BP) with ACEi or ARB. Dietary omega-3 fatty acid (FA) is associated with a slower deterioration of albumin excretion in patients with diabetic nephropathy and in model diabetic rats [4–6]. It is not clear whether omega-3 FA has an additional effect on decreasing proteinuria in patients treated with ACEi or ARB.

Diabetic nephropathy is a microvascular complication, and patients with diabetic nephropathy often suffer from accompanying macrovascular complications [7]. Patients with diabetic nephropathy show higher rates of cardiovascular events compared to the general population [8]. Omega-3 FA has been shown to be beneficial in the treatment of cardiovascular disease (CVD), and this cardioprotective effect may be explained by the anti-inflammatory, antioxidative, and antithrombotic abilities of omega-3 FA [9–11]. Several studies have reported that increased intake of omega-3 FA is linked to

decreased incidence of atherosclerotic CVD, arrhythmia, and sudden death, although recent meta-analysis did not proved these effects [12–14]. The cardioprotective effects of omega-3 FA are more prominent in diabetics than in nondiabetics [15]. Erythrocyte membrane oleic acid content is significantly higher in patients with acute coronary syndrome than in control subjects [16–18]. However, there are no reports regarding changes of the FA contents of the erythrocyte membrane, including oleic acid, caused by omega-3 FA supplementation in diabetic nephropathy patients with overt proteinuria.

In this study, we hypothesized that omega-3 FA supplementation may decrease proteinuria in patients with BP controlled by ACEi or ARB. In addition, we evaluated the status of erythrocyte membrane FA contents and the effect of omega-3 FA on erythrocyte membrane FA contents, including oleic acid, in diabetic nephropathy patients with overt proteinuria.

2. Materials and Methods

2.1. Study Design and Patients. We conducted a randomized, double-blind, placebo-controlled study of Dong-A University Nephrology outpatients between June 2009 and October 2010. Nineteen diabetic nephropathy patients, with a proteinuria level > 0.3 g/day and undergoing treatment with ACEi or ARB for at least 6 months, were included. Diabetic nephropathy was defined as diabetic renal disease with proteinuria, with or without elevation of serum creatinine (Cr) levels [19]. Patients matching any of the following criteria were excluded: history of active infection within 3 months; fish oil or omega-3 FA supplementation within 3 months; history of allergies to fish, omega-3 FA, and/or olive oil; history of hospital admission within 3 months; history of bleeding within 3 months; thrombocytopenia; current use of warfarin; an albumin level < 2.5 g/dL; and malignancy and/or liver cirrhosis. Enrolled patients were randomly selected for 12 weeks of treatment with either omega-3 FAs (Omacor, 3 g/day) or a placebo treatment (olive oil, 3 g/day). One gram of Omacor contained 460 mg of eicosapentaenoic acid (EPA) and 380 mg of docosahexaenoic acid (DHA). Randomization was performed using a random number table.

In addition, 32 healthy volunteers were included as normal controls [20]. Healthy volunteers were defined as those with no diabetes mellitus (DM), no urinary abnormalities, and a glomerular filtrate rate (GFR) of more than 60 mL/min/1.73 m^2. The mean eGFR of healthy volunteers was 87.3 ± 10.6 mL/min/1.73 m^2. This study was approved by the Dong-A University Hospital Institutional Review Board. Informed consent was obtained from all enrolled patients, and the study was conducted in accordance with the Declaration of Helsinki.

2.2. Survey of Food Consumption. Food consumption was surveyed to gather data on the average frequency and portion size at the start of the study and after 12 weeks using a semiquantitative food frequency questionnaire including 121 foods, which was used in the Korean Cancer Research Survey. Three-dimensional food models and full-scale photographs were used to estimate portion size. Nutrient intake was estimated by the Computer Aided Nutritional Analysis Program (Can-Pro 3.0, The Korean Nutrition Society), which includes 1,823 food items.

2.3. Laboratory Measurements. Fasting blood samples were obtained, subsequently processed, immediately refrigerated, and stored at −70°C until analysis. Routine laboratory tests were performed, including hemoglobin (Hb), glucose, glycated hemoglobin (HbA1c), blood urea nitrogen (BUN), Cr, GFR, albumin, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL). Body mass index (BMI) was calculated using weight and height measurements (weight (kg)/height (m^2)). Proteinuria was measured using random spot urine samples, and urine samples were collected over 24 hours. The levels of urinary neutrophil gelatinase-associated lipocalin (NGAL, R&D systems, Minneapolis, MN, USA) and prostaglandin E_2 (PGE_2, R&D systems) were measured by enzyme-linked immunoassay.

2.4. Gas Chromatography Procedure. Erythrocyte membrane FA contents were analyzed using gas chromatography (Shimadzu 2010AF, Shimadzu Scientific Instrument, Japan). Isolated erythrocytes were methylated by the addition of boron trifluoride (BF_3) methanol-benzene for 10 min at 100°C. FA methyl esters were analyzed by gas chromatography with a 100 m SP2560 capillary column (Supelco, Bellefonte, PA, USA). FA was identified by comparison with known standards (GLC-727; Nu-Chek Prep, Elysian, MN, USA). The omega-3 index is a measure of EPA and DHA in erythrocyte membranes. Erythrocyte membrane FA contents were expressed as weight percentages.

2.5. Statistical Analysis. Data were expressed as mean ± SD or frequency. Characteristics were analyzed using the Mann-Whitney U test or Wilcoxon exact rank sum test for nonparametric data and the Chi-squared test for categorical variables. A P value < 0.05 was considered to be statistically significant. All statistical calculations were performed with SPSS software (SPSS version 18.0, Chicago, IL).

3. Results

3.1. Baseline Characteristics. All 19 enrolled patients completed the trial. The mean age of patients was 60.4 ± 10.7 years, and 63.2% of the study population was male. The mean systolic and diastolic BP readings were 121.1 ± 14.5 mmHg and 72.1 ± 11.8 mmHg, respectively. There were no significant differences between the omega-3 FA group and the placebo group (Table 1).

3.2. Comparison of Erythrocyte Membrane FA Contents. We compared the erythrocyte membrane FA contents between healthy volunteers and DM patients (Table 2). When comparing DM patients to healthy controls, the erythrocyte membrane contents of omega-3 FA, DHA, and the omega-3 index were significantly lower, while the erythrocyte membrane contents of oleic acid and the omega-6 FA : omega-3 FA ratio were significantly higher.

Table 1: Clinical blood biochemical analyses of the subjects.

	Olive oil ($n = 8$)	Omega-3 FA ($n = 11$)	P value[1]
Age (years)	62.0 ± 8.6	59.2 ± 12.2	0.771
Male, n (%)	5 (62.5%)	7 (63.6%)	0.960
Systolic BP	121.3 ± 14.6	120.9 ± 15.1	0.966
Diastolic BP	70.0 ± 10.7	73.6 ± 12.9	0.764
BMI	25.0 ± 2.3	25.5 ± 2.8	0.396
Glucose (mg/dL)	158.5 ± 67.6	148.5 ± 64.8	0.680
HbA1c (%)	7.2 ± 1.2	7.2 ± 0.9	0.934
BUN (mg/dL)	19.3 ± 6.1	22.1 ± 6.5	0.320
Creatinine (mg/dL)	1.2 ± 0.2	1.3 ± 0.2	0.116
Estimated GFR	62.7 ± 7.4	55.0 ± 8.0	0.057
Total cholesterol (mg/dL)	162.5 ± 46.8	162.5 ± 36.4	0.967
Triglyceride (mg/dL)	127.0 ± 85.3	142.9 ± 66.9	0.283
HDL (mg/dL)	47.6 ± 13.7	44.7 ± 7.9	0.836
LDL (mg/dL)	88.1 ± 43.4	88.9 ± 33.4	0.934
CRP (mg/dL)	0.15 ± 0.13	0.12 ± 0.13	0.772
Spot urine PCR (g/g)	0.65 ± 0.46	0.79 ± 0.77	0.964
24 hr urine protein (g/g)	0.46 ± 0.44	0.60 ± 0.63	1.000
PGE_2 (pg/mL)	1494.0 ± 489.8	1308.4 ± 526.5	0.643
NGAL (ng/mL)	30.0 ± 31.2	33.3 ± 60.9	1.000

Data are expressed as means ± SD.
[1] P value for nonparametric Mann-Whitney U test comparing baseline data between olive oil group and omega-3 FA group. The difference in frequency was tested using Pearson χ^2.
BP: blood pressure; BMI: body mass index; HbA1c: glycated hemoglobin; BUN: blood urea nitrogen; GFR: glomerular filtration rate; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; CRP: C-reactive protein; PCR: protein to creatinine ratio; PGE_2: prostaglandin E_2; NGAL: neutrophil gelatinase-associated lipocalin.

Table 2: Comparison of erythrocyte membrane fatty acids content.

	Control ($n = 32$)	DM ($n = 19$)	P value[1]
Saturated	39.0 ± 6.1	42.7 ± 7.3	0.081
Myristic	0.57 ± 0.29	0.72 ± 0.32	0.065
Palmitic	24.4 ± 4.0	26.6 ± 4.8	0.159
Stearic	13.9 ± 2.9	15.1 ± 3.5	0.097
Lignoceric	0.23 ± 0.12	0.27 ± 0.17	0.614
Monounsaturated	16.2 ± 2.6	17.6 ± 2.9	0.085
Palmitoleic	1.8 ± 1.7	1.2 ± 0.8	0.437
Trans-oleic	1.7 ± 0.7	1.2 ± 0.9	0.036*
Oleic	13.9 ± 2.4	15.9 ± 3.0	0.020*
Polyunsaturated	42.0 ± 7.6	37.4 ± 8.3	0.038*
Omega-6	28.9 ± 5.7	27.2 ± 5.7	0.166
Linoleic	13.5 ± 3.0	14.4 ± 4.4	0.785
AA	11.7 ± 3.1	9.4 ± 3.0	0.014*
Omega-3	13.1 ± 3.4	10.2 ± 3.9	0.011*
Alpha-linolenic	0.35 ± 0.19	0.49 ± 0.21	0.026*
EPA	2.0 ± 0.9	1.9 ± 1.1	0.556
DHA	8.3 ± 2.3	6.0 ± 2.2	0.002*
Omega-3 index	10.3 ± 2.9	7.9 ± 3.1	0.008*
AA/EPA	7.3 ± 4.4	7.1 ± 6.0	0.644
Omega-6/Omega-3	2.3 ± 0.7	3.0 ± 1.1	0.025*

Data are expressed as means ± SD.
[1] P value for nonparametric Mann-Whitney U test comparing baseline data between control group and DM group.
* P value <0.05 (mean values are significantly different from control group).
AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; DM: diabetes mellitus.

3.3. Dietary Consumption Data. There were no significant differences in dietary consumption between the omega-3 FA group and the placebo group at baseline. In addition, there were no significant changes in dietary consumption between the omega-3 FA group and the placebo group after 12 weeks compared with baseline values (Table 3).

3.4. Changes in Biochemical Data. Glucose, total cholesterol, and LDL levels were significantly decreased in the placebo group after 12 weeks compared with baseline values ($P = 0.025$, $P = 0.021$, and $P = 0.017$, resp.). Analysis of spot urine protein to creatinine ratio, 24 h urine protein, and Cr revealed no alterations after 12 weeks compared with baseline values in both groups. There was a tendency for increased GFR in the omega-3 FA group after 12 weeks compared with baseline values, but this was not statistically significant. In the placebo group, the urinary levels of PGE_2 and NGAL increased and decreased, respectively, after 12 weeks compared with baseline values, but these changes were not statistically significant. In the omega-3 FA group, urinary levels of PGE_2 and NGAL were increased and decreased, respectively, after 12 weeks compared with baseline values, but these changes were also not statistically significant (Table 4).

3.5. Changes in Erythrocyte Membrane FA Content. The erythrocyte membrane FA contents at baseline showed no significant difference between the 2 groups (Table 5). In the omega-3 FA group, the erythrocyte membrane contents of omega-3 FA and EPA were significantly increased after 12 weeks compared with baseline values ($P = 0.025$, $P = 0.050$, resp.). The erythrocyte membrane contents of oleic acid, arachidonic acid (AA) : EPA ratio, and omega-6 FA : omega-3 FA ratio were significantly decreased after 12 weeks compared with the baseline values in the omega-3 FA group ($P = 0.036$, $P = 0.012$, and $P = 0.012$, resp.). In the placebo group, the erythrocyte membrane contents of palmitoleic acid and AA were significantly increased after 12 weeks compared with baseline values ($P = 0.046$, $P = 0.046$, resp.). The erythrocyte membrane content of oleic acid was decreased in the placebo group after 12 weeks compared with baseline values, but this was not statistically significant.

Table 3: Dietary consumption of foods and nutrients.

	Olive oil ($n = 8$)		Omega-3 FA ($n = 11$)	
	Baseline	12 weeks	Baseline	12 weeks
Kcal (kcal)	1801.3 ± 420.8	1717.2 ± 428.9	1658.4 ± 356.1	1613.4 ± 397.0
Animal protein (g)	29.1 ± 15.9	29.3 ± 17.8	25.2 ± 11.9	22.2 ± 13.3
Vegetable protein (g)	42.5 ± 8.5	39.4 ± 14.6	37.7 ± 12.9	37.1 ± 12.1
Animal lipid (g)	18.2 ± 9.1	21.1 ± 14.8	16.4 ± 6.7	16.7 ± 10.9
Vegetable lipid (g)	16.8 ± 6.4	16.0 ± 9.3	18.8 ± 8.8	17.4 ± 8.1
Carbohydrate (g)	302.8 ± 70.4	278.8 ± 67.1	275.8 ± 51.4	267.3 ± 66.3
Fiber (g)	25.8 ± 8.0	22.7 ± 10.8	24.8 ± 12.1	20.7 ± 8.7
Retinol (μg)	68.5 ± 37.6	89.8 ± 77.3	57.3 ± 35.5	66.5 ± 47.1
Niacin (mg)	16.4 ± 4.1	15.6 ± 5.4	14.9 ± 6.3	13.2 ± 4.3
Vitamin E (mg)	12.3 ± 4.9	11.7 ± 6.0	12.6 ± 6.6	10.5 ± 4.2
Cholesterol (mg)	210.6 ± 108.0	218.8 ± 128.9	192.8 ± 111.6	184.9 ± 111.4

Data are expressed as means ± SD.
The nonparametric Wilcoxon exact rank sum test was used to compare baseline data with 12 weeks data.

Table 4: Changes in biochemical data.

	Olive oil ($n = 8$)		Omega-3 FA ($n = 11$)	
	Baseline	12 weeks	Baseline	12 weeks
Systolic BP (mmHg)	121.3 ± 14.6	118.8 ± 14.6	120.9 ± 15.1	123.0 ± 15.7
Diastolic BP (mmHg)	70.0 ± 10.7	70.0 ± 10.7	73.6 ± 12.9	69.0 ± 8.8
BMI (kg/m^2)	25.0 ± 2.3	25.2 ± 3.1	25.5 ± 2.8	25.4 ± 2.7
Glucose (mg/dL)	158.5 ± 67.6	121.4 ± 50.7*	148.5 ± 64.8	128.9 ± 41.8
HbA1c (%)	7.2 ± 1.2	7.0 ± 1.1	7.2 ± 0.9	7.3 ± 1.0
BUN (mg/dL)	19.3 ± 6.1	21.8 ± 6.7	22.1 ± 6.5	22.0 ± 4.8
Creatinine (mg/dL)	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.3 ± 0.3
Estimated GFR (mL/min/1.73 m^2)	62.7 ± 7.4	63.4 ± 8.4	55.0 ± 8.0	59.2 ± 10.8
Total cholesterol (mg/dL)	162.5 ± 46.8	151.3 ± 42.3*	162.5 ± 36.4	161.8 ± 32.7
Triglyceride (mg/dL)	127.0 ± 85.3	113.8 ± 61.3	142.9 ± 66.9	122.9 ± 52.4
HDL (mg/dL)	47.6 ± 13.7	46.3 ± 12.3	44.7 ± 7.9	45.3 ± 11.6
LDL (mg/dL)	88.1 ± 43.4	79.1 ± 39.8*	88.9 ± 33.4	88.7 ± 31.3
CRP (mg/dL)	0.15 ± 0.13	0.12 ± 0.10	0.12 ± 0.13	0.18 ± 0.20
Spot urine PCR (g/g)	0.65 ± 0.46	0.66 ± 0.46	0.79 ± 0.77	0.73 ± 0.67
24 hr urine protein (g/g)	0.46 ± 0.44	0.47 ± 0.41	0.60 ± 0.63	0.69 ± 0.65
PGE_2 (pg/mL)	1494.0 ± 489.8	1355.0 ± 440.1	1308.4 ± 526.5	1548.4 ± 541.8
NGAL (ng/mL)	30.0 ± 31.2	38.5 ± 35.9	33.3 ± 60.9	24.6 ± 18.9

Data are expressed as means ± SD.
The nonparametric Wilcoxon exact rank sum test was used to compare baseline data with 12 weeks data.
* P value <0.05 (mean values are significantly different from baseline).
BP: blood pressure; BMI: body mass index; HbA1c: glycated hemoglobin; BUN: blood urea nitrogen; GFR: glomerular filtration rate; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; CRP: C-reactive protein; PCR: protein to creatinine ratio; PGE_2: prostaglandin E_2; NGAL: neutrophil gelatinase-associated lipocalin.

4. Discussion

In this study, we found that omega-3 FA supplementation decreased the erythrocyte membrane oleic acid content in patients with diabetic nephropathy and overt proteinuria. This effect may be induced by elevated erythrocyte levels of omega-3 FA and EPA. Several studies have identified the association between omega-3 FA and diabetes risk. In particular, a recent study has reported that the erythrocyte membrane contents of omega-3 FA were correlated with the risk of type 2 diabetes in a Korean population [21]. They identified that the erythrocyte levels of oleic acid and the omega-6 : omega-3 ratio were positively associated with diabetes risk and that erythrocyte levels of omega-3 FA were negatively associated with diabetes risk. Similar results between the healthy control group and the diabetic nephropathy group were found in the present study. Our previous studies showed that omega-3 FA supplementation decreased erythrocyte membrane oleic acid content in patients treated with hemodialysis and peritoneal dialysis [22, 23]. Therefore, omega-3 FA definitely

Table 5: Changes in erythrocyte membrane fatty acids content.

	Olive oil ($n = 8$)		Omega-3 FA ($n = 11$)	
	Baseline	12 weeks	Baseline	12 weeks
Saturated	42.5 ± 6.1	37.7 ± 5.3	42.8 ± 8.3	39.8 ± 6.9
Myristic	0.62 ± 0.28	0.44 ± 0.10	0.79 ± 0.34	0.84 ± 0.48
Palmitic	25.2 ± 3.9	23.0 ± 2.9	27.5 ± 5.4	24.6 ± 2.9
Stearic	16.4 ± 3.7	14.0 ± 3.1	14.3 ± 3.1	14.1 ± 5.0
Lignoceric	0.27 ± 0.13	0.25 ± 0.14	0.27 ± 0.20	0.27 ± 0.25
Monounsaturated	17.2 ± 3.6	15.1 ± 2.3	17.8 ± 2.5	15.7 ± 1.4
Palmitoleic	1.2 ± 0.9	1.9 ± 1.7*	1.2 ± 0.7	1.8 ± 1.6
Transoleic	1.7 ± 1.1	2.0 ± 0.7	0.92 ± 0.61	1.9 ± 0.9
Oleic	15.5 ± 3.7	12.7 ± 1.3	16.2 ± 2.7	13.1 ± 2.2*
Polyunsaturated	37.4 ± 7.5	43.9 ± 6.6	37.5 ± 9.2	41.5 ± 7.4
Omega-6	26.7 ± 4.6	29.4 ± 5.1	27.6 ± 6.6	23.3 ± 4.7
Linoleic	13.9 ± 4.7	13.2 ± 2.7	14.7 ± 4.4	11.8 ± 3.8
AA	8.9 ± 3.2	11.7 ± 2.9*	9.8 ± 2.9	7.9 ± 2.9
Omega-3	10.7 ± 3.1	14.5 ± 2.8	9.8 ± 4.4	18.2 ± 8.4*
Alpha-linolenic	0.49 ± 0.24	0.45 ± 0.27	0.49 ± 0.21	0.49 ± 0.23
EPA	1.8 ± 0.6	2.9 ± 1.2	1.9 ± 1.4	4.2 ± 1.5*
DHA	6.1 ± 2.0	8.1 ± 1.2	6.0 ± 2.5	7.2 ± 3.2
Omega-3 index	7.9 ± 2.3	11.0 ± 2.1	7.9 ± 3.7	11.3 ± 4.6
AA/EPA	5.4 ± 3.0	4.7 ± 2.2	8.3 ± 7.4	1.9 ± 0.4*
Omega-6/Omega-3	2.7 ± 0.7	2.1 ± 0.5*	3.2 ± 1.3	1.5 ± 0.7*

Data are expressed as means ± SD.
The nonparametric Wilcoxon exact rank sum test was used to compare baseline data with 12 weeks data.
* P value <0.05 (mean values are significantly different from baseline).
AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

decreases erythrocyte membrane oleic acid contents in CKD patients regardless of dialysis treatment. The current study is the first to demonstrate the effect of omega-3 FA on the erythrocyte membrane oleic acid content in patients with diabetic nephropathy. It is of note that patients with a high risk of CVD, such as patients with diabetic nephropathy, and those undergoing dialysis have elevated erythrocyte membrane oleic acid contents. We suggest that the possible cardioprotective effect of omega-3 FA supplementation may be related to these changes in erythrocyte membrane FA contents.

Interestingly, we found that the erythrocyte membrane contents of omega-3 FA, EPA, and DHA, in addition to the omega-3 index, tended to increase, and oleic acid tended to decrease, in the control group supplemented with olive oil after 12 weeks compared with baseline values. This result suggests that enough supplementation with olive oil also has a cardioprotective effect in diabetic nephropathy patients. Previous studies have reported that olive oil may affect lipid profiles [24, 25]. In this study, total cholesterol, triglyceride, and LDL levels were decreased in the olive oil group. Thus, the actions of olive oil on lipid metabolism may have potential cardioprotective effects. In addition, omega-3 FA levels were increased after not only omega-3 FA supplementation but also olive oil supplementation. One study has reported that omega-3 FAs could be elevated in olive oil-fed rats [26]. Therefore, olive oil may not have been a good choice for the control treatment in the present study. Another notable result in this study is that although AA levels tended to be lower after omega-3 FA supplementation, there was a significant rise in AA levels after olive oil supplementation. This appears to be a major difference between olive oil and omega-3 FA supplementation. In addition, palmitoleic acid levels were increased by olive oil supplementation. The reason for this may be the increased activity of desaturases, which constitute the rate-limiting step in the process of de novo FA synthesis [27], but this might not be the unique factor affecting this particular finding. Further studies are needed to identify the dose-dependent effects of olive oil on changes in erythrocyte membrane FA.

Proteinuria is a strong predictor of progressive renal dysfunction [28]. It is well recognized that ACEi and ARB are the first-line treatments in diabetic nephropathy with proteinuria [2]. These drugs decrease intraglomerular pressure, systemic arterial pressure, and urinary protein excretion and delay the deterioration in renal function [29–31]. Despite the renoprotective effects of ACEi and ARB, it is difficult to fully halt the progression of proteinuria or reverse existing disease [32, 33]. Therefore, a number of efforts to reduce proteinuria have been attempted. Recently, a growing body of clinical data has demonstrated the antiproteinuric effects of omega-3 FA. Studies in animals have shown that omega-3 FA can be beneficial in retarding the progression of renal failure [34, 35]. Epidemiological studies suggest that omega-3 FA can slow the progression of renal dysfunction [36, 37] and prevent the decline in creatinine clearance in healthy elderly people [38].

In addition, omega-3 FA could slow the progression of proteinuria in patients with type 2 diabetes, but the study in question included patients who were not controlling their BP [4]. In the present study, proteinuria was not significantly decreased in the omega-3 FA supplementation group after 12 weeks compared with baseline values. One possible explanation is that the included patients had already been administrated the maximum dose of ACEi or ARB. This result may suggest that it is difficult to obtain additional antiproteinuric benefits from omega-3 FA. To see the effect of omega-3 FA on proteinuria, further studies separating patients with heavier proteinuria or adding some patients with heavier proteinuria are needed. Alternatively additional studies could estimate the effect of omega-3 FA by using them without ACEi or ARB.

High blood glucose levels lead to diabetic complications, including diabetic nephropathy. Therefore, studies suggest that good glucose control is the most important factor in managing diabetes and its related end-organ damage. The glucose levels were decreased in the olive oil group in this study. These effects may be induced by the actions of olive oil on glucose metabolism and insulin sensitivity [39, 40]. The effect of omega-3 FA on blood glucose control is still uncertain, but it is currently believed that omega-3 FA do not have any adverse effect on this [41, 42]. In this study, glucose levels seemed to be lowered by omega-3 FA supplementation. These results were not related to changes in diet or diabetes medication. Despite decreased glucose levels, HbA1c levels were not significantly altered after omega-3 FA supplementation. This lack of change may be related to the short study period, because HbA1c levels reflect plasma glucose levels over the preceding weeks and months. Further long-term studies are needed to identify the alterations in insulin resistance or glucose levels caused by omega-3 FA supplementation.

NAGL is a 25-kDa lipocalin protein and is produced in epithelial cells and neutrophils in most tissues. It is commonly recognized as a marker of renal tubular damage. Previous studies have reported that NGAL levels may reflect renal impairment and be associated with proteinuria in diabetic patients [43, 44]. Therefore, we hypothesized that omega-3 FA supplementation may affect NGAL levels in patients with diabetic nephropathy. However, our data did not show any statistically significant changes after 12 weeks, despite some apparent decreases in NGAL levels in the omega-3 FA group. One possible explanation is that NGAL levels are highly associated with proteinuria levels [43]. Because our study did not control for the level of proteinuria in both groups, the patients had a wide range of proteinuria extents.

AA is metabolized by 3 major pathways, namely, cyclooxygenase, lipoxygenase, and cytochrome P450, into bioactive eicosanoids. The major Cox-derived eicosanoids are thromboxane and prostaglandins, such as PGE_2, and have roles in increasing renal blood flow and inhibiting GFR decline in kidney disease [45, 46]. In our study, there were no statistically significant differences in Cr levels and GFR between the 2 study groups, but GFR tended to be higher after omega-3 FA supplementation. PGE_2 plays an important role as a regulator of pancreatic β-cell dysfunction and destruction [47, 48]. Omega-3 FA may protect against β-cell dysfunction and destruction and improve insulin sensitivity [49–51]. However, our data did not show any statistically significant changes in PGE_2 levels after omega-3 FA supplementation. Further large-scale studies are needed.

This study had a number of limitations. First, the poor efficacy of omega-3 FA in preserving renal function and decreasing proteinuria may be due to the smaller number of enrolled patients and the shorter study period, compared to those of other studies. Second, although ethnicity can affect the erythrocyte membrane contents of omega-3 FA, data were only obtained from Korean patients [52, 53].

In summary, although there appears to be no beneficial effect of omega-3 FA on proteinuria, the FA contents of the erythrocyte membrane were significantly altered by omega-3 FA supplementation over 12 weeks in patients with diabetic nephropathy and blood pressure controlled by ACEi or ARB. We conclude that the role of omega-3 FA in altering the FA (including oleic acid) contents of the erythrocyte membrane is not completely clear. Therefore, larger controlled studies evaluating cardiovascular end-points are essential.

5. Conclusions

Our results indicate that omega-3 FAs may affect the modification of erythrocyte membrane FA contents in patients with diabetic nephropathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Su Mi Lee and Seuk Hee Chung contributed equally to this work.

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References

[1] I. H. de Boer, T. C. Rue, Y. N. Hall, P. J. Heagerty, N. S. Weiss, and J. Himmelfarb, "Temporal trends in the prevalence of diabetic kidney disease in the United States," *Journal of the American Medical Association*, vol. 305, no. 24, pp. 2532–2539, 2011.

[2] E. Imai, S. Ito, M. Haneda, J. C. N. Chan, and H. Makino, "Olmesartan reducing incidence of endstage renal disease in diabetic nephropathy trial (orient): Rationale and study design," *Hypertension Research*, vol. 29, no. 9, pp. 703–709, 2006.

[3] D. de Zeeuw, G. Remuzzi, H.-H. Parving et al., "Proteinuria, a target for renoprotection in patients with type 2 diabetic nephropathy: lessons from RENAAL," *Kidney International*, vol. 65, no. 6, pp. 2309–2320, 2004.

[4] H. Shimizu, K.-I. Ohtani, Y. Tanaka, N. Sato, M. Mori, and Y. Shimomura, "Long-term effect of eicosapentaenoic acid ethyl

(EPA-E) on albuminuria of non-insulin dependent diabetic patients," *Diabetes Research and Clinical Practice*, vol. 28, no. 1, pp. 35–40, 1995.

[5] A. V. Möllsten, G. G. Dahlquist, E.-L. Stattin, and S. Rudberg, "Higher intakes of fish protein are related to a lower risk of microalbuminuria in young swedish type 1 diabetic patients," *Diabetes Care*, vol. 24, no. 5, pp. 805–810, 2001.

[6] J. H. Garman, S. Mulroney, M. Manigrasso, E. Flynn, and C. Marie, "Omega-3 fatty acid rich diet prevents diabetic renal disease," *American Journal of Physiology—Renal Physiology*, vol. 296, no. 2, pp. F306–F316, 2009.

[7] A. J. Krentz, G. Clough, and C. D. Byrne, "Interactions between microvascular and macrovascular disease in diabetes: pathophysiology and therapeutic implications," *Diabetes, Obesity and Metabolism*, vol. 9, no. 6, pp. 781–791, 2007.

[8] H. C. Gerstein, J. F. E. Mann, Q. Yi et al., "Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals," *The Journal of the American Medical Association*, vol. 286, no. 4, pp. 421–426, 2001.

[9] M. Svensson, E. B. Schmidt, K. A. Jørgensen, and J. H. Christensen, "N-3 fatty acids as secondary prevention against cardiovascular events in patients who undergo chronic hemodialysis: a randomized, placebo-controlled intervention trial," *Clinical journal of the American Society of Nephrology*, vol. 1, no. 4, pp. 780–786, 2006.

[10] F. B. Hu, L. Bronner, W. C. Willett et al., "Fish and omega-3 fatty acid intake and risk of coronary heart disease in women," *The Journal of the American Medical Association*, vol. 287, no. 14, pp. 1815–1821, 2002.

[11] S. M. Lee and W. S. An, "Cardioprotective effects of ω-3 PUFAs in chronic kidney disease," *BioMed Research International*, vol. 2013, Article ID 712949, 8 pages, 2013.

[12] M. L. Burr, A. M. Fehily, J. F. Gilbert et al., "Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and reinfarction trial (DART)," *The Lancet*, vol. 2, no. 8666, pp. 757–761, 1989.

[13] R. Marchioli, "Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin e after myocardial infarction: results of the gissi-prevenzione trial. Gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico," *The Lancet*, vol. 354, no. 9177, pp. 447–455, 1999.

[14] E. C. Rizos, E. E. Ntzani, and M. S. Elisaf, "Omega-3 fatty acid supplementation and cardiovascular disease events—*q*eply," *JAMA - Journal of the American Medical Association*, vol. 309, no. 1, p. 29, 2013.

[15] J. H. Christensen, H. Aarup Skou, T. Madsen, I. Tørring, and E. Berg Schmidt, "Heart rate variability and n-3 polyunsaturated fatty acids in patients with diabetes mellitus," *Journal of Internal Medicine*, vol. 249, no. 6, pp. 545–552, 2001.

[16] E. N. Siguel and R. H. Lerman, "Altered fatty acid metabolism in patients with angiographically documented coronary artery disease," *Metabolism: Clinical and Experimental*, vol. 43, no. 8, pp. 982–993, 1994.

[17] F. Paganelli, J. M. Maixent, M. J. Duran, R. Parhizgar, G. Pieroni, and S. Sennoune, "Altered erythrocyte n-3 fatty acids in Mediterranean patients with coronary artery disease," *International Journal of Cardiology*, vol. 78, no. 1, pp. 27–32, 2001.

[18] R. C. Block, W. S. Harris, K. J. Reid, and J. A. Spertus, "Omega-6 and trans fatty acids in blood cell membranes: a risk factor for acute coronary syndromes?" *American Heart Journal*, vol. 156, no. 6, pp. 1117–1123, 2008.

[19] J. L. Gross, M. J. de Azevedo, S. P. Silveiro, L. H. Canani, M. L. Caramori, and T. Zelmanovitz, "Diabetic nephropathy: diagnosis, prevention, and treatment," *Diabetes Care*, vol. 28, no. 1, pp. 164–176, 2005.

[20] J. S. Oh, S. M. Kim, Y. H. Sin et al., "Comparison of erythrocyte membrane fatty acid contents in renal transplant recipients and dialysis patients," *Transplantation Proceedings*, vol. 44, no. 10, pp. 2932–2935, 2012.

[21] S. Jo, W.-S. An, and Y. Park, "Erythrocyte n-3 polyunsaturated fatty acids and the risk of type 2 diabetes in koreans: a case-control study," *Annals of Nutrition and Metabolism*, vol. 63, no. 4, pp. 283–290, 2014.

[22] W. S. An, S. M. Lee, Y. K. Son et al., "Effect of omega-3 fatty acids on the modification of erythrocyte membrane fatty acid content including oleic acid in peritoneal dialysis patients," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 86, no. 1-2, pp. 29–34, 2012.

[23] W. S. An, S. M. Lee, Y. K. Son et al., "Omega-3 fatty acid supplementation increases 1,25-dihydroxyvitamin D and fetuin-A levels in dialysis patients," *Nutrition Research*, vol. 32, no. 7, pp. 495–502, 2012.

[24] S. M. Namayandeh, F. Kaseb, and S. Lesan, "Olive and sesame oil effect on lipid profile in hypercholesterolemic patients, which better?" *International Journal of Preventive Medicine*, vol. 4, no. 9, pp. 1059–1062, 2013.

[25] M. Trevisan, V. Krogh, J. Freudenheim et al., "Consumption of olive oil, butter, and vegetable oils and coronary heart disease risk factors," *The Journal of the American Medical Association*, vol. 263, no. 5, pp. 688–692, 1990.

[26] M. D. Navarro, J. L. Periago, M. L. Pita, and P. Hortelano, "The n-3 polyunsaturated fatty acid levels in rat tissue lipids increase in response to dietary olive oil relative to sunflower oil," *Lipids*, vol. 29, no. 12, pp. 845–848, 1994.

[27] C. D. Green, C. G. Ozguden-Akkoc, Y. Wang, D. B. Jump, and L. K. Olson, "Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species," *Journal of Lipid Research*, vol. 51, no. 7, pp. 1871–1877, 2010.

[28] B. A. Perkins, L. H. Ficociello, B. E. Ostrander et al., "Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes," *Journal of the American Society of Nephrology*, vol. 18, no. 4, pp. 1353–1361, 2007.

[29] E. J. Lewis, L. G. Hunsicker, R. P. Bain, and R. D. Rohde, "The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy," *The New England Journal of Medicine*, vol. 329, no. 20, pp. 1456–1462, 1993.

[30] E. J. Lewis, L. G. Hunsicker, W. R. Clarke et al., "Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes," *The New England Journal of Medicine*, vol. 345, no. 12, pp. 851–860, 2001.

[31] N. Nakao, A. Yoshimura, H. Morita, M. Takada, T. Kayano, and T. Ideura, "Combination treatment of angiotensin-II receptor blocker and angiotensin-converting-enzyme inhibitor in non-diabetic renal disease (COOPERATE): a randomised controlled trial," *The Lancet*, vol. 361, no. 9352, pp. 117–124, 2003.

[32] M. Cortinovis, D. Cattaneo, N. Perico, and G. Remuzzi, "Investigational drugs for diabetic nephropathy," *Expert Opinion on Investigational Drugs*, vol. 17, no. 10, pp. 1487–1500, 2008.

[33] P. Fioretto, M. L. Caramori, and M. Mauer, "The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi Lecture 2007," *Diabetologia*, vol. 51, no. 8, pp. 1347–1355, 2008.

[34] U. O. Barcelli, J. Miyata, Y. Ito et al., "Beneficial effects of polyunsaturated fatty acids in partially nephrectomized rats," *Prostaglandins*, vol. 32, no. 2, pp. 211–219, 1986.

[35] W. S. An, H. J. Kim, K.-H. Cho, and N. D. Vaziri, "Omega-3 fatty acid supplementation attenuates oxidative stress, inflammation, and tubulointerstitial fibrosis in the remnant kidney," *American Journal of Physiology—Renal Physiology*, vol. 297, no. 4, pp. F895–F903, 2009.

[36] F. Lauretani, M. Maggio, F. Pizzarelli et al., "Omega-3 and renal function in older adults," *Current Pharmaceutical Design*, vol. 15, no. 36, pp. 4149–4156, 2009.

[37] J. V. Donadio Jr., T. S. Larson, E. J. Bergstralh, and J. P. Grande, "A randomized trial of high-dose compared with low-dose omega-3 fatty acids in severe IgA nephropathy," *Journal of the American Society of Nephrology*, vol. 12, no. 4, pp. 791–799, 2001.

[38] F. Lauretani, R. D. Semba, S. Bandinelli et al., "Plasma polyunsaturated fatty acids and the decline of renal function," *Clinical Chemistry*, vol. 54, no. 3, pp. 475–481, 2008.

[39] K. Esposito, R. Marfella, M. Ciotola et al., "Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial," *The Journal of the American Medical Association*, vol. 292, no. 12, pp. 1440–1446, 2004.

[40] F. Perez-Jimenez, G. A. de Cienfuegos, L. Badimon et al., "International conference on the healthy effect of virgin olive oil," *European Journal of Clinical Investigation*, vol. 35, no. 7, pp. 421–424, 2005.

[41] C. E. Friedberg, M. J. F. M. Janssen, R. J. Heine, and D. E. Grobbee, "Fish oil and glycemic control in diabetes. A meta-analysis," *Diabetes Care*, vol. 21, no. 4, pp. 494–500, 1998.

[42] V. M. Montori, A. Farmer, P. C. Wollan, and S. F. Dinneen, "Fish oil supplementation in type 2 diabetes: a quantitative systematic review," *Diabetes Care*, vol. 23, no. 9, pp. 1407–1415, 2000.

[43] S. E. Nielsen, K. J. Schjoedt, A. S. Astrup et al., "Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM1) in patients with diabetic nephropathy: a cross-sectional study and the effects of lisinopril," *Diabetic Medicine*, vol. 27, no. 10, pp. 1144–1150, 2010.

[44] D. Bolignano, A. Lacquaniti, G. Coppolino et al., "Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 4, no. 2, pp. 337–344, 2009.

[45] R. C. Harris, "COX-2 and the kidney," *Journal of Cardiovascular Pharmacology*, vol. 47, supplement 1, pp. S37–S42, 2006.

[46] N. O. S. Câmara, J. O. Martins, R. G. Landgraf, and S. Jancar, "Emerging roles for eicosanoids in renal diseases," *Current Opinion in Nephrology and Hypertension*, vol. 18, no. 1, pp. 21–27, 2009.

[47] M. L. McDaniel, G. Kwon, J. R. Hill, C. A. Marshall, and J. A. Corbett, "Cytokines and nitric oxide in islet inflammation and diabetes," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 211, no. 1, pp. 24–32, 1996.

[48] S. J. Persaud, D. Muller, V. D. Belin et al., "The role of arachidonic acid and its metabolites in insulin secretion from human islets of langerhans," *Diabetes*, vol. 56, no. 1, pp. 197–203, 2007.

[49] D. Wei, J. Li, M. Shen et al., "Cellular production of n-3 PUFAs and reduction of n-6 -to-n-3 ratios in the pancreatic β-cells and islets enhance insulin secretion and confer protection against cytokine-induced cell death," *Diabetes*, vol. 59, no. 2, pp. 471–478, 2010.

[50] J. Bellenger, S. Bellenger, A. Bataille et al., "High pancreatic n-3 fatty acids prevent STZ-induced diabetes in fat-1 mice: inflammatory pathway inhibition," *Diabetes*, vol. 60, no. 4, pp. 1090–1099, 2011.

[51] J. C. Lovejoy, "The influence of dietary fat on insulin resistance," *Current Diabetes Reports*, vol. 2, no. 5, pp. 435–440, 2002.

[52] T. Jafari, A. A. Fallah, and L. Azadbakht, "Role of dietary n-3 polyunsaturated fatty acids in type 2 diabetes: a review of epidemiological and clinical studies," *Maturitas*, vol. 74, no. 4, pp. 303–308, 2013.

[53] J.-S. Zheng, T. Huang, J. Yang, Y.-Q. Fu, and D. Li, "Marine n-3 polyunsaturated fatty acids are inversely associated with risk of type 2 diabetes in asians: a systematic review and meta-analysis," *PLoS ONE*, vol. 7, no. 9, Article ID e44525, 2012.

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Research Article

Investigation of the Protective Effects of Taurine against Alloxan-Induced Diabetic Retinal Changes via Electroretinogram and Retinal Histology with New Zealand White Rabbits

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The purpose of this study was to investigate the protective role of orally administered taurine against diabetic retinal changes via electroretinogram (ERG) and retinal histology on rabbits. Rabbits were randomly assigned into groups: Group I (vehicle administration only); Group II (diabetes: induced by 100 mg/kg alloxan injection); Group III (diabetes and fed with 200 mg/kg taurine); and Group IV (diabetes and fed with 400 mg/kg taurine). The body weight and blood glucose levels of the rabbits were monitored weekly. The ERG was measured on weeks 5 and 15. Retinal histology was analyzed in the end of the experiment. Results revealed that a taurine supplement significantly ameliorates the alloxan-induced hyperglycemia and protects the retina from electrophysiological changes. Group II showed a significant ($P < 0.05$) change in the mean scotopic b-wave amplitude when compared to that of Group I, whereas the diabetic rabbits treated with taurine (Group III and IV) were analogous to Group I. Histologically, the amount of Bipolar and Müller cells showed no difference ($P > 0.05$) between all groups and when compared with those of Group I. Our study provides solid evidences that taurine possesses an antidiabetic activity, reduced loss of body weight, and less electrophysiological changes of the diabetic retina.

1. Introduction

Diabetes mellitus is one of the most serious medical issues around the world. Untreated diabetes ultimately leads to a variety of secondary complications, such as neuropathy, heart disease, kidney failure, and retinopathy [1]. In the United States, among those adults aged between 20 and 74 years, diabetic retinopathy has been shown to be the leading cause of new cases of blindness [2, 3]. Fong and colleagues [4] described that the prevalence of any signs of retinopathy was as high as 80% at 15 years of having diabetes.

The clinical signs of diabetic retinopathy within the retinal circulation include microaneurysms, haemorrhages, intraretinal microvascular abnormalities, and neovascularization [5, 6]. Microaneurysms are usually the first clinically detectable lesion of diabetic retinopathy; they represent weakening of the capillary walls and may be associated with retinal oedema due to serum leakage from

the vessels. Haemorrhages are also an early sign of diabetic damage to blood vessels. They may include "dot and blot" haemorrhages that occur deeper in the retina and shallow flame-shaped haemorrhages that follow the retinal nerve fibre layer. Intraretinal microvascular abnormalities (IRMA) may also be present and caused by poor functioning or nonperfusion of capillaries which prevent normal blood flow. Neovascularization can occur anywhere within the retina as a response to ischaemia and is the hallmark of the advanced and proliferative stage of diabetic retinopathy. The occurrence of neovascularization increases the risk of vision loss in the diabetic patient. In addition to the clinical signs in the retina that can be visualized via ophthalmoscopic view, evidence from previous studies also suggests that choroidal angiopathy may coexist along with retinal vascular damage [7, 8].

Other than the clinical signs mentioned above in detection of diabetic retinopathy, several studies have found that diabetes affects the electrophysiological aspects of vision. Electroretinogram (ERG) is one of the tests that have been well described in the detection of early functional changes in diabetic retinas. In fact, previous studies have demonstrated that ERG abnormalities (i.e., changes in b-wave amplitude) occur before any signs of structural abnormalities can be detected by fundus photography [9], fluorescein angiography [10], and morphological examinations [11].

Taurine (2-aminoethanesulfonic acid) is a conditionally essential amino acid that is present in the retina in a high concentration and is widely distributed in mammalian tissues. The main source of taurine *in vivo* is from a dietary intake of meat or seafood and biosynthesis that is derived from methionine and cysteine metabolism. However, previous study reported that biosynthetic capacity of taurine in humans is very low and absent in cats [12]. Taurine has many biological roles and is involved in several physiological actions, such as the formation of bile acid, osmoregulation, antioxidation, maintaining the structural integrity of the membrane, and modulation of calcium binding and transport [13–15].

In various experimental models, taurine has been shown to protect against alloxan-induced hyperglycemia in type I diabetes [16] and to inhibit cataractogenesis in rabbit lenses exposed to 30 mM galactose [17]. Previous studies have established that taurine is essential for visual development and those deficiencies are associated with retinal degeneration [18]. The physiological role of taurine has been paid attention since reports of cats developing central retinal degeneration when they have been fed to induce a chronic deficiency of taurine, which is similar to the retinitis pigmentosa in humans [19, 20].

Since taurine has been demonstrated to have such excellent bioactivity properties, we hypothesized that taurine administration can protect rabbits from alloxan-induced diabetic retinal changes. The extent of alloxan-induced diabetic retinal changes and protective effects of taurine were measured by electroretinogram (ERG) and histological observations.

2. Methods

2.1. Animals. Twenty-two male New Zealand White Rabbits (10 weeks old) from Ta Tsung Farm (Changhua City, Taiwan) were used in this study. The animals were quarantined and allowed to acclimatize for one week prior to the experiment phase. The animals were housed one rabbit per cage under standard laboratory conditions with a 12-hour light/dark cycle. The temperature of the animal room was maintained at 25 ± 2°C with a relative humidity of 55 ± 5%. The air-handling units were set to provide approximately 12 fresh air changes per hour. Food and water were available *ad libitum*. The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee (IACUC), Chung Shan Medical University Experimental Animal Center (approval number: 684), and the animals were cared for in accordance with the institutional ethical guidelines. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Experimental Design. The rabbits were randomly assigned into groups. Group I served as the control ($n = 6$). The experimental groups (Groups II, III, and IV) received an intravenous injection of alloxan [21, 22] in 0.9% sodium chloride at a dosage of 100 mg/kg body weight to induce diabetes. The blood glucose levels were monitored weekly. Those animals with blood glucose levels >200 mg/dL in a consecutive 3-week period were included in the experiment and were distributed into the following groups: Group II diabetes (untreated diabetic rabbits, $n = 5$); Group III (DT200; diabetic rabbits treated with 200 mg/kg taurine in the drinking water, $n = 5$); Group IV (DT400; diabetic rabbits treated with 400 mg/kg taurine in the drinking water, $n = 6$). The food and water intake were checked daily, and body weight was measured weekly.

2.3. Blood Glucose Measurement. The blood was collected weekly during 15 weeks of experiment from the marginal ear vein to measure the blood glucose levels (Accu-Chek Active blood glucose meter; Roche Diagnostics Gmbha, Germany).

2.4. Electroretinogram (ERG) Analyses. The electroretinogram was measured in both photopic (350 Lux of ambient light) and scotopic (after 5 minutes of dark adaptation) conditions. The instruments used in this study were Eickemeyer ERG, HP Compaq 6230 Notebook, ERG-jet, and ERG-probe by Universo Plastique. The instruments were tested prior to the animals' preparation to ensure that the electrodes were in good order and then the rabbits were prepared in the ambient light with the following steps.

(1) One to two drops of tropicamide 1% mydriatic were instilled into rabbits' eyes for at least 30 min prior to ERG.

(2) Subcutaneously atropine sulphate was injected at 0.05 mg/kg prior to anesthesia to reduce the salivary and bronchial secretions. Pupils were checked to

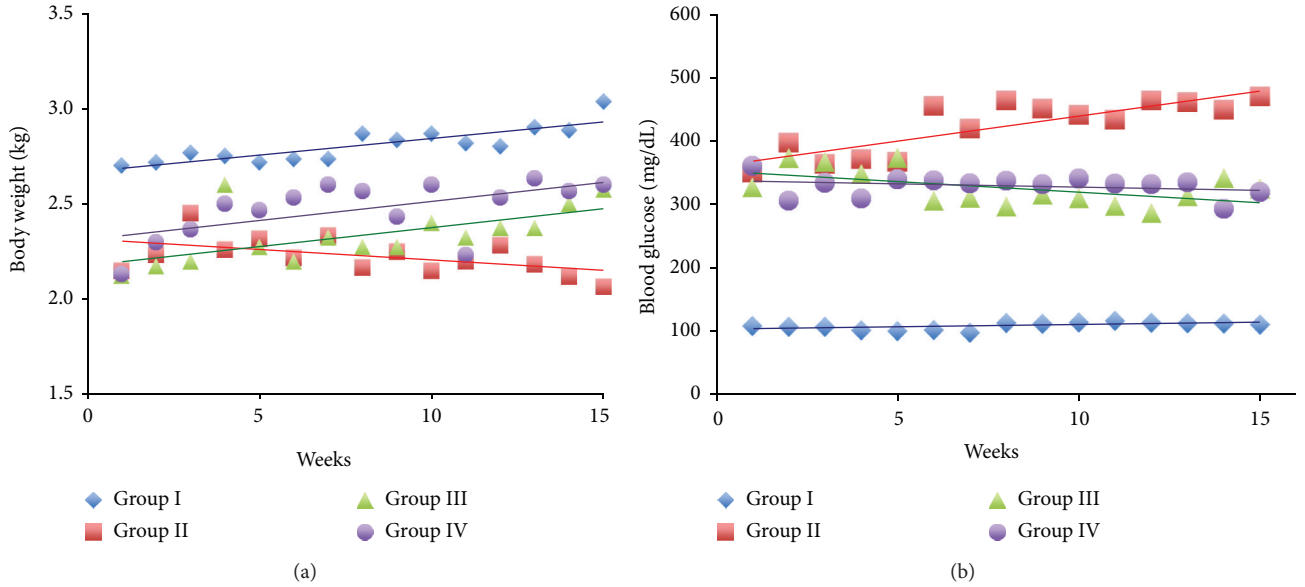

FIGURE 1: The effect of the taurine on body weight (a) and blood glucose levels (b) in alloxan-induced diabetic rabbits over the 15 weeks of experimentation.

confirm pupil dilation, and mydriatic drops were repeated at this stage if necessary.

(3) Zoletil 50 anesthetics were injected into rabbit's thigh muscles, and then 1 to 2 drops of proparacaine hydrochloride 0.5% local anesthetics were instilled into the rabbits' eyes. Both were done 5 minutes prior to the ERG measurements.

(4) Electrodes were placed. Artificial tears were used with contact lens electrode to ensure better contacts of electrode and cornea.

(5) Light stimulus was placed 1 cm away from the cornea with standard flash of 2-3 cd/m^2/s.

(6) The electroretinogram was measured with ambient light and after 5 minutes of dark adaptation.

(7) Results of ERG including a-wave and b-wave were recorded.

2.5. Histological Evaluation. The rabbits were sacrificed at the end of the experiment with 100–150 mg/kg pentobarbital injection and CO_2; the eyes were removed, weighed, and fixed in Davidson's fixative. The eyes were processed for paraffin embedding following standard microtechniques. Four- to five-micron sections of the eyes tissue were stained with hematoxylin and eosin to estimate the retinal damage and were observed under a microscope (IX71S8F-2, Olympus, Tokyo, Japan).

2.6. Statistical Analysis. All results are expressed as mean ± SD. The comparison between any two groups was performed using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests using the statistical software SPSS (Drmarketing Co., Ltd., New Taipei City, Taiwan). Statistically significant differences between groups were defined as $P < 0.05$.

3. Results

3.1. Effects of Taurine on Body Weights. The body weight losses are well diagnosed in alloxan-induced diabetic animal studies. The body weights were recorded weekly over the 15 weeks of study, and the results are shown in Figure 1(a). The trend of the body weight gain revealed an increase in the weights of Group I, Group III, and Group IV over time. Over the 15 weeks of study, the mean increase in body weight for Group I was 0.33 kg, Group III increased by 0.45 kg, and Group IV increased by 0.47 kg. In contrast, Group II lost on average 0.08 kg over the course of the experiment. Although the final body weight of the taurine treated groups was still lower than Group I, they were 24.6% (Group III) and 25.6% (Group IV) heavier than Group II.

3.2. Effects of Taurine on Blood Glucose Levels. Blood glucose levels are commonly used as an indicator for alloxan-induced diabetes in experimental animals. They indicate whether or not diabetes was successfully induced. The results of blood glucose levels over the 15 weeks are shown in Figure 1(b). As shown in Figure 1(b), the mean glucose levels of Group I, Group III, and Group IV remained constant over time, whereas Group II's mean blood glucose levels increased over time. This indicated that alloxan is very effective ($P < 0.0001$) in elevating the blood glucose levels in comparison to Group I. This is also as an indication of successfully induced diabetes in rabbits. Our results also demonstrated that taurine treatments alleviate increases of blood glucose levels effectively ($P < 0.05$). On average at 15th week of the study, the glucose levels were lowered by 145 mg/dL

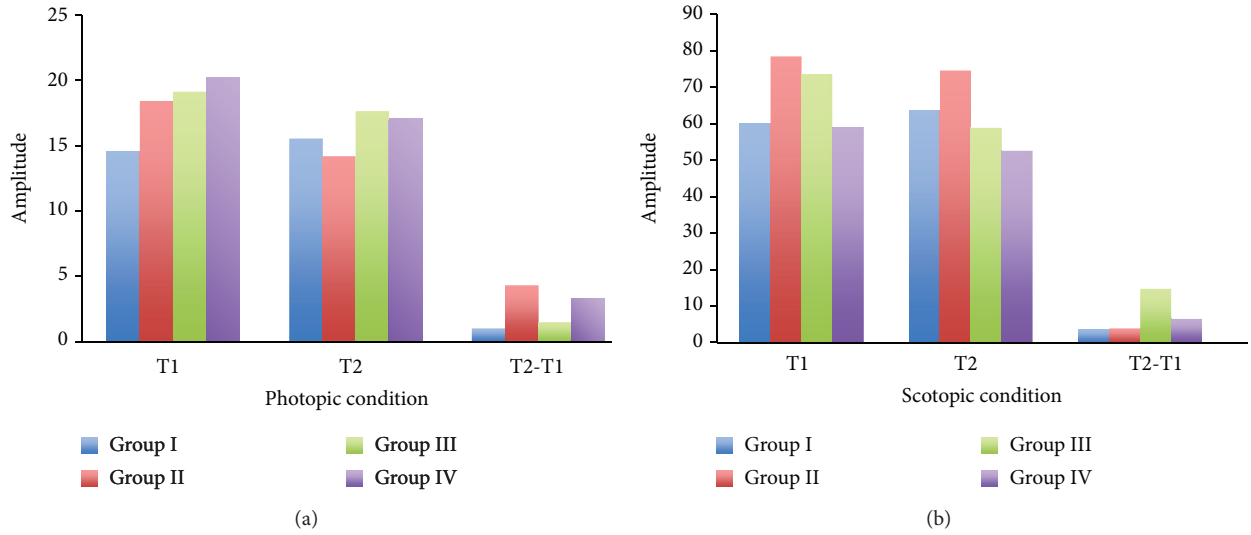

Figure 2: The electroretinogram (ERG) was performed on the control (Group I), diabetes (Group II), and diabetes + taurine (Group III, Group IV) groups on week 5 (T1) and week 15 (T2). The averaged a-wave amplitude under the photopic (a) and scotopic (b) conditions was recorded and analyzed.

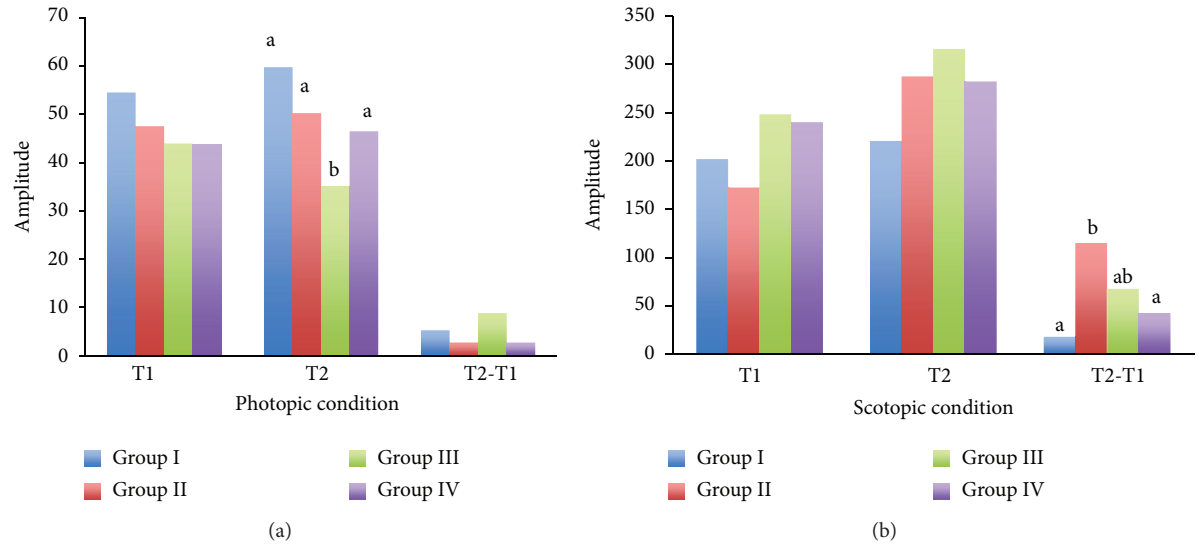

Figure 3: The electroretinogram (ERG) was performed on the control (Group I), diabetes (Group II), and diabetes + taurine (Group III, Group IV) groups on week 5 (T1) and week 15 (T2). The averaged b-wave amplitude under the photopic (a) and scotopic (b) conditions was recorded and analyzed. a, b: bars with different alphabetic letters differ ($P < 0.05$).

(30.9%) in Group III and 151 mg/dL (32.1%) in Group IV when compared to the mean glucose levels of Group II. These findings suggested that the hyperglycemia was induced successfully by alloxan and was effectively ameliorated by taurine treatments.

3.3. Electroretinogram. The electroretinogram (ERG) was performed for all groups on weeks 5 and 15 to investigate the possible electrophysiological changes of the diabetic retina. The mean amplitude of a-wave and b-wave was analyzed and is shown in Figures 2(a) and 2(b) (a-wave) and Figures 3(a) and 3(b) (b-wave). Over the 15 weeks of study, the mean amplitude changes in both photopic and scotopic a-wave were insignificant ($P > 0.05$). In regard to the b-wave, the mean amplitude under photopic condition was also insignificant ($P > 0.05$). However, the mean amplitude of scotopic b-wave in Group II showed a significantly higher change over time during the experimental period ($P < 0.05$), whilst Group III and Group IV's scotopic changes were not significantly different from animals in the control condition. Group III showed a slightly greater change in scotopic b-wave than Group IV, which may mean that Group IV is more superior in minimizing the electrophysiological changes of the retina over time.

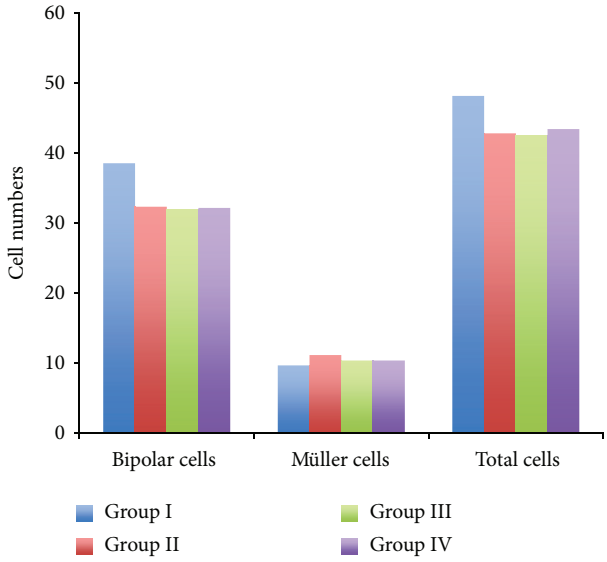

Figure 4: The comparison of Bipolar and Müller cells number among all groups. The cross-section of the retinas was prepared and examined under the microscope for histological evaluation.

3.4. Histological Evaluation. The cross-section of the retinas was prepared and examined under the microscope for histological evaluation. The Bipolar and Müller cells were counted and results are shown in Figure 4. Our results revealed that there are not significant differences ($P > 0.05$) in Bipolar and Müller cells' count among all groups when compared with control group. We then further added the Bipolar and Müller cells' count all together to see if there were any clearer trends of cell counts. However, the results still revealed a statistically insignificant difference ($P > 0.05$). These results indicated that there were no obvious cell losses over the 15 weeks of diabetes in rabbits.

4. Discussion

Taurine is an organic acid that has many fundamental biological roles in our human body and in animals. Lombardini [23] found that taurine has several functions in the retina, which includes the regulation of Ca^{2+} transport, protection of the photoreceptor, and regulation of signal transduction. A previous study demonstrated that taurine treatments attenuate the induction of retinal VEGF that associates with vascularization in STZ-diabetic rats, suggesting that taurine may normalize the retinal vascular function in diabetes [24]. While elevation of glutamate in the retina is associated with the development of diabetic retinopathy, taurine appeared to be able to regulate Müller cells' glutamate uptake and degradation under diabetic conditions via its antioxidant mechanism [25].

Our study showed that taurine has significant hypoglycemic properties, which is in line with previous work. For example, Gavrovskaya et al. [16] found a decrease in glucose concentration, together with the protection of b-cells of the islets of Langerhans in experimental insulin dependent diabetes mellitus, and other researches have demonstrated the effectiveness of taurine treatments against both insulin dependent and non-insulin dependent diabetes mellitus [26, 27].

In electroretinogram (ERG), the a-wave is a negative, maximal combined response that is believed to reflect the membrane potential in photoreceptors. The b-wave is a positive, maximal combined response that is thought to originate from the Bipolar and Müller cells that postsynapse to the photoreceptors [28]. However, in various animal and human studies, ERG measured under scotopic conditions showed that the large b-wave appeared to be directly generated by the Bipolar cells.

Previous studies investigated the relationship between the ERG changes and diabetic eyes. Holopigian et al. [29] found that the b-wave activity in electroretinogram might indicate retinal changes in early diabetic retinopathy. Coupland [30] and Hardy et al. [31] have demonstrated that the amplitude of the scotopic b-wave reflects the abnormal activity of the Bipolar cells in diabetes even with the absence of visible fundus signs of retinopathy. Kern et al. [32] looked at the streptozotocin-induced diabetes in different species of rats and observed that all strains tended to show diabetes-induced impairment of the dark-adapted (scotopic) b-wave amplitude.

The ERG results from our study revealed that the changes in mean b-wave amplitude of Group II are significantly higher in comparison to Group I, whereas the rabbits with taurine treatments (both Group III and Group IV) reveal no statistical difference against control. Therefore, based on our ERG results, one can assume the following. Firstly, Bipolar cells are probably more susceptible to the alloxan-induced diabetic damages, as the scotopic b-wave appears to be directly generated by the Bipolar cells. Secondly, taurine has protective effects on the diabetic retinas, as it minimizes the retinal electrophysiological changes in the diabetic rabbits. We have also noticed that the rabbits fed daily with 400 mg/kg of taurine had fewer electrophysiological changes over time than the animals assigned to 200 mg/kg of taurine intake. Therefore, the 400 mg/kg taurine supplement may be more superior than the 200 mg/kg of taurine.

In regard to the Bipolar and Müller cells' counts in a retinal histological evaluation, our results revealed no significant difference in Bipolar and Müller cells' counts among all groups ($P > 0.05$). This is probably due to the short experimental period which was unable to observe the retinal histology changes caused by diabetes. This finding confirms previous research findings that the electroretinogram is able to detect abnormalities before any structural changes.

The body weight losses in alloxan-induced diabetes animals were well demonstrated in our study as well. On average, the diabetes group exhibited a 31.7% weight loss when compared with the control group at the end of the 15-week experiment. However, those rabbits treated with 200 mg/kg and 400 mg/kg of taurine daily showed a similar trend of weight gain as the control group over the study period. This also implies that Group III and Group IV end up with much less weight loss compared to those in Group II.

Therefore, we can conclude that treatment of taurine protects the alloxan-induced diabetes rabbits from body weight losses.

There are a number of limitations to this study. Firstly, we were unable to determine whether the protective effect of taurine on the retina in diabetic rabbits acted directly on the retina or the effect was indirect and due to hypoglycemic. Secondly, we found no significant differences between the groups for the histological evaluation. This may be because period of 15 weeks is too short to cause the rabbits' retinal tissues change. Future studies could extend the experimental period to further investigate histological differences over time including fluorescein angiography and optical coherence tomography (OCT), particularly for the diabetic group.

In conclusion, our study demonstrated that taurine possesses antidiabetic activities, especially in the hypoglycemic effect, reduces body weight loss, and meanwhile directly or indirectly minimizes the electrophysiological changes in diabetic retina. Future studies are needed to determine whether these results can be replicated in other animal models. If these results hold for human models, taurine could be a cheap and effective way to prevent the damage to retina caused by diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] "UK Prospective Diabetes Study VIII: study design, progress and performance," *Diabetologia*, vol. 34, pp. 877–890, 1991.

[2] M. I. Harris, "Diabetes in America: epidemiology and scope of the problem," *Diabetes Care*, vol. 21, no. 12, pp. C11–C14, 1998.

[3] M. F. Rabb, D. A. Gagliano, and H. E. Sweeney, "Diabetic retinopathy in blacks," *Diabetes Care*, vol. 13, no. 11, pp. 1202–1206, 1990.

[4] D. S. Fong, L. Aiello, T. W. Gardner et al., "Retinopathy in diabetes," *Diabetes Care*, vol. 27, no. 1, pp. S84–S87, 2004.

[5] R. Engerman, D. Finkelstein, G. Aguirre et al., "Ocular complications," *Diabetes*, vol. 31, no. 1, pp. 82–88, 1982.

[6] C. P. Wilkinson, F. L. Ferris III, R. E. Klein et al., "Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales," *Ophthalmology*, vol. 110, no. 9, pp. 1677–1682, 2003.

[7] M. Adhi, E. Brewer, N. K. Waheed, and J. S. Duker, "Analysis of morphological features and vascular layers of choroid in diabetic retinopathy using spectral-domain optical coherence tomography," *JAMA Ophthalmology*, vol. 131, no. 10, pp. 1267–1274, 2013.

[8] I. Fukushima, D. S. McLeod, and G. A. Lutty, "Intrachoroidal microvascular abnormality: a previously unrecognized form of choroidal neovascularization," *The American Journal of Ophthalmology*, vol. 124, no. 4, pp. 473–487, 1997.

[9] S. Juen and G. F. Kieselbach, "Electrophysiological changes in juvenile diabetics without retinopathy," *Archives of Ophthalmology*, vol. 108, no. 3, pp. 372–375, 1990.

[10] R. D. Levin, H. C. Kwaan, J. G. Dobbie, C. L. Fetkenhour, H. S. Traisman, and C. Kramer, "Studies of retinopathy and the plasma co-factor of platelet hyperaggregation in Type 1 (insulin-dependent) diabetic children," *Diabetologia*, vol. 22, no. 6, pp. 445–449, 1982.

[11] Q. Li, E. Zemel, B. Miller, and I. Perlman, "Early retinal damage in experimental diabetes: electroretinographical and morphological observations," *Experimental Eye Research*, vol. 74, no. 5, pp. 615–625, 2002.

[12] S. H. Hansen, "The role of taurine in diabetes and the development of diabetic complications," *Diabetes/Metabolism Research and Reviews*, vol. 17, no. 5, pp. 330–346, 2001.

[13] J. G. Jacobsen and L. H. Smith, "Biochemistry and physiology of taurine and taurine derivatives," *Physiological Reviews*, vol. 48, no. 2, pp. 424–511, 1968.

[14] R. J. Huxtable, "Taurine in the central nervous system and the mammalian actions of taurine," *Progress in Neurobiology*, vol. 32, no. 6, pp. 471–533, 1989.

[15] R. J. Huxtable, "Physiological actions of taurine," *Physiological Reviews*, vol. 72, no. 1, pp. 101–163, 1992.

[16] L. Gavrovskaya, O. Ryzhova, A. Safonova, A. K. Matveev, and N. S. Sapronov, "Protective effect of taurine on rats with experimental insulin-dependent diabetes mellitus," *Bulletin of Experimental Biology and Medicine*, vol. 146, no. 2, pp. 226–228, 2008.

[17] J. I. Malone, S. A. Benford, and J. Malone Jr., "Taurine prevents galactose-induced cataracts," *Journal of Diabetes and Its Complications*, vol. 7, no. 1, pp. 44–48, 1993.

[18] T. C. Birdsall, "Therapeutic applications of taurine," *Alternative Medicine Review*, vol. 3, no. 2, pp. 128–136, 1998.

[19] K. C. Hayes, R. E. Carey, and S. Y. Schmidt, "Retinal degeneration associated with taurine deficiency in the cat," *Science*, vol. 188, no. 4191, pp. 949–951, 1975.

[20] J. A. Sturman, "Taurine in development," *Physiological Reviews*, vol. 73, no. 1, pp. 119–147, 1993.

[21] Y.-W. Hsu, S.-M. Yeh, Y.-Y. Chen, Y.-C. Chen, S.-L. Lin, and J.-K. Tseng, "Protective effects of taurine against alloxan-induced diabetic cataracts and refraction changes in New Zealand White rabbits," *Experimental Eye Research*, vol. 103, pp. 71–77, 2012.

[22] S. H. Mir and M. M. Darzi, "Histopathological abnormalities of prolonged alloxan-induced diabetes mellitus in rabbits," *International Journal of Experimental Pathology*, vol. 90, no. 1, pp. 66–73, 2009.

[23] J. B. Lombardini, "Taurine: retinal function," *Brain Research Reviews*, vol. 16, no. 2, pp. 151–169, 1991.

[24] I. G. Obrosova, A. G. Minchenko, V. Marinescu et al., "Antioxidants attenuate early up regulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats," *Diabetologia*, vol. 44, no. 9, pp. 1102–1110, 2001.

[25] K. Zeng, H. Xu, K. Chen et al., "Effects of taurine on glutamate uptake and degradation in Müller cells under diabetic conditions via antioxidant mechanism," *Molecular and Cellular Neuroscience*, vol. 45, no. 2, pp. 192–199, 2010.

[26] F. Franconi, M. A. Di Leo, F. Bennardini, and G. Ghirlanda, "Is taurine beneficial in reducing risk factors for diabetes mellitus?" *Neurochemical Research*, vol. 29, no. 1, pp. 143–150, 2004.

[27] S. W. Schaffer, J. Azuma, and M. Mozaffari, "Role of antioxidant activity of taurine in diabetes," *Canadian Journal of Physiology and Pharmacology*, vol. 87, no. 2, pp. 91–99, 2009.

[28] I. Perlman, *The Electroretinogram: ERG. Webvision: The Organization of the Retina and Visual System*, Online Textbook of the Visual System, University of Utah, Salt Lake City, Utah, USA, 2011.

[29] K. Holopigian, W. Seiple, M. Lorenzo, and R. Carr, "A comparison of photopic and scotopic electroretinographic changes in early diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 33, no. 10, pp. 2773–2780, 1992.

[30] S. G. Coupland, "A comparison of oscillatory potential and pattern electroretinogram measures in diabetic retinopathy," *Documenta Ophthalmologica*, vol. 66, no. 3, pp. 207–218, 1987.

[31] K. J. Hardy, C. Fisher, P. Heath, D. H. Foster, and J. H. B. Scarpello, "Comparison of colour discrimination and electroretinography in evaluation of visual pathway dysfunction in aretinopathic IDDM patients," *The British Journal of Ophthalmology*, vol. 79, no. 1, pp. 35–37, 1995.

[32] T. S. Kern, C. M. Miller, J. Tang, Y. Du, S. L. Ball, and L. Berti-Matera, "Comparison of three strains of diabetic rats with respect to the rate at which retinopathy and tactile allodynia develop," *Molecular Vision*, vol. 16, pp. 1629–1639, 2010.

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Research Article

The Effects of Soy Bean Flour Enriched Bread Intake on Anthropometric Indices and Blood Pressure in Type 2 Diabetic Women: A Crossover Randomized Controlled Clinical Trial

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Previous studies showed that soy bean has the potential to improve many aspects of diabetes state and provide metabolic benefits that aid in weight management. We aimed to determine the effects of soy bean flour enriched bread on anthropometric indices and blood pressure among type 2 diabetic patients. This randomized, crossover, clinical trial was performed in 30 type 2 diabetic women. There were two trial periods for 6 weeks and a wash-out period for 4 weeks. In the soy bread diet period, 120 g of soy bean flour enriched bread was consumed each day instead of the same amount of their usual bread or other cereal products. After a 4-week wash-out period, participants were crossed over for another 6 weeks. Mean (±SD) age of study participants was 45.7 ± 3.8 years. The results of our study showed no significant effects of soy bean flour enriched bread on anthropometric indices and blood pressure among diabetic patients. Despite the slight reduction in BMI, waist circumference, and percent of body fat, there were no significant differences in changes of these values between two groups. No significant changes in waist to hip ratio and blood pressure were seen.

1. Introduction

Diabetes mellitus is one of the most common chronic diseases in the world [1] and has become a major threat for global health [2]. It has been estimated that the prevalence of diabetes for all age-groups worldwide was 2.8% in 2000 and will reach 4.4% in 2030 [3]. According to the World Health Organization (WHO) estimates, more than 2 million diabetic patients were living in Iran in 2000 and has been estimated to increase to more than 6.4 million in 2030 [4]. Currently, the effects of soy and its components on many chronic diseases have been studied. Soy bean is a legume which is a unique source of protein, fiber, vitamins, minerals, polyunsaturated fatty acids, isoflavones, and phytoestrogens [5, 6]. The effects of soy on body composition are not well understood. In a cross-sectional study conducted in postmenopausal women, individuals who received a high soy diet had a lower BMI and waist circumference compared with individuals who received no soy [7]. In contrast, several studies have failed to reach such significant effects. In a randomized trial in perimenopausal women, soy protein did not affect total body fat or lean mass [8]. The same results were found in a 6-week crossover study in overweight and obese female youths, which soy drink replacement had no significant effects on

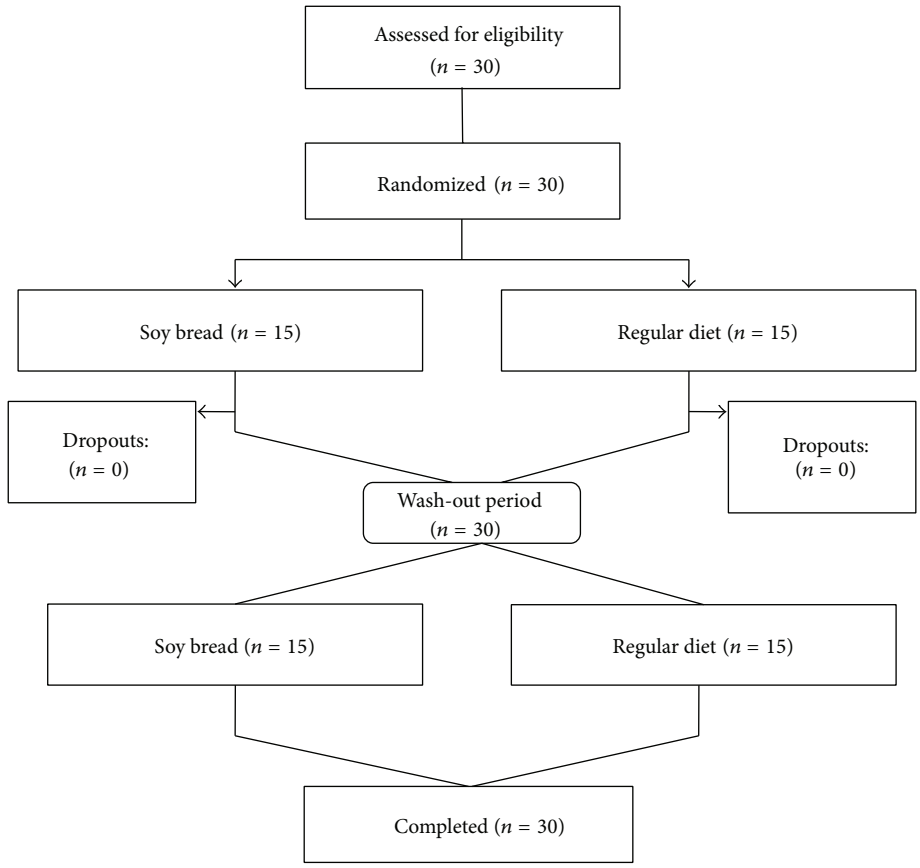
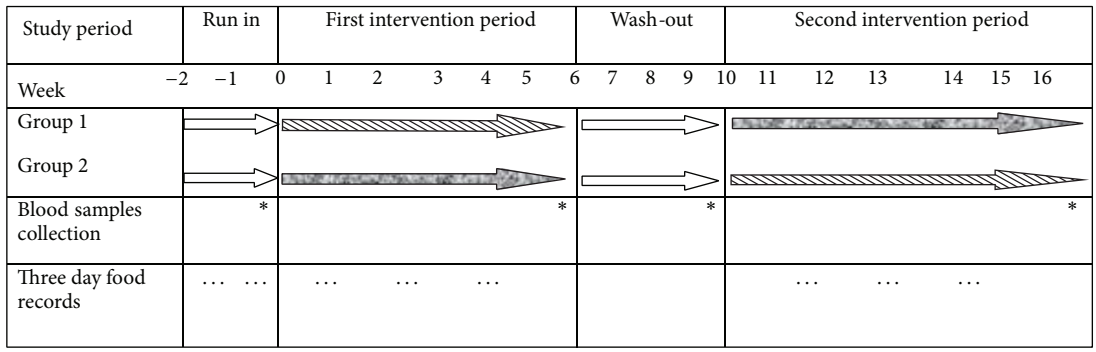


Figure 1: Participants flow diagram.

weight and waist circumference [9]. Soy contains high fiber content. Earlier studies have shown that higher intake of dietary fiber is linked to suppressed appetite and enhanced satiety [6]. Besides, soy is a rich source of protein (~35–40%) [10]. Recently, protein intake is considered as a major determinant in weight control [11]. High protein content of foods can reduce appetite and decrease food intake [12]. Also soy protein can reduce appetite by stimulating cholecystokinin [13]. Soy polyphenols can affect endothelial function and as a consequence, blood pressure [14]. Prevalence of type 2 diabetes is increasing in Iran [4]. It seems that the inclusion of soy foods in the diet is particularly relevant for diabetic patients because soy foods have beneficial effects on chronic diseases and soy can help them in weight management and then can improve many aspects of diabetes state [5, 15]. In addition, bread is the staple food in Iranian diet. Fortification of bread with soy flour can increase the quality of protein and improve its effects on human health. According to our knowledge, there is no study examining the effects of soy bean flour enriched bread on weight and blood pressure control among women with type 2 diabetes. Therefore, With regard to beneficial effects of soy on chronic disease including type 2 diabetes, we aimed to fortify bread with soy bean flour and determine its effects on anthropometric indices and blood pressure among type 2 diabetic patients.

2. Subjects and Methods

2.1. Participants. This randomized, crossover, controlled clinical trial was undertaken in 30 premenopausal women with type 2 diabetes. This study was conducted in Isfahan, Iran, from April 2013 to September 2013. Being in the range age of 30–50, diagnosis of type 2 diabetes and having body mass index >25 were the inclusion criteria. Exclusion criteria were insulin injection, use of hormone replacement therapy, use of supplements, hypo- and hyperthyroidism, smoking, and allergy to soy bean. Participants who were pregnant or breastfeeding were excluded. All participants were recruited from Endocrine and Metabolism Research Center of Isfahan University of Medical Sciences. Participants were not undergoing dietary changes in the last 3 months and had no current weight loss. Sample size for this study was calculated based on suggested formula for crossover trials [16]: $n = [(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot s^2]/2\Delta^2$; we considered type 1 error of 5% and type 2 error of 20% (power = 80%) and BMI as a key variable [9]. According to the previous formula, 19 participants were needed for adequate power. Since there are high dropouts in crossover trials, we enrolled 30 women in this study based on the above mentioned inclusion criteria. All participants completed the entire crossover study. Participants diagram is shown in Figure 1. This study



Group 1: intervention-control; treatment from soy bean flour enriched bread diet to regular diet

Group 2: control-intervention; treatment from regular diet to soy bean flour enriched bread diet

Figure 2: Study diagram.

was approved by Ethical Committee of Isfahan University of Medical Sciences, Isfahan, Iran. All participants provided informed written consent. The clinical trial registration code was obtained from the center for registration of clinical trial (IRCT2013061613684N1).

2.2. Study Procedures. At first, a 2-week run-in period was conducted. This period was conducted to assess diet and physical activity level and to evaluate the compliance of study participants to soy bean flour enriched bread. Participants were asked not to alter their habitual diet and level of physical activity. Also, to improve compliance, participants were asked to consume 1 serving/day of soy bean flour enriched bread during this period of the study. In the run-in period, all participants completed two dietary records (nonconsecutive days) and a 2-day physical activity record. After run-in period, all measurements were done. Then, participants were randomly assigned to either intervention or control groups, each one for 6 weeks. Participants were not blinded because of texture and taste of the bread. After the first period of intervention, a 4-week wash-out period was conducted. Then participants were crossed over for another 6 weeks. During the wash-out period, subjects consumed the same diet they consumed before the study. All measurements were done at baseline and at 6, 10, and 16 weeks. Compliance of the participants monitored once a week through face-to-face visits and phone interviews. In addition, all participants completed a 3-day (2 weekdays and one weekend day) dietary and physical activity records once every two weeks during the study. Study diagram is shown in Figure 2.

2.3. Interventions. Soy bean flour enriched bread was prepared by replacing 30% of the wheat flour by soy bean flour. Participants in the intervention group (soy bread) were asked to consume 120 g of soy bean flour enriched bread each day instead of the same amount of their usual bread intake and if necessary other carbohydrate rich foods such as rice, pasta and other cereal products. On average, each bread was 120 g. Participants were supplied with enough fresh packaged bread weekly. Bread packages used fresh or were frozen before use. Individuals in the control group were asked to remain on their habitual diet. The dietitian monitored bread intake weekly, if bread intake was outside the recommended amount. Participants were trained on how to use bread properly. Soy bean flour enriched bread characteristics are shown in Table 1.

Table 1: Characteristics of soy bean flour enriched bread used in the intervention.

Nutrient	Amount per 100 g
Fat	7.2
Carbohydrate	44.31
Protein	14.1
Moisture	28.24
Ash	2.5

2.4. Anthropometric Assessments. Height was measured to the nearest 0.1 cm in a standing position without wearing shoes, using a measuring tape while shoulders were relaxed. Weight was measured to the nearest 0.1 kg using a digital scale with minimal clothes and without shoes (Seca, Hamburg, Germany). Body mass index (BMI) was calculated as weight (in kg) divided by height^2 (in m). Waist circumference (WC) was measured to the nearest 0.1 cm at the narrowest level over light clothing, by using a nonstretchable tape measure, without any pressure to the body surface. Hip circumference (HC) was measured in the largest part of the hip over light clothing. Percent of body fat (PBF) was measured by body composition analyzer (Jawon Medical Company, Korea). Blood pressure was measured after 15 minutes of rest in the seated position by the use of a mercurial sphygmomanometer.

2.5. Statistical Methods. Dietary records were assessed using Nutritionist IV software. Statistical analyses were done using SPSS 18 statistical software package (SPSS Inc., Chicago, IL). All subjects were included in the final analysis. At first,

Table 2: The effects of soy bean flour enriched bread intake on anthropometric indices and blood pressure in type 2 diabetic women[1].

Variables	First period		Second period		Change differences	P^4
	Baseline	6th week	10th week	16th week		
Weight (kg): intervention-control[2]	71.1 ± 10	71.1 ± 9.7	70.8 ± 9.5	70.5 ± 10.1	0.12	0.7
Weight (kg): control-intervention[3]	76 ± 11.2	75.7 ± 9.5	75.6 ± 9.9	76 ± 9.6		
BMI (kg/m^2): intervention-control	28.6 ± 3.6	28.6 ± 3.5	28.5 ± 3.3	28.1 ± 3.8	−0.05	0.8
BMI (kg/m^2): control-intervention	30.2 ± 4.3	30.1 ± 3.7	30 ± 3.8	30.2 ± 3.8		
WC (Cm): intervention-control	87 ± 6.7	86.6 ± 6.3	86.5 ± 6.3	86.7 ± 6.3	−0.55	0.26
WC (Cm): control-intervention	89.4 ± 7.7	88.8 ± 7.2	88.6 ± 6.9	89.1 ± 6.5		
HC (Cm): intervention-control	98.5 ± 4.6	98.6 ± 4.6	98.6 ± 4.5	99.08 ± 4	−0.4	0.25
HC (Cm): control-intervention	99.3 ± 4.8	99.1 ± 4.7	99.8 ± 5.5	99.2 ± 4.3		
WHR: intervention-control	0.88 ± 0.04	0.88 ± 0.04	0.88 ± 0.03	0.88 ± 0.04	0.006	0.08
WHR: control-intervention	0.88 ± 0.04	0.88 ± 0.04	0.88 ± 0.04	0.88 ± 0.04		
PBF: intervention-control	36.8 ± 3.8	36.4 ± 3.6	36.5 ± 3.6	36.6 ± 3.5	−0.36	0.45
PBF: control-intervention	37 ± 4	36.6 ± 4.1	36.5 ± 3.9	36.9 ± 3.6		
DBP: intervention-control	76.7 ± 10.5	74.7 ± 8.3	73.3 ± 6.2	73.3 ± 4.9	3	0.1
DBP: control-intervention	72.7 ± 5.9	70 ± 6.5	73.3 ± 9.7	74.7 ± 7.4		
SBP: intervention-control	114.7 ± 11.2	114.7 ± 9.1	114.7 ± 6.4	114 ± 7.4	2	0.3
SBP: control-intervention	112.7 ± 7	112 ± 6.8	110 ± 8.4	114 ± 9.8		

[1] All data are means ± SD.
[2] Intervention-control; treatment from soy bean flour enriched bread diet to regular diet.
[3] Control-intervention; treatment from regular diet to soy bean flour enriched bread diet.
[4] Results of paired t-test (mean differences between groups).
BMI, body mass index; WC, waist circumference; WHR, waist to hip ratio; PBF, percent of body fat; DBP, diastolic blood pressure; SBP, systolic blood pressure.

normal distribution of all variables was checked with the QQ-Plot test. Paired t-test was used to examine the main effects by comparing the mean differences of variables in two groups. Carry over effect and period effect were checked using t-test. All means are presented as mean ± standard deviation ($\overline{x}$ ± SD). Values of $P < 0.05$ were considered as statistically significant.

3. Results

Mean (±SD) age of study participants was 45.7 ± 3.8 years. Mean BMI and waist circumference was 29.5 ± 3.9 kg/m^2 and 87.4 ± 6.7 cm, respectively. Dietary intakes of participants throughout the study showed that participants in the intervention group received higher energy compared with the control group, but the difference was not significant. No significant differences in dietary intakes of macronutrients, SFA, PUFA, MUFA, calcium, magnesium, folate, and vitamin C were observed between the two groups.

No adverse effects from soy bean flour enriched bread consumption were reported during the study. The effects of soy bean flour enriched bread intake on anthropometric indices and blood pressure are presented in Table 2. The results of our study showed no significant effects of soy bean flour enriched bread on anthropometric indices and blood pressure. Despite the slight reduction in BMI (change difference: −0.05, $P = 0.8$), waist circumference (change difference: −0.55, $P = 0.26$), and percent of body fat (change difference: −0.36, $P = 0.45$), there were no significant differences in changes of these values between two groups. No significant changes in waist to hip ratio and blood pressure were seen.

4. Discussion

The results of the present study conducted among type 2 diabetic women showed that daily consumption of 120 g soy bean flour enriched bread for 6 weeks could not significantly affect anthropometric indices and blood pressure. To our knowledge, this is the first interventional study that examined the effects of soy bean flour enriched bread intake on anthropometric indices and blood pressure among diabetic women.

Obesity is a growing concern due to its increasing prevalence and obesity related health problems [17]. Recently, nutritional strategies for weight management have attracted a great deal of attention. Due to high protein and low cholesterol content, soy is often used as a dietary component in weight loss diets [18–20]. Most of diabetic patients are overweight or obese. Studies showed that dry beans intake such as soy bean has the potential effect to improve many aspects of diabetes state and provide metabolic benefits that aid in weight management. In the current study, we found that soy bean flour enriched bread intake for 6 weeks could decrease BMI, WC, and percent of body fat but these changes were not significant. In line with our study, findings of Kok et al.'s study do not support the hypothesis that soy isoflavones have favorable effects on body composition [21]. In contrast to our study, findings from cross-sectional studies have demonstrated an inverse association between soy genistein

intake and BMI, waist circumference, and total body fat among postmenopausal women [7, 22]. Such findings have also been reported by another interventional study [23]. Several studies support the hypothesis that soy protein or soy phytoestrogens may be beneficial in prevention of obesity and diabetes [24, 25]. In one study conducted in overweight and obese subjects, meal replacement diet with high soy protein drink was effective in reducing weight and improving anthropometric indices [26]. In line with our study, there are some studies that showed no significant effects of soy intake on anthropometric measures and fat mass loss [27, 28]. Several mechanisms have been proposed to underlie the beneficial effects of soy intake on body weight and satiety regulation. Soy peptides may play a role on body weight control, possibly by increasing energy utilization [29]. Also soy phytoestrogens might have beneficial effects on reducing fat accumulation [30]. Soy may exert its favorable effects through its high fiber content. Dietary fiber intake stimulates gastrointestinal hormones secretion that may act as satiety factors [31]. Furthermore, soy proteins can manage appetite. Proteins suppress food intake and contribute to satiety and delay return of hunger compared with fat and carbohydrate. Mechanisms of protein that act on food intake include slowing gastric emptying and direct or indirect stimulation of gastrointestinal hormones such as cholecystokinin and glucagon like peptide-1 [13].

In the present study, we found that soy bean flour enriched bread for 6 weeks could not substantially affect blood pressure among diabetic women. This finding is in contrast to an earlier study in overweight and obese women, where soy drink consumption could favorably influence blood pressure [9]. In a cross-over clinical trial by Miraghajani et al., soy milk consumption for 4 weeks in 29 type 2 diabetic patients with nephropathy could decrease blood pressure [32]. Soy polyphenols can affect endothelial function and then blood pressure [14]. Evidence suggests a small beneficial effect of protein on blood pressure, especially for plant protein [33]. Soy protein intake increases nitric oxide levels that have vasodilatory effects. High amount of arginine, nitric oxide precursor, in the amino acid profile of soy protein might explain soy protein effects on nitric oxide levels [34]. Angiotensin-converting enzyme inhibitory peptides exist in plant proteins such that soy can be enzymatically released from precursor proteins. These peptides can reduce blood pressure by decreasing the vasoconstrictory effects of angiotensin II and enhancing the vasodilatory effects of bradykinin [35]. Our study has strengths as well as limitations. The main strength point of our study is the cross-over design of the study. Other strength points are high percentage of participants who completed the study and took 3-day food records throughout the study to assess compliance of participants to soy bean flour enriched bread. Furthermore, among different soy products, we used soy bean flour enriched bread, a novel food ingredient, for this intervention. Despite the strength points, there are some limitations which deserve attention. Soy effects may be associated with the duration of intervention. Short duration of our intervention might result in the lack of observing any significant effect of soy bean flour enriched bread on anthropometric indices and blood pressure. Further studies with longer duration might be needed.

It was not possible for us to design a double-blind study due to texture and taste of the bread. Also, daily distribution of bread package was impossible. The effects of soy on anthropometric indices and blood pressure may be linked to the dosage of soy. We were not capable of increasing the soy bean flour dosage in bread more than 30% because of unfavorable effects on texture and taste of the bread. In addition, as the trial performed among only women, we cannot generalize the results to the general population.

5. Conclusion

In conclusion, our findings suggest that daily intake of 120 g soy bean flour enriched bread for 6 weeks had no significant effects on anthropometric indices and blood pressure among type 2 diabetic women. Short duration of our intervention might result in the lack of observing any significant effect. Further studies with longer duration are warranted.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] N. Horri, M. Farmani, M. Ghassami, S. Haghighi, and M. Amini, "Visual acuity in an Iranian cohort of patients with type 2 diabetes: the role of nephropathy and ischemic heart disease," *Journal of Research in Medical Sciences*, vol. 16, pp. 419–426, 2011.

[2] Z.-M. Liu, Y.-M. Chen, S. C. Ho, Y. P. Ho, and J. Woo, "Effects of soy protein and isoflavones on glycemic control and insulin sensitivity: a 6-mo double-blind, randomized, placebo-controlled trial in postmenopausal Chinese women with prediabetes or untreated early diabetes," *The American Journal of Clinical Nutrition*, vol. 91, no. 5, pp. 1394–1401, 2010.

[3] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[4] M. Amini, A. Esmaillzadeh, M. Sadeghi, N. Mehvarifar, and M. Zare, "The association of hypertriglyceridemic waist phenotype with type 2 diabetes mellitus among individuals with first relative history of diabetes," *Journal of Research in Medical Sciences*, vol. 16, no. 2, pp. 156–164, 2011.

[5] B. Yang, Y. Chen, T. Xu et al., "Systematic review and meta-analysis of soy products consumption in patients with type 2 diabetes mellitus," *Asia Pacific Journal of Clinical Nutrition*, vol. 20, no. 4, pp. 593–602, 2011.

[6] J. W. Anderson, B. M. Smith, and C. S. Washnock, "Cardiovascular and renal benefits of dry bean and soybean intake," *The American Journal of Clinical Nutrition*, vol. 70, no. 3, pp. 464–474, 1999.

[7] D. Goodman-Gruen and D. Kritz-Silverstein, "Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women," *Journal of Nutrition*, vol. 131, no. 4, pp. 1202–1206, 2001.

[8] L. E. Moeller, C. T. Peterson, K. B. Hanson et al., "Isoflavone-rich soy protein prevents loss of hip lean mass but does not prevent the shift in regional fat distribution in perimenopausal women," *Menopause*, vol. 10, no. 4, pp. 322–331, 2003.

[9] L. Azadbakht and S. Nurbakhsh, "Effect of soy drink replacement in a weight reducing diet on anthropometric values and blood pressure among overweight and obese female youths," *Asia Pacific Journal of Clinical Nutrition*, vol. 20, no. 3, pp. 383–389, 2011.

[10] N. Torres, I. Torre-Villalvazo, and A. R. Tovar, "Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders," *Journal of Nutritional Biochemistry*, vol. 17, no. 6, pp. 365–373, 2006.

[11] P. Deibert, D. König, A. Schmidt-Trucksaess et al., "Weight loss without losing muscle mass in pre-obese and obese subjects induced by a high-soy-protein diet," *International Journal of Obesity*, vol. 28, no. 10, pp. 1349–1352, 2004.

[12] C. R. Cederroth, M. Vinciguerra, F. Kühne et al., "A phytoestrogen-rich diet increases energy expenditure and decreases adiposity in mice," *Environmental Health Perspectives*, vol. 115, no. 10, pp. 1467–1473, 2007.

[13] G. H. Anderson and S. E. Moore, "Dietary proteins in the regulation of food intake and body weight in humans," *Journal of Nutrition*, vol. 134, no. 4, pp. 974S–979S, 2004.

[14] M. Galleano, O. Pechanova, and C. G. Fraga, "Hypertension, nitric oxide, oxidants, and dietary plant polyphenols," *Current Pharmaceutical Biotechnology*, vol. 11, no. 8, pp. 837–848, 2010.

[15] C. W. Xiao, "Health effects of soy protein and isoflavones in humans," *Journal of Nutrition*, vol. 138, no. 6, pp. 1244S–1249S, 2008.

[16] J. L. Fleiss, *The Design and Analysis of Clinical Experiments*, John Wiley&Sons, London, UK, 1986.

[17] A. H. Mokdad, E. S. Ford, B. A. Bowman et al., "Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001," *The Journal of the American Medical Association*, vol. 289, no. 1, pp. 76–79, 2003.

[18] K. R. Fontaine, D. Yang, G. L. Gadbury et al., "Results of soy-based meal replacement formula on weight, anthropometry, serum lipids & blood pressure during a 40-week clinical weight loss trial," *Nutrition Journal*, vol. 2, article 14, 2003.

[19] D. B. Allison, G. Gadbury, L. G. Schwartz et al., "A novel soy-based meal replacement formula for weight loss among obese individuals: a randomized controlled clinical trial," *European Journal of Clinical Nutrition*, vol. 57, no. 4, pp. 514–522, 2003.

[20] J. W. Anderson, J. Fuller, K. Patterson, R. Blair, and A. Tabor, "Soy compared to casein meal replacement shakes with energy-restricted diets for obese women: randomized controlled trial," *Metabolism*, vol. 56, no. 2, pp. 280–288, 2007.

[21] L. Kok, S. Kreijkamp-Kaspers, D. E. Grobbee, and Y. T. van der Schouw, "Soy isoflavones, body composition, and physical performance," *Maturitas*, vol. 52, no. 2, pp. 102–110, 2005.

[22] D. Goodman-Gruen and D. Kritz-Silverstein, "Usual dietary isoflavone intake and body composition in postmenopausal women," *Menopause*, vol. 10, no. 5, pp. 427–432, 2003.

[23] C. K. Sites, B. C. Cooper, M. J. Toth, A. Gastaldelli, A. Arabshahi, and S. Barnes, "Effect of a daily supplement of soy protein on body composition and insulin secretion in postmenopausal women," *Fertility and Sterility*, vol. 88, no. 6, pp. 1609–1617, 2007.

[24] M. T. Velasquez and S. J. Bhathena, "Role of dietary soy protein in obesity," *International Journal of Medical Sciences*, vol. 4, no. 2, pp. 72–82, 2007.

[25] S. J. Bhathena and M. T. Velasquez, "Beneficial role of dietary phytoestrogens in obesity and diabetes," *The American Journal of Clinical Nutrition*, vol. 76, no. 6, pp. 1191–1201, 2002.

[26] D. König, P. Deibert, I. Frey, U. Landmann, and A. Berg, "Effect of meal replacement on metabolic risk factors in overweight and obese subjects," *Annals of Nutrition and Metabolism*, vol. 52, no. 1, pp. 74–78, 2008.

[27] M.-P. St-Onge, N. Claps, C. Wolper, and S. B. Heymsfield, "Supplementation with soy-protein-rich foods does not enhance weight loss," *Journal of the American Dietetic Association*, vol. 107, no. 3, pp. 500–505, 2007.

[28] J. D. Wagner, M. J. Jorgensen, J. M. Cline et al., "Effects of soy vs. casein protein on body weight and glycemic control in female monkeys and their offspring," *The American Journal of Primatology*, vol. 71, no. 9, pp. 802–811, 2009.

[29] N. Vaughn, A. Rizzo, D. Doane, J. L. Beverly, and E. Gonzalez De Mejia, "Intracerebroventricular administration of soy protein hydrolysates reduces body weight without affecting food intake in rats," *Plant Foods for Human Nutrition*, vol. 63, no. 1, pp. 41–46, 2008.

[30] C. R. Cederroth and S. Nef, "Soy, phytoestrogens and metabolism: a review," *Molecular and Cellular Endocrinology*, vol. 304, no. 1-2, pp. 30–42, 2009.

[31] M. O. Weickert and A. F. H. Pfeiffer, "Metabolic effects of dietary fiber consumption and prevention of diabetes," *Journal of Nutrition*, vol. 138, no. 3, pp. 439–442, 2008.

[32] M. S. Miraghajani, M. M. Najafabadi, P. J. Surkan, A. Esmaillzadeh, M. Mirlohi, and L. Azadbakht, "Soy milk consumption and blood pressure among type 2 diabetic patients with nephropathy," *Journal of Renal Nutrition*, vol. 23, no. 4, pp. 277.e1–282.e1, 2013.

[33] W. Altorf-van der Kuil, M. F. Engberink, E. J. Brink et al., "Dietary protein and blood pressure: a systematic review," *PLoS ONE*, vol. 5, no. 8, Article ID e12102, 2010.

[34] A. N. C. Simão, M. A. B. Lozovoy, T. N. C. Simão, J. B. Dichi, T. Matsuo, and I. Dichi, "Nitric oxide enhancement and blood pressure decrease in patients with metabolic syndrome using soy protein or fish oil," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 54, no. 6, pp. 540–545, 2010.

[35] F. de Leo, S. Panarese, R. Gallerani, and L. R. Ceci, "Angiotensin converting enzyme (ACE) inhibitory peptides: Production and implementation of functional food," *Current Pharmaceutical Design*, vol. 15, no. 31, pp. 3622–3643, 2009.

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Clinical Study

Factors Associated with Utilization of Dipeptidyl-4 Inhibitors in Patients with Type 2 Diabetes Mellitus: A Cross-Sectional Retrospective Study

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Dipeptidyl-4 (DPP-4) inhibitors are oral antidiabetic agents recently introduced to Malaysia. Thus, limited data is available on their utilization patterns and factors associated with their use. This study aims to analyse the utilization patterns of DPP-4 inhibitors, factors that influenced the choice of agent, and the rationale for treatment with DPP-4 inhibitors in patients with type 2 diabetes mellitus. This retrospective study was conducted to address the utilization pattern of DPP-4 inhibitors and factors that influence choice in type 2 diabetes mellitus patients. 299 subjects taking either sitagliptin or vildagliptin from September 2008 to September 2012 were included in the study. Sitagliptin was more frequently prescribed than vildagliptin. Of the patients prescribed DPP-4 inhibitors, 95% received combinations of these and other agents, whereas only 5% were prescribed DPP-4 inhibitors as monotherapy. Factors affecting the utilization of DPP-4 inhibitors included age ($P = 0.049$) and concomitant use of beta blockers ($P = 0.045$) and aspirin ($P = 0.008$). Early identification of factors associated with DPP-4 inhibitors is essential to enhance quality use of the drugs.

1. Introduction

The International Diabetes Federation (IDF) has estimated that 371 million people worldwide had diabetes in 2012 and that 552 million will have this disease by 2030 [1]. Moreover, the costs involved in managing patients with diabetes worldwide were estimated to be approximately 471 billion USD. At least 90% of individuals with diabetes have type 2 diabetes mellitus (T2DM) [2]. In Malaysia, the prevalence of T2DM has increased markedly, from 11.6% of the population in 2006 to 15.2% in 2011, a relative increase of 31% over 5 years [3]. A 2007 report stated that antidiabetic medications were the second highest healthcare expenditures for medications in Malaysia [4].

According to the American Diabetes Association (ADA), management of T2DM involves a combination of life-style modifications and pharmacological approaches, consisting of oral antidiabetic (OAD) agents and insulin injection [5]. At present, six classes of OADs are used to treat patients with T2DM, with dipeptidyl peptidase-4 (DPP-4) inhibitors being one of the newer drug classes. DPP-4 inhibitors inhibit the degradation of the hormone GLP-1, which stimulates insulin release immediately after a meal. Currently available DPP-4 inhibitors include sitagliptin, vildagliptin, saxagliptin, alogliptin, and linagliptin, which differ in pharmacokinetic and pharmacodynamics profiles [6]. Generally, OADs have excellent safety profiles, with a low rate of adverse effects [7].

Sitagliptin was the first DPP-4 inhibitor approved for use in Malaysia to treat patients with T2DM [8]. At present, three DPP4 inhibitors are available for use in Malaysia: sitagliptin, saxagliptin, and vildagliptin [9], but their use is not widespread. These agents are more likely to be prescribed

to patients in private health institutions than in government hospitals and clinics [4]. Guidelines for the appropriate utilization of these agents include those of the National Institute for Health and Clinical Excellence (NICE) and the Scottish Medicine Consortium (SMC). However, there are no specific guidelines in Malaysia on the appropriate use of these drugs. Thus, it is important to determine factors that may influence physicians to prescribe DPP-4 inhibitors.

Little is known about the utilization pattern of DPP-4 inhibitors, both within Malaysia and in other countries. This study was, therefore, designed to determine the patterns of DPP-4 inhibitor utilization and factors that influence their utilization.

2. Materials and Methods

2.1. Sample Population. The study population consisted of all UMMC patients aged ≥18 years who were diagnosed with T2DM and had received sitagliptin or vildagliptin at any time from September 2008 to September 2012.

2.2. Study Procedures. This retrospective, cross-sectional study was performed in accordance with the Declaration of Helsinki and was approved by the medical ethics committee (MEC) of UMMC (reference number 956.29), which waived the requirement for written informed consent from the participants. Patients were included if they were aged ≥18 years, fulfilled the requirements of the ICD-10 code for T2DM (E11.0–E11.9), and were prescribed either sitagliptin or vildagliptin at any time from September 2008 to September 2012, either as monotherapy or in combination with other antidiabetic medications. Patients were excluded if they had been diagnosed with type 1 diabetes mellitus (T1DM), had never received any DPP4 inhibitor, or were diagnosed with a psychiatric illness that may compromise compliance with diabetic treatment.

Demographic and clinical parameters were collected for each eligible patient. Demographic parameters included age, gender, ethnicity, weight, height, and body mass index (BMI). Characteristics of T2DM included the year of diagnosis, HbA1c concentration, and either FPG or RPG concentration. Characteristics of T2DM treatment included class and name of drug and dosage and date of prescribed DPP-4 inhibitors.

Other clinical parameters included the number and type of comorbidities, with comorbidities defined as chronic diseases requiring long-term treatment; concurrent medications, including medications prescribed to manage comorbidities; renal impairment, defined as creatinine clearance <50 mL/min [10], and hepatic impairment, defined as chronic hepatitis, liver cirrhosis, or elevation of liver enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) to more than 3 times the upper limit of normal value.

2.3. Statistical Methods

2.3.1. Determination of Sample Size. The sample size required was calculated using Epi Info Program Version 7.0 (CDC, Atlanta, GA, USA). The level of significance, α, was set as 0.05 and the desired power of the study ($1-\beta$) was 80%. Assuming an expected proportion of T2DM patients of 20.8% and a confidence limit of 5%, the minimum number of patients required was 108.

All data extracted were analyzed using the Statistical Package for Social Sciences (SPSS) software version 20.0 (Armonk, New York, USA). All continuous data were tested for normality using the Kolmogorov-Smirnov test. Normally distributed parameters were expressed as mean ± standard deviation, and nonnormally distributed parameters were expressed as median and range. Categorical data were analyzed by Pearson's chi square test or Fisher's exact tests, as appropriate. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Study Subject's Disposition. Convenience sampling identified 370 eligible subjects, but the medical records for only 316 of these subjects (85.4%) could be successfully retrieved from the Medical Records Unit (MRU) of UMMC. Of these 316 patients, 17 were excluded for serious psychiatric illness. Thus, 299 patients were analyzed.

3.2. Demographic Characteristics. Of the 299 subjects, 133 (44.5%) were males and 166 (55.5%) were females. Ethnically, 103 subjects (34.4%) were Chinese, 99 (33.1%) were Malay, and 97 (32.4%) were Indian. Mean subject age was 63.1 ± 11.4 years (range, 22 to 89 years), with the Kolmogorov-Smirnov test showing a normal distribution by age. We found that 161 subjects (53.8%) were <65 years old and 138 (46.2%) were aged ≥65 years.

Height and weight were available for only 220 subjects (73.6%). BMI was not normally distributed in these 220 subjects, with median BMI of 26.9 kg/m^2 (range, 18.4 kg/m^2 to 49.3 kg/m^2). BMI was classified into four categories: underweight (<18.5 kg/m^2), normal weight (18.5–22.9 kg/m^2), overweight (23–27.4 kg/m^2), and obese (≥27.5 kg/m^2). Using these criteria, 99 subjects (33.1%) were overweight and 90 (30.1%) were obese (Table 1).

3.3. Clinical Characteristics. Information on duration of T2DM since diagnosis was available for only 220 (73.6%) patients. The Kolmogorov-Smirnov test showed that the duration of T2DM for these subjects was not normally distributed. The median duration was 13.5 years (range, 2 to 37 years). In most subjects (77.6%), the duration of T2DM was >10 years.

Information on HbA1c concentration was available for 266 (89%) of the 299 patients. HbA1c concentration in these 266 patients was not normally distributed. Median HbA1c concentration was 7.75% (range, 5.2% to 19.4%), with more than 89% of the subjects having HbA1c concentrations ≥6.5% (Table 2).

Of the 299 subjects, 255 (85.3%) were taking 5 or more concurrent medications. Hypertension and hyperlipidemia were the most frequent comorbidities, observed in 251 (83.9%) and 210 (70.2%) subjects, respectively, followed by

Table 1: Demographic characteristics of the patients.

Demographic characteristic	*N*	Number of patients (percentage, %)
Gender		
Male	299	133 (44.5)
Female	299	166 (55.5)
Age		
Nonelderly	299	161 (53.8)
Elderly	299	138 (46.2)
Ethnicity		
Malay	299	99 (33.2)
Chinese	299	103 (34.4)
Indian	299	97 (32.4)
BMI		
Underweight	220	1 (0.4)
Normal body weight	220	30 (13.6)
Overweight	220	99 (45.0)
Obese	220	90 (41.0)

Table 2: Clinical characteristics of the patients.

Clinical characteristics	*N*	Number of patients (percentage, %)
Duration since the diagnosis of T2DM		
≤10 years	220	67 (22.4)
11–20 years	220	98 (32.8)
21–30 years	220	46 (15.4)
>30 years	220	9 (3.0)
HbA1c		
Less than 6.5%	266	29 (10.9)
6.5% or more	266	237 (89.1)
Number of concurrent medication		
No polypharmacy	299	44 (14.7)
Polypharmacy	299	255 (85.3)

obesity, observed in 86 subjects (28.8%). Not surprisingly, the most frequently prescribed concurrent medications were HMG-CoA inhibitors and salicylates, prescribed to 251 (83.9%) and 131 (43.8%) subjects, respectively. Diuretics included thiazides (27.8%), furosemide (11.0%), and spironolactone (2.3%).

3.4. Pattern of Use of DPP-4 Inhibitors. Sitagliptin was the DPP-4 inhibitor most frequently utilized at UMMC, prescribed to 86% of patients, with vildagliptin prescribed to the other 14%. The most frequent doses of sitagliptin were 100 mg OD (58.7%) and 50 mg OD (28.4%). The combination of sitagliptin/metformin in one tablet was prescribed to 50% of patients, with 28.6% taking a dosage regimen of 50/850 mg OD and 21.4% taking 50/500 mg OD. Vildagliptin was available only as 50 mg tablets, with most patients taking this dose OD. The two fixed dose combinations of vildagliptin/metformin, both given twice daily, were equally preferred (36.8%).

Both DPP-4 inhibitors were administered in combination with other antidiabetic medications. Only 4.3% of patients were prescribed sitagliptin monotherapy and only 2.4% were prescribed vildagliptin monotherapy. Combinations of DPP-4 inhibitors with metformin and sulfonylureas were prescribed to 224 (74.9%) and 209 (69.9%) of the subjects, respectively. Fewer DPP-4 treated patients were also treated with thiazolidinediones (TZD), meglitinides, insulin, and acarbose.

Table 3: Association between age and use of DPP-4 inhibitors.

Agent	*n*	Age	
		Nonelderly	Elderly
Sitagliptin	257	132 (51.4%)	125 (48.6%)
Vildagliptin	42	29 (69.0%)	13 (31.0%)

Chi square = 3.860, df = 1, $P = 0.049$.

Table 4: Association between use of a DPP-4 inhibitor and a beta blocker.

Agent	*n*	Beta blocker	
		Yes	No
Sitagliptin	257	86 (33.5%)	171 (66.5%)
Vildagliptin	42	7 (16.6%)	35 (83.4%)

Chi square = 4.001, df = 1, $P = 0.045$.

Table 5: Association between use of a DPP-4 inhibitor and aspirin.

Agent	*n*	Aspirin	
		Yes	No
Sitagliptin	257	121 (47.1%)	136 (52.9%)
Vildagliptin	42	10 (23.8%)	32 (76.2%)

Chi square = 7.025, df = 1, $P = 0.008$.

3.5. Factors Significantly Associated with Utilization of DPP-4 Inhibitors

3.5.1. Age. The majority of patients taking either sitagliptin or vildagliptin were aged <65 years. A significant association between age and DPP-4 inhibitor use was observed ($P = 0.049$) (Table 3).

3.5.2. Concurrent Medications

(1) Beta Blockers. Of the 299 subjects, 93 (31.1%) were taking a beta blocker along with a DPP-4 inhibitor. A significant association was observed between utilization of DPP-4 inhibitors and beta blockers ($P = 0.045$) (Table 4).

(2) Aspirin. Of the 299 patients, 131 (43.8%) were taking aspirin concomitantly. Aspirin use was significantly associated with prescription of DPP-4 inhibitors (Table 5). Table 6

Table 6: Parameters not significantly associated with the use of DPP-4 inhibitors.

Patient characteristic	Number of patients (percentage, %)		P value
	Sitagliptin (n = 257)	Vildagliptin (n = 45)	
Gender			
Male	111 (43.2%)	22 (52.4%)	0.345[a]
Female	146 (56.8%)	20 (47.6%)	
Ethnicity			
Malay	85 (33.1%)	14 (33.3%)	
Chinese	85 (33.1%)	18 (42.9%)	0.345[a]
Indian	87 (33.9%)	10 (23.8%)	
BMI			
Underweight	0 (0%)	1 (4.0%)	
Normal weight	28 (14.4%)	2 (8.0%)	0.160[a]
Overweight	87 (44.6%)	12 (48.0%)	
Obese	80 (41.0%)	10 (40.0%)	
Duration since the diagnosis of T2DM			
≤10 years	58 (29.6%)	9 (37.5%)	
11–20 years	88 (44.9%)	10 (41.7%)	0.661[a]
21–30 years	41 (20.9%)	5 (20.8%)	
>30 years	9 (4.6%)	0 (0.0%)	
A1c			
<6.5%	25 (10.7%)	4 (12.5%)	0.762[b]
≥6.5%	209 (89.3%)	28 (87.5%)	
Polypharmacy			
<5 drugs	38 (14.8%)	6 (13.3%)	1.000[a]
≥5 drugs	219 (85.2%)	36 (86.7%)	
Renal impairment	70 (27.2%)	8 (19.0%)	0.352[a]
Hepatic impairment	6 (2.3%)	0 (0%)	1.000[b]
Heart disease	77 (29.9%)	9 (21.4%)	0.343[a]
Obesity	77 (29.9%)	9 (21.4%)	0.343[a]
Hypertension	219 (85.2%)	32 (76.19%)	0.211[a]
Hyperlipidemia	180 (70.0%)	30 (71.4%)	1.000[a]
Metformin	188 (73.1%)	36 (85.7%)	0.121[a]
Sulphonylurea	179 (69.6%)	30 (71.4%)	0.959[a]
Acarbose	34 (13.2%)	7 (16.6%)	0.720[a]
Thiazolidinedione	1 (0.39%)	0 (0%)	1.000[b]
Meglitinides	1 (0.39%)	1 (2.3%)	0.262[b]
Insulin	65 (25.3%)	5 (11.9%)	0.089[a]
ACE inhibitor	88 (34.2%)	19 (45.2%)	0.228[a]
ARB	95 (36.9%)	11 (26.1%)	0.238[a]
CCB	111 (43.2%)	19 (45.2%)	0.936[a]
Thiazide	74 (28.8%)	9 (21.4%)	0.422[a]
Loop diuretics	31 (12.0%)	2 (4.7%)	0.194[b]
Spironolactone	7 (2.7%)	0 (0%)	0.599[b]
Statin	215 (83.6%)	36 (85.7%)	0.912[a]
Fibrates	27 (10.5%)	4 (9.5%)	1.000[b]

[a]By Pearson chi square.
[b]By Fisher's exact test.

shows parameters not significantly associated with the use of DPP-4 inhibitors.

4. Discussion

4.1. Clinical Characteristics. We found that the time from diagnosis of T2DM to treatment with DPP-4 inhibitors was longer than 10 years in 69% of the subjects, with a median duration of 13.5 years, longer than the mean 10.8 years and 9.27 years reported in earlier studies [11, 12]. However, the duration of T2DM could not be determined in 79 of the 299 (26.4%) subjects, which may have altered the median duration in the entire cohort.

Glycemic control, as indicated by HbA1c level, was poorer in patients treated at UMMC than in other studies. We found that 13% of subjects assessed for HbA1c had HbA1c levels <6.5%, similar to findings showing that 11.4% and 11.6% of patients with T2DM had HbA1c levels <6.5% [13, 14]. In contrast, a cross-sectional survey reported blood glucose control (HbA1c < 6.5%) in 18% of patients, but HbA1c concentrations were measured in only 52.6% of that patient cohort [15].

Hypertension was the most frequent comorbidity in our patient population, followed by hyperlipidemia, obesity, and heart problems. A previous study reported similar results for hypertension and hyperlipidemia, but since overweight and obesity were pooled, a comparison with our results was impossible [15]. In addition, Mafauzy reported that hypertension was the most common comorbid condition in diabetic patients [16]. The Malaysian NHMS IV conducted in 2011 reported that hyperlipidemia (35.1%) and hypertension (32.7%) were the most prevalent noncommunicable diseases [17]. However, the NHMS was a survey of the entire population, not only of patients with T2DM.

We found that more than 85% of the subjects in this study were taking five or more medications (polypharmacy) and that older age was significantly associated with polypharmacy ($P = 0.000$). HMG-CoA inhibitors (statins) were the most frequently drug class prescribed concomitantly to these subjects. More than 97% of our subjects were aged ≥40 years and were given statins regardless of baseline LDL concentration [18]. Moreover, a local study performed at a university primary care center reported that statins (69%) were the class of drugs most frequently prescribed concomitantly to diabetic patients [16]. The same study reported that 33% of patients were prescribed salicylate, likely because of the relatively low percentage of patients with coronary artery disease (9.9%) and stroke (5.2%). A cross-sectional study found that cardiovascular drugs were the most frequently prescribed to 27.3% of patients [19].

We found that the four leading classes of antidiabetic agents prescribed to our T2DM patients were metformin, sulphonylureas, insulin, and acarbose, which were given to 224 (74.9%), 209 (69.9%), 70 (23.4%), and 41 (13.7%) subjects, respectively. This finding is in good agreement with a previous study showing that the four classes of drugs most prescribed for patients with T2DM were metformin (84%), sulphonylureas (81%), insulin (16%), and acarbose (8%) [16].

4.2. Utilization Pattern of DPP-4 Inhibitors by T2DM Patients. Two DPP-4 inhibitors were used to treat T2DM patients at UMMC, sitagliptin and vildagliptin. More than 85% of our patients were prescribed sitagliptin, whereas only 15% received vildagliptin, similar to findings showing that 57 of 66 subjects (86.4%) prescribed a DPP-4 inhibitor were taking sitagliptin [20]. At the time of this study, these two agents were the only DPP-4 inhibitors available at UMMC, although, currently, saxagliptin and linagliptin are also available in Malaysia. Sitagliptin was the first DPP-4 inhibitor approved for use in Malaysia in June 2007 [9]. Although vildagliptin has been licensed for use in Malaysia, it has not yet been approved by the U.S. FDA. Sitagliptin and vildagliptin are comparable clinically, both in effectiveness and incidence of hypoglycemia [21]. Sitagliptin is available as a single drug, at doses of 25 mg, 50 mg, and 100 mg, and in combination with metformin, at a dose of 50 mg. Vildagliptin is available at one dosage (50 mg), both as a single drug and in combination with metformin. Assessment of dosage regimens of sitagliptin monotherapy found that 100 mg once daily was the preferred dosage, administered to 58.7% of patients, in line with dosage recommended by the Malaysian Clinical Practice Guideline on Management of Type 2 Diabetes Mellitus [22]. In contrast, 50% of subjects taking sitagliptin/metformin were not prescribed the dosage recommended in the guidelines.

The Malaysian Drug Formulary for 2013 recommends that the dose of vildagliptin be 50 mg BD if taken with metformin and 50 mg OD if taken with sulphonylureas [9]. When combined with metformin, the Formulary recommends a maximum daily dose of 100 mg vildagliptin and 2000 mg metformin. Most of the subjects in this study prescribed vildagliptin were given appropriate dosage regimens.

Few studies to date have assessed patterns of utilization of DPP-4 inhibitors or of individual agents. Thus, our results can only be compared with general utilization patterns of OAD agents. A study of patients hospitalized at a tertiary care referral hospital found that the most widely used OAD was biguanides (23%), followed by sulphonylureas (22.5%) and thiazolidinediones (11%) [23]. Only 9.5% of those patients were treated with DPP-4 inhibitors.

Combinations of DPP-4 inhibitors with metformin and sulphonylureas were the most popular, followed by combinations with insulin. Sitagliptin can be prescribed as monotherapy as well as in combination with metformin, a sulphonylurea, or a thiazolidinedione [9, 22], although taking these combinations may enhance the risk of hypoglycemia. Guidelines have recommended that vildagliptin be used as second-line therapy in combination with either metformin or sulphonylureas whenever the latter two agents are not sufficient to provide glycemic control or are not tolerated [9, 24].

4.3. Factors Significantly Associated with the Use of DPP-4 Inhibitor in Type 2 Diabetes Mellitus

4.3.1. Age. We found that age was weakly associated with the utilization of DPP-4 inhibitors with patients aged ≥65 years more likely to be given DPP-4 inhibitors ($P = 0.049$). The

Malaysian Drug Formulary 2013 has recommended the use of sitagliptin in T2DM patients, especially in elderly patients with multiple comorbidities who frequently experience hypoglycemia while on other OADs. Vildagliptin has been recommended for T2DM patients with poor glycemic control on the maximal tolerated dose of metformin monotherapy and at high risk of hypoglycemia [9]. Sitagliptin and vildagliptin were well tolerated by elderly patients and are associated with a low risk of hypoglycemia, as well as having similar efficacy in both older and younger patients [25]. However, the association between these variables was very weak, which may be due to the limited use of DPP-4 inhibitors in elderly individuals. These drugs are rather costly, limiting their availability to older individuals. Indeed, we found that the majority of patients in our study (53.8%) were nonelderly.

4.3.2. Concurrent Medications. This study found significant associations between the use of DPP-4 inhibitors and the concomitant use of beta blockers ($P = 0.045$). Most subjects taking sitagliptin and vildagliptin were not prescribed beta blockers, in line with recommendations that beta blockers are prescribed to T2DM patients with hypertension only when alternative agents cannot be used or when there are concomitant compelling indications (e.g., effort angina, tachyarrhythmias, and previous myocardial infarction) [18]. The effects of beta blockers in patients with T2DM are nonselective and may include masking the early symptoms of hypoglycemia and slowing recovery from hypoglycemia attacks [18]. Nevertheless, three beta blockers, bisoprolol, carvedilol, and metoprolol succinate, have been recommended for T2DM patients with heart failure. Indeed, an observational study in patients with T2DM and systolic heart failure found that carvedilol and bisoprolol significantly improved glycemic control [26].

The concurrent use of aspirin was found to be negatively associated with the use of DPP-4 inhibitors ($P = 0.008$), in that less aspirin was prescribed to patients taking either sitagliptin or vildagliptin. This may be due to the relatively low prevalence of comorbidities that warrant the use of aspirin as treatment or prophylaxis. The three most common comorbidities in our patient cohort were hypertension, hyperlipidemia, and obesity, all three of which do not require treatment or prophylaxis with aspirin [18, 27, 28]. Nevertheless, aspirin was the second most frequent drug prescribed to our subjects, being taken by more than 43%. This was likely due to the high proportion of our patients with ischemic heart disease (IHD) and cerebrovascular accidents (CVA).

4.4. Factors Not Significantly Associated with Utilization of DPP-4 Inhibitors

4.4.1. Duration of Diabetes. As for duration since the diagnosis of T2DM, both sitagliptin and vildagliptin had a higher proportion of subjects that had the duration since diagnosis of T2DM of more than 10 years. Despite this, the association failed to achieve significance level of $P < 0.05$ ($P = 0.661$). Besides, there was no literature found that studies the effect of these factors on prescribing of DPP-4 inhibitors. There was also no guideline found that advocates the use of DPP-4 inhibitors according to the duration of T2DM diagnosis. Therefore, it reflects that duration since the diagnosis of T2DM did not influence the prescriber in prescribing DPP-4 inhibitors.

4.4.2. HbA1C. World Health Organization has announced in 2011 that the new level of HbA1c <6.5% is the new cut-off point for diagnosing diabetes [29]. Formerly, it was agreed that the level of HbA1c was less than 7% as a diagnostic criterion. HbA1c level is used as an indicator that reflects the glycemic control for the past 2-3 months. An individual with A1c of less than 6.5% is considered to have achieved good glycemic control. In this study, the same parameter with the newly recommended cut-off point was used. Subjects on both sitagliptin and vildagliptin had a poor glycemic control with A1c level equal to or more than 6.5%. However, when tested for association, this parameter failed to be associated significantly with the use of DPP-4 inhibitors ($P = 0.995$). Hence, glycemic control, as demonstrated by A1c level, was not a determinant in prescribing DPP-4 inhibitors.

4.4.3. BMI. As for the BMI, most of the overweight and obese patients were taking sitagliptin and vildagliptin. There were nonsignificant associations between utilisation of sitagliptin and BMI. According to the Malaysian guidelines, the first-line treatment option for obese type 2 diabetes patients is metformin [22]. As other oral antidiabetic agents are acceptable alternatives to metformin, other OADs inclusive DPPIV inhibitors will be used in a case of metformin intolerability/ineffectiveness and contraindicated in overweight and obese patients. Therefore, DPPIV inhibitors would not be selected by the prescribers as the first-line treatment in overweight and obese patients. Metformin will still be the mainstay treatment for these patients.

5. Conclusions

Factors identified as significantly associated with prescribing DPP-4 inhibitors in this study included patient age and concurrent use of beta blockers and aspirin. Identification of factors underlying the use of DPP-4 inhibitors may enhance rational use of drugs and, thus, diabetes care in T2DM patients.

6. Limitation of Study

This study had several limitations, including its retrospective design. Only the subject characteristics affecting the prescribing of DPP-4 inhibitors were analyzed. In contrast, physician-associated factors were not assessed. In addition, since all information came from patient's medical records, errors may be caused by the absence of information or incorrect information.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hasniza Zaman Huri and Shireene Ratna Vethakkan have made substantial contributions to the conception and design of the study. Hasniza Zaman Huri has been involved in acquisition of data, analysis and interpretation of data, and drafting the paper or revising it critically for important intellectual content. NorFarahen Selamat has been involved in acquisition of data and analysis and interpretation of data. Hasniza Zaman Huri, NorFarahen Selamat, and Shireene Ratna Vethakkan have given final approval for the version to be published.

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References

[1] International Diabetes Federation, *IDF Diabetes Atlas Update 2012*, 2012, http://www.idf.org/sites/default/files/EN_6E_Atlas_ Full_0.pdf.

[2] International Diabetes Federation, 2012, http://www.idf.org/ types-diabetes.

[3] Ministry of Health Malaysia, *The Future of Diabetes in Malaysia*, Malaysian Diabetes Educators Society Seminar, 2012.

[4] Pharmaceutical Services Division and the Clinical Research Centre, "Malaysian Statistics on Medicine," 2007, http://www .pharmacy.gov.my/v2/sites/default/files/document-upload/ msom2007.pdf.

[5] American Diabetes Association, "Standard of medical care in diabetes," *Diabetes Care*, vol. 36, no. 1, pp. S11–S66, 2013.

[6] H. Duez, B. Cariou, and B. Staels, "DPP-4 inhibitors in the treatment of type 2 diabetes," *Biochemical Pharmacology*, vol. 83, no. 7, pp. 823–832, 2012.

[7] R. Pathak and M. B. Bridgeman, "Dipeptidyl peptidase-4 (DPP-4) inhibitors in the management of diabetes," *Pharmacy and Therapeutics*, vol. 35, no. 9, pp. 509–513, 2010.

[8] Malaysian Dia betes Association, "A New Approach to Type 2 Diabetes Drug Treatment is Now Available in Malaysia," 2007, http://www.diabetes.org.my/article.php?aid=202.

[9] Ministry of Health Malaysia, *Drug Formulary*, Ministry of Health Malaysia, 2013.

[10] A. J. Bergman, J. Cote, B. Yi et al., "Effect of renal insufficiency on the pharmacokinetics of sitagliptin, a dipeptidyl peptidase-4 inhibitor," *Diabetes Care*, vol. 30, no. 7, pp. 1862–1864, 2007.

[11] M. Mafauzy, "Diabetes control and complications in public hospitals in Malaysia," *Medical Journal of Malaysia*, vol. 61, no. 4, pp. 477–483, 2006.

[12] M. Eid, M. Mafauzy, and A. R. Faridah, "Glycaemic control of type 2 diabetic patients on follow up at Hospital Universiti Sains Malaysia," *The Malaysian Journal of Medical Sciences*, vol. 10, no. 2, pp. 40–49, 2003.

[13] M. Mafauzy, Z. Hussein, and S. P. Chan, "The status of diabetes control in Malaysia: results of DiabCare 2008," *Medical Journal of Malaysia*, vol. 66, no. 3, pp. 175–181, 2011.

[14] B. H. Chew, I. Mastura, P. Y. Lee et al., "Ethnic differences in glycaemic control and complications: the adult diabetes control and management (ADCM), Malaysia," *Medical Journal of Malaysia*, vol. 66, no. 3, p. 245, 2011.

[15] J. E. Shaw, R. A. Sicree, and P. Z. Zimmet, "Global estimates of the prevalence of diabetes for 2010 and 2030," *Diabetes Research and Clinical Practice*, vol. 87, no. 1, pp. 4–14, 2010.

[16] M. Mafauzy, "Diabetes control and complications in private primary healthcare in Malaysia," *Medical Journal of Malaysia*, vol. 60, no. 2, pp. 212–217, 2005.

[17] Malaysian Ministry of Healt, "National Health Morbidity Survey IV Kuala Lumpur," Institute of Public Health Malaysia, 2012.

[18] Ministry of Health Malaysia, Malaysian Society of Hypertension, and Academy of Medicine, *Clinical Practice Guideline. "Management of Hypertension"*, Ministry of Health Malaysia, Kuala Lumpur, Malaysia, 3rd edition, 2008.

[19] I. Truter, "An investigation into antidiabetic medication prescribing in South Africa," *Journal of Clinical Pharmacy and Therapeutics*, vol. 23, no. 6, pp. 417–422, 1998.

[20] C. H. Po and A. Todd, "Drug utilization review of dipeptidyl peptidase-4 (DPP-4) inhibitors in Alice Ho Miu Ling Nethersole Hospital (AHNH)," in *Proceedings of the Hong Kong Pharmacy Conference*, 2012.

[21] B. Ahrén, "Clinical results of treating type 2 diabetic patients with sitagliptin, vildagliptin or saxagliptin—diabetes control and potential adverse events," *Best Practice & Research: Clinical Endocrinology & Metabolism*, vol. 23, no. 4, pp. 487–498, 2009.

[22] Ministry of Health Malaysia/Malaysian Endocrine & Metabolic Society/Academy of Medicine & Malaysian Diabetes Association: Clinical Practice Guideline, *Management of Type 2 Diabetes Mellitus*, Ministry of Health Malaysia, Kuala Lumpur, Malaysia, 4th edition, 2009.

[23] A. Khalam, C. Dilip, and C. Shinu, "Drug use evaluation of diabetes mellitus in hospitalized patients of a tertiary care referral hospital," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 23, no. 4, pp. 173–177, 2012.

[24] National Institute for Health and Clinical Excellence (NICE), *Type 2 Diabetes: The Management of Type 2 Diabetes*, 2009, http:// www.nice.org.uk/nicemedia/pdf/CG66NICEGuideline.pdf.

[25] S. L. Schwartz, "Treatment of elderly patients with type 2 diabetes mellitus: a systematic review of the benefits and risks of dipeptidyl peptidase-4 inhibitors," *The American Journal Geriatric Pharmacotherapy*, vol. 8, no. 5, pp. 405–418, 2010.

[26] B. Wai, L. G. Kearney, D. L. Hare, M. Ord, L. M. Burrell, and P. M. Srivastava, "Beta blocker use in subjects with type 2 diabetes mellitus and systolic heart failure does not worsen glycaemic control," *Cardiovascular Diabetology*, vol. 11, no. 1, pp. 1–5, 2012.

[27] Ministry of Health Malaysia, *Malaysian Society for The Study of Obesity Clinical Practice Guideline*, Management of Obesity Ministry of Health Malaysia, Kuala Lumpur, Malaysia, 2004.

[28] Ministry of Health Malaysia, and National Heart Association of Malaysia & Academy of Medicine, *Clinical Practice Guideline: Management of Dyslipidemia (4th Ed)*, Ministry of Health Malaysia, Kuala Lumpur, Malaysia, 2011.

[29] World Health Organization & International Diabetes Association, *Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus*, WHO, Geneva, Switzerland, 2011.

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Review Article

A Review on the Relationship between SGLT2 Inhibitors and Cancer

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Risk of increasing breast and bladder cancer remains a safety issue of SGLT2 (sodium glucose cotransporter type 2) inhibitors, a novel class of antidiabetic agent. We reviewed related papers published before January 29, 2014, through Pubmed search. Dapagliflozin and canagliflozin are the first two approved SGLT2 inhibitors for diabetes therapy. Although preclinical animal toxicology did not suggest a cancer risk of dapagliflozin and overall tumor did not increase, excess numbers of female breast cancer and male bladder cancer were noted in preclinical trials (without statistical significance). This concern of cancer risk hindered its approval by the US FDA in January, 2012. New clinical data suggested that the imbalance of bladder and breast cancer might be due to early diagnosis rather than a real increase of cancer incidence. No increased risk of overall bladder or breast cancer was noted for canagliflozin. Therefore, the imbalance observed with dapagliflozin treatment should not be considered as a class effect of SGLT2 inhibitors and the relationship with cancer for each specific SGLT2 inhibitor should be examined individually. Relationship between SGLT2 inhibition and cancer formation is still inconclusive and studies with larger sample size, longer exposure duration, and different ethnicities are warranted.

1. Introduction

The prevalence of type 2 diabetes mellitus is increasing globally with time [1, 2]. According to an epidemiological study with 370 country years and 2.7 million participants, prevalence of diabetes in adults aged 25 years and older was 9.8% in men and 9.2% in women in 2008, leading to an estimated 173 million men and 173 million women with diabetes [1]. Intensive glycemic control was associated with significantly decreased rates of microvascular and neuropathic complications in type 2 diabetic patients [3, 4]. More intensive glycemic control in newly diagnosed type 2 diabetic patients may reduce long-term cardiovascular disease rates [5]. Along with lifestyle modification, medications with different mechanisms may be needed to achieve glycemic goal.

SGLT2 (sodium glucose cotransporter type 2) inhibitor is a novel class of antidiabetic agent acting independent of effects on insulin resistance or insulin insufficiency. Glucose filtered through the kidneys is reabsorbed into the blood in the proximal tubules via SGLT1 and SGLT2 [6]. SGLT2 accounts for 90% and SGLT1 accounts for 10% of the glucose reabsorbed from the kidneys [7, 8]. SGLT2 is expressed mainly in the kidney cortex [9]. Beside the kidney, SGLT1 is mainly expressed in small intestine and also in trachea, heart, and plasma membranes [6, 9]. Blocking these transporters in the kidney inhibits renal glucose reabsorption and may lower blood glucose levels in diabetic patients. However, nonselective inhibition of SGLT1 and SGLT2 can cause severe diarrhea [10, 11]. Oral agents with high selectivity of SGLT2 have been developed to overcome the shortcoming [11]. Use of selective SGLT2 inhibitors significantly lowers HbA1c levels by 0.5–1.5% without hypoglycemia [12–17]. Use of SGLT inhibitors also showed significant weight loss and reduction of systemic blood pressure [13, 14, 16–18]. Increased genital

Table 1: SGLT2 inhibitors under phase II and phase III clinical trials.

Drug	Company	Phase of clinical trial
Dapagliflozin (BMS-512148)	Bristol-Myers Squibb/AstraZeneca	Phase III (approved in Australia (Oct 2012), Europe (Nov 2012), Mexico (Mar 2013), New Zealand (June 2013), Brazil (July 2013), Argentina (Sep 2013), US (Jan 2014), and Japan (Mar 2014))
Canagliflozin (TA-7284, JAJ-28431754)	Johnson and Johnson and Mitsubishi Tanabe Pharma	Phase III (approved in US (Mar 2013) and Europe (Nov 2013))
Empagliflozin (BI-10773)	Boehringer Ingelheim/Lilly	Phase III (approved in Europe (May 2014))
Ipragliflozin (ASP-1941)	Astellas Pharma and Kotobuki Pharmaceutical Company	Phase III (approved in Japan (Jan 2014)
Tofogliflozin (CSG452)	Roche/Chugai	Phase III
Luseogliflozin (TS-071)	Taisho	Phase III
BI 44847	Boehringer Ingelheim	Phase III
LX4211	Lexicon Pharmaceutical	Phase II
PF-04971729	Pfizer	Phase II
EGT0001442	Theracos	Phase II
GW 869682	GlaxoSmithKline	Phase II

From [17, 19].

infection and possibly increased urinary tract infection were noticed but rarely caused drug discontinuation [7, 11, 16, 17, 19].

Currently, more than ten SGLT2 inhibitors are under development [17, 19]. SGLT2 inhibitors under phase II and III clinical trials are listed in Table 1. Dapagliflozin was the first SGLT2 inhibitor approved in the world. It was approved in Europe in November 2012 and in USA in January 2014. Dapagliflozin should be given at an initial dose of 5 mg per day and may be increased to 10 mg per day and it is not recommended in patients with limited renal function (eGFR < 60 mL/minute/1.73 m^2) [20]. Canagliflozin was approved in Europe and in USA in 2013. It should be given at an initial dose of 100 mg per day and may be increased to 300 mg per day in a once daily dose. Canagliflozin was not recommended in patients with severe renal impairment and end stage renal disease [14].

Patients with diabetes are at an increased risk of bladder cancer compared with those without diabetes [21–26]. Use of some antidiabetic agents (e.g., pioglitazone) might be associated with increased risk of bladder cancer [27–33] and some (e.g., metformin) were associated with lower risk [34]. An imbalance of male bladder cancer and female breast cancer was observed in the treatment arms of dapagliflozin [35]. This ever hindered the approval of dapagliflozin by the FDA in January, 2012. Therefore, it is important to clarify the cancer risk associated with the use of SGLT2.

Publications before January 29, 2014, were searched in the Pubmed using key words including "SGLT1 and cancer," "SGLT2 and cancer," "canagliflozin and cancer," "dapagliflozin and cancer," "empagliflozin and cancer," "ipragliflozin and cancer," "tofogliflozin and cancer," "luseogliflozin and cancer," and "BI 44847 and cancer." Except for the two approved SGLT2 inhibitors (i.e., dapagliflozin and canagliflozin), the incidence of cancer for the other SGLT2 inhibitors was not available. Hence, the present review will focus on the cancer risk associated with dapagliflozin and canagliflozin.

2. Dapagliflozin

Among phase 2b and phase 3 trials presented to FDA in 2011, event rates for male bladder cancer and female breast cancer in the active treatment arms of dapagliflozin exceeded the rates expected in an age-matched reference of diabetic population [35]. However, the imbalance was not statistically significant.

More updated data until November, 2013, were gathered through 21 phase 2b and phase 3 trials [20]. The incidence rate ratios of malignancy by tumor type compared to dapagliflozin and nondapagliflozin groups were listed in Table 2. There was no overall imbalance of malignancies [20]. Though not significant, some tumor types (such as bladder cancer and female breast cancer) were more common while others (such as renal tract cancer) were less common in the dapagliflozin group.

2.1. Bladder Cancer. At the time of the FDA advisory committee meeting in 2011, 9 cases of bladder cancer were reported on dapagliflozin out of 5,478 patients and one was reported on control out of 3,156 patients among 14 phase 2b and 3 clinical trials [35].

Updated data presented in November, 2013, were gathered from 21 phase 2b and 3 clinical trials of dapagliflozin. With 2000 additional patient-years of exposure compared to those in 2011, no additional case of bladder cancer was identified. All previously detected cases of bladder cancer were males. Subsequent follow-up after the integrated database was locked, a new case of bladder cancer was reported in a female patient who participated in an ongoing add-on sulfonylurea and metformin study [20]. This additional female patient of bladder cancer was a smoker who had hematuria at baseline, and the bladder cancer was diagnosed only 3.5 months after initiation of treatment [20].

Till November, 2013, a total of 10 cases of bladder cancer out of 6,045 patients (0.17%) were noted in the dapagliflozin

Table 2: Incidence rate ratio of malignancy by tumor type comparing dapagliflozin versus nondapagliflozin in 21 phase 2b and phase 3 clinical trials (dapagliflozin $N = 5936$ and control $N = 3403$).

Tumor origin	Patients with events	Incidence rate ratio	95% confidence interval
Overall	140	1.03	0.71, 1.51
Bladder	10	5.17	0.68, 233.55
Breast (female only)	15	2.47	0.64, 14.10
Pancreas	8	1.84	0.31, 19.46
Prostate (male only)	17	1.50	0.53, 5.35
Hepatobiliary	3	0.92	0.04, 61.49
Thyroid and endocrine	10	0.88	0.19, 4.46
Skin	31	0.83	0.37, 1.91
Respiratory and mediastinal	15	0.79	0.24, 2.81
Female reproductive (female only)	4	0.74	0.05, 10.74
Metastases and site unspecified	5	0.56	0.07, 8.96
Gastrointestinal	10	0.51	0.13, 3.19
Renal tract	5	0.40	0.03, 3.82
Blood/lymphatic	7	0.37	0.05, 2.35
Musculoskeletal and soft tissue	1	∞	0.010, ∞

Source: reference [20, 35]: data obtained from 21 phase 2b and 3 clinical trials, data cutoff date: Nov 4, 2013; a new case of bladder cancer found in an ongoing add-on to sulfonylurea and metformin phase 3 trial is not included. The incidence rate ratio with 95% CI including the case is 6.11 (0.827–272.00).

treatment group compared to 1 case of bladder cancer out of 3,512 patients (0.03%) in the placebo arms [20]. All 10 cases of bladder cancer were reported within 2 years of starting dapagliflozin. All but one showed hematuria, which was the first clinical sign of bladder cancer, within 6 months of starting treatment [20]. The incidence rate remained stable over the first 2 years of drug exposure then fell, with no additional case detected between 2–4 years of exposure. The characteristics of bladder cancer differed from low grade to high grade and from noninvasive to widely metastatic [20]. The biological heterogeneity argued against a single triggering cause for the cancer [20].

A recently published study by Reilly et al. disclosed that, in mice and rats, exposure to dapagliflozin for up to 2 years at greater than a 100-fold human clinical exposure did not increase tumor incidence or urinary bladder proliferative/preneoplastic lesions [36]. In their study, dapagliflozin and its primary metabolite did not affect *in vitro* transitional cell carcinoma (TCC) proliferation rates and dapagliflozin did not enhance tumor growth in nude mice heterotopically implanted with human bladder TCC cell lines [36].

Therefore, the clinical observation of imbalance in bladder cancer associated with dapagliflozin was not supported by animal or *in vitro* studies, and the early diagnosis of bladder cancer might be due to detection bias rather than true causal relationship.

2.2. Breast Cancer. In an animal carcinogenicity study, dapagliflozin was administered to Sprague-Dawley rats up to 186-fold human exposure for 90 weeks in males and 105 weeks in females [36]. No mammary tumor was noted in the male rats during study. In the female rats, the most common causes of death in treatment and control groups were benign and malignant mammary and pituitary tumors. However, there was no dapagliflozin related increase in the incidence of these tumors and there was no difference in the time to onset of the tumors [36].

In the data presented in the FDA advisory committee meeting in 2011, 9 cases of breast cancer out of 2,223 female patients (0.4%) were noted in the dapagliflozin treatment group compared to 1 case out of 1,053 female patients (0.09%) in the placebo group [35, 37].

Till November, 2013, the total number of breast cancer cases on dapagliflozin was 12 and was 3 for the control group [20]. Exposure adjusted incidence rate was 0.40 per 100 patient-years (95% CI = 0.21, 0.70) on dapagliflozin and 0.19 (95% CI = 0.04, 0.56) in the control group [20]. The incidence rate ratio decreased from 4.41 (95% CI = 0.57, 200.86) as estimated in 2011 to 2.47 (95% CI = 0.64, 14.10) in 2013 when more patient exposure was included [20].

All female breast cancers were diagnosed within the first year of treatment and were heterogeneous in patient age, tumor type, stage, progesterone/estrogen receptor status, and HER2/neu status [20]. These may not support a causative role for dapagliflozin.

3. Canagliflozin

In an animal study, lifetime exposure to canagliflozin in CD-1 mice at doses up to a 14-fold human clinical exposure did not increase the incidence of neoplasms or preneoplastic histological lesions [14].

Canagliflozin was associated with increased neoplasms of renal tubules, adrenals, and testicular Leydig cells (LCT) in Sprague-Dawley rats, which were preceded by carbohydrate malabsorption and disrupted calcium homeostasis [14, 38, 39]. However, canagliflozin does not result in significant carbohydrate malabsorption or calcium imbalance in humans [14].

Table 3: Incidence rates of bladder and breast cancer with canagliflozin exposure.

Canagliflozin	N	Subjects with events (%)	Rate per 1000 patient-year
Bladder Cancer			
Canagliflozin 100 mg	3139	2 (0.06)	0.44
Canagliflozin 300 mg	3506	3 (0.09)	0.63
All canagliflozin	6645	5 (0.07)	
All noncanagliflozin	3640	4 (0.11)	0.84
Breast Cancer			
Canagliflozin 100 mg	1313	5 (0.38)	2.61
Canagliflozin 300 mg	1514	7 (0.46)	3.39
All canagliflozin	2827	12 (0.42)	
All noncanagliflozin	1501	6 (0.4)	3.05

Source: reference [14], data obtained from 8 phase 3 clinical trials, data cutoff date: Nov 15, 2012.

Due to the increased renal, adrenal, and LCT tumor associated with canagliflozin in rat studies and the clinical imbalance of bladder and breast cancer associated with dapagliflozin, the incidence of these five cancers was followed through 8 phase 3 trials with canagliflozin. There were 6,645 patients who underwent canagliflozin and 3,640 patients who underwent noncanagliflozin treatment.

Till November 15, 2012, there were no reported cases of pheochromocytoma or malignant adrenal tumors in the treatment group [14]. One case of testicular cancer was reported 2 months after starting canagliflozin 100 mg. The patient had an enlarged scrotum a year prior to trial entry and had scrotal pain before trial entry. The prior scrotal abnormality and the short latency period did not support a drug-related causality [14].

The overall incidence of bladder, breast, and renal cancers did not increase in canagliflozin treatment groups while being compared to the noncanagliflozin groups (Table 3) [14, 37].

4. Discussion

4.1. Class Effect of Cancer Risk Unlikely for SGLT2 Inhibitors. Statistical data did not reveal an overall imbalance of cancer in both canagliflozin and dapagliflozin [14, 20, 35]. An increased incidence of bladder cancer without statistical significance was observed in patients treated with dapagliflozin. However, this was not similarly observed in patients treated with canagliflozin, which actually showed a trend of decreased risk (Table 3). Therefore, the risk of bladder cancer associated with SGLT2 inhibitors is not conclusive and such a risk is unlikely a class effect.

4.2. Cancer Risk Not Increased in SGLT2 Knockout Animals. Reilly et al. studied the potential carcinogenic risk of inhibiting SGLT2 in SGLT2$^{-/-}$ mice [36]. The 15-month study included 36 (23 male and 13 female) SGLT knockout mice and 33 (16 male, 17 female) wild type mice. At 15 months, 86% of knockout mice and 85% wild type mice survived. Knockout mice exhibited substantial glucosuria. Microscopic evaluation of urinary bladder, kidneys, liver, heart, pancreas, adrenal glands, thyroids, spleen, female reproductive tract, male sex glands, skin, brain, and skull did not reveal adverse effect of SGLT2 gene deletion. Neither hyperplasia nor neoplasia was observed in the urinary bladder mucosa, urogenital tract, or kidneys of SGLT2 knockout mice [36].

4.3. Cancer Cells May Overexpress SGLT. In humans, SGLT1 is overexpressed in many cancers [19, 40]. Inhibition of SGLT1 sensitizes prostate cancer cells to treatment with EGFR (epidermal growth factor receptor) tyrosine kinase inhibitor [40]. High SGLT1 level combined with high MAP17 (membrane-associated protein 17) is a marker for good prognosis in patients with cervical cancer after chemotherapy and radiotherapy [41]. High SGLT1 expression in pancreatic adenocarcinomas was significantly correlated with disease free survival, especially in younger patients [42]. On the other side, overexpression of SGLT1 is related to tumor development and poor prognosis of ovarian carcinoma [43]. In oral squamous cell carcinoma, SGLT1/EGFR expression was inversely related to tumor differentiation [44]. SGLT1/EGFR overexpression in colorectal cancer was related to higher clinical stages though SGLT1 expression was not associated with the prognosis [45].

In a study of lung cancer, there were no significant differences in the level of SGLT1 or SGLT2 gene expression between the primary lung cancers and the normal lung tissues [46]. However, higher SGLT2 expression was found in metastatic lesions of lung cancer compared to primary tumor [46].

Studies above imply that SGLT, especially SGLT1, plays a role in glucose uptake in many cancers. From this point of view, inhibition of SGLT1 and SGLT2 might even be protective in certain cancer types. If there was any positive link between dapagliflozin and bladder cancer, mechanisms other than inhibition of SGLT2 should be considered. Again, relationship between different SGLT2 inhibitors and bladder cancer should be examined individually.

4.4. Detection Bias and Increased Bladder Cancer Risk Induced by Glucosuria and Urinary Tract Infection Related to SGLT2 Use. SGLT2 inhibitors block renal glucose reabsorption resulting in increased glucosuria. Increased urinary tract and genital infections were observed in patients treated with SGLT2 inhibitors [7, 8, 15–17, 19]. The increased use of urinalysis in these patients may lead to a detection bias for bladder cancer. However, the possibility of an increased risk of bladder cancer induced by chronic glucosuria and urinary tract infection among patients who use SGLT2 inhibitors cannot be excluded.

In SGLT2$^{-/-}$ mice, lifelong glucosuria did not increase the incidence of bladder tumor [36]. However, patients with diabetes are indeed at an increased risk of bladder cancer compared with those without diabetes [21–26]. Recurrent or chronic urinary tract infection may cause chronic irritation to bladder epithelium and is a potential risk factor of bladder cancer in humans [47–50]. The duration of current human studies on SGLT2 inhibitors is probably not long enough to

answer the question and careful postmarketing surveillance should be conducted.

4.5. Ethnicity and Sexual Differences. Ethnicity should be taken into consideration while evaluating the risk of bladder cancer. Diabetes associated bladder cancer risk was obviously higher in Asia populations [21]. A population-based matched case—control study performed in the USA, suggested a reduced risk of bladder cancer in women, but not in men, with a history of urinary tract infection [51]. However, a large population-based study in Taiwan disclosed that urinary tract infection is significantly associated with increased bladder cancer risk in both sexes [25].

In phase 2b and phase 3 studies of dapagliflozin presented to the FDA, Asian ethnicities account for less than 20% of the study population [20]. The sample size of the studied Asian population was probably too small to evaluate the effect in this specific ethnic group of patients.

5. Conclusions

Molecular evidences and animal studies do not suggest a positive link between exposure to SGLT2 inhibitors and cancer risk. The imbalance of bladder cancer and breast cancer with dapagliflozin treatment in humans could be a result of early diagnosis of preexisting cancer. However, long-term effects should be examined carefully by including larger sample size, with longer exposure duration, and in different ethnicities.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] G. Danaei, M. M. Finucane, Y. Lu et al., "National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants," *The Lancet*, vol. 378, no. 9785, pp. 31–40, 2011.

[2] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[3] Y. Ohkubo, H. Kishikawa, E. Araki et al., "Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study," *Diabetes Research and Clinical Practice*, vol. 28, no. 2, pp. 103–117, 1995.

[4] UK Prospective Diabetes Study (UKPDS) Group, "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

[5] R. R. Holman, S. K. Paul, M. A. Bethel, D. R. Matthews, and H. A. W. Neil, "10-Year follow-up of intensive glucose control in type 2 diabetes," *New England Journal of Medicine*, vol. 359, no. 15, pp. 1577–1589, 2008.

[6] E. M. Wright, "Renal Na(+)-glucose cotransporters," *American Journal of Physiology—Renal Physiology*, vol. 280, no. 1, pp. F10–F18, 2001.

[7] M. Bhartia, A. A. Tahrani, and A. H. Barnett, "SGLT-2 inhibitors in development for type 2 diabetes treatment," *Review of Diabetic Studies*, vol. 8, no. 3, pp. 348–354, 2011.

[8] E. Ferrannini and A. Solini, "SGLT2 inhibition in diabetes mellitus: rationale and clinical prospects," *Nature Reviews Endocrinology*, vol. 8, no. 8, pp. 495–502, 2012.

[9] E. M. Wright and E. Turk, "The sodium/glucose cotransport family SLC5," *Pflugers Archiv*, vol. 447, no. 5, pp. 510–518, 2004.

[10] J. R. L. Ehrenkranz, N. G. Lewis, C. R. Kahn, and J. Roth, "Phlorizin: a review," *Diabetes/Metabolism Research and Reviews*, vol. 21, no. 1, pp. 31–38, 2005.

[11] J. E. Gerich and A. Bastien, "Development of the sodium-glucose co-transporter 2 inhibitor dapagliflozin for the treatment of patients with Type 2 diabetes mellitus," *Expert Review of Clinical Pharmacology*, vol. 4, no. 6, pp. 669–683, 2011.

[12] J. Rosenstock, N. Aggarwal, D. Polidori et al., "Dose-ranging effects of canagliflozin, a sodium-glucose cotransporter 2 inhibitor, as add-on to metformin in subjects with type 2 diabetes," *Diabetes Care*, vol. 35, no. 6, pp. 1232–1238, 2012.

[13] S. A. Nisly, D. M. Kolanczyk, and A. M. Walton, "Canagliflozin, a new sodium-glucose cotransporter 2 inhibitor, in the treatment of diabetes," *The American Journal of Health-System Pharmacy*, vol. 70, no. 4, pp. 311–319, 2013.

[14] U.S. Food and Drug Administration, "FDA Briefing Document, NDA 204042 Canagliflozin tablets. Advisory Committee Meeting: 2013," http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/EndocrinologicandMetabolicDrugsAdvisoryCommittee/UCM334550.pdf.

[15] E. Ferrannini, A. Berk, S. Hantel et al., "Long-term safety and efficacy of empagliflozin , sitagliptin , and metformin: an active-controlled, parallel-group, randomized, 78-week open-label extension study in patients with type 2 diabetes," *Diabetes Care*, vol. 36, no. 12, pp. 4015–4021, 2013.

[16] J. Rosenstock, L. J. Seman, A. Jelaska et al., "Efficacy and safety of empagliflozin, a sodium glucose cotransporter 2 (SGLT2) inhibitor, as add-on to metformin in type 2 diabetes with mild hyperglycaemia," *Diabetes, Obesity and Metabolism*, vol. 15, no. 12, pp. 1154–1160, 2013.

[17] R. F. Rosenwasser, S. Sultan, D. Sutton, R. Choksi, and B. J. Epstein, "SGLT-2 inhibitors and their potential in the treatment of diabetes," *Journal of Diabetes, Metabolic Syndrome and Obesity*, vol. 6, pp. 453–467, 2013.

[18] N. Inagaki, K. Kondo, T. Yoshinari, N. Maruyama, Y. Susuta, and H. Kuki, "Efficacy and safety of canagliflozin in Japanese patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, 12-week study," *Diabetes, Obesity and Metabolism*, vol. 15, no. 12, pp. 1136–1145, 2013.

[19] S. Cangoz, Y. Y. Chang, S. J. Chempakaseril et al., "The kidney as a new target for antidiabetic drugs: SGLT2 inhibitors," *Journal of Clinical Pharmacy and Therapeutics*, vol. 38, no. 5, pp. 350–359, 2013.

[20] U.S. Food and Drug Administration, "FDA background document, BMS-512148 NDA 202293 dapagliflozin," in *Proceedings of the Endocrinologic & Metabolic Drug Advisory Committee Meeting*, 2013, http://www.fda.gov/downloads/drugs/endocrinologicandmetabolicdrugsadvisorycommittee/ucm378079.pdf.

[21] X.-Q. Yang, C. Xu, Y. Sun, and R.-F. Han, "Diabetes mellitus increases the risk of bladder cancer: an updated meta-analysis," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 4, pp. 2583–2589, 2013.

[22] H. Fang, B. Yao, Y. Yan et al., "Diabetes mellitus increases the risk of bladder cancer: an updated meta-analysis of observational studies," *Diabetes Technology & Therapeutics*, vol. 15, no. 11, pp. 914–922, 2013.

[23] Z. Zhu, X. Wang, Z. Shen, Y. Lu, S. Zhong, and C. Xu, "Risk of bladder cancer in patients with diabetes mellitus: an updated meta-analysis of 36 observational studies," *BMC Cancer*, vol. 13, article 310, 2013.

[24] C.-H. Tseng, C.-K. Chong, C.-P. Tseng, and T.-T. Chan, "Age-related risk of mortality from bladder cancer in diabetic patients: a 12-year follow-up of a national cohort in Taiwan," *Annals of Medicine*, vol. 41, no. 5, pp. 371–379, 2009.

[25] C.-H. Tseng, "Diabetes and risk of bladder cancer: a study using the National Health Insurance database in Taiwan," *Diabetologia*, vol. 54, no. 8, pp. 2009–2015, 2011.

[26] C.-H. Tseng, "Benign prostatic hyperplasia is a significant risk factor for bladder cancer in diabetic patients: a population-based cohort study using the National Health Insurance in Taiwan," *BMC Cancer*, vol. 13, article 7, 2013.

[27] C.-H. Tseng, "Pioglitazone and bladder cancer: a population-based study of Taiwanese," *Diabetes Care*, vol. 35, no. 2, pp. 278–280, 2012.

[28] R. Mamtani, K. Haynes, W. B. Bilker et al., "Association between longer therapy with thiazolidinediones and risk of bladder cancer: a cohort study," *Journal of the National Cancer Institute*, vol. 104, no. 18, pp. 1411–1421, 2012.

[29] L. Azoulay, H. Yin, K. B. Filion et al., "The use of pioglitazone and the risk of bladder cancer in people with type 2 diabetes: nested case-control study," *BMJ*, vol. 344, Article ID e3645, 2012.

[30] L. Wei, T. M. Macdonald, and I. S. Mackenzie, "Pioglitazone and bladder cancer: a propensity score matched cohort study," *British Journal of Clinical Pharmacology*, vol. 75, no. 1, pp. 254–259, 2013.

[31] C. H. Tseng, "A review on thiazolidinediones and bladder cancer in human studies," *Journal of Environmental Science and Health C*, vol. 32, no. 1, pp. 1–45, 2014.

[32] J. D. Lewis, A. Ferrara, T. Peng et al., "Risk of bladder cancer among diabetic patients treated with pioglitazone: interim report of a longitudinal cohort study," *Diabetes Care*, vol. 34, no. 4, pp. 916–922, 2011.

[33] A. Neumann, A. Weill, P. Ricordeau, J. P. Fagot, F. Alla, and H. Allemand, "Pioglitazone and risk of bladder cancer among diabetic patients in France: a population-based cohort study," *Diabetologia*, vol. 55, no. 7, pp. 1953–1962, 2012.

[34] C. H. Tseng, "Metformin may reduce bladder cancer risk in Taiwanese patients with type 2 diabetes," *Acta Diabetol*, vol. 51, no. 2, pp. 295–303, 2014.

[35] U.S. Food and Drug Administration, FDA Briefing Document, NDA 202293 Dapagliflozin tablets, 5 and 10 mg. Advisory Committee Meeting, 2011, http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/endocrinologicandmetabolicdrugsadvisorycommittee/ucm262994.pdf.

[36] T. P. Reilly, M. J. Graziano, E. B. Janovitz et al., "Carcinogenicity risk assessment supports the chronic safety of dapagliflozin, an inhibitor of sodium-glucose co-transporter 2, in the treatment of type 2 diabetes mellitus," *Diabetes Therapy*, vol. 5, no. 1, pp. 73–96, 2014.

[37] D. Jones, "Diabetes field cautiously upbeat despite possible setback for leading SGLT2 inhibitor," *Nature Reviews Drug Discovery*, vol. 10, no. 9, pp. 645–646, 2011.

[38] D. E. Prentice and A. W. Meikle, "A review of drug-induced Leydig cell hyperplasia and neoplasia in the rat and some comparisons with man," *Human and Experimental Toxicology*, vol. 14, no. 7, pp. 562–572, 1995.

[39] F. J. C. Roe, "Relevance for man of the effects of lactose, polyols and other carbohydrates on calcium metabolism seen in rats: a review," *Human Toxicology*, vol. 8, no. 2, pp. 87–98, 1989.

[40] J. Ren, L. R. Bollu, F. Su et al., "EGFR-SGLT1 interaction does not respond to EGFR modulators, but inhibition of SGLT1 sensitizes prostate cancer cells to EGFR tyrosine kinase inhibitors," *Prostate*, vol. 73, no. 13, pp. 1453–1461, 2013.

[41] M. Perez, J. M. Praena-Fernandez, B. Felipe-Abrio et al., "MAP17 and SGLT1 protein expression levels as prognostic markers for cervical tumor patient survival," *PLoS ONE*, vol. 8, no. 2, Article ID e56169, 2013.

[42] V. F. Casneuf, P. Fonteyne, N. van Damme et al., "Expression of SGLT1, Bcl-2 and p53 in primary pancreatic cancer related to survival," *Cancer Investigation*, vol. 26, no. 8, pp. 852–859, 2008.

[43] B. Lai, Y. Xiao, H. Pu, Q. Cao, H. Jing, and X. Liu, "Overexpression of SGLT1 is correlated with tumor development and poor prognosis of ovarian carcinoma," *Archives of Gynecology and Obstetrics*, vol. 285, no. 5, pp. 1455–1461, 2012.

[44] Y. Hanabata, Y. Nakajima, K. I. Morita, K. Kayamori, and K. Omura, "Coexpression of SGLT1 and EGFR is associated with tumor differentiation in oral squamous cell carcinoma," *Odontology*, vol. 100, no. 2, pp. 156–163, 2012.

[45] G. F. Guo, Y. C. Cai, B. Zhang et al., "Overexpression of SGLT1 and EGFR in colorectal cancer showing a correlation with the prognosis," *Medical Oncology*, vol. 28, no. 1, pp. S197–S203, 2011.

[46] N. Ishikawa, T. Oguri, T. Isobe, K. Fujitaka, and N. Kohno, "SGLT gene expression in primary lung cancers and their metastatic lesions," *Japanese Journal of Cancer Research*, vol. 92, no. 8, pp. 874–879, 2001.

[47] C. Pelucchi, C. Bosetti, E. Negri, M. Malvezzi, and C. La Vecchia, "Mechanisms of disease: the epidemiology of bladder cancer," *Nature Clinical Practice Urology*, vol. 3, no. 6, pp. 327–340, 2006.

[48] S. Janković and V. Radosavljević, "Risk factors for bladder cancer," *Tumori*, vol. 93, no. 1, pp. 4–12, 2007.

[49] R. L. Jacobs, C. T. Lee, and J. E. Montie, "Bladder cancer in 2010 how far have we come?" *CA Cancer Journal for Clinicians*, vol. 60, no. 4, pp. 244–272, 2010.

[50] D. Volanis, T. Kadiyska, A. Galanis, D. Delakas, S. Logotheti, and V. Zoumpourlis, "Environmental factors and genetic susceptibility promote urinary bladder cancer," *Toxicology Letters*, vol. 193, no. 2, pp. 131–137, 2010.

[51] X. Jiang, J. E. Castelao, S. Groshen et al., "Urinary tract infections and reduced risk of bladder cancer in Los Angeles," *British Journal of Cancer*, vol. 100, no. 5, pp. 834–839, 2009.

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Research Article

Relative Validity and Reproducibility of a Quantitative Food Frequency Questionnaire for Adolescents with Type 1 Diabetes: Validity of a Food Frequency Questionnaire

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Background. Food frequency questionnaires are used to assess dietary intake in epidemiological studies. *Objective.* The aim of the study was to assess the relative validity and reproducibility of a quantitative food frequency questionnaire (QFFQ) for adolescents with type 1 diabetes. Methods: Validity was evaluated by comparing the data generated by QFFQs to those of 24-hour recalls (24 hrs). QFFQs were applied twice per patient to assess reproducibility. Statistical analysis included performing *t*-tests, obtaining Pearson correlation coefficients when necessary, correcting measurements for randomness by the weighted *kappa* method, calculating intraclass correlation coefficients, and generating Bland-Altman plots ($P < 0,05$). *Results.* The total energy and nutrient intake as estimated by the QFFQs were significantly higher than those from 24 hrs. Pearson correlation coefficients for energy-adjusted, deattenuated data ranged from 0.32 (protein) to 0.75 (lipid, unsaturated fat and calcium). Weighted *kappa* values ranged from 0.15 (vitamin C) to 0.45 (calcium). Bland-Altman plots indicated acceptable validity. As for reproducibility, intraclass correlation coefficients ranged from 0.24 (calcium) to 0.65 (lipid), and the Bland-Altman plots showed good agreement between the two questionnaires. Conclusion: The QFFQ presented an acceptable ability to classify correctly and with good reproducibility, adolescents with type 1 diabetes according to their levels of dietary intake.

1. Introduction

The goals of type 1 diabetes treatment in adolescents are to keep patients free of symptoms, prevent acute and chronic complications of hyperglycaemia, avert episodes of hypoglycaemia, control weight, prevent dyslipidemia, and maintain normal growth and development rates. To ensure the success of this approach, it is essential to know their dietary habits in order to modify them, as well as for continued evaluation and follow-up of patients. Despite its importance, few studies about food consumption and its relation with the control and management of this disease are found in the literature [1].

Several methods have been developed to assess dietary intake of individuals and populations. Since each method has its advantages and limitations, choice must be guided by its adequacy to the target population and the goals of the study. Furthermore, for the reliability of the analysis the method must be highly reproducible and also must have been tested in a posterior validation process [2].

The use of food frequency questionnaires is a low cost method to assess dietary intake and is often used in epidemiological studies, since it allows correlating diet and the occurrence of nontransmissible chronic illnesses. The aim of this method is to evaluate the frequency of intake of certain foods, or food groups, over a specific period of time [3–5].

Considering factors as the prevalence of diabetes among adolescents, its substantial correlation with dietary intake and impact on growth and development, including also the lack of specific assessment tools for this population, the present study evaluated the reproducibility and relative validity of a quantitative food frequency questionnaire for type 1 diabetes adolescent patients.

2. Materials and Methods

2.1. Development of the QFFQ. The participants in this study were DM1 adolescents of both sexes who were followed as patients at the Endocrinology Outpatient Clinic of the Clinical Hospital (CH) of the Goiás Federal University (EOC-CH). All were volunteers whose consent was requested, in the presence of their legal guardians, about their interest in taking part of the research and, upon being informed about the study, they signed the Free and Informed Consent Formulary (FICF) in accordance with the guidelines of the Medical Research Ethics Committee of Goiás Federal University (GFU).

At the time the study was outlined, there were 170 adolescents registered at the EOC-CH, 20% of whom (34 adolescents) were selected to evaluate the food items that were to be included in the quantitative food frequency questionnaire (QFFQ).

In order to identify the food items consumed by the study cohort, an adult QFFQ was used (validated previously) [6]. This questionnaire covered the previous three months of food consumption, using open questions that allowed the inclusion of new food items, portion sizes, and usual preparations. Each patient also filled two 24-hour recalls (24hRs), one on the day of the interview and another 15–20 days later. The interviewers were undergraduate students of Nutrition at the GFU who had been previously trained. Interviews were carried out under supervision of the researchers.

Nutrient content calculations were performed on the data from dietary surveys based on the Brazilian reference tables for the chemical composition of food items [7, 8]. Lists were compiled with the percentage contribution (PC) of all food items towards each nutrient, in accordance with the statistical analysis technique of weighted proportions according to Block et al. [9], Haile et al. [10], Willett [11], and Flegal et al. [12]. To this end, the following formula was used: PC (%) $= 100 \times \Sigma$(specific nutrient content per food item)/Σ(nutrient content in all food items).

All food items were classified by PC value, and those with a PC equal to or lower than 85% were included into the questionnaire. The preliminary list resulting from this selection included few dietetic and low calorie food items, due to the low frequency of consumption by the study group.

To identify the consumption frequency (cf) for each food item that was in the QFFQ, we defined nine frequency unit categories of classification. This assessment was quantified by attributing weights (Sn, where n is the category number) to each category (cf) based on the frequency in the previous three months [13].

The mean value of $S6 = 1$ was defined for items ingested daily. Weights for the other categories were obtained according to the following formula for a given food item ingested between a and b times in the past three months [13]: $Sn = (1/90) \times (a + b)/2$. Consumption frequency categories (f) and their respective weights are as follows:

(f1) never or less than once a month; $S1 = 0$;

(f2) once a month; $S2 = 0{,}016[6/2 = 3 * 0.0055 = 0.016]$;

(f3) twice to four times a month; $S3 = 0{,}099[12 + 24/2 = 18 * 0.0055 = 0.09]$;

(f4) twice to four times a week; $S4 = 0{,}43[52 + 104/2 = 78 * 0.0055 = 0.43]$;

(f5) five or six times a week; $S5 = 0{,}79[130 + 156/2 = 143 * 0.0055 = 0.79]$;

(f6) once a day; $S6 = 1$;

(f7) twice or thrice a day; $S7 = 2{,}5[360 + 540/2 = 450 * 0.0055 = 2.5]$;

(f8) four or five times a day; $S8 = 4{,}5[720 + 900/2 = 810 * 0.0055 = 4.5]$;

(f9) six times a day; $S9 = 6[1080 * 0.0055 = 5.9]$.

The usual portion sizes were defined from the reported portions in the two 24hRs. These were classified relative to the 25th, 50th, and 75th percentiles, which marked the thresholds for small, medium, and large portions, respectively.

The final format of the QFFQ, corresponding to the three preceding months, comprised 106 food items divided into eleven groups, namely, dairy products, legumes, meat and eggs (with or without apparent fat), cereals and derivative products, pasta and snacks, sugar and sweets, fruits, green leaves, fats, spices and seasonings, and nonalcoholic beverages. The reported dietary intake for a given product, thus, is quantified by multiplying the quantities in grammas or milliliters by the aforementioned weights (Sn), according to the consumption frequency category.

2.2. Relative Validity and Reproducibility. Relative validity was assessed by comparing QFFQ results to those of a reference method, the 24-hour recall (24hR). Reproducibility was evaluated by comparing results of two separate applications of the same questionnaire. The overall design of the study is outlined in Figure 1.

Participants were adolescents of both sexes who were regular patients at the Endocrinology Outpatient Clinics of the GGH and EOC-CH, both in the city of Goiânia, Brazil. Inclusion criteria were of ages between ten and 18 years and a positive DM1 diagnosis; patients were not included if they had other types of diabetes, celiac disease, growth hormone deficiency, or chromosomal abnormalities. Girls who were

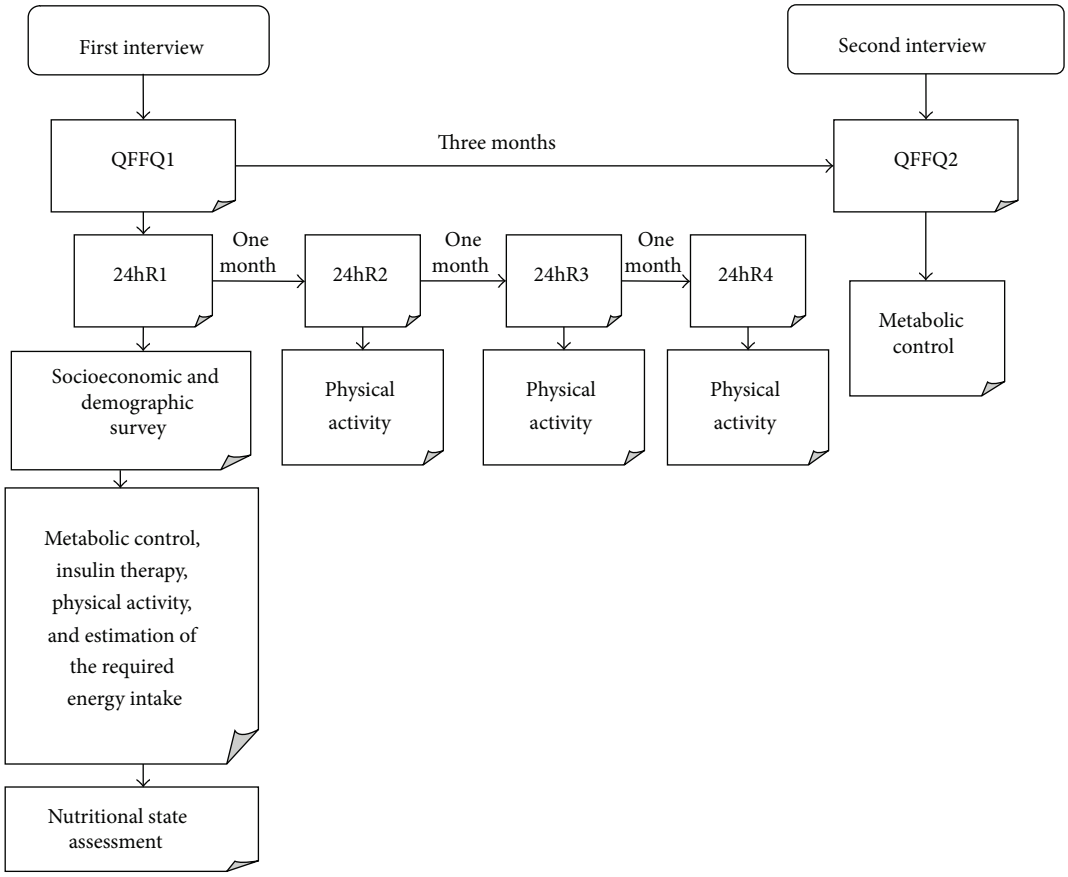

Figure 1: Protocol for validation and reproducibility analysis of the proposed QFFQ for type 1 diabetes adolescent.

pregnant at the time of selection or became pregnant at any time during the course of the study were also not included.

We recruited 84 patients. Data were collected from April 2008 to July 2009. A guideline basis was constructed in order to standardize the procedures of data collection.

The 24hR, a validated method [14] used in national [2, 15] and international [14, 16] validation studies, allowed us to register meal times and types, as well as the food items, their preparations, and quantities. The customary measures reported were converted to grammas or milliliters. Nutritional content was calculated by reference to the national tables of chemical composition of food items [7, 8] and to package labels.

The following dietary variables were analyzed: total energetic content, carbohydrates, protein, lipids, saturated fats, unsaturated fats, total cholesterol, dietary fiber, vitamin C, calcium, iron, and zinc.

Variables that did not follow a normal distribution underwent Neperian logarithmic transformation to generate an approximate Gaussian distribution, which was successfully accomplished for all of them. Mean and standard deviation values for total energy, macronutrients, and micronutrients were determined for both QFFQ and the four 24hRs. The differences between mean values obtained by each survey technique were calculated by Student's t-test.

Validity was assessed by using the Pearson correlation coefficient for crude, deattenuateddata, and again after they were adjusted for energy content. Deattenuation, which corrects intrapersonal variability, was carried out according to the procedure described by Beaton and colleagues [17]: $r_d = r_p \times (1 + \lambda_x/n_x)^{1/2}$, where r_d is the deattenuated coefficient, r_p is the observed coefficient, λ_x is the ratio of intrapersonal variation, and n_x is the number of surveys per subject. Values were adjusted for energy by means of regression using the residue method, whereby the total energy content was considered an independent variable and the nutrient itself was considered a dependent one [11].

The nutrients under study were categorized in quartiles of intake in order to correlate the mean values of the QFFQ and those of the 24hR. Concordance and extreme discordance between methods were estimated by the percentage of patients classified into the same quartile, in opposed quartiles and in adjoining quartiles. Reliability of analyses was assessed by using the weighed *kappa* method, which corrects concordance measurements for chance events; results were considered acceptable when more than 50% of individuals were correctly classified, less than 10% fell on opposite quartiles, and the weighed *kappa* was more than 0.4, so that the possibility of false-negative associations between diet and illness in epidemiological studies would be kept to a minimum.

Table 1: Total energy and nutrient intake means. The means of energy and nutrient intake were estimated from results of two quantitative food frequency questionnaires (QFFQ) and four 24-hour recalls (24hR) filled by adolescents with type 1 diabetes. Goiânia, Brazil, 2009.

Energy and nutrients	QFFQ ($n = 70$)		24hR ($n = 70$)		QFFQ : 24hR ratio	P value[†]
	Mean	SD[*]	Mean	SD[*]		
Total energy						
kJ	10946.22	3673.80	7885.58	2246.09	1.39	0.0001
kcal	2616.21	878.06	1884.70	536.83		
Carbohydrate (g)	312.89	109.94	199.42	63.07	1.57	0.0001
Protein (g)	114.25	45.74	92.39	32.05	1.24	0.0004
Lipid (g)	100.85	40.51	79.71	28.92	1.26	0.0001
Saturated fat (g)	29.84	12.97	23.35	8.79	1.28	0.0001
Unsaturated fat (g)	57.18	22.79	45.77	15.85	1.25	0.0001
Total cholesterol (mg)	327.53	185.21	286.15	127.75	1.14	0.0001
Dietary fibre (g)	51.88	17.37	33.08	13.43	1.57	0.0001
Vitamin C (mg)	314.62	294.94	106.59	86.77	2.95	0.0001
Calcium (mg)	983.28	699.37	542.23	299.52	1.81	0.0001
Iron (mg)	13.30	4.63	9.81	3.71	1.35	0.0001
Zinc (mg)	17.54	8.43	15.36	6.93	1.14	0.0354

[*] SD: standard deviation; [†] Student's t-test (significant if $P < 0.05$).

The intraclass correlation coefficient (ICC) was used to assess reproducibility of the QFFQ in two different forms, that is, for energy-adjusted and unadjusted values. To assess the agreement between both QFFQ, differences were compared to mean values and a plot comparing the two measurements was drawn as suggested by Bland and Altman [18].

P values lower than 0.05 were considered significant and correlations were found to be moderate between 0.4 and 0.7. Statistical analyses were conducted using two different programs: *Statistical Analysis System* (SAS), version 9.2, and *Statistical Package of Social Sciences* (SPSS), version 18.0.

The study was approved by the Ethics Committee in Human and Animal Medical Research of both CH (protocol 042/07) and GGH (protocol 383/08). All patients and legal guardians were informed about the goals and procedures of the study and, upon consent on volunteer participation, signed the FICF.

3. Results

At the end of the data collection period, from the 84 patients that were initially included in the study group, 14 (17.0%) returned incomplete dietary reports, leaving 70 adolescents (58.6% females) for the study to be carried out on. The mean age was 14 years (SD = ±2.5 years), the mean monthly *per capita* income was 99.68 USD (ranging from 28.48 to 360.28 USD), and the mean school attendance was seven years (7–16).

Table 1 presents the means for the estimated total energy and nutrient intake for QFFQs and 24hRs. It is of note that QFFQ overestimated intake relative to 24hR. This trend was statistically significant for all analysed measurements. The QFFQ : 24hR ratio of individual means varied from 1.14 (total cholesterol and zinc) to 2.95 (vitamin C).

As for Pearson correlation analyses, the estimates for total energetic intake, carbohydrate, lipids, saturated fat and unsaturated fats, dietary fibre, calcium, iron, and zinc all fell in the moderate range (values between 0.4 and 0.68) when data were analysed crude and nonadjusted for energy content (Table 2). Only the values for protein, cholesterol, and vitamin C were below 0.4. All correlations were significant and the best results were for unsaturated fats (0.68), lipids (0.66), and calcium (0.61). Deattenuation increased all correlation coefficients. A similar effect was verified when values were adjusted for energy content, except for protein, fibre, iron, and zinc.

The agreement of QFFQs and 24hRs was assessed by quartile categorization of adolescents according to energy and nutrient intake. The exact agreement varied from 31.4% (cholesterol) to 47.1% (lipid; Table 3). Extreme disagreement (classification in opposite quartiles) ranged from zero (calcium) to 8.5% (vitamin C). Approximately 70.0% (vitamin C) to 85.7% (calcium) of participants were classified either in the same or in adjacent quartiles. The mean values of exact and exact/adjacent agreement and disagreement were 38.4%, 78.5%, and 4.1%, respectively. Agreements assessed with the weighted *kappa* correction ranged from 0.15 (weak correlation) for vitamin C to 0.45 (moderate) for calcium, with a mean of 0.3.

Figures 2 and 3 show the Bland-Altman analysis of our measurements, which plots the difference in intake between the two methods against the mean in intake of the two measures for each individual intake. Both axes are logarithmic. The highest validity on visual inspection is that for cholesterol (Figure 2). The major bias is observed for the vitamin C measurements (Figure 2). Validity was acceptable for both protein, which showed the lowest correlation value (0.32), and calcium, which showed the highest (0.75; Figure 3).

Results for the reproducibility test are presented in Table 4. The ICC ranged from 0.25 (calcium) to 0.65 (lipid), with a mean of 0.46 for nonadjusted values. With the adjustment for energy content, values became lower for lipid,

Table 2: Pearson correlation coefficients for the comparison between energy and nutrient intake. These values were estimated by QFFQ and 24hR from type 1 diabetes adolescent patients. Goiânia, Brazil, 2009.

Energy and nutrients	Unadjusted				Energy-adjusted			
	Crude	(CI 95%)*	*Deattenuated***	(CI 95%)*	Crude	(CI 95%)*	*Deattenuated***	(CI 95%)*
Total energy	0.47	(0.26–0.64)	0.48	(0.28–0.64)	—	—	—	—
Carbohydrate	0.44	(0.22–0.61)	0.45	(0.24–0.62)	0.58	(0.40–0.72)	0.60	(0.43–0.73)
Protein	0.36	(0.14–0.55)	0.38	(0.16–0.56)	0.31	(0.08–0.50)	0.32	(0.09–0.52)
Lipid	0.66	(0.50–0.77)	0.67	(0.51–0.78)	0.74	(0.61–0.83)	0.75	(0.63–0.84)
Saturated fat	0.57	(0.39–0.71)	0.59	(0.41–0.72)	0.58	(0.40–0.72)	0.60	(0.42–0.73)
Unsaturated fat	0.68	(0.53–0.79)	0.69	(0.55–0.80)	0.74	(0.61–0.83)	0.75	(0.62–0.84)
Total cholesterol	0.36	(0.13–0.55)	0.37	(0.15–0.56)	0.38	(0.16–0.56)	0.40	(0.19–0.58)
Dietary fibre	0.56	(0.38–0.70)	0.59	(0.41–0.72)	0.56	(0.37–0.70)	0.58	(0.40–0.72)
Vitamin C	0.33	(0.10–0.52)	0.34	(0.12–0.54)	0.42	(0.20–0.60)	0.44	(0.23–0.61)
Calcium	0.61	(0.44–0.74)	0.62	(0.45–0.75)	0.73	(0.60–0.82)	0.75	(0.62–0.84)
Iron	0.47	(0.27–0.64)	0.49	(0.29–0.65)	0.45	(0.24–0.62)	0.47	(0.26–0.63)
Zinc	0.40	(0.18–0.58)	0.42	(0.20–0.59)	0.38	(0.16–0.57)	0.40	(0.18–0.58)

*CI = confidence interval; **intraclass correlation coefficient.

Table 3: Agreement measures for energy and nutrient intake. The measures for energy and nutrient intake were estimated by QFFQ and 24hR from type 1 diabetes adolescent patients, categorized into intake quartiles. Results were assessed by the *kappa* test. Goiânia, Brazil, 2009.

Energy and nutrients	Exact agreement (%)	Exact plus adjacent-quartile agreement (%)	Extreme disagreement (%)	Weighted *Kappa*
Total energy	32.8	84.2	5.7	0.29
Carbohydrate	37.1	77.1	5.7	0.27
Protein	32.8	75.7	5.7	0.22
Lipid	47.1	80.0	4.2	0.38
Saturated fat	41.4	84.2	2.8	0.38
Unsaturated fat	45.7	80.0	2.8	0.38
Total cholesterol	31.4	74.2	2.8	0.22
Dietary fibre	37.1	75.7	4.2	0.27
Vitamin C	32.8	70.0	8.5	0.15
Calcium	45.7	85.7	0.0	0.45
Iron	41.4	77.1	4.2	0.31
Zinc	35.7	78.5	2.8	0.29
Mean	38.4	78.5	4.1	0.30

Table 4: Reproducibility assessment as measured by the intraclass correlation coefficient (ICC) for energy and nutrient intake. This intraclass correlation coefficient was calculated by the application of two QFFQs per individual on type 1 diabetes adolescent patients. Goiânia, Brazil, 2009.

Energy and nutrients	ICC	CI 95%*	Adjusted ICC	CI 95%*
Total energy	0.59	0.416–0.725	—	—
Carbohydrate	0.54	0.348–0.684	0.44	0.234–0.613
Protein	0.33	0.105–0.522	0.29	0.061–0.490
Lipid	0.65	0.493–0.767	0.52	0.330–0.674
Saturated fat	0.56	0.375–0.700	0.41	0.201–0.591
Unsaturated fat	0.65	0.485–0.763	0.56	0.373–0.700
Total cholesterol	0.42	0.211–0.597	0.48	0.280–0.643
Dietary fibre	0.41	0.198–0.588	0.55	0.363–0.694
Vitamin C	0.38	0.166–0.566	0.21	−0.030–0.419
Calcium	0.25	0.014–0.453	0.35	0.125–0.538
Iron	0.38	0.165–0.566	0.41	0.192–0.585
Zinc	0.40	0.184–0.578	0.46	0.254–0.626
Mean	0.46	—	0.43	—

*CI: confidence interval.

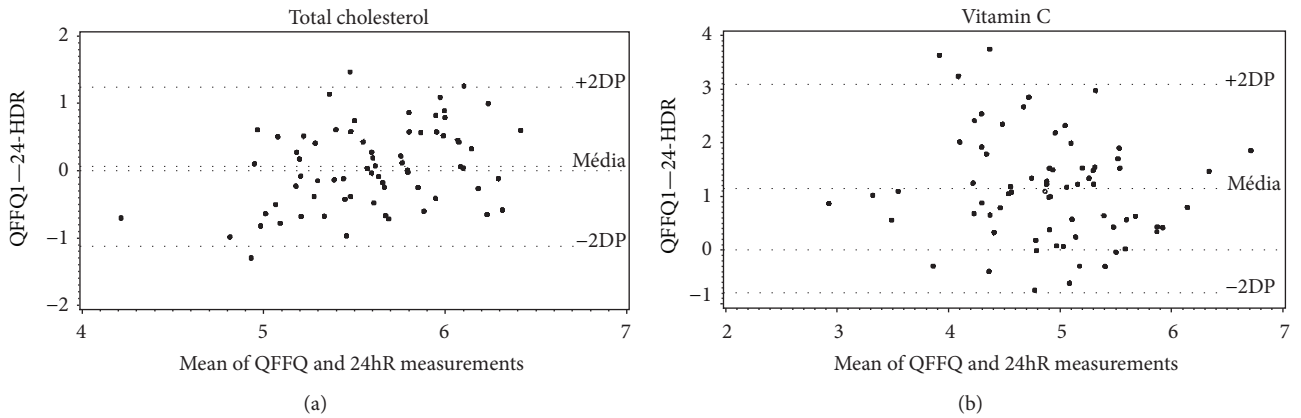

Figure 2: Bland-Altman plots for mean values of total cholesterol and vitamin C intake. These values were obtained from the application of QFFQ and 24hR on type 1 diabetes adolescent patients. Goiânia, Brazil, 2009.

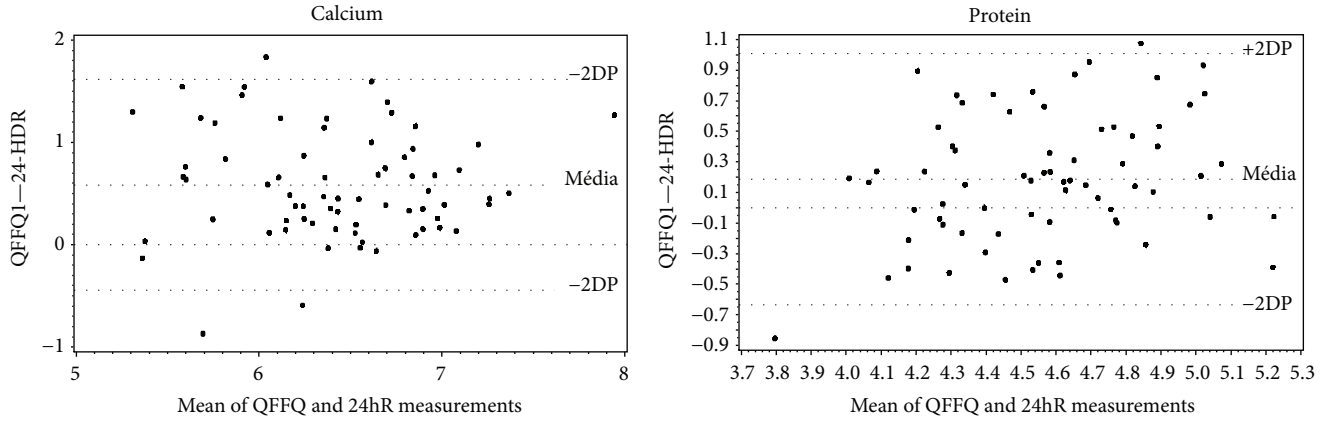

Figure 3: Bland-Altman plots for mean values calcium and protein intake. These values were obtained from the application of QFFQ and 24hR on type 1 diabetes adolescent patients. Goiânia, Brazil, 2009.

saturated and unsaturated fats, carbohydrate, and protein and higher for cholesterol, fibre, calcium, iron, and zinc. The ICC was not significant for vitamin C. Bland-Altman analysis showed good agreement between both questionnaires of each method for all nutrients (Figure 4).

4. Discussion

The applied questionnaire, designed specifically for evaluating the population from our study (Brazilian type 1 diabetic adolescents of a low income population), was able to measure the food intake in the subjects analyzed, with a good reproducibility and high agreement when compared to standard (reference) methods. Adolescents in this study had a *per capita* income lower than half the minimum wage and therefore below the poverty line, whose expenses with health and medications, according to the Brazilian Family Budget Survey (BFBS) for years 2002-3 [19], reach, respectively, 12.45% and 8.78% of familial income. Regarding dietary habits surveys, quantitative frequency of food consumption questionnaires, when compared to reference methods, tends to overestimate food and nutrient intake, especially relative to fruits and thus to vitamin C, as shown in this study [2, 6]. Slater et al. [20], validating a questionnaire for adolescents, observed that intake overestimation may occur when the questionnaire is applied to adolescents. Watson et al. [21], who validated a questionnaire for adolescents in Australia, also reported that the method overestimated energy, macronutrients, and fibres.

Crude Pearson correlation coefficients were similar to those described by Willet [11] (0.5–0.7) to be accepted in validation studies. They were close to those of Watson et al. [21], lower than those of Slater et al. [20], and higher than those of Rodriguez et al. [16] and Kobayashi et al. [22], all of which were carried out in adolescents. Correlation coefficients lower than 0.4 were similar to the results of Rockett et al. [14] for cholesterol, of Riley and Blizzard [23] for protein, and of Rodriguéz et al. [24] for vitamin C. Despite limitations of methods due to their reliance on memory and difficulties in calculating portion size, Hernández-Avila et al. [25] suggest that the observed differences among methods are more likely due to intake frequency rather than portion size.

Energy adjustment corrects nutrient intake for caloric content. When it was applied, the crude correlation coefficients for some nutrients rose, which indicates that energy content is a source for the observed variability. However, the values for protein, zinc, and iron became lower, which can be explained by systematic over- or underestimation of intake.

The intraindividual variation may have influenced results significantly. de Costa et al. [26] noted lower correlation coefficients for protein relative to other macronutrients and energy intake in an adolescent population from Piracicaba, Brazil. They reported that raising the coefficient to 0.9 demanded eleven days of food intake measurements, which is impracticable for validation studies. They nevertheless recommend a minimal of six days for this kind of population.

Epidemiological studies seek to find evidence of association between nutrient intake and the development of chronic diseases. To this end individuals must be classified according to intake levels so that risk factors can be correctly estimated. Therefore, assessing the ability of QFFQ to do this by the agreement between repeated applications of the questionnaire may be more important than correlation analysis. The means for exact agreement and extreme disagreement found in this study were similar to those of Slater et al. [20] for adolescents. The low agreement and high disagreement found for vitamin C are similar to the ones reported by Rodriguéz et al. [24] in adults and by Giacomello et al. [27] in pregnant women with up to seven years of school education.

Rodriguéz et al. [24] contend that 24hRs record dietary intake more precisely. They note that vitamin C is a nutrient difficult to measure due to the high daily variation in the intake of specific items such as fruits and vegetables and to the limited number of applied questionnaires, thus resulting in low correlation coefficients and agreement rates between methods.

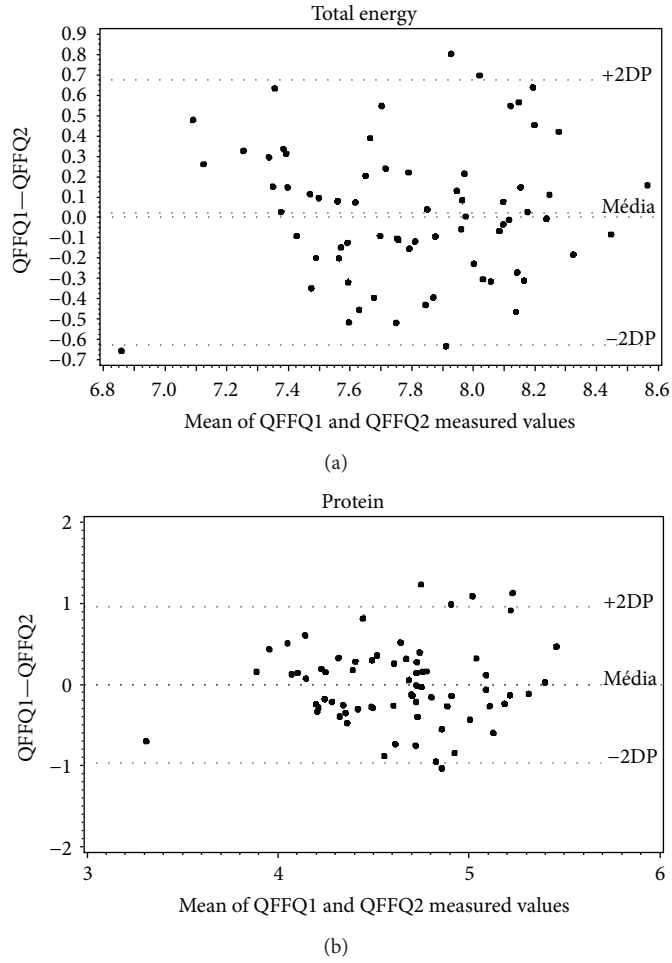

Figure 4: Bland-Altman plots for the mean total energy and protein intake of two QFFQs on type 1 diabetes adolescent patients. Goiânia, Brazil, 2009.

Percent agreement values between methods may be a random occurrence. Therefore, alternative measurements are necessary to complement the comparison. The weighted *kappa* is an indicator that corrects agreement values for randomness. In the present study results fell below the recommendation for exact agreement, but all values for extreme disagreement were below 10%. As for the *kappa*, even if vitamin C showed poor agreement scores, the mean value was regular. Voci et al. [15] assessed the validity of a questionnaire by food groups and observed low agreement for fruits and meat. In contrast, Assis et al. [28] found a mean *kappa* of 0.85 for a previous-day questionnaire applied in school-age individuals, but in this case the surveys were carried out at shorter intervals.

Watson et al. [21] found weak to regular agreement in questionnaires retrieved from Australian adolescents which they attributed to the intrinsic limitation of three 24hRs in faithfully registering the intake of items for which there is great within-individual variation. However, Xia et al. [29] showed moderate agreement.

As for the evaluation of agreement by Bland-Altman plots, which are seldom used in QFFQ validation studies, Voci et al. [15], Robinson et al. [30], and the present study found that the questionnaire overestimated values relative to the reference method. Giacomello et al. [27] showed that vitamin C is the nutrient with which methods disagree the most.

ICC values indicate the degree of association and agreement between values from each questionnaire. Our results for this reproducibility assessment were close to those reported by Marchioni et al. [31] and by Robinson et al. [30]. Rodriguez et al. [16] found values higher than 0.5 and Nahas et al. [32] found values higher than 0.8, but in both cases the interval was shorter between consecutive applications of the questionnaire.

It is recommended that the interval between applications should not be too short, lest the individual remembers the answers, or too long, lest there are changes in dietary habits. Our study found results similar to another Brazilian survey that used the same three-month interval [28]. McPherson et al. [33] suggest that the higher the correlation coefficients are, the shorter the interval between applications is, which probably allows for fewer changes in dietary patterns and a higher recall of answers.

Reproducibility studies in adults found higher correlation coefficients. Values between 0.5 and 0.7 are acceptable for

such studies, even if they are considered low relative to laboratory studies under controlled conditions [11]. However, children and adolescents are expected to yield lower coefficients, since the difference in nutrient ingestion in adolescent is twice that of adults [20, 24].

Children at seven or eight years of age can report their dietary intake, since by this time they already notice the passage of time and the ingestion of meals. Nevertheless, both older children and adolescents have difficulty in reporting portion size. It is therefore suggested that the ability to quantify ingestion does not depend on age since even adults have problems estimating the food quantities. A complicating factor is that this age range experiences quick changes in feeding habits. It must be considered that studies with adolescents rely on cognitive skills to record intake, but on subjective assessments of portion size [20].

Other reproducibility studies have also reported a reduction of the ICC values when they were adjusted for energy content [20, 31, 34] and this phenomenon is an indicator of systematic error in estimating ingestion, which is common in adolescents [21, 31].

The results plotted on Bland-Altman charts confirm the good agreement between the two QFFQ measurements for each individual. Marchioni and colleagues [31] reported an overestimation bias in the first application, but it was not significant. In the present study, there was a small positive difference for lipids, saturated and unsaturated fats, calcium, and total energy, but fibre, vitamin C, and iron were not underestimated.

Some limitations of our study must be remarked. Due to the sample size it was not possible to divide it according to age, gender, and nutritional status, all of which are factors that can influence intake estimates [31]. To be used on other populations, even on adolescent ones, this QFFQ must be validated again considering socioeconomic characteristics and health status of patients (considering that our population was based on a low income rate, e.g.). Other points considered by the authors as drawbacks are as follows. Although correlation coefficients fell in the "moderate" range, all were significant, especially the values for protein intake. Since the QFFQ significantly overestimated intake relative to the reference method, the obtained results must be analysed cautiously. The greater variability in consumption found in adolescents, which would account for the weaker correlation values, could be minimized by the application of more 24hRs and the use of familiar tools that help respondents to estimate portions and record intake and by motivating patients and helping them to develop skills that facilitate adherence to the survey. Even if perfect agreement is not reached, it must be taken into consideration that, for epidemiological studies, estimates of habitual dietary intake, even if less precise, are more relevant than more accurate measurements of current intake, which fail to capture general exposure trends [34].

5. Conclusion

In conclusion, the QFFQ was able to measure usual intake of energy, macronutrients, cholesterol, vitamin C, calcium, iron, and zinc by type 1 diabetes adolescent patients and, as required by epidemiological studies, showed good ability to classify them by intake level. Reproducibility results were also acceptable, confirming that this questionnaire will enable the execution of longitudinal studies that help us to understand clinical outcomes and better to orient and accompany these patients with a view to promoting their healthy growth and development.

Abbreviations

24hR: 24-hour recall
BFBS: Brazilian family budget survey
CF: Consumption frequency
DM1: Type 1 diabetes
EOC-CH: Endocrinology outpatient clinic, Clinical Hospital of the Federal University of Goiás
F: Consumption frequency categories
FICF: Free and informed consent formulary
GFU: Goiás Federal University
GGH: Goiânia General Hospital
ICC: Intraclass correlation coefficient
n_x: Number of surveys per subject
PC: Percentage contribution
QFFQ: Quantitative food frequency questionnaire
r_d: Deattenuated coefficient
r_p: Observed coefficient
SAS: Statistical Analysis System
Sn: Weights
SPSS: Statistical Package of Social Sciences
λ_x: Ratio of intrapersonal variation.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Rosana de Moraes Borges Marques conceived of and designed the study, did the literature review, analysed data, critically interpreted results, and wrote the paper. Amanda Cristine de Oliveira did the literature review and wrote the paper. Sheylle Almeida da Silva Teles conceived of and designed the study and analysed data. Maria Luiza Ferreira Stringuini wrote the paper and critically reviewed results. Giulliano Gardenghi critically interpreted results and wrote the paper. Nélida Shimid Fornés conceived of and designed the study, provided guidance during statistical analysis, and reviewed results critically. All authors read and approved the final paper.

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the conception of this paper and therefore it is cited as "*in memoriam*."

References

[1] M. Gabbay, P. R. Cesarini, and A. S. Dib, "Diabetes melito do tipo 2 na infância e adolescência," *Journal of Pediatrics*, vol. 75, pp. 201–208, 2003.

[2] R. Sichieri and J. E. Everhart, "Validity of a Brazilian food frequency questionnaire against dietary recalls and estimated energy intake," *Nutrition Research*, vol. 18, no. 10, pp. 1649–1659, 1998.

[3] U. Noethlings, K. Hoffmann, M. M. Bergmann, and H. Boeing, "Portion size adds limited information on variance in food intake of participants in the EPIC-potsdam study," *Journal of Nutrition*, vol. 133, no. 2, pp. 510–515, 2003.

[4] N. S. Fernés, I. S. Martins, M. Hernan, G. Velásquez-Meléndez, and A. Ascherio, "Food frequency consumption and lipoproteins serum levels in the population of an urban area, Brazil," *Revista de Saude Publica*, vol. 34, no. 4, pp. 380–387, 2000.

[5] M. Jain, G. R. Howe, and T. Rohan, "Dietary assessment in epidemiology: comparison of a food frequency and a diet history questionnaire with a 7-day food record," *The American Journal of Epidemiology*, vol. 143, no. 9, pp. 953–960, 1996.

[6] N. Schmid Fornés, M. L. Ferreira Stringhini, and B. Müller Elias, "Reproducibility and validity of a food-frequency questionnaire for use among low-income Brazilian workers," *Public Health Nutrition*, vol. 6, no. 8, pp. 821–827, 2003.

[7] Núcleo de Estudos e Pesquisas em Alimentação (NEPA), *Tabela Brasileira de Composição de Alimentos*, Nepa-Unicamp, Campinas, Brazil, 2nd edition, 2006.

[8] Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Alimentos e Nutrição Experimental/BRASILFOODS, Tabela Brasileira de Composição de Alimentos-USP, Versão 5.0, Rio de Janeiro, Brazil, 2009, http://www.fcf.usp.br/tabela/.

[9] G. Block, J. C. Norris, R. M. Mandel, and C. DiSogra, "Sources of energy and six nutrients in diets of low-income Hispanic-American women and their children: Quantitative data from HHANES, 1982–1984," *Journal of the American Dietetic Association*, vol. 95, no. 2, pp. 195–208, 1995.

[10] R. W. C. Haile, I. F. Hunt, J. Buckley, B. L. Browdy, N. J. Murphy, and D. Alpers, "Identifying a limited number of foods important in supplying selected dietary nutrients," *Journal of the American Dietetic Association*, vol. 86, no. 5, pp. 611–616, 1986.

[11] W. C. Willett, *Nutritional Epidemiology*, Oxford University Press, New York, NY, USA, 2nd edition, 1998.

[12] K. M. Flegal, F. A. Larkin, H. L. Metzner, F. E. Thompson, and K. E. Guire, "Counting calories: Partitioning energy intake estimates from a food frequency questionnaire," *The American Journal of Epidemiology*, vol. 128, no. 4, pp. 749–760, 1988.

[13] N. S. Fornés, I. S. Martins, G. Velasquez-Meléndez, and M. R. D. O. Latorre, "Escores de consumo alimentar e níveis lipêmicos em população de São Paulo, Brasil," *Revista de Saúde Pública*, vol. 32, pp. 12–18, 2002.

[14] H. R. H. Rockett, M. Breitenbach, A. L. Frazier et al., "Validation of a youth/adolescent food frequency questionnaire," *Preventive Medicine*, vol. 26, no. 6, pp. 808–816, 1997.

[15] S. M. Voci, C. C. Enes, and B. Slater, "Validação do Questionário de Freqüência Alimentar para Adolescentes (QFAA) por grupos de alimentos em uma população de escolares," *Revista Brasileira de Epidemiologia*, vol. 11, no. 4, pp. 561–572, 2008.

[16] I. T. Rodriguez, J. F. Ballart, G. C. Pastor, E. B. Jorda, and V. A. Val, "Validación de un cuestionnario de frecuencia de consume alimentario corto: reproducibilidad y validez," *Nutrición Hospitalaria*, vol. 23, pp. 242–252, 2008.

[17] G. H. Beaton, J. Milner, P. Corey et al., "Sources of variance of 24-hour dietary recall data: Implications for nutrition study designing and interpretation," *The American Journal of Clinical Nutrition*, vol. 32, no. 12, pp. 2546–2559, 1979.

[18] J. M. Bland and D. G. Altman, "Statistical methods for assessing agreement between two methods of clinical measurement," *The Lancet*, vol. 1, no. 8476, pp. 307–310, 1986.

[19] Ministério da Saúde, "Rede Interagencial de Informações para a Saúde. Indicadores e dados Básicos-Brasil," 2008, http://tabnet.datasus.gov.br/cgi/idb2008/matriz.htm.

[20] B. Slater, S. T. Philippi, R. M. Fisberg, and M. R. D. O. Latorre, "Validation of a semi-quantitative adolescent food frequency questionnaire applied at a public school in São Paulo, Brazil," *European Journal of Clinical Nutrition*, vol. 57, no. 5, pp. 629–635, 2003.

[21] J. F. Watson, C. E. Collins, D. W. Sibbritt, M. J. Dibley, and M. L. Garg, "Reproducibility and comparative validity of a food frequency questionnaire for Australian children and adolescents," *International Journal of Behavioral Nutrition and Physical Activity*, vol. 6, article 1479, p. 62, 2009.

[22] T. Kobayashi, M. Kamimura, S. Imai et al., "Reproducibility and validity of the food frequency questionnaire for estimating habitual dietary intake in children and adolescents," *Nutrition Journal*, vol. 10, article 27, no. 1, 2011.

[23] M. D. Riley and L. Blizzard, "Comparative validity of a food frequency questionnaire for adults with IDDM," *Diabetes Care*, vol. 18, no. 9, pp. 1249–1254, 1995.

[24] M. M. Rodriguéz, H. Méndez, B. Torún, D. Shroeder, and A. D. Stein, "Validation of a food frequency questionnaire for use among adults in Guatemala," *Public Health Nutrition*, vol. 5, pp. 691–698, 2002.

[25] M. Hernández-Avila, I. Romieu, S. Parra, J. Hernández-Avila, H. Madrigal, and W. Willett, "Validity and reproducibility of a food frequency questionnaire to assess dietary intake of women living in México City," *Salud Publica de Mexico*, vol. 40, no. 2, pp. 133–140, 1998.

[26] M. M. F. de Costa, L. Takeyama, S. M. Voci, B. Slater, and M. V. Silva, "Within- and between-person variations as determinant factors to calculate the number of observations to estimate usual dietary intake of adolescents," *Revista Brasileira de Epidemiologia*, vol. 11, no. 4, pp. 541–548, 2008.

[27] A. Giacomello, M. I. Schmidt, M. A. A. Nunes et al., "Validação relativa de questionário de frequência alimentar em gestantes usuárias de serviços do Sistema Único de Saúde em dois municípios no Rio Grande do Sul, Brasil," *Revista Brasileira de Saúde Materno Infantil*, vol. 8, pp. 445–454, 2008.

[28] M. A. A. Assis, D. Guimaraes, M. C. M. Calvo, M. V. G. Barros, and E. Kupek, "Reprododutibilidade e validade de questionário de consumo alimentar para escolares," *Revista de Saúde Pública*, vol. 41, pp. 1054–1057, 2007.

[29] W. Xia, C. Sun, L. Zhang et al., "Reproducibility and relative validity of a food frequency questionnaire developed for female adolescents in Suihua, North China," *PLoS ONE*, vol. 6, no. 5, Article ID e19656, 2011.

[30] S. Robinson, R. Skelton, M. Barker, and C. Wilman, "Assessing the diet of adolescent girls in the UK," *Public Health Nutrition*, vol. 2, no. 4, pp. 571–577, 1999.

[31] D. M. L. Marchioni, S. M. Voci, F. E. L. Lima, R. M. Fisberh, and B. Slater, "Reproducibility of a food frequency questionnaire for adolescents," *Cadernos de Saúde Pública*, vol. 23, pp. 2187–2196, 2007.

[32] M. V. Nahas, M. V. G. Barros, A. A. Florindo, J. C. Farias Junior, P. C. Hallal, L. Konrad et al., "Reprodutibilidade e validade do questionário saúde na boa para avaliar atividade física e hábitos alimentares em escolares do ensino médio," *Revista Brasileira de Atividade Física & Saúde*, vol. 12, pp. 12–20, 2007.

[33] R. S. McPherson, D. M. Hoelscher, M. Alexander, K. S. Scanlon, and M. K. Serdula, "Dietary assessment methods among school-aged children: validity and reliability," *Preventive Medicine*, vol. 31, no. 2, pp. S11–S33, 2000.

[34] R. A. G. Souza, A. C. S. Rangel, C. M. Rogério, and R. Sichieri, "Confiabilidade de um questionário de frequência de consumo alimentar em mulheres," *Ciência, Cuidado e Saúde*, vol. 6, pp. 103–109, 2007.