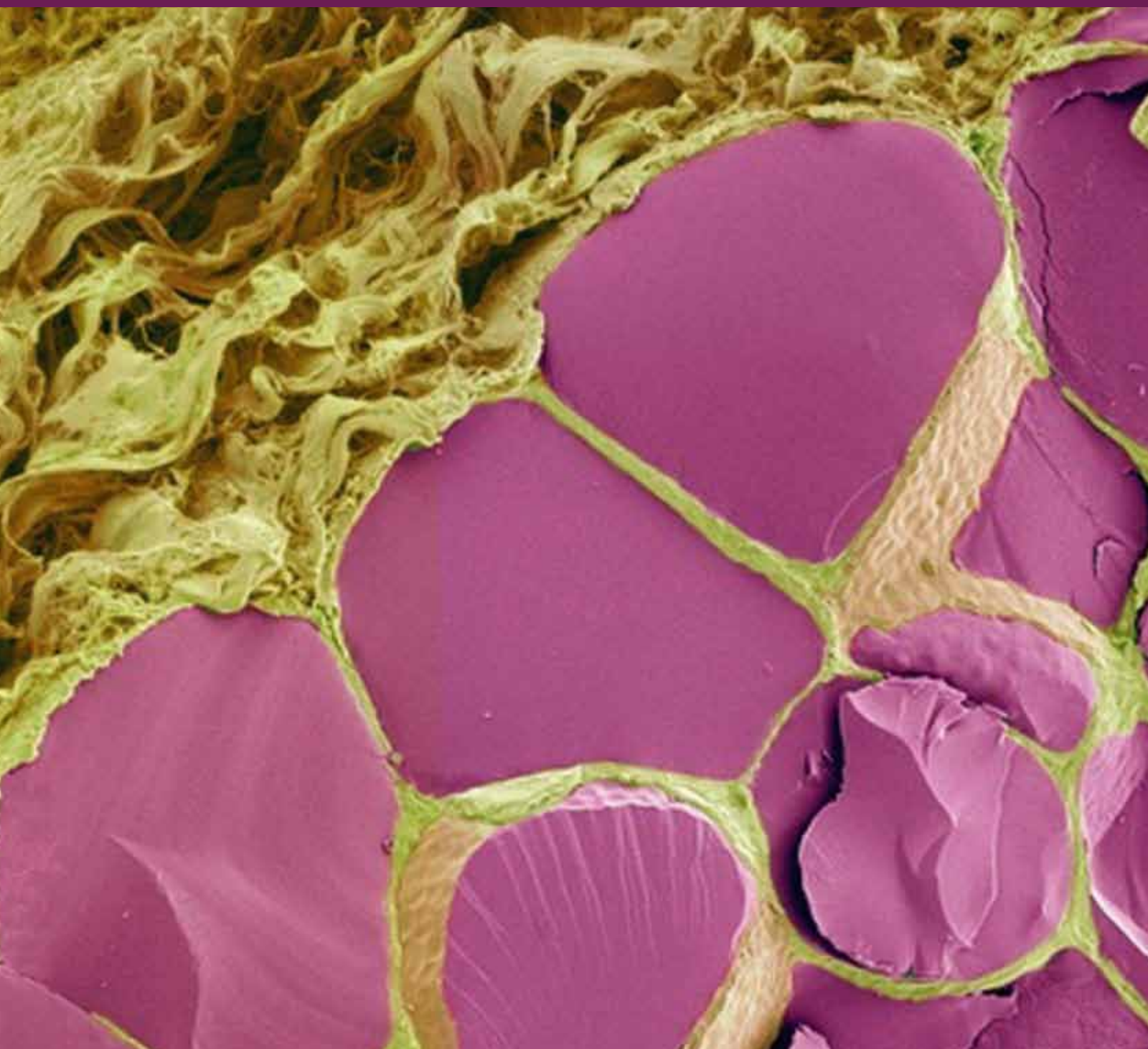


Diabetes Mellitus

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





Diabetes Mellitus

International Journal of Endocrinology

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Editorial

Diabetes Mellitus

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There is ongoing progress in the understanding, diagnosis, and management of diabetes mellitus [1]. The present special issue is devoted to the recent progress. There are 28 articles in total, which cover 6 thematic areas: epidemiology, pathogenesis, treatment, complications, nonalcoholic fatty liver (NAFL), and sleep disorders.

Epidemiology. In the context of the diabetes epidemic, W. Nseir et al. in their paper titled “*Seeking out high risk population: the prevalence characteristics and outcome of diabetic patients of Arab ethnicity hospitalized in internal medical and acute coronary units in Israel*” have looked at the prevalence of diabetes and issues relating to treatment efficacy among inpatients of hospitals with predominant Arab patients in Northern Israel. The prevalence of diabetes in this setting was 39% with a preponderance of women. Importantly, diabetic patients experienced prolonged hospitalisation, increased readmission rates, and suboptimal control. This data is important because it highlights the issues needing improvement.

Pathogenesis. M. Zhang et al. in their paper titled “*Fish and marine omega-3 polyunsaturated fatty acid consumption and incidence of type 2 diabetes: a systematic review and meta-analysis*” have conducted a systematic review and meta-analysis on the relationship between the intake of fish and marine long-chain omega-3 polyunsaturated fatty acids and

the incidence of type 2 diabetes mellitus (T2DM). They noted significant heterogeneity between included studies and could find no significant correlation between the effect of fish/seafood or marine polyunsaturated fatty acid consumption and risk of T2DM in general. However, oily fish polyunsaturated fatty acid, in particular, significantly reduced the risk of T2DM by 11% ($P = 0.005$). This work adds to the growing body of literature on the impact of polyunsaturated fatty acids on T2DM and attempts to take us beyond the conflicting results of the individual cohort studies [2, 3]. It now remains to scrutinise the selective beneficial effect of oily fish fatty acids by clinical trials with longer follow-up and determine any differences between high- and low-risk populations.

Turning their attention to type 1 diabetes (T1DM), K. Blaslov et al. in their paper titled “*Relationship between adiponectin level, insulin sensitivity, and metabolic syndrome in type 1 diabetic patients*” have explored insulin resistance and the metabolic syndrome in this condition. For this purpose, they have evaluated the relationship between adiponectin concentrations, metabolic syndrome, and insulin sensitivity. Adiponectin is known to exert a protective action against the metabolic syndrome, T2DM, and vascular injury [4]. At the same time, features of the metabolic syndrome may, albeit occasionally, be present in T1DM as well, while the role of adiponectin in this situation is less clear [5]. K. Blaslov et al. have now shown higher adiponectin levels to

be associated with significantly lower waist circumference and serum glucose, along with a more favourable lipid profile. Logistic regression analysis confirmed adiponectin to bear a significant negative correlation with the metabolic syndrome ($P = 0.014$). Obviously, these results are promising and may have useful clinical implications. Nonetheless, it is rather premature to uphold a potential protective effect of adiponectin against development of metabolic syndrome in T1DM until further confirmation is obtained.

A. Al-Shukaili et al. in their paper titled “*Analysis of inflammatory mediators in type 2 diabetes patients*” have investigated cytokine levels and lymphocyte subsets in patients with T2DM. In comparison to healthy volunteers, T2DM patients exhibited significantly reduced interleukin (IL)-1 β , IL-6, IL-15, and tumour necrosis factor- α (TNF- α), as well as significantly elevated IL-10, interferon- γ (IFN- γ), and caspase-1, while lymphocyte subsets did not differ. Moreover, the presence of hypertension was linked with diminution of IL-1 β and caspase-1. Overall, HbA_{1c} was positively correlated with IL-6 ($P = 0.005$), and body-mass index (BMI) was positively correlated with c-reactive protein (CRP) ($P = 0.001$) and TNF- α ($P = 0.013$). These results confirm the activation of cytokines, primarily IL-6, in insulin resistance and T2DM [6, 7]. They also add new useful information on the contribution of hypertension to this inflammatory activity.

B. Liu et al. in their paper titled “*Ketosis-onset diabetes and ketosis-prone diabetes: same or not?*” have provided new knowledge on diabetes mellitus presenting with ketosis. This atypical form of diabetes, first described in Afro-Americans 25 years ago, is now known to present in two slightly different forms, that is, ketosis-prone diabetes and ketosis-onset diabetes [8]. In such patients, there may be considerable heterogeneity in terms of autoantibodies, which permits classification into 4 subgroups ($A + \beta^-$, $A - \beta^+$, $A - \beta^-$, and $A + \beta^+$) [8]. B. Liu et al. now document that most patients with ketosis-prone diabetes had greater age and longer duration than ketosis-onset diabetes, although this does not apply to all subgroups. Moreover, in some of the subgroups, lower fasting plasma glucose and lower HbA_{1c} in ketosis-prone versus ketosis-onset diabetes was noted. The authors conclude that ketosis-prone and ketosis-onset diabetes are not uniformly the same in presentation. Clearly, there is a lot to learn about this rare form of diabetes in the future.

X.-F. Zhang et al. in their paper titled “*The ETS-domain transcription factor elk-1 regulates COX-2 gene expression and inhibits glucose-stimulated insulin secretion in the pancreatic β -cell line INS-1*” have used the pancreatic β -cell line INS-1 to study the association between the transcription factor Elk-1 and Cyclooxygenase-2 (COX-2). The rationale is that Elk-1 can significantly enhance the activation of COX-2 gene promoter, thereby impairing β -cell secretion via production of Prostaglandin E2 [9]. The authors report that Elk-1 can upregulate COX-2 expression and that excessive expression of the former can impair pancreatic glucose-stimulated insulin secretion. It may be hoped that these new findings can help towards defining new target molecules for potential future pharmaceutical intervention to hinder β -cell malfunction.

Treatment. Nowadays, the challenges in the management of T2DM arise not only from the abundance of different drugs and expert guidelines but also from the need for a modern patient-centred approach [10, 11]. In this issue, L. Chhabra et al. in their paper titled “*Challenges in the management of type 2 diabetes mellitus and cardiovascular risk factors in obese subjects: what is the evidence and what are the myths?*” discuss the goals, priorities, and fallacies in the treatment of T2DM (including improvement of cardiovascular risk factors) in obese subjects. They summarise recent evidence showing that intensive lifestyle modification does not succeed in reducing cardiovascular morbidity [12], but this does not negate the role of patient education on healthy dietary choices. They also emphasise the need to intensify antidiabetic treatment strategies in subjects with newly diagnosed T2DM, as these are most likely to revert to normoglycemia and gain a reduction in cardiovascular morbidity. Moreover, they maintain that stringent, as opposed to lenient, targets achieve more efficacious and durable weight loss, and they discuss the growing importance of bariatric surgery [13]. The latter can drastically improve serum glucose before actual weight loss and deserves to be considered not only in morbid but also in moderate obesity, at least in selected patients [13].

M. Haluzík et al. in their paper titled “*Renal effects of DPP-4 inhibitors: a focus on microalbuminuria*” have examined the effects of dipeptidyl peptidase-4 (DPP-4) inhibitors on the kidney, focusing on microalbuminuria. There is evidence to suggest that hyperglycemia, among other perturbations, interferes with glucagon-like peptide-1 (GLP-1) signalling in the kidney, which, in turn, may promote the expression of angiotensin II and TNF- α , thereby perpetuating glomerular injury [14]. In this context, DPP-4 inhibitors may contribute not only to the reduction of oxidative stress and inflammation but also to the improvement of endothelial dysfunction in the kidney, as discussed by the authors. It appears that their beneficial actions may be exerted by both GLP-1 dependent and GLP-1 independent mechanisms. Taken together, the data presented by M. Haluzík et al. cherish some hope that DPP-4 inhibitors may prove more useful in renal protection, while ongoing trials are anticipated to shed more light on this issue.

W.-H. Xiao et al. in their paper titled “*The effects of pioglitazone on biochemical markers of bone turnover in the patients with type 2 diabetes*” have investigated the potential untoward effects of pioglitazone on bone turnover in T2DM. They have demonstrated a significant reduction of procollagen type 1N-terminal propeptide (PINP) and total alkaline phosphatase (BAP) in women but not in men, following 3 months of treatment. This effect was most pronounced in the post-rather than premenopausal group. Conversely, there was no change in osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) levels in either gender. As already shown with thiazolidinediones [15], the present findings point to an adverse effect of pioglitazone on bone turnover. Specifically, this effect involves inhibition of bone formation but not of bone resorption, and postmenopausal women are most amenable. Clearly, these observations should find more application when choosing antidiabetic regimens in clinical

practice, as it is now beginning to be appreciated in the era of individualised treatment [10].

A. Colatrella et al. in their paper titled "*Comparison of insulin lispro protamine suspension with NPH insulin in pregnant women with type 2 and gestational diabetes mellitus: maternal and perinatal outcomes*" have retrospectively compared maternal and perinatal outcomes of insulin lispro protamine suspension with neutral protamine Hagedorn (NPH) insulin in 25 pregnant women with T2DM and 64 with gestational diabetes (GDM). Insulin lispro protamine suspension is a relatively new formulation, which has hitherto been evaluated in T1DM and T2DM but not GDM [16]. More knowledge in GDM with this preparation is desirable, given the worldwide increase of GDM and the favourable outcomes achieved with detemir, another recent insulin analogue [17]. In the present analysis, there was no difference between the two treatment arms in terms of maternal outcomes (mode and time of delivery). Similarly, there was no difference in neonatal outcomes (e.g., newborn weight, neonatal hypoglycaemia rates, and congenital malformations) except for excessive ponderal index, which was more frequent with NPH. Moreover, fasting blood glucose, maternal hypoglycaemia rates, and weight gain did not differ between the two insulin regimens. Finally, insulin dose was lower with lispro protamine suspension. Thus, the new insulin formulation was not inferior to traditional NPH insulin.

J. Nicoll et al. in their paper titled "*Subetta treatment increases adiponectin secretion by mature human adipocytes in vitro*" have investigated the effect of Subetta (a novel composite preparation containing antibodies to the beta subunit of the insulin receptor and antibodies to endothelial nitric oxide synthase) on the production of adiponectin by human mature adipocytes. This was compared to rosiglitazone. It was found that Subetta significantly promoted adiponectin secretion. Although preliminary *in vitro* evidence only, the present results encourage further enquiry into the efficacy and the modes of action of Subetta as a potential emerging hypoglycaemic agent.

Complications. S. Pruhova et al. in their paper titled "*Chronic mild hyperglycemia in GCK-MODY patients does not increase carotid Intima-Media Thickness*" have examined carotid intima media thickness (CIMT) in patients with glucokinase-mutation maturity onset diabetes of the young (GCK-MODY or MODY2). In comparison to controls, subjects with GCK-MODY had insignificantly increased CIMT, adjusted for age, gender, and family status, while there were also no differences in frequency of myocardial infarction and ischaemic stroke between the two groups. The authors take this data as evidence for a low risk of developing macrovascular complications in GCK-MODY, despite chronic mild hyperglycaemia. However, caution is needed to avoid physician negligence in pursuing glycaemic targets in such patients.

M. Cetin et al. in their paper titled "*Relation of epicardial fat thickness with carotid intima-media thickness in patients with type 2 diabetes mellitus*" have examined the association of CIMT with epicardial fat thickness, an emerging potential additional indicator of cardiovascular risk [18], in T2DM. Both parameters were increased in T2DM patients versus

controls. In T2DM, epicardial fat thickness was correlated with CIMT, waist circumference, BMI, age, and diabetes duration. In linear regression analysis, CIMT and waist circumference emerged as independent predictors of epicardial fat thickness. Interestingly, the authors defined a 6.3 mm cut-off for epicardial fat thickness, which yielded 72.5% sensitivity and 71.7% specificity for the diagnosis of increased CIMT denoting incipient atherosclerosis. This work adds to the growing evidence that epicardial fat thickness may prove useful as a marker of cardiovascular risk, and further experience is now awaited.

Y. Zhang et al. in their paper titled "*Assessment of carotid atherosclerosis in type 2 diabetes mellitus patients with microalbuminuria by high-frequency ultrasonography*" present data from high-frequency ultrasound employed in the assessment of CIMT and atherosclerotic plaques in T2DM patients with micro-versus those with normoalbuminuria. IMT was predictably higher in the former. Overall, IMT exhibited significant correlations with urinary albumin excretion rate, age, diabetes duration, HbA_{1c}, waist circumference, BMI, and systolic blood pressure. Importantly, urinary albumin excretion rate was among the independent predictors of CIMT.

Analysing insulin sensitivity and antioxidant enzyme activity in patients with or without T2DM and various stroke subtypes, A. Jotic et al. in their paper titled "*Type 2 diabetic patients with ischemic stroke: decreased insulin sensitivity and decreases in antioxidant enzyme activity are related to different stroke subtypes*" show that reduced insulin sensitivity and glutathione reductase are associated with atherothrombotic and lacunar stroke in T2DM. These results open up a new vista linking insulin resistance with stroke via diminished antioxidant enzyme activity, but the attractive question why this particularly applies to some kinds of stroke remains unanswered.

Wound healing may be impaired in diabetes, especially in the foot [19]. C. Shrestha et al. in their paper titled "*Enhanced healing of diabetic wounds by subcutaneous administration of human umbilical cord derived stem cells and their conditioned media*" have used mesenchymal stem cells from the umbilical cord and their conditioned media to promote wound closure in the experimental model (dorsal wound in db/db mice). Phosphate buffer solution was used as control. Both stem cells and their conditioned media accelerated healing (especially the latter). The results are encouraging and further clinical experience is desirable.

Going from the experimental setting to humans, diabetic foot infections increase the risk of lower-limb amputations [20]. Tissue cultures are the gold standard for the identification of true pathogens [21]. Using these, M. Demetriou et al. in their paper titled "*Determinants of microbial load in infected diabetic foot ulcers: a pilot study*" have attempted to define parameters predicting a high microbial load in diabetic foot infections. The number of isolates on tissue cultures and white blood cell count were found as the most powerful predictors of microbial load. Other predictors included platelet count and clinical severity of infection. The authors suggest that high blood cell and platelet count, as well as a sinister clinical manifestation, call for a more aggressive

initial antibiotic regimen to cover a diversity of pathogens, and this sounds quite reasonable.

Polyneuropathy is a cardinal aetiological factor in the pathogenesis of the diabetic foot [19, 20], and its early diagnosis with accurate clinical tests is of utmost importance [22]. T. Mete et al. in their paper titled "*Comparison of efficiencies of Michigan neuropathy screening instrument, neurothesiometer, and electromyography for diagnosis of diabetic neuropathy*" have used the Michigan Neuropathy Screening Instrument (MNSI), the neurothesiometer, and nerve conduction study (NCS) to detect diabetic polyneuropathy. The neurothesiometer and NCS yielded higher rates of polyneuropathy (74.5% and 46.2%, resp.), as compared to clinical examination by MNSI (32.1%). The authors use these findings as argument that neurothesiometer and NCS should be employed to increase timely detection of polyneuropathy.

P. Thomakos et al. in their paper titled "*Cigarette smoking is associated with prolongation of the QTc interval duration in patients with type 2 diabetes mellitus*" have assessed the effect of smoking on autonomic nerve function and QTc interval in T2DM. They could demonstrate significant prolongation of the QTc interval during both day and night in smokers versus nonsmokers. Conversely, there was no difference in attributes of autonomic nerve function between the two groups. It was concluded that smoking prolonged the QTc interval by mechanism(s) independent of autonomic dysfunction. Whether this effect contributes to increased cardiovascular risk remains to be further queried.

S. Meguro et al. in their paper titled "*Past obesity as well as present body weight status is a risk factor for diabetic nephropathy*" have sought the association between prevalence of nephropathy and past obesity status in a large series of Japanese patients with T2DM. Nephropathy was significantly ($P < 0.017$) more frequent in the event of prior or current obesity than in constantly normal-weight subjects. Both past and present obesity belonged to the independent predictors of neuropathy in logistic regression analysis. These observations are important, given the increasing appreciation of an obesity-related nephropathy, and it would be highly welcome to have studies providing more information on duration of obesity, fluctuations of BMI, and the effect of anti-diabetic treatment and lifestyle changes on the development and/or evolution of diabetic kidney disease.

N. Grandfils et al. in their paper titled "*Glucose lowering therapeutic strategies for type 2 diabetic patients with chronic kidney disease in primary care setting in France: a cross-sectional study*" have scrutinised the strategies and priorities used by French general practitioners when deciding on anti-diabetic treatment for T2DM patients with moderate/severe chronic kidney disease. Perceived severity of diabetes, rather than of kidney disease, was the most important factor in choosing treatment. Of note, most practitioners tended to underestimate the risk of hypoglycaemia in this vulnerable population. In pursuit of stringent glycaemic targets, 2/3 of patients received drugs not safe enough for use in moderate/severe kidney disease. This observational study highlights the importance of modern individualised treatment, based on comorbidities, life expectancy, diabetes duration, hypoglycaemia awareness, and other patient parameters [10, 11].

Clearly, we need to improve physicians' understanding of these intricate issues.

B. Xu et al. in their paper titled "*Low serum magnesium level is associated with microalbuminuria in Chinese diabetic patients*" have addressed the relationship between low serum magnesium levels and microalbuminuria in Chinese subjects with diabetes mellitus. Microalbuminuria was significantly ($P < 0.0001$) more frequent in subjects with low serum magnesium. Even after adjustment for several covariates, there was an almost twofold increase in the frequency of microalbuminuria among those with the lowest magnesium levels. Given that magnesium harbours antioxidant actions [23], an arguable explanation may be that low magnesium contributes to oxidative stress, which, in turn, increases kidney damage [24]. We should, however, bear in mind that dietary habits and the effect of diuretics may be confounding factors, and so further experience regarding the aforementioned association is needed.

V. Jegdic et al. in their paper titled "*Physical fitness in children with type 1 diabetes measured with six-minute walk test*" have addressed the question whether children with T1DM exhibit lower physical fitness and whether, should this be the case, increased HbA_{1c} plays a contributory role. By means of the 6-minute walk test, they demonstrated lower physical fitness in T1DM, but this did not appear to be dependent on HbA_{1c}. This rather surprising result calls for increased medical attention offered to T1DM children and, therefore, merits prompt replication in other populations.

NAFL. A. N. Mavrogiannaki and I. N. Migdalis in their paper titled "*Nonalcoholic Fatty liver disease, diabetes mellitus and cardiovascular disease: newer data*" present new information on NAFL, T2DM, and cardiovascular disease. They review the evidence on the prevalence of NAFL in T2DM (and, more rarely, in T1DM) and on the prevalence of cardiovascular morbidity in subjects with NAFL. Pathogenesis of NAFL mainly involves insulin resistance. New imaging techniques (such as elastography), emergent breath tests, and new biomarkers represent the progress that is being achieved in diagnosis. Weight loss, antioxidant drugs, and hypolipidaemic agents have been used in the management. As regards anti-diabetic treatment, some favourable outcomes have been achieved with metformin, thiazolidinediones, and GLP-1 analogues [25], but there is no uniformly accepted therapeutic modality.

Following on, E. Bacchi and P. Moghetti in their paper titled "*Exercise for hepatic fat accumulation in type 2 diabetic subjects*" discuss the role of exercise in the management of NAFL in T2DM. Indeed, there is interesting accumulating data that exercise can, independently of dietary changes, contribute to the reduction of hepatic steatosis. Of relevance, this appears to hold true both for aerobic and for anaerobic exercise. However, the magnitude of achieved effects is quite variable, and the precise effect on liver histology need further examination.

T. Hirata et al. in their paper titled "*Effect of telmisartan or losartan for treatment of nonalcoholic fatty liver disease: fatty liver protection trial by telmisartan or losartan study*"

(FANTASY)” have examined the efficacy of losartan and telmisartan in improving NAFL in the presence of hypertension and T2DM. Telmisartan-treated patients exhibited significant reductions in serum-free fatty acids and liver fat (as evaluated by Computed Tomography). These promising results may be ascribed to the unique activation of peroxisome proliferator-activated receptors gamma (PPAR- γ) by telmisartan [26] and call for replication in larger trials and different ethnic groups. If established, this action of telmisartan will improve our therapeutic interventions for NAFL.

Sleep Disorders. Sleep disorders are now increasingly being appreciated in diabetes and prediabetes. Indeed, somnolence, obstructive sleep apnoea, and sleep deprivation are now frequently examined in T2DM patients [27, 28]. W.A.Wan Mahmood et al. in their paper titled “Association between sleep disruption and levels of lipids in Caucasians with type 2 diabetes” have examined lipid profile in T2DM patients with poor sleep quality. They have shown elevated total cholesterol in subjects with long sleep duration and elevated triglycerides in those with short sleep duration. Sleep duration and quality were identified as major contributors to adverse serum lipids. This study adds to our knowledge on the disadvantageous effect of sleep disorders on serum lipids, as already shown for obstructive sleep apnoea [29].

J. Liu et al. in their paper titled “The association of sleep disorder, obesity status, and diabetes mellitus among US adults—the NHANES 2009–2010 survey results” have used the National Health and Nutrition Examination Survey (NHANES) 2009–2010 data to examine the relationship of sleep disorders with T2DM. In summary, after adjustment for several covariates including BMI, they have found that sleep disorders increase the risk of diabetes by 38%. Importantly, most of this increased risk is driven through subjects’ obesity. These observations come from a large robust database and consolidate our knowledge pertaining to sleep perturbations in diabetes.

Conclusions. There is ongoing progress in diabetes research and care, as exemplified in the areas covered in this special issue. Several other fields are also showing progress, such as management of the diabetic foot [30], but these are not discussed in the present issue. From a clinical perspective, it is now important to integrate this progress into clinical reality, and this remains an ongoing challenge.

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Research Article

Chronic Mild Hyperglycemia in GCK-MODY Patients Does Not Increase Carotid Intima-Media Thickness

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Aim. GCK-MODY is an autosomal dominant form of diabetes caused by heterozygous mutations in the glucokinase gene leading to a lifelong mild hyperglycemia. The risk of macrovascular complications is considered low, but studies are limited. We, therefore, investigated the carotid intima-media thickness (CIMT) as an indicator of macrovascular complications in a group of patients with GCK-MODY. **Methods.** Twenty-seven GCK mutation carriers and 24 controls recruited among their first-degree relatives were compared, all aging over 35 years. The CIMT was tested using a high-resolution B-mode carotid ultrasonography. Medical history, anthropometry, and biochemical blood workup were obtained. **Results.** The mean CIMT was 0.707 ± 0.215 mm (mean \pm SD) in GCK mutation carriers and 0.690 ± 0.180 mm in control individuals. When adjusted for age, gender, and family status, the estimated mean difference in CIMT between the two groups increased to 0.049 mm ($P = 0.19$). No difference was detected for other characteristics, with the exception of fasting blood glucose (GCK-MODY 7.6 mmol/L ± 1.2 (136.4 mg/dL); controls 5.3 mmol/L ± 0.3 (95.4 mg/dL); $P < 0.0001$) and glycated hemoglobin HbA_{1c} (GCK-MODY $6.9\% \pm 1.0\%$, 52 mmol/mol ± 10 ; controls $5.7\% \pm 0.4\%$, 39 mmol/mol ± 3 ; $P < 0.0001$). The frequency of myocardial infarction and ischemic stroke did not differ between groups. **Conclusion.** Our data indicate that the persistent hyperglycemia in GCK-MODY is associated with a low risk of developing diabetic macrovascular complications.

1. Introduction

GCK-MODY (GCK diabetes, glucokinase diabetes, or MODY2) is a monogenic condition caused by heterozygous

mutations in the gene encoding glucokinase (GCK) [1]. It is characterized by chronic, lifelong, and mild hyperglycemia present from birth, and less than 50% of patients fulfil

the criteria for overt diabetes. GCK-MODY is not associated with insulin resistance or dyslipidemia [2].

The increased risk of atherosclerotic vascular disease—as compared with healthy individuals without diabetes—is known in subjects with impaired glucose tolerance, patients with type 2 diabetes, and patients with metabolic syndrome [3, 4]. Noninvasive imaging techniques, such as carotid intima-media thickness (CIMT) measurements, may help to stratify this risk of atherosclerosis, as well as the risk of myocardial ischemia [5]: the CIMT has been shown to independently predict coronary events in type 2 diabetes and cardiovascular diseases [4]. However, the situation in GCK-MODY is likely to be different from that in type 2 diabetes: patients with GCK-MODY have increased fasting blood glucose and relatively low 2-hour post-OGTT blood glucose without other components of metabolic syndrome or insulin resistance [6]. Such a blood glucose profile has been shown to be associated with lower rates of cardiovascular mortality among patients with type 2 diabetes [7]. Importantly, Niskanen et al. [8] demonstrated that components of insulin resistance syndrome, including hyperinsulinemia after an oral glucose load, serum lipid abnormalities, and elevated blood pressure, are major determinants of CIMT in patients with diabetes.

The risk of macrovascular complications in GCK-MODY is considered low, but the data are scarce. We aimed to evaluate the carotid intima-media thickness (CIMT) as an indicator of this risk in GCK-MODY patients aging 35 years or older and their unaffected relatives, who share a similar environment and lifestyle.

2. Materials and Methods

We studied 27 patients from 20 Czech families with genetically confirmed GCK-MODY (age 35–75 years; median 46 years) and 24 unaffected family members (siblings, parents, and partners) representing the control group (age 35–79 years; median 50 years). Each of the 20 participating families contributed 1 to 3 patients with GCK-MODY and 1 to 3 control individuals matched by age and gender. Control individuals with fasting blood glucose more than 5.6 mmol/L (100 mg/dL) and/or with a known history of diabetes were excluded from the study. The identification of families with GCK-MODY has been reported previously [9, 10]. Informed consent was obtained from all study participants. The study protocol was approved by the Ethics Committee of the 3rd Faculty of Medicine, Charles University in Prague, Czech Republic.

All study participants were examined in a fasting state. The structured assessment included a questionnaire, anthropometric examination and blood sampling for biochemical analysis. The laboratory methods used have been described previously [11].

High-resolution B-mode carotid ultrasonography (using Phillips iU22 ultrasound) was performed to measure the CIMT of the distant wall for 1 cm lengths of the carotid bifurcation and the internal carotid and right and left common carotid arteries. The mean CIMT values of 10 sites were combined in an unweighted average to produce

an overall CIMT. The upper normal limit of CIMT was set to 0.7 mm [12, 13]. The patient history of coronary heart disease and ischemic stroke was obtained from medical records. All participants were further investigated with echocardiography and ECG.

The clinical and demographic characteristics of GCK mutation carriers and control individuals were compared using Welch's two-sample *t*-tests (continuous variables) and Fisher's exact tests (categorical variables). Mixed linear regression models with CIMT, blood pressure, and serum creatinine as outcomes were used to estimate and test the adjusted effects of GCK mutation status. Age, gender, and mutation status were included as fixed effects, and families were included as random effects. All analyses were performed using the R statistical package [14]. *P* value ≤ 0.05 was considered statistically significant.

3. Results

No significant differences in baseline characteristics were found between patients and control individuals, with the exception of fasting blood glucose (GCK-MODY 7.6 mmol/L, SD \pm 1.2 (136.4 mg/dL); controls 5.3 mmol/L, SD \pm 0.3 (95.4 mg/dL); *P* < 0.0001) and glycated hemoglobin HbA_{1c} (GCK-MODY 6.9% (SD \pm 1.0), 52 mmol/mol (SD \pm 10); controls 5.7% (SD \pm 0.4), 39 mmol/mol (SD \pm 3); *P* < 0.0001) (Table 1). The prevalence of smokers and hypertensive patients was similar in both samples (*P* = 1).

The measured CIMT values for participants with and without GCK mutations are shown in Figure 1. The mean CIMT was 0.707 mm (range: 0.4–1.1) in GCK-MODY patients and was 0.692 mm (range: 0.4–1.1) in healthy control individuals. According to the published recommendations [12, 13], these values did not indicate an increased risk of accelerated atherosclerosis. After adjusting for age, gender, and family status, the estimated mean difference in CIMT between patients and healthy individuals increased slightly to 0.049 mm (95% CI from –0.026 to 0.123; *P* = 0.19). As expected, the estimated trends of mean CIMT indicated a moderate increase in CIMT with age and mutation status (see regression lines plotted in Figure 1). Carotid plaques (local intima-media thickening exceeding 1 mm and protruding into the lumen) were identified in 7 (25.9%) patients and 3 (12.5%) control individuals (*P* = 0.1), but all of these plaques were hemodynamically insignificant.

Myocardial changes typical of ischemic heart disease described on echocardiography and/or ECG were detected in three of 27 patients with GCK-MODY and two of the 24 healthy control individuals (*P* = 0.866). Three study participants had suffered from myocardial infarction (two with GCK-MODY and one control individual) (*P* = 0.895) in the past, and two had suffered from ischemic stroke (one with GCK-MODY and one control individual). A similar proportion of participants (35%) from both groups were treated for hypertension with one or more antihypertensive drugs. Four of the 27 patients with GCK-MODY (14.8%) were treated with oral hypoglycemic agents, and one was treated with insulin.

TABLE 1: Clinical and biochemical characteristics of participants with GCK-MODY and control individuals.

	Controls <i>n</i> = 24	GCK-MODY <i>n</i> = 27	<i>P</i> value
Gender			
Female	13 (54.2%)	12 (44.4%)	0.58
Male	11 (45.8%)	15 (55.6%)	
Smoking			
Nonsmoker	20 (83.3%)	22 (81.5%)	1
Smoker	4 (16.7%)	5 (18.5%)	
Age [yr]	53 (12.2)	49.8 (12.1)	0.35
Weight [kg]	81.7 (14)	77.2 (12.9)	0.23
Height [cm]	172 (7.43)	170 (7.34)	0.35
BMI [kg/m ²]	27.7 (4.32)	26.8 (4.04)	0.43
Waist circumference [cm]	96.8 (13.6)	92.1 (12.3)	0.20
Hip circumference [cm]	108 (7.97)	104 (9.88)	0.081
Waist-to-hip ratio	0.892 (0.083)	0.881 (0.121)	0.73
Systolic blood pressure [mmHg]	124 (12.8)	122 (20.8)	0.8
Diastolic blood pressure [mmHg]	75.6 (9.7)	72.6 (12.5)	0.34
Glycemia [mmol/L], [mg/dl]	5.26 (0.33), 95.4	7.58 (1.17), 136.4	<0.001
HbA _{1c} [%]	5.74 (0.381)	6.92 (0.957)	<0.001
HbA _{1c} [mmol/mol]	39 (3)	52 (10)	<0.001
C-peptide [pmol/L]	871 (301)	853 (545)	0.89
Total cholesterol [mmol/L]	5.42 (0.866)	5.15 (0.856)	0.26
HDL cholesterol [mmol/L]	1.51 (0.368)	1.52 (0.366)	0.94
LDL cholesterol [mmol/L]	3.16 (0.66)	2.85 (0.757)	0.13
Triglycerides [mmol/L]	1.54 (0.858)	1.58 (1.33)	0.9
Creatinine [mmol/L]	70.5 (19.1)	73.6 (22.1)	0.59
GMT [μ kat/L]	0.468 (0.307)	0.647 (0.785)	0.28
Microalbuminuria [μ g/mg creatinine]	6.58 (10.1)	24.8 (61)	0.15
Albumin/creatinine ratio	0.818 (1.56)	5.11 (14.6)	0.17
Intima-media thickness [mm]	0.692 (0.189)	0.707 (0.215)	0.78

Numbers and percentages are shown for categorical variables; means and standard deviations are shown for numerical variables. *P* values compare the unadjusted means/percentages between the two groups. The bold font refers to statistically significant *P* values; *P* values less than 0.05 were considered as statistically significant.

4. Discussion

To the best of our knowledge, the present study is the first case-control study including the CIMT measurements of GCK-MODY patients older than 35 years. The results are consistent with the mild natural course of GCK-MODY and confirm that GCK mutation is not associated with an increased risk of developing macroangiopathic complications. The 95% confidence interval of the CIMT difference, adjusted for age, family, and gender, is -0.026 to $+0.123$ mm, indicating that the possible increase in CIMT associated with GCK mutation is low and most likely clinically insignificant.

The absence of serious chronic microvascular complications in GCK-MODY was observed by Page et al. [15] and Velho et al. [16, 17], who described proliferative retinopathy in less than 4%, proteinuria in 6%, and peripheral neuropathy in 5% of patients with hyperglycemia at more than 5 years after diagnosis. A major problem with assessing diabetic complications in those patients is the differentiation between patients who only have GCK-MODY and those who develop type 2 diabetes in addition to GCK-MODY. It is generally

assumed that patients with GCK-MODY do not necessarily develop insulin resistance or dyslipidemia in the natural disease course [2], and their glucose tolerance remains stable over many years [18]. Nevertheless, carrying a GCK mutation does not protect against the development of type 2 diabetes, which occurs at a similar prevalence in GCK-MODY patients and in the general population [19].

It has been reported that metabolic syndrome and insulin resistance are the major components of atherosclerosis risk in patients with diabetes and also in individuals without diabetes with insulin resistance [3, 20, 21], whereas the role of hyperglycemia in cardiovascular disease associated with type 2 diabetes is less clear [22]. In contrast with patients with type 2 diabetes, patients with GCK-MODY have mild hyperglycemia without other components of metabolic syndrome or insulin resistance [6]. Therefore, our study adds to the accumulating evidence that chronic mild hyperglycemia without additional components of metabolic syndrome has a milder effect on the development of macrovascular complications compared with the same glycemic levels associated with metabolic syndrome components. Admittedly, more subtle

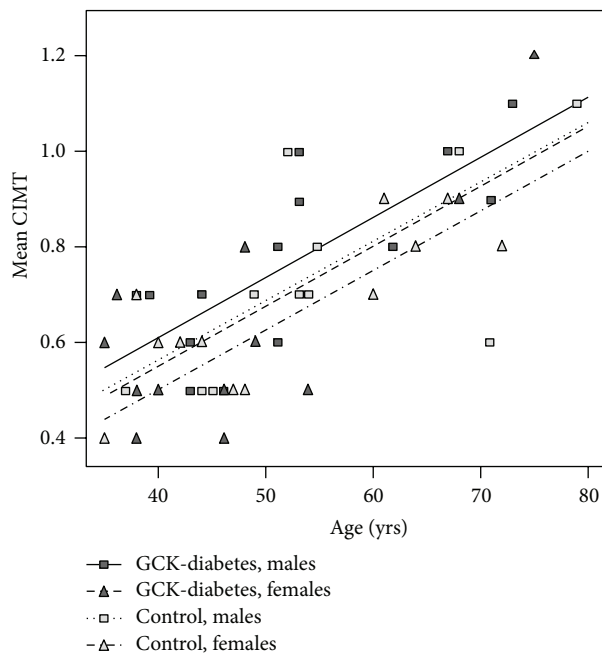


FIGURE 1: Estimated trends of mean CIMT by gender and GCK mutation status (adjusted for family).

effects on CIMT would remain undetected, as the present study is moderately sized. The numbers of available patients in other studies are, however, comparable—a detailed analysis of the effects of p.Gly299Arg mutation in the GCK gene was limited to a single large pedigree [15], whereas another study reporting selected metabolic parameters and history data included 35 families, but these subjects were not examined at a single centre, and the data did not include CIMT [16, 17]. Thus, the number of participants may reflect a compromise between the depth of the acquired data and the subjects' willingness to undergo a complicated set of investigations.

Patients with GCK-MODY exhibit only a small increase in glucose levels after oral glucose loading [2]. This might explain the observed lack of complications in GCK-MODY. By contrast, patients with type 2 diabetes have relatively high 2-hour glucose levels (as a proxy for postprandial glucose levels), indicating that postprandial glucose levels could be the most pathogenic glycemic factor for developing micro- and macrovascular complications. The CIMT has been shown to correlate more strongly with postprandial glycemia than with fasting hyperglycemia [23]. Additionally, the serum hs-CRP levels are lower in GCK-MODY patients than in patients with type 2 diabetes [24].

However, a common variant in the pancreatic GCK promoter has been shown to influence the risk of diabetes complications. März et al. showed [25] that the A allele at c.-30G>A of GCK was associated with an increased risk of coronary artery disease in not only patients with type 2 diabetes but also individuals who did not have diabetes, albeit with a much weaker association (OR = 1.27; 95% CI 1.02–1.59). Additionally, the SNP rs4607517, which is in linkage disequilibrium with c.-30G>A, has been associated with fasting glucose in genome-wide association studies. The association between components of the fasting glucose genetic

risk score (represented by five SNPs, including rs4607517) and CIMT has been described with an increment of 0.0048 mm in carriers [26].

In conclusion, our data indicate that the natural course of mild lifelong hyperglycemia is associated with a low risk of developing diabetic macrovascular complications. However, patients with GCK-MODY should take steps to reduce the risk of developing “classical” type 2 diabetes in addition to GCK-MODY that is, avoid obesity and maintain a high level of physical activity. Our data support a conservative therapeutic approach for hyperglycemia in nonpregnant patients with GCK-MODY. Other risk factors for micro- and macrovascular complications should be treated according to present guidelines.

Conflict of Interests

None of the authors declare having any conflict of interests.

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Review Article

Fish and Marine Omega-3 Polyunsaturated Fatty Acid Consumption and Incidence of Type 2 Diabetes: A Systematic Review and Meta-Analysis

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Objective. To examine the association between fish and marine long-chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA) consumption and incidence of type 2 diabetes (T2D) in prospective cohort studies. **Methods.** Meta-analytic procedures were used to estimate the relative risk (RR) using random effects or fixed effects generic inverse variance model. Publication bias and study heterogeneity were assessed using Egger's test and I^2 statistic. **Results.** We found no significant association between the intake of fish/seafood (pooled RR: 1.04; $P = 0.63$, 95% CI: 0.9 to 1.2, 549, 955 participants) or marine LC n-3 PUFA (pooled RR: 1.08, $P = 0.39$, 95% CI: 0.90 to 1.30, 346, 710 participants) and T2D risk. Significant study heterogeneity was observed in fish/seafood and marine LC n-3 PUFA studies ($P < 0.00001$). Subgroup analysis revealed no obvious sources for high heterogeneity. We also found a significant protective effect of oily fish intake on T2D risk (pooled RR = 0.89, $P = 0.005$, 95% CI: 0.82 to 0.96). Dose-response analysis suggested that every 80 g per day intake of oily fish may reduce 20% risk of T2D. **Conclusion.** We found no significant effect of fish/seafood or marine LC n-3 PUFA intake on risk of T2D but a significant effect of oily fish intake on risk of T2D.

1. Introduction

Type 2 diabetes (T2D) is the most common form of diabetes and its prevalence is steadily increasing by about 6.4% annually worldwide [1]. However the etiology of T2D is still unclear. Both genetic and environmental factors have been shown to be involved in T2D incidence. It is well established that obesity and low physical activity are high risk factors for T2D [2]. Importantly, dietary factors are also risk modulators for this disease [3]. Meat consumption [4] and western diet [5] have been linked to increased T2D risk, while carbohydrates, fiber [6, 7], green leaf vegetables [8], and dietary pattern [9] have all been reported to reduce T2D risk. Interestingly, high intake of fish has been associated with a reduced incidence of mortality due to cardiovascular disease [10], which shares many common risk factors with T2D. Currently, the association between fish intake and T2D risk is still not fully understood.

It has been reported that in countries with a high prevalence of obesity, the incidence of T2D is significantly reduced with high fish and seafood consumption ($2.5 \pm 1.8\%$ versus $0.9 \pm 0.7\%$; $P = 0.007$ and $11.0 \pm 3.9\%$ versus $6.2 \pm 4.1\%$; $P = 0.041$ for the 20 to 44 and 45 to 64 year age groups, resp.) [11]. The associations between high intake of fish/seafood and marine long-chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA) and incidence of T2D have been investigated in several prospective cohort studies [12–22]. However the conclusions are inconsistent. Three cohort studies showed reduced risk of T2D with high intake of fish [12, 13, 16], while seven studies showed no difference or increased risk of T2D with high fish intake [14, 15, 17, 18, 20–22]. Fish types, cooking methods, selenium, mercury, and other environmental contaminants in fish were potential factors influencing the results [12]. High intake of marine LC n-3 PUFA was reported to reduce the risk of T2D in two cohort studies [13, 19] but to increase T2D risk in

four others [14, 15, 18, 20]. To clarify these associations, we conducted a meta-analysis of fish/seafood and marine LC n-3 PUFA intake and T2D incidence in prospective cohort studies.

2. Methods

2.1. Search Strategy. We searched PubMed, OViD, and EMBASE databases from their respective launch dates to May 2013. The searching subject terms in heads, abstracts, or texts were specified to T2D, fish, seafood, omega-3 fatty acid, follow-up, prospective studies, and cohort studies. Cross-references of studies or reviews that were included in the analysis were also examined.

2.2. Study Selection and Assessment. The eligible studies had to meet the following criteria: (1) to be a prospective cohort design and study the association between fish/seafood, omega-3 fatty acid intake, and the incidence of T2D; (2) risk ratios or odds ratios have to be available with 95% confidence intervals (CI), or otherwise the case numbers and participant numbers in both highest and lowest intake groups should be available; (3) the method of dietary assessment had to be reported, and participants should consume either fish/seafood that includes fish (such as salmon, tuna, trout, and tilapia) and shellfish (such as shrimp, crab, and oysters), and/or LC n-3 PUFA (e.g., EPA and DHA); and (4) the participants at baseline were not already diagnosed as being diabetic.

We assessed all studies for quality using a scoring system that accounted for participants (1 point if a power calculation had been conducted to give the numbers of participants needed to detect an effect of fish/seafood intake on risk of T2D and 1 point for appropriate inclusion and exclusion criteria), outcome (1 point if T2D was confirmed by clinical criteria or blood tests), assessment of diet (1 point if a validated FFQ was used), relative risk (RR) adjustment for seven T2D risk factors (age, BMI, family history of diabetes, physical activity, vegetable intake, fruit intake, and meat intake) (1 point for each risk factor), and RR adjustment for other factors such as energy intake (1 point). This scoring system was designed with reference to [8]. Studies were assessed as high quality if they had a score of 9–12 points and moderate quality if they had a quality score of 5–8 points.

2.3. Data Extraction. We extracted data on the diagnosis of T2D, intake of fish/seafood and marine LC n-3 PUFA, the adjusted RR, and 95% CI. For those with odds ratio (OR) data, we converted OR to RR using a previously published formula [23], and the corresponding CI values were also converted. For studies that had separate results for men and women, we generated a pooled RR for the total population. We also extracted other information from each eligible paper, including the country of the study, the sample size (participants' numbers) at baseline, the age of participants, the method of assessing diabetes status, follow-up years, the types of fish/seafood and marine LC n-3 PUFA they measured, and the highest and lowest intake amounts of

fish/seafood or marine LC n-3 PUFA (Table 1). M. Z and E. P. D conducted study selection, data extraction, and quality assessment independently, with disagreements resolved by consensus after discussion with A. M.

2.4. Statistical Methods. We transformed the RRs by using their natural logarithms and calculating standard errors and corresponding CI. Heterogeneity was assessed with the I^2 statistic. We calculated the summary RRs and 95% CI for the highest versus the lowest intake according to Dersimonian and Laird for the random effects generic inverse variance model [24] when heterogeneity was found significant ($P < 0.05$); otherwise the fixed effects generic inverse variance model was used according to Hedges and Olgin. We also conducted meta-analysis of stratified samples according to gender (men and women) and fish/seafood types (e.g., shellfish, oily fish, and lean fish). The publication bias was assessed by the asymmetry of funnel plot and Egger's regression test [25]. The meta-analysis was conducted by Review Manager 5.1 (The Nordic Cochrane Centre, The Cochrane Collaboration). A two-tailed $P < 0.05$ was considered as statistically significant, and 95% confidence intervals were quoted where available.

Dose-response analysis for consumption of fish/seafood and marine LC n-3 PUFA was conducted by using a previously reported method [10]. We included intake, adjusted RR, and CI from all related studies except one [17] that had no quartile information. The median or mean level of fish or marine omega-3 fatty acid intake was assigned to the corresponding RR for each study. For those reported ranges of intake, we estimated the mean intake in each category. When the lowest dose was open-ended, we set the lower boundary to zero. When the highest dose was open-ended, we assumed that the interval length was the same as the adjacent interval [4]. For publications that provided servings per day for fish intake, we transformed them into g/day by 100 g per serving [10]. Linear regression was used to estimate the relationship between total fish, oily fish and marine omega-3 fatty acid intake, and incidence of T2D. STATA 11.0 was used for dose-response analysis.

Subgroup analysis was conducted based on ethnicity (Asian versus US/European), length of follow-up (<10 years versus ≥ 10 years), assessment of T2D (confirmed by physician/phone interview/hospital records versus confirmed by standard criteria/plasma glucose level), sample size (<10,000 versus $\geq 10,000$), and study quality score (high quality (9–12 points) versus moderate quality (5–8 points)), as these factors are possible sources of study heterogeneity. The Mann Whitney U test was used to calculate the significance of differences within subgroups and to detect factors contributing to heterogeneity.

3. Results

3.1. Selection of Studies. We identified 178 candidate publications related to fish/seafood intake and risk of T2D in prospective cohort studies through searching PubMed, OViD, and EMBASE databases. Among them, 155 articles

TABLE 1: Characteristics of included studies regarding fish/seafood, fish, and LC n-3 PUFA intake and risk of type 2 diabetes.

(a)										
Fish/seafood	Ethnicity	Age	Follow-up years	Fish/seafood type	Fish/seafood consumption (highest versus lowest) (range)	Number of participants	Quartile	Adjustment for T2D risk factors	Quality score (0–12)	Adjusted RR (95% CI)
Nanri et al. 2011 ^A	Japan	40–59	10	Total fish/seafood	166.6 g/d versus 35.9 g/d (130.7 g/d)	52680	4	Age, BMI, family history of diabetes mellitus, total physical activity, vegetable, fruit, and meat	11	0.84 (0.62–1.15)
Villegas et al. 2011 ^B	China	40–74	4.1–8.9	Total fish/shellfish	99.2 g/d versus 14.0 g/d (85.2 g/d)	116129	5	Age, BMI, physical activity, family history of diabetes, and dietary pattern (vegetable, fruit, and meat)	11	0.87 (0.78–0.97)
Patel et al. 2009 ^C	Norwich, England	40–79	7.5	Total fish/seafood	>14.28 g/d versus <14.28 g/d (14.28 g/d)	21984	2	Age, BMI, family history of diabetes, and physical activity	7	0.76 (0.59–0.96)
Patel et al. 2012 ^K	Europe	55–63	6.9	Total fish/seafood	>51.8 g/d versus <15 g/d	24813	4	Age, BMI, physical activity, fruit, and vegetable intake	8	0.99 (0.86–1.15)
Montonen et al. 2005 ^J	Finland	40–69	23	Total fish	>0 versus 0 g/d	4304	4	Age, BMI, and family history of diabetes	6	0.96 (0.71–1.29)
Schulze et al. 2003 ^D	US	26–46	8	Total fish	>28.57 g/d versus <14.28 g/d (28.57 g/d)	91246	N/A	Age, BMI, physical activity, and family history of diabetes	8	1.04 (0.82–1.32)
Djoussé et al. 2011 ^E	US	>45	12.4	Total fish/seafood	56.14 g/d versus 6.71 g/d (49.43 g/d)	36328	5	Age, BMI, parental history of diabetes, physical activity, and meat	8	1.49 (1.30–1.70)
Djoussé et al. 2011 ^F	US	>65	15	Total fish	>71.43 g/d versus <3.3 g/d (71.43 g/d)	2831	5	Age, BMI, and physical activity	6	1.07 (0.35, 3.33)
Kaushik et al. 2009 ^G	US	26–78	14.5–18.5	Total fish (finfish)	>71.43 g/d versus <3.3 g/d (78.57 g/d)	195204	5	Physical activity, family history of diabetes mellitus, and BMI	7	1.22 (1.08–1.38)
Van Woudenberg et al. 2009 ^H	Dutch	>55	12	Total fish/seafood	35.6 g/d versus 0 g/d (35.6 g/d)	4472	4	Age	5	1.32 (1.02–1.70)
(b)										
Fish	Ethnicity	Age	Follow-up years	Fish type	Fish consumption	Number of participants	Quartile	Adjustment for T2D risk factors	Quality score	Adjusted RR (95% CI)
Nanri et al. 2011 ^A	Japan	40–59	10	Fresh fish	95.4 g/d versus 13.6 g/d (81.8 g/d)	52680	4	Age, BMI, family history of diabetes mellitus, physical activity, vegetable, fruit, and meat	11	0.91 (0.73–1.13)
Villegas et al. 2011 ^B	China	40–74	4.1–8.9	Fish	79.6 g/d versus 9.6 g/d (70 g/d)	116156	5	Age, BMI, physical activity, family history of diabetes, and dietary pattern (vegetable, fruit, and meat)	11	0.90 (0.80–1.01)
Kaushik et al. 2009 ^G	US	26–78	14.5–18.5	Finfish	>71.43 g/d versus <3.3 g/d (78.57 g/d)	195204	5	Physical activity, family history of diabetes, and BMI	7	1.22 (1.08–1.38)
Montonen et al. 2005 ^J	Finland	40–69	23	Total fish	>0 versus 0 g/d	4304	4	Age, BMI, and family history of diabetes	6	0.96 (0.71–1.29)
Patel et al. 2009 ^C	Norwich, England	40–79	7.5	White fish, oily fish	>14.28 g/d versus <14.28 g/d (14.28 g/d)	21984	2	Age, BMI, family history of diabetes, and physical activity	7	0.90 (0.79–1.03)

(b) Continued.

Fish	Ethnicity	Age	Follow-up years	Fish type	Fish consumption	Number of participants	Quartile	Adjustment for T2D risk factors	Quality score	Adjusted RR (95% CI)
Patel et al. 2012 ^K	Europe	55–63	6.9	Total fish	>34.9 g/d versus <2.9 g/d	24813	4	Age, BMI, physical activity, fruit, and vegetable intake	8	1.06 (0.92–1.22)
Van Woudenberg et al. 2009 ^H	Dutch	>55	12	Lean fish, fatty fish	46.3 g/d versus 0 (46.3 g/d)	4472	4	Age	5	1.16 (0.89–1.51)

(c)

LC n-3 PUFA	Ethnicity	Age	Follow-up years	LC n-3 PUFA type	LC n-3 PUFA consumption	Number of participants	Quartile	Adjustment for T2D risk factors	Quality score	Adjusted RR (95% CI)
Villegas et al. 2011 ^B	China	40–74	4.1–8.9	EPA, DHA	0.2 g/d versus 0.02 g/d (0.18 g/d)	64193	5	Age, BMI, physical activity, family history of diabetes, and dietary pattern (vegetable, fruit, and meat)	11	0.85 (0.76–0.95)
Brostow et al. 2011 ^I	Singapore, Chinese	45–74	6	EPA, DHA	0.6 g/d versus 0.11 g/d (0.49 g/d)	43175	5	Age, BMI, and physical activity	7	0.93 (0.77–1.11)
Kaushik et al. 2009 ^G	US	26–78	14.5–18.5	EPA, DHA	0.46 g/d versus 0.07 g/d (0.37 g/d)	195204	5	Physical activity, family history of diabetes, and BMI	7	1.24 (1.09–1.41)
Djoussé et al. 2011 ^E	US	>45	12.4	EPA, DHA	0.39 g/d versus 0.05 g/d (0.34 g/d)	36328	5	Age, BMI, parental history of diabetes, physical activity, and meat	8	1.45 (1.32–1.59)
Djoussé et al. 2011 ^F	US	>65	15	EPA, DHA	>0.56 g/d versus <0.17 g/d (0.6 g/d)	3088	4	Age, BMI, and physical activity	6	1.04 (0.67–1.61)
Van Woudenberg et al. 2009 ^H	Dutch	>55	12	EPA, DHA	0.237 g/d versus 0.024 g/d (0.21 g/d)	4472	3	Age	5	1.22 (0.97–1.53)

A–I: A: T2D was assessed by Japanese criteria [12]; B: T2D was assessed by ADA criteria [13]; C: T2D was assessed by other resource and hospital records [16]; D: T2D was assessed by NDDG criteria [17]; E: T2D was assessed by ADA criteria [14]; F: T2D was assessed by medication use and fasting/nonfasting glucose level in plasma [20]; G: T2D was assessed by NDDG criteria [15]; H: T2D was assessed by ADA/WHO criteria [18]; I: T2D was self-reported [19]; J: T2D was assessed by physician according to diabetic criteria [22]; K: T2D was assessed by multiple sources [21].

were excluded based on our inclusion criteria and duplicated reports. A further 12 studies were excluded with more specific criteria, including cross-section, studies, case-control studies, those without original data or with odds ratio only, those with fatty acid levels in blood samples, those with outcomes reported as glycated haemoglobin, and those with unknown type of LC n-3 PUFA (Figure 1). The remaining 11 studies were assessed in the current meta-analysis. 10 prospective studies (549,955 participants at baseline) [12–18, 20–22] were used for measuring the relationship between fish/seafood intake and risk of T2D (Table 1). Six prospective studies [13–15, 18–20] (346,710 participants at baseline) were selected for assessing the association between marine LC n-3 PUFA intake and risk of T2D quality (Table 1).

3.2. Fish/Seafood Intake and Risk of T2D. No significant association between high fish/seafood intake and T2D incidence was observed (pooled RR: 1.04; 95% CI: 0.890 to 1.20; $P = 0.63$) (Figure 2). However, there was a significant study heterogeneity ($I^2 = 83\%$, $P < 0.00001$). Subgroup analysis showed no clear sources of this high heterogeneity (ethnicity, $P = 0.18$; follow-up years, $P = 0.17$; assessment of T2D, $P = 0.18$; sample size, $P = 0.51$; study quality, $P = 0.18$, MWU test) (Table 2). Two studies using Asian populations showed a beneficial effect of fish/seafood intake on risk of T2D (pooled RR = 0.87, $P = 0.006$), but six studies of western populations demonstrated no significant effect of fish/seafood intake on T2D risk (pooled RR = 1.10, $P = 0.22$). Four studies with less than 10 years of follow-up showed a protective effect of fish/seafood intake against development of T2D (Pooled RR = 0.91, $P = 0.11$), while six studies with more than 10 years of follow-up indicated an increased risk of T2D with high fish/seafood intake (Pooled RR = 1.17, $P = 0.04$). The high quality studies demonstrated a significant protective effect of high fish/seafood intake on incidence of T2D (pooled RR = 0.87, 95% CI: 0.78 to 0.96, $P = 0.006$), while the moderate quality studies showed no obvious effect of fish/seafood intake on risk of T2D (pooled RR = 1.10, 95% CI: 0.95 to 1.04, $P = 0.22$). Egger's regression test and funnel plot showed no significant publication bias (Egger's $P > 0.05$). Dose-response analysis for fish/seafood intake showed no significant linear relationship between fish/seafood intake and risk of T2D ($R^2 = 0.11$, P for regression = 0.076).

3.3. Fish Types Intake and Risk of T2D. We also conducted meta-analysis of stratified samples based on fish/seafood types (oily fish and lean fish, fish, and shellfish). We found a significant protective effect of high oily fish intake on T2D risk (pooled RR = 0.89, $P = 0.005$, 95% CI: 0.82 to 0.96, 103,949 participants), but lean fish intake had no significant effect on T2D risk (pooled RR = 1.02, $P = 0.66$, 95% CI: 0.93 to 1.12) (Figure 3). In order to better understand the effect of oily fish intake on risk of T2D, we also conducted a dose-response analysis by linear regression (Figure 4). We found that 80 g/day oily fish intake may reduce 20% risk of T2D. Meanwhile, we observed no significant effect of high consumption of fish (including oily fish and lean fish, fresh or canned) (pooled RR = 1.01, $P = 0.89$, 95% CI: 0.90 to 1.12)

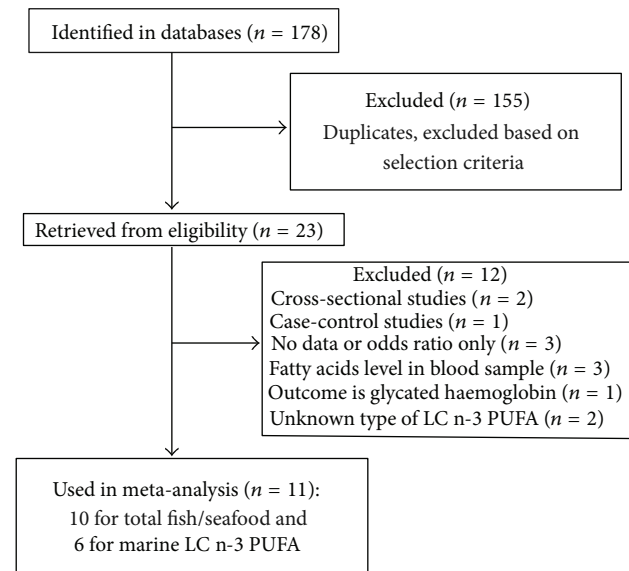


FIGURE 1: Process of study selection.

or shellfish (pooled RR = 1.03, $P = 0.78$, 95% CI: 0.83 to 1.29) on incidence of T2D (Figure 5). Egger's regression test and funnel plot showed no significant publication bias (Egger's $P > 0.05$).

3.4. Marine LC n-3 PUFA Intake and Risk of T2D. The meta-analysis showed no significant association between high intake of marine LC n-3 PUFA (EPA and DHA) and incidence of T2D (pooled RR = 1.08, 95% CI: 0.90, 1.30, $P = 0.39$) (Figure 6). Significant heterogeneity ($I^2 = 85\%$, $P < 0.00001$) caused by ethnicity and follow-up years in the trend level ($P = 0.064$, MWU test) could explain the inconsistency of the results. In the subgroup analysis, two studies [13, 19] in Asian populations with shorter follow-up time (<10 years) showed reduced risk of T2D with high intake of marine LC n-3 PUFA (pooled RR = 0.87, heterogeneity $P = 0.42$). Conversely, four studies [14, 15, 18, 20] with western participants and longer follow-up periods showed increased risk of T2D with increased LC n-3 PUFA consumption (pooled RR = 1.27, heterogeneity $P = 0.7$) (Table 2). Egger's regression test and funnel plot showed no significant publication bias (Egger's $P > 0.05$). Dose-response analysis for marine LC n-3 PUFA studies showed no significant dose-response relationship with the risk of T2D.

4. Discussion

4.1. Heterogeneity Exploration and Risk of Bias. Meta-analysis allows us to increase the power of detecting associations between exposures and outcomes by increasing sample size. However, it may be complicated by study heterogeneity. Our subgroup analysis showed no significant source for the observed high heterogeneity between fish/seafood and marine LC n-3 PUFA studies, but ethnicity may be partially contributing to high heterogeneity. Two studies using Asian populations [13, 19] reported protective effects of LC n-3

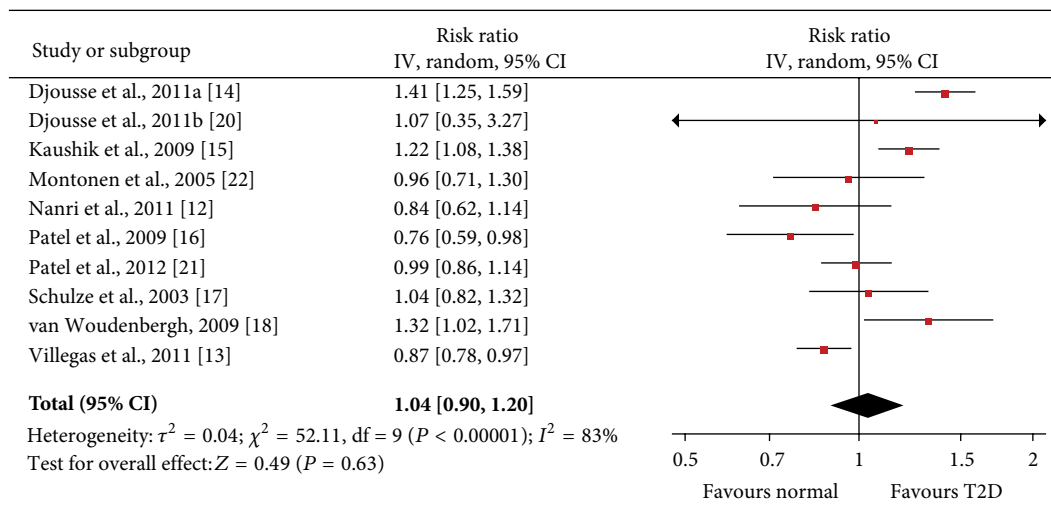


FIGURE 2: Forest plot of the meta-analysis for fish/seafood intake and incidence of type 2 diabetes.

PUFA intake on risk of T2D, while four studies using western populations [14, 15, 18, 20] showed opposite results. This may be related to differences in overall dietary patterns or genetic background between eastern and western populations.

Studies of different quality showed variable results. Two high quality studies [12, 13] showed a beneficial effect (RR = 0.87) of fish/seafood intake on risk of T2D with significance ($P = 0.006$). But it cannot be excluded that the beneficial effect of fish/seafood intake is related to ethnicity or fish/seafood consumption range instead of study quality because these two studies used Asian populations and had higher fish/seafood consumption (>80 g/day). Meanwhile, moderate quality fish/seafood studies yielded no significant conclusions and pointed to a pooled RR of 1.15. Publication bias (reporting bias) is unlikely because funnel plot and Egger's regression tests showed no significance, but other nonrandom biases are possible because of the failure to adjust some known T2D risk factors (e.g., physical activity, dietary pattern, age, BMI, or family history of diabetes) or lack of validation of the FFQ method.

For the association between marine LC n-3 PUFA intake and risk of T2D, only one high quality study [13] showed a beneficial effect of marine LC n-3 PUFA intake on risk of T2D, while five studies with moderate quality showed a slightly increased risk pooled effect with weak significance ($P = 0.04$). Funnel plot and Egger's regression tests showed no significant publication bias for marine LC n-3 PUFA studies. However, there may be some nonrandom bias in moderate quality studies, as some confounding factors (such as family history of diabetes and dietary factors) were not well adjusted when calculating the RR. In the study of [19], for example, the RR may bias to risk because the vegetable/fruit intake (22.5 versus 14.3 g/100 kcal, highest versus lowest quartile) was not adjusted.

Other possible sources of heterogeneity may include the amount of fish consumed, fish types, and gender. Two studies [12, 13] with more than 80 g/d of fish/seafood intake showed reduced incidence of T2D. Among six studies with less than

80 g/d fish/seafood intake, five showed either no effect or increased risk of T2D except for one study [16] that may be biased by a nonstandard outcome measurement. The range of EPA and DHA intake across studies may not explain the high heterogeneity in the marine LC n-3 PUFA studies (Table 1). Our dose-response analysis could reduce the bias caused by different doses of fish/seafood or marine LC n-3 PUFA intake set as the highest level within publications. The dose-response analysis for the fish/seafood intake studies showed a trend towards an inverse linear relationship between fish/seafood intake and risk of T2D, supporting that the range of fish/seafood intake may contribute to heterogeneity. The method of preparing the fish and the amount and type of fat added may also alter the effects of fish on glucose metabolism. There is only one included cohort study [16] that showed the effect of fried fish on risk of T2D (pooled RR = 0.91, 95% CI: from 0.75 to 1.10), which makes it in sufficient to conduct stratified meta-analysis according to the method of fish preparation. Most included prospective cohort studies used fish and seafood as exposure, which include fish (oily and lean fish) and other seafood (shellfish, octopus, and other fish products). This makes it difficult to clarify their relationships with T2D because different types of fish and seafood contain different ratios of nutrients and different levels of contaminants. More studies will be required to strengthen conclusions regarding the individual impact of fish versus seafood on T2D risk.

4.2. Limitations. Publication bias is an important concern with meta-analysis. Although we found no significant publication bias in the current meta-analysis using Egger's test, the results should still be considered with caution. The statistical power for publication bias might be low because there are only ten studies for fish/seafood and six studies for marine n-3 PUFA in the meta-analysis. The likelihood of selection bias and recall bias is low because of the design of prospective studies, but the observational studies are limited because

TABLE 2: Subgroup analysis to investigate heterogeneity source in meta-analysis.

Fish/seafood intake	Publications	Pooled RR, ¹ <i>P</i> value	Heterogeneity ² <i>P</i> value	³ <i>P</i> value	Marine LC n-3 PUFA	Publications	Pooled RR, ¹ <i>P</i> value	Heterogeneity ² <i>P</i> value	³ <i>P</i> value
<i>Ethnicity</i>				0.18	<i>Ethnicity</i>				0.064
Asian (Chinese, Japanese)	2 [12, 13]	0.87, 0.006	0.83		Asian (Chinese, Japanese)	2 [13, 19]	0.87, 0.005	0.42	
US/European	⁸ [14–18, 20–22]	1.10, 0.22	0.0001		US/European	4 [14, 15, 18, 20]	1.27, <0.00001	0.7	
<i>Length of follow-up (years)</i>				0.17	<i>Length of follow-up (years)</i>				0.064
<10	4 [13, 16, 17, 21]	0.91, 0.11	0.16		<10	2 [13, 19]	0.87, 0.005	0.42	
≥10	⁶ [13–15, 18, 20, 22]	1.17, 0.04	0.02		≥10	4 [14, 15, 18, 20]	1.27, <0.00001	0.7	
<i>Assessment of T2D</i>				0.18	<i>Assessment of T2D</i>				NA
Confirmed by physician/phone interview/hospital records	3 [16, 21, 22]	0.91, 0.28	0.2		Confirmed by physician/phone interview/hospital records	0	NA	NA	
Confirmed by standard criteria or plasma glucose measurement	⁷ [12–15, 17, 18, 20]	1.10, 0.31	<0.0001		Confirmed by standard criteria or plasma glucose measurement	6 [13–15, 18–20]	1.09, 0.36	<0.00001	
<i>Sample size</i>				0.51	<i>Sample size</i>				1
<10000	3 [18, 20, 22]	1.14, 0.26	0.29		<10000	2 [18, 20]	1.18, 0.11	0.53	
≥10000	7 [12–17, 21]	1.01, 0.88	<0.0001		≥10000	4 [13–15, 19]	1.07, 0.57	<0.0001	
<i>Quality score</i>				0.18	<i>Quality score</i>				0.33
High (9–12)	2 [12, 13]	0.87, 0.006	0.83		High (9–12)	1 [13]	0.85, NA	NA	
Moderate (5–8)	⁸ [14–18, 20–22]	1.1, 0.22	0.0001		Moderate (5–8)	5 [14, 15, 18–20][16, 17, 20–22]	1.17, 0.01	0.03	

¹ *P* value calculated by *Z* statistic.² *P* value calculated by *I*² statistic.³ *P* value calculated by Mann-Whitney *U* test.

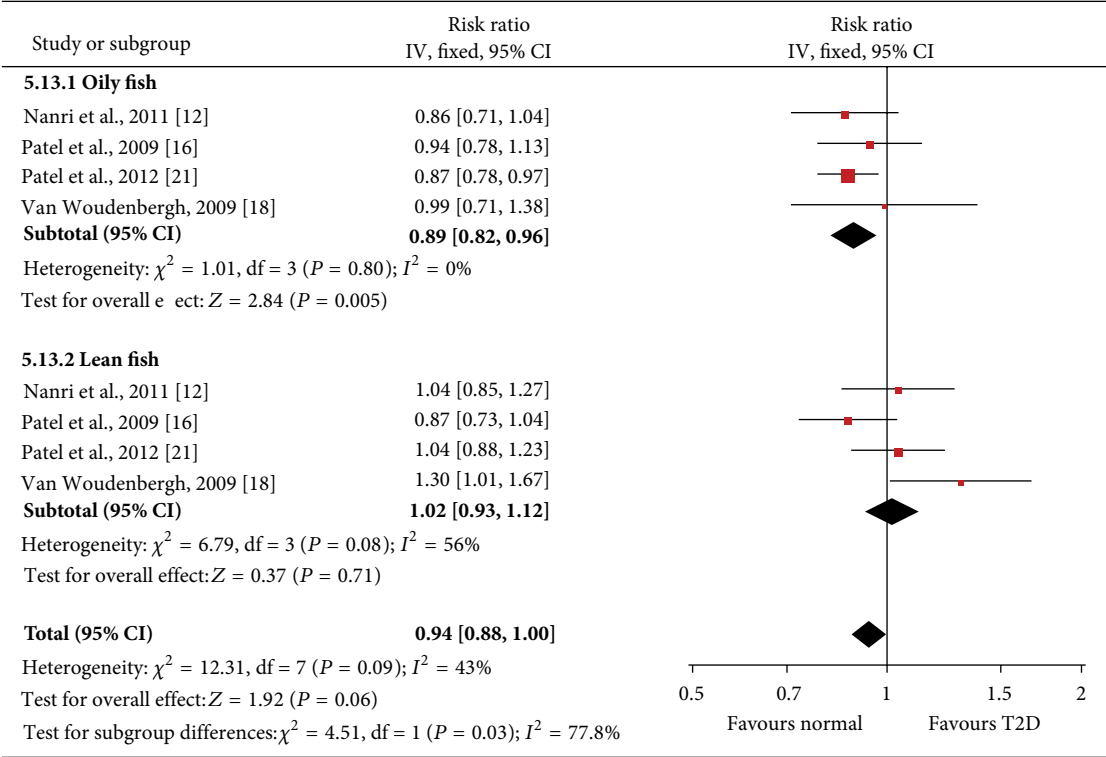


FIGURE 3: Forest plot of the meta-analysis for oily fish and lean fish intake and incidence of type 2 diabetes.

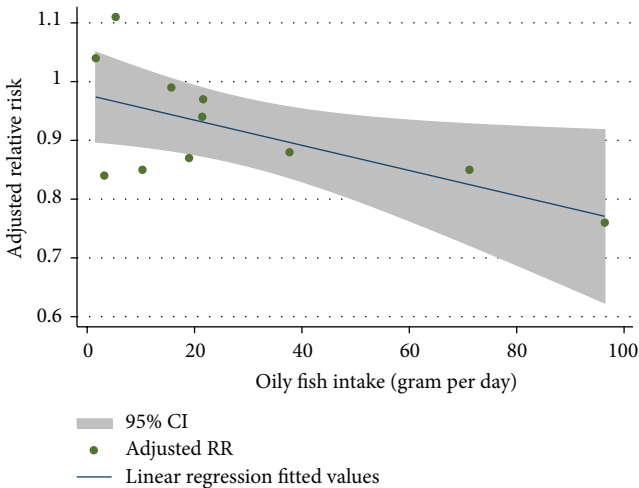


FIGURE 4: Linear regression of adjusted RR of T2D versus oily fish intake. The upper and lower bands denote the 95% confidence interval on mean of the predicted value, $R^2 = 0.40$.

confounders were adjusted in different studies, making them difficult to compare.

The method of dietary assessment may also affect the results. FFQ is related to random and systematic errors [26], which will underestimate the true associations between diet and diseases. Nutritional biomarkers in plasma should be monitored and will minimize this problem. One prospective study [19] also measured plasma levels of EPA and DHA,

which were significantly associated with reduced risk of T2D ($RR = 0.64$ for EPA and DHA) in those with high plasma levels of EPA and DHA. However, three other studies showed no significant relationship between plasma/serum LC n-3 PUFA levels and risk of T2D [27–29].

4.3. Potential Mechanism Underlying the Beneficial Effects of Oily Fish Intake on Risk of T2D. Our current meta-analysis showed significant beneficial effect of oily fish intake on risk of T2D, corresponding to previous clinical trial findings that fish intake was associated with reduced fasting glycemia [30] and improved glucose tolerance [31]. One recent cross-sectional study in a Spanish population also showed that the high fish intake is related to low plasma level of glucose and low incidence of diabetes [32]. But ours and other 5 meta-analyses [33–37] showed no significant effects of fish intake on risk of T2D, although two prospective cohort studies [12, 13] showed that the beneficial effects of fish/seafood intake on risk of T2D, which used an Asian population, had high study quality and had high range of amount of fish intake (>80 g/d, highest versus lowest quartile). However high heterogeneity for fish/seafood studies remains to be clarified before any conclusions are to be made regarding the effects of high fish/seafood intake.

As oily fish has high amount of LC n-3 PUFA (EPA and DHA), we are wondering if EPA and DHA intake may contribute to the beneficial effect of oily fish intake. But ours and other 5 meta-analyses studies showed no significant association between LC n-3 PUFA intake and T2D

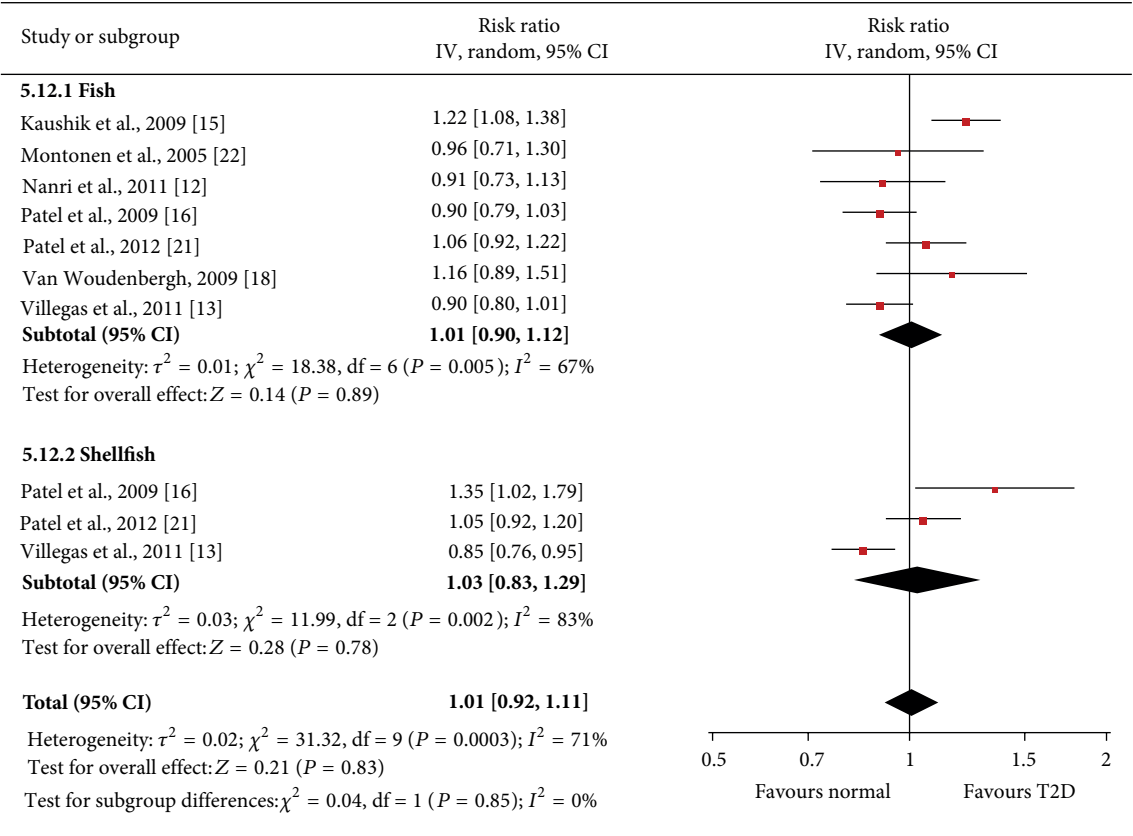


FIGURE 5: Forest plot of the meta-analysis for fish and shellfish intake and incidence of type 2 diabetes.

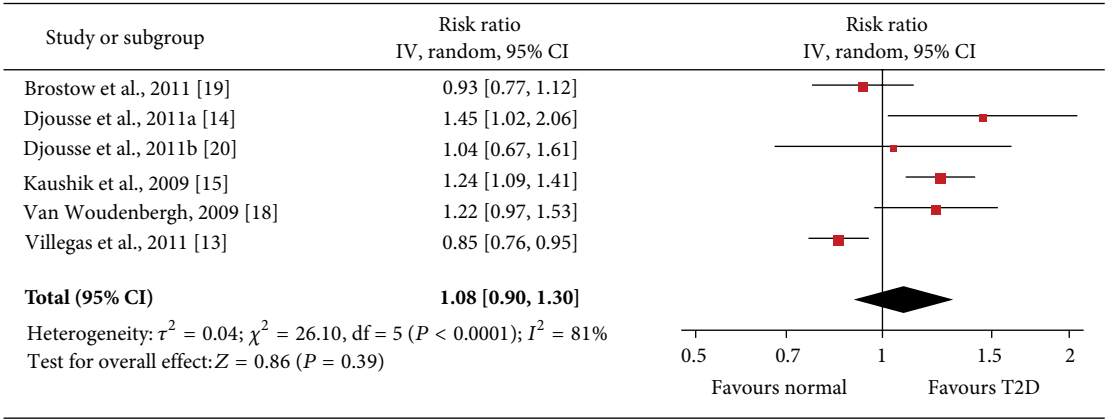


FIGURE 6: Forest plot of the meta-analysis for LC n-3 PUFA intake and incidence of type 2 diabetes.

risk. This is corresponding to one randomized double-blind placebo-controlled study which showed that fish oil has no significant effect on improving glucose control and insulin sensitivity in diabetic patients [38]. However, there are also contaminations in fish which may disrupt insulin signaling and glucose homeostasis, such as selenium and mercury [39, 40]. In the current meta-analysis, only one study [18] adjusted selenium level for RR and showed much lower RR after adjustment when measuring the effects of EPA and DHA intake. Nonetheless, there are other oily fish nutrients (such

as vitamin D and fish protein) that may contribute to the beneficial effects of oily fish intake on T2D risk. Oily fish is a major diet source of vitamin D, and several recent cohort studies have demonstrated the protective effects of vitamin D on T2D incidence in various populations [41, 42].

5. Conclusions

Our meta-analysis showed no significant effect of fish/seafood or marine LC n-3 PUFA intake on risk of

T2D. However high heterogeneity was found in the current meta-analysis, which may include the bias from different ethnicities, follow-up years, and amount of fish intake. In addition, our stratified meta-analysis showed a significant weak effect of oily fish intake on risk of T2D. Dose-response analysis suggested that 80 g per day intake of oily fish may reduce 20% risk of T2D. But no significant association between EPA and DHA intake and risk of T2D was found, suggesting that other nutrients from oily fish may contribute to the beneficial effects of oily fish intake, such as vitamin D and oily fish protein. However, more high quality prospective cohort studies will be needed to support our conclusion for beneficial effects of oily fish intake on T2D risk and to clarify the association between fish/seafood intake and marine LC n-3 PUFA intake and T2D incidence.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

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Research Article

Enhanced Healing of Diabetic Wounds by Subcutaneous Administration of Human Umbilical Cord Derived Stem Cells and Their Conditioned Media

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Objective. Mesenchymal stem cells (MSCs) isolated from the umbilical cord and their conditioned media (CM) can be easily obtained and refined compared with stem cells from other sources. Here, we explore the possibility of the benefits of these cells in healing diabetic wounds. **Methodology and Results.** Delayed wound healing animal models were established by making a standard wound on the dorsum of eighteen db/db mice, which were divided into three groups with six mice in each: groups I, II, and III received PBS, UC-MSC, and CM, respectively. UC-MSC and their CM significantly accelerated wound closure compared to PBS-treated wounds, and it was most rapid in CM-injected wounds. In day-14 wounds, significant difference in capillary densities among the three groups was noted ($n = 6$; $P < 0.05$), and higher levels of VEGF, PDGF, and KGF expression in the CM- and UC-MSC-injected wounds compared to the PBS-treated wounds were seen. The expression levels of PDGF- β and KGF were higher in CM-treated wounds than those in UC-MSC-treated wounds. **Conclusion.** Both the transplantation of UC-MSC and their CM are beneficial to diabetic wound healing, and CM has been shown to be therapeutically better than UC-MSC, at least in the context of diabetic wound healing.

1. Introduction

Diabetes has undoubtedly become a major public health concern of the twenty-first century. Various studies have estimated the impact of diabetes, and it seems that the numbers are growing at unprecedented rates. The International Diabetes Federation claims that 366 million people had diabetes in 2011 and by 2030 this number will have increased to 552 million, and that this caused 4.6 million deaths in 2011 [1]. About 15% of people with diabetes suffer from foot ulcerations [2]. Diabetic wounds that resist healing are also associated with decreased peripheral blood flow and often resist current therapies. These achieve only 50% healing rates even with the best treatment available, that too, for a short-term [2], and 4/5 of these cases eventually succumb to amputation of the lower extremity [3, 4]. Normal wounds,

without underlying pathological defects, heal readily, but the healing deficiency of diabetic wounds can be attributed to a number of factors, including decreased production of growth factors and reduced revascularization. Mesenchymal stem cells are multipotent, nonhematopoietic progenitor cells that hold great promise for tissue regeneration. Mesenchymal stem cells isolated from the umbilical cord and their conditioned media can be easily obtained and refined compared to stem cells from other sources. Although the therapeutic potential of transplanted human umbilical cord derived stem cells has been widely explored as a promising tool in the treatment of several human diseases, including graft versus host disease [5–8], diabetes [9, 10], Crohn's disease [8], heart disease [11–13], and solid tumor cancers [14, 15], its effect on healing diabetic wounds has not been studied to the same degree.

2. Materials and Methods

2.1. Materials

2.1.1. Cell Culture

Isolation and Culture of Umbilical Cord Derived Mesenchymal Stem Cells. Umbilical cords of gestational ages (39–40 weeks) were obtained from the Department of Obstetrics and Gynecology of The Third Xiangya Hospital of Central South University after normal deliveries. Tissue collection for research was approved by the institutional review board of The Third Xiangya Hospital of Central South University. After having been minced into 1–2 mm³ fragments, umbilical cord was incubated with 0.075% collagenase type II for 30 minutes and then 0.125% trypsin for 30 minutes with gentle agitation at 37 degrees centigrade. The digested mixture was then passed through a 100 micrometre filter to obtain cell suspensions. Cells were plated at a density of 1×10^6 cells/cm² in noncoated cell culture flasks. Growth medium consisted of Dulbecco's modified Eagle medium with low glucose and 20% fetal bovine serum supplemented with 4 ng/mL bFGF and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% carbon dioxide at 37 degrees centigrade. After 3 days of culture, the medium was replaced, and nonadherent cells were removed. The medium was then changed twice weekly then after. Once 80% of confluence had been reached, adherent cells were replated at a density of 1×10^4 /cm² in umbilical cord growth medium for expansion. The primary culturing of UC-MSC in Dulbecco's modified eagle Medium until near confluence (passage 1) was approximately one week. The time course of UC-MSC was amplified for two weeks until the third passage, and UC-MSC were then applied to the wound bed.

Immunophenotype Analysis. The morphology of cells derived from umbilical cord was fibroblast-like as observed under the microscope. Surface markers including CD29, CD44, CD73, CD90, and CD105 were positive. CD34, CD45, CD31, and HLA-DR were negative. Cells were stained in a single label and then analyzed by flow cytometry with a fluorescent-activated cell sorter (FACS).

Preparation of Conditioned Media. Conditioned media was derived from culturing of UC-MSCs in serum-free M199 media at 37°C for 24 hours and was cleared out and concentrated by centrifugation. After 24 hours, the supernatant was collected as conditioned media and filtered through a 0.2 µm filter for immediate use.

Mesenchymal Stem Cells: Characterization and Their Differentiation Activity. After mesenchymal stem cells were tested for their characterization, they were tested for their ability to differentiate into different mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes. Adipogenic differentiation was induced with 1×10^{-8} mM dexamethasone and 5 microgram/mL insulin, and droplet staining was performed using oil red O. Osteogenic differentiation was induced by treating mesenchymal stem cells with

10^{-8} M dexamethasone, 10 mM beta-glycerol-phosphate, and 50 microgram/mL ascorbic acid, and differentiated cells were identified by Alizarin red staining. Chondrogenic differentiation medium was composed of high-glucose Dulbecco's modified Eagle medium supplemented with 40 microgram/mL proline, 50 mg/mL ITS-plus, 100 microgram/mL sodium pyruvate, GlutaMAX, 50 microgram/mL ascorbate-2-phosphate, 10 ng/mL transforming growth factor-beta 3, and 1×10^{-8} mM dexamethasone. Chondrogenic differentiation was visualized by Alcian blue staining.

Animal Model for Diabetic Wound Healing. Eighteen male db/db mice (BKS.Cg-m +/+ *Lepr*^{db}/J, db⁺/db⁺; 10–14 weeks old; male; body weight, 42.4 ± 2.1 g; blood glucose > 16.67 mM) were obtained from Model Animal Research Center of Nanjing University. The animals were randomly divided into three groups ($n = 6$ per group), and the excisional wound-healing model was generated. The glucose levels were measured every alternate day during this experimental procedure in wound healing. The glucose level remained more than 16.67 mM in all mice in all groups, and there was no significant difference in the blood glucose levels among the three groups throughout the entire 14-day period of the experiment. The mice were sedated by intraperitoneal administration of 6% chloral hydrate (4 mL/kg body weight) before the procedure. Then the mice were shaved on the dorsum, and a full-thickness dorsal skin defect was created on the dorsal midline using a 6 mm diameter biopsy punch for the evaluation of wound healing. All the animals were treated humanely according to the guidelines provided in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. All animals were housed individually under standard conditions. The study was approved by the Institutional Animal Care and Use committee of The Third Xiangya Hospital of Central South University.

Criteria for Inclusion. Animals with random blood glucose which exceeded 16.67 mM were included in the study. The measurements were taken in triplicate.

2.2. Methods

2.2.1. Cell Administration at the Wound Site. Right after wound induction, each wound received 2.0×10^6 cells in 60 µL of PBS injected subcutaneously along the margin of the dorsal wound at four injection sites applied onto the wound bed ($n = 6$). Conditioned medium was administered immediately and on every alternate day ($n = 6$), whereas an equivalent volume of PBS was administered in the control group ($n = 6$) in the same fashion. A transparent bioocclusive adhesive tape (Comfeel Plus Transparent Dressing) was placed over the wounds. The adhesive tape on the skin in mice was tested prior to this experiment for any skin irritation or allergic reaction, and there was none. The transparent dressing was changed every alternate day to maintain wet wound conditions. Wounded animals were housed individually under standard conditions. Wound healing was assessed

TABLE 1: Criteria for wound scoring.

Score	Epidermal and dermal regeneration	Granulation tissue	Angiogenesis (day-14 wounds only)
1–3	Minimal to moderate reepithelialization	Granulation around wound edges only	Capillary density <300/mm ²
4–6	Complete reepithelialization	Granulation around wound edge and in 30%–50% of wound bed	Capillary density 300–500/mm ²
7–9	Complete reepithelialization	Thick granulation around wound edge and in >50% of wound bed	Capillary density >500/mm ²

<http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0041105#pone-0041105-t001>, at doi: 10.1371/journal.pone.0041105.t001.

by measuring the epithelial gap every alternate day for two weeks. A ruler was placed next to the wound, and the wounds were photographed from an equidistant arbitrary level at all times. Then the area was calculated using Image Pro Plus. Scale bar was taken as 1 mm.

2.2.2. Estimation of Wound-Healing Area. The wound-healing area was assessed once every alternate day after the procedure. At different time points (0, 2, 4, 6, 8, 10, 12, and 14 days) after wounding, lesion closure was documented using a digital camera. Images were processed and analyzed by tracing the wound margin and calculating the pixel area using the Image Pro Plus. Reepithelialization was reported as a percentage of the initial wound area and calculated as reepithelialization percentage = $[1 - (\text{area on day of analysis}/\text{area on day 0})] \times 100$. The day in which the full-thickness wound is seen to be completely closed was taken as the day of complete healing. The healed area was calculated from the original wound area (diameter = 6 mm) and the unhealed area once every alternate day for two weeks.

2.2.3. Histological Examination. A full-thickness 3 mm punch biopsy was performed from the wound margin after the wounds healed completely, and euthanized. Six samples were randomly examined and analyzed in each group. Specimens were fixed in 10% formalin and embedded in paraffin. Sections were cut from the paraffin-embedded specimens and stained with hematoxylin and eosin. Images of hematoxylin and eosin stained slides of each wound obtained from maximal cross sections were digitally acquired, and then wound scoring was done (Table 1).

2.2.4. RT-PCR Analysis. Total RNA (1 μ g) was extracted from wound tissues harvested at day 14 after wound induction and was processed for cDNA synthesis using the Superscript first-strand synthesis system (Invitrogen), after which the cDNA was amplified with 40 cycles of PCR using gene-specific primers as shown in Table 2. Real-time PCR was performed using Transtart Green qPCR SuperMix UDG system. Data analysis was based on the $\Delta\Delta C_t$ method with normalization of the raw data to housekeeping gene, GAPDH, included in the experiment. All reactions were performed in triplicate.

2.2.5. Immunohistochemistry. Wound sections were treated with 0.3% hydrogen peroxide to quench the endogenous

TABLE 2: Primers used in PCR.

Primer name	Primer sequence (5'-3')
VEGF	F: CAAGGCCAGCACATAGGAGA R: AGGGAACGCTCCAGGACTTA
PDGF- β	F: TCGAGA TTGTGCGGAAGAAG R: GTGTGCTTGAATTTCCGGTG
PDGF- α	F: CCATTTCGGAGGAAGAGAAGC R: GTATTCCACCTTGGCCACCT
KGF	F: TTCACATTATCTGTCTAGTGGGT R: TGGGTCCCTTTTACTTTGCC
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC

peroxidase, and then antigen epitopes were retrieved by heating in Target Retrieval Solution. After sections were blocked in 10% normal goat serum, they were treated with anti-von Willebrand factor (vWF; Abcam, USA).

2.2.6. Data Management and Statistical Analysis. Data were analyzed by SPSS 16.0 software and are presented as mean \pm SEM. Student's paired *t*-test was performed for data comparison of paired samples, and analysis of variance followed by Bonferroni's post hoc multiple comparison test was performed to determine the significant differences among the three groups. A probability (*P*) value of less than 0.05 was considered statistically significant.

3. Results

3.1. The Transplantation of UC-MSCs and Their CM Accelerate Wound Closure in Diabetic Mice. The therapeutic effect of transplantation of umbilical cord derived mesenchymal stem cells and their conditioned media to heal wounds in genetically diabetic *db/db* mice was significant (Table 3). *db/db* mice in which PBS was injected displayed markedly delayed wound healing. When UC-MSCs and their CM were injected subcutaneously around full-thickness dermal wounds created on the diabetic mice, wound closure was significantly accelerated as early as day four after injury in the CM-treated wounds and at day eight after injury in the UC-MSC-treated wounds compared to PBS-treated ones and became more evident at day 14 (Figures 1(a), 1(b), and 1(c)). This significant increase in the healed wound area was consistently observed until day 14 (CM (94.38 \pm 0.80)%,

TABLE 3: Analysis of wound closure rate of all groups (mean \pm SEM).

Duration (days)	Area CM (%)	Area UC-MSC (%)	Area PBS (%)
0	0	0	0
2	3.31 \pm 0.67	4.13 \pm 0.81	2.51 \pm 0.09
4	12.01 \pm 1.77***	9.10 \pm 0.82	3.84 \pm 0.32
6	19.98 \pm 1.41***	14.75 \pm 0.66	6.9 \pm 0.60
8	33.72 \pm 1.67***	27.63 \pm 0.59**	8.11 \pm 0.61
10	61.20 \pm 2.12***	59.70 \pm 0.41**	11.64 \pm 0.68
12	78.62 \pm 1.69*	70.71 \pm 1.39**	16.67 \pm 0.96
14	94.38 \pm 0.80*	83.22 \pm 1.54**	18.63 \pm 1.13

Note. Comparison of wound closure rates ($n = 6$; * $P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; ** $P < 0.05$, UC-MSC versus PBS); ($n = 6$; *** $P < 0.05$, CM versus PBS).

UC-MSC (70.71 \pm 1.39)%, PBS (18.63 \pm 1.13)% on day 14). Statistically significant difference in the wound-healing rate among all groups was observed on day 12 and day 14 ($P < 0.05$). At day 14, all 6 wounds in CM-treated *db/db* mice and 3 of 6 wounds in UC-MSC-treated wounds achieved complete closure, but no completely closed wound was seen in PBS-treated mice ($n = 6$). In addition, substantially reduced cross-sectional area of granulation tissue among all groups was observed at day 14 ($P < 0.05$) (Figure 1(d)).

Histological evaluation of wounds in *db/db* mice at 14 days disclosed enhanced re-epithelialization in conditioned media treated wounds (complete epithelialization in all 6 wounds examined; $n = 6$) compared with UC-MSC-treated (complete reepithelialization in 3 of 6 wounds; $n = 6$) or PBS-treated wounds (complete reepithelialization in none; $n = 6$). Analysis of wounds on day 14 indicated wounds treated with conditioned media and UC-MSC had increased vasculature compared to PBS-treated controls (Figure 2(a)). In addition, granulation tissue in conditioned media and UC-MSC-treated wounds appeared to be thicker but lesser in area. Consistent with these findings, the wound scores of the day-14 wounds among three groups were statistically significant (Figure 2(b)).

3.2. Injection of UC-MSC and CM Increases Neovascularization of Wounded Tissue. Capillary densities in day-14 wounds were assessed after immunohistochemical staining for vWF. Immunohistological staining of tissue sections for vWF showed increased vasculature in conditioned media treated wounds at 14 days compared with UC-MSC-treated or PBS-treated wounds (Figures 3(a) and 3(b)). Significantly higher capillary density in conditioned media treated (725 \pm 47.87/mm²) and in UC-MSC-treated (475 \pm 47.87/mm²) wounds compared to PBS-treated (133.3 \pm 21.02/mm²) wounds was found ($n = 6$; * $P < 0.05$).

3.3. Secreted Factors from Mesenchymal Stem Cells and CM Directly Stimulate Growth Factors That Are Potentially Relevant to Dermal Healing. To determine whether mesenchymal stem cells and conditioned media derived from culturing of UC-MSCs in serum-free M199 media for 24 hours could

enhance angiogenesis through a paracrine effect, real-time polymerase chain reaction analysis was performed on wound tissues harvested at 14 days and normalized as its relative ratio to GAPDH. It revealed higher levels of VEGF, PDGF, and KGF expression in the CM- and UC-MSC-injected wounds compared to the PBS-treated wounds. The expression of PDGF- β and KGF was higher in CM-treated wounds compared to UC-MSC-treated wounds. The data were obtained with samples from three independent preparations and are expressed as mean \pm SEM (Figure 4).

4. Discussion

There is certainly no room for doubt that innovative treatments for the prevention, alleviation, and/or total cure of the diabetic wounds are in high demand. Here, we show that umbilical cord derived mesenchymal stem cells enhance wound healing in diabetic mice by promoting reepithelialization, secretion of paracrine factors, and neovascularization.

Isogenic strains of mice were used in the experiment. These are considered immortal clones of genetically identical animals. The purity of the mouse stock can assure a research scientist of a true and sure experiment, and in experimental medicine today, the use of inbred genetic materials is just as necessary as the use of the aseptic and antiseptic precautions in surgery [16]. Gruneberg in 1952 further emphasized the use of inbred strains saying that the introduction of inbred strains into biology is probably comparable in importance with that of the analytical balance into chemistry [17]. We used an excisional wound-healing model in genetically diabetic *db/db* mice, which has been known to have markedly impaired wound healing and is an established model to study the effect of therapeutic reagents on wound healing [18–22]. *db/db* mouse model is characterized by early hyperinsulinemia with marked hyperglycemia progressing with age to slowly developing islet failure. These animals exhibit hyperglycemia over 16.67 mM by six weeks of age which increases in severity over time. Our study shows that wound closure is significantly delayed in diabetic mice, and umbilical cord derived mesenchymal stem cell transplantation significantly accelerates wound healing in diabetic mice in this study. Consistent to our findings, similar studies, albeit with different sources of mesenchymal stem cells, including those from the bone marrow [18, 21], umbilical cord blood [23], and adipose tissue [24], also accelerated wound healing in diabetic mice. Other studies which reiterate the efficacy of these autologous stem cells in wound healing in patients have also been reported [25–30].

Allogeneic mesenchymal cells have been administered by various routes in animal wound-healing models [31–36]; however, the optimal type has not been well defined. These include topical [37, 38], intravenous [36], local injection [18, 31, 34, 36], and systemic administration [27, 37]. In most studies, the cells were delivered in a single dose; however, one study reported a study with multiple doses [28]. No significant adverse effect has been reported so far in case of allogeneic lineage negative bone marrow cells, despite being effective in wound healing in a murine diabetic

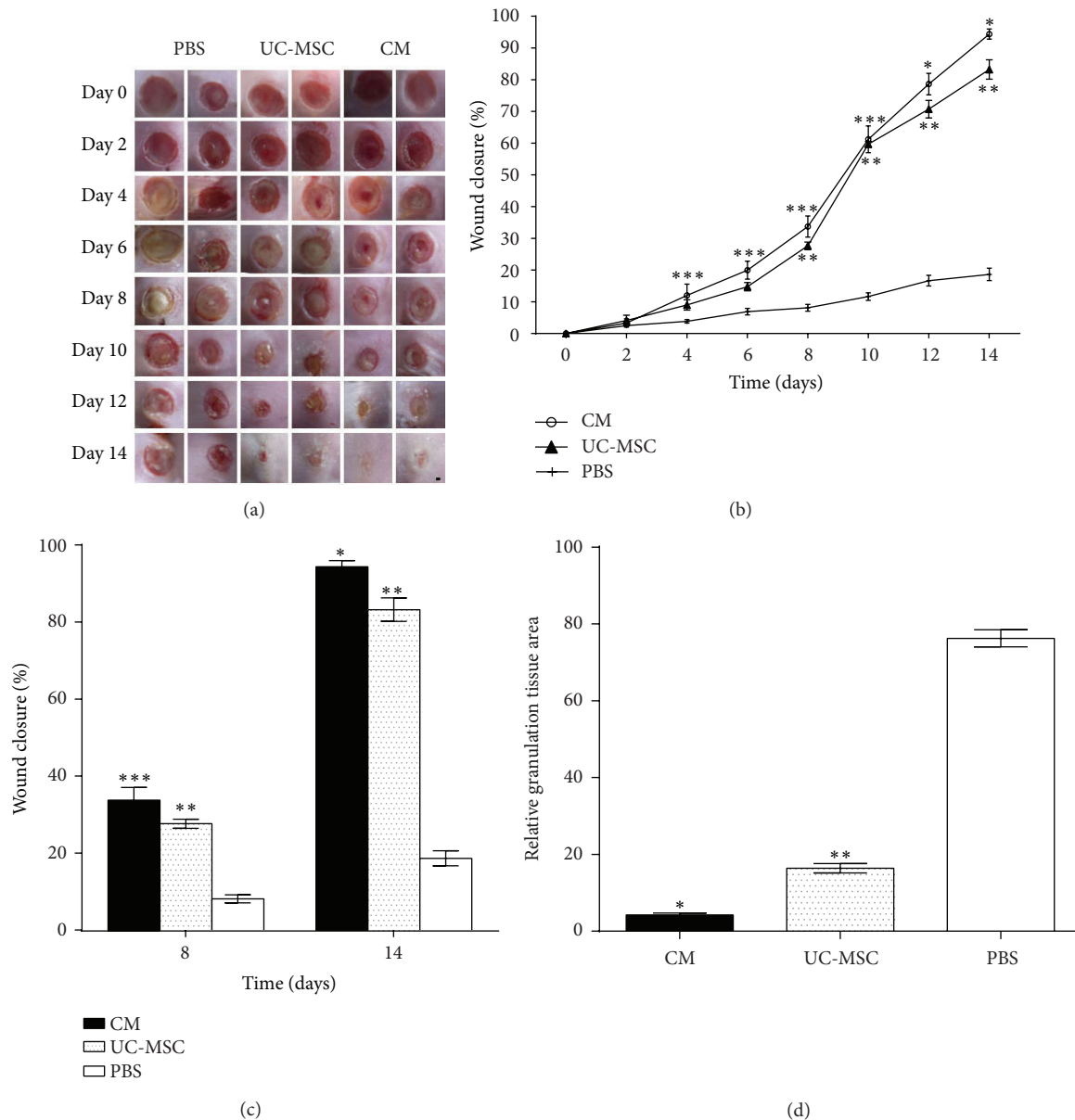


FIGURE 1: Effects of CM, UC-MSC, and PBS on wound closure. (a) Representative photographs of the wounds of every alternate day from day 0 to day 14 after injury. Scale bar is 1 mm. (b) Comparison of wound closure rates expressed as a percentage of its initial wound area every alternate day after wounding ($n = 6$; $*P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; $**P < 0.05$, UC-MSC versus PBS); ($n = 6$; $***P < 0.05$, CM versus PBS). (c) Wound measurement of all groups at day 8 and day 14 ($n = 6$; $*P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; $**P < 0.05$, UC-MSC versus PBS). (d) Relative granulation tissue area of wounds treated with CM, UC-MSC, and PBS at day 14 ($n = 6$; $*P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; $**P < 0.05$, UC-MSC versus PBS).

wound-healing model [38]. In addition, in consistency with to our findings, human adipose tissue derived stem cells delivered to diabetic (*db/db*) mice have shown effectiveness in wound healing, also without adverse effect [39]. In studies by Badiavas et al., mixed population of bone marrow cells were used to treat chronic wound patients via local injection and topical application in saline, and they state that no adverse events were noted [40, 41]. Variation in dosing in these studies range from single to multiple applications with doses up to 2×10^8 cells per administration. Falanga et al. came up with the

finding that dosages exceeding 1×10^6 cells·cm⁻² were directly related to accelerated wound closure [28].

The immunomodulatory properties of mesenchymal stem cells allow for an allogeneic source for therapy. Possible drawbacks of autologous source of the same can be reflected in chronic disorders associated with nonhealing wounds such as diabetes and autoimmune disease, owing to the abnormalities in bone marrow cells, including mesenchymal stem cells [42–46]. Bone marrow derived cells from chronic wound patients showed reduced growth in

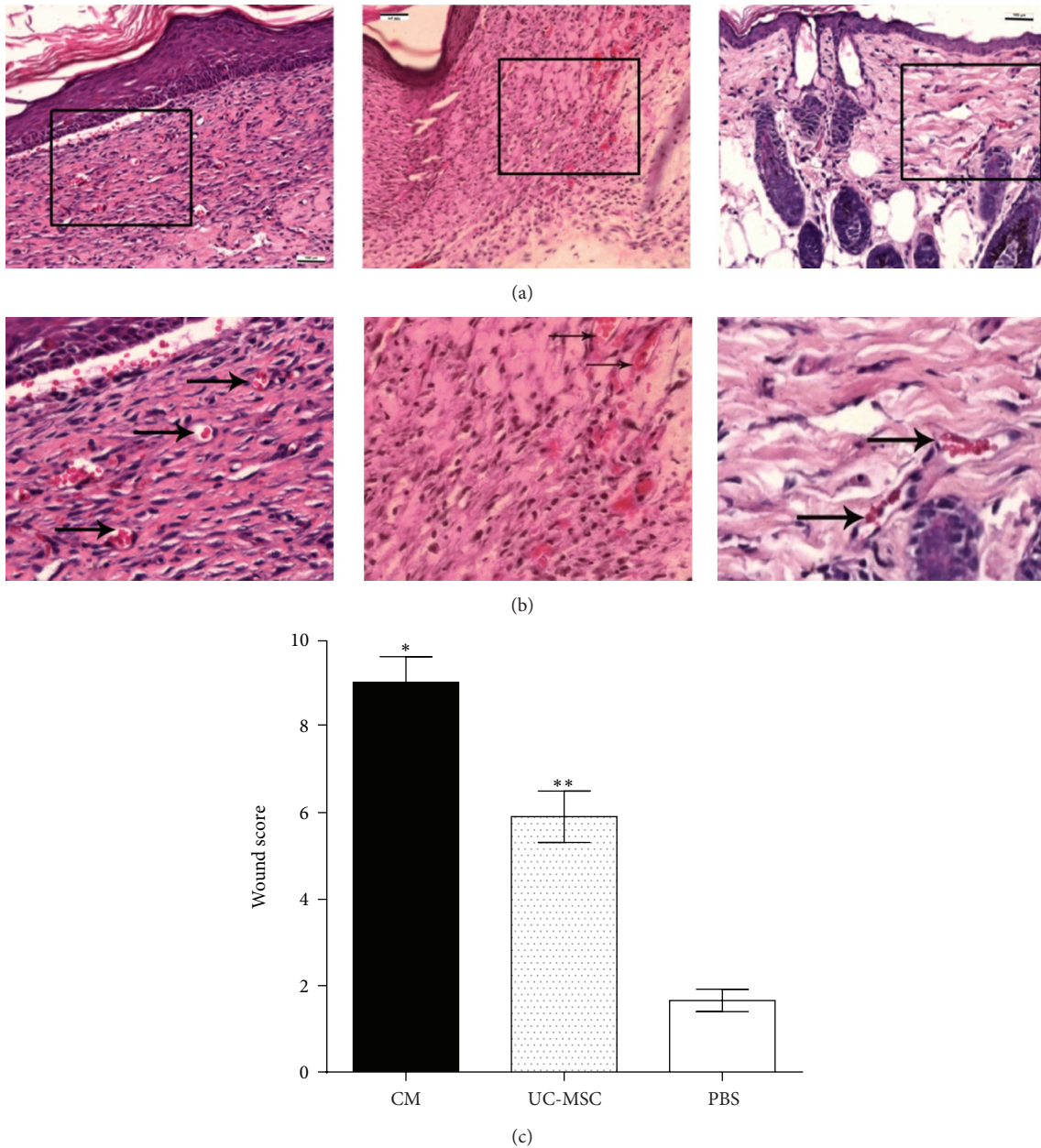


FIGURE 2: Histological analysis of day-14 wounds in *db/db* mice. Scale bar is 100 μ m. (a) H and E stained images of CM-, UC-MSC- and PBS-injected tissues from left to right, respectively (20x). (b) Magnified images of CM-, UC-MSC-, and PBS-injected tissues from left to right, respectively. (c) Wound scores at day 14 ($n = 6$; * $P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; ** $P < 0.05$, UC-MSC versus PBS).

culture compared to their normal counterparts [41], and the administration of allogeneic MSCs could be preferred under such circumstances [44–47]. The therapeutic benefits from healthy donors of allogeneic mesenchymal stem cells might surpass those that can be accomplished by the transplantation of autologous mesenchymal stem cells derived from chronic wound patients.

Neovascularization, the formation of new blood vessels which is necessary to sustain the newly formed granulation tissue and the survival of keratinocytes, is considered as one of the important processes in wound healing [3, 48, 49].

In this study, we demonstrated that MSC-treated wounds had enhanced capillary density, suggesting that these cells promote angiogenesis. It was also found that UC-MSC and CM injection resulted in increased amounts of KGF and PDGF in the wounds. The levels of PDGF- β and KGF were even more pronounced in CM-injected wounds than UC-MSC-injected wounds in this study. Indeed, another important role in angiogenesis is played by vasoendothelial growth factor, which does so by stimulating endothelial cell proliferation, migration, and organization into tubules [49, 50]. Moreover, VEGF increases circulating endothelial

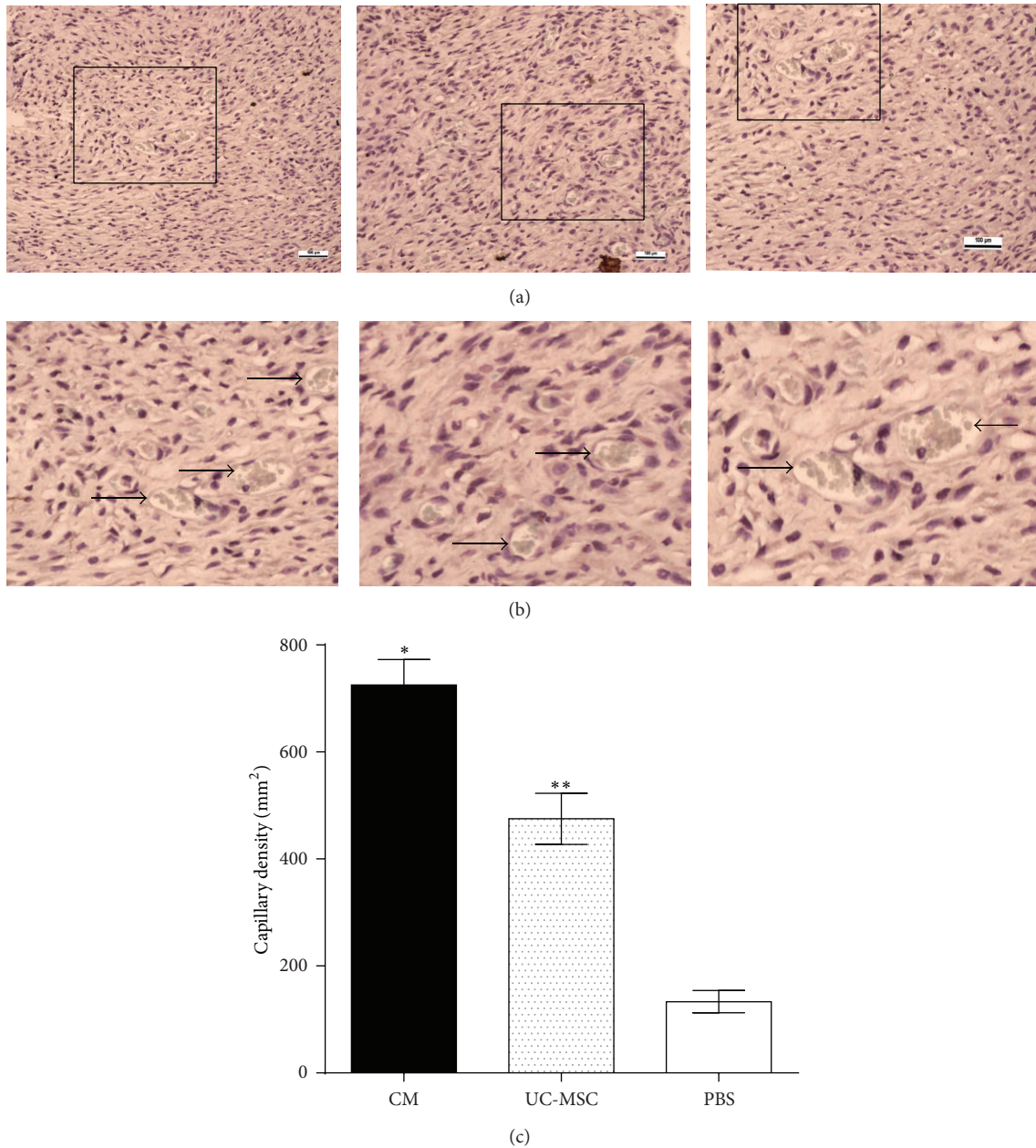


FIGURE 3: Capillary density in 14-day-old wounds was determined after immunohistochemistry. Scale bar is 100 μm . (a) Images of CM-, UC-MSC-, and PBS-injected tissues from left to right, respectively (20x). (b) Magnified images of CM-, UC-MSC-, and PBS-injected tissues from left to right, respectively. (c) Capillary density as the number of vWF-positive vessels per mm^2 ($n = 6$; * $P < 0.05$, CM versus UC-MSC or PBS); ($n = 6$; ** $P < 0.05$, UC-MSC versus PBS).

progenitor cells [50]. VEGF was comparatively more in UC-MSC- and CM-injected wounds than in PBS-treated wounds in this study too. Angiogenesis was more pronounced in CM-injected wounds compared to UC-MSC, but the levels of VEGF were found to be similar in both groups. VEGF levels were measured in day-14 wounds only. One possible reason could be that CM was administered every alternate day, and it enabled stable and effective long-term release of VEGF compared to UC-MSC and, as a consequence, more pronounced angiogenesis compared to UC-MSC.

Angiogenesis is a complex process controlled by the balance of proangiogenic and antiangiogenic factors [50]. Our results suggest that UC-MSCs engrafted in the wound release proangiogenic factors, subsequently leading to MSC-mediated enhanced angiogenesis. Differentiation of MSCs into keratinocytes found in our study was consistent with the findings of similar other studies [51, 52]. In our study, we found that injection of CM could accelerate wound closure, and the enhancement was even better and more rapid than that achieved by UC-MSC transplantation in contrast to

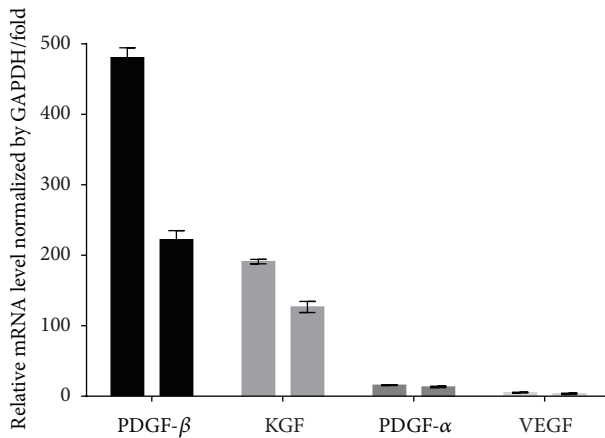


FIGURE 4: The mRNA profile of wound-healing related growth factors analyzed by real-time PCR and normalized by its relative ratio to GAPDH in CM-treated (left) and UC-MSC-treated (right) wounds.

the results obtained by Wu et al. [18], although they used MSC from a different source. These results suggest that differentiation of MSCs may be one of the major role players in MSC-mediated cutaneous repair/regeneration, although paracrine factors are important.

Several limitations do exist in our study. Human MSCs are likely to play unique roles in delivery to nonhealing wounds that cannot be fully duplicated in animal models. Animal models of chronic wounds are just delayed-healing models, with marked differences in pathophysiology [53–55]. Wounds of size surpassing 5 cm² in humans and lasting for a duration exceeding six months [56, 57] and age-related changes and chronic disorders common in chronic wound patients are next to impossible to be studied in animals [58]. Elderly patients with chronic wounds are more likely to respond to allogeneic therapy owing to the immune dysregulation in such patients [59]. These important factors of mesenchymal stem cells, compounded with the imperfections inherent to animal model of chronic wounds, show the importance of more extensive investigation in humans.

Controversial data concerning the effects of MSCs on regulation of tumor growth have been reported for animal and *in vitro* models [60–63]. The tumorigenicity of transplanted cells needs thorough assessment, although no tumor formation has been reported so far, after UC-MSCs transplantation [64].

Our study demonstrates the beneficial effect of UC-MSCs and CM in cutaneous regeneration and wound healing in diabetic mice through angiogenesis and paracrine effects. UC-MSCs and CM represent a defined and expandable population of cells with potential therapeutic use in the treatment of diabetic wounds, and CM has proved to be therapeutically better, at least, in the context of diabetic wound healing in this study.

5. Conclusion

Both the transplantation of UC-MSC and CM accelerate wound closure in diabetic mice, and even more rapid rate of wound healing is achieved in CM-treated wounds than MSC-treated wounds. Secreted factors from UC-MSC and CM directly stimulate growth factors (VEGF, PDGF, and KGF) that are potentially relevant to dermal healing. PDGF-β and KGF levels were more pronounced in CM-injected wounds than in UC-MSC-injected wounds. Injection of UC-MSC and CM increases angiogenesis of wounded tissue. The angiogenesis in wounded tissue after CM administration was more than that achieved by UC-MSC administration.

Abbreviations

CM:	Conditioned media
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
KGF:	Keratinocyte growth factor
MSCs:	Mesenchymal stem cells
PBS:	Phosphate buffer solution
PCR:	Polymerase chain reaction
PDGF-α:	Platelet-derived growth factor-alpha
PDGF-β:	Platelet-derived growth factor-beta
UC-MSCs:	Umbilical cord derived mesenchymal stem cells
VEGF:	Vascular endothelial growth factor
vWF:	von Willebrand factor.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Renal Effects of DPP-4 Inhibitors: A Focus on Microalbuminuria

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Incretin-based therapies represent one of the most promising options in type 2 diabetes treatment owing to their good effectiveness with low risk of hypoglycemia and no weight gain. Other numerous potential beneficial effects of incretin-based therapies have been suggested based mostly on experimental and small clinical studies including its beta-cell- and vasculo-protective actions. One of the recently emerged interesting features of dipeptidyl peptidase-4 (DPP-4) inhibitors is its possible protective effect on the diabetic kidney disease. Here, we review the renal effects of DPP-4 inhibitors with special focus on its influence on the onset and progression of microalbuminuria, as presence of microalbuminuria represents an important early sign of kidney damage and is also associated with increased risk of hypoglycemia and cardiovascular complications. Mechanisms underlying possible nephroprotective properties of DPP-4 inhibitors include reduction of oxidative stress and inflammation and improvement of endothelial dysfunction. Effects of DPP-4 inhibitors may be both glucagon-like peptide-1 (GLP-1) dependent and independent. Ongoing prospective studies focused on the nephroprotective effects of DPP-4 inhibitors will further clarify its possible role in the prevention/attenuation of diabetic kidney disease beyond its glucose lowering properties.

1. Introduction

Increasing prevalence of diabetes worldwide, leading to a steep rise of patients with chronic complications, represents one of the major health problems of the current medicine [1]. Since both micro- and macrovascular complications contribute in the increasing morbidity and mortality of patients with type 2 diabetes, novel antidiabetic therapies are intensively studied with respect to their possible beneficial effects on the long-term complications beyond their glucose-lowering properties [2]. Incretin-based therapies represent one of the most promising options in type 2 diabetes treatment owing to their good effectiveness with low risk of hypoglycemia and no weight gain [3]. These therapeutics either increase concentrations of endogenous glucagon-like peptide-1 (GLP-1) by the inhibition of its degradation (dipeptidyl peptidase-4 inhibitors) or directly stimulate GLP-1 receptor (GLP-1 receptor agonists) [4]. Stimulation of GLP-1 receptor in turn increases insulin secretion and suppresses excessive glucagon release leading to improved glucose control. Other numerous potential beneficial effects

of incretin-based therapies have been suggested based mostly on experimental and small clinical studies including its beta-cell- and vasculoprotective actions and also numerous others pleiotropic positive effects such as neuroprotection and others [5]. One of the interesting possibilities that have emerged from experimental studies is the protective effect of DPP-4 inhibitors on the diabetic kidney disease [6]. Here, we review the renal effects of DPP-4 inhibitors with special focus on its influence on the onset and progression of microalbuminuria. We will discuss potential mechanism of these effects, the differences between various DPP-4 inhibitors, and future perspectives of its use in patients with diabetic kidney disease. We performed a primary Medline search using combinations of keywords: sitagliptin, vildagliptin, saxagliptin, linagliptin, exenatide, liraglutide, and GLP-1, DPP-4 with albuminuria, and we consequently used all relevant articles published in English language in this review. Due to a limited number of results, we performed secondary searches using combinations of additional keywords like diabetic kidney disease and nephropathy.

2. Diabetic Kidney Disease: Basic Pathophysiology

Diabetic kidney disease (DKD) represents one of the most frequent microvascular complications of diabetes with an overall prevalence of approximately 40% in type 2 diabetes population [7]. DKD is defined by the presence of albuminuria and decreased glomerular filtration rate (GFR) into 5 chronic kidney disease (CKD) stages. CKD stage 1 is characterized by normal GFR and urine findings (mostly albuminuria) or structural abnormalities of the kidney. Stages 2–5 are defined by specific values of GFR [7]. Patients with diabetic kidney disease, even in stage 1, have a markedly increased risk of cardiovascular complications and hypoglycemia compared to patients without DKD [8, 9]. Numerous studies have shown that the risk of diabetic kidney disease is tightly linked to poor glucose control in both type 1 and type 2 diabetes [10, 11]. The adverse effects of hyperglycemia are generally mediated through diverse metabolic pathways including increased reactive oxygen species formation, excessive production of advanced glycation end products (AGEs), and the activation of polyol, protein kinase C (PKC), and hexosamine pathways, respectively [12]. Activation of these pathways leads to a complex dysregulation of various effector molecules resulting in cellular damage and dysfunction [12]. Experimental studies have shown that some of these pathophysiological mechanisms are potentially modifiable by DPP-4 inhibition [6]. Activation of PKC in the kidney by hyperglycemia reduces GLP-1 signaling while enhancing angiotensin II and nuclear factor- κ B (NF- κ B) signaling pathways with subsequent development of glomerular endothelial dysfunction [12]. This process that typically occurs in the early stages of diabetic kidney disease is characterized by decreased local production of nitric oxide and enhanced production of reactive oxygen species, proinflammatory and cell adhesive molecules including tumor necrosis factor- α (TNF- α), plasminogen activator inhibitor-1 (PAI-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and others [13]. Increased expression of ICAM-1 on glomerular endothelial cells promotes local macrophage infiltration into glomeruli with subsequent development of local inflammation and increased production of the renal profibrotic cytokine transforming growth factor β (TGF- β) [14]. Chronic overproduction of TGF- β markedly contributes to local degenerative changes and progressive fibrosis in diabetic kidney [14]. Additional important players contributing to kidney damage especially in patients with type 2 diabetes include arterial hypertension and dyslipidemia that commonly cluster with glucose metabolism disturbances in these patients [15].

3. Mechanism of Albuminuria

The presence of microalbuminuria represents an important early sign of kidney damage in patients with diabetes [16]. Associations between microalbuminuria, increased risk of cardiovascular complications, and progressive renal impairment are well described but the underlying pathophysiological mechanisms are only partially understood [16]. In

general, the presence of microalbuminuria implies either dysfunction of the glomerular filtration barrier and/or dysfunction in tubular reabsorption [17]. At the time of overt microalbuminuria, established glomerular structural changes are present in diabetic kidney [18]. Studies have shown that local changes in glomerular morphology and the extent of matrix accumulation in glomeruli and interstitium correlate with extent of albuminuria [19]. Glomerular filtration barrier is characterized by three-layer structure: endothelium with glycocalyx, glomerular basement membrane, and podocytes [12]. Endothelial glycocalyx forms a barrier to protein permeability in both systemic and glomerular capillaries. The total systemic glycocalyx volume is reduced by acute hyperglycemia in humans [20]. Podocyte loss is one of the first changes contributing to increased glomerular permeability for albumin. Nevertheless, albuminuria may also occur in the complete absence of structural changes of podocytes [21, 22]. Tubular dysfunction is another important player promoting albuminuria by lysosomal dysfunction which is promoted by renin-angiotensin system activation and TGF- β overproduction [23]. A number of experimental studies demonstrated that TGF- β neutralization improved renal function and reversed morphological changes associated with disease progression in diabetic nephropathy models [24]. TGF- β is typically elevated in serum, urine, and glomerular tissue since early stages of both type 1 and 2 diabetes. Its levels correlate with the degree of mesangial expansion, interstitial fibrosis, and renal dysfunction, but not with the extent of microalbuminuria [25]. On the contrary, urinary levels of TNF- α and vascular endothelial growth factor (VEGF) tightly correlate with microalbuminuria [26, 27]. Both of these cytokines are upregulated in patients with type 2 diabetes [26, 27]. TNF- α directly increases endothelial permeability and disrupts the endothelial glycocalyx [27]. Albuminuria also positively correlates with markers of endothelial dysfunction and chronic low-grade inflammation including C-reactive protein [28, 29].

4. GLP-1 Dependent and Independent Actions of DPP-4 Inhibitors in the Kidney

The main action of DPP-4 inhibitors is to increase the levels of endogenous incretin hormones, especially GLP-1. DPP-4 inhibitors are known to exert also GLP-1 independent effects as DPP-4 cleaves a wide range of other substrates such as neuropeptides, hormones, cytokines, and chemokines [6, 30]. DPP-4 is also bound on the surface of many cell types including kidney proximal tubular cells and endothelial cells [31]. Microvesicle-bound DPP-4 secreted from tubular epithelial cells is found in urine and may be an early marker of renal damage before the onset of albuminuria [31]. Sun et al. also described higher urinary microvesicle DPP-4 levels in patients with diabetes compared to nondiabetic controls that positively correlated with extent of albuminuria in patients [31]. Upregulation of DPP-4 expression in renal glomeruli occurs during inflammation and usually accompanies the development of diabetes-induced glomerulosclerosis [6].

Renal effects of DPP-4 inhibitors appear to be, at least in part, mediated by increased GLP-1 levels [32]. In addition

to pancreas, GLP-1 receptor (GLP-1R) is expressed in other numerous tissues including glomerular endothelial cells, mesangial cells, podocytes and also proximal tubular cells. Its expression was decreased in diabetic compared with non-diabetic mice [32]. Studies have shown that GLP-1 has anti-inflammatory properties and decreases AGEs production by activation of protein kinase A (PKA). The stimulation of nitric oxide (NO) production and inhibition of production of angiotensin II, PAI-1, ICAM-1, and VCAM-1 seems to be both GLP-1 dependent and independent [33–37].

Several candidates for GLP-1 independent effects of DPP-4 inhibitors in the kidney have been identified among known substrates of DPP-4 cleavage including high mobility group protein-B1 (HMGB1), Meprin β , neuropeptide Y (NPY), and peptide YY (PYY). Nevertheless, their exact role and importance in renoprotective effects of DPP-4 inhibitors has not yet been tested [38]. HMGB1 is a known ligand of advances glycation end products receptor (RAGE), as well as Toll-like receptor 2 (TLR2) and TLR4, which are involved in the inflammatory process of diabetic nephropathy leading to NF- κ B activation [38]. Meprin β has been associated with several types of renal pathology [39]. Both NPY and PYY are important mediators of various kidney functions including natriuresis [40–44]. Experimental studies have also shown that DPP-4 participates in the extracellular catabolism of proteins in the kidney such as catabolism/degradation proline-containing peptides [45].

5. Preclinical Data of DPP-4 Inhibitors with Nephroprotective Outcomes

Preclinical data suggesting nephroprotective effects of DPP-4 inhibitors are available for sitagliptin [46], vildagliptin [47], and linagliptin [48]. The study with sitagliptin administration assessed its effects on metabolic profile and renal lesions in a rat model of type 2 diabetic nephropathy [46]. Diabetic and controls rats were treated with sitagliptin or vehicle for 6 weeks. Sitagliptin treatment of diabetic rats lowered glycemia and ameliorated glomerular, tubulointerstitial, and vascular lesions. It also reduced kidney lipid peroxidation as measured by decreased malondialdehyde content.

The study with vildagliptin was performed on rats with streptozotocin-induced diabetes. In this insulinopenic model, vildagliptin increased GLP-1 levels but did not affect blood glucose levels [47]. Light and electron microscopies of renal tissue revealed that vildagliptin treatment dose dependently inhibited interstitial expansion, glomerulosclerosis, and thickening of the glomerular basement membrane [47]. It also significantly decreased both albuminuria and proteinuria and reduced TGF- β overexpression. Expression of GLP-1R was demonstrated by immunohistochemical analysis in both glomeruli and tubules. The 24-week duration of hyperglycemia in untreated diabetic rats resulted in decrease of GLP-1R staining [47]. This decrease was prevented by treatment with vildagliptin. Furthermore, increased urinary excretion rates of 8-Oxo-2'-deoxyguanosine (major product of DNA oxidation and marker of oxidative stress) in diabetic rats were markedly attenuated by vildagliptin treatment.

These results suggest prevention of oxidative DNA damage and renal cell apoptosis by activating the GLP-1R.

Streptozotocin-induced diabetes was used also in an experimental study with linagliptin [48]. Diabetes was induced in endothelial nitric oxide synthase (eNOS) knock-out mice which were used as an experimental model of nephropathy [48]. The effect of linagliptin on the progression of nephropathy alone and in combination with the angiotensin receptor blocker telmisartan was tested. After 12 weeks of administration, linagliptin or telmisartan had no effect on glycemic control, while telmisartan reduced systolic blood pressure by 5.9 mmHg compared to no change in linagliptin group. Combined treatment with linagliptin and telmisartan significantly reduced albuminuria compared with untreated diabetic mice, while monotherapy with either telmisartan or linagliptin had no effect. Reduced glomerulosclerosis and normalization of tissue immune reactivity of malondialdehyde, a biomarker of oxidative stress, were seen in linagliptin and combined linagliptin/telmisartan group while angiotensin II receptor blockade alone failed to reduce the markers of oxidative stress.

Another data suggesting possible nephroprotective effects of DPP-4 inhibitors come from the study on the experimental model of renal ischemia/reperfusion injury [49]. In this study, vildagliptin was administered intravenously 15 minutes before surgery, and animals were sacrificed after 2, 12, and 48 hours of reperfusion. DPP-4 inhibition dose dependently decreased serum creatinine, tubular necrosis, serum malondialdehyde levels, and mRNA expression of proinflammatory chemokine CXCL10. These data suggest that the nephroprotection by DPP-4 inhibition was mediated by antiapoptotic, anti-inflammatory, and antioxidant changes.

In addition to experimental data for DPP-4 inhibitors, animal studies suggest also possible nephroprotective effects of GLP-1R agonists. Both exendin-4 [35] and liraglutide [50] ameliorated albuminuria decreased oxidative stress and inflammatory cytokines in a rat model of diabetic nephropathy. In the exendin-4 study, glomerular macrophage infiltration was prevented by suppression of ICAM-1 production on glomerular endothelial cells and by inhibition of proinflammatory cytokine release from macrophages.

6. Clinical Studies of DPP-4 Inhibitors with Albuminuria Outcomes

Many diabetic patients develop diabetic kidney disease despite intensive efforts to achieve optimal control of blood pressure and glycemia. In addition to being a marker of renal damage, albuminuria has emerged as a predictive marker of increased risk of cardiovascular disease [51]. According to current guidelines, the primary intervention in patients with detected albuminuria is the blockade of renin-angiotensin-aldosterone system (RAAS) with an angiotensin-converting enzyme inhibitor (ACEi) or angiotensin II receptor blocker (ARB) [52]. Proven additive effect of any antihyperglycemic agent on albuminuria lowering would be therefore of a high interest.

First study indicating possible beneficial effect of a DPP-4 inhibitor on the kidney in Man was a small observational study with sitagliptin [53]. 36 patients with HbA1c > 6.5%, despite lifestyle measures and antidiabetic treatment for at least 6 months, were enrolled and treated by sitagliptin (50 mg/day) for 6 months. Sitagliptin significantly lowered HbA1c and systolic and diastolic blood pressure. Significant reductions of C-reactive protein and soluble VCAM-1 were also observed. After 6 months of sitagliptin treatment, albuminuria measured by urinary albumin creatinine ratio (UACR) significantly decreased both in the patients with relatively modest microalbuminuria and the patients with more pronounced microalbuminuria at baseline. Albuminuria was also one of secondary endpoints in the study of Harashima et al. performed with sitagliptin [54]. 82 subjects were enrolled to the 52-week, prospective, single-arm study where sitagliptin was added-on to sulphonylureas (glimepiride or gliclazide) with or without metformin. The primary endpoint was a change in HbA1c. The secondary endpoints were changes in BMI, insulin secretory capacity, blood pressure, UACR, unresponsive rate, and hypoglycemia. After 52 weeks sitagliptin treatment reduced HbA1c by 0.8% and UACR from 76.2 ± 95.6 to 33.0 ± 48.1 mg/g along with slight decreases in BMI blood pressure.

In 2012, a comprehensive analysis of randomized, double-blind, placebo-controlled trials (duration 24–52 weeks) with linagliptin was published [55]. The analysis included studies with both linagliptin monotherapy or add-on therapy to various glucose-lowering agents. Inclusion criteria for the analysis were diabetic patients with persistent albuminuria defined as $30 \leq \text{UACR} \leq 3000$ mg/g and stable treatment with ACE inhibitor or angiotensin II receptor blocker at baseline. The analysis included 168 patients treated with linagliptin and 59 patients on placebo, respectively. The endpoint of the analysis was the percentage change in geometric mean of UACR after 24 weeks of treatment compared to baseline values. Placebo-corrected reduction of HbA1c reached -0.71% blood pressure, and renal function remained unchanged. In linagliptin-treated group, UACR significantly decreased by 33% with a between-group difference versus placebo of -29% . Interestingly, the degree of microalbuminuria reduction did not correlate with the magnitude of change in HbA1c suggesting that the effects may have been independent of improvement in glycemic control.

In a recently published meta-analysis of 13 linagliptin trials including 5466 patients focused on composite renal outcome, the hazard ratio of 0.84 in favor of linagliptin compared to placebo or comparator was found [56]. The risk ratios for individual renal endpoints were 0.85 for microalbuminuria, 0.88 for macroalbuminuria, 0.44 for new onset of DKD, 0.77 for worsening of DKD, 0.93 for acute renal failure, and 0.77 for death, respectively.

Although none of the studies was primarily designed to test the effect of linagliptin on microalbuminuria and renal function, they collectively suggest its possible nephroprotective effects. Furthermore, two small studies with sitagliptin and the experimental studies described previously open the possibility that nephroprotection might be a class effect of DPP-4 inhibition. On the other hand, DPP-4 inhibitors have

different pharmacokinetics, and their nonglucose lowering effects may vary in man due to different concentrations in various organs and due to distinct substrate selectivity of binding to DPP-4 enzyme [57].

7. Clinical Studies of Other Glucose Lowering Agents with Albuminuria Outcomes

In general, all long-term studies with antidiabetic agents suggest that good glucose control can prevent or delay the development of microvascular complications in both type 1 and type 2 diabetes [10, 11]. In short-term studies, the specific influence of different antidiabetic medications on microalbuminuria might differ substantially. In a small 16 weeks study comparing GLP-1R agonist exenatide with glimepiride, no differences in improvement of glucose control were found but a 24-hour urinary albumin was reduced by 40% in exenatide group compared to 5% reduction in glimepiride group. Furthermore, urinary TGF- β and type IV collagen in the exenatide group were also significantly reduced compared to no change in glimepiride-treated group [58]. Another antidiabetic medication affecting microalbuminuria is thiazolidinediones that share several mechanisms of action with incretin-based therapies including amelioration of increased activation of protein kinase C pathway in glomerular mesangial cells, improvement of impaired endothelial function, inhibition of mesangial and tubular cell proliferation by suppressing TGF- β expression, anti-inflammatory actions by attenuation of interleukin-1, 6, and TNF- α in renal mesangial cells, and reduction of oxidative stress in the kidney [59, 60]. In a 52-week, open-label, cardiac safety study comparing rosiglitazone to glyburide, only the rosiglitazone group showed a significant reduction of albuminuria from baseline [59]. For patients with microalbuminuria at baseline, reductions in UACR did not correlate with reductions in HbA1c or fasting plasma glucose but showed strong correlation with changes in mean 24 h systolic and diastolic blood pressures in rosiglitazone-treated patients. Another small randomized study comparing pioglitazone versus metformin in patients with baseline albuminuria treated with RAAS blockade showed similar effects [60]. After 52 weeks of treatment, the changes in UACR from baseline were -8.3% in pioglitazone group and $+4.2\%$ in metformin group ($P = 0.01$) with similar glycemic and blood pressure changes in both arms. These results suggest that metformin does not share the albuminuria-lowering potential of thiazolidinediones and incretin-based therapies [60].

Collectively, these data suggest a possibility of specific and glucose-lowering independent effects of incretin-based therapies and thiazolidinediones on the renal damage in patients with diabetes. Nevertheless, larger trials designed primary on testing renal outcomes are necessary to confirm this interesting possibility.

8. Conclusion and Perspectives

The results of published preclinical and clinical studies suggest that DPP-4 inhibitors may have a potential to lower

albuminuria and to also possess other more complex nephro-protective properties. Possible mechanisms underlying these effects include reduction of oxidative stress and inflammation and improvement of endothelial dysfunction in the kidney. Effects of DPP-4 inhibitors may be both GLP-1 dependent and GLP-1 independent. At the moment, the data are too scarce and incomplete to make definite conclusions with respect to DPP-4 inhibitors induced nephroprotection. Ongoing studies, such as the MARLINA study comparing, prospectively, the effects of linagliptin to placebo on albuminuria may shed some light in this field [61]. Furthermore, numerous ongoing long-term cardiovascular trials with DPP-4 inhibitors can bring novel crucial information about relationships among glucose control and macrovascular and microvascular complications and further elucidate the role of albuminuria in these processes.

Abbreviations

ACEi:	Angiotensin-converting enzyme inhibitor
AGEs:	Advanced glycation end-products
ARB:	Angiotensin II receptor blocker
CKD:	Chronic kidney disease
DKD:	Diabetic kidney disease
DPP-4:	Dipeptidyl peptidase-4
eNOS:	Endothelial nitric oxide synthase
GFR:	Glomerular filtration rate
GLP-1:	Glucagon like peptide-1
HMGB1:	High mobility group protein-B1
ICAM-1:	Intercellular adhesion molecule-1
GLP-1R:	GLP-1 receptor
NF- κ B:	Nuclear factor- κ B
NO:	Nitric oxide
NPY:	Neuropeptide Y
PAI-1:	Plasminogen activator inhibitor-1
PKA:	Protein Kinase A
PKC:	Protein Kinase C
PYY:	Peptide YY
RAAS:	Renin-angiotensin-aldosterone system
RAGE:	Advances glycation endproducts receptor
TGF- β :	Transforming growth factor β
TLR-2:	Toll-like receptor 2
TLR-4:	Toll-like receptor 4
TNF- α :	Tumor necrosis factor- α
UACR:	Urinary albumin creatinine ratio
VCAM-1:	Vascular cell adhesion molecule-1
VEGF:	Vascular endothelial growth factor.

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Review Article

Exercise for Hepatic Fat Accumulation in Type 2 Diabetic Subjects

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Type 2 diabetes is characterized by frequent ectopic fat accumulation in several tissues and organs. In particular, a number of studies showed that these subjects frequently have hepatic fat accumulation, which may play a role in the metabolic abnormalities typical of diabetes and has been also linked to increased risk for cardiovascular disease. In the last decade, the effect of exercise on ectopic fat content of type 2 diabetic patients has raised growing interest. However, there are only a few small randomized controlled trials on this topic. Results from these intervention studies indicate that exercise training, independent of dietary modifications, may reduce hepatic fat content and serum transaminases in these patients, suggesting that exercise *per se* may be an effective strategy to be combined with the traditional dietary interventions. As regards the different training modalities, there is recent evidence that both aerobic and resistance exercise may equally reduce hepatic fat accumulation in type 2 diabetic subjects. However, information regarding the effect of exercise on liver histology and fat accumulation in other ectopic sites is still very limited.

1. Introduction

Type 2 diabetes is characterized by frequent ectopic fat accumulation in several tissues and organs (liver, skeletal muscle, heart, and pancreas) and unfavorable adipose tissue distribution [1]. Ectopic fat accumulation is defined by the deposition of triglycerides within cells of nonadipose tissues, which normally contain only small amounts of fat. Over the past decade, magnetic resonance (MR) spectroscopy has been used as the gold standard technique for noninvasive quantification of intramyocellular, hepatic, and more recently myocardial and pancreatic lipids. However, ectopic fat content can also be assessed by other methods, such as computed tomography and magnetic resonance imaging, which provide data on hepatic fat content that correlate very well with those obtained by the proton MR spectroscopy as well as with the histopathologic findings [2].

In particular, a number of studies have shown that subjects with type 2 diabetes frequently suffer from nonalcoholic fatty liver disease (NAFLD), a term which includes a variety of pathological conditions, from simple steatosis

to nonalcoholic steatohepatitis and cirrhosis. Available data suggest that approximately 50% to 70% of patients with type 2 diabetes have NAFLD, whereas the frequency of ectopic fat infiltration in other tissues has yet to be fully investigated. Interestingly, this phenomenon seems to have prominent clinical implications. In particular, there is evidence that NAFLD may play a role in the progression of insulin resistance and in several other metabolic abnormalities typical of diabetes, as well as in the increased risk for cardiovascular disease of these subjects [3].

The mechanisms underlying the relationships between type 2 diabetes and ectopic fat accumulation in the body are still largely unclear, and the specific approach to this phenomenon has yet to be determined. However, it is widely accepted that lifestyle changes, that is, diet and exercise, are the mainstay of treatment, as they may improve metabolic control and reduce body fat in these patients. Conversely, limited information is available on the effects of pharmacological treatments on ectopic fat infiltration.

In this paper we review the literature regarding the role of exercise on ectopic fat accumulation in subjects with type

2 diabetes. It should be noted that most studies on this issue specifically analyzed NAFLD.

2. Strategies in the Treatment of Ectopic Fat Accumulation

According to the present knowledge about pathophysiology of ectopic fat accumulation, there are several potential targets for this phenomenon [4]. Weight loss in overweight/obese patients is an obvious target and the widely accepted milestone of treatment. In a recent meta-analysis or randomized trials, it was observed that a weight loss of at least 7% is effective in improving histological disease activity, although it was achieved by less than 50% of patients [5]. Additional proposed strategies include targeting insulin resistance, hyperglycemia, dyslipidemia, oxidative stress, and inflammation. Research focusing on specific dietary components suggests that both macronutrients and micronutrients may play a role in the development of NAFLD [6]. From this point of view, there is evidence of adverse effects of fructose and favourable effects of polyunsaturated fatty acids (PUFA), possibly linked to opposite effects of these nutrients on inflammation. The role of vitamins and minerals in this field is also under investigation. Interestingly, lifestyle improvement may favourably affect all these potential mechanisms underlying ectopic fat accumulation.

Among the pharmacological options, statins showed some efficacy in the treatment of NAFLD [5]. As regards antidiabetic medications, insulin sensitizers and glucagon-like peptide-1 (GLP-1) analogs appear to be the most interesting options. However, the literature on this topic is still limited. Several studies assessed the potential efficacy on NAFLD of the insulin sensitizer metformin, which is the first-line medication in the treatment of type 2 diabetes and primarily improves hepatic insulin sensitivity. These studies consistently showed that metformin may reduce serum levels of liver enzymes, a surrogate marker of NAFLD. However, most of these studies were uncontrolled and liver histology results were inconsistent [4, 5].

The effect on NAFLD of thiazolidinediones, another class of insulin sensitizers, which are agonists of peroxisome proliferator activated gamma (PPAR-gamma) receptor, was also investigated by several studies. These drugs improve insulin action primarily at the skeletal muscle level. However, PPAR-gamma receptor is highly expressed in adipose tissue. Moreover, these medications induce a redistribution of body fat depots, making these molecules of particular interest for targeting ectopic fat accumulation. Available data from a few randomized trials support this hypothesis, showing that thiazolidinediones may improve liver histology [5]. In particular, these drugs consistently reduced hepatic steatosis and inflammation; in addition, in patients with stable stage fibrosis, they significantly reduced progression of fibrosis [5]. Nonetheless, large RCTs are needed before we can consider thiazolidinediones a specific treatment for ectopic fat accumulation.

As regards GLP-1 analogs, which belong to the incretin class and favour weight loss, there are some ongoing randomized trials. Preliminary data have shown the reduction of liver enzyme levels and hepatic steatosis after treatment with these drugs, suggesting they could be potentially useful in targeting NAFLD in diabetic patients.

3. The Role of Exercise Training in Type 2 Diabetes

Although increased physical activity has for decades been considered a first-line issue in the treatment of type 2 diabetes, the role of exercise training in these subjects has recently raised renewed and considerable interest in both clinical and scientific terms. In this regard, a recent joint position statement of the American College of Sport Medicine and the American Diabetes Association suggested that, whenever possible, both aerobic and resistance exercise training for subjects with type 2 diabetes, should be used to improve glycemic control, cardiovascular risk factors, and body composition [7]. Aerobic and resistance exercise training share some general effects but differ in their specific characteristics. Aerobic training, such as walking or cycling, involves repetitive and rhythmic contraction of large muscle groups, promoting cardiorespiratory fitness. Conversely, resistance training, such as weightlifting, typically engages relatively slow, high force contractions, promoting musculoskeletal fitness and stimulating the increase in muscle proteins and muscle cross-sectional area.

In patients with type 2 diabetes, some recent head-to-head RCTs have shown that both aerobic and resistance training may elicit similar results in terms of a number of endpoints, such as glucose control, insulin sensitivity, and body composition [8–10]. Although the results of some of these studies suggested that combination training could be more effective in these subjects than aerobic or resistance training alone [9, 10], it should be pointed out that in these trials exercise volume was higher in the combination groups, precluding a definitive answer to this crucial question.

As regards ectopic fat, regular physical activity may reduce its content through several different mechanisms, including increased hepatic and muscle fatty acid oxidation, reduced postprandial hepatic lipogenesis, and reduced fatty acid and proinflammatory molecule flow to the liver and other organs. However, to date only a few intervention studies have assessed the effect of exercise, either alone or in combination with diet, on ectopic (especially hepatic) fat content [11–16] (Table 1). Moreover, most of these studies included a combination of exercise and caloric restriction, making it difficult to assess the role of exercise *per se*.

4. Effect of Exercise Training on Liver Fat Content in Type 2 Diabetes

Aerobic exercise is usually recommended in the management of NAFLD. However, not all subjects are able to perform this type of exercise. Moreover, until now only one randomized controlled trial has assessed the effect of exercise alone on

TABLE 1: Summary of RCTs which assessed the effect of exercise training on hepatic fat content of subjects with type 2 diabetes.

Study	Patients	Design and duration	Intervention	Technique for fat assessment	BMI	Effect of intervention Body composition	Hepatic fat content
Tamura et al., (2005) [11]	14, overweight	Diet versus diet plus exercise; 2 weeks	AER exercise 50–60% $\text{VO}_{2\text{max}}$, 30 min, 5–6 times a week; low-fat diet	H-MRS	D: -0.4 kg/m^2 D + EX: -0.7 kg/m^2	Body fat D: -2.4% D + EX: -2.8%	D: -2.18% D + EX: -1.48% (absolute change)
Bonekamp et al., (2008) [12]	45, obese	Exercise versus control group; 6 months	Moderate AER and weightlifting exercise, 45 min, 3 times a week; (Diet: ?)	H-MRS	No change	Body fat EX: -1.3% SAT EX: -12 cm^2	EX: -2.5% (absolute change)
Lazo et al., (2010) [13]	96, obese	Intensive lifestyle versus standard diabetes support and education; 12 months	Moderate AER exercise, 175 min per week; moderate caloric restriction	H-MRS	-2.6 kg/m^2	Body fat: -8.8% SAT: -6.7% VAT: -12.7%	D + EX: -50.8% DSE: -22.8% (percent change)
Albu et al., (2010) [14]	58, obese	Intensive lifestyle versus standard diabetes support and education; 12 months	Moderate AER exercise, 175 min per week; moderate caloric restriction	CT scan	D + EX: -4 kg/m^2 in men -2.8 kg/m^2 in women	Body fat D + EX: -27.7% in men -14% in women	D + EX: -18% in both men and women (percent change)
Bozzetto et al., (2012) [15]	45, obese	CHO/fibers versus MUFA versus CHO/fibers + exercise versus MUFA + exercise; 8 weeks	AER exercise 70% of baseline $\text{VO}_{2\text{peak}}$, 45 min 2 times a week	H-MRS	No change	No change	MUFA: -29% MUFA + EX: -25% (percent change)
Bacchi et al., (2013) [16]	31, overweight or obese	Aerobic versus resistance training; 4 months	AER exercise 60–65% HRR 60 min, 3 times a week; RES exercise: 9 exercises, 3 series, 10 repetitions at 70–80% 1RM, 60 min, 3 times a week; habitual diet	MRI	AER: -0.70 kg/m^2 RES: -0.55 kg/m^2	Body fat AER: -1.90 kg RES: -1.76 kg VAT AER: -66.8 cm^2 RES: -38.0 cm^2 SAT AER: -175 cm^2 RES: -22.5 cm^2	AER: -32.8% RES: -25.9% (percent change)

AER: aerobic training; CHO: carbohydrate; CT: computed tomography imaging; D: diet only; D + EX: diet plus exercise; DES: standard diabetes support and education; EX: exercise; HRR: heart rate reserve; H-MRS: proton magnetic resonance spectroscopy; MRI: magnetic resonance imaging; RES: resistance training; 1RM: one repetition maximum; SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; MUFA: multi-unsaturated fatty acid diet.

hepatic fat content in subjects with type 2 diabetes [16], while four RCTs have compared the effect of combined interventions with both aerobic exercise and hypocaloric diet versus diet alone or standard diabetes support and education programs [11, 13–15].

We have recently randomized 31 type 2 diabetic patients with NAFLD to regular training with either aerobic or resistance exercise. Interestingly, after 4 months hepatic fat content was markedly reduced, to a similar extent, in both groups, showing that these exercise modalities are equally effective in type 2 diabetic patients with NAFLD [16]. It is noteworthy that hepatic steatosis disappeared in about 25% of these diabetic subjects. This reduction in hepatic fat accumulation was accompanied by concurrent, mild but significant improvements in both anthropometric [BMI, total and truncal body fat, abdominal visceral (VAT), and subcutaneous (SAT) adipose tissue] and metabolic features (insulin sensitivity, HbA1c, triglycerides). These results were independent of the effect of diet, as participants were instructed to maintain their baseline calorie intake by consuming healthy self-selected foods, and compliance to dietary recommendations was confirmed by a stable body weight in the pre-intervention period and the results of the food recall at the end of intervention.

Conversely, Bonekamp et al. in a sample of 45 subjects with type 2 diabetes have assessed the effect of combined, moderate intensity training with both aerobic and resistance exercise, without any associated caloric restriction [12]. Their preliminary results suggested that combined exercise training may reduce hepatic fat content, independently of any changes in body composition and metabolic features.

In agreement with these findings, a one-month aerobic training intervention, carried out in a sample of 19 non-diabetic obese individuals, reported a 21% reduction of hepatic fat content, in the absence of any significant body weight reduction [17]. Similarly, Hallsworth et al. [18] have recently reported that in 18 subjects with NAFLD, some of them with type 2 diabetes, there was a 13% reduction of hepatic fat content after 8 weeks of resistance exercise, without any changes in body weight.

Other studies reported that, in the short term, hepatic fat content is similarly reduced after diet only or exercise *plus* diet interventions [11, 13–15]. Tamura et al. [11], in 14 type 2 diabetic patients, showed a similar decrease of hepatic fat content, with a reduction of body mass index, after two weeks of diet only (60% carbohydrate, 25% fat, 15% protein, and mean total energy intake of 27.9 kcal/kg ideal body weight) or diet *plus* exercise (30 minutes of moderate intensity aerobic exercise, 5–6 times a week). More recently, in 45 obese subjects with type 2 diabetes, Bozzetto et al. [15] compared the effect of high-carbohydrate/high fiber/low glycemic index diet or multi-unsaturated fatty acid (MUFA) diet, with or without a concurrent moderate intensity aerobic exercise program of 45 minutes 2 times a week. After 8 weeks, the authors reported that MUFA intervention, as compared with the control diet, was associated with a significant and clinically relevant reduction of hepatic fat content, without reduction in body weight. These findings were independent of the associated training program, as the reduction of liver fat

content was similar in both the MUFA diet alone and the MUFA diet *plus* exercise protocols. Nonetheless, it could be hypothesized that the duration of the interventions was too short to detect differences in changes of hepatic fat content between the exercise *plus* diet group and the diet only group. Even more important, these authors reported a negligible improvement of only 1 mL/kg/min of peak oxygen uptake in the MUFA *plus* exercise group compared with the MUFA only group. Thus, it can be speculated that training volume and/or intensity were not sufficiently adequate to improve cardiorespiratory fitness of these subjects.

Two studies [13, 14] carried out in the cohort of the Look AHEAD Study—a multicenter prospective study comparing intensive lifestyle intervention, including caloric restriction and at least 175 min of moderate aerobic physical activity per week, versus standard care in diabetic subjects—reported that after 1 year of intervention there was a significant decrease in hepatic fat content and a reduced incidence of NAFLD in patients randomized to the intensive lifestyle group. In particular, the median percent decrease in steatosis was 50.8% in the intervention lifestyle group versus 22.8% in the control group [13]. In addition, 3% of participants in the intervention group versus 26% of those in the control group, who were without NAFLD at baseline, developed NAFLD during the study. Interestingly, similar changes in hepatic fat content were found in both men and women [14].

Overall, these data support the recommendation for weight loss using lifestyle changes as the first step in patients with NAFLD, including those with type 2 diabetes. In addition, they suggest that either aerobic or resistance exercise *per se* is effective in reducing hepatic fat content. Unfortunately, due to the limited information available, it remains unclear what amount/volume and what intensity of exercise is optimal in targeting a reduction of hepatic fat content.

5. Summary

In the last decade, the effect of exercise on ectopic, especially hepatic, fat content of type 2 diabetic patients has raised growing interest. However, there are still only a few small RCTs on this topic. Results from these intervention studies are promising, as they indicate that exercise training, independent of dietary modifications, can reduce hepatic fat content in these patients, suggesting that exercise *per se* may be an effective strategy to be combined with the traditional low calorie diet interventions.

The extent of improvement induced by exercise training in hepatic fat content appears to be quite different in these studies. These differences might be explained by differences in exercise volume and intensity, as well as in the duration of the interventions, although they can also be linked to the different techniques used for the measurement of fat content.

Future research should further address the effects of both aerobic and resistance exercise, alone or in combination, on ectopic fat accumulation of these subjects, by focusing on the differences between training protocols in terms of exercise frequency, duration, and intensity, in order to establish which

training model is more effective in reducing ectopic fat accumulation and thereby counteracting the multiple adverse effects of this phenomenon.

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Research Article

Association between Sleep Disruption and Levels of Lipids in Caucasians with Type 2 Diabetes

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Aim. To investigate the association between sleep quality and duration with lipid and glycaemic control in Caucasian subjects with type 2 diabetes. **Methods.** Sleep quality was assessed using the Pittsburgh Sleep Quality Index (PSQI) in 114 type 2 diabetes (T2DM) subjects. Comparisons were made between subjects with different sleep quality and sleep duration. Hierarchical multiple regression analyses were used to determine contributors to metabolic parameters. **Results.** Subjects with poor sleep quality (PQ; $PSQI \geq 6$) had higher systolic blood pressure, glycated haemoglobin, urine albumin : creatinine ratio (UAC), total cholesterol (TC), and triglycerides (TG) ($P < 0.05$ for all) compared to those with good sleep quality (GQ; $PSQI \leq 5$). Long sleep duration (LSD) subjects had higher TC and short sleep duration (SSD) subjects had higher TG compared to those with medium sleep duration. Sleep duration and PSQI score were independent predictors of TC and low-density lipoprotein cholesterol (LDL), contributing to 14.0% and 6.1% of the total variance, respectively. **Conclusions.** In this Caucasian T2DM population, PQ is associated with adverse cardiovascular risk markers, and long and short sleep disruptions have an independent negative impact on lipids. Sleep assessment should be included as part of a diabetes clinic review.

1. Introduction

In the latest NHANES survey [1], the estimated 10-year UKPDS risk for developing coronary heart disease among people with diabetes was 22% lower in the 2007-2008 survey compared to the 1999-2000 surveys. This decrease has been attributed to pharmacological interventions such as statins, which have become the cornerstone of treatment in patients with hyperlipidaemia. However, statins only reduce the risk of cardiovascular events by 30–40% [2] leaving a considerable residual cardiovascular risk in T2DM patients effectively treated to targets. Continuing efforts are needed to improve this residual risk by identifying any additional risk factors and improvement of the treatment modalities.

In 2008, The US National Sleep Foundation Report revealed that sleep duration on a typical workday averaged 6 hours and 40 minutes [3] and that this was significantly less than the 8.0–8.9 hours reported in a 1960 survey. This change

has coincided with a dramatic increase in the prevalence of obesity, metabolic diseases, and increased cardiovascular mortality although no conclusion can be made regarding cause and effect.

Studies in sleep and mortality date back to the late 1960s when a “U” shaped relationship between short and long sleep durations and mortality was shown [4, 5]. This was further confirmed in subsequent large-scale studies [6, 7]. In the last decade, “U” shaped associations have been demonstrated between sleep duration and risk for diabetes mellitus [8], obesity [9], hypertension [10], coronary heart disease (CHD) [11], and atherosclerosis [12].

Specifically in T2DM subjects, a cross-sectional study in 935 women demonstrated lower high-density lipoprotein (HDL) level in normotensive subjects with short and long sleep durations compared to mid-range sleep duration [13]. A cross-sectional study in African Americans with T2DM revealed that sleep duration and sleep quality were significant

predictors of glycated haemoglobin (HbA_{1c}) after controlling for age, sex, BMI, insulin use, and the presence of major complications [14]. The latter study did not include data on lipid parameters, and both studies involved a homogeneous population.

Based on the evidence above, we hypothesize that sleep disruption is a potential contributor to cardiovascular disease (CVD) risk through worsening of metabolic markers and glycaemic control in male and female Caucasians with T2DM. We performed a cross-sectional study to assess associations between self-reported sleep quality, sleep duration, diabetes control, and metabolic parameters in a Caucasian diabetes cohort.

2. Patients and Methods

All patients with T2DM attending the diabetes clinic and diabetes day centre were eligible for the study. Inclusion criteria for the study included T2DM diagnosis based on the American Diabetes Association (ADA) criteria [15]: Caucasian in origin and able to give consent. Exclusion criteria included having other types of diabetes and inability to give consent to the study. Patients were recruited consecutively within 6-month period as they visited the centre for their diabetes review. This study was approved by the Connolly Hospital Ethics Committee. In total, 134 T2DM patients attending for annual clinic review of diabetes agreed to take part in the study.

We performed a clinical history and recorded anthropometric data including blood pressure (BP), weight, and BMI in all patients attending our diabetes clinic. Patients were approached about the study while waiting for their turn for review. Patients who agreed to participate were brought into an interview room, where details of the study were explained, consent form was signed, and a questionnaire was filled in. At the same time, medical notes were carefully reviewed to identify any history of hypertension, hyperlipidaemia, and all current antidiabetic, lipid-lowering and antihypertensive medications were recorded. HbA_{1c}, fasting glucose, fasting lipid profile, renal profile, and urine albumin: creatinine ratio (UAC) performed in our local laboratory prior to the clinic visit were also recorded.

All patients were asked to fill a questionnaire to assess their sleep quality. The Pittsburgh Sleep Quality Index (PSQI) was used. This is a validated 19-item questionnaire covering 7 components of sleep that produces a global sleep quality score that ranges from 0 to 21 [16]. A global score greater or equal to 6 distinguishes poor sleepers from good sleepers [17, 18]. As part of the PSQI questionnaire, patients were asked if they had trouble sleeping during the past month due to pain. Patients who responded positively to the question (less than once a week, once or twice weekly, or three times weekly) were identified as possibly having pain-disturbed sleep and were excluded from the analysis as pain is a known confounder [14]. Patients with GQ (PSQI score ≤ 5) were compared with patients with PQ (PSQI ≥ 6). We extracted sleep duration from the PSQI questionnaire and categorized patients into 3 groups based on their sleep duration within the last one

month: short (SSD, <6 hours daily) medium (MSD, 6–8 hours daily), and long sleep durations (LSD, >8 hours daily) and comparisons between the three groups were made.

All statistical analyses were carried out using SPSS 18.0 (SPSS Inc. Chicago, IL, USA). Continuous data are expressed as mean \pm standard deviation (SD) for parametric data or median and interquartile (IQ) range for nonparametric data. Normally distributed means were compared using Student's independent *t*-test, and nonnormally distributed medians were compared using the Mann-Whitney test. Analysis of variance (ANOVA) was used to compare parametric means across 3 groups of differing sleep duration. The Kruskal-Wallis test was used when comparing nonparametric data across 3 groups with Bonferroni adjustment for significance. Categorical data were compared using the chi square test.

For correlation and regression analyses, any nonnormally distributed variables were transformed logarithmically. Pearson's correlation was used to examine the relationship between PSQI score and sleep quality status and variables of interest. Standard multiple linear regression analysis was used to examine the crude associations between sleep duration (hours) and sleep quality (PSQI score) to glucose, HbA_{1c}, lipid parameters, and blood pressure (Model 1). Using hierarchical multiple regression, we repeated the analysis adjusted for age, sex, BMI, and lipid lowering treatment (Model 2). Results are presented with part correlation and β value, with a significance value of <0.05 . We used *R* square change value to determine to what extent the variable is explained by both sleep duration and sleep quality. We squared the part correlation to determine the individual contribution of sleep duration and sleep quality to other variables.

3. Results

Of the 134 patients who completed the study, 20 patients reported pain-disturbed sleep and were excluded as pain is a known confounder for disturbed sleep [14]. Therefore, 114 patients were included for analysis. All patients were Caucasians and 52 (45.6%) were females. A nightly average of nine hours of sleep within the last one month was reported in 7% of patients, 8 hours in 16.7%, 7 hours in 31.6%, 6 hours in 25.4%, 5 hours in 7.9%, 4 hours in 6.1%, and 3 hours in 5.3%, respectively. Overall, 51 patients (44.7%) had a PSQI score ≥ 6 , indicative of poor sleep quality.

Table 1 demonstrates the comparison of baseline characteristics in patients with PQ and GQ. Patients with PQ were more likely to be females ($P = 0.002$) (Table 1). The PQ group was more likely to have higher systolic blood pressure (SBP; 147.16 ± 17.67 versus 137.30 ± 18.75 mm Hg, $P = 0.005$), HbA_{1c} (50.8 (44.3–59.6) versus 43.2 (36.6–55.2) mmol/mol, $P = 0.026$), UAC (18.02 (8.19–44.27) versus 8.11 (3.12–33.1) mg/mmol, $P = 0.03$), total cholesterol (TC; 4.87 ± 0.87 versus 4.50 ± 0.87 mmol/L, $P = 0.025$), and TG (1.69 (1.26–2.44) versus 1.38 (0.97–2.07) mmol/L, $P = 0.026$). There are no significant differences between the use of insulin or oral antidiabetic medications in patients with PQ compared to GQ.

Table 2 demonstrates the comparison of characteristics in patients with SSD, MSD, and LSD. The LSD group had

TABLE 1: Baseline characteristics based on sleep quality.

	GQ (<i>n</i> = 62)	PQ (<i>n</i> = 52)	Sig
Female	20 (31.7)	32 (62.7)	0.002
Antihypertensive	45 (71.4)	43 (84.3)	0.16
Lipid lowering	30 (47.6)	22 (43.1)	0.773
Oral antidiabetic	41 (66.1)	34 (65.4)	1.0
Insulin	14 (22.6)	8 (15.4)	0.522
Diet controlled	13 (21)	8 (15.4)	0.664
Age (years)	64.0 ± 11.32	66.0 ± 10.98	0.339
Weight (kg)	86.63 ± 21.65	83.25 ± 16.26	0.358
BMI (kg/m ²)	30.59 ± 7.3	30.84 ± 4.36	0.835
SBP (mmHg)	137.30 ± 18.75	147.16 ± 17.67	0.005
DBP (mmHg)	76.43 ± 12.26	80.04 ± 10.35	0.097
Glucose (mmol/L)	7.2 (5.9–8.5)	7.3 (6.7–9.4)	0.169
HbA _{1c}			
(%)	6.1 (5.5–7.2)	6.8 (6.2–7.6)	0.026
mmol/mol	43.2 (36.6–55.2)	50.8 (44.3–59.6)	
UAC (mg/mmol)	8.11 (3.12–33.1)	18.02 (8.19–44.27)	0.03
Total cholesterol (mmol/L)	4.50 ± 0.87	4.87 ± 0.87	0.025
TG (mmol/L)	1.38 (0.97–2.07)	1.69 (1.26–2.44)	0.026
HDL (mmol/L)	1.21 ± 0.29	1.28 ± 0.34	0.249
LDL (mmol/L)	2.51 ± 0.62	2.73 ± 0.72	0.097
Sleep duration (h)	7.25 ± 0.95	5.57 ± 1.49	<0.001

SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; HbA_{1c}: glycated haemoglobin; UAC: urinary albumin:creatinine ratio; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein. Genders, treatment with antihypertensive, lipid-lowering agents, oral antidiabetic medications, insulin, and diet controlled are expressed in *n* (%).

higher TC compared to the MSD (5.5 ± 1.18 versus 4.55 ± 0.79 mmol/L, $P = 0.009$) while SSD group had a higher TG level compared to the MSD group (2.0 (1.44 – 3.02) versus 1.43 (1.0 – 2.08) mmol/L, $P = 0.013$, Table 2).

PSQI score correlated positively with SBP ($r = 0.187$, $P = 0.047$), TC ($r = 0.212$, $P = 0.024$), and female gender ($r = 0.249$, $P = 0.007$) and negatively with sleep duration ($r = -0.802$, $P < 0.001$). Poor sleep quality status correlated positively with female gender ($r = 0.309$, $P = 0.001$), SBP ($r = 0.261$, $P = 0.005$), Log HbA_{1c} ($r = 0.191$, $P = 0.042$), TC ($r = 0.21$, $P = 0.025$), and Log triglycerides (Log TG; $r = 0.198$, $P = 0.035$) and negatively with sleep duration ($r = -0.57$, $P < 0.001$).

The results of multiple regression analyses examining the association between sleep measures (sleep duration and PSQI score) and glucose, HbA_{1c}, lipid profiles, and blood pressure are presented in Table 3. Longer sleep duration and higher PSQI score were associated with higher TC in both the unadjusted and adjusted models contributing to 14% of the variance (R square change = 0.14, $P = 0.001$). Individually, in the adjusted model, sleep duration made a significant contribution of 10.4% (part correlation = 0.322) and PSQI

score contributed 13.8% (part correlation = 0.372) of the variance of TC.

In the unadjusted model, longer sleep duration was associated with higher HDL and higher PSQI score was associated with higher SBP, but these associations were no longer statistically significant in the fully adjusted model (i.e., model two). Longer sleep duration showed a trend towards correlation with higher LDL while higher PSQI score correlated with higher LDL in model one. The combination of sleep duration and PSQI score, however, was associated with higher LDL in the fully adjusted model explaining 6.1% of the variance (R square change = 0.061, $P = 0.03$). Individually, in the adjusted model, sleep duration contributed 5.1% (part correlation = 0.226) and PSQI score contributed 5.9% (part correlation = 0.242) of the variance of LDL cholesterol.

In this analysis, there were no other significant associations between sleep measures and glucose, HbA_{1c}, TG, level and DBP.

4. Discussion

Our results have shown that sleep disruption has a potentially detrimental effect on clinical and biochemical risk markers of cardiovascular disease (CVD) in Caucasian people with T2DM. Subjects with poorer quality sleep were more likely to be females, have shorter sleep duration, higher HbA_{1c}, SBP, total cholesterol, TG, and UAC. Subjects with short sleep duration had higher TG levels and those with long sleep duration had high TC levels compared to subjects who slept between 6 and 8 hours daily. However, adjusting for age, sex, BMI, and lipid-lowering treatment, we found significant associations between longer sleep duration and poorer sleep quality and TC and LDL. There was no significant difference in HbA_{1c} based on sleep duration, and there was no association between PSQI score or sleep duration with HbA_{1c} in multiple regression analyses. Our study is the first study to illustrate the independent association between PSQI score and sleep duration and TC and LDL cholesterol in Caucasians with diabetes.

There have only been two other studies investigating lipid parameters and sleep quality or quantity in subjects with T2DM. The Nurse's Health Study involving 935 T2DM women showed that HDL was lower in normotensive women with SSD and LSD, and frequent snoring was associated with higher TG and inversely related to HDL and adiponectin levels [13]. Whilst our study did not show any difference in HDL between groups of different sleep duration, we showed that TG was higher in SSD and nonsignificantly increased in the LSD group compared to MSD. We also showed that TC was higher in LSD compared to MSD group. The only other study in T2DM assessing sleep and metabolic risk factors was the Sleep AHEAD Study [19]. Ten sleep parameters were analyzed with six dependent metabolic variables. Apart from a weak association of sleep duration and HbA_{1c}, there were no associations between other sleep parameters including sleep duration and TC, HDL, LDL, and TG. Whilst our study did not show any association between sleep measurements and HbA_{1c}, we showed that

TABLE 2: Characteristics based on sleep duration.

	SSD (<6 hours, <i>n</i> = 22)	MSD (6–8 hours, <i>n</i> = 84)	LSD (>8 hours, <i>n</i> = 8)	Sig
Age (years)	64.61 ± 11.06	65.13 ± 10.82	63.17 ± 15.94	0.887
Weight (kg)	85.45 ± 18.04	85.56 ± 19.96	79.51 ± 18.66	0.702
BMI (kg/m ²)	30.67 ± 4.6	30.91 ± 6.59	28.6 ± 4.85	0.6
SBP (mmHg)	141.14 ± 21.38	142.02 ± 18.39	140 ± 18.71	0.948
DBP (mmHg)	77.68 ± 12.05	78.37 ± 11.47	75.63 ± 12.08	0.805
Glucose (mmol/L)	7.3 (6.55–9.25)	7.2 (6.1–8.57)	8.45 (7.0–9.2)	0.304
HbA _{1c}				
(%)	6.9 (6.2–7.63)	6.3 (5.6–7.18)	6.18 (5.48–8.95)	0.162
mmol/mol	51.9 (44.3–59.9)	45.4 (37.7–55.0)	44.0 (36.4–74.3)	
UAC (mg/mmol)	16.6 (8.85–38.88)	14.41 (4.1–38.27)	5.5 (2.36–9.19)	0.109
Total cholesterol (mmol/L)	4.81 ± 1.01	4.55 ± 0.79*	5.5 ± 1.18*	0.009
TG (mmol/L)	2.0 (1.44–3.02)**	1.43 (1.0–2.08)**	1.94 (1.45–2.28)	0.013
HDL (mmol/L)	1.16 ± 0.28	1.25 ± 0.32	1.35 ± 0.31	0.312
LDL (mmol/L)	2.5 ± 0.76	2.61 ± 0.64	2.86 ± 0.8	0.439

* Indicates difference between groups ($P < 0.05$). ** Indicates difference between groups ($P < 0.017$ after Bonferonni adjustment).

sleep duration and sleep quality significantly contributes to TC and LDL cholesterol. Compared to our study, the Sleep AHEAD Study had significant methodological differences such as including subjects from a heterogeneous ethnicity, having short to normal sleep duration (mean 5.96 ± 1.21 hours/night) only, and the use of home polysomnography which has its own limitations.

A number of other studies examining the association between sleep duration and lipid metabolism were done in nondiabetic subjects. The Hordaland Health Study, a population-based cross-sectional study in 8860 subjects without T2DM, concluded that SSD was associated with higher BMI, TC, TG, SBP, and DBP [20]. In another study involving 3995 Japanese nondiabetic subjects, women with SSD and LSD were more likely to have higher TG and lower HDL levels compared to those with MSD [21].

Our data have shown that compared to MSD, TC was higher in those with LSD and TG were higher in the SSD subjects. Despite the differences in patient sample and methods, our results in diabetic patients largely support the notion that long and shorter than normal sleep durations are associated with lipid abnormalities. This is significant in light of the accumulating evidence that there is a “U” shaped association between sleep duration and the risk of diabetes, obesity, hypertension, and coronary heart disease. Our results also lend further support to this emerging theory by showing independent contributions between sleep duration and sleep quality and TC and LDL.

Based on experimental and clinical data, a number of mechanisms have been suggested linking the relationship between sleep disruption and lipid metabolism. Reduced leptin, reduced insulin sensitivity, increased sympathetic nervous system activation, and increased cortisol production have all been proposed as explanations [22–24]. Further studies are needed to clearly demonstrate the pathophysiology of these findings especially in the area of adipocyte function and regulation [22].

Our results have demonstrated that subjects with PQ had higher HbA_{1c} compared to GQ. However, there was no significant difference in HbA_{1c} in groups with different sleep duration, and there were no associations between PSQI score or sleep duration and HbA_{1c} in the regression analyses. This is in contrast to a previous study in an African American type 2 diabetes cohort, whereby higher perceived sleep debt (the difference between a patient's preferred sleep duration and reported sleep duration) or lower sleep quality (modified PSQI score) was associated with poorer glycaemic control after controlling for confounding factors [14]. However, differences in the method used and ethnicity of the subjects may explain the different findings compared to our study. Firstly, by subtracting sleep duration from the PSQI questionnaire, a modified PSQI score was used. Secondly, it has been suggested that African Americans take longer to fall asleep, report poorer sleep quality, less deep sleep, nap more often, and have higher prevalence of sleep-disordered breathing compared to Caucasians [25]. The findings of the current study are in agreement with a more recent study involving patients with and without diabetes, whereby habitual sleep duration assessed by wrist actigraphy was not found to be associated with markers of glucose metabolism in normal and diabetic subjects [26]. In the same study, insomnia and snoring contribute to poor sleep quality, and these factors have been shown to predict hyperglycemia and insulin resistance in type 2 diabetes.

Our study has some limitations that need to be considered. Firstly, as this is a cross-sectional study, determination of causality cannot be established. Secondly, our sample size was small. The largest study ($n = 935$) that examined the effect of sleep on lipid metabolism in T2DM subject was the Nurses' Health Study. However, this study included only female subjects and only includes sleep duration but not sleep quality. The Sleep AHEAD Study was also larger ($n = 305$). However, as described above, the subject cohorts were different. Thirdly, we do not have any data on smoking,

TABLE 3: Standard and hierarchical multiple regression analyses predicting the natural log glucose and log HbA_{1c} (Table 3(a)), SBP and DBP (Table 3(b)), total cholesterol and LDL (Table 3(c)), and Log TG and HDL (Table 3(d)), from sleep duration and sleep quality.

(a)					
Outcome: Log glucose			Outcome: Log HbA _{1c}		
	Model 1 (unadjusted)	Model 2 (adjusted)*		Model 1 (unadjusted)	Model 2 (adjusted)*
Sleep duration (h)			Sleep duration (h)		
β	0.184	0.21	β	0.038	-0.024
Part correlation	0.11	0.13	Part correlation	0.022	-0.014
<i>P</i>	0.25	0.19	<i>P</i>	0.813	0.886
Sleep quality (PSQI score)			Sleep quality (PSQI score)		
β	0.187	0.24	β	0.107	0.038
Part correlation	0.11	0.14	Part correlation	0.064	0.021
<i>P</i>	0.24	0.16	<i>P</i>	0.499	0.826
(b)					
Outcome: SBP			Outcome: DBP		
	Model 1 (unadjusted)	Model 2 (adjusted)*		Model 1 (unadjusted)	Model 2 (adjusted)*
Sleep duration (h)			Sleep duration (h)		
β	0.167	0.076	β	0.084	0.051
Part correlation	0.1	0.043	Part correlation	0.05	0.029
<i>P</i>	0.284	0.64	<i>P</i>	0.593	0.759
Sleep quality (PSQI score)			Sleep quality (PSQI score)		
β	0.32	0.194	β	0.187	0.126
Part correlation	0.92	0.107	Part correlation	0.112	0.07
<i>P</i>	0.041	0.249	<i>P</i>	0.237	0.465
(c)					
Outcome: total cholesterol			Outcome: LDL		
	Model 1 (unadjusted)	Model 2 (adjusted)*		Model 1 (unadjusted)	Model 2 (adjusted)*
Sleep duration (h)			Sleep duration (h)		
β	0.506	0.561	β	0.294	0.393
Part correlation	0.302	0.322	Part correlation	0.176	0.226
<i>P</i>	0.001	0.001	<i>P</i>	0.065	0.016
Sleep quality (PSQI score)			Sleep quality (PSQI score)		
β	0.617	0.675	β	0.334	0.438
Part correlation	0.369	0.372	Part correlation	0.2	0.242
<i>P</i>	0.001	0.002	<i>P</i>	0.036	0.01
(d)					
Outcome: HDL			Outcome: Log TG		
	Model 1 (unadjusted)	Model 2 (adjusted)*		Model 1 (unadjusted)	Model 2 (adjusted)*
Sleep duration (h)			Sleep duration (h)		
β	0.34	0.304	β	0.142	0.122
Part correlation	0.203	0.174	Part correlation	0.085	0.07
<i>P</i>	0.032	0.06	<i>P</i>	0.364	0.453
Sleep quality (PSQI score)			Sleep quality (PSQI score)		
β	0.285	0.214	β	0.285	0.279
Part correlation	0.17	0.118	Part correlation	0.17	0.154
<i>P</i>	0.072	0.201	<i>P</i>	0.07	0.101

Bold face type indicates statistically significant results. * Model 2 is adjusted for age, sex, BMI, and treatment with lipid-lowering agent.

alcohol consumption, and physical activity/inactivity. In particular, physical activity is associated with improvement in weight and cholesterol levels [27]. Although we did not have data on self-reported activity levels, since the weight in our patients was similar in both GQ and PQ groups as well as across the three groups of different sleep duration, we do not think that this is a significant confounder. We also do not have any data on other comorbidities, depression and the use of antidepressant. In The Netherlands Study of Depression and Anxiety (NESDA) [28], lower HDL and higher TG levels were demonstrated in subjects with major depressive disorder compared to control and subjects in remission from depression. However, this association was lost after adjustment suggesting that the unfavourable lipid pattern was mainly secondary to lifestyle factors. Fourthly, we measured self-reported sleep habits using a well-validated sleep questionnaire. While some studies have reported that self-reporting of sleep habits is a reliable tool in predicting the risk of developing T2DM [29] and is considered reproducible, it has also been shown to be only moderately correlated with objectively measured sleep duration using wrist actigraphy. It has been suggested that self-reported sleep may be biased by a systematic overreporting of an average of 34 minutes for each additional hour of measured sleep [30]. Lastly, we do not have data on possible sleep-disordered breathing such as obstructive sleep apnoea (OSA), which is prevalent in patients with T2DM and is associated with poorer glycaemic control [31]. Severity of OSA has been shown to have an independent contribution to poor glycaemic control in T2DM, and studies of the effect of treating OSA on glycaemic control in T2DM have been performed. However, the results were inconsistent largely due to differences in design and duration of therapy [32].

Despite these limitations, to the best of our knowledge, our study is the first study to illustrate the independent association between PSQI score and sleep duration and TC and LDL cholesterol in Caucasians with diabetes. We included both genders and we have used a well-validated questionnaire to assess sleep quality and duration.

In summary, in a Caucasian diabetic population, sleep disruption is associated with an unfavourable lipid pattern and could be an additional unrecognized risk factor for macrovascular complications in diabetes. We recommend that subjective or objective sleep assessment should be considered as part of the overall management of patients with T2DM.

Abbreviations

DBP:	Diastolic blood pressure
GQ:	Good sleep quality
HbA _{1c} :	Glycated haemoglobin
HDL:	HDL cholesterol
LDL:	LDL cholesterol
LSD:	Long sleep duration
MSD:	Medium sleep duration
PSQI:	Pittsburgh Sleep Quality Index
PQ:	Poor sleep quality
SSD:	Short sleep duration

SBP:	Systolic blood pressure
TC:	Total cholesterol
TG:	Triglycerides
T2DM:	Type 2 diabetes
UAC:	Urine albumin: creatinine ratio.

Conflict of Interests

The authors have no conflict of interests to declare.

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Wan Aizad Wan Mahmood researched data, analyzed data, and wrote the paper. Mohd Shazli Draman Yusoff analyzed data and reviewed the paper. Andrea Di Perna and Lucy Ann Behan researched data. Tommy Kyaw Tun contributed to discussion and reviewed/edited the paper. John McDermott and Seamus Sreenan reviewed/edited the paper. Seamus Sreenan is the guarantor for the content of the paper.

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Clinical Study

Past Obesity as well as Present Body Weight Status Is a Risk Factor for Diabetic Nephropathy

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Aims. We analyzed the prevalence of nephropathy according to past body weight status in Japanese subjects with type 2 diabetes because the influence of past obesity on diabetic complications is not certain. **Methods.** We examined the prevalence of nephropathy in 2927 subjects with type 2 diabetes mellitus according to current BMI and maximum BMI in the past. We defined “current obesity” as BMI on hospitalization of 25 or more, “previous obesity” as BMI on hospitalization of less than 25 and self-reported maximum BMI in the past of 25 or more, and “continuously lean” as maximum BMI of less than 25. **Results.** The prevalence of nephropathy was significantly higher in subjects with current obesity (40.6%) or previous obesity (35.6%) than in those who were continuously lean (24.3%) ($P < 0.017$). In logistic regression analysis, previous obesity, as well as current obesity, was a significant risk factor for nephropathy, independent of sex, age, disease duration, hypertension, dyslipidemia, HbA1c, and diabetic retinopathy. **Conclusions.** Obesity in the past, as well as the present body weight status, was a risk factor for diabetic nephropathy.

1. Introduction

The majority of Japanese patients with type 2 diabetes mellitus are not obese, as reported by Kosaka and Ogata more than 50 years ago [1]. This is a well-known fact about Japanese type 2 diabetes mellitus [2]. Eastern Asian subjects might share common characteristics of the disease, comparable to those reported in Korean people [3]. It is, therefore, debatable whether we can apply epidemiological evidence obtained from studies of Caucasian subjects with type 2 diabetes and obesity to eastern Asian people, especially in the fields of diabetic complications, which are influenced not only by hyperglycemia but also by obesity. For example, type 2 diabetes is a relative risk factor for cardiovascular disease in Asians, as in Western societies, but the absolute risk differs greatly between these populations [4–7].

Recently, the “legacy effect” of intensive glycemic control early after the diagnosis of diabetes was advocated based on the UKPDS follow-up study [8]. However, there is no report about the legacy effect of past obesity over a lifetime on diabetic complications. Although there have been inconsistent results as to whether obesity is a risk for diabetic nephropathy [9–12], it was reported that current obesity and maximum past body mass index (BMI) were significant risk factors for diabetic nephropathy in the Japanese [12]. Although Caucasian subjects with type 2 diabetes usually maintain their body weight status during their disease course, the majority of patients of eastern Asian ethnicity begin to lose body weight from around the time of diagnosis of diabetes [3, 13]. This was easily overlooked, even if the effect of obesity in the past persisted for a long time, because they were already nonobese at the start of clinical follow-up. To clarify the influence of

past obesity on diabetic complications is an important clinical concern in patients of eastern Asian ethnicity.

We therefore analyzed the difference in the prevalence of diabetic nephropathy in Japanese type 2 diabetics according to their history of body weight status to clarify the effect of past obesity on diabetic nephropathy.

2. Materials and Methods

We examined subjects with type 2 diabetes whose estimated glomerular filtration rate (eGFR) was 30 mL/min/1.73 m² or more and who were admitted to Saiseikai Central Hospital from January 1999 to December 2004 ($n = 1834$) or Keio University Hospital from April 1998 to September 2010 ($n = 1093$) for the management of metabolic control. The study protocol was reviewed and approved by the ethics committee of both hospitals. Those subjects in whom the etiology of renal disease was strongly suspected to be other than diabetic nephropathy were excluded. According to the history of body weight status and the Japanese criteria for obesity [14], we defined “current obesity” as BMI on hospitalization of 25 or more, “previous obesity” as BMI on hospitalization of less than 25 and self-reported maximum BMI in the past of 25 or more, and “continuously lean” as maximum BMI of less than 25.

HbA1c level on admission was determined by high-performance liquid chromatography (HPLC: Arkray Inc., Kyoto, Japan) according to the recommended method by the Japan Diabetes Society (JDS) at that time and converted to the National Glycohemoglobin Standardization Program (NGSP) value [15]. eGFR (mL/min/1.73 m²) was calculated as $194 \times \text{Cr}^{-1.094} \times \text{Age}^{-0.287}$ (with further multiplication by 0.739 for female subjects) using the equation provided by the Japanese Society of Nephrology [16].

Subjects with albumin excretion rate (AER) of 20 µg/min or more in 24-hour urine were considered to have diabetic nephropathy. All subjects underwent funduscopy examination by trained ophthalmologists during or just before admission. The diagnosis of diabetic retinopathy was made based on the Davis classification [17]. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or the prescription of antihypertensive medication. Dyslipidemia was defined as LDL cholesterol >3.63 mmol/L, triglyceride >1.72 mmol/L, HDL cholesterol <1.04 mmol/L, or the prescription of lipid-lowering medication.

Continuous variables are expressed as mean ± SD. Differences in baseline characteristics among the obesity categories were analyzed by ANOVA and chi-squared test. Chi-squared test was also performed to evaluate differences in prevalence, with Bonferroni's correction for post hoc multiple comparisons. As a result, the probability equivalent to the usual $P = 0.05$ was $P = 0.017$. Logistic regression analysis with forced entry method was performed to detect significant independent predictors of diabetic nephropathy. We adopted as covariates factors such as sex, age, disease duration, hypertension, dyslipidemia, HbA1c, and obesity status (current obesity, past obesity, and continuously lean) in this study. Obesity status was converted to two dichotomous variables

with dummy coding. $P < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS software ver. 18.0 (SPSS Inc., an IBM company, Japan).

3. Results

We analyzed a total of 2927 persons (males 2038, females 889, age 59.3 ± 10.6 years, BMI 24.0 ± 4.0 , duration of diabetes 10.4 ± 28.0 years, HbA1c $9.3 \pm 2.8\%$). The subjects' characteristics are shown in Table 1. The prevalence of current obesity in the total subjects was 33.6%, previous obesity 41.5%, and a continuous lean state 24.8%. The prevalence of nephropathy was significantly different among the categories, and both currently obese (40.6%) and previously obese (35.6%) patients had a significantly higher prevalence of diabetic nephropathy than that in continuously lean patients (24.3%) ($P < 0.017$).

When we divided the patients into quartiles according to current BMI, the prevalence of nephropathy significantly increased as current BMI increased (Figure 1, $P < 0.001$). When we similarly divided them into quartiles according to previous maximum BMI, the prevalence of nephropathy significantly increased as previous maximum BMI increased (Figure 1, $P < 0.001$). Current BMI and previous maximum BMI were highly correlated with each other ($r = 0.785$, $P < 0.001$).

In logistic regression analysis, both current obesity and previous obesity revealed a significant odds ratio for nephropathy, as well as diabetic retinopathy, independent of sex, age, disease duration, hypertension, dyslipidemia, and HbA1c (Table 2).

4. Discussion

We confirmed that previous obesity, as well as present obesity, was closely associated with nephropathy in type 2 diabetes. The notable finding of this study was that obesity is an independent risk factor, not only if it is present, but also if it was present in the past. This might indicate a legacy effect of obesity on nephropathy. The mechanism is the theme for investigation of how obesity in the past can influence diabetic complications over time.

Both current BMI and previous maximum BMI were associated with the nephropathy, as demonstrated in Figure 1. However, current BMI and previous maximum BMI were highly correlated with each other. So, generally, the higher the previous BMI, the higher the current BMI. When we analyzed the effects of previous obesity, we had to separate the effects of current obesity from those of previous obesity. This was the reason we analyzed the effect of previous obesity according to the categories defined as previous maximum BMI and current BMI. As a result, we could elucidate the effect of previous obesity on diabetic nephropathy.

Whether nephropathy in type 2 diabetes really derives from diabetes has always been a point of discussion. However, type 2 diabetes *per se* is a disease so closely linked with obesity and the metabolic syndrome that we cannot strictly distinguish the cause among the components of the syndrome. We here found that the effect of obesity was independent of the

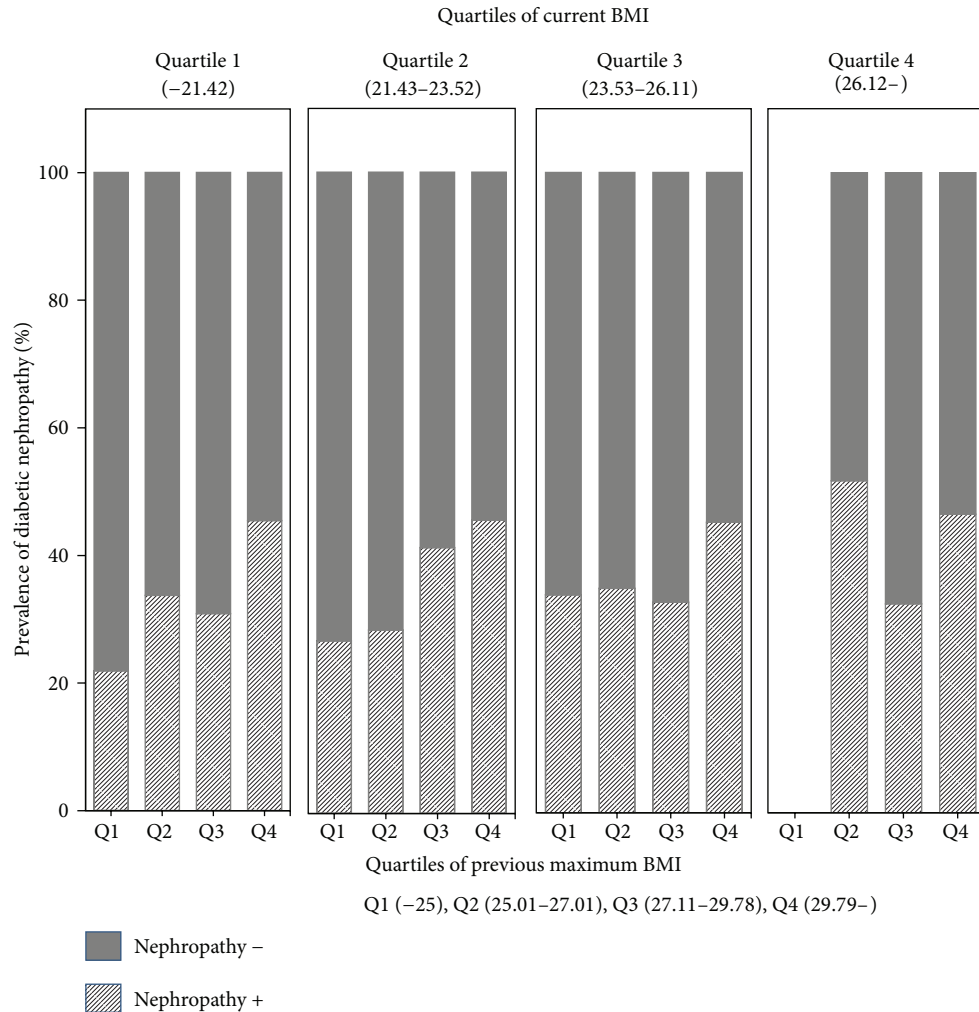


FIGURE 1: Prevalence of diabetic nephropathy divided by quartiles of current BMI and quartiles of previous maximum BMI. As for quartiles of current BMI, Quartile 1; BMI ≤ 21.42 , Quartile 2; BMI: 21.43–23.52, Quartile 3; BMI: 23.53–26.11, and Quartile 4; BMI > 26.11 . As for quartiles of previous maximum BMI, Quartile 1 (Q1); BMI ≤ 25.00 , Quartile 2 (Q2); BMI: 25.01–27.10, Quartile 3 (Q3); BMI: 27.11–29.78, and Quartile 4 (Q4); BMI > 29.78 . Prevalence of diabetic nephropathy was significantly different among the quartile groups of current BMI ($P < 0.001$ by chi-squared analysis). Prevalence of diabetic nephropathy was significantly different among the quartile groups of previous maximum BMI ($P < 0.001$ by chi-squared analysis).

existence of diabetic retinopathy, and we expect this result can be applied to all type 2 diabetes patients.

Several groups, including us, have reported that albuminuria was the strongest predictor of the progression of diabetic nephropathy [18–22]. We did not chronologically follow the decline of glomerular filtration rate (GFR) in this study. However, as we defined diabetic nephropathy by albuminuria, obesity, either current or past, might relate to the GFR decline through albuminuria.

Recently, the concept of obesity-related nephropathy has been advocated [23]. Although this concept is strictly defined with exclusion of both nephrosclerosis and diabetic nephropathy, the suspected mechanisms, including the contraction of efferent glomerular arterioles by the activated renin-angiotensin system (RAS) and glomerular hyperfiltration, as well as glomerular hypertrophy due to insulin resistance, are very similar to those of diabetic nephropathy.

The border between the concepts of diabetic nephropathy and obesity-related nephropathy is unclear, and they cannot be distinguished clinically if a patient has both. Vivante et al. reported that overweight state and obesity in adolescents were associated with significantly increased risk for both diabetic and nondiabetic ESRD during a 25-year period [24]. If obesity even before the diagnosis of diabetes influences kidney function later, these concepts are continuous and indivisible.

There are some limitations of this study. One is that this study was retrospective, based on self-reported body weight in the past. As for the other limitation, we might have to consider how they lost their body weight because the study subjects required metabolic interventions for poor glycemic status. The duration of obesity must be a factor of interest affecting the results. However, we only have the data of maximum body weight and the body weight on admission but

TABLE 1: Characteristics of study subjects.

	Total	Continuously lean	Previous obesity	Current obesity	P value
Age	59.3 ± 10.6	61.1 ± 9.4	60.4 ± 10.0	56.7 ± 11.6	<0.01
Sex (male/female)	2038/889	486/243	870/345	682/301	ns
BMI	24.0 ± 4.0	20.5 ± 2.1	22.6 ± 1.7	28.3 ± 3.2	<0.01
Max. BMI	27.7 ± 4.3	23.0 ± 1.7	27.5 ± 2.3	31.4 ± 4.1	<0.01
Disease duration (years)	10.4 ± 28.0	10.4 ± 8.4	12.2 ± 42.2	8.2 ± 7.8	<0.01
SBP (mmHg)	132 ± 18	131 ± 17	132 ± 18	133 ± 18	ns
DBP (mmHg)	75 ± 11	74 ± 11	75 ± 11	75 ± 10	ns
FPG (mmol/L)	9.9 ± 4.0	9.5 ± 4.0	9.9 ± 4.1	10.2 ± 3.9	ns
HbA1c (%)	9.3 ± 2.8	9.1 ± 2.7	9.5 ± 3.4	9.3 ± 2.0	<0.05
TC (mmol/L)	5.3 ± 1.2	5.3 ± 1.0	5.2 ± 1.0	5.5 ± 1.6	<0.01
HDL (mmol/L)	1.3 ± 0.5	1.5 ± 0.5	1.3 ± 0.4	1.2 ± 0.3	<0.01
TG (mmol/L)	1.6 ± 1.4	1.4 ± 0.2	1.5 ± 1.1	1.9 ± 1.6	<0.01
Cr (mmol/L)	66.3 ± 19.4	64.5 ± 16.8	66.3 ± 19.4	68.9 ± 20.3	<0.01
eGFR (mL/min/1.73 m ²)	81.8 ± 28.8	81.4 ± 20.5	83.1 ± 31.1	80.6 ± 31.0	ns
Retinopathy (none/simple/proliferative)	2111/505/311	558/116/55	824/225/166	729/164/90	<0.01

Continuous variables are expressed as mean ± SD. Differences in baseline characteristics among the obesity categories were analyzed by ANOVA and chi-squared test. The numbers of each stage of diabetic retinopathy are noted on the row of retinopathy. SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting blood glucose, TC: total cholesterol, HDL: HDL cholesterol, TG: triglyceride, ns: not significant.

TABLE 2: Logistic regression analysis with forced entry method for diabetic nephropathy.

	Odds ratio	95% confidence interval	P value
Age	1.011	1.002–1.019	<0.05
Disease duration	1.001	0.998–1.004	ns
Sex (male: 1, female: 0)	1.955	1.606–2.378	<0.001
Hypertension	1.232	1.023–1.484	<0.05
Dyslipidemia	1.306	1.096–1.556	<0.01
HbA1c	1.014	0.984–1.044	ns
Diabetic retinopathy	3.856	3.214–4.626	<0.001
Previous obesity: 1, other: 0	1.656	1.323–2.073	<0.001
Current obesity: 1, other: 0	2.480	1.959–3.141	<0.001

Adjusted $R^2 = 0.166$, $P < 0.001$. ns: not significant.

not the duration. So the hypothesis needs to be confirmed in a future large cohort study with more detailed information. In spite of these limitations, this was an analysis of a large population over 10 years. We therefore believe it includes important suggestions on the effect of obesity on diabetic nephropathy.

5. Conclusion

Our study indicated that obesity in the past, as well as present obesity, was a risk factor for diabetic nephropathy. We should consider the effect of earlier obesity on diabetic nephropathy even if it has been present before the diagnosis of diabetes.

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Research Article

Low Serum Magnesium Level Is Associated with Microalbuminuria in Chinese Diabetic Patients

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Whether serum magnesium deficiency is independently associated with the prevalence of microalbuminuria is still unclear. The objective of the present study was to elucidate the association between serum magnesium and microalbuminuria in diabetic patients. A cross-sectional study was conducted in 1829 diabetic subjects (aged ≥ 40 years) from Shanghai, China. Subjects were divided into three groups according to serum magnesium tertiles. A first-voided early-morning spot urine sample was obtained for urinary albumin-creatinine ratio (UACR) measurement. Microalbuminuria was defined as $30 \text{ mg/g} \leq \text{UACR} < 300 \text{ mg/g}$. Overall, 208 (11.37%) of the study population had microalbuminuria, with similar proportions in both genders ($P = 0.44$). The prevalence of microalbuminuria in tertile 1 of serum magnesium was higher than the prevalence in tertile 2 and tertile 3 (15.98%, 9.72%, and 8.46%, resp.; P for trend < 0.0001). After adjustment for age, sex, BMI, blood pressure, lipidaemic profile, HbA1c, eGFR, history of cardiovascular disease, HOMA-IR, antihypertensive and antidiabetic medication, and diabetes duration, we found that, compared with the subjects in tertile 3 of serum magnesium, those in tertile 1 had 1.85 times more likeliness to have microalbuminuria. We concluded that low serum magnesium level was significantly associated with the prevalence of microalbuminuria in middle-aged and elderly Chinese.

1. Introduction

Magnesium (Mg) is the fourth most abundant cation in the human body and is a critical cofactor in many enzymatic reactions [1, 2]. It plays an important role in many fundamental biological processes. Mg depletion is a common feature in diabetic patients [3, 4]. An Australian study demonstrated that hypomagnesaemia was 10.51-fold more common between patients with new diabetes and 8.63-fold more common between patients with known diabetes as compared with control subjects without diabetes [3]. In another large cohort of young American adults participating in the Coronary Artery Risk Development in Young Adults (CARDIA) study, it was shown that Mg intake was inversely longitudinally associated with the incidence of diabetes [4].

Microalbuminuria was first reported in diabetic patients by Viberti et al. in 1982 [5]. It has been shown to be associated with increased risk of cardiovascular morbidity and mortality in diabetic patients [6]. Furthermore, the presence of microalbuminuria is generally associated with a poorer glycometabolic control and a higher prevalence of chronic complications including diabetic retinopathy, peripheral vascular disease, and diabetic neuropathy [7].

The association between microalbuminuria and Mg depletion is a controversial issue. A previous report showed that high doses of Mg reduce microalbuminuria in traumatic critically ill patients at 36 hour, after infusion [8]. Conversely, there were no significant differences between patients with hypomagnesaemia and normal subjects with respect to microalbuminuria [9]. Therefore, the aim of the present study

was to evaluate the association between serum Mg and microalbuminuria in diabetic patients in China.

2. Materials and Methods

2.1. Research Design and Subjects. This community-based cross-sectional study was conducted in Jiading district, Shanghai, China, from March to August, 2010. In brief, 10375 subjects, aged 40 years or above, were enrolled to participate in the survey. Among those subjects, there were 1872 diabetic patients, those with fasting plasma glucose (FPG) ≥ 7.0 mmol/L and/or 2 h plasma glucose (2 h-PG) ≥ 11.1 mmol/L or with a history of diabetes. The diagnosis of diabetes was defined according to the 1999 World Health Organization criteria [10]. Microalbuminuria was defined as 30 mg/g \leq urinary albumin-creatinine ratio (UACR) < 300 mg/g [11]. For analysis, we excluded subjects who had missing data on serum Mg or urine albumin or urine creatinine ($n = 5$), those who had urinary tract infection, glomerulonephritis, nephritic syndrome, or kidney cancer ($n = 17$), and those who had UACR ≥ 300 mg/g ($n = 21$). Finally, a total of 1829 diabetic subjects (775 males and 1054 females) were included in the analysis.

This study was conducted with the approval of the institutional review board of Ruijin Hospital affiliated to Shanghai Jiao-Tong University School of Medicine. All participants provided informed consent.

2.2. Clinical Data Collection and Biochemical Measurements. The information about demographic characteristics, lifestyle, the history of chronic diseases, and current use of medication, including antihypertensive drugs and antidiabetic drugs, were obtained by a standard interview questionnaire. Current smokers or drinkers were defined as subjects who smoked cigarettes or consumed alcohol regularly in the past 6 months, while subjects who never or formerly smoked cigarettes or consumed alcohol were defined as noncurrent smokers or noncurrent drinkers.

Blood pressure was measured at the nondominant arm three times consecutively at 1 min intervals after subjects had rested for at least 5 min in a sitting position, using an automated electronic device (OMRON Model HEM-752; Omron, Dalian, China). The average of the three measurements was used in the analysis. Subjects with systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg or taking antihypertensive drugs were defined as having hypertension [12]. Body height and body weight were recorded to the nearest 0.1 cm and 0.1 kg while participants were wearing light indoor clothing without shoes. Body mass index (BMI) was calculated as body weight divided by squared body height (kg/m²).

After at least 10 hours of overnight fasting, venous blood samples were collected for the measurements of serum insulin, blood glucose, lipid profile, serum creatine and glycated hemoglobin A1c (HbA1c). Blood glucose was measured with the use of the glucose oxidase method on an auto-analyser (Modular P800, Roche, Basel, Switzerland). Fasting serum insulin, serum creatinine, Mg, triglycerides (TG), total

cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were measured by an autoanalyser (Modular E170, Roche, Basel, Switzerland). HbA1c was assessed by high-performance liquid chromatography (HPLC, BIO-RAD D-10, USA). The insulin resistance index (homeostasis model assessment of insulin resistance, HOMA-IR) was calculated as fasting insulin (μ IU/mL) \times fasting glucose (mmol/L)/22.5 [13]. GFR was estimated based on serum creatinine concentration using the modification of diet in renal disease (MDRD) formula: $eGFR = [186 \times \text{serum creatinine (umol/L)} \times 0.0113]^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ for women})$ [14].

A first-morning spot urine sample was obtained at the survey center. Women experiencing menstruation on the survey day were not included in the present study. Urine albumin and creatinine were measured by immunoturbidimetric method (Beijing Atom High-Tech, Beijing, China) and the Jaffe's kinetic method on an automatic analyser (Hitachi 7600-020, Tokyo, Japan), respectively. The UACR in mg/g was calculated as urine albumin concentration divided by urine creatinine concentration.

2.3. Statistical Analysis. Participants were divided into tertiles according to serum Mg concentration as tertile 1: Mg < 0.86 mmol/L, tertile 2: $0.86 \text{ mmol/L} \leq \text{Mg} < 0.92$ mmol/L, and tertile 3: Mg ≥ 0.92 mmol/L. Baseline characteristics of subjects were calculated as mean and standard deviation (SD), median and interquartile range, or percentage. Trends in means and proportions were tested using linear regression and χ^2 tests, respectively. HbA1c, HOMA-IR, TG, and UACR were logarithmically transformed before analysis due to a nonnormal distribution.

Logistic regression was used to evaluate the association between serum Mg and the prevalence of microalbuminuria. Model 1 was unadjusted. In Model 2, we adjusted for age, sex, and BMI. In Model 3, we further adjusted for SBP, DBP, LDL-c, HDL-c, TC, TG, HbA1c and history of cardiovascular disease. In Model 4, we additionally adjusted for HOMA-IR, eGFR, antihypertensive drugs, antidiabetic drugs, and diabetes duration. Relationship between serum Mg and the prevalence of microalbuminuria was also explored in stratified analysis. The factors associated with serum Mg levels or UACR were considered as the strata factors. Odds ratios were calculated for each tertile decline of serum Mg levels in subgroups of the strata variables.

All analysis were performed with SAS (version 9.3; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of the Study Population. Demographic and clinical characteristics and biochemical measurements of 1829 subjects according to tertiles of serum Mg are shown in Table 1. Compared with subjects in the higher serum Mg group, those with lower serum Mg level were more likely to be females and had higher prevalence of antidiabetic drugs use, higher level of FPG, 2 h-PG, HbA1c, HOMA-IR, UACR, and eGFR, and lower level of LDL-c, TC, and serum creatinine

TABLE 1: General characteristics of the study population.

Variables	Serum magnesium levels (mmol/L)			P for trend
	Tertile 1 Mg < 0.86	Tertile 2 0.86 ≤ Mg < 0.92	Tertile 3 Mg ≥ 0.92	
N	607	607	615	
Age (year)	61.11 ± 10.01	61.53 ± 9.18	61.98 ± 9.98	0.10
Female (%)	62.11	57.00	53.82	0.003
BMI (kg/m ²)	26.31 ± 3.50	26.24 ± 3.40	26.08 ± 3.50	0.24
Current smoking (%)	19.28	20.59	19.84	0.81
Current drinking (%)	9.72	10.71	9.92	0.91
Antidiabetic drugs (%)	47.12	40.53	35.45	<0.0001
Antihypertensive drugs (%)	41.19	41.19	46.18	0.08
FPG (mmol/L)	8.34 ± 2.97	7.35 ± 2.18	7.02 ± 1.89	<0.0001
2h-PG (mmol/L)	16.60 ± 5.86	15.35 ± 4.66	14.24 ± 4.33	<0.0001
HbA1c (%)	6.9 (6.2–8.3)	6.6 (6.0–7.4)	6.3 (5.8–7.0)	<0.0001
HOMA-IR	2.93 (1.91–4.92)	2.86 (1.77–4.35)	2.67 (1.63–4.12)	0.002
SBP (mmHg)	149.60 ± 19.95	149.53 ± 20.23	148.09 ± 18.57	0.18
DBP (mmHg)	84.40 ± 10.30	83.92 ± 10.58	83.64 ± 9.90	0.20
LDL-c (mmol/L)	3.23 ± 0.94	3.31 ± 0.92	3.34 ± 0.91	0.03
HDL-c (mmol/L)	1.28 ± 0.30	1.27 ± 0.31	1.26 ± 0.30	0.20
TC (mmol/L)	5.44 ± 1.12	5.49 ± 1.09	5.58 ± 1.08	0.02
TG (mmol/L)	1.60 (1.19–2.37)	1.67 (1.19–2.23)	1.74 (1.22–2.44)	0.11
UACR (mg/g)	7.68 (3.94–17.94)	6.15 (3.43–13.58)	5.74 (3.00–12.72)	<0.0001
Serum creatinine (umol/L)	58.85 ± 14.95	61.15 ± 14.37	64.01 ± 16.98	<0.0001
eGFR (mL/min per 1.73 m ²)	114.90 ± 27.58	110.16 ± 23.05	105.85 ± 22.73	<0.0001

Data are means ± SD, medians (interquartile range), or percentages of subjects.

(all *P* for trend <0.05). However, age, BMI, SBP, DBP, HDL-c, TG, antihypertensive drugs use, smoking status, and drinking status were not statistically different among the three groups.

3.2. Prevalence of Microalbuminuria in Different Serum Mg Levels. Overall, 208 (11.37%) of the study population had microalbuminuria, with similar proportions in males and females (10.71% versus 11.86%; *P* = 0.44). As shown in Figure 1, across the serum Mg tertiles, the prevalence of microalbuminuria was 15.98%, 9.72%, and 8.46%, respectively (*P* for trend <0.0001). Strikingly, a significant increase was observed in tertile 1 compared with tertile 2 (*P* = 0.001) and tertile 3 (*P* < 0.0001), respectively. However, the difference of the prevalence of microalbuminuria between tertile 2 and tertile 3 was not statistically significant (*P* = 0.44).

3.3. Serum Mg in relation to Microalbuminuria. As shown in Table 2, declined serum Mg was strongly associated with an increased prevalence of microalbuminuria in both univariate and multivariate analyses. In the univariate model, the presence of microalbuminuria was significantly more frequent among the participants in tertile 1 than those in tertile 3 (OR = 2.06, 95% CI: 1.44–2.95). After further adjustment for age, sex, BMI, SBP, DBP, LDL-c, HDL-c, TC, TG, HbA1c, eGFR, history of cardiovascular disease, HOMA-IR, antihypertensive drugs, antidiabetic drugs and diabetes duration (Model 4), the ORs for microalbuminuria among patients in tertile 1, and

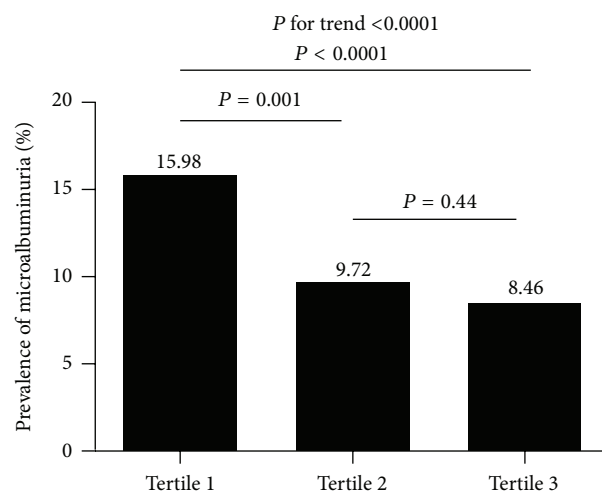


FIGURE 1: Prevalence of microalbuminuria by serum Mg tertiles (the serum Mg tertiles cutoff points were tertile 1: Mg < 0.86 mmol/L, and tertile 2: 0.86 mmol/L ≤ Mg < 0.92 mmol/L, tertile 3: Mg ≥ 0.92 mmol/L).

2 in comparison with tertile 3 were 1.85 (95% CI: 1.26–2.72) and 1.11 (95% CI: 0.74–1.67), respectively.

Multivariate-adjusted OR for microalbuminuria with each tertile decrease of serum Mg in different subgroups is shown in Table 3. The associations between serum Mg and the prevalence of microalbuminuria were not subgroup

TABLE 2: Association between serum Mg levels and the prevalence of microalbuminuria.

	Tertile 3	Serum Mg levels Tertile 2	Tertile 1
Model 1	1.00 (reference)	1.17 (0.79–1.72)	2.06 (1.44–2.95)
Model 2	1.00 (reference)	1.17 (0.79–1.73)	2.09 (1.45–3.00)
Model 3	1.00 (reference)	1.06 (0.71–1.59)	1.74 (1.19–2.54)
Model 4	1.00 (reference)	1.11 (0.74–1.67)	1.85 (1.26–2.72)

Model 1: unadjusted.

Model 2: adjusted for age, sex, and BMI.

Model 3: adjusted for age, sex, BMI, SBP, DBP, LDL-c, HDL-c, TC, TG, HbA1c, and history of cardiovascular disease.

Model 4: adjusted for age, sex, BMI, SBP, DBP, LDL-c, HDL-c, TC, TG, HbA1c, eGFR, history of cardiovascular disease, HOMA-IR, antihypertensive drugs, antidiabetic drugs, and diabetes duration.

TABLE 3: The risk of microalbuminuria with each tertile decline of serum Mg in different subgroups of diabetic patients.

	Number of microalbuminuria/number of subjects	Multivariate ^a OR (95% CI)	P
Sex			
Male	83/775	1.71 (1.22–2.38)	0.002
Female	125/1054	1.20 (0.93–1.53)	0.16
HbA1c			
6.5%	81/861	1.11 (0.82–1.51)	0.48
≥6.5%	127/968	1.69 (1.30–2.20)	<0.0001
Hypertension			
Absence	24/392	1.85 (1.00–3.40)	0.05
Presence	184/1436	1.37 (1.11–1.69)	0.003
Duration of diabetes			
<10 year	187/1684	1.42 (1.16–1.74)	0.0008
≥10 year	21/145	0.98 (0.48–1.98)	0.95

^aadjusted for age, sex, BMI, SBP, DBP, LDL-c, HDL-c, TC, TG, HbA1c, eGFR, history of cardiovascular disease, HOMA-IR, antihypertensive drugs, antidiabetic drugs, and diabetes duration (except for the strata variables).

consistent. Significant associations were detected in males, subjects with higher HbA1c (≥6.5%), subjects with or without hypertension, and subjects with shorter duration of diabetes (<10 year).

4. Discussion

In this cross-sectional study, we found a significant inverse association between serum Mg concentration and the prevalence of microalbuminuria in middle-aged or elderly Chinese. Moreover, the relationship was independent of other confounding factors.

Our findings are generally consistent with the results from some previous studies. For instance, Corsonello et al. demonstrated that diabetic patients with microalbuminuria or overt proteinuria showed a significant decrease in serum Mg compared with normoalbuminuria group [7]. It has been reported that, compared with type 1 diabetic patients with normoalbuminuria, a significant reduction in serum Mg levels has been found in type 1 diabetic patients with microalbuminuria or clinical proteinuria [15]. Evidence also suggested that non-insulin-dependent diabetic patients with hypomagnesemia

showed an increased urinary albumin excretion rate with respect to normomagnesemic diabetic patients [16].

In contrast, other studies did not find any significant associations between serum Mg and microalbuminuria. A previous study on type 1 diabetic patients has shown that there was no association between microalbuminuria and serum total Mg concentration [17]. In addition, a cross-sectional study in Brazil did not find any significant difference in microalbuminuria between type 2 diabetic patients with plasma Mg <0.75 mmol/L and type 2 diabetic patients with plasma Mg ≥0.75 mmol/L [9].

The possible reasons for the inconsistency of our results with the above previous studies are shown as follows. (1) The JACC study from Japan demonstrated that dietary magnesium intake was associated with reduced mortality from cardiovascular disease [18]. On the other hand, microalbuminuria is considered as an independent predictor of cardiovascular disease [19]. Thus, different habits of food intake from different countries may affect the association between serum Mg concentration and microalbuminuria. (2) The sample size of above studies were too small to demonstrate the relationship between serum Mg and microalbuminuria.

One of the potential pathophysiological mechanisms linking serum Mg to microalbuminuria is amplification of insulin resistance. It was said that low serum Mg plays an important role in pathogenesis of insulin resistance. Mg can function as a mild, natural calcium antagonist. So the level of intracellular calcium is increased in Mg-deficiency subjects. This increased intracellular calcium may compromise the insulin responsiveness of adipocytes and skeletal muscles leading to the development of insulin resistance [20]. Another study has also found that insulin deficiency or insulin resistance can affect the tubular absorption of Mg, leading to hypomagnesemia in diabetic subjects [21]. We speculated that a vicious circle formed by mutual influence between insulin resistance and hypomagnesemia results in aggravation of insulin resistance which can increase the risk of microalbuminuria [22].

Oxidative stress is becoming increasingly recognized as an important causative factor for microalbuminuria [23]. Mg has been reported to possess antioxidant property [24]. Hence, oxidative stress may be one of the mechanisms that underlie the association between low serum Mg and microalbuminuria. Study has also shown that Mg intake and serum Mg concentration were inversely associated with systemic inflammation markers [4], which also play an crucial role in the pathogenesis of microalbuminuria [8].

Our study adds evidence to the association between low serum Mg and microalbuminuria. However, there are several limitations that require consideration. First, lack of dietary Mg measurement is one limitation of the present study which may impede us to determine the effect of low dietary Mg intake on serum Mg level and risk of prevalent microalbuminuria. Second, no causal inference can be drawn due to the cross-sectional design of the current study. Further prospective studies are needed to illustrate the precise relationship between Mg depletion and incident of microalbuminuria. Third, UACR levels were determined by a single measurement and may not be accurately representative of the status of study subjects.

5. Conclusions

In summary, serum Mg was inversely associated with the prevalence of microalbuminuria. Further large-scale clinical trials are needed to be carried out to determine whether correction of Mg deficiency, through medications or dietary intake, could be effective to reduce the incidence of microalbuminuria and elucidate the mechanisms underlying the association between serum Mg and microalbuminuria.

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Clinical Study

Effect of Telmisartan or Losartan for Treatment of Nonalcoholic Fatty Liver Disease: Fatty Liver Protection Trial by Telmisartan or Losartan Study (FANTASY)

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Aim. This study compared the effects of telmisartan and losartan on nonalcoholic fatty liver disease (NAFLD) and biochemical markers of insulin resistance in hypertensive NAFLD patients with type 2 diabetes mellitus. **Methods.** This was a randomized, open-label, parallel-group comparison of therapy with telmisartan or losartan. Nineteen hypertensive NAFLD patients with type 2 diabetes were randomly assigned to receive telmisartan at a dose of 20 mg once a day ($n = 12$) or losartan at a dose of 50 mg once a day ($n = 7$) for 12 months. Body fat area as determined by CT scanning and hepatic fat content based on the liver-to-spleen (L/S) ratio, as well as several parameters of glycemic and lipid metabolism, were compared before and after 12 months. **Results.** The telmisartan group showed a significant decline in serum free fatty acid (FFA) level (from 0.87 ± 0.26 to 0.59 ± 0.22 mEq/L (mean \pm SD), $P = 0.005$) and a significant increase in L/S ratio ($P = 0.049$) evaluated by CT scan, while these parameters were not changed in the losartan group. **Conclusion.** Although there was no significant difference in improvement in liver enzymes with telmisartan and losartan treatment in hypertensive NAFLD patients with type 2 diabetes after 12 months, it is suggested that telmisartan may exert beneficial effects by improving fatty liver.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver disease throughout the world [1]. NAFLD is characterized by hepatic steatosis in the absence of significant alcohol use, hepatotoxic medication, or other known liver diseases [2]. NAFLD represents a spectrum ranging from simple fatty liver to nonalcoholic steatohepatitis (NASH), which is an aggressive form of NAFLD leading to cirrhosis and hepatocellular carcinoma [3–6]. Recently, it has been established that NAFLD is commonly associated with metabolic syndrome, including type 2 diabetes, obesity, dyslipidemia, and hypertension and consequently is associated with cardiovascular mortality [6–10]. The potential need

for treatment of NAFLD is recognized, in order to improve cardiovascular and liver-related outcomes, and several therapeutic interventions to treat various components of metabolic syndrome have been evaluated [10–12].

Angiotensin II receptor blockers (ARBs), which are highly selective for the angiotensin II type 1 (AT1) receptor and block diverse effects of angiotensin II, are commonly used to treat hypertension [13]. Recently, ARBs have been expected to be effective for treatment of NAFLD, due to targeting of the mechanisms of insulin resistance and hepatic injury via suppression of the renin-angiotensin system (RAS), which has been suggested to be involved in the pathways of liver damage. It has been reported that an ARB, losartan, showed significant improvement in aminotransferase levels

and serum markers of fibrosis in hypertensive patients with NASH [14]. Moreover, losartan has been reported to decrease the number of activated hepatic stellate cells, which play a pivotal role in the progression of hepatic fibrosis [15]. These results suggest that losartan might be therapeutically efficacious for NASH.

Telmisartan, another ARB, has been reported to have a partial agonistic effect on peroxisome proliferator-activated receptor (PPAR)- γ in addition to the effect of angiotensin II blockade [16, 17]. So, telmisartan is expected to have more potent effects in NAFLD than those of losartan, via PPAR γ activation, which promotes hepatic fatty acid oxidation, decreases hepatic lipogenesis, and increases peripheral and hepatic insulin sensitivity [18, 19]. In fact, it is reported that telmisartan attenuated steatohepatitis progression in an animal model [20]. In addition, telmisartan has been reported to improve insulin resistance and liver injury, based on measurement of homeostasis model assessment-insulin resistance (HOMA-IR) and serum aminotransferase (ALT) levels in humans [21].

In the present study, we tested the hypothesis that telmisartan might have a more potent effect on NAFLD and biochemical markers of insulin resistance than does losartan.

2. Materials and Methods

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Review Board of Keio University. Written informed consent was obtained from each subject before participation in the study. This study was assigned the UMIN-ID, UMIN000000540.

2.1. Subjects. We screened patients with type 2 diabetes between 20 to 80 years of age with both NAFLD and hypertension. NAFLD was defined as fatty liver on ultrasonography, and aspartate aminotransferase (AST) level over 30 IU/L, and/or alanine aminotransferase (ALT) level over 40 IU/L. A detailed history of alcohol consumption was taken by physicians. All patients consumed less than 20 g of pure alcohol per day, and were negative for hepatitis B serological tests, antibody to hepatitis C virus, and autoantibodies, including anti-mitochondrial antibody and anti-nuclear antibody. Hypertension was defined as systolic blood pressure (SBP) over 140 mmHg and/or diastolic blood pressure (DBP) over 90 mmHg. Patients using antihypertensive agents were also included. Exclusion criteria included the presence of AST > 100 IU/L and/or ALT > 100 IU/L, severe hypertension (i.e., SBP > 200 mmHg, DBP > 120 mmHg), malignancy and recent major cardiovascular disease (i.e., cardiovascular disease or stroke within past 3 months), insulin, biguanide or thiazolidinedione treatment for diabetes mellitus, and drug allergy to ARBs.

2.2. Study Design. This was a randomized, open-label, parallel-group comparison of therapy with telmisartan or losartan. Nineteen hypertensive NAFLD patients with type 2 diabetes were randomly assigned to the telmisartan (T) group (receiving a standard dose of 20 mg once daily, $n = 12$)

or losartan (L) group (receiving a standard dose of 50 mg once daily, $n = 7$). Patients using other antihypertensive agents were randomly switched to telmisartan or losartan. Medication was not masked, and treatment had to be taken daily at the same hour in the morning, with no concomitant medication or alcohol consumption allowed. Either the patient or the medical staff was aware of the treatment group allocation. All 19 subjects received dietary instructions using a meal-exchange plan from nutritionists. The ideal dietary caloric intake for each patient was calculated as the ideal body weight (kg) \times 25 kcal/kg. It was confirmed by questionnaire that the physical activity level was almost constant in each subject throughout the study period.

The included patients were followed for 12 months, with two-monthly visits.

Anthropometric measurements, blood pressure (BP), heart rate (HR), and several clinical and biochemical parameters of glycemic control, lipid metabolism, and liver function were checked at every visit. Body fat area as determined by computed tomographic (CT) scanning at the umbilical level, hepatic fat content based on the liver-to-spleen (L/S) ratio according to CT attenuation values, inflammatory markers, and serum bile acid level were determined before and after 12 months.

2.3. Measurements. Blood pressure was determined in the sitting position after a 10-minute rest. Body weight was measured at the clinic under the same conditions for each patient. Blood samples were taken from each subject before breakfast in the early morning, after overnight bed rest.

Fasting plasma glucose (FPG) was determined by the glucose oxidase method. Hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography (Toso, Tokyo, Japan) and presented as the equivalent value for the National Glycohemoglobin Standardization Program (NGSP). Serum immunoreactive insulin (IRI) was measured by an enzyme immunoassay using a commercially available kit. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated by the formula: fasting plasma insulin (μ U/mL) \times fasting plasma glucose (mg/dL)/405. HOMA- β was calculated by the formula: fasting plasma insulin (μ U/mL) \times 360/(fasting plasma glucose (mg/dL) – 63) [22]. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and free fatty acids (FFAs) were measured enzymatically by an autoanalyzer (Hitachi, Tokyo, Japan). As biochemical parameters, AST, ALT, gamma glutamyl transpeptidase (γ GT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CR), uric acid (UA), sodium (Na), potassium (K), ferritin, and creatine phosphokinase (CPK) were measured. Inflammatory markers such as hyaluronic acid (Hyal), 7S domain of type IV collagen (4Col7S), high-sensitivity C-reactive protein (hs-CRP), procollagen III peptide (P-3-P), zinc (Zn), total adiponectin, and interleukin (IL)-6 were analyzed at the Special Reference Laboratory (SRL, Tokyo, Japan). We also measured bile acid (BA) components by high-performance liquid chromatography, because BAs might be related to lipid absorption and cholesterol catabolism [23].

Subcutaneous and visceral fat distribution was determined by measuring a -150 Hounsfield unit (HU) to -50 HU area using the method of CT scanning at the umbilical level as described previously [24]. V/S ratio was calculated as visceral fat area (VFA)/subcutaneous fat area (SFA). An index of fat deposition in the liver based on the liver-to-spleen (L/S) ratio according to CT attenuation values was also determined. The mean HU values of the liver and spleen were determined in the parenchyma of the right (CT-L1) and left lobe (CT-L2) of the liver and approximately the same size area of the spleen (CT-Spleen), avoiding blood vessels, artifacts, and heterogeneous areas. L/S ratio was calculated as $[(CT-L1) + (CT-L2))/2]/(CT-Spleen)$.

2.4. Statistical Analyses. Continuous variables are presented as mean \pm standard deviation. Continuous variables were compared between the telmisartan group and losartan group using the Mann-Whitney *U* test for independent samples. Differences in each baseline treatment between groups were analyzed by chi-squared test. Differences in each parameter between the start and after 12 months in each group were analyzed using the Wilcoxon's matched-pair signed-rank test. A *P* value less than 0.05 was considered to be statistically significant. Statistical analyses were carried out using StatView 5.0 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Baseline Characteristics. Baseline characteristics of the subjects in both groups are shown in Table 1. There were no significant differences in most parameters including duration of diabetes, anthropometric measurements, BP, biochemical measurements, and inflammatory markers between the T group and L group. In spite of randomization, there were significant differences in two parameters between the two groups at baseline; serum FFA (0.87 ± 0.26 mEq/L in T group versus 0.50 ± 0.26 mEq/L in L group ($P = 0.001$)) and L/S ratio (0.82 ± 0.25 in T group versus 1.01 ± 0.23 in L group ($P = 0.035$)).

3.2. Changes in Anthropometric Measurements and BP. No subject terminated the trial because of adverse events.

Body weight and waist and hip measurements did not change in both groups. Both groups showed a significant decrease in SBP (139.4 ± 11.1 versus 130.8 ± 15.0 mmHg in T group ($P = 0.045$), 136.4 ± 13.9 versus 127.4 ± 10.6 mmHg in L group ($P = 0.046$)) after 12 months. Concerning DBP, a statistically significant decrease was found in the T group (86.0 ± 8.5 versus 75.4 ± 12.7 mmHg ($P = 0.032$)), whereas the decrease in the L group did not reach statistical significance (81.6 ± 13.0 versus 75.3 ± 7.9 mmHg ($P = 0.116$)) (Table 2).

3.3. Changes in Biochemical Measurements. Liver enzyme levels such as AST, ALT, and γ GT did not show significant change in both groups after 12 months. While TC, HDL-C, and TG levels did not show significant change in both groups after 12 months, FFA level showed a significant decrease in

the T group (0.87 ± 0.26 versus 0.59 ± 0.22 mEq/L ($P = 0.005$)) whereas the change in the L group was not significant (0.50 ± 0.26 versus 0.66 ± 0.22 mEq/L ($P = 0.237$)).

FPG level did not change in both groups after 12 months. Regarding HbA1c level, the L group showed a significant increase (6.7 ± 1.0 versus $7.2 \pm 1.2\%$ ($P = 0.017$)), while the change in the T group was not significant (6.4 ± 0.6 versus $6.4 \pm 0.4\%$ ($P = 0.552$)).

UA level showed a significant decrease in the L group (5.7 ± 1.5 versus 5.2 ± 1.3 mg/dL ($P = 0.046$)), while it showed a significant increase in the T group (5.8 ± 1.4 versus 6.3 ± 1.2 mg/dL ($P = 0.016$)). Consequently, the difference in changes was also statistically significant.

Levels of other inflammatory markers and bile acids did not show significant change in both groups after 12 months (Tables 3 and 4).

3.4. Changes in Fat Distribution and Fat Deposition in Liver. Visceral and subcutaneous fat area did not change in both groups after 12 months. Consequently, V/S ratio did not change in both groups. Regarding L/S ratio, a significant increase was found in the T group (0.82 ± 0.25 versus 0.97 ± 0.22 ($P = 0.049$)), while it did not change in the L group (1.01 ± 0.23 versus 1.01 ± 0.21 ($P > 0.999$)) (Table 5).

4. Discussion

In the present study, we evaluated the effects of ARBs (telmisartan and losartan) on NAFLD in hypertensive patients with type 2 diabetes and compared their effect to improve liver function after 12 months of treatment.

There was no significant improvement in liver function in either group. However, serum FFA level was significantly decreased in the telmisartan group, leading to a significant improvement in L/S ratio, which reflects the severity of fatty change in the liver, compared to that in the losartan group. This finding suggests that telmisartan might improve fat deposition in the liver.

Unlike other ARBs, telmisartan is known to activate PPAR γ [16, 17, 25]. Its activation induces insulin sensitization through an increase in adiponectin in adipose tissue. In fact, several reports have been published concerning the efficacy of the PPAR γ agonist, pioglitazone, in the treatment of NASH. It is known that pioglitazone improves liver histological features, including steatosis, hepatocellular ballooning degeneration, lobular inflammation, and fibrosis [26–28]. In studies using several strains of animal models, telmisartan inhibited fat deposition, inflammation, and fibrosis in the liver [20, 29–31]. Also, these effects of telmisartan were greater than those of another ARB, valsartan [32], with the expectation of its efficacy in the liver also in humans.

In the present study, liver enzyme levels were not significantly improved in either the telmisartan or losartan group over 12 months. In a previous study, 48-week treatment with losartan significantly improved liver enzyme levels [14]. However, liver enzyme levels before ARB administration in the study were higher compared with those in our study, and after one year of administration of ARB they were only

TABLE 1: Baseline characteristics in each group.

Parameters	Telmisartan	Losartan	P-value
N (male/female)	12 (6/6)	7 (3/4)	
Age (years)	57.7 ± 12.8	60.3 ± 14.3	0.612
Duration of diabetes (years)	5.4 ± 6.0	6.1 ± 6.9	0.523
Height (cm)	161.8 ± 11.0	158.9 ± 13.5	0.673
Body mass index (kg/m ²)	29.2 ± 5.8	27.8 ± 3.8	0.735
Waist circumference (cm)	97.0 ± 14.9	94.4 ± 8.1	0.899
Hip circumference (cm)	111.9 ± 20.9	99.6 ± 8.7	0.257
Systolic blood pressure (mmHg)	139.4 ± 11.1	136.4 ± 13.9	0.526
Diastolic blood pressure (mmHg)	86.0 ± 8.5	81.6 ± 13.0	0.611
Pulse (beats/min)	74.5 ± 11.8	85.0 ± 9.5	0.205
Biochemical markers			
Fasting plasma glucose (mg/dL)	116.5 ± 20.3	122.8 ± 20.2	0.571
Hemoglobin A1c (%)	6.4 ± 0.6	6.7 ± 1.0	0.444
Glycoalbumin (%)	15.9 ± 2.6	17.0 ± 3.0	0.447
Immunoreactive insulin (μU/mL)	12.5 ± 6.1	12.6 ± 6.4	0.955
Total cholesterol (mg/dL)	217.8 ± 42.3	201.0 ± 38.9	0.447
High-density lipoprotein cholesterol (mg/dL)	52.8 ± 13.1	46.7 ± 9.2	0.290
Triglyceride (mg/dL)	122.3 ± 54.3	120.9 ± 45.7	0.866
Free fatty acids (mEq/L)	0.87 ± 0.26	0.50 ± 0.26	0.001
Aspartate aminotransferase (IU/L)	30.6 ± 13.9	32.0 ± 10.3	0.372
Alanine aminotransferase (IU/L)	39.8 ± 26.6	43.7 ± 26.2	0.583
γ Glutamyl transpeptidase (IU/L)	58.9 ± 43.0	60.9 ± 63.8	0.612
Alkaline phosphatase (IU/L)	249.2 ± 56.9	256.9 ± 97.0	0.800
Lactate dehydrogenase (IU/L)	193.4 ± 23.7	207.6 ± 40.3	0.353
Blood urea nitrogen (mg/dL)	13.5 ± 3.6	12.8 ± 3.1	0.704
Creatinine (mg/dL)	0.8 ± 0.2	0.8 ± 0.3	0.283
Uric acid (mg/dL)	5.8 ± 1.4	5.7 ± 1.5	0.582
Na (mEq/L)	141.1 ± 2.2	140.2 ± 2.1	0.447
K (mEq/L)	4.2 ± 0.4	4.2 ± 0.3	0.966
u-Microalbumin (μg/mL)	23.9 ± 43.9	76.1 ± 113.7	0.375
Ferritin (ng/mL)	184.4 ± 167.9	159.9 ± 130.6	0.767
Creatine phosphokinase (IU/L)	108.2 ± 44.1	124.4 ± 80.4	0.899
HOMA-IR	3.55 ± 1.68	3.84 ± 2.30	0.865
HOMA-β	98.1 ± 71.2	84.5 ± 53.6	0.865
Complete blood count			
White blood cells (×10 ³ /μL)	6.6 ± 1.2	5.4 ± 0.9	0.052
Hemoglobin (g/dL)	14.7 ± 1.4	14.9 ± 1.3	0.865
Platelets (×10 ³ /μL)	23.6 ± 5.4	20.1 ± 4.6	0.163
Inflammatory markers			
Hyaluronic acid (ng/mL)	38.3 ± 29.1	57.5 ± 46.2	0.446
7S domain of type 4 collagen (ng/mL)	4.4 ± 0.7	4.4 ± 0.9	0.445
High-sensitivity CRP (mg/dL)	0.131 ± 0.099	0.130 ± 0.122	0.964
Procollagen-3-peptide (U/mL)	0.57 ± 0.09	0.51 ± 0.05	0.175
Zn (μg/dL)	88.8 ± 13.9	88.0 ± 17.1	0.612
Total adiponectin (μg/mL)	7.3 ± 1.5	7.8 ± 1.1	0.400
Interleukin-6 (pg/mL)	3.4 ± 5.5	1.7 ± 1.0	0.309
Bile acids (BA)			
Total BA (μmol/L)	2.51 ± 1.82	5.34 ± 4.87	0.331
Primary BA (μmol/L)	1.32 ± 1.74	2.99 ± 3.16	0.135
Secondary BA (μmol/L)	1.18 ± 0.97	2.36 ± 3.41	0.966

TABLE 1: Continued.

Parameters	Telmisartan	Losartan	P-value
CT scan			
Visceral fat (cm ²)	188.8 ± 73.7	170.8 ± 51.1	0.673
Subcutaneous fat (cm ²)	257.1 ± 150.4	252.9 ± 76.1	0.877
V/S ratio	0.92 ± 0.57	0.71 ± 0.26	0.612
CT-L1 (HU)	39.9 ± 10.5	50.2 ± 11.9	0.025
CT-L2 (HU)	40.2 ± 13.8	50.7 ± 11.3	0.063
CT-Spleen (HU)	49.2 ± 2.9	49.9 ± 4.7	0.866
L/S ratio	0.82 ± 0.25	1.01 ± 0.23	0.035
Baseline treatment for hypertension [<i>n</i> (%)]			0.973
Naive	5 (41.7)	3 (42.9)	
Other ARB or ACE inhibitor	3 (25.0)	2 (28.6)	
Calcium channel blocker	4 (33.3)	2 (28.6)	
Baseline treatment for diabetes mellitus [<i>n</i> (%)]			0.123
Diet only	12 (100.0)	5 (71.4)	
Sulphonylurea+α-glucosidase inhibitor	0 (0.0)	2 (28.6)	
Baseline treatment for lipid abnormality [<i>n</i> (%)]			0.603
Diet only	10 (83.3)	5 (71.4)	
HMG-CoA reductase inhibitor (statin)	2 (16.7)	2 (28.6)	

Data are mean ± SD. Parameters were compared between groups (telmisartan versus losartan) by Mann-Whitney *U* test or chi-squared test.

TABLE 2: Changes in anthropometric measurements and blood pressure.

Parameters	Group	0 month	12 months	P-value	Difference	P-value
Body mass index (kg/m ²)	T	29.2 ± 5.8	29.0 ± 5.9	0.875	−0.2 ± 1.1	
	L	27.8 ± 3.8	28.1 ± 4.2	0.398	0.3 ± 0.8	0.447
Waist circumference (cm)	T	97.0 ± 14.9	98.2 ± 15.5	0.247	1.3 ± 3.4	
	L	94.4 ± 8.1	99.6 ± 8.6	0.091	5.3 ± 7.8	0.310
Hip circumference (cm)	T	111.9 ± 20.9	107.9 ± 15.0	0.500	−2.1 ± 8.3	
	L	99.6 ± 8.7	98.3 ± 9.1	0.655	−0.1 ± 0.4	0.754
Systolic blood pressure (mmHg)	T	139.4 ± 11.1	130.8 ± 15.0	0.045	−8.6 ± 15.2	
	L	136.4 ± 13.9	127.4 ± 10.6	0.046	−9.0 ± 10.4	0.933
Diastolic blood pressure (mmHg)	T	86.0 ± 8.5	75.4 ± 12.7	0.032	−10.6 ± 13.7	
	L	81.6 ± 13.0	75.3 ± 7.9	0.116	−6.3 ± 9.1	0.446
Pulse (beats/min)	T	74.5 ± 11.8	73.2 ± 9.4	0.397	−1.2 ± 5.8	
	L	85.0 ± 9.5	76.4 ± 11.5	0.273	−5.0 ± 10.2	0.397

Data are presented as mean ± SD. Parameters at 0 and 12 months of treatment were compared by Wilcoxon's matched-pair signed-rank test. Differences are shown as [value at 12 months − value at 0 month]. Differences between groups (telmisartan (T) versus losartan (L)) were compared by Mann-Whitney *U* test.

reduced to around the same levels as found in our study. Because the subjects had mild high levels of liver enzymes in the present study, it might have been difficult to observe marked improvement of liver enzyme levels.

It is notable that there was a significant decrease in serum FFA level in the telmisartan group compared to that in the losartan group in the present study. Reduction in serum FFA can improve insulin resistance and reduce fat deposition in the liver as ectopic fat [33, 34]. Here, the L/S ratio, which indicates fat deposition in the liver [35], was significantly increased in the telmisartan group but not in the losartan group, suggesting that this might be associated with the ability of telmisartan to activate PPARγ [16, 17]. However, in the losartan group, a low serum FFA level and a L/S ratio were

found at baseline compared to those in the telmisartan group, suggesting low insulin resistance and less fat deposition in the liver. Thus, this suggests that it would not be possible to observe improvement of FFA and L/S ratio in the losartan group. In addition, glycemic control deteriorated over 12 months in the losartan group.

It is reported that telmisartan, but not losartan, displayed insulin-sensitizing activity in a clinical study, which may be explained by its partial PPARγ activity [36]. Telmisartan might have a preventive effect against progressive deterioration of beta-cell function.

Although a few clinical trials have examined the effects of ARBs on NAFLD, they were mostly conducted in patients with NAFLD with markedly elevated liver enzyme levels.

TABLE 3: Changes in biochemical measurements.

Parameters	Group	0 month	12 months	P-value	Difference	P-value
Aspartate aminotransferase (IU/L)	T	30.6 ± 13.9	35.3 ± 19.0	0.583	4.8 ± 17.6	
	L	32.0 ± 10.3	32.3 ± 12.3	>0.999	0.3 ± 5.6	0.672
Alanine aminotransferase (IU/L)	T	39.8 ± 26.6	50.3 ± 32.3	0.261	10.5 ± 28.3	
	L	43.7 ± 26.2	46.4 ± 28.7	0.344	2.7 ± 8.6	0.672
γ Glutamyl transpeptidase (IU/L)	T	58.9 ± 43.0	69.2 ± 72.7	0.683	10.3 ± 56.8	
	L	60.9 ± 63.8	57.6 ± 57.8	0.463	-3.3 ± 9.2	0.471
Total cholesterol (mg/dL)	T	217.8 ± 42.3	212.4 ± 29.7	0.505	-5.3 ± 19.8	
	L	201.0 ± 38.9	198.9 ± 31.8	0.345	-2.1 ± 23.8	0.525
Triglyceride (mg/dL)	T	122.3 ± 54.3	128.5 ± 55.1	0.433	6.2 ± 54.3	
	L	120.9 ± 45.7	122.0 ± 50.9	0.866	1.1 ± 31.8	0.899
HDL cholesterol (mg/dL)	T	52.8 ± 13.1	51.1 ± 12.9	0.283	-1.8 ± 4.8	
	L	46.7 ± 9.2	45.7 ± 11.0	0.343	-1.0 ± 3.1	>0.999
Free fatty acids (mEq/L)	T	0.87 ± 0.26	0.59 ± 0.22	0.005	-0.28 ± 0.27	
	L	0.50 ± 0.26	0.66 ± 0.22	0.237	0.16 ± 0.29	0.007
Fasting plasma glucose (mg/dL)	T	116.5 ± 20.3	112.3 ± 11.1	0.247	-4.2 ± 15.5	
	L	122.8 ± 20.2	137.0 ± 28.6	0.078	14.2 ± 12.9	0.031
IRI (μ U/mL)	T	12.5 ± 6.1	14.5 ± 11.1	0.720	2.1 ± 7.5	
	L	12.6 ± 6.4	10.2 ± 3.8	0.498	-2.4 ± 4.8	0.396
Hemoglobin A1c (%)	T	6.4 ± 0.6	6.4 ± 0.4	0.552	0.0 ± 0.4	
	L	6.7 ± 1.0	7.2 ± 1.2	0.017	0.5 ± 0.2	0.001
HOMA-IR	T	3.55 ± 1.68	3.98 ± 2.92	>0.999	0.43 ± 2.45	
	L	3.84 ± 2.30	3.43 ± 1.46	0.686	-0.41 ± 1.63	0.770
HOMA- β	T	98.1 ± 71.2	115.5 ± 103.6	0.374	17.4 ± 42.8	
	L	84.5 ± 53.6	58.5 ± 33.6	0.043	-26.0 ± 29.6	0.062
Creatinine (mg/dL)	T	0.8 ± 0.2	0.9 ± 0.2	0.149	0.03 ± 0.06	
	L	0.8 ± 0.3	0.8 ± 0.3	0.655	0.00 ± 0.06	0.274
Uric acid (mg/dL)	T	5.8 ± 1.4	6.3 ± 1.2	0.016	0.4 ± 0.5	
	L	5.7 ± 1.5	5.2 ± 1.3	0.046	-0.5 ± 0.5	0.002
Primary bile acids (μ mol/L)	T	1.3 ± 1.7	2.0 ± 2.3	0.534	0.7 ± 3.0	
	L	3.0 ± 3.2	2.8 ± 2.6	0.345	-0.2 ± 1.6	0.447
Secondary bile acids (μ mol/L)	T	1.2 ± 1.0	1.4 ± 1.8	0.906	0.2 ± 1.3	
	L	2.4 ± 3.4	2.4 ± 2.5	0.735	0.0 ± 2.1	0.554
Microalbumin in urine (μ g/mL)	T	23.9 ± 43.9	24.5 ± 46.6	0.173	-0.5 ± 53.0	
	L	76.1 ± 113.7	39.5 ± 46.2	0.173	-36.6 ± 58.5	0.884

Data are presented as mean ± SD. Parameters at 0 and 12 months of treatment were compared by Wilcoxon's matched-pair signed-rank test. Differences are shown as [value at 12 months – value at 0 month]. Differences between groups (telmisartan (T) versus losartan (L)) were compared by Mann-Whitney *U* test. HDL: high-density lipoprotein, IRI: immunoreactive insulin, and HOMA-IR: homeostasis model assessment-insulin resistance.

However, epidemiologic studies conducted in Japan showed that liver enzyme levels remained only slightly elevated in many patients [37]. The present study included patients with NAFLD, which is often seen in daily clinical practice, and thus was meaningful in regard to examining the effect of ARBs in a more realistic setting. Furthermore, the present study is thought to be meaningful since the effects of telmisartan and losartan in treating NAFLD have not been examined in a randomized controlled study.

Our study has several limitations. First, the small number of patients and the deviation between groups in spite of randomization made it difficult to detect differences in outcomes between groups. Especially, differences in BMI and

duration of diabetes between groups might affect the results. Therefore, randomized controlled studies with larger numbers of patients might be needed in the future. Secondly, in this study, dietary instruction and exercise therapy were left entirely to the discretion of the outpatient attending physicians rather than implementing specific patient education programs. For this reason, although it was uncertain whether dietary and exercise therapy were sufficient or not in either group, an increase in BMI was not observed at least in the telmisartan group, suggesting that dietary and exercise therapy were probably sufficient. Lastly, we did not perform histological examination of fat deposition, inflammation, or fibrosis in the liver. It is thus unclear whether histological

TABLE 4: Changes in inflammatory markers.

Parameters	Group	0 month	12 months	P-value	Difference	P-value
Hyaluronic acid (ng/mL)	T	38.3 ± 29.1	51.9 ± 41.3	0.091	13.6 ± 23.9	
	L	57.5 ± 46.2	60.1 ± 44.5	0.345	2.6 ± 5.8	0.310
7S domain of type 4 collagen (ng/mL)	T	4.4 ± 0.7	4.5 ± 1.7	0.723	0.07 ± 1.46	
	L	4.4 ± 0.9	4.4 ± 0.9	0.834	0.03 ± 0.44	0.419
High-sensitivity C-reactive protein (mg/dL)	T	0.13 ± 0.10	0.20 ± 0.14	0.155	0.09 ± 0.15	
	L	0.13 ± 0.12	0.11 ± 0.09	0.176	−0.02 ± 0.04	0.077
Procollagen-3-peptide (U/mL)	T	0.57 ± 0.09	0.57 ± 0.13	0.656	−0.003 ± 0.134	
	L	0.51 ± 0.05	0.49 ± 0.11	0.351	−0.023 ± 0.098	0.766
Zinc (μg/dL)	T	88.8 ± 13.9	85.1 ± 17.7	0.139	−3.8 ± 7.8	
	L	88.0 ± 17.1	89.1 ± 15.6	0.735	1.1 ± 12.3	0.374
Total adiponectin (μg/mL)	T	7.3 ± 1.5	7.1 ± 2.1	0.553	−0.2 ± 0.9	
	L	7.8 ± 1.1	8.3 ± 1.5	0.753	0.5 ± 2.1	0.561
Interleukin-6 (pg/mL)	T	3.4 ± 5.5	2.5 ± 0.8	0.158	−0.9 ± 5.9	
	L	1.7 ± 1.0	2.3 ± 1.8	0.173	0.6 ± 1.0	0.766

Data are presented as mean ± SD. Parameters at 0 and 12 months of treatment were compared by Wilcoxon's matched-pair signed-rank test. Differences are shown as [value at 12 months – value at 0 month]. Differences between groups (telmisartan (T) versus losartan (L)) were compared by Mann-Whitney *U* test.

TABLE 5: Changes in fat distribution on CT scanning.

Parameters	Group	0 month	12 months	P-value	Difference	P-value
Visceral fat area (cm ²)	T	188.8 ± 73.7	188.8 ± 92.3	0.695	0.0 ± 47.5	
	L	170.8 ± 51.1	181.5 ± 23.6	0.345	10.7 ± 35.5	0.353
Subcutaneous fat area (cm ²)	T	257.1 ± 150.4	252.7 ± 171.5	0.875	−4.4 ± 52.0	
	L	252.9 ± 76.1	253.8 ± 65.5	0.834	0.9 ± 27.2	0.933
Visceral to subcutaneous fat ratio	T	0.92 ± 0.57	1.12 ± 1.02	0.754	0.19 ± 0.74	
	L	0.71 ± 0.26	0.75 ± 0.19	0.176	0.05 ± 0.15	0.398
Liver to spleen ratio	T	0.82 ± 0.25	0.97 ± 0.22	0.049	0.16 ± 0.24	
	L	1.01 ± 0.23	1.01 ± 0.21	>0.999	0.00 ± 0.16	0.272

Data are presented as mean ± SD. Parameters at 0 and 12 months of treatment were compared by Wilcoxon's matched-pair signed-rank test. Differences are shown as [value at 12 months – value at 0 month]. Differences between groups (telmisartan (T) versus losartan (L)) were compared by Mann-Whitney *U* test. CT: computer tomography.

changes occurred in the liver tissue due to treatment with either drug.

5. Conclusion

In this randomized controlled study that examined the effect of telmisartan and losartan in improving steatosis in hypertensive NAFLD patients with type 2 diabetes, significant improvement in liver function was not observed in either group. However, serum FFA level was significantly reduced in the telmisartan group compared to the losartan group. In addition, unlike losartan, telmisartan improved the L/S ratio. Due to its potential to improve fat deposition in the liver, telmisartan could be a therapeutic option in the treatment of NAFLD. In the future, a large-scale clinical study is needed to determine the utility of telmisartan in the treatment of NAFLD.

Authors' Contribution

Takumi Hirata, Kengo Tomita, and Toshihide Kawai contributed equally to the work described in this manuscript.

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Research Article

Relationship between Adiponectin Level, Insulin Sensitivity, and Metabolic Syndrome in Type 1 Diabetic Patients

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Objective. Adiponectin is known to be decreased in insulin resistance (IR) and metabolic syndrome (MS) which can be present in patients with type 1 diabetes mellitus (T1DM). The aim of this study was to evaluate the relationship between adiponectin level, MS, and insulin sensitivity in T1DM. **Research Design and Methods.** The study included 77 T1DM patients divided into two groups based on the total plasma adiponectin median value. Insulin sensitivity was calculated with the equation for eGDR, and MS was defined according to International Diabetes Federation criteria. **Results.** Patients with higher adiponectin level ($n = 39$) had significantly lower waist circumference ($P < 0.002$), fasting venous glucose levels ($P < 0.001$), higher HDL3-cholesterol ($P = 0.011$), and eGDR ($P = 0.003$) in comparison to the group with lower adiponectin who showed higher prevalence of MS ($P = 0.045$). eGDR increased for $1.09 \text{ mg/kg}^{-1} \text{ min}^{-1}$ by each increase of $1 \mu\text{g/mL}$ total fasting plasma adiponectin ($P = 0.003$). In the logistic regression model, adiponectin was inversely associated with the presence of MS ($P = 0.014$). **Conclusion.** Higher adiponectin concentration is associated with lower prevalence of MS in T1DM. Whether higher adiponectin concentration has a protective role in the development of the MS in T1DM needs to be clarified in future follow-up studies.

1. Introduction

White adipose tissue is a major site of energy storage and is important for energy homeostasis. It has been increasingly recognised as an important endocrine organ that secretes a number of biologically active “adipokines” like tumor necrosis factor- α , plasminogen activator inhibitor-1, leptin, resistin, angiotensinogen, and adiponectin [1]. Some of these adipokines are associated with obesity-related conditions. Adiponectin has attracted much attention because of its antidiabetic and antiatherogenic effect, and it might become a novel therapeutic tool for type 2 diabetes mellitus (T2DM) and metabolic syndrome (MS) [2, 3]. According to several studies, plasma concentration of adiponectin is reduced in human obesity, particularly visceral, and negatively correlated with insulin resistance (IR) [4, 5]. Additionally, hypoadiponectinemia is independently associated with the MS, surrogate marker of IR, and significantly related to T2DM development [5–7]. MS represents an important risk factor for mortality and micro- and macrovascular complications

development both in patients with type 1 diabetes mellitus (T1DM) and T2DM [8]. Adiponectin was found to accumulate in damaged vascular walls and beneficially modulate the endothelial inflammatory response to vascular injury as it possesses antiinflammatory and antiatherogenic properties [9]. Consequently, normal adiponectin concentrations or even induction of elevated concentrations are considered to be beneficial. However, increased adiponectin concentrations have been found to be associated with an increased cardiovascular mortality in T1DM [10, 11], but in cross-sectional data, the elevation in adiponectin levels has been hypothesised as a compensatory response in T1DM patients who have microvascular complications [10]. In addition, there are a growing number of patients with T1DM and MS who appear to be at increased risk of cardiovascular mortality and development of diabetes related complications, a greater need for higher insulin doses, and multifactorial intervention, thus, more aggressive treatment [12, 13]. Therefore, the purpose of this study was (1) to determine the relationship between total plasma adiponectin concentrations and parameters

associated with the MS: body mass index (BMI), waist circumference, lipid profile, blood pressure levels, fasting glucose levels, and insulin sensitivity, (2) to determine the relationship between adiponectin concentrations and MS prevalence, and (3) to evaluate the possible pathophysiological role of adiponectin in MS development in T1DM patients defined according to International Diabetes Federation (IDF) criteria.

2. Subjects, Materials, and Methods

This cross-sectional study was undertaken at the University Clinic for Diabetes, Endocrinology, and Metabolic Diseases Vuk Vrhovac, Zagreb, Croatia. The study population consisted of 77 T1DM patients coming for their comprehensive annual review with following characteristics: age of 18–65 years, minimum duration of type 1 diabetes of 1 year, no medical history of cardiovascular diseases or electrocardiogram (ECG) evidence of ischemic heart disease, absence of any systemic disease, and absence of any infections in the previous month. T1DM was defined as age at onset of diabetes younger than 35 years, positive autoantibodies, and time to definite insulin therapy less than a year. Patients were excluded from the study if they had taken any of the following: thyroid hormone therapy, medications that might affect glucose metabolism and insulin sensitivity such as glucocorticoids, oral contraceptives, and patients taking oral glucose-lowering medication. They could be using antihypertensive or lipid lowering drugs (i.e., statins: atorvastatin and simvastatin). Acute and chronic inflammation was excluded on the basis of medical history, physical examination, and routine laboratory tests, including measurement of temperature and urinalysis.

All subjects were studied in the morning between 08:00 am and 09:30 am after an overnight fast. Waist circumference was measured on bare skin as the narrowest circumference between the 10th rib and the iliac crest with tailor meter. Weight was measured by the physician using a balanced-beam scale with light clothing without shoes and expressed in kilograms (kg). Height was measured using a wall-mounted stadiometer and expressed in centimetres (cm). Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Blood pressure was measured in the sitting position with a mercury sphygmomanometer after a resting period of 10 minutes and expressed in mmHg. Fasting venous blood samples were collected for the determination of biochemistry panel, lipid profile status, HbA1c, liver biochemistry, and adiponectin. HbA1c was measured spectrophotometrically by turbidimetric immunoinhibition (Olympus AU600, Beckman Coulter, USA). Glucose, cholesterol, and triglycerides in serum were measured by an enzymatic colorimetric method. Total fasting adiponectin was measured using BioVendor's Human ELISA (sandwich) commercial kit for research use only. Insulin sensitivity was calculated using the equation derived from euglycemic-hyperinsulinemic clamp studies estimated glucose disposal rate (eGDR): $24.31 - 12.2 \times (\text{WHR}) - 3.29 \times (\text{AHT}) - 0.57 \times (\text{HbA1c})$, where the units are $\text{mg}/\text{kg}^{-1} \text{min}^{-1}$, WHR indicates the waist to hip ratio,

AHT indicates blood pressure and is expressed as 0—no, 1—yes. Those on blood pressure medications or with blood pressure $>140/90$ mmHg were considered to have hypertension [14]. This equation was derived from a substudy of 24 EDC (Epidemiology of Diabetes Complications) participants (12 men and 12 women drawn from low, middle, and high age-specific tertiles of insulin resistance risk factors in order to represent the spectrum of insulin resistance) who underwent euglycemic-hyperinsulinemic clamp studies [15]. It should be emphasized that lower eGDR levels indicate greater insulin resistance. According to the IDF guidelines, participants were defined as having MS if they had central obesity defined as waist circumference with ethnicity specific values (>80 cm in female and 94 cm in male population) plus any two of the following four factors: raised TG concentration: >150 mg/dL (1.7 mmol/L), specific treatment for this lipid abnormality, reduced HDL cholesterol: <40 mg/dL (1.03 mmol/L) in males and <50 mg/dL (1.29 mmol/L) in females, specific treatment for this lipid abnormality, raised blood pressure: systolic BP >130 or diastolic BP >85 mmHg, treatment of previously diagnosed hypertension, raised fasting plasma glucose (FPG): >100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes [16].

The study protocol complies with the Declaration of Helsinki as well as with local institutional guidelines and was approved by the local ethics committees. Written informed consent was obtained from all participants.

Baseline data were reported using descriptive statistics. Normality of distribution for continuous variables was analysed using Shapiro-Wilk test. Normally distributed variables were described with mean and standard deviation (SD), while variables that were not normally distributed were described with median, minimum, and maximum. The nominal variables were reported with absolute numbers and/or percentages. Differences between groups were examined, depending on the nature of the data, using parametric (*t*-test) or nonparametric tests (Mann-Whitney). Correlations between parameters of adiponectin with anthropometric and metabolic variables were determined using Pearson's or Spearman's correlation coefficient. The association between total fasting adiponectin concentrations and eGDR value was further evaluated in univariate linear regression, and in order to evaluate the association of total fasting adiponectin concentration with MS presence, we constructed multivariable binary logistic regression models to assess whether circulating total adiponectin concentration was independently associated with MS. Adjustments were performed for age, gender, disease duration, and the use of statins since they are shown to affect adiponectin secretion [17]. Level of statistical significance was chosen to be 0.05. Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) ver. 17.0 for Windows.

3. Results

The characteristics of the study subjects are listed in Table 1. The average age was approximately 46 years, median BMI was $25 \text{ kg}/\text{m}^2$, and 61% of patients were males with median diabetes duration of 21 years. Forty-five (58.4%) were using

TABLE 1: Demographic, anthropometric, and clinical characteristics of the 77 patients with type 1 diabetes.

Variable	Mean/median	SD/range
Age (years)	45.66	11.87
Gender (m/f)	47/30	
Duration of diabetes (years)	21.0	1.0–47.0
Weight (kg)	76	52–112
BMI (kg/m ²)	25	18–36
Waist circumference (cm)	85.6	11.8
HbA1c (%)	7.3	5.4–12.2
SBP (mmHg)	130	100–165
DBP (mmHg)	80	60–110
eGDR (mg/kg ⁻¹ min ⁻¹)	6.77	3.78–11.65
LDL cholesterol (mmol/L)	2.95	1.49–5.32
Total HDL cholesterol (mmol/L)	1.56	0.78–3.83
HDL2 cholesterol (mmol/L)	0.44	0.2–2.8
HDL3 cholesterol (mmol/L)	1.13	0.39–2.28
Triglycerides (mmol/L)	1.19	0.61
AST (U/L)	23.5	13–57
ALT (U/L)	24	12–95
GGT (U/L)	20	10–92
ALP (U/L)	76	18–155
Adiponectin (μg/mL)	14.10	3.4–31.4

ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: γ -glutamyltransferase, BMI: body mass index, DBP: diastolic blood pressure, HbA1c: glycated hemoglobin, eGDR: estimated glucose disposal rate, and SPB: systolic blood pressure.

antihypertensive therapy and 33 (42.9%) hypolipemic medications. Patients were divided according to the median adiponectin value (14.1 (3.4–31.4) μg/mL). The group of patients ($n = 38$) with lower adiponectin concentrations (<14.1 μg/mL) had significantly shorter disease duration, higher weight, BMI, waist circumference, GGT, and fasting venous glucose levels (8.45 versus 6.80 mmol/L, $P < 0.001$), while lower HDL3-cholesterol and eGDR compared to the group ($n = 39$) with higher adiponectin concentrations (>14.1 μg/mL) (Table 2). No significant difference was found in the use of antihypertensive (60.5% versus 56.4%) or hypolipemic (39.5% versus 46.2%) therapy between the two groups. Associations of adiponectin concentrations with anthropometric and metabolic variables are presented in Table 3. Disease duration, total HDL and HDL3 cholesterol showed significantly positive correlation with adiponectin concentrations; weight, BMI, waist circumference, systolic blood pressure, and eGDR showed significantly negative correlation with adiponectin concentrations. Between all study patients, 26 (33.8%) met the metabolic syndrome criteria.

In linear regression model, the unadjusted eGDR increased for 1.09 mg/kg⁻¹ min⁻¹ by each increase of 1 μg/mL total plasma adiponectin (CI (1.03–1.16), $P = 0.003$). In the multivariate binary logistic regression, total fasting plasma adiponectin was inversely associated with presence of MS after adjustment for age, sex, disease duration, and the statin

use (Table 4). The other variables included in the final model were not shown to be significant predictors of the MS presence (age OR = 0.977, $P = 0.459$; gender OR = 0.639, $P = 0.418$; disease duration OR = 1.022, $P = 0.452$; and the use of statins OR = 0.886, $P = 0.140$).

4. Discussion

Although pathophysiology of T1DM is defined with an autoimmune destruction of pancreatic β cells, increased IR has an important role in the development of so called “double diabetes” and greater risk for micro- and macrovascular complications [18]. MS and increased IR can be found in about 30%–40 % of T1DM patients depending on the used MS definition [19–21]. The association of lower adiponectin concentrations with the risk of MS development was previously shown in both healthy population and T2DM patients [5–7, 22].

Our results show that patients with higher plasma adiponectin concentrations had significantly lower BMI and waist circumference and higher HDL 3 cholesterol. The levels of systolic blood pressure, fasting plasma glucose, and triglyceride were also lower although they did not reach statistical significance. Additionally, the group with higher plasma adiponectin showed higher eGDR levels indicating higher insulin sensitivity which is in accordance with Pereira et al. (2012) [23] who reported that both total and high molecular weight (HMW) adiponectin positively correlate with insulin sensitivity assessed by hyperinsulinemic-euglycemic clamp in T1DM patients. Pham et al. (2013) [24] also investigated the association of total plasma adiponectin, anthropometric and metabolic parameters and found an inverse association of total plasma adiponectin and fasting glucose levels as well as BMI. About 35%–40% of the observed patient population met MS criteria, which is in accordance with the literature data [12, 18]. In the multivariate logistic regression after adjustment for age, gender, disease duration, and the use of statins, adiponectin concentration was significantly inversely associated with the prevalence of the MS. Those data suggest that higher adiponectin concentrations might play an important protective role in the development of MS in T1DM patients.

Adiponectin has been shown to have insulin-sensitizing effects through activation of AMPK in the peripheral tissues [25]. These effects include stimulation of fatty acid oxidation and glucose uptake in skeletal muscle and suppression of glucose production in the liver [26]. Several experimental studies suggested that administration of adiponectin ameliorates IR in lipoatrophic and T2DM while it decreases plasma glucose levels in healthy mice [26, 27]. For those reasons, adiponectin is considered to be one of the major insulin-sensitizing hormones strongly associated with IR related disorders. Additionally, regarding the glucose metabolism, Berg et al. (2001) have shown that the administration of adiponectin lowers circulating glucose levels without stimulating insulin secretion in both healthy and diabetic mice [28], and the data from two independent studies performed by Kriketos et al. (2004) as well as Kim et al. (2007) show that exercise induced increased insulin sensitivity in humans could be due

TABLE 2: Clinical and metabolic characteristics of patients depending on adiponectin concentrations.

Variable	Adiponectin < 14.10 $\mu\text{g/mL}$	Adiponectin > 14.10 $\mu\text{g/mL}$	P
Age (years)	43.8 \pm 13.72	46.26 \pm 9.79	NS
Gender (m/f)	31/7	16/23	NS
Duration of diabetes (years)	17.5 (1–41)	25 (2–47)	0.015
Weight (kg)	83 (52–112)	68 (54–103)	<0.001
BMI (kg/m^2)	26 (18–33)	24 (20–36)	0.027
Waist circumference (cm)	90.5 \pm 10.4	80.9 \pm 11.3	<0.002
HbA1c (%)	7.3 (5.41–11.1)	7.2 (5.4–12.2)	NS
SBP (mmHg)	130 (110–160)	125 (100–165)	NS
DBP (mmHg)	80 (60–110)	80 (60–100)	NS
eGDR ($\text{mg/kg}^{-1} \text{min}^{-1}$)	6.06 (3.79–10.01)	7.67 (3.78–11.65)	0.003
LDL cholesterol (mmol/L)	2.79 (1.83–5.20)	2.74 (1.49–5.32)	NS
Total HDL cholesterol (mmol/L)	1.46 (0.83–3.83)	1.66 (0.78–2.62)	NS
HDL2 cholesterol (mmol/L)	0.43 (0.20–2.80)	0.44 (0.21–1.05)	NS
HDL3 cholesterol (mmol/L)	1.06 (0.56–1.53)	1.23 (0.39–2.28)	0.011
Triglycerides (mmol/L)	1.27 \pm 0.64	1.11 \pm 0.58	NS
AST (U/L)	24 (15–42)	22 (13–57)	NS
ALT (U/L)	27.5 (12–91)	22 (12–95)	NS
GGT (U/L)	25 (13–92)	16 (10–59)	0.042
ALP (U/L)	77 (18–155)	75 (36–140)	NS
MS presence (n, %)	17 (44.7%)	9 (23.1%)	0.045

ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: γ -glutamyltransferase, BMI: body mass index, DBP: diastolic blood pressure, HbA1c: glycated hemoglobin, eGDR: estimated glucose disposal rate, and SPB: systolic blood pressure.

TABLE 3: Correlation analysis of adiponectin concentrations with metabolic and anthropometric variables.

Variable	
Duration of diabetes (years)	0.370*
Weight (kg)	−0.502*
BMI (kg/m^2)	−0.367*
Waist circumference (cm)	−0.512*
HbA1c (%)	0.076
SBP (mmHg)	−0.238*
DBP (mmHg)	−0.149
eGDR ($\text{mg/kg}^{-1} \text{min}^{-1}$)	0.332*
LDL cholesterol (mmol/L)	−0.143
Total HDL cholesterol (mmol/L)	0.332*
HDL2 cholesterol (mmol/L)	0.049
HDL3 cholesterol (mmol/L)	0.222*
Triglycerides (mmol/L)	−0.129
AST (U/L)	−0.062
ALT (U/L)	−0.107
GGT (U/L)	−0.199
ALP (U/L)	−0.34

* $P < 0.05$.

ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: γ -glutamyltransferase, BMI: body mass index, DBP: diastolic blood pressure, HbA1c: glycated hemoglobin, eGDR: estimated glucose disposal rate, and SPB: systolic blood pressure.

to elevation in total plasma adiponectin concentration in humans [29, 30].

TABLE 4: Logistic regression analysis of adiponectin concentrations and diabetes duration with development of metabolic syndrome in type 1 diabetic patients according to the IDF criteria.

Independent variable	Model A	Model B	Model C
(a) Adiponectin	0.91 (0.84–0.98)*	0.89 (0.82–0.97)*	0.89 (0.81–0.97)*

Data are OR (95% CI) from separate models. Model A crude; model B adjusted for age and sex, model C adjusted for age, sex, duration of diabetes, and the use of statins.

* $P < 0.05$.

Moreover, our finding of lower LDL cholesterol and TG as well as higher total HDL, HDL2, and HDL3 cholesterol in the group with higher plasma adiponectin is in accordance with a growing body of literature data suggesting that adiponectin has a direct effect on the regulation of lipid metabolism. Decreased adiponectin concentrations have been linked to higher LDL cholesterol and TG concentrations probably due to adiponectin directly affecting lipoprotein lipase [31, 32]. Data from two large cross-sectional studies indicate that circulating adiponectin concentrations are negatively correlated with triglyceride concentrations and strongly positively correlated with plasma HDL concentrations [32, 33]. There is even an assumption based on several study reports that treatment with some of the most used lipid lowering drugs such as statins and fibrates increases plasma adiponectin concentration which might contribute to their beneficial effect in LDL and triglyceride lowering effect [34]. The LDL cholesterol particle is the major cholesterol transporter

shown to be a strong independent risk factor for atherosclerotic events associated with MS [35]. Regarding HDL cholesterol and the process of atherogenesis, it has been shown that both quantitative and qualitative particle alterations play an important role [36]. In particular, circulating HDL2 cholesterol was found to protect from atherosclerosis [37, 38], while we previously observed that HDL3 cholesterol might protect from progression of renal disease in type 1 diabetes [38]. That might be of particular importance in this population as T1DM represents a chronic condition with an increased cardiovascular mortality [10, 11]. Beyond observed, adiponectin might exert a protective effect on atherogenesis by preventing endothelial damage and decreasing blood pressure levels.

There are several studies suggesting the inverse correlation of circulating adiponectin concentrations and elevated arterial blood pressure levels in healthy subjects as well as in diabetic population which is also in concordance with our results [39, 40]. It has recently been reinforced by a 5-year prospective study by Chow et al. (2007) who found hypoadiponectinemia to be a good predictor of hypertension development even after adjustment for risk factors such as BMI, sex, and age [41]. The inverse correlation between adiponectin concentration and waist circumference was previously described in several studies on MS [8, 13], that is, intra-abdominal fat mass was found to influence the concentration of circulating adiponectin more than subcutaneous fat. Despite whether hypoadiponectinemia is a cause or a consequence of intraabdominal obesity has not been fully addressed yet, so the significantly lower waist circumference and BMI in our group of patients with higher adiponectin concentrations might possibly be explained with an independent adiponectin effect on weight loss. As suggested by Fruebis et al. (2001), adiponectin administration induces weight loss without decreasing food intake in mice consuming a high-fat high-sucrose diet [42].

Additionally, in agreement with adiponectin modulating liver function previously mentioned, we also found a negative correlation between plasma adiponectin concentrations and hepatic biomarkers, that is, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl-transferase (GGT), and alkaline phosphatase (ALP). This is with accordance with the results of López-Bermejo et al. (2004) who reported that adiponectin concentrations were associated with plasma concentrations of various liver function tests in healthy humans suggesting that adiponectin deficiency is an important risk factor for the development of fatty liver, steatohepatitis, and other forms of liver injury [43].

The present study has a number of potential limitations. First, our study was cross-sectional, which limited our ability to infer a causal relation between adiponectin concentrations and development of MS in T1DM. Second, we measured insulin sensitivity using clinical parameters with eGDR and did not have access to direct, detailed measures of insulin resistance using euglycemic-hyperinsulinemic clamp test. Third, our analyses were based on a single measurement of total fasting adiponectin that may not reflect the relation over time.

In conclusion, our results highlight the importance of the relationship between adiponectin, IR, and the presence of the MS in T1DM. Whether adiponectin is a marker of MS as well as all components of MS according to the IDF criteria in T1DM patients and whether higher adiponectin concentrations have a protective role in the development of the MS in T1DM need to be clarified in future follow-up studies.

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Research Article

The Association of Sleep Disorder, Obesity Status, and Diabetes Mellitus among US Adults—The NHANES 2009-2010 Survey Results

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To examine the association between sleep disorders, obesity status, and the risk of diabetes in adults, a total of 3668 individuals aged 40+ years from the NHANES 2009-2010 without missing information on sleep-related questions, measurements related to diabetes, and BMI were included in this analysis. Subjects were categorized into three sleep groups based on two sleep questions: (a) no sleep problems; (b) sleep disturbance; and (c) sleep disorder. Diabetes was defined as having one of a diagnosis from a physician; an overnight fasting glucose > 125 mg/dL; Glycohemoglobin > 6.4%; or an oral glucose tolerance test > 199 mg/dL. Overall, 19% of subjects were diabetics, 37% were obese, and 32% had either sleep disturbance or sleep disorder. Using multiple logistic regression models adjusting for covariates without including BMI, the odds ratios (OR, (95% CI)) of diabetes were 1.40 (1.06, 1.84) and 2.04 (1.40, 2.95) for those with sleep disturbance and with sleep disorder, respectively. When further adjusting for BMI, the ORs were similar for those with sleep disturbance 1.36 (1.06, 1.73) but greatly attenuated for those with sleep disorders (1.38 [0.95, 2.00]). In conclusion, the impact of sleep disorders on diabetes may be explained through the individuals' obesity status.

1. Introduction

Over the past two decades, a dramatic increase in prevalence of obesity has been witnessed globally. As the result, the incidence and prevalence rates of type 2 diabetes (T2D) have also been elevated significantly. In the United States, it has been estimated that currently approximately a third of adults are obese (defined as BMI ≥ 30 kg/m²) [1], and over 11% of people aged 20 years and older have diabetes [2]. Among those who are obese, approximately 60% have T2D [3]. The increasingly common early onset of T2D is not surprising as more children become overweight and obese [4]. The precise mechanisms linking obesity status and T2D remain unclear, but the progress of insulin resistance is considered as the key to this link [5]. Sleep problems are common among those obese subjects who are at a great risk for developing T2D [6]. The emerging evidence indicates that sleep-related problems may play a role in the development of these concurrent illnesses [7]. A clear understanding of the interrelationships between sleep-related problems, obesity,

and T2D may provide information that would allow effective strategies for prevention and/or intervention of these two diseases. In this report, the National Health and Nutrition Examination Survey (NHANES) 2009-2010 data was used to examine the associations between sleep status, obesity status, and T2D.

2. Methods

2.1. NHANES 2009-2010 and Its Participants. The NHANES 2009-2010 is a stratified, multistage probability design survey conducted between 2009 and 2010 with approximately 10,500 people aged 0 year and over being involved. Details of the survey design and measurement procedures can be found elsewhere [8]. We restricted our sample to those whose age was 40 years and older since T2D is the most common type of diabetes mellitus among adults and diabetes among individuals aged 40 and above is most likely T2D. A total of 4,009 subjects aged 40 years and older were identified

in the NHANES 2009-2010 data set. After removing individuals with missing information on sleep-related questions, variables related to diabetes, body mass index, and pregnant women, a total of 3,668 individuals (1,813 men and 1,855 women) were included in this analysis.

2.2. Sleep Disorders Measurements. Three sleep-related questions were asked, in the home, by trained interviewers using the Computer-Assisted Personal Interviewing (CAPI) system. They are “how much sleep do you usually get at night on weekdays or workdays?” “Have you ever told a doctor or other health professionals that you have trouble sleeping?” and “Have you ever been told by a doctor or other health professional that you have a sleep disorder?” The first question was measured by hours in sleep and the other two questions as “yes” versus “no” responses. Using the last two questions, all subjects were grouped into three categories; that is, group one, no sleep problems if the answers for both questions were “no”; group two, having a sleep disturbance if “yes” to the first question, but “no” to the second question; and group three, having a sleep disorder if “yes” to the second question. Since it has been suggested that either shorter sleep hours or longer sleep hours are associated with the increased risk for cardiovascular disease [9], sleeping hour responses are also categorized into three groups <7, 7-8, and >8 hours.

2.3. Body Mass Index Related Measurements. Body mass index (BMI) was derived from body weight and standing height and was used to determine obesity status of the participants in the survey. Both weight and height are part of body measurements, which were measured in the Mobile Examination Center (MEC), by trained health technicians following the 2009-2010 NHANES anthropometry protocol [10]. Participants wore the standard MEC examination gown, which consists of a disposable shirt, pants, and slippers. Weight was measured using a digital weight scale and measured to near 0.1 kg. Standing height was measured using a stadiometer and measured to 0.1 cm. BMI was calculated as weight (kg) divided by the square of their standing height (m^2) and then categorized into three groups, that is, BMI < 25.0 kg/ m^2 , BMI: 25.0–29.9 kg/ m^2 , and BMI \geq 30.0 kg/ m^2 . The last two groups were considered as overweight and obese, respectively [11]. There are 95 subjects (51 men and 44 women), about 2.6% of the study sample, whose BMI were below 18.5 kg/ m^2 , which is considered as underweight [12]. Similar results are found when excluding these subjects; therefore, we reported our results with them included.

2.4. Diabetes Mellitus Related Measurements. Diabetes mellitus is defined as either having been diagnosed with diabetes mellitus by a physician, or a fasting glucose level >125 mg/dL (6.9 mmol/L), or a 2-hour value in the oral glucose tolerance test (OGTT) >199 mg/dL (11.1 mmol/L), or HbA1C level \geq 6.5% [13]. Blood was drawn at the MEC, and blood specimens were processed, stored, and shipped to Fairview Medical Center Laboratory at the University of Minnesota, Minneapolis Minnesota for analysis. The concentration of glucose was measured using enzymatic method, and the

level of HbA1C was measured using G7 Glycohemoglobin Analyzer [14]. Diabetes mellitus was the main outcome and was coded as 1 = yes and 0 = no in the analysis.

2.5. Measurements of Covariates. Covariates in the analysis included age (years), gender (male versus female), ethnicity (non-Hispanic white versus others), education (less than high-school versus high-school or higher), marital status (living with a spouse or partner: yes versus no), ratio of family income to poverty threshold, currently cigarette smoking (yes versus no), alcohol drinking (average drinks per day), sedentary activity time per day (minutes), total to HDL cholesterol ratio, systolic blood pressure (mmHg), and C-reactive protein (mg/dL).

2.6. Statistical Analysis. All analyses were conducted using survey procedures in SAS 9.3 (SAS Institute Inc., Cary, NC, USA), and take into account the weighted and clustered sampling design of NHANES. The significant level was defined at 2-tailed alpha equal or less than 0.05. Odds ratio (OR) and its 95% confidence interval (CI) from logistic regression models were used to examine the relationship between diabetes and sleep disorder status, and four models have been used for this purpose. Diabetes mellitus is the dependent variable in each model, and two indicator variables were created for people having a sleep disturbance and having a sleep disorder, respectively. Subjects who reported no sleep issues are considered as the reference in the analyses. The covariates in Model One were age, gender, race, education, ratio of family income to poverty, and marital status; in Model Two were the covariates in Model One plus total to HDL cholesterol ratio, systolic blood pressure, sedentary active time, alcohol drinking, and cigarette smoking; in Model Three were the covariates in Model Two plus C-reactive protein and sleep duration; and in Model Four, BMI was added to the analysis.

To examine the impact of interaction of sleep disorder and obesity status on diabetes mellitus, we further created eight indicators to indicate people's sleep disorder and obesity status, that is, (1) reporting a sleep disturbance and BMI < 25.0 kg/ m^2 ; (2) reporting a sleep disorder and BMI < 25.0 kg/ m^2 ; (3) reporting no sleep problems and BMI: 25.0–29.9 kg/ m^2 ; (4) reporting a sleep disturbance and BMI: 25.0–29.9 kg/ m^2 ; (5) reporting a sleep disorder and BMI: 25.0–29.9 kg/ m^2 ; (6) reporting no sleep problem and BMI \geq 30.0 kg/ m^2 ; (7) reporting a sleep disturbance and BMI \geq 30 kg/ m^2 ; and (8) reporting a sleep disorder and BMI \geq 30 kg/ m^2 . Those people reporting no sleep issues and BMI < 25 kg/ m^2 were used as the reference, and all covariates in the Model Three in previous analyses were included.

3. Results

Approximately 19% individuals in this sample were categorized as diabetes mellitus. The prevalence rates of having sleep disturbance and sleep disorder were 22.1% and 9.4%, respectively. Based on BMI measurements, approximately 35% of people were in the overweight group and 37% were in

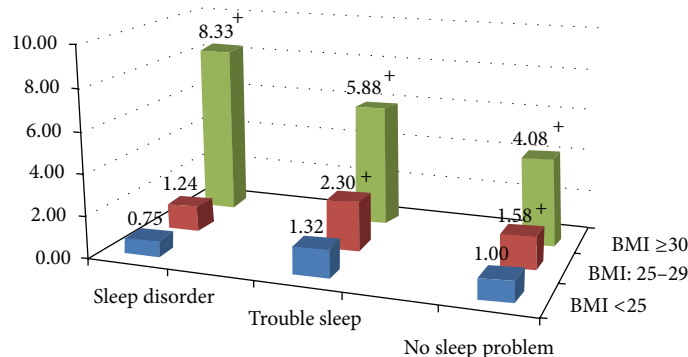


FIGURE 1: The ORs of diabetes by different combinations of sleep and obesity status after adjustment for age, gender, race, education, ratio of family income to poverty, marital status, total to HDL cholesterol ratio, systolic blood pressure, sedentary activity time, alcohol drinking, cigarette smoking, C-reactive protein, and sleep duration. The group of BMI < 25.0 with no sleep problem is the reference group. ⁺ *P* value < 0.05.

obese group. Characteristics of participants by sleep disorder status are shown in Table 1. Compared to people without sleep problem, individuals with sleep disturbance had similar profiles apart from being more likely to be female, non-Hispanic white, less likely to live with a spouse or partner, having a higher level of C-reactive protein, and shorter sleep duration. Those having sleep disorder were statistically different from those without sleep problems except for age, ratio of family income to poverty, lifestyle variables, total to HDL cholesterol ratio, blood pressure measurements, and percentage male.

The ORs and their 95% CIs derived from logistic regression models are presented in Table 2. The odds of diabetes for the first three models are similar. Compared to those having no sleep problem, the ORs of diabetes in Model Three for those with a sleep disturbance and those with a sleep disorder were 1.40 (95% CI: 1.06, 1.84) and 2.04 (95% CI: 1.40, 2.95), respectively. When further adjusting for BMI in Model Four, the OR for those with a sleep disturbance did not change much (OR (95% CI): 1.36 (1.06, 1.73)) but was greatly attenuated for those having a sleep disorder (1.38 (0.95, 2.00)).

Figure 1 shows the ORs of diabetes for different combinations of sleep and obesity status. After adjustment for all covariates used in Model three in the previous analysis and using those reporting no sleep problems and BMI < 25 kg/m² as the referent group, the odds of diabetes for those with a sleep disturbance or a sleep disorder were similar if their BMI was < 25 kg/m². The ORs (95% CIs) of T2D for sleep disturbance and sleep disorder were 1.32 (0.69, 2.49) and 0.75 (0.28, 1.99), respectively, when they were in the group of BMI < 25 kg/m². The ORs (95% CIs) for those with BMI: 25.0–29.9 kg/m² were 1.58 (1.16, 2.15) in the group of reporting no sleep problem, 2.30 (1.37, 3.86) in the group of reporting a sleep disturbance, and 1.24 (0.57, 2.68) in the group of reporting a sleep disorder. While the ORs (95% CIs) for those with a BMI ≥ 30 kg/m² were 4.08 (2.59, 6.41), 5.88 (3.53, 9.80), and 8.33 (4.81, 14.49) for those in the groups of reporting no sleep problem, a sleep disturbance, and a sleep disorder, respectively.

To confirm that the results from the previous analyses were not due to the change of the sample size from model to model, we used a multiple imputation method [15] to create a database with all missing values imputed, and the results are similar (data not shown).

4. Discussion

Although evidence indicates that sleep problems increase the risk of cardiovascular disease (CVD) [16–18], and a similar risk association has been observed between sleep problems and diabetes mellitus [19–21], it is unclear whether the impact of sleep problems on T2D may be mediated through obesity. Because sleep disorders are prevalent among people with overweight and obesity, which is also a risk for T2D [22–24]; when examining the risk association of T2D with sleep problems in cohort studies, researchers previously took into account the impact of BMI measured at baseline [25–28]. Yet, the results from these cohort studies are not consistent. For instance, a cohort of women from Gothenburg, Sweden, were followed for 32 years, and no significant associations or trends were observed between incidence of diabetes and any of the available sleep indicators; however, obesity status, using either BMI or waist circumference, was highly associated with sleep problems [26]. The results from the Nurses Health Study suggested that sleep restriction might mediate the effect of weight change on diabetes mellitus [25]. However to date, no study has closely examined these associations. Using recent NHANES data, we explored this issue and found that people with sleep disorders are indeed associated with a great risk of diabetes mellitus independently from a number of known risk factors without including BMI. However, after taking BMI into account the odds ratio of diabetes mellitus is similar for those with sleep disturbance but significantly attenuated for those with sleep disorders. This suggests that the impact of sleep disorders on diabetes mellitus may be confounded by BMI. Results from the model with different combinations of sleep and obesity status (Figure 1) further demonstrate that the risk association of diabetes mellitus with sleep disorders may be mediated through obese status. Subjects not in the

TABLE 1: Basic characteristics of 3668 participants in the NHANES 2009-2010 by sleep disorder status.

	None	Sleep disturbance	Sleep disorders
<i>n</i>	2551	773	344
Demographic measures			
Age (yrs, mean [S.E.])	57.9 [0.3]	58.2 [0.7]	56.9 [0.7]
Male (%)	50.5	37.3 [^]	51.5
Non-Hispanic white (%)	72.1	79.9 [^]	76.3 [^]
Less than high school (%)	20.9	19.6	17.6 [^]
Living with spouse or partner (%)	70.9	63.2 [^]	61.3 [^]
Ratio of family income to poverty (mean [S.E.])	3.23 [0.04]	3.17 [0.09]	3.1 [0.14]
Cigarette smoking (%)	14.4	15.6	14.9
Alcohol drinking (average drinks/day, mean (S.E.))	1.9 [0.4]	1.4 [0.1]	1.5 [0.14]
Body composition measures			
Body mass index (kg/m ² , mean [S.E.])	28.5 [0.2]	29.3 [0.3]	32.9 [0.5] [^]
Waist circumference (cm, mean [S.E.])	99.2 [0.5]	100.8 [0.8]	109.4 [1.3] [^]
Measurements from blood sample			
Total to HDL cholesterol ratio (mean [S.E.])	4.06 [0.03]	4.0 [0.06]	4.1 [0.11]
C-reactive protein (mg/dL, mean [S.E.])	0.33 [0.01]	0.51 [0.06] [^]	0.54 [0.06] [^]
Glycohemoglobin (% mean [S.E.])	5.77 [0.03]	5.86 [0.04]	5.98 [0.06] [^]
Blood pressure and pulse measures			
Systolic blood pressure (mmHg, mean [S.E.])	124.7 [0.6]	125.1 [0.9]	125.0 [1.3]
Diastolic blood pressure (mmHg, mean [S.E.])	70.1 [0.7]	69.8 [0.7]	69.8 [1.3]
Sedentary activity (min, mean [S.E.])	336.4 [6.0]	351.9 [10.7]	389.9 [11.6] [^]
Sleep duration (hours, mean [S.E.])	7.09 [0.03]	6.51 [0.08] [^]	6.54 [0.11] [^]
Diabetes (%)	17.1	20.9	27.2 [^]

[^]P < 0.05 when compared to "none."

TABLE 2: Adjusted odds ratio of diabetes for sleep disorder status, NHANES 2009-2010.

	<i>N</i>	None	OR (95% confidence interval)	
			With a sleep disturbance	With a sleep disorder
Model 1	3300	1.00	1.43 (1.07, 1.89)	2.08 (1.39, 3.09)
Model 2	3188	1.00	1.44 (1.05, 1.99)	2.14 (1.44, 3.17)
Model 3	3185	1.00	1.40 (1.06, 1.84)	2.04 (1.40, 2.95)
Model 4	3148	1.00	1.36 (1.06, 1.73)	1.38 (0.95, 2.00)

Model 1: adjustment for age, gender, race, education, ratio of family income to poverty, and marital status.

Model 2: further adjustment for total to HDL cholesterol ratio, systolic blood pressure, sedentary activity time, alcohol drinking, and cigarette smoking.

Model 3: further adjustment for C-reactive protein and sleep duration.

Model 4: further adjustment for BMI.

overweight or obese groups but having a sleep disturbance or disorder had similar odds for diabetes mellitus as those with no sleep problems. However, for those in the overweight group (BMI: 25–29.9 kg/m²), the odds of T2D are higher regardless of their sleep status, while those in the obese group had over four folds higher odds of T2D and those with a sleep disorder had the highest one.

Laboratory studies conducted among healthy adults have shown that short-term sleep deprivation could increase each of insulin resistance, sympathetic tone, blood pressure, and C-reactive protein level [29–31]. Observational studies have reported that children with sleep problems are more likely to be overweight and obese [32, 33]. It appears that having a healthy weight through exercise may be the key variable in both overweight/obesity prevention and intervention [34]

and that the preschool years are a key time for shaping relevant attitudes and behaviours [35]. As physical activity also has a positive effect on sleep pattern, its benefits may go beyond simple weight control [36, 37]. Although in this report, we cannot test the role of physical activity on the association between sleep disorders, obesity status, and diabetes mellitus, the significantly longer sedentary time among those individuals with sleep disorders in comparison to those reporting no sleep problems suggests that physical activity is a critical element for intervention.

Compared to waist circumference, BMI has been criticized for not being a good indicator of central obesity in the risk prediction of cardiovascular disease [38]. Central obesity is a prevalent trait among people with sleep disorders, but results from a meta-analysis indicated that both BMI and

waist circumference are similarly associated with the risk of diabetes since they are highly correlated to each other [39]. The Pearson correlation coefficient between the body mass index and waist circumference in our data is 0.9, and the replacement of BMI with waist circumference in the Model 4 shows similar results—ORs (95% CI) of diabetes for sleep disturbance and sleep disorders are 1.31 (1.01, 1.68) and 1.28 (0.88, 1.85), respectively. This suggests that the impact of sleep problems on diabetes is indeed affected by individual's obesity status though different sleep problems show different interactions.

Several limitations must be recognized when interpreting the results of this study. First, this was a cross-sectional study, and therefore conclusions about causal associations cannot be made. Second, the sleep-related problems were self-reported and may not reflect the true picture of sleep status. However, the results from a study indicate that self-reported sleep duration was relatively accurate [40]. The “yes/no” questions on whether one has reported having trouble sleeping to a physician or whether a physician has told the person that he or she has a sleep disorder are quite straightforward and should be reliable. Third, we did not control for the impact of physical activity in this study, but we did control for the self-reported sedentary activity time, which may be underreported due to a social desirability bias. However, we assume that this underreported time is random and should not differ with different sleep status. The strengths of this study are (1) the data are from a well-designed nationally representative survey; (2) the laboratory measurements are validated and reliable; and (3) we have adjusted for a number of known risk factors including sleeping duration.

In conclusion, sleep disturbance is independently associated with an increased risk for T2D. However, the impact of sleep disorders on T2D may be explained by obesity status. Longitudinal research is needed to examine the relationship between sleep problems, changes in obesity status, and their impacts on the development of T2D.

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Clinical Study

Physical Fitness in Children with Type 1 Diabetes Measured with Six-Minute Walk Test

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Aim/Hypothesis. To examine whether children with DM1 are less physically fit than healthy children and to assess whether an elevated level of HbA1c was associated with decreased physical fitness among children with diabetes. **Methods.** The study was conducted using case-control methodology. The cases were 100 children with T1DM, 7–17 years. Study subjects underwent a 6MWT, where distance measured, heart rate, and oxygen saturation was recorded. **Results.** Results of the 6MWT for children with T1DM and controls were 601.3 ± 86.1 meters versus 672.1 ± 60.6 meters, respectively ($P < 0.001$). The cases were divided into two subgroups, one with HbA1c levels $>8\%$ and one with HbA1c $<8\%$. Results for both groups were inferior to the controls ($P < 0.001$). The posttest pulse rate in all subjects was higher than the pretest pulse rate ($P < 0.001$). Pulse oxygen levels were lower than controls at the pretest measurement ($P < 0.001$), and for both cases and controls, pulse oxygen levels decreased after test ($P = 0.004$). However, the change in oxygen saturation did not differ between the groups ($P = 0.332$). **Conclusions.** Children with T1D are less fit than matched controls. The level of HbA1c did not affect the physical fitness of children with T1D.

1. Introduction

Diabetes mellitus type 1 is a multifactorial autoimmune disease characterized by complete destruction of pancreatic beta cells and loss of insulin production, caused by multiple genetic and environmental influences [1].

Young people with type 1 diabetes (T1D) have been found to have decreased aerobic capacity and lower cardiorespiratory fitness levels compared to nondiabetic control subjects [2, 3].

Serum concentration of HbA1c is the “gold standard” for the assessment of both therapeutic efficacy and the risk of development of diabetic microvascular and macrovascular complications [4].

Various factors are associated with glycemic control including age, sex, diabetes duration, and management (frequency of blood glucose monitoring, insulin regimen, and dose adjustments), as well as those indirectly connected to diabetes care (family history, dietary, cultural habits, etc.) [5].

Physical activity also plays an important role of glycemic control. Nondiabetic individuals have a reduction in insulin

secretion and an increase in glucose counterregulatory hormones that facilitate an increase in liver glucose production that matches skeletal muscle glucose uptake during exercise, and consequently glucose levels during physical activity remain stable [6, 7].

In patients with T1D the pancreas does not regulate insulin levels in response to exercise, and there may be impaired glucose counterregulation, making normal fuel regulation nearly impossible [8]. In T1D patients with poor glycaemic control, there is insufficient amount of insulin, and counterregulatory hormones induced by physical activity will cause a further increase in blood glucose levels. In contrast, increased amounts of insulin present in the circulation will reduce or even prevent the mobilization of glucose which can result with hypoglycemia [9]. Regular physical activity has a particularly positive effect by improving insulin sensitivity and allows for a better use of the synthesis of glycogen and fat as energy sources [7, 10].

The six-minute walk test (6MWT) is a quick, simple and inexpensive method of determining physical fitness. It is also an important clinical test used to determine the quality of life

[11]. It measures the distance achieved during a smooth walk with a constant effort for six minutes on a flat, hard surface. This is a simple method for estimating the physical stamina during submaximal effort. It is routinely used to assess the condition of people with cardiovascular and pulmonary diseases [12]. Initially the test was twelve minutes long and was used to test the healthy population. Cooper used it in the study of physical fitness of American pilots [13]. The test has been used in evaluation of patients with chronic obstructive pulmonary disease and the American Thoracic Society has developed guidelines for its use [14, 15]. Additionally the 6MWT has been used for monitoring the course of disease and response to therapy in patients with rheumatism, pulmonary, cardiovascular, and endocrine diseases [14–25]. Factors that have been shown to affect the test results include age, gender, leg length, body weight, various diseases, and the motivation of the subjects undergoing testing [15]. In children the 6MWT is being increasingly used [26–28].

2. Objective

The objectives of this research are

- (1) investigate whether children with T1D are less physically fit than healthy children;
- (2) establishing the influence of HbA1c level on physical fitness.

3. Subjects and Methods

3.1. Patients. We examined all children (186 children) aged 7–18 from Split—Croatia and Mostar—Bosnia and Herzegovina who have type 1 diabetes.

After examining medical history from the study children with cardiorespiratory disease and anemia were excluded. Also, patients with blood glucose levels were excluded (below 4, 0 and above 14 mmol/L), just prior to exercise testing or who tested positive for ketone.

The study group consisted of 100 children with T1D aged 7–18 years from the Mostar—Bosnia and Hercegovina and Split—Croatia, without clinical cardiopulmonary disease or anemia.

The control group consisted of the same number of healthy individuals from primary and secondary school, of equal age and sex, with a similar height (not greater than 2 cm), weight (not greater than 2 kg), length of the lower extremities, BMI, and who were not acutely ill a month before the test. Table 1 presents the demographic and anthropometric measures for the cases and controls.

At a predetermined arrangement with the principal and class, children were provided with informational flyers that were submitted to parents for review and eventual approval. Signed approval was given by parents and the participant. The study was approved by the Ethics Committees of the University Hospital Mostar and the University Hospital Split. The diagnosis of T1D was made based on the criteria of the American Diabetes Association [29].

TABLE 1: Presentation of the groups studied in relation to body height, weight, length of the lower extremities, and BMI.

Variables	Mean \pm standard deviation		<i>P</i> *
	Test group	Control group	
Number of participants	100	100	
Height (cm)	159.9 \pm 14.3	160.1 \pm 13.9	0.942
Weight (kg)	51.1 \pm 15.0	50.98 \pm 14.7	0.964
Lower extremities length (cm)	78.9 \pm 8.1	78.8 \pm 7.5	0.964
BMI (kg/m ²)	19.4 \pm 3.5	19.5 \pm 3.3	0.955

* Student *t*-test.

3.2. Anthropometric and Other Measurements. The participants weight (kg) and height (cm), body mass index (BMI—weight in kg divided by height in meters squared), heart rate (beats per minute), blood pressure (mm Hg), the lower segment of the body from the upper edge of the symphysis to the floor in upright position (cm), and blood oxygen saturation SpO₂ (%) (CMS-50E fingertip Pulse Oximeter OLED) were determined in order to match participants and compare their performance.

In a group of type 1 diabetic patients HbA1c was measured within ± 10 days of 6MWT by spectrophotometry (DCA 2000+, Siemens, Germany). Capillary blood glucose (CBG) level measured by a glucose meter was performed before and after the 6MWT. All patients had breakfast and an insulin injection administered 2 h to 2.5 h earlier.

3.3. 6MWT. The test consisted of the six-minute walking on a flat, hard surface 20 meters long (20 meters forward and 20 meters back). Participants used the measuring wheel (Nedo GmbH & Co. KG, Dornstetten, Germany) which was held by hand. The measuring wheel has different handle lengths (240, 370, and 560 mm) that were changed depending on the height of participant. Each participant chose the handle of appropriate length. Participant had not engaged in serious physical activity for at least two hours before the test.

The test was performed in a separate room, and a dial on the wheel was covered during the performance test to rule out the possibility of competition between participants.

On the floor a distance of 20 meters was measured and marked by cones. Each participant was given the opportunity to experience the wheel and select the optimal handle size. Then he was given instructions for the test which read: “The purpose of this test is to walk as fast as you can in six minutes and cross as many meters you can. You will walk around the set cones. You are allowed to slow down and stop, if necessary, and lean on the wall and when you can, continue. It is forbidden to run. Again, the purpose is to cross as many meters as you can. If you’re ready, go!” Time was measured by a stopwatch, and every minute the respondents were informed by standard phrases: after the first minute, “Well done, you have five minutes to the end,” after another minute, “Keep it up, you got four more minutes,” after three minutes, “Well done, you’re half-way,” after four minutes, “Keep it up,

TABLE 2: Presentation of the groups studied in relation to the mileage meter.

Variables	Mean \pm standard deviation		P^*
	Test group	Control group	
Number of participants	100	100	
Crossed distance	601.3 \pm 86.1 m	672.1 \pm 60.6 m	<0.001

* Student t -test = 6.718; $P < 0.001$.

you got two more minutes,” and after five minutes, “Just keep going, you have another minute.” No other words of encouragement as well as body language were used to avoid the possibility of encouraging the participant to speed up or slow down. The examiner at all times stood in the middle of the walking track to control the accuracy of the test. After six minutes the participant was asked to stop. Immediately after that participant was placed in a sitting position where blood oxygen saturation and heart rate were measured. Then we noted how many meters the participant had crossed in six minutes.

3.4. Statistical Analysis. Statistical analysis included the Kolmogorov-Smirnov test for symmetry of distribution of continuous variables. For a description of their assembly and disintegration the arithmetic mean and standard deviation was used. Comparison of two normally distributed independent variables was performed using Student's t -test. Comparison of continuous variables measured at multiple time points was made by the test of repeated measurements. Unlike the distribution of nominal variables an ordinal χ^2 test was used. The possibility of a type I error was set at $\alpha < 0.05$ and differences between groups were accepted as statistically significant at $P < 0.05$.

For statistical analysis we used SPSS for Windows (version 13.0, SPSS Inc. Chicago, IL, USA) and Microsoft Excel (version 11, Microsoft Corporation, Redmond, WA, USA).

4. Results

The study and control groups included 49 girls (49.0%) and 51 boys (51.0%), and there were no significant differences in sex ratio (χ^2 test = 0.040, $df = 1$, $P = 0.841$). The average age was 13.0 ± 2.9 years (mean \pm SD), range 7.0 to 17.9 years.

Test and control groups did not differ significantly in average height (Student t -test = 0.073, $P = 0.942$), in average body weight (Student t -test = 0.046, $P = 0.964$), in the average length of the lower extremities (Student t -test = 0.045, $P = 0.964$), and in mean body mass index (Student's t -test = 0.043, $P = 0.955$) (Table 1).

Combining both cases and controls, the average value of the 6MWT was 636.7 ± 82.3 meters, range (360.3–808.0) meters.

The cases had significantly shorter distance measured in meters compared to the control group (Student t -test = 6.718, $P < 0.001$) (Table 2).

According to the values of HbA1c test group was divided into two subgroups. The first group consisted of children with

TABLE 3: Presentation of the groups studied with HbA1c levels lower than 8.0% and their controls in relation to the mileage meter.

Variables	Mean \pm standard deviation		P^*
	Test group	Control group	
Number of participants	40	40	
Crossed distance	599.8 \pm 86.7 m	669.2 \pm 62.3 m	<0.001

* Student t -test = 4.109; $P < 0.001$.

TABLE 4: Presentation of the groups studied with the level of HbA1c $\geq 8.0\%$ and their controls in relation to the mileage meter.

Variables	Mean \pm standard deviation		P^*
	Test group	Control group	
Number of participants	60	60	
Crossed distance	602.4 \pm 86.4 m	674.1 \pm 59.9 m	<0.001

* Student t -test = 5.279; $P < 0.001$.

the values of HbA1c $< 7.9\%$ ($n = 40$). In the second group children with the values of HbA1c $\geq 8.0\%$ ($n = 60$) were included. Both subgroups were compared with their pairs in the control group.

Children with lower levels of HbA1c of 8.0% exceeded by significantly shorter distance the corresponding control group (Student's t -test = 4.109, $P < 0.001$) (Table 3).

Children with levels of HbA1c $\geq 8.0\%$ walked a significantly shorter distance than the corresponding control group (Student t -test = 5.279, $P < 0.001$) (Table 4).

Observing the entire sample, cases, and control groups pretest heart rate was significantly slower (88.3 ± 14.7), compared to the heart rate after the test (131.2 ± 20.7) ($F(1.198) = 931.573$; $P < 0.001$).

There were no significant differences in heart rate before and after the test between the two groups ($F(1.198) = 1.733$, $P = 0.190$) (Figure 1).

Observing the entire sample, cases, and control group, oxygen saturation (%) before the test was significantly higher (98.8 ± 1.0) compared to the oxygen saturation (%) after the test (98.6 ± 1.0) ($F(1.198) = 8.529$, $P = 0.004$).

A downward trend in the oxygen saturation between the two groups, before and after the test, showed no statistically significant difference ($F(1.198) = 0.948$, $P = 0.332$).

Taking into consideration the overall value of oxygen saturation before and after the test, the test group had significantly lower values of saturation (98.3 ± 1.1) compared to the control group (99.1 ± 1.1) ($F(1.198) = 51.238$, $P < 0.001$) (Figure 2).

5. Discussion

In our study, we compared the physical fitness of children with T1D with controls. The results of our study indicate that children with T1D are less physically fit than a matched set of healthy control children (Table 1).

Physical activity has a positive effect on health [30]. The ability of trained muscle to take and oxidize free fatty acid

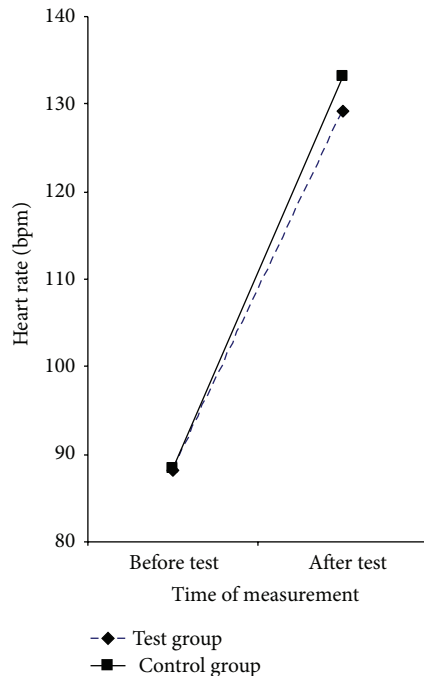


FIGURE 1: Presentation of the groups studied in relation to the value of the heart rate before and after the test.

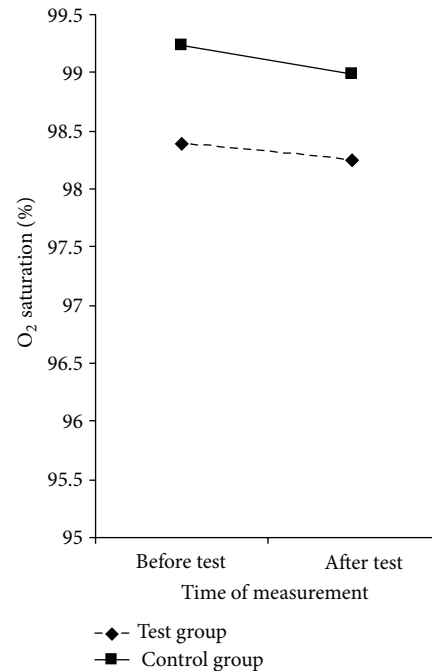


FIGURE 2: Presentation of the groups studied compared to the value of oxygen saturation before and after the test.

reduces blood lipid levels. The positive effect on blood pressure significantly reduces the overall risk of cardiovascular disease [31]. Maintaining fitness and ideal body weight promotes self-esteem and self-satisfaction [32]. All these effects are particularly important in patients with diabetes who have chronic hyperglycemia as they are exposed to additional risks. Regular physical activity improves insulin sensitivity and reduces the daily need for exogenous insulin. In children with T1D the frequency of regular physical activity was associated with lower HbA1c without increasing the risk of severe hypoglycemia [33].

Despite the positive effect that physical activity has on blood glucose, controlled studies have not confirmed a long-term improvement in metabolic control in patients with T1D. Physical activity may be just one element in the complex therapy of diseases. Another possible explanation is that patients avert the risk of hypoglycemia before physical activity by underdosing insulin or by injecting carbohydrate, and this offsets the benefits of exercise [34].

The 6MWT is a reliable test for the assessment of physical fitness [11]. In many countries all over the world the standards of normal values for different age groups have been established [26–28, 35]. The test can be used to monitor the progress of disease and response to therapy in patients with various diseases. Dos Santos Alves et al. used the 6MWT for monitoring lung capacity in patients with idiopathic scoliosis [16]. Otto Lelieveld used the 6MWT in children with juvenile idiopathic arthritis [17]. However, the most common use of 6MWT is in pulmonary and cardiovascular diseases [18–23]. Novak et al. applied 6MWT in diabetics who have neuropathy as a complication of their primary disease and showed that patients with severe leg pain have more difficulty in walking

than patients with mild pain or no pain which significantly affects their quality of life [24]. 6MWT represents a practical and reliable assessment tool for exercise performance in overweight and obese children and adolescents [36, 37].

To the best of our knowledge in the literature, we have not found studies that used the 6MWT in children with T1D. We found results documented from one pilot study. Physical condition of seven type 1 diabetic girls aged 8–10 years was given exercise scheme activities and examined with a 6MWT before and three months after [25].

Thanks to the precise regulation in nondiabetic individuals glucose levels during physical activity remain stable [6, 7]. In T1D the pancreas does not regulate insulin levels in response to exercise, and there may be impaired glucose contraregulation. In poor controlled diabetic patients, there is an insufficient amount of insulin, and contraregulatory hormones in physical activity cause a further rise in blood glucose levels. In contrast, the increased amount of insulin present in the circulation will reduce or even prevent the mobilization of glucose which can result in hypoglycemia; it may explain the weaker results of physical fitness of the group with T1D [38]. Although we hypothesized that that subjects with well-controlled diabetes would perform better on the 6MWT than those with poorly controlled diabetes [39], our results did not confirm this (Tables 2 and 3). The study group was divided into two groups: well and poorly controlled. A HbA1c of 8.0% was taken as the dividing boundary. Then these groups were compared with their controls. It is known that HbA1c reflects levels of glycemia over the preceding 4–12 weeks [7]. The reason why is this value taken as the limit comes in the following facts. From the clinical side of view, we can say that the value of HbA1c of 7.9% and

less is acceptable especially if it is known that the majority of respondents were in puberty, when many adolescents experience a deterioration in metabolic control. One of the reasons for this is the greater insulin resistance at puberty [40].

The difference in heart rate frequency was statistically significant when comparing values before and after the test, which means that all respondents gave their maximum during the performance test (Figure 1). It is also very important to note that there was no difference in heart rate frequency between the groups suggesting that both groups were equally motivated and goal-directed in the test. The better result of physical fitness in the control group is not a result of greater effort than the test group. Given that the cases were free of vascular complications of diabetes, the difference in oxygen saturation between the groups was surprising (Figure 2). This could be explained by possible functional and structural abnormalities in the peripheral blood vessels caused by diabetes and poor cardiorespiratory fitness of these patients, although the trend of oxygen saturation in both groups remained the same. The question remains whether the reduced performance of children with T1D is associated with lower levels of physical activity or is it a result of their illness [3, 41]. Children with T1D have been found to have decreased aerobic capacity measured by VO₂ max and also to have lower heart rate at exercise exhaustion compared to nondiabetic control subjects. The authors postulated that individuals with T1D had decreased lung ventilation associated with decreased maximal O₂ consumption and exercising capacity [2]. Children with T1D aged 5–14 years had reduced cardiorespiratory fitness levels compared to nondiabetic control children [3]. Recent research in patients with type 2 diabetes reports less blood flow to the periphery in physical activity [42]. As the oxygen saturation is measured at the fingertip, this may explain our result.

6. Conclusions

We found that children with T1D are less physically fit than matched healthy controls as measured by the 6MWT.

We also found that the level of HbA_{1c} did not affect the physical fitness of children with T1D.

Future research is needed to confirm these results and should investigate whether the reduced physical fitness in children with T1D is attributable to physiological changes resulting from the diabetes pathology itself.

Conflict of Interests

All previously mentioned authors disclose that there was not any financial or personal conflict of interests that might influence their work. All previously listed authors state that the submitted paper has not been previously published or is not under review in any other journal.

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Research Article

Determinants of Microbial Load in Infected Diabetic Foot Ulcers: A Pilot Study

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We examined the determinants of microbial load in infected diabetic foot ulcers in 62 patients (38 men and 24 women, mean age: 65.63 ± 12.71 years) with clinically infected diabetic foot ulcers. Tissue cultures were taken from ulcers by 4 mm punches. Ulcer grade (University of Texas classification), neuropathy disability score (NDS), neuropathy symptom score (NSS), ankle-brachial index (ABI), perfusion, extent, depth, infection, and sensation (PEDIS) grade of diabetic foot infection, and laboratory parameters were evaluated in all patients. Total microbial load was positively correlated with the number of isolates on tissue cultures ($r_s = 0.544$, $P < 0.001$), white blood cell count (WBC) ($r_s = 0.273$, $P = 0.032$), and platelet count (PLT) ($r_s = 0.306$, $P = 0.015$). It also exhibited a borderline insignificant positive correlation with PEDIS infection grade ($r_s = 0.246$, $P = 0.053$). In stepwise linear regression analysis, the number of isolates on tissue cultures and WBC were identified as the only two significant parameters accounting for 38% of the variation in the log of total microbial load (adjusted $R^2 = 0.380$, $P < 0.001$). In conclusion, patients with infected diabetic foot ulcer exhibit a positive correlation of total microbial load with the number of isolates on tissue cultures, WBC and PLT.

1. Introduction

In both epidemiological surveys and everyday clinical practice, the diabetic foot remains a major cause of patient morbidity [1–3] and nontraumatic lower extremity amputations [4–6]. Ischaemia, neuropathy, and infection are the three cardinal aetiological factors predisposing to diabetic foot ulcers [3, 7]. Some progress has been accomplished in the management of these conditions [7], including revascularisation [8] and improved pharmacology for peripheral arterial disease [9], neuroprotective agents [10, 11], new antibiotics [12, 13], growth factors [14, 15], and adjunctive treatment modalities [16–18], but there is a considerably long way to go to improve outcomes [2, 4, 7].

In particular, diabetic foot infections may be extremely challenging to cure [3, 7, 19]. Some of the therapeutic difficulty arises from late diagnosis (due to blunted clinical signs [3, 7, 13]), presence of ischaemia [7, 20], difficult-to-treat Methicillin-resistant *Staphylococcus aureus* (MRSA) [21, 22] or other multidrug-resistant pathogens, and spread

of infection to the bones, leading to osteomyelitis [23–25]. Characteristics of foot ulcers (chronicity, extension, and depth), prior antibiotic use, and presence of peripheral arterial disease, generally, have a considerable impact on bacterial pathogens in infected foot ulcers [7, 25–28], but there is no reliable way of predicting types of pathogens and microbial load [7, 13]. This is important because a high number of foot ulcers are nowadays already infected at initial presentation [29]. Therefore, the aim of the present study is to examine the determinants of microbial load in infected diabetic foot ulcers.

2. Patients and Methods

This study included 62 patients with clinically infected diabetic foot ulcers presenting to the Outpatient Clinic of the Diabetic Foot of the Second Department of Internal Medicine at Democritus University of Thrace, Greece. Patient characteristics are presented in Table 1. The study was approved by

TABLE 1: Patient characteristics.

Parameter	Value
Age (years, mean \pm SD)	65.63 \pm 12.71
Sex (males/females)	38/24
Diabetes type (2/1)	59/3
Diabetes duration (years, mean \pm SD)	16.31 \pm 8.07
Ulcer duration (median, IQR)*	2 (1.0–4.25)
ABI (mean \pm SD)	0.94 \pm 0.34
NDS (mean \pm SD)	7.02 \pm 2.33
NSS (median, IQR)	2 (0–4)
PEDIS infection grade (median, IQR)	2 (2–3)
Total microbial load (CFU _s , median, IQR)	275000 (0.0–24900000)
Number of isolates on tissue culture (median, IQR)	1 (0–2)
WBC (mean \pm SD)	8142.58 \pm 2174.97
PLT (mean \pm SD)	271685.48 \pm 81581.41
Ht% (mean \pm SD)	36.77 \pm 4.56
CRP (mg/dL, median, IQR)	1.2 (0.72–2.93)
Urea (mg/dL, median, IQR)	41.50 (34.00–56.25)
Creatinine (mg/dL, median, IQR)	1.00 (0.80–1.20)
AST (U/L, mean \pm SD)	22.85 \pm 9.84
ALT (U/L, mean \pm SD)	24.39 \pm 10.79
HbA _{1c} (% , mean \pm SD)	8.29 \pm 1.50
LDL (mg/dl, mean \pm SD)	114.53 \pm 38.65
HDL (mg/dl, mean \pm SD)	44.07 \pm 10.82
TC (mg/dl, mean \pm SD)	189.32 \pm 44.83
TGs (mg/dl, mean \pm SD)	159.61 \pm 50.26
SUA (mg/dl, mean \pm SD)	5.24 \pm 1.62
CPK (U/L, mean \pm SD)	126.03 \pm 78.18
FPG (mg/dl, mean \pm SD)	171.18 \pm 56.65

*Duration of ulcer in months, as based on medical history.

ABI: ankle-brachial index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CFUs: colony-forming units; CPK: creatine phosphokinase; CRP: c-reactive protein; FPG: fasting plasma glucose; HDL: high-density lipoprotein cholesterol; Ht%: haematocrit; IQR: interquartile range; LDL: low-density lipoprotein cholesterol; NDS: neuropathy disability score; NSS: neuropathy symptom score; PEDIS: grade of diabetic foot infection, according to the International Working Group on the Diabetic Foot; PLT: platelet count; SD: standard deviation; SUA: serum uric acid; TC: total cholesterol; TGs: triglycerides; WBC: white blood cell count.

the institutional ethics committee and all patients gave their informed consent.

Foot ulcers were defined as skin defects located beneath the malleoli and extending through all skin layers [30, 31]. Infection of foot ulcers was based on clinical presentation, requiring ≥ 2 of the following criteria: local swelling or induration; erythema greater than 0.5 cm in any direction around the ulcer; local tenderness or pain; local increase of temperature; purulent discharge [13]. Ulcer grade was based on the University of Texas (UT) classification system [32],

and clinical severity of infection was quantified according to the PEDIS system proposed by the International Working Group on the Diabetic Foot [31]. Ulcer duration was measured in months, as based on medical history of tissue breakdown. Diagnosis of osteomyelitis was based on positive probe-to-bone test and/or positive magnetic resonance imaging [33]. Patients with osteomyelitis were excluded.

Moreover, patients were examined for diabetic polyneuropathy by the neuropathy disability score (NDS), a standardised clinical examination system incorporating loss of ankle reflexes and sensory deficits in the feet [34]. Peripheral arterial supply was evaluated by the ankle-brachial index (ABI) measured by a Doppler apparatus [35, 36]. Neuropathic symptoms were assessed by the neuropathy symptom score (NSS) [34]. Glycated haemoglobin (HbA_{1c}), c-reactive protein (CRP), full blood count, and biochemical parameters were measured in blood samples.

Patients had not been treated with antibiotics for 1 week prior to examination. Following appropriate debridement, deep-tissue cultures were taken from ulcers by 4 mm biopsy punches (Kai Europe GmbH, Solingen, Germany), as previously described [28]. Specimens were placed in sterile transport containers, which were delivered to the Microbiology Laboratory within 20 minutes.

Quantitative tissue cultures were performed using standardised procedures [37], as described in [28]. Tissue specimens were weighed, homogenised, and diluted with 5 mL of Thioglycolate broth. Serial 10-fold dilutions to 10⁴ were made with 0.85% NaCl, and 0.1 mL of each dilution was plated onto the appropriate media. Samples for aerobic cultures were inoculated into Columbia sheep blood agar and MacConkey agar plates and were then incubated at 35°C for 24–48 hours. Samples for anaerobic cultures were inoculated into Brucella agar with 5% sheep blood supplemented with vitamin K and haemin and were then incubated at 35°C for 48–72 hours in anaerobic jars (Gas Pak EZ Gas Generating Container System, Becton Dickinson, Sparks, MD, USA). Identification of species was based on the automated system Vitek 2 and the Api 20A (BioMerieux, Marcy l'Etoile, France) [28]. Total microbial load was expressed as number of colony-forming units (CFUs) per g of tissue.

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 19. Normality of distribution was evaluated by Kolmogorov-Smirnov test. Normally distributed variables were expressed as mean \pm Standard Deviation and variables without normal distribution as median and range. Correlations of isolate numbers and total microbial load were examined by Spearman's rank coefficient. We also performed stepwise linear regression analysis using log of total microbial load as independent variable. Significance was defined at the 5% level (two-tailed $P < 0.05$).

3. Results

Correlations of total microbial load are presented in Table 2. Total microbial load was positively correlated with the

TABLE 2: Correlations of total microbial load.

Correlations of total microbial load	<i>P</i> value
Number of isolates on tissue cultures	$r_s = 0.544, P < 0.001$
WBC	$r_s = 0.273, P = 0.032$
PLT	$r_s = 0.306, P = 0.015$
PEDIS infection grade	$r_s = 0.246, P = 0.053$
Age	$r_s = -0.147, P = 0.253$
Diabetes duration	$r_s = -0.20, P = 0.880$
Ulcer duration*	$r_s = 0.048, P = 0.712$
UT ulcer stage	$r_s = 0.202, P = 0.116$
NDS	$r_s = -0.119, P = 0.359$
ABI	$r_s = -0.032, P = 0.806$
NSS	$r_s = -0.101, P = 0.436$
Ht%	$r_s = 0.051, P = 0.694$
CRP	$r_s = 0.140, P = 0.278$
Urea	$r_s = -0.005, P = 0.969$
Creatinine	$r_s = 0.048, P = 0.710$
AST	$r_s = 0.000, P = 1.000$
ALT	$r_s = -0.068, P = 0.599$
HbA _{1c}	$r_s = 0.228, P = 0.075$
LDL	$r_s = 0.113, P = 0.382$
HDL	$r_s = 0.051, P = 0.692$
TC	$r_s = 0.130, P = 0.315$
TGs	$r_s = 0.104, P = 0.420$
SUA	$r_s = 0.096, P = 0.458$
CPK	$r_s = -0.197, P = 0.125$
FPG	$r_s = 0.015, P = 0.908$

*Duration of ulcer in months, as based on medical history.

ABI: ankle-brachial index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CPK: creatine phosphokinase; CRP: c-reactive protein; FPG: fasting plasma glucose; HDL: high-density lipoprotein cholesterol; Ht%: haematocrit; LDL: low-density lipoprotein cholesterol; NDS: neuropathy disability score; NSS: neuropathy symptom score; PEDIS: grade of diabetic foot infection, according to the International Working Group on the Diabetic Foot; PLT: platelet count; SUA: serum uric acid; TC: total cholesterol; TGs: triglycerides; UT: University of Texas; WBC: white blood cell count.

number of isolates on tissue cultures ($r_s = 0.544, P < 0.001$), white blood cell count (WBC) ($r_s = 0.273, P = 0.032$), and platelet count (PLT) ($r_s = 0.306, P = 0.015$). It also exhibited a borderline insignificant positive correlation with PEDIS grade of diabetic foot infection ($r_s = 0.246, P = 0.053$). Moreover, the number of isolates on tissue cultures exhibited a positive correlation with platelet count ($r_s = 0.339, P = 0.007$).

In stepwise linear regression analysis, log of total microbial load was used as independent variable, while dependent variables included number of isolates on tissue cultures, age, diabetes duration, ulcer duration, PEDIS grade of diabetic foot infection, ulcer grade, WBC, PLT, HbA_{1c}, ABI, NDS, and NSS. The number of isolates on tissue cultures and WBC were identified as the only two significant parameters accounting for 38% of the variation in the log of total microbial load (adjusted $R^2 = 0.380, F = 19.712, P < 0.001$).

4. Discussion

The present study found a positive correlation between total microbial load and the number of isolates on tissue cultures. Indeed, the latter was one of the 2 parameters significantly influencing the variation of the former. This is not surprising, given that total microbial load was calculated by adding CFUs per g tissue of each isolate. In practice, our finding shows a high microbial load to be encountered in polymicrobial infections. Consequently, the clinician should be alerted that a high microbial load calls for aggressive antibiotic management with use of agents targeting multiple pathogens.

Moreover, total microbial load exhibited a positive correlation with some markers of inflammation, that is, WBC and PLT, though not with CRP. WBC was the other parameter significantly contributing to the variation of total microbial load in stepwise linear regression analysis. Inflammatory markers have been used in the study of foot infections [38–41]. The commonest applications include diagnosis of infection [39, 40], distinction between soft-tissue infection and osteomyelitis [38, 41], as well as differential diagnosis of infection from Charcot osteoarthropathy, in which serum markers are, generally, normal [42]. This study adds the association of WBC and PLT with high microbial load in patients with clinically infected diabetic foot ulcers.

We also found a borderline insignificant correlation of total microbial load with clinical severity of infection, as expressed by the PEDIS grade of diabetic foot infection [31]. This finding should be interpreted in the light of available evidence that type and number of microorganisms cannot be reliably predicted on the basis of clinical manifestation [1, 7, 12, 13]. Essentially, a number of factors may influence the clinical manifestation of infection, notably ischaemia and neuropathy, both of which may blunt inflammatory response [3, 7, 43]. An alternative explanation for the absence of significant correlation is the homogeneity of our study population, inasmuch as patients had infection grade PEDIS 2 or 3 only.

Conversely, total microbial load exhibited no association with ulcer duration. Generally, long-standing ulcers are pre-disposed to colonisation and infection [1, 7, 12, 13]. However, this propensity does not equate to development of high microbial load, as shown by our findings. Indeed, infection has now been documented to be very common even at initial presentation of diabetic foot ulcers [29].

Of note, there was no association between total microbial load and ulcer stage. This is most likely due to the fact that all patients had UT stage 1 or 2 ulcers and not more severe lesions, so that such relationship could not be documented. We also found no association of total microbial load with age and diabetes duration. This agrees with current knowledge that the aforementioned factors do not relate to the severity of foot infections [3, 7].

Interestingly, total microbial load did not correlate with ABI. We have previously found no difference in the number of isolates on tissue cultures, in the frequency of high microbial load, and in the number of CFUs/g tissue between patients with neuropathic and those with neuroischaemic infected foot ulcer [28]. Based on the new and on the prior data, it is plausible that the adequacy of arterial blood flow itself is

not of paramount importance in determining microbial load among patients with infected diabetic foot ulcer. Instead, the impact of ischaemia is crucial on treatment outcomes [7, 44], which should not be overlooked in clinical practice [2].

Similarly, total microbial load did not correlate with clinical severity of neuropathy (NDS) and of neuropathic symptoms (NSS). Arguably, this novel finding may be seen as increasing our knowledge on the pathogenic role of neuropathy. While patients with more severe neuropathy are at increased risk of foot ulceration [1, 2, 30, 45], and while ulceration, in turn, increases the risk of superimposed infection, severity of neuropathy per se appears not to affect the total microbial load. This does not negate the pivotal role of neuropathy in the development of foot ulceration [1–3, 45–47] but shows that other factors determine microbial load in the case of infection complicating ulceration.

The strengths of this study are the use of quantitative tissue cultures and the homogeneous study population. Indeed, microbial load was quantified on deep-tissue specimens and not on superficial swabs, which may, generally, be criticised for harbouring contaminating skin flora as well [12, 13]. Of particular importance, we excluded patients with osteomyelitis, in whom the microbial load of infected bones and not of soft tissues would be relevant [13, 23, 33]. The limitations include the relatively small patient series and the underrepresentation of type 1 diabetes, given that the vast majority of patients had type 2 diabetes. Of additional note, all patients presented with clinically infected foot ulcers. Hence, our results cannot be readily extrapolated to subjects with uncomplicated foot lesions.

The clinical implications of our findings may be summarised as follows. In patients with clinically infected diabetic foot ulcers, microbial load is associated with increased number of isolates on tissue cultures, as well as elevated WBC, and PLT counts. It is conceivable that this information may prove useful for the choice of initial empirical antibiotic regimen, inasmuch as patients with elevated WBC and/or PLT may be taken to require more aggressive antibiotic regimen with broad coverage for a polymicrobial infection. However, more experience with this interpretation is desirable.

In conclusion, our results indicate that patients with infected diabetic foot ulcers exhibit a positive correlation of total microbial load with number of isolates on tissue cultures, WBC and PLT. Conversely, no such association is seen with severity of ischaemia and peripheral neuropathy. These findings should be seen in the context of the clinician's attempt to estimate severity of infection and choose the initial antibiotic regimen. Findings reported herein might prove useful in this endeavour, but further confirmation is awaited.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Seeking Out High Risk Population: The Prevalence Characteristics and Outcome of Diabetic Patients of Arab Ethnicity Hospitalized in Internal Medical and Acute Coronary Units in Israel

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Aims. To seek high risk population for diabetes and to improve their health care by investigating the characteristics and outcome of hospitalization in hospitals with predominant Arab patients in Northern Israel. **Methods.** Retrospective analysis of the prevalence of diabetes and the outcome of diabetic in comparison to nondiabetic patients hospitalized in the internal medicine and intensive cardiac units in two major hospitals with one-year postdischarge data between 1.1.2009 and 31.12.2009. **Results.** Thirty-nine percent of the patients were diagnosed with diabetes. The preponderance of women in the diabetes group was noted. Diabetic patients had an increase in the duration of hospitalization ($P = 0.0008$), with one hospital having a high readmission rate for the diabetic patients. The average glycemia during hospitalization exceeded the recommended threshold of 180 mg% without major changes in the therapeutic regimens in comparison to preadmission regimens. **Conclusions.** Arab populations, women in particular, in westernizing societies are at high risk for diabetes which exemplifies as high rate of patients with diabetes among hospitalized patients. Areas for intervention during hospitalization and at predischage have been identified to improve health outcomes and prevent readmissions.

1. Introduction

The prevalence of diabetes is increasing globally, associated with an increase in obesity and in sedentary lifestyle [1–3]. This is associated with morbidity and mortality due to the effects of hyperglycemia related complication and by its association with atherosclerosis-heart disease and stroke, as the leading causes for mortality in diabetics. Hospitalization for acute internal and coronary disorders is therefore high among patients with diabetes; estimates from several studies show that the percentage of patients with diabetes admitted is up to three times the percent of diabetes in the relevant population [4].

The increase in the prevalence diabetes and obesity is, however, not distributed evenly between different racial and

ethnic groups, with some groups showing higher susceptibility to environmental changes brought by immigration or rapid socioeconomic changes.

The Arab population in Israel has recently been observed to have an alarming high prevalence of diabetes, reaching 50% in women above 50 years old [5]. This high prevalence seems to be higher than observed in Arab countries or in Arab immigrants to the USA or Western Europe. The unique situation of this population calls for both studies and intervention on a national level to promote health and prevent the consequences of the metabolic perturbation [6].

Considering the high prevalence of diabetes in the Arab population in Israel, we hypothesized that we will find substantial number of patients in the internal and the intensive coronary unit (ICU) wards diagnosed and undiagnosed

TABLE 1: Analysis between controls and diabetes patients (2 hospitals), demographic data.

	Control		Diabetes		P value
	N	%	N	%	
Gender					
Male	421	55.0	701	47.1	0.0003
Female	344	45.0	788	52.9	
Ethnicity					
Arab	710	92.8	1351	90.7	0.1551
Jew	26	3.4	76	5.1	
Other	29	3.8	63	4.2	
Dwelling					
Urban	447	58.4	364	24.4	<0.0001
Non urban	317	41.4	1121	75.2	
UK	1	0.1	5	0.3	
Smoker					
No	374	48.9	930	65.9	<0.0001
Yes	244	31.9	372	26.3	
UK	147	19.2	110	7.8	

diabetic patients. Seeking such a high risk population will enable the characterization of demographic, clinical state and diabetes related parameters. This data will serve to form intervention strategy in order to enhance health related outcomes.

2. Material and Methods

2.1. Population. The study was performed at the Internal Medicine wards and ICU of the Holy Family (HFH) Hospital and the Nazareth Hospital in Nazareth. The HFH hospital (established 1882) has 32 beds in the Internal Ward and ICU. The Nazareth Hospital was established by the Edinburgh Medical Mission Society in 1861 in which 40 beds are in the internal ward and the ICU. Both wards have affiliated with the Bar Ilan medical school, Zefat. These two hospitals serve the population of Nazareth and surrounding villages and towns. Estimated population served is 463,000. The majority of the population is urban. The rural population has shifted from mainly an agrarian society toward an urban like society. Both hospitals serve mainly the Arab population in Northern Israel with an estimated population of 253,000.

2.2. Definitions. Diabetes was defined as either known or unknown diabetes.

Known Diabetes: pervious diagnosis and/or treatment with diabetes related medication.

Unknown Diabetes: two measurements of blood glucose > 200 mg% and/or HbA1c > 6.5%.

Dwelling was defined as urban residing in the areas of major cities Nazareth and Acre; rural-residing in the areas of smaller towns and villages.

2.3. Data Retrieved. Data on all patients hospitalized in the Internal Medicine and ICU units in HFH and Nazareth

hospitals between 1.1.2009 and 31.12.2009 were reviewed. Charts of all diabetics were probed for data concerning demographics and previous diagnosis, and data pertaining to the hospitalization cause and the outcomes. Population census was searched for one-year after discharge for mortality data. All patients who were hospitalized during the months: January, April, July, and October served as a control group. Data was collected similarly to the diabetes cohort.

2.4. Statistical Methods. All measured variables and derived parameters were tabulated by descriptive statistics. For descriptive statistics summary tables were provided giving sample size, absolute and relative frequency for categorical variables and sample size, arithmetic mean, standard deviation, median, minimum, and maximum for continuous variables.

The following statistical tests were used in the analysis of the data presented in this study.

Chi-square test was applied for testing the statistical significance of the differences in frequency of categorical variables between the study groups.

The two-sample t-test was applied for testing the statistical significance of the differences of continuous variables between the study groups.

Analysis of Covariance (ANOVA) was applied for comparing the differences of continuous variables between the outcomes of patients.

Logistic Regression was applied using patient's death as outcome and glucose on day 1 as predictor variable.

Multiple Regression was applied using days of hospitalization as outcome and glucose on day 1 as predictor variable.

All tests applied were two-tailed, and *P* value of 5% or less was considered statistically significant. The data was analyzed using the SAS version 9.1 (SAS Institute, Cary, NC).

3. Results

Population: there were 1489 diabetic patients admitted during the study year. The number of all patients hospitalized one or more during the study year was 3784. Thus, the proportion of diabetic patients hospitalized for one or more times during the study year was 39%.

Patient demographics show the preponderance of the Arab patients admitted: 92.8% of diabetic and 90.7% in the control group (*P* = nonsignificant (NS)). The minority were Jews (3.45, 5.1%, resp.) or other ethnic groups (*P* = NS). There was a significant difference between groups regarding patients domicile. More patients with diabetes reported residence in rural versus urban areas than in the control group (Table 1).

Clinical characteristics at admission differed between patients with diabetes and controls (Table 2). Diabetic patients admitted were significantly older than control 66.53 ± 12.72 years and 54.26 ± 21.53 years, respectively (*P* < 0.0001). Gender was significantly different between

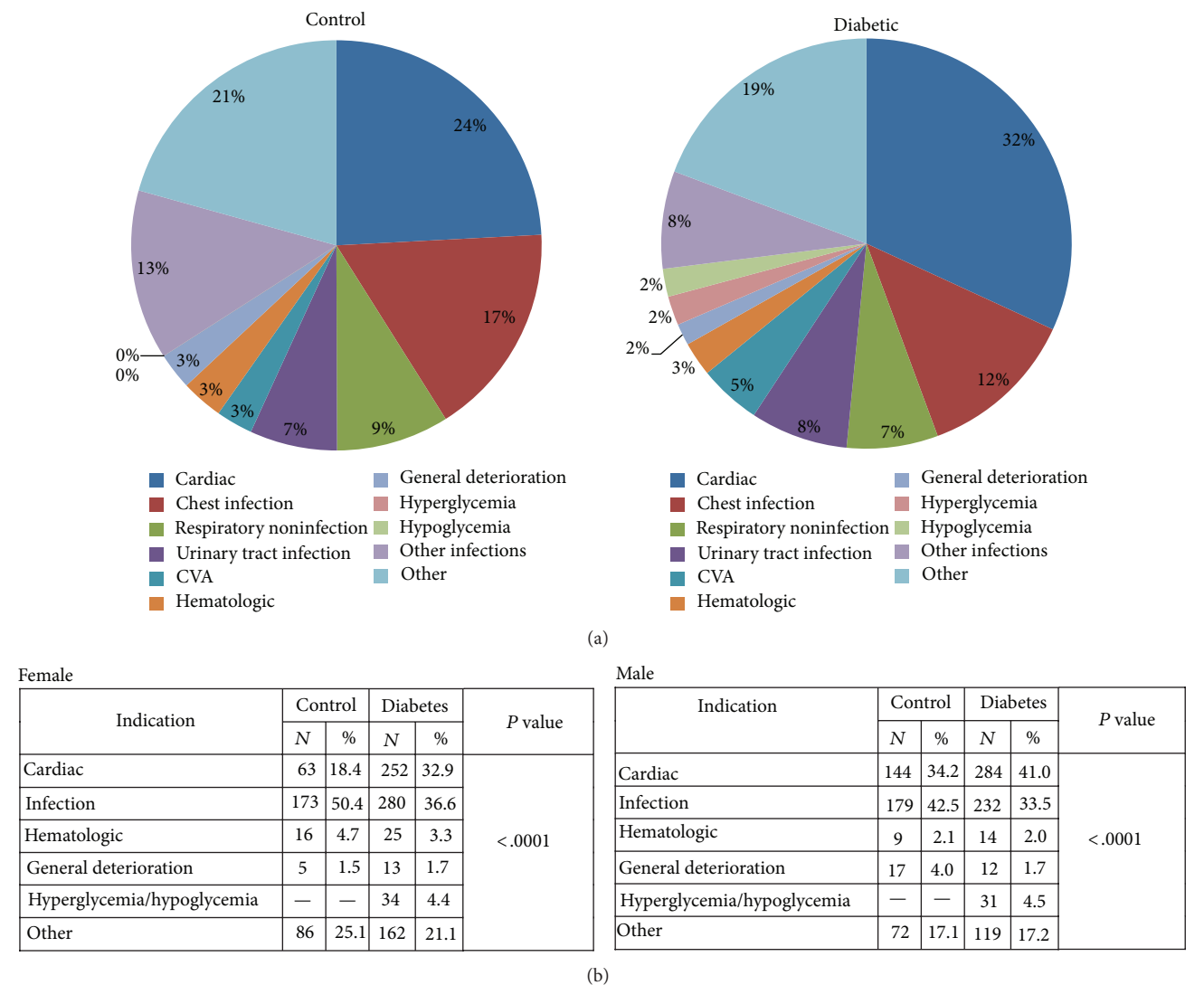


FIGURE 1: Analysis between controls to diabetes patients (2 hospitals). Summary of admission indications in percentage for the nondiabetic and diabetic groups (a) and according to gender by the diabetic and nondiabetic groups (b).

TABLE 2: Analysis between controls and diabetes patients (2 hospitals); summary of data at admission.

Baseline data	Control						Diabetes						P value
	N	Mean	Std.	Median	Min.	Max.	N	Mean	Std.	Median	Min.	Max.	
Age at hospitalization (year)	758	54.26	21.53	53.79	14.01	100.3	1392	66.53	12.72	67.21	16.18	98.90	<0.0001
Weight	179	82.39	49.12	76.00	42.00	676.0	795	85.08	19.08	83.00	45.00	169.0	0.4722
Height	170	164.5	12.79	164.0	95.00	193.0	670	167.5	101.5	162.0	54.00	2007	0.4545
HbA1c							106	8.84	2.51	8.15	5.10	17.70	
Duration of diabetes							821	10.80	5.89	10.00	0.00	48.00	
Days of hospitalization	761	3.27	2.97	3.00	0.00	29.00	1486	3.71	2.99	3.00	0.00	30.00	0.0008

the control and the diabetic groups. Of the diabetic patients admitted, 52.9% were women in comparison to 45.0% of the control group ($P = 0.0003$).

Weight did not differ between groups 82.39 ± 49.12 versus 85.08 ± 19.08 ($P = 0.47$). There were significantly less smokers among the diabetic patients (Table 2).

The indications for hospitalization were different between diabetes and controls and between genders: the main difference was in the higher occurrence of hospitalization due to atherosclerotic related diseases—both cardiovascular disease and strokes in the diabetic group versus controls (37% versus 27%, resp., $P < 0.001$) (Figure 1). Significant Differences in

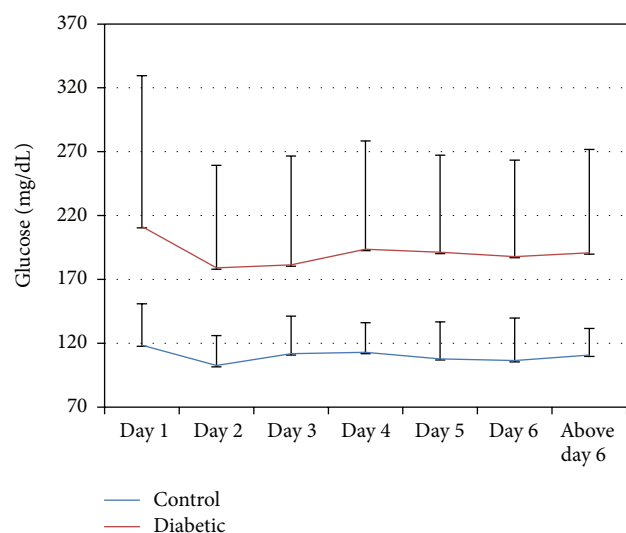


FIGURE 2: Analysis between Controls to Diabetes patients (2 hospitals). Blood glucose levels by hospitalization day.

TABLE 3: Analysis between controls and Diabetes patients (2 hospitals), frequency of comorbidity.

Comorbidity	Control		Diabetes		P value
	N	%	N	%	
Carcinoma	31	6.8	62	4.9	<0.0001
Neurological	34	7.5	64	5.0	
Psychiatric	19	4.2	12	0.9	
Renal	36	7.9	211	16.6	
Respiratory	72	15.9	119	9.4	
Cardiac	135	29.7	397	31.3	
Hypertension	127	28.0	404	31.8	

TABLE 4: Analysis between controls and diabetes patients (2 hospitals), frequency of outcome.

	Control		Diabetes		P value
	N	%	N	%	
Outcome					
Home discharge	727	95.0	1419	95.2	0.8734
Institutional discharge	18	2.4	37	2.5	
Death	20	2.6	34	2.3	
Outcome one year					
Alive	460	68.7	990	66.5	0.4805
Dead	77	11.5	197	13.2	
Readmission	133	19.9	302	20.3	

the indications for hospitalization were maintained within the gender in each group. While only 18.4% of control women were hospitalized for cardiac indications, 32.9% of diabetic women were hospitalized for cardiac reasons—a similar rate as in the nondiabetic men (34.2%). In comorbidities, we also detected significant difference between groups with a noticeable higher renal disease in the diabetic group (16.6% versus 7.9%) (Table 3).

3.1. Outcome Results. Hospitalization: duration of hospitalization was significantly longer for the diabetic patients than for control patients: 3.71 ± 2.99 days versus 3.27 ± 2.97 days, respectively ($P = 0.0008$) (Table 2). No significant differences in discharge status were noted between the two groups with the majority of patients being home discharged and with a low rate of in-hospital death—2.6% control and 2.3% diabetic (Table 4). One-year outcome was poorer in the diabetic group but without reaching statistical significance with a major difference in readmission rates in the diabetic group between the two hospitals. Mortality during the first year following discharge was 11.5% in the control versus 13.2% in the diabetic group ($P = 0.48$). A high readmission rate for both groups 19.9% and 20.3% control and diabetic group respectively, but there was an inequality between the two hospitals with one hospital demonstrating a significant difference between the two groups in the rate of readmission being significantly higher in the diabetic group during the first year after discharge in comparison to the control readmission rate.

3.2. Intrahospital Glycemic Control. Average daily glucose levels during hospitalization as described in Figure 2 demonstrate a decline in values from day one $211.4 (\pm 118.1)$ mg/dL to less than 200 mg/dL and thereafter stable levels while glucose levels were stable in the control group (Figure 2). The glucose level at admission was a significant and an independent predictor for longer hospitalization and death in the nondiabetic but not in the diabetic group (data not shown).

3.3. Indication for Hospitalization. As a group, indications for hospitalization differed between patients with diabetes and controls. For both groups, the leading indication for hospitalization was cardiovascular disease, but cardiovascular disease and urinary tract infection were more prevalent as an indication for hospitalization in the diabetic group (36.8% versus 27.1% and 7.7% versus 6.9%, resp.) while chest infections were significantly less prevalent in the diabetic group versus control group (12.5% versus 16.9%, resp.).

3.4. Diabetes Related Treatment Modifications. The glucose lowering treatment for the patients with diabetes, prior to their hospitalization, was diet alone in 13.1%, oral therapy in 60%, insulin in 19%, and combination therapy in 7% of the patients. At discharge, there was no significant shift in the treatment paradigm in the total group (Table 5).

4. Discussion

This study sheds light on the characteristics of hospitalized patients with diabetes in hospitals serving a high risk for diabetes population. We found high percentage of diabetes among the patients hospitalized in the internal medicine wards and cardiac intensive units, reflecting the high prevalence of diabetes in the population. 39.3% of total patient admitted to the internal medical ward and intensive care unit were diabetic. There was a female preponderance among patients admitted with diabetes 52.9% while only 45.0% of

TABLE 5: Analysis between controls and diabetes patients (2 hospitals), frequency of diabetes treatments.

	N	%
Preadmission diabetes treatment		
None	178	13.1
Oral	823	60.6
Insulin	262	19.3
Combination	95	7.0
Discharge diabetes treatment		
None	216	16.1
Oral	755	56.3
Insulin	277	20.6
Combination	94	7.0

patients without diabetes, hospitalized, were women ($P = 0.0003$). All this reflects the alarming prevalence of obesity and diabetes among adult Arab women in northern Israel. It has been recently reported that the prevalence of overweight and obesity among Arab woman over 40 years of age who attend primary health clinic is 74% [7–9] with diabetes near 50% [5]. This novel observation is seminal by bringing attention to this specific group of women with high morbidity risk and now observing high rate of hospitalization. Interestingly, diabetic women tend to have similar prevalence of coronary heart disease as main indication for admission, as for nondiabetic men (32.9% versus 34.2%, resp.).

As cardiovascular disease and urinary tract infection were more prevalent as an indication for hospitalization in the diabetic group, prevention of cardiovascular disease and urinary tract infection should therefore be reinforced particularly in the group of Arab diabetic women within diabetic population.

Patients with diabetes had a significant greater length of stay than hospitalized patients without diabetes, similar to data described in previous publications [10–12]. The reasons might be multifactorial, and it is important to note that interventions with chronic disease management programs for outpatients [13] and diabetes team consultation during hospitalization [10] have been proved to be an effective means to reduce the likelihood and duration of hospitalizations for individuals with diabetes. Implementing similar strategies in this high risk group and evaluating the impact of this intervention on outcomes of rate and recurrence of hospitalization and hospital length of stay is therefore of paramount importance. In this study, we can additionally identify areas for intervention; as is evident in Table 5, the stay in the hospital did not affect the patients treatment regimens, although glycemic levels at discharge were above the target (mean glucose levels at discharge >190 mg/dL). Furthermore, in only a minority of patients, an HbA1c measurement was performed during hospitalization. These issues reflect the current practice and constraint on hospitalization length and on the performance of blood tests, pushing toward shorter hospital stay and cutting back on performing blood tests not directly relevant to the immediate hospital care. It will be of interest to examine whether interventions as described

above [13] will cause changes in treatment regimens with improvement in long-term outcomes and readmissions.

The glycemic control goals in nonsurgical hospitalized patients continue to be debated, but a consensus defining upper limits below 180 m/dL in noncritical patients is well based on outcomes studies and is therefore recommended by recent position papers [14]. Average glucose levels during hospitalization were above 180 mg% throughout the hospital stay (Figure 2); therefore, as noted there is a need for team consultation during hospitalization tailored to hospitals that provide medical care to populations in high risk for diabetes.

Results of this study show the impact of the high prevalence of diabetes on the hospitalization of Arab patients in Northern Israel, with specific emphasis on the burden of diabetes of the Arab women. It calls attention to the need of in-hospital consultation team and postdischarge plan. Information from the cohort described within this study might be applicable to other medical centers providing large population of Arab descent [15–17].

Author's Contribution

Shehadeh Haj and William Nseir contributed equally to this paper.

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Clinical Study

The Effects of Pioglitazone on Biochemical Markers of Bone Turnover in the Patients with Type 2 Diabetes

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Aim. To investigate whether pioglitazone had detrimental effects on biochemical markers of bone turnover in patients with type 2 diabetes (T2DM). **Methods.** Seventy patients with T2DM were included in this study. The patients remained on their previous antihyperglycemic therapies during the trial. Pioglitazone was then added on their regimen for 3 months. **Results.** After 3 months of treatment with pioglitazone, the levels of fasting blood glucose and HbA_{1c} were significantly decreased (7.9 ± 1.5 mmol/L versus 9.1 ± 1.6 mmol/L and $7.1 \pm 1.0\%$ versus $8.2 \pm 1.4\%$, resp., $P < 0.01$), compared with baseline in the overall patients. Serum concentrations of PINP and BAP were significantly decreased from baseline (45.0 ± 20.0 μ g/L versus 40.6 ± 17.9 μ g/L and 13.23 ± 4.7 μ g/L versus 12.3 ± 5.0 μ g/L, resp., $P < 0.01$) in female group, but not in male group. The serum levels of OC and CTX were unchanged in both female and male subgroups. In addition, the levels of serum BAP and PINP were significantly decreased after pioglitazone treatment in postmenopausal subgroup, comparing with baseline. **Conclusion.** Pioglitazone inhibits bone formation and does not seem to affect bone resorption. Postmenopausal female patients rather than premenopausal or male patients are particularly vulnerable to this side effect of pioglitazone.

1. Introduction

Recent studies have shown that type 2 diabetes (T2DM) is associated with abnormality in bone metabolism including bone loss and osteoporosis [1–4]. The risk of fragility fracture, in particular of the hip, proximal humerus, and foot, was also increased in diabetic patients [3, 5]. Thiazolidinediones (TZDs), selective agonists of peroxisome proliferators-activated receptor (PPAR- γ), are worldwide prescribed oral antihyperglycemic agents. The safety of TZDs, however, is questioned because of their adverse effects on bone metabolism and high risk of fracture in patients receiving the treatment of TZDs. The data from ADOPT (A Diabetes Outcome Progression Trial) suggested that the incidence of fractures in lower and upper limbs significantly increased in diabetic women treated with rosiglitazone (9.3% in rosiglitazone group versus 5.1% in metformin group and 3.5% in glyburide group) [6]. In a prospective cohort study, 4 year-follow-up data from elderly American (mean age > 70 years)

showed that treatment with TZDs including troglitazone, pioglitazone, and rosiglitazone was associated with additional whole-body bone loss in older diabetic women [7]. Moreover, the side effects of TZDs on bone metabolism were demonstrated in animal experiments as well. Trabecular bone volume and bone mineral density (BMD) were found to be decreased in rosiglitazone-treated mice [8–11].

In humans, several randomized controlled trials were performed to observe the changes of biochemical markers of bone metabolism after treatment with rosiglitazone. The results indicated that the levels of bone formation markers were significantly decreased in healthy or diabetic postmenopausal women, whereas the levels of bone resorption markers remained unchanged [12, 13]. On the other hand, several *in vitro* studies demonstrated that TZDs increased the allocation of mesenchymal stem cells toward adipocytes and decreased differentiation toward osteoblasts, and therefore inhibited osteoblastogenesis [14–16]. Although the adverse effects of TZDs on bone metabolism are well known and

possibly attributable to the inhibition of bone formation, the exact mechanism of the effects is little known and needs to be elucidated. Moreover, the majority of available clinical data about the effects of TZDs on bone metabolism come from rosiglitazone, and the data from pioglitazone are very limited. In this regards, we designed the present study to investigate the effects of pioglitazone on biochemical markers of bone turnover in patients with T2DM.

2. Materials and Methods

Seventy patients with T2DM, including 33 males and 37 females, were screened and enrolled in this study. Ten cases out of 37 females were premenopausal, while 27 cases were postmenopausal with mean menopausal duration of 5.9 ± 2.6 years. Inclusion criteria were 25 to 70 years of age, 19 to 35 kg/m^2 of body mass index (BMI), previous therapy regimen including lifestyle modification with or without oral antihyperglycemic agents (monotherapy or combination therapy for at least 2 months), and fasting plasma glucose $>7.0 \text{ mmol/L}$ and $\leq 13 \text{ mmol/L}$. The patients were also required to be free of diabetic symptom, diabetic ketoacidosis, nonketotic hyperosmolar coma, renal dysfunction (serum creatinine levels $>136 \text{ mmol/L}$), hepatic dysfunction (serum alanine aminotransferase or aspartate aminotransferase >2 times over the upper limit of normal range), and severe heart disease. Additionally, patients receiving previous therapy of any TZDs and pregnant or breast-feeding women or taking oral contraceptive pills were excluded from this study. The patients treated by vitamin D and/or bisphosphonate were also excluded. The study protocol was approved by the Ethical Committee of Peking University Health Science Center. All subjects gave informed consent.

Pioglitazone (East China Pharmaceutical Company, Hangzhou, China) 15–45 mg once daily was added on the previous therapy regimen for 12 weeks in all eligible patients, and the previous antihyperglycemic agent, if any, should remain unchanged. Pioglitazone dosage was adjusted according to the fasting blood glucose levels tested once every 4 weeks. All subjects underwent complete clinical examinations and laboratory tests at baseline and at the end of the study. Laboratory tests included the measurement of fasting plasma glucose, glycosylated hemoglobin (HbA_{1c}), lipid profiles, serum calcium, and phosphate, as well as serum levels of bone formation markers, procollagen type I N-terminal propeptide (PINP), osteocalcin (OC), total alkaline phosphatase (BAP), and bone resorption marker, C-terminal telopeptide of type I collagen (CTX).

Serum PINP was analyzed by competitive RIA (Uniq-PINP RIA, Orion Diagnostica, Espoo, Finland); Serum bone specific ALP (BAP) and Serum OC and Serum CTX were determined by ELISA (IDS Ltd, Boldon, UK). The intra- and interassay coefficients of variation were as follows: BAP intra-assay CV 2.6%–6.5% and interassay CV 3.7%–6.4%; PINP intraassay CV 6.5%–10% and inter-assay CV 6.0%–9.8%; OC intraassay CV 1.3%–2.2% and interassay CV 2.5%–7.1%; CTX intraassay CV 1.7%–3.0% and interassay CV 2.5–10.9%. Plasma glucose levels were measured using the glucose-oxidase method, while HbA_{1c} values were assessed by

high-performance liquid chromatography (HPLC). Serum lipid profiles, calcium, and phosphate were analyzed by automatic biochemical analyzer.

Data were expressed as mean \pm SD. Statistical analysis for the comparisons of mean values was performed using paired Student's *t*-test. Because the values of serum PINP, OC, and CTX did not follow the Gaussian distribution, the values were presented as median (interquartile range) and comparisons were carried out using Wilcoxon signed-rank test as appropriate. Logarithmic (log) transformation of PINP, OC, and CTX values was carried out before performing correlation analysis. Statistical analysis was carried out using the Statistical Program for Social Sciences (Version 20.0; SPSS Inc., Chicago, IL, USA). The two-tailed value of $P < 0.05$ was considered statistically significant.

3. Results

Seventy patients, including 33 males and 37 females with T2DM, were enrolled in and completed the study. The mean age was 53.6 ± 8.8 years. The mean duration of disease was 6.0 ± 4.6 years. At baseline, the age, BMI, and diabetic duration showed no statistically significant differences between male and female subgroups.

3.1. Changes in Metabolic Profiles after Pioglitazone Treatment. Compared to those before pioglitazone treatment, the levels of fasting plasma glucose, HbA_{1c} , triglycerides, and diastolic blood pressure were significantly decreased after 12 weeks of pioglitazone treatment in the overall T2DM patients ($P < 0.05$). However, BMI, total cholesterol, and low-density lipoprotein cholesterol (LDL-C) levels were significantly increased after 12 weeks of pioglitazone treatment ($P < 0.05$). Furthermore, there were no significant differences in all metabolic parameters including BMI, blood glucose control, blood pressure, and lipid profiles between male and female subgroups at the end of 12-week treatment with pioglitazone (Table 1).

3.2. Effects of Pioglitazone on Biochemical Markers of Bone Turnover. Compared to baseline, both serum BAP and PINP levels declined markedly after 12 weeks of pioglitazone treatment in the overall T2DM patients ($P < 0.01$). It was noteworthy that serum BAP and PINP levels were significantly decreased after pioglitazone treatment in the female subgroup rather than in the male subgroup. Although serum levels of BAP and PINP had a trend to decline after pioglitazone treatment, there were no significant differences in the male subgroup. In addition, no statistically significant changes were observed in serum OC, CTX, calcium, and phosphate levels after pioglitazone treatment (Table 1).

3.3. Subgroup Analyses of Bone Turnover Markers after Pioglitazone Treatment in Patients with Type 2 Diabetes. In order to investigate whether menopause in female or the age factor in male affect the bone turnover markers after pioglitazone therapy, subgroup analyses were conducted. 37 female patients were divided into two subgroups, premenopause subgroup

TABLE 1: Changes of metabolic profiles and bone turnover markers after pioglitazone treatment.

	Overall (<i>n</i> = 70)		Male (<i>n</i> = 33)		Female (<i>n</i> = 37)	
	Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy
Age (yr)	53.6 ± 8.8		52.3 ± 8.7		54.7 ± 8.8	
Duration (yr)	6.0 ± 4.6		6.3 ± 5.4		5.9 ± 3.8	
BMI (kg/m ²)	26.8 ± 2.9	27.0 ± 2.9	26.7 ± 2.9	26.9 ± 2.8	26.8 ± 2.9	27.1 ± 3.0
FBG (mmol/L)	9.1 ± 1.6	7.9 ± 1.5 [#]	9.2 ± 1.6	8.1 ± 1.8 [#]	9.1 ± 1.7	7.8 ± 1.2 [#]
HbA _{1c} (%)	8.2 ± 1.4	7.1 ± 1.0 [#]	8.0 ± 1.1	7.0 ± 0.9 [#]	8.3 ± 1.7	7.2 ± 1.1 [#]
BMI (kg/m ²)	26.8 ± 2.9	27.0 ± 2.9 [*]	26.7 ± 2.9	26.9 ± 2.8	26.8 ± 2.9	27.1 ± 3.0
SBP (mmHg)	122.2 ± 12.0	121.9 ± 12.4	120.0 ± 13.3	120.0 ± 12.7	123.8 ± 11.5	123.2 ± 12.1
DBP (mmHg)	77.1 ± 7.5	74.5 ± 6.5 [*]	77.0 ± 8.4	74.0 ± 6.3	77.6 ± 6.7	75.0 ± 6.8
T-CHO (mmol/L)	4.9 ± 0.8	5.3 ± 0.9 [#]	4.7 ± 0.8	5.2 ± 1.0 [#]	5.1 ± 0.8	5.4 ± 0.9 [*]
TG (mmol/L)	2.4 ± 1.6	1.9 ± 1.1 [#]	2.3 ± 1.3	2.1 ± 1.3	2.5 ± 1.8	1.7 ± 0.9 [#]
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.2	1.2 ± 0.3	1.2 ± 0.3
LDL-C (mmol/L)	3.1 ± 0.8	3.2 ± 0.9 [*]	3.0 ± 0.7	3.2 ± 0.9 [*]	3.2 ± 0.8	3.2 ± 0.9
Ca ²⁺ (mmol/L)	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.9	2.3 ± 0.8	2.4 ± 0.1	2.3 ± 0.1
P ³⁻ (mmol/L)	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.1
BAP (μgU/L)	13.2 ± 4.7	12.3 ± 5.0 [#]	13.6 ± 6.0	13.2 ± 5.5	12.9 ± 3.8	11.5 ± 3.5 [#]
PINP (μg/L)	40.1 ± 16.8	36.6 ± 15.3 [#]	34.6 ± 10.2	32.1 ± 10.3	45.0 ± 20.0	40.6 ± 17.9 [#]
OC (ng/mL)	10.5 ± 5.1	10.2 ± 4.7	9.5 ± 4.2	9.0 ± 3.3	11.5 ± 5.7	11.3 ± 5.5
CTX (ng/mL)	0.35 ± 0.20	0.35 ± 0.19	0.33 ± 0.13	0.31 ± 0.14	0.37 ± 0.24	0.39 ± 0.22

Data are mean ± SD. * *P* < 0.05; [#] *P* < 0.01, compared to before therapy. FBG: fasting blood glucose; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; T-CHO: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; BAP: bone-specific alkaline phosphatase; PINP: procollagen type 1 N-terminal propeptide; OC: osteocalcin; CTX: C-terminal telopeptide of type 1 collagen.

TABLE 2: Subgroup analyses of bone turnover after pioglitazone treatment in female patients with type 2 diabetes.

	Premenopause (<i>n</i> = 10)		Postmenopause (<i>n</i> = 27)	
	Before therapy	After therapy	Before therapy	After therapy
Ca ²⁺ (mmol/L)	2.3 ± 0.08	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.07
P ³⁻ (mmol/L)	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.3 ± 0.1
BAP (μgU/L)	13.7 ± 5.3	13.0 ± 4.6	12.6 ± 4.8	10.9 ± 3.6 [*]
PINP (μg/L)	28.1 ± 8.5	25.0 ± 8.8	51.2 ± 19.4	46.4 ± 16.9 [*]
OC (ng/mL)	6.1 ± 1.2	6.3 ± 2.1	13.2 ± 5.5	13.1 ± 5.1
CTX (ng/mL)	0.15 ± 0.06	0.18 ± 0.07	0.46 ± 0.23	0.47 ± 0.20

Data are mean ± SD. * *P* < 0.05, compared to before therapy. BAP: bone-specific alkaline phosphatase; PINP: procollagen type 1 N-terminal propeptide; OC: osteocalcin; CTX: C-terminal telopeptide of type 1 collagen.

(*n* = 10) and postmenopause subgroup (*n* = 27). Similarly, the 33 male patients were also divided into two subgroups, age <50 years (*n* = 14) and age ≥50 years. Subgroup analysis showed that in postmenopause subgroup the levels of serum BAP and PINP were significantly decreased after pioglitazone treatment, compared with those before pioglitazone treatment (Table 2). However, the bone turnover markers remained unchanged in two male subgroups after pioglitazone treatment (Table 3).

3.4. Correlation Analysis of Relationships between Baseline Values of Demographic and Biochemical Parameters versus Baseline Bone Turnover Markers or Changes in Bone Turnover Markers after Pioglitazone Treatment. Correlation analysis revealed that only changes in HbA_{1c} are negatively correlated with the changes in CTX (*r* = −0.360, *P* = 0.002) rather than changes in BAP or PINP after pioglitazone therapy, while

age, BMI, baseline glucose, HbA_{1c}, and lipid profiles are not correlated with changes in BAP, PINP, or OC.

4. Discussion

The present study demonstrated that pioglitazone treatment could significantly decrease the serum concentrations of PINP and BAP, two of biochemical markers of bone formation, in the overall patients with T2DM. In addition, the detrimental effects found in the overall patients should be attributable to decreased serum PINP and BAP levels in the postmenopausal female patients rather than in premenopausal female patients or male patients. The results of correlation analysis showed that the changes in bone turnover markers were not associated with the changes in HbA_{1c} by pioglitazone treatment, suggesting independent effects of pioglitazone on bone turnover.

TABLE 3: Subgroup analyses of bone turnover after pioglitazone treatment in male patients with type 2 diabetes.

	<50 yrs (<i>n</i> = 14)		≥50 yrs (<i>n</i> = 19)	
	Before therapy	After therapy	Before therapy	After therapy
Ca ²⁺ (mmol/L)	2.3 ± 0.1	2.3 ± 0.08	2.3 ± 0.07	2.3 ± 0.07
P ³⁻ (mmol/L)	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
BAP (μgU/L)	13.2 ± 5.7	13.0 ± 6.2	14.1 ± 5.9	13.5 ± 5.5
PINP (μg/L)	38.1 ± 9.5	35.8 ± 9.9	31.7 ± 10.0	29.1 ± 9.8
OC (ng/mL)	10.8 ± 4.8	9.9 ± 3.5	8.4 ± 3.2	8.2 ± 3.1
CTX (ng/mL)	0.37 ± 0.11	0.35 ± 0.14	0.29 ± 0.14	0.27 ± 0.14

Data are mean ± SD. BAP: bone-specific alkaline phosphatase; PINP: procollagen type 1 N-terminal propeptide; OC: osteocalcin; CTX: C-terminal telopeptide of type 1 collagen.

Pioglitazone plays a critical pharmacological role as regulators of glucose homeostasis, lipid metabolism, and cell proliferation by activating the nuclear receptor PPAR- γ [17]. In 2007, Takeda pharmaceutical company declared that long-term treatment with pioglitazone increased the incidence of fracture in women with T2DM. This declaration hinted that pioglitazone may have clinically important adverse effects on bone. The data from Health ABC Study also suggested that treatment with TZDs, including pioglitazone, rosiglitazone and troglitazone, was associated with greater bone loss at the whole body, lumbar spine, and trochanter in women, but not men, with diabetes [7]. Owing to limited information available in human diabetes studies, the mechanism regarding the effects of TZDs on human bone is little known and needs to be clarified. In a small clinical study of troglitazone, urine type 1 collagen N-telopeptide and serum bone-specific alkaline phosphatase were reduced after the first month of treatment but returned to baseline levels by 12 months of treatment [18]. By now, a few studies have reported the effects of pioglitazone on bone metabolic markers in patients with diabetes. It is described that pioglitazone decreased significantly the alkaline phosphatase and induced on average a 45% increase in urinary calcium excretion [19]. Decreased OC after 6-month treatment with pioglitazone also was reported although the change at 3 months was not significant [20]. In our study, we found that serum levels of BAP and PINP, but not OC, were significantly reduced after 12-week treatment with pioglitazone in the overall T2DM patients, suggesting decreased osteoblast activity. The present data are in agreement with a pioglitazone study in polycystic ovary syndrome (PCOS) [21]. Similar results were also seen in some troglitazone studies [16, 22, 23]. The effects of pioglitazone on different bone formation markers may be related to their expression at different stages of osteoblastic differentiation. Both BAP and PINP are markers of early bone formation. The former is representative early marker of osteoblastic differentiation and bone formation during the matrix maturation phase, and the latter is product of extracellular processing of procollagen before fiber assembly and appears during osteoblast proliferation. OC, however, is expressed in mature osteoblasts and involved in the arrangement of the mineral phase in bone. In addition, our findings are supported by several basic studies. *In vitro* studies show that activation of PPAR- γ signaling by pioglitazone or other TZDs could promote differentiation of pluripotent mesenchymal stem cells

into adipocytes at the expense of osteoblasts and suppress osteoblast differentiation and OC expression in osteoblastic cell lines [14, 24]. Taken together, the present study and other reported studies suggested that pioglitazone or other TZDs may affect bone formation at an earlier stage and eventually reduce bone mineral density. Inhibition of bone formation could be responsible for TZDs-induced human bone loss.

CTX is well known as an important biochemical marker of bone resorption. In the present study, serum CTX level was not significantly changed after 12-week treatment with pioglitazone, which was supported by a clinical study in obese premenopausal patients with PCOS [21]. In addition, an *in vitro* study showed that pioglitazone did not stimulate bone resorption in cultured mouse calvarial bones [25]. These data suggested that pioglitazone may not affect bone resorption. Likewise, two randomized controlled trials demonstrated that the markers of bone resorption β -CTX and deoxypyridinoline were not altered either in healthy or in diabetic postmenopausal women after rosiglitazone administration [16, 22]. Although controversy exists with regard to the effects of TZDs on bone resorption in several *in vitro* studies, the data from human studies seemed to be consistent and suggested that rosiglitazone or pioglitazone had no effects on bone resorption.

Interestingly, the present study showed that pioglitazone led to significant reduction of serum BAP and PINP levels in female subgroup, but not in male subgroup of the T2DM patients, suggesting that the effects of TZDs on bone formation seemed to be sex-specific. This finding is supported by almost all of TZD clinical studies, including the ADOPT [6] and Health ABC Study [7]. Up to now, most of the human data showing harmful impact of TZDs on bone were consistently obtained from women, especially from older or postmenopausal women. Whether it is also the case in men remains unknown. In fact, there are only a few clinical studies available and the results were conflicted. However, recent evidences from several studies focused on male TZDs' user demonstrated that rosiglitazone was associated with significant decrease in BMD of both spine and hip, and with an increased prevalence of fractures in males with T2DM [26, 27]. Considering that bone turnover rate is greater in older women than in older men and that TZDs had been reported to cause a decrease in estrogen levels by inhibiting the aromatase pathway [28], older women seem more likely to be at higher risk of bone metabolic abnormalities than older men

in response to treatment with TZDs. Nevertheless, due to the lack of large-scale trials focused on bone metabolic markers, whether the effects of TZDs on bone turnover are sex-specific or not remains to be clarified.

In agreement with previous reports, the present study confirmed that pioglitazone as an add-on treatment to failing monotherapy or combine therapy is efficient in T2DM patients with poorly controlled glucose levels [29, 30]. Pioglitazone was also shown to affect lipid metabolism in our study where the levels of triglycerides were markedly decreased and LDL-C levels were slightly but significantly increased after pioglitazone treatment. In fact, the beneficial effect of pioglitazone on triglycerides has been well established, whereas the effects of pioglitazone on total cholesterol and LDL-C are still a little inconsistent in the literatures [31]. The reason why pioglitazone elevates LDL-C levels is unclear but is likely to represent an increase in LDL particle size [32].

Limitations of the present study are its short duration of trial and lack of a control group. Additionally, there was no BMD measurement for comparison due to the short term of followup.

5. Conclusions

The present study suggests that pioglitazone reduces bone formation at earlier stages but may have no impact on bone resorption in the patients with T2DM. This effect was not associated with decrease in HbA_{1c} by pioglitazone treatment. The reduction in bone formation is speculated to be the main reason for bone metabolic abnormality in the diabetic patients receiving pioglitazone therapy. Moreover, our study also showed that the detrimental effect of pioglitazone on bone formation was more obvious in postmenopausal women than in men or in premenopausal women with T2DM. The potential side effects of pioglitazone on bone metabolism should be given more attention in clinical practice.

Conflict of Interests

The authors stated that they have no interests which might be perceived as posing a conflict or bias.

Acknowledgments

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Research Article

Type 2 Diabetic Patients with Ischemic Stroke: Decreased Insulin Sensitivity and Decreases in Antioxidant Enzyme Activity Are Related to Different Stroke Subtypes

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We analyzed (a) insulin sensitivity (IS) and (b) glutathione peroxidase (GSH-Px), glutathione reductase (GR), and superoxide dismutase (SOD) antioxidant enzyme activity in type 2 diabetic (T2D) patients with atherothrombotic infarction (ATI) (group A), lacunar infarction (LI) (B), or without stroke (C) and in nondiabetics with ATI (D), LI (E), or without stroke (F). ATI and LI were confirmed by brain imaging IS levels were determined by minimal model (Si index), and the enzyme activity by spectrophotometry. In T2D patients, Si was lower in A and B versus C (1.14 ± 0.58 , 1.00 ± 0.26 versus $3.14 \pm 0.62 \text{ min}^{-1}/\text{mU/l} \times 10^4$, $P < 0.001$) and in nondiabetics in D and E versus F (3.38 ± 0.77 , 3.03 ± 0.72 versus $6.03 \pm 1.69 \text{ min}^{-1}/\text{mU/l} \times 10^4$, $P < 0.001$). Also, GSH-Px and GR activities were lower in A and B versus C (GSH-Px: 21.96 ± 3.56 , 22.51 ± 1.23 versus 25.12 ± 1.67 ; GR: 44.37 ± 3.58 , 43.50 ± 2.39 versus $48.58 \pm 3.67 \text{ U/gHb}$; $P < 0.001$) and in D and E versus F (GSH-Px: 24.75 ± 3.02 , 25.57 ± 1.92 versus 28.56 ± 3.91 ; GR: 48.27 ± 6.81 , 49.17 ± 6.24 versus $53.67 \pm 3.96 \text{ U/gHb}$; $P < 0.001$). Decreases in Si and GR were significantly related to both ATI and LI in T2D. Our results showed that decreased IS and impaired antioxidant enzymes activity influence ischemic stroke subtypes in T2D. The influence of insulin resistance might be exerted on the level of glutathione-dependent antioxidant enzymes.

1. Introduction

It was previously suggested that atherothrombotic infarction (ATI) and lacunar infarction (LI), as two different subtypes of ischemic stroke, might also differ in the set of the relevant risk factors, with ATI being more associated to the atherogenic risk factors in contrast to LI [1]. In addition, the risk factors for both ischemic stroke subtypes remain still largely unclarified.

However, decreased insulin sensitivity (IS), that is, insulin resistance, was observed both in ATI and LI, which was

frequently accompanied with compensatory hyperinsulinemia in T2D patients as well as in nondiabetics [2, 3].

Simultaneously, it has been shown that impaired balance between products of oxidative stress and the level of antioxidant enzyme activities might be the important mechanism underlying the occurrence of ischemic stroke [4]. Moreover, it was elucidated that the brain has only moderate content of glutathione-dependent enzymes, for example, glutathione peroxidase (GSH-Px), glutathione reductase (GR), and superoxide dismutase (SOD), together with the fact that the intact antioxidant defense could provide first line of

protection from initiation and exacerbation of ischemic cerebral injury [5].

In addition, changes in enzymatic antioxidative defense mechanisms in patients with stroke are still controversial. Previous results implied that the majority of antioxidant enzyme activity was significantly reduced in acute ischemic stroke, possibly as a consequence of increased oxidative stress [5] while the recent finding suggested increased levels of glutathione dependent enzymes as an adaptive mechanisms during acute cerebral ischemia [6]. Finally, due to novel facts, oxidative stress can be an important component for astrocytic cell death following metabolic stress [7].

Until now, experimental studies provided evidence of an association between ischemic stroke and increased oxidative stress [8, 9], but data in humans are still heterogeneous and limited. Therefore, our study was aimed to determine IS levels and three different types of antioxidant enzyme activities GSH-Px, GR, and SOD, in T2D with ATI and LI.

2. Materials and Methods

2.1. Patients. In this study we included a total of 93 patients with T2D, ascribed to the following groups: T2D patients with ATI (group A, $N = 30$), and T2D with LI (group B, $N = 30$), and T2D without ischemic stroke (group C, $N = 33$). Simultaneously, we involved a total of 93 nondiabetics, matched with the T2D patients regarding gender and age, and also comprising the following groups: nondiabetics with ATI (group D, $N = 30$), nondiabetics with LI (group E, $N = 30$), and nondiabetics without stroke (group F, $N = 33$).

T2D was diagnosed in accordance with the criteria of the World Health Organization [10]. Diagnosis of ATI and LI was done by a neurologist due to clinical features and brain imaging methods such as cranial computerized scan and/or magnetic resonance imaging in two consecutive examinations, during the first 7 days from the appearance of ischemic stroke [11]. The patients with ATI or LI were included in the study provided that they had not shown signs of cardioembolic cerebral infarction, or coronary heart disease based on a history of myocardial infarction with definite elevation of serum cardiac enzymes or coronary angiography. T2D patients were treated with insulin therapy, and/or ingestion of antioxidant supplements and drugs, which might affect free radical and antioxidant activity potential; likewise patients who had other endocrine disease or autoimmune diseases, renal or hepatic failure, current infections, neoplasms, polycythemia, or rheumatic diseases were also excluded as well as the patients with history of trauma or operation within the last 3 months. No patient had uncontrolled hypertension, severe alcohol consumption, acute infection, or an inflammatory disease during the last 4 weeks. All the patients, with or without ATI and LI, showed the similar level of their physical activity. In addition, they were required not to smoke at least 12 hr before the tests were performed.

The patients were fully informed about the study and gave the inform consent to participate.

The study was conducted 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 interview, physical examination, metabolic test, and evaluation of antioxidant enzyme activities were performed in all the patients included in the study, for each patient within the same day. The interview comprised the questions about medical conditions, current medication, and habits. Hypertension was diagnosed according to World Health Organisation criteria (systolic/diastolic blood pressure $\geq 140/\geq 90$ mm Hg) or by the use of antihypertensive agents [12].

2.3. Metabolic Evaluations. The metabolic tests were implemented at least after 6 months from the occurrence of the ischemic stroke.

The evaluation of insulin sensitivity was done by Frequently Sampled Intravenous Glucose Tolerance Test (FSIGT) with minimal model analysis [13]. Briefly, before testing, each patient was required to be at a 12 hr fasting state. During the FSIGT, 0.3 g/kg body weight of glucose was injected and the blood samples for plasma glucose (PG) and plasma insulin (PI) determination were taken immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, and 180 minutes after intravenous glucose stimulation. Insulin was injected as a continuous infusion 4 mU/kg/min between minutes 20 and 25 in order to avoid the effect of the potentially blunted insulin response. The insulin sensitivity index (Si) was calculated from the results of PG and PI levels by computerized minimal model analysis, using the MINMOD program (kindly provided by Dr. Richard Bergman from the University of Southern California, Los Angeles) [13].

2.4. Laboratory Analyses. Determination of the antioxidant enzymes SOD, GSH-Px, GR was conducted using the commercial assays (produced by Randox Laboratories Ltd., UK), based on spectrophotometer determination methods as described previously [14].

PG was determined by glucose oxidase method using a Beckman Glucose Analyser (Beckman Instruments, Fullerton, CA). PI was tested by radioimmunoassay (INEP, Zemun, RS, double antibody kits). Total cholesterol, HDL cholesterol, and triglyceride concentrations were determined with the chromatography method using commercial kits (produced by Boehringer Mannheim). LDL cholesterol concentrations were calculated using the Friedewald formula.

2.5. Statistical Analyses. Data are presented as mean \pm SE. The categorical variables were analyzed with Kruskal-Wallis Test. The continuous variables within each subtype of ischemic stroke were analyzed with analysis of variance (ANOVA) with a post hoc Bonferroni test. Multiple logistical regression analysis was performed. Differences were considered statistically significant at $P < 0.05$. All analyses were performed with the SPSS statistical package (version 16.0 for Windows).

TABLE 1: Clinical characteristics and laboratory analyses in type 2 diabetic patients and nondiabetics with different subtypes of ischemic stroke: atherothrombotic infarction (ATI) and lacunar infarction (LI).

	Groups						P value
	A	B	C	D	E	F	
	T2D+ ATI+	T2D+ LI+	T2D+ Stroke–	T2D– ATI+	T2D– LI+	Healthy Controls	
n (M/F)	30 (16/14)	30 (16/14)	33 (15/18)	30 (15/15)	30 (15/15)	33 (15/18)	NS
Age (years)	56.9 ± 1.67	56.03 ± 2.51	56.42 ± 3.05	57.07 ± 2.88	56.00 ± 2.03	56.97 ± 2.42	NS
Duration of diabetes (years)	4.73 ± 1.53	5.34 ± 1.00	4.65 ± 2.01	—	—	—	NS
Duration from onset of ischaemic stroke (years)	1.23 ± 0.43	1.30 ± 0.24	—	1.03 ± 0.21	1.09 ± 0.21	—	NS
HbA1c (%)	7.39 ± 0.20	7.30 ± 0.14	7.31 ± 0.43	5.52 ± 0.31	5.37 ± 0.31	4.89 ± 0.26	NS
Total cholesterol (mmol/L)	6.98 ± 0.91	6.81 ± 0.68	7.03 ± 0.62	6.25 ± 0.71	6.15 ± 0.63	6.14 ± 0.74	NS
Triglyceride (mmol/L)	2.20 ± 0.37	2.26 ± 0.38	2.18 ± 0.26	1.90 ± 0.22	2.00 ± 0.29	1.87 ± 0.35	NS
LDL-c (mmol/L)	5.21 ± 0.42*	4.81 ± 0.14	4.47 ± 0.29	4.34 ± 0.43*	4.05 ± 0.55	3.71 ± 0.49	P < 0.05
HDL-c (mmol/L)	0.94 ± 0.17	1.01 ± 0.40	1.01 ± 0.15	0.99 ± 0.27	1.05 ± 0.16	1.11 ± 0.21	NS
Hypertension (n, %)	22 (73.3%)**	21 (70.0%)**	23 (69.7%)**	22 (73.3%)**	22 (66.7%)**	0 (0%)	P < 0.001
Smoking (n, %)	10 (33.3%)	11 (36.7%)	12 (36.4%)	10 (33.3%)	11 (36.7%)	12 (36.4%)	NS

Data are n, means ± SEM.

*P < 0.05 A versus B, C and D versus E, F.

**P < 0.001 A, B, C, D, E versus F.

3. Results

3.1. Clinical Characteristics. The clinical characteristics and biochemistry parameters of T2D patients and nondiabetics with ATI or LI as different subtypes of ischemic stroke are shown in Table 1. The age, gender, duration of diabetes, and duration from the onset of ischemic stroke were similar in T2D patients and nondiabetics with different subtypes of ischemic stroke, together with HbA_{1c} levels, implying satisfactory metabolic control before metabolic investigation was done. However, LDL-c level was significantly higher in T2D patients with ATI compared to the T2D patients with LI and T2D patients without stroke and also in nondiabetics with ATI compared to nondiabetics with LI and healthy controls. There is no difference in prevalence of hypertension in patients with T2D and ATI or LI and T2D without ischemic stroke, while it was significantly higher in nondiabetics with ATI or LI than in healthy controls. The percentage of patients who were smokers was similar in all investigated groups.

3.2. Insulin Sensitivity. We found that Si levels were significantly lower in T2D patients with ATI (group A) and LI (group B) compared to T2D patients without ischemic stroke (group C) (1.14 ± 0.58 and 1.00 ± 0.26 versus 3.14 ± 0.62 min⁻¹/mU/L × 10⁴, resp., P < 0.001). Also, the results showed significantly lower Si levels in nondiabetics with ATI (group D) and LI (group E) compared to healthy controls (group F) (3.38 ± 0.77 and 3.03 ± 0.72 versus 6.03 ± 1.69 min⁻¹/mU/L × 10⁴, resp., P < 0.001) (Figure 1). Simultaneously, PI levels were higher in T2D patients, ATI (group A), and LI (group B) than in T2D patients without ischemic stroke (group C) (20.94 ± 4.31 and 20.07 ± 0.88 versus 16.06 ± 0.91 mU/L, respectively, P < 0.001) and in nondiabetics with

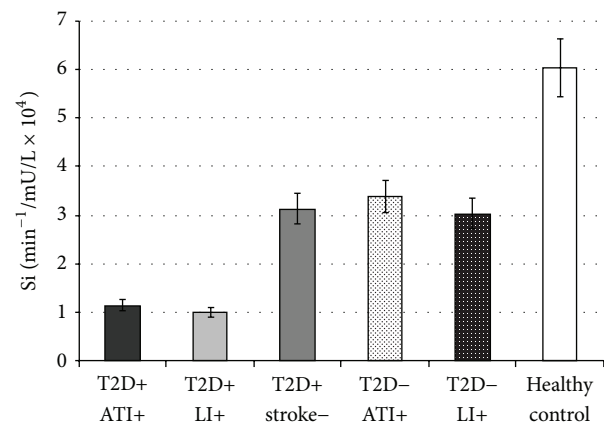


FIGURE 1: Values are expressed as mean ± SE. Bar graphs show the values of insulin sensitivity index (Si) determined by minimal model analysis. Si levels were significantly lower in type 2 diabetic (T2D) patients with different subtypes of ischemic stroke: atherothrombotic infarction (ATI) and lacunar infarction (LI) compared to T2D patients without ischemic stroke, and the same relationship is found in the respective groups in nondiabetics (P < 0.001) (ANOVA test).

ATI (group D) or LI (group E) in comparison to healthy controls (group F) (15.57 ± 1.86 and 15.59 ± 1.26 versus 7.54 ± 2.03 mU/L, respectively, P < 0.001) (Figure 2).

3.3. Antioxidant Enzyme Activity. When we evaluated antioxidant enzyme activities in T2D patients with ATI (group A) and LI (group B) and without ischemic stroke (group C) and in nondiabetics with ATI (group D) and LI (group E) and healthy controls (group F), we detected the levels

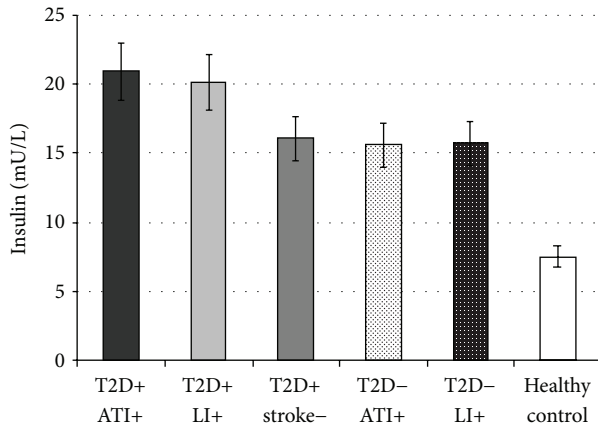


FIGURE 2: Values are expressed as mean \pm SE. Bar graphs show the values of basal plasma insulin (PI) level. PI levels were higher in type 2 diabetic (T2D) patients with different subtypes of ischemic stroke: atherothrombotic infarction (ATI) and lacunar infarction (LI) compared to T2D patients without ischemic stroke, and the same relationship is found in the respective groups in nondiabetics ($P < 0.001$) (ANOVA).

of the GSH-Px and GR activity being significantly lower in group A and B versus C and in group D and E versus F (GSH-Px: A: 21.96 ± 3.56 versus B: 22.51 ± 1.23 versus C: 25.12 ± 1.67 U/gHb, $P < 0.001$; D: 24.75 ± 3.02 versus E: 25.57 ± 1.92 versus F: 28.56 ± 3.91 U/gHb, $P < 0.001$; GR: A: 44.37 ± 3.58 versus B: 43.50 ± 2.39 versus C: 48.58 ± 3.67 U/gHb, $P < 0.001$; D: 24.75 ± 3.02 versus E: 25.57 ± 1.92 versus F: 28.56 ± 3.91 U/gHb, resp., $P < 0.001$) (Figure 3), while the SOD levels did not differ between the investigated groups (A: 769.57 ± 72.36 versus B: 768.97 ± 34.50 versus C: 789.18 ± 60.28 , D: 795.23 ± 48.28 versus E: 797.80 ± 69.21 versus F: 813.88 ± 45.80 mU/mgHb, resp., $P = \text{NS}$).

3.4. Multiple Logistic Regression Analysis. This analysis identified that decreased insulin sensitivity Si and the decreases of GR were related to both ATI and LI in T2D patients. Simultaneously, this model identified decreased insulin sensitivity Si and the level of GR and GSH-Px in nondiabetics with ATI, but predominantly decreased insulin sensitivity Si in nondiabetics with LI (Table 2).

4. Discussion

In this study, we directly measured the IS level, together with three different types of antioxidant enzyme activities, GSH-Px, SOD, and GR, in T2D patients and nondiabetic individuals with two different subtypes of ischemic stroke in type 2 diabetics.

Our results have shown decreased IS level in T2D patients with two different subtypes of ischemic stroke, ATI and LI, compared to T2D without stroke, while we could not demonstrate the difference between the subtypes, and the same pattern of IS changes was found in the nondiabetics.

To our knowledge, there are scarce data regarding the changes in IS in T2D with ischemic stroke subtypes.

TABLE 2: Independent factors related to different subtypes of ischemic stroke in diabetics and nondiabetics in multiple logistic regression analysis.

	Odds ratio (95% CI)	P value
Related to T2D+ ATI+		
Si	0.002 (0.000–0.017)	$P = 0.000$
GR	0.613 (0.422–0.889)	$P = 0.01$
GSH-Px	0.599 (0.332–1.113)	$P = 0.105$
Related to T2D+ LI+		
Si	0.001 (0.0006–0.08)	$P = 0.000$
GR	0.549 (0.369–0.817)	$P = 0.003$
GSH-Px	0.685 (0.362–1.297)	$P = 0.245$
Related to T2D– ATI+		
Si	0.159 (0.056–0.454)	$P = 0.001$
GSH-Px	0.606 (0.428–0.858)	$P = 0.005$
GR	0.795 (0.643–0.982)	$P = 0.033$
Related to T2D– LI+		
Si	0.071 (0.022–0.236)	$P = 0.000$
GSH-Px	0.760 (0.536–1.077)	$P = 0.123$
GR	0.840 (0.680–1.039)	$P = 0.108$

Nagelkerke $R^2 = 0.844$.

Cox and Snell $R^2 = 0.821$.

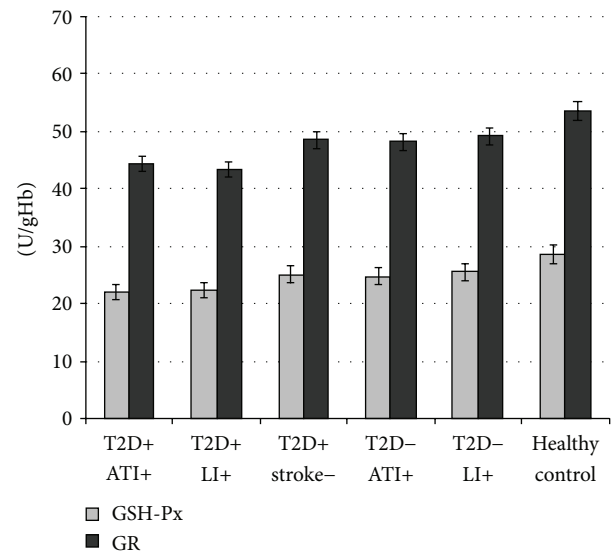


FIGURE 3: Values are expressed as mean \pm SE. Bar graphs show the values of antioxidant enzyme glutathione peroxidase (GSH-Px) and glutathione reductase (GR) activity. GSH-Px and GR levels were lower in type 2 diabetes (T2D) patients with different subtypes of ischemic stroke: atherothrombotic infarction (ATI) and lacunar infarction (LI) compared to T2D patients without ischemic stroke, and the same relationship is found in the respective groups in nondiabetics ($P < 0.001$) (ANOVA test).

Previous study also suggested that insulin resistance measured using different methodology, the short insulin tolerance test, is independently associated with markers of atherosclerosis detected on carotid arteries in T2D patients [15]. Simultaneously, an association has been documented between insulin resistance and other markers at the large vessels, such as the pulsatility index on cerebral arteries [16].

On the other hand, it has been shown that insulin resistance, evaluated by the homeostasis model assessment (HOMA) index, was higher in diabetic in contrast to nondiabetic patients with LI being a small vessel disease [17]. Additionally, it has been suggested that diabetes, hypertension, and metabolic syndrome which share insulin resistance as a common mechanism, contribute to LI occurrence [18–20]. However, detailed mechanisms of the possible link between the presence of ischemic stroke subtypes and insulin resistance remain to be clarified.

In addition, when we measured the level of insulinemia in these patients, the significantly higher level of insulinemia was detected in both groups of T2D patients, with ATI and LI. The increases in insulin levels might be primarily consequence of the simultaneous presence of insulin resistance in the relevant groups. However, the increases in insulin might contribute to the appearance of the ischemic stroke subtypes. Insulin, as a growth factor, might interfere with the beneficial effects of nitric oxide (NO) on the vasculature [21]. Moreover, insulin infusion during euglycemic insulin clamp was able to suppress endothelium-dependent vasodilation in large arteries, which is reported to be based on increased availability of endothelin-1, leading to downregulation of NAD(P)H oxidase and superoxide anion production [22], and endothelial dysfunction is proposed to play an important role in the pathogenesis of cerebral small vessel disease [23, 24].

Also, our results demonstrated lower values of antioxidant enzymes in T2D patients with ATI or LI than in type 2 diabetics without ischemic stroke. The patients with ATI and LI did not differ regarding the level of antioxidant enzymes. These findings could be explained by the fact that in diabetes there is already reduced capacity of antioxidant protection, which is further significantly disturbed in the acute phase of ischemic stroke [4, 5].

In our data, in patients with ATI and LI, we found the decreases in glutathione-dependent enzymes activity in contrast to other types of antioxidant enzyme activity, for example, SOD. These results imply a prolonged and severe depression of glutathione dependent antioxidative defense mechanisms irrespective of stroke subtypes.

Since it has been documented that free radicals are extremely difficult to measure directly, antioxidant enzymes have been proposed to represent indirect markers of oxidative stress [5].

Numerous, but mostly experimental, studies provided evidence of an association between ischemic stroke and decreased antioxidant enzyme activity, although this possible association in humans has been less investigated [9, 25]. Moreover, the analyses of treatment with agents in stroke showing an ability to prevent further depression of antioxidant protection and scavenging reactive free radicals were reported to fail to restore GSH-Px and GR activities [26, 27].

Generally, a recently study that aimed to assess total antioxidant capacity and oxidative stress in diabetic and nondiabetic acute stroke patients with 2 different stroke subtypes, large and small vessel disease strokes, concluded that oxidative stress and counterbalancing antioxidant capacity

are more pronounced in diabetic acute stroke patients than in nondiabetics [28].

The study provided different data in comparison to the previous investigations showing decreased GSH-Px activity in both diabetic and nondiabetic patients with coronary heart disease when compared to controls [17, 29, 30].

However, the results from our study have shown the diminished activity of both GSH-Px and GR in T2D patients with ATI and LI, which is consistent with previous reports of decreased GSH-Px levels patients with stroke [6, 8, 31, 32].

The levels of SOD are found to exhibit great variations in the previous study in patients with the stroke [6, 8, 26, 29, 31–37]. Our results could not detect the differences in the SOD levels in different groups of patients, and thus they are in line with data showing no changes in respect to SOD level in both diabetics and nondiabetics irrespective of different subtypes of ischemic stroke [33, 34, 37]. The tentative explanation for the inconsistent findings regarding SOD might reflect the predominant role of intracellular versus extracellular fraction of SOD in free radical scavenging [38].

In our study, the detected antioxidant enzyme activities were not affected by other important factors potentially influencing the enzyme levels, for example, by hyperglycemia, aging, duration of diabetes, and presence of macrovascular complications, due to the fact that in all groups of T2D patients had similar levels of metabolic and satisfactory metabolic control and that patients were matched in respect of age, duration of diabetes, and the prevalence of macrovascular complications.

The multiple regression analysis applied to our data has demonstrated that decreased IS together with the decreases in GR are related to both ATI and LI in T2D patients. In nondiabetics, the decreases in Si levels and the diminished GR are found to be related only to ATI. The analysis reveals the potential difference in the mechanisms underlying the onset of the two subtypes of the stroke in T2D patients compared to nondiabetics. The results imply that higher levels of insulin resistance combined with lower levels of GR, detected in T2D patients compared to nondiabetics, were underlying the onset of LI together ATI in T2D in contrast to the findings in nondiabetics. In this context, our results are consistent with the findings that LI represents the most frequent ischemic stroke subtype in T2D. Taken together, our data imply that insulin resistance exerts its pathogenic influence on the level of glutathione dependent antioxidant enzymes, especially in T2D.

5. Conclusions

In conclusion, our results suggest that the presence of different subtypes of ischemic stroke is associated with insulin resistance and diminished antioxidant enzyme activity in both subtypes of ischemic stroke in T2D. The results also imply that atherogenic influence of decreased IS in the different subtypes of stroke in T2D might be exerted through a significantly reduced glutathione dependent antioxidant enzyme activity, while the mechanisms relating the effects of insulin resistance and decreased antioxidant enzyme activity remain to be clarified.

Conflict of Interests

The authors have no financial interests or other conflict of interests.

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Review Article

Challenges in the Management of Type 2 Diabetes Mellitus and Cardiovascular Risk Factors in Obese Subjects: What Is the Evidence and What Are the Myths?

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The increasing worldwide prevalence of diabetes mellitus and obesity has projected concerns for increasing burden of cardiovascular morbidity and mortality. The dangers of obesity in adults and children have received more attention than ever in the recent years as more research data becomes available regarding the long-term health outcomes. Weight loss in obese and overweight subjects can be induced via intensive lifestyle modifications, medications, and/or bariatric surgery. These methods have been shown to confer overall health benefits; however, their effect on remission of preexisting diabetes mellitus and reduction in cardiovascular risk has been variable. Recent research data has offered a much better understanding of the pathophysiology and outcomes of these management strategies in obese patients. In this paper, the authors have summarized the results of major studies on remission of type 2 diabetes mellitus and reduction of cardiovascular events by weight loss induced by different methods. Furthermore, the paper aims to clarify various prevailing myths and practice patterns about obesity management among clinicians.

1. Introduction

Historically, among various cultures, weight gain has been viewed as a sign of wealth and prosperity but as the dynamics of food production and consumption have changed, the world now faces an epidemic of obesity. According to the World Health Organization (WHO), worldwide prevalence of obesity has doubled since 1980 with estimated 1.5 billion adults with obesity in 2008 [1]. In the United States, more than 64% of the population is overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$), and more than 33% of the adult population meets criteria for obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$) [2]. On a similar note, more than 25 million US adults have type 2 diabetes mellitus, and this figure will likely reach 50 million by 2050 given the current demographic trends and continued progression of obesity [3, 4]. The increasing worldwide prevalence of diabetes mellitus and obesity has projected concerns for increasing burden of cardiovascular morbidity and mortality. The dangers of

obesity in adults and children have received more attention than ever in the recent years as more research data becomes available regarding the long-term health outcomes. The increasing rates of diabetes in children and adolescents and the limited capacity of the current therapeutic treatments to slow the disease progression raise the concern for a full blown diabetes tsunami for the generations to come [5]. Weight loss in obese and overweight subjects can be induced via intensive lifestyle modifications, medications, and/or bariatric surgery. These methods have been shown to confer overall health benefits; however, their effect on remission of preexisting diabetes mellitus and reduction in cardiovascular risk has been variable. Recent research data has offered a much better understanding of the pathophysiology and outcomes of these management strategies in obese patients. In this paper, the authors have summarized the results of major studies on remission of type 2 diabetes mellitus and reduction of cardiovascular events by weight loss induced

by different methods. Furthermore, the paper aims to clarify various prevailing myths and practice patterns about obesity management among clinicians.

2. Methods

In preparation for this paper, several online search engines were used to gather journal articles that focused on studying the efficacy of surgical weight loss as compared to the conventional medical therapies and intensive lifestyle modifications. An initial advanced literature search was conducted by PubMed and MEDLINE using a combination of key words that included “epidemiology,” “obesity,” “weight loss,” “diabetes mellitus,” and “bariatric surgery” which yielded 831 articles. The search was then narrowed to articles published in the last two decades. Furthermore, an individual screening of the articles was conducted for prospective, randomized, and controlled trials comparing the effectiveness of surgical versus medical/lifestyle intervention for weight loss and long-term cardiovascular and diabetes outcomes. Also, the Cochrane collaborative database was utilized to obtain articles on the latest information on weight loss and its effects on cardiovascular risk. To write our paper, we finally selected 56 articles, based on the study design and power, which were relevant to our current discussion. Studies referred were original research, both prospective and retrospective controlled studies, and three large meta-analyses. The current review aims to draw evidence based conclusions, utilizing the latest research data, which can be utilized to guide the treatment of the obese diabetic patient population.

3. Data and Discussion

Recent data has suggested that diabetes mellitus may be reversed or prevented with weight loss strategies in obesity [4, 6]. The exact definition of diabetes remission remains an area of debate [7]; however, the American Diabetes Association (ADA) currently defines this as the achievement of euglycemia without pharmacological treatment for at least 1 year [8]. Partial diabetes remission is defined as a transition from meeting diabetes criteria to a prediabetes glycemia level (i.e., fasting plasma glucose of 100–126 mg/dL and HbA1c of 5.7–6.5%) and complete diabetes remission as the transition from diabetes criteria to full glucose normalization (i.e., fasting plasma glucose < 100 mg/dL and HbA1c < 5.7%) [9]. Although this is a good operational definition, according to glycemic and pharmacological criteria, it does not account the underlying beta-cell function and insulin action and thus may be inappropriate to define a cure of diabetes [10].

The most recently published data from the Look AHEAD trial showed that the intensive lifestyle intervention (ILI) is superior to conventional diabetes education in inducing weight loss and partial or complete remission of diabetes mellitus [9]. ILI included weekly group and individual counseling in the first 6 months, followed by 3 sessions per month for the second 6 months and twice-monthly contact and regular refresher group series and campaigns for 3 years. The ILI aimed to limit total caloric intake to 1200

to 1800 kcal/d through reductions in total and saturated fat intake and by increasing physical activity levels to a goal of 175 min/wk. Liquid meal replacements were provided to assist dietary goals. Participants in the conventional diabetes education were offered 3 group sessions each year focusing on diet, physical activity, and social support. At 2, 3, and 4 years, respectively, 9.2%, 6.4%, and 3.5% of intensive lifestyle intervention participants ($n = 2262$) had partial diabetes remission compared with 1.7%, 1.3%, and 0.5% of participants in the diabetes support and education group ($n = 2241$). Complete remission was; however, rare: 1.3% and 0.7% at 1 and 4 years. Participants with early stage diabetes (shortest duration, not treated with insulin, good baseline glycemic control) were the most to benefit from ILI. Furthermore, the study also showed that ILI leads to significant improvements in other health indicators, such as body weight, fitness, blood pressure, glycemic control, and lipids. Despite improvements in risk factor profiles, ILI did not result in decrease of cardiovascular events (nonfatal myocardial infarction, hospitalization for angina, nonfatal stroke, or death) compared to conventional diabetes education and therapy. As the primary end point was not met, it led to premature termination of the Look AHEAD trial. The results of the Look AHEAD trial are somewhat similar to Action in Diabetes and Vascular Disease (ADVANCE) trial, Action to Control Cardiovascular Disease in Diabetes (ACCORD) trial, and Veterans Affairs Diabetes Trial (VADT). In these trials, intensive medical/antiglycemic therapy did not show any cardiovascular benefit despite better diabetes control and improvement in cardiovascular risk factors. In the ACCORD trial, the mortality rate was about 19% higher in the intensive glucose management group as compared to standard glucose management group [11–13]. Nevertheless, it should be noted that most patients in the ACCORD, ADVANCE, and VADT trials had longstanding diabetes with preexisting overt cardiovascular disease. In contrast to these studies, intensive antiglycemic therapy in newly diagnosed type 2 diabetics (UKPDS study) without overt coronary heart disease resulted in reduction in coronary events. Nonetheless, the effect on cardiovascular risk reduction became apparent after several years of treatment [14]. One may argue that the results of the look AHEAD trial could be similar to the UKPDS study with longer followup, but the results could have been affected by the use of statins and medical therapy including angiotensin converting enzyme inhibitors in both the study groups.

Medical therapy for obesity with orlistat and sibutramine results in modest weight loss. Orlistat leads to about 3 kg weight loss; however, no reduction in cardiovascular events has been shown despite the weight loss and improvement in diabetes mellitus and lipid parameters [15]. Sibutramine use has been linked to increased number of nonfatal myocardial infarction and stroke despite weight loss [14], and for this reason, sibutramine was withdrawn from the market in October 2010.

Two other studies, the UKPDS and STENO trials, reported important findings in regards to reduction in cardiovascular events with better diabetes control. The open, parallel STENO trial studied a composite end point of death from cardiovascular causes, nonfatal myocardial infarction,

nonfatal stroke, revascularization and amputation in patients with type 2 diabetes. The study concluded that a focused multifactorial intervention that includes pharmacological therapy along with behavior modification and aspirin therapy decreased the risk of cardiovascular disease and the overall levels of glycosylated hemoglobin, blood pressure, cholesterol, and triglycerides levels as well as urinary albumin excretion [16]. Interestingly, the 13-year followup to the STENO trial (STENO-2) did not reveal any significant weight loss in either study groups despite the sustained reduction in cardiovascular events in the intensive therapy group posing a question for whether weight loss plays a key role in cardiovascular risk reduction [17]. The study showed a 50% overall risk reduction in cardiovascular and microvascular events with the implementation of the intensive multifactorial treatment [16]. The UKPDS trial on the other hand, randomly assigned 4209 patients with newly diagnosed type 2 diabetes to either conventional therapy (dietary restriction) or intensive therapy (sulfonylurea or insulin or in obese subjects metformin). The study's 10-year followup concluded that despite the loss of glycemic differences between the two groups, a continued reduction in microvascular risk and an overall risk reduction for myocardial infarction and death from any cause were observed [18]. A summary of the main findings of various research studies is enclosed in Table 1, whereas Table 2 gives a summary of the baseline characteristics of the study participants for the ACCORD, UKPDS, Look AHEAD, VADT, and ADVANCE trials.

There is increasing evidence that bariatric surgery is more effective than medical or lifestyle interventions for weight loss, reduction in cardiovascular risk, and diabetes remission. The reduction in comorbidities by bariatric surgery appears to translate into a 29% mortality reduction rate [19, 20]. A large cohort study of nearly 8000 patients who had undergone Roux-en-Y surgery was matched to a similar sized cohort. In this study the patients with Roux-en-Y had a reduction of all-cause mortality by 40%, diabetes-related mortality by 92%, coronary artery disease related mortality by 56%, and mortality from cancers by 60% [21]. A large meta-analysis published in 2009 based on the results of 621 studies (from 1990 to 2006) reported that average weight loss by a bariatric surgery was about 38.5 kg or 55.9% excess body weight loss. Overall, 78.1% of diabetes patients had complete resolution and diabetes was improved or resolved in about 86.6% of the patients. Weight loss and diabetes resolution were found to be the greatest for patients undergoing biliopancreatic diversion/duodenal switch, followed by gastric bypass, and the least with banding procedure [22]. In a recent analysis of severely obese patients with diabetes, the adjusted probability of initial remission of diabetes mellitus was found to be 12-to-24-fold greater for patients who underwent bariatric surgery than for those who underwent medical care alone. Bariatric subjects also experienced lower relapse rates of diabetes compared to the nonsurgical subjects and without higher risk of death [23].

Another large multisite retrospective cohort study of adults with uncontrolled or medication-controlled type 2 diabetes who underwent gastric bypass reported that 68% patients experienced an initial complete diabetes remission

within 5 years after surgery, of which 35% redeveloped diabetes within 5 years. Significant negative predictors of diabetes remission were poor preoperative glycemic control, insulin use, and longer diabetes duration. Weight trajectories after surgery were significantly different for never remitters, relapsers, and durable remitters [24]. Furthermore, Adams et al. recently reported the results of a prospective study which gathered data 6 years after Roux-en-Y gastric bypass (RYGB) surgery and aimed to examine the association of the surgery with weight loss, diabetes mellitus, and other health risks. They found that remission of type 2 diabetes was significantly more common among patients who underwent RYGB (62%) than among severely obese subjects with diabetes who initially sought but did not undergo bariatric surgery (6%) and those who never sought bariatric surgery (8%) [25].

The Swedish Obese Subjects (SOS) study is an ongoing, nonrandomized, controlled, prospective, and matched study conducted at 25 public surgical departments and 480 primary health care centers in Sweden of 2010 obese participants who underwent bariatric surgery and 2037 contemporaneously matched obese controls who received usual care. Sjöström et al. published the 2-year and 10-year follow-up results of SOS study in 2004 where they found that after two years, the weight had increased by 0.1 percent in the control group and had decreased by 23.4 percent in the surgery group ($P < 0.001$) and after 10 years, the weight had increased by 1.6 percent in the control group and decreased by 16.1 percent in the surgery group ($P < 0.001$). Energy intake was lower and the proportion of physically active subjects higher in the surgery group than in the control group throughout the observation period. Two- and 10-year rates of recovery from diabetes, hypertriglyceridemia, low levels of high-density lipoprotein cholesterol, hypertension, and hyperuricemia were more favorable in the surgery group than in the control group, whereas recovery from hypercholesterolemia did not differ between the groups. The surgery group had lower 2- and 10-year incidence rates of diabetes, hypertriglyceridemia, and hyperuricemia than those of the control group; differences between the groups in the incidence of hypercholesterolemia and hypertension were; however, undetectable [26]. Sjöström et al. published another followup results of the SOS study in 2012 where they reported that bariatric surgery was associated with reduced incidence of fatal and total cardiovascular events, myocardial infarction and stroke even after the adjustment of baseline conditions (adjusted hazard ratio of 0.47; 95% CI 0.29–0.76; $P = 0.002$ for all cardiovascular deaths and adjusted hazard ratio of 0.67; 95% CI 0.54–0.83; $P < 0.001$ for all first time fatal or nonfatal cardiovascular events) [27].

Another prospective, nonrandomized, and controlled one year clinical trial aimed to compare the effects of Roux-en-Y gastric bypass surgery and comprehensive lifestyle intervention on type 2 diabetes and obesity-related cardiovascular risk factors reported mean one-year weight loss of 30% and 8% respectively in the surgical and medical intervention groups [28]. Beneficial effects on glucose metabolism, blood pressure, lipids, and low-grade inflammation were observed in both groups. Remission rates of type 2 diabetes and hypertension were significantly higher in the surgery group

TABLE 1: Summary of the results of the major trials.

Look AHEAD trial	Intensive lifestyle intervention (ILI) is superior to conventional diabetes education in inducing weight loss and partial or complete remission of diabetes mellitus. No benefit of ILI, however, on cardiovascular outcomes was noted
ACCORD trial	Five-year results confirm that neither more intensive lowering of blood glucose levels and more intensive lowering of blood pressure nor treatment of blood lipids with a fibrate and a statin drug reduces cardiovascular risk in people with established type 2 diabetes who are at severely high risk for cardiovascular events. However, the study did find improvements to microvascular conditions. Also, more deaths from any cause and fewer nonfatal myocardial infarctions were observed
VADT	Intensive glucose control in patients with poorly controlled type 2 diabetes had no significant effect on the rates of major cardiovascular events, death, or microvascular complications, with the exception of progression of albuminuria
ADVANCE trial	Intensive medical/antiglycemic therapy did not show any macrovascular benefit despite better diabetes control and improvement in cardiovascular risk factors, but it did show a reduction in microvascular events
UKPDS study	Intensive antiglycemic therapy in newly diagnosed type 2 diabetics without overt coronary heart disease resulted in reduction in coronary events. Study's 10-year follow up concluded that despite the loss of glycemic differences between the two groups, a continued reduction in microvascular risk and an overall risk reduction for myocardial infarction and death from any cause were observed
STENO trial	It concluded that a focused multifactorial intervention that includes pharmacological therapy along with behavioral modification and aspirin therapy decreased the risk of cardiovascular disease and the overall levels of glycosylated hemoglobin, blood pressure, and cholesterol and triglycerides levels as well as urinary albumin excretion

than the lifestyle intervention group; 70 versus 33%, $P = 0.027$, and 49 versus 23%, $P = 0.016$. The surgery group experienced a significantly greater reduction in the prevalence of metabolic syndrome, albuminuria, and electrocardiographic left ventricular hypertrophy than the lifestyle group. Gastrointestinal symptoms and symptomatic postprandial hypoglycemia developed more frequently after gastric bypass surgery than after lifestyle intervention, though mortality in either group was not significantly different [28]. One very recent prospective, randomized, and nonblinded clinical trial with one-year follow-up period, performed at the Cleveland Clinic, evaluated the efficacy of intensive medical therapy alone versus medical therapy combined with Roux-en-Y gastric bypass or sleeve gastrectomy in 150 obese patients with uncontrolled diabetes mellitus and reported HbA1c reduction to $\leq 6\%$ in 12% patients receiving medical therapy versus 42% in gastric-bypass group and 37% in sleeve-gastrectomy group [29]. Weight loss and improvement in insulin resistance was significantly higher in both the surgical groups than in medical therapy only group. The use of drugs to lower glucose, lipid, and blood-pressure levels decreased significantly after both surgical procedures but increased in patients receiving medical therapy only [29]. Mingrone et al. conducted a single-center, nonblinded, randomized, and controlled trial comparing the efficacy of Roux-en-Y gastric bypass and biliopancreatic diversion versus medical therapy for the remission of diabetes mellitus in morbidly obese diabetic patients. At 2 years, diabetes remission occurred in no patients in the medical-therapy group versus 75% in the gastric-bypass group and 95% in the biliopancreatic-diversion group ($P < 0.001$). Age, sex, baseline BMI, duration of diabetes, and weight changes were not significant predictors of diabetes remission or of improvement in glycemia. Though the average baseline glycated hemoglobin level had decreased in all groups, patients in both the surgical groups

had greater degree of glycemia improvement than those of medical therapy group, the greatest improvement to have noted in the biliopancreatic-diversion group [30].

Though previous studies have offered a strong evidence of benefit of bariatric surgery in patients with a BMI $> 40 \text{ kg/m}^2$, the benefits in patients with a lower BMI obese patients, that is, mild (BMI = $30\text{--}35 \text{ kg/m}^2$) or moderate obesity (BMI = $35\text{--}40 \text{ kg/m}^2$), have remained unclear until recently [31]. Current indications of bariatric surgery in obese patients as defined by the National Institute of Health are: BMI $> 40 \text{ kg/m}^2$ OR BMI $> 35 \text{ kg/m}^2$ associated with serious comorbidities like diabetes, sleep apnea, obesity-related cardiomyopathy, or severe joint disease [32]. However, recent data also suggests benefit of bariatric surgery in mild and moderate obesity patients. A recent prospective study compared the effects of laparoscopic sleeve gastrectomy (LSG) versus medical therapy on patients with type 2 DM and a BMI of $< 35 \text{ kg/m}^2$ and reported diabetes mellitus and hypertension remission rate of 88.8% (8 of 9 patients) without undesirable excessive weight loss as compared to none of the patients (0 of 9 patients) undergoing remission in the medical therapy group [33]. Another prospective, consecutive, and nonrandomized trial of 79 patients investigate the role of sleeve gastrectomy for patients with class I obesity (BMI $30\text{--}35 \text{ kg/m}^2$) and reported promising early weight loss and quality of life improvement in patients [34]. One of the largest, prospective randomized trials published to date studying the long-term impacts of RYGB (followup being 6 years) on patients with diabetes and only class I obesity reported durable diabetes remission occurred in 88% of cases, with glycemic improvement in 11% [35]. Mean HbA1c fell from 9.7 ± 1.5 to $5.9 \pm 0.1\%$ ($P < 0.001$), despite diabetes medication cessation in the majority. Weight loss failed to correlate with several measures of improved

TABLE 2: Summary of participants' baseline characteristics in the reviewed studies.

Group	Look AHEAD trial		VADT		ACCORD trial		ADVANCE trial		UKPDS 38 trial	
	ILI	DSE	Standard therapy	Intensive therapy	Intensive therapy	Standard therapy	Intensive control	Standard control	Intensive therapy	Moderate therapy
Subjects (n)	2570	2575	899	892	5128	5123	5571	5569	758	390
Age (mean ± SD)	58.6 ± 6.8	58.9 ± 6.9	60.3 ± 9.0	60.5 ± 9.0	62.2 ± 6.8	62.2 ± 6.8	66 ± 6	66 ± 6	56.4 ± 8.1	56.5 ± 8.1
Male (n)	1044	1038	873	866	3143	3156	3195	3212	410	227
Female (n)	1526	1537	26	26	1985	1967	2376	2357	348	163
Average HbA1c (%)	7.25 ± 1.14	7.31 ± 1.20	9.4 ± 2.0	9.4 ± 2.0	8.3 ± 1.1	8.3 ± 1.1	7.51 ± 1.57	7.52 ± 1.54	6.9 ± 1.7	6.8 ± 1.5
Weight (mean ± SD)	F	F								
	94.8 ± 17.9	95.4 ± 17.3	214 ± 36	214 ± 36	93.5 ± 18.7	93.6 ± 18.7	78.2 ± 16.8	78.0 ± 16.8		
	M	M								
BMI (mean ± SD)	108.9 ± 19.0	109.0 ± 18.0								
	F	F								
	36.3 ± 6.2	36.6 ± 6.0	31.2 ± 4.0	31.3 ± 3.0	32.2 ± 5.5	32.2 ± 5.5	28 ± 5	28 ± 5	29.8 ± 5.5	29.3 ± 5.5
Systolic BP (mean ± SD)	M	M								
	35.3 ± 5.7	35.1 ± 5.2								
	128.1 ± 17.3	129.45 ± 17.1	132 ± 17	131 ± 17	136.2 ± 16.9	136.4 ± 17.2	145.0 ± 21.7	145.0 ± 21.4	159 ± 20	160 ± 18
Diastolic BP (mean ± SD)	69.69 ± 9.55	70.4 ± 9.72	76 ± 10	76 ± 10	74.8 ± 10.6	75.0 ± 10.7	80.8 ± 11.0	80.5 ± 10.8	94 ± 10	94 ± 9

ILI represents intensive lifestyle intervention; DSE represents diabetes support and education (conventional management); M and F represent males and females, respectively. Data are presented as (n) or mean ± standard deviation (SD).

glucose homeostasis, consistent with weight-independent antidiabetes mechanisms of RYGB. C-peptide responses to glucose increased substantially, suggesting improved β -cell function. There was no mortality, major surgical morbidity, or excessive weight loss. Hypertension and dyslipidemia also improved, yielding 50–84% reductions in predicted 10-year cardiovascular disease risks of fatal and nonfatal coronary heart disease and stroke [35]. In addition, post hoc study findings reported by Sjöström et al. demonstrated that a higher baseline BMI may not be associated with a greater benefit of bariatric surgery [27].

Other important comorbidities that deserve to be mentioned in this paper are nonalcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) as they may frequently be considered a part of the metabolic syndrome (insulin resistance). Some studies have reported improvement in NAFLD, adipokines (used to measure fatty liver disease), and insulin resistance after weight loss with bariatric surgery [36, 37]. However, randomized trials to confirm these findings are lacking and thus use of surgical approach for these conditions remains controversial [38].

Though our current discussion mainly focuses on the review and comparison of effectiveness of medical versus surgical weight loss strategies; however, we wanted to mention here the results of another most recent randomized trial which showed that the use of Mediterranean diet results in the reduction of major cardiovascular events especially in obese patient population [39]. This study mainly compared the cardiovascular outcomes in patients receiving Mediterranean diet versus those receiving regular controlled diet. None of the study patients were advised either total calorie restriction or promotion of physical activity. The effects of Mediterranean diet on the weight/BMI were also not the major focus of this study. To best of our knowledge, this study is the first largest prospective study which showed significant cardiovascular risk reduction in obese patients with the use of Mediterranean diet and thus this opens up a consideration for recommending the use of this dietary lifestyle intervention which may potentially reduce cardiovascular risk in obese patients.

4. Summary and Future Directions

Look AHEAD trial, the largest prospective trial comparing efficacy of ILI with standard diabetes education and management (SDE) in obese diabetics, failed to show any additive benefit of ILI over SDE in the improvement of cardiovascular outcomes despite an observed improvement in weight loss, body fitness, and diabetes remission. Complete diabetes remission with ILI was rare, but partial diabetes remission even though significant in the initial years was not long lasting. Partial diabetes remission with ILI was most effectively achieved in patients with short duration diabetes history, who have lower HbA1c levels and those who do not yet require insulin therapy, suggesting that emphasis on early diabetes screening and probably intensive/aggressive management in the early course of the disease warrants more attention.

Some prior studies of medication based therapy have shown that reductions in HbA1c and fasting glucose, if sustained, are likely to considerably reduce the risks of microvascular complications [40, 41]. One could argue that failure of demonstrating any benefit in the cardiovascular outcomes with ILI in the Look AHEAD trial could be because of improved cardiovascular risk factor control in both the study groups through medical treatment, improved clinical care after acute cardiovascular events, enrollment of a healthier-than-expected patient cohort and excluding patients with high baseline cardiovascular risk factors [10]. This may need to be confirmed with future long-term prospective studies. However, based on the current evidence, intensive medical therapy alone does not appear to be very promising in improving cardiovascular outcomes or producing long-term diabetes remission.

In contrast to weight loss by medications or intensive lifestyle modification, bariatric surgery has shown to be very effective in improving cardiovascular health and in producing diabetes remission in obese diabetic patients by the majority of both long-term and short-term cohort and prospective studies. Although one may assume that the degree of postoperative weight loss might be the major driver for improvement in diabetes mellitus in obese patients undergoing bariatric surgery, the weight loss and type 2 diabetes are often not in a direct cause-and-effect relationship. The improvement of diabetes mellitus occurs within days after gastric bypass, before there is significant weight loss [42–44]. These findings suggest that bariatric surgeries may affect the diabetes course independent of the weight. Rubino et al. suggested that changes in the gut hormonal milieu after bypass of the distal stomach, duodenum, and proximal jejunum can influence the mechanism of type 2 diabetes [45]. Other mechanisms involved may be changes in neurohumoral regulation, increased expression of glucose transporter type 4 (GLUT4) [46], and normalization of whole-body insulin resistance [30].

Though BMI is still the most widely used criteria for selection of patients for bariatric surgery in clinical practice, this practice pattern may change as the recent data shows significant benefit of bariatric surgery in mild and moderately obese patients. Also, one study findings suggested that high insulin levels may be a better selection criterion for bariatric surgery than high BMI, as far as cardiovascular events are concerned [27]. As seen with intensive lifestyle treatment, patients with early stage diabetes appeared to benefit most from bariatric surgery; so a strong argument can be made for considering earlier surgical intervention in moderately obese patients (body mass index ≥ 30) with early stage diabetes. At the same time, offering bariatric surgery to every obese diabetic individual may impose serious practical concerns which include nonbenign nature of the surgical intervention (including some associated long-term mechanical and nutritional complications), nonsustained beneficial long-term effects, as shown by Holman et al. [18] and Arterburn et al. [24], and significant increase in the nationwide healthcare burden related to the cost of intervention.

5. Common Myths about Obesity among Clinicians and Patients

- (1) *I should advise my obese diabetic patients to follow intensive dietary and exercise (lifestyle) interventions as this would decrease their cardiovascular risk as compared to routine interventions and medical therapy.*

Answer. This is the most common myth which prevails among clinicians, may it be general internists or cardiologists. Though this statement sounds very conceptual, the prospective studies have jolted this prevailing belief as intensive lifestyle interventions have not shown to decrease cardiovascular morbidity and mortality in these patients despite improvement in other health indicators like weight loss, body fitness and diabetes remission.

Though with that being said, data in these studies have their own limitations as discussed earlier and the authors feel it should not preclude clinicians from advising their patients to adopt healthier lifestyle measures including intensive lifestyle interventions for management of diabetes in obesity.

- (2) *Poorly controlled diabetics with a long standing history of disease are most likely to benefit from intensive lifestyle therapy for their diabetes control or remission.*

Answer. This is another common myth. As we discussed earlier, data from prospective studies have shown that newly diagnosed diabetics or diabetics with better glycemic control are in fact more likely to achieve a partial or transient complete diabetes remission with intensive lifestyle modification and medical therapy, as compared to patients with long standing history of disease.

- (3) *Bariatric surgery is more effective than medical management in inducing diabetes remission because it induces rapid and more weight loss.*

Answer. The improvement of diabetes mellitus occurs within days after gastric bypass, before there is significant weight loss. Changes in the gut hormonal milieu after gastric bypass, changes in neurohumoral regulation, and normalization of whole body insulin resistance have been proposed to be additional mechanisms providing therapeutic benefit.

- (4) *Weight loss strategies in obesity should include setting of realistic goals because otherwise patients will become frustrated and lose less weight.*

Answer. Although this sounds a very reasonable concept, several studies have offered contrasting data and support that more ambitious goals are associated with better weight-loss outcomes [47, 48]. Study results were more supportive of the idea that higher goals motivate patients to lose more weight than of the hypothesis that high goals undermine effort.

- (5) *Large amount and rapid weight loss is associated with poorer long-term outcomes, as compared with slow, gradual weight loss.*

Answer. This belief probably emerged over five decades ago in reaction to the adverse effects of nutritionally insufficient very-low-calorie diets (<800 kcal per day) and has persisted to the extent that it may be common advice by many regular dietitians. In several prospective trials, patients often undergo more rapid and greater initial weight loss and this has been associated with lower body weight at the end of long-term followup. Some obese persons have a greater initial weight loss than others do due to unclear reasons, thus a recommendation to lose weight more slowly might interfere with the ultimate success of weight-loss efforts [49].

- (6) *Sexual activity is a good means to burn extra calories.*

Answer. People often may feel that they spend a lot of energy (about 100–300 kcal) while indulging in one sexual intercourse. It is a myth. Studies have shown that an average bout of sexual activity lasting for about 6 minutes approximately expends only 21 kcal [50].

- (7) *Breastfeeding is protective against childhood obesity.*

Answer. Breastfeeding has been advocated to offer a protective benefit against the development of childhood obesity, for a long time. Previously published review report from the World Health Organization also supported this fact [51]. The incidence of type 1 diabetes is reduced by 30% in infants exclusively breastfed for at least 3 months. A reduction of 40% in type 2 diabetes also is reported, possibly reflecting the long-term positive effect of breastfeeding on weight control and feeding self-regulation [52]. However, more recent data from prospective randomized controlled studies do not support the theory of antiobesity effects of breast milk to the children [53–55].

- (8) *“My obesity is hereditary. Everyone in my family is obese. Dietary or behavioral modifications are not going to work, so why even bother?”*

Answer. Although genetic factors play a large role in obesity, heritability is not horoscope. Studies have shown that even moderate environmental changes can promote significant weight loss [56]. Identification of key environmental factors and then successful interventions can achieve clinically significant reductions in obesity.

- (9) *Bariatric surgery offers benefits only in patients with BMI >40 or BMI >35 with associated severe comorbidities.*

Answer. Though BMI is still the most widely used criteria for selection of patients for bariatric surgery in clinical practice, this practice pattern may change as the recent data from multiple studies shows significant benefit of bariatric surgery in mild and moderate obese patients.

6. Conclusions

- (1) The association of diabetes mellitus, obesity, and cardiovascular risk factors is very complex. Recent research data certainly provides a better understanding; however, many pathophysiologic mechanisms associated with the benefits of medical and surgical interventions remain poorly understood. Future long-term prospective studies are warranted for a better clinical decision making.
- (2) Clinical education, research, and health policies need to increase their focus towards primary prevention of risk factors associated with diabetes mellitus and obesity especially in children and adolescents who are deemed to be at high risk.
- (3) Though ILI did not appear to reduce the risk of cardiovascular events significantly in prospective studies, the authors feel that it should be recommended to all the patients given the absence of long-term follow-up data and significant health benefits achieved in a subset of patients.
- (4) Several myths prevail regarding obesity not only among general population but also among clinicians which may affect practice patterns. More education among the patients and general clinicians may produce better healthcare outcomes in obese subjects.
- (5) Bariatric surgery certainly appears to have an emerging and promising future role in the management of cardiac risk factors and diabetes remission in obese diabetic patients, especially in the subset of the newly diagnosed diabetics in this patient population. Furthermore, bariatric surgical intervention has also shown some promising outcomes in the less severely obese patient population who have high cardiovascular risk factors. However, appropriate selection criteria need a consensus opinion involving possible reformulation of the existing guidelines.

Conflict of Interests

The authors declare that they have no conflict of interests.

Disclosure

Nitin Trivedi is a member of the Speaker Bureau for Novo Nordisk, Merck, and Novartis.

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Research Article

Comparison of Insulin Lispro Protamine Suspension with NPH Insulin in Pregnant Women with Type 2 and Gestational Diabetes Mellitus: Maternal and Perinatal Outcomes

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Insulin therapy is still the gold standard in diabetic pregnancy. Insulin lispro protamine suspension is an available basal insulin analogue. *Aim.* To study pregnancy outcomes of women with type 2 and gestational diabetes mellitus when insulin lispro protamine suspension or human NPH insulin was added to medical nutrition therapy and/or short-acting insulin. *Methods.* In this retrospective study, for maternal outcome we recorded time and mode of delivery, hypertension, glycaemic control (fasting blood glucose and HbA1c), hypoglycemia, weight increase, and insulin need. For neonatal outcome birth weight and weight class, congenital malformations was recorded and main neonatal complications. Two-tail Student's *t*-test and chi-square test were performed when applicable; significant $P < 0.05$. *Results.* Eighty-nine pregnant women (25 with type 2 diabetes and 64 with gestational diabetes mellitus; 53 under insulin lispro protamine suspension and 36 under human NPH insulin) were recruited. Maternal and neonatal outcomes were quite similar between the two therapeutic approaches; however, insulin need was higher in NPH. At the end of pregnancy, eight women with gestational diabetes continued to use only basal insulin analogue. *Conclusions.* Pregnancy outcome in type 2 and gestational diabetes mellitus with insulin lispro protamine suspension was similar to that with NPH insulin, except for a lower insulin requirement.

1. Introduction

Insulin therapy is still the gold standard in the treatment of diabetes in pregnancy when medical nutrition therapy (MNT) and lifestyle cannot reach and maintain the metabolic targets [1, 2].

Studies using lispro and aspart in pregnancy have shown that both rapid-acting insulin analogues are safe and effective in reducing the postprandial glycaemia [3–8].

However, human neutral protamine Hagedorn (NPH) insulin still remains the primary basal insulin choice [9–13], even though, recently, a randomized controlled trial demonstrated noninferiority of detemir versus NPH insulin in 310 pregnant women with type 1 diabetes [14].

Insulin lispro protamine suspension (ILPS), formulated by cocrystallizing insulin lispro with protamine, is a basal insulin analogue with pharmacokinetics and glucodynamics comparable to those of NPH insulin [15]. Current evidence suggests that ILPS may represent a valuable option in the management of diabetic patients, primarily those with type 2 diabetes, requiring insulin treatment regimens [16]. There are no studies about ILPS use during pregnancy, during which the continuous adjustment of the hormonal pattern causes several metabolic and circulatory changes in the mother's body to accommodate fetal needs [17], so its metabolic effectiveness in pregnancy has not yet been demonstrated. To this aim, we retrospectively studied pregnancies in women with type 2 and gestational diabetes mellitus (GDM) when

ILPS or NPH insulin was added to MNT and/or rapid insulin analogues.

Our primary objective was to compare the main maternal outcomes (mode and time of delivery, preterm delivery, and hypertensive disorders) and perinatal outcomes (birth weight and weight class, congenital malformations, neonatal hypoglycaemia, and other perinatal morbidities). As a secondary objective, we evaluated other clinical and glycaemic outcomes (fasting blood glucose and HbA1c, hypoglycemic episodes, insulin need, and weight gain) between the two therapeutic approaches (ILPS or NPH insulin).

2. Patients and Methods

This is a multicentre retrospective observational study of a cohort of pregnant women affected by type 2 or gestational diabetes mellitus (GDM) which was not being effectively controlled with MNT and/or rapid insulin analogues, who were additionally treated with an intermediate-acting insulin (ILPS or NPH).

All women were recruited consecutively from January 2008 to August 2010 in two hospitals located in Rome (S. Andrea and Sandro Pertini).

3. Study Protocol

Pregnancy dating, based on menstrual history and physical examinations, was definitely confirmed by an early ultrasound examination before the 16th week of gestation.

GDM was diagnosed between the 24th and 28th weeks of gestation with an (75 or 100 g) oral glucose tolerance test (OGTT); results were interpreted according to the Carpenter and Coustan criteria [18] and the recommendations of the 4th International Workshop Conference on Gestational Diabetes Mellitus [19]. In cases with one or more GDM risk factors (family history of type 2 diabetes, history of GDM and/or impaired glucose tolerance, obesity, and glycosuria), diagnosis was done earlier, as soon as it was feasible [19] to do so. All women with type 2 diabetes were treated with diet and/or oral hypoglycemic agents before pregnancy. At conception, 8 of these women were treated with metformin, 1 with metformin + sulphonylurea, 2 with sulphonylurea, 2 with repaglinide, 2 with metformin + repaglinide, 1 with metformin + rosiglitazone, 1 with insulin, and 8 with diet only. At the first visit, oral hypoglycaemic agents were shifted to diet only or diet plus insulin.

Glycaemic control was obtained when the following standardized goals were reached: fasting and preprandial ≤ 95 mg/dL (5.3 mmol/L) and 1 h after-meal ≤ 140 mg/dL (7.8 mmol/L) [20].

The individualized MNT was prescribed according to the patient's own preferences (ethnic, cultural, financial, etc.), physical activity level, gestational age, and prepregnancy BMI group with a distribution of carbohydrate intake of 45–50%, 30–35% of lipids, 20% of protein, and 28 g/day of fibers [9]. When MNT was not sufficient to control postprandial hyperglycemia, short-acting insulin analogues such as aspart or lispro were injected before meals; when MNT was not sufficient to control fasting hyperglycemia, basal insulins

such as ILPS or NPH were prescribed at bed time; in those few cases in which preprandial glucose values were higher than targets, ILPS or NPH was added before breakfast as well [9].

Taking into account the safety of lispro, ILPS and NPH insulins were autonomously prescribed, often on the basis of their different commercial availability in the area where the women came from, that is, the consequence of some brands' policy which is removing NPH insulin from the Italian market.

At the moment of enrollment (coinciding with the introduction of basal insulin), within the ILPS group, 36 patients were already being treated with rapid analogue, while 17 were being treated only with diet; within the NPH group, all the patients were already being treated with rapid analogue.

Patients were taught to self-monitor their plasma glucose levels 4–6 times a day, using the same type of glucometer, given to them by our staff. All data was recorded in a diary kept by the patients at each control visit. Maternal glycohemoglobin (HbA1c) was checked every 4–6 weeks.

The diabetic women were visited at regular intervals (1–2 weeks). At each visit, home capillary blood glucose profiles, insulin requirement and adjustments, hypoglycemic episodes, and body weight were recorded. Capillary blood glucose profiles during the previous 1 or 2 weeks were recorded as mean values \pm standard deviation (SD).

Regarding the maternal outcomes, we recorded time and mode of delivery, hypertensive disorders, glycaemic control (as fasting capillary blood glucose, FCBG, and HbA1c), hypoglycemic episodes, weight increase, and insulin need.

Preterm deliveries were those occurring before the 37th gestational week.

Hypertension was defined according to the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [21] and classified as follows: chronic hypertension as hypertension before the 20th week of gestation (CR); gestational hypertension (PIH) as hypertension after the 20th week of gestation; preeclampsia (PE) as gestational hypertension + proteinuria (0.3 g/24 h). We considered hypertensive disorders as CR+PIH+PE.

The degree of hypoglycaemic episodes was graded as follows: mild if slight symptoms spontaneously were resolved; moderate if symptoms resolved by taking oral carbohydrate; severe if symptoms were resolved requiring assistance from another person; serious if they required admittance to hospital.

Prepregnancy BMI was calculated according to our patients' height/reported pregestational weight (kg^2) [22]. Insulin need (as units/kg/day) was calculated as total daily insulin doses (rapid + intermediate-acting insulin divided body weight), daily rapid insulin (daily rapid insulin/body weight), and daily intermediate-acting insulin (daily ILPS or NPH insulin/body weight) at the last visit before delivery; daily rapid insulin doses were also calculated before the introduction of ILPS or NPH (enrollment).

For neonatal outcomes, we noted the length and the weight at birth, APGAR score at 5 minutes, congenital malformations, hypoglycemia, hyperbilirubinemia, other neonatal morbidity (as obstetric trauma, respiratory disorders, and

need for intensive care—NICU—), stillbirths, and neonatal mortality.

We calculated the ponderal index (PI) as the ratio of weight to length cubed (g/cm^3), considering a PI higher than $2.85 \text{ g}/\text{cm}^3$ as excessive [23]. Babies were defined large for gestational age (LGA) if their birth weight was above the 97th percentile and small for gestational age (SGA) if their birth weight was below the 3rd percentile, based on standard growth and development tables for the Italian population [24]. Malformations were classified according to the EUROCAT (<http://www.eurocat-network.eu/>) and fetal morbidity to the Obstetrical Quality Indicator [25]. Stillbirths were children born dead beyond 180 days of pregnancy. Neonatal mortality was the rate of deaths before the 28th day of life.

Women gave their written consent for the anonymous use of their clinical data at the first visit, as previously approved by our ethics committee.

4. Statistics

All data was presented as means \pm standard deviation (SD) for continuous variables and as percentages for categorical variables. Data was processed with the Apple software program (Stat View). Two-tail unpaired and paired Student's *t*-tests were used when applicable to compare the pairs of means or longitudinal values. Chi-square (χ^2), as nonparametric test, was performed to compare percentages. *P*-values of <0.05 were considered significant.

5. Results

Eighty-nine diabetic pregnant women treated with a basal insulin (ILPS or NPH) were consecutively recruited from January 2008 to August 2010 in two diabetes units in Rome (S. Andrea and Sandro Pertini).

Twenty-five of them were affected by type 2 diabetes and sixty-four by GDM. ILPS (ILPS group or ILPSg, $n = 53$) or NPH (NPH group or NPHg, $n = 36$) was introduced in addition to the current MNT \pm rapid insulin analogues therapy when fasting plasma glucose was above the ADA goal.

Because there was a higher number of women with type 2 diabetes in NPHg ($\chi^2 0.0002$), we separated those with type 2 diabetes from those with GDM; no significant differences of main clinical characteristics between treatment groups (ILPSg versus NPHg) at baseline were reported (see Table 1).

The duration of type 2 diabetes was 7.0 ± 4.8 years with no differences between groups (ILPSg 8.0 ± 5.2 versus NPHg 6.6 ± 4.7 yrs, ns); GDM was diagnosed at 21.9 ± 7.3 weeks with no differences between ILPSg and NPHg (respectively, 22.6 ± 7.4 versus 20.3 ± 6.7 wks, ns). At enrollment, in the ILPSg, 17 women (16 with GDM and one with type 2 diabetes) needed only a basal insulin ($\chi^2 0.002$) and 36 had already been treated by short-acting insulin analogues, with no difference between ILPS and NPH groups (Table 1). At the end of pregnancy; eight of these patients (all with GDM) continued to use ILPS

only, with an insulin need of $0.06 \pm 0.02 \text{ IU}/\text{kg}/\text{day}$ ($P < 0.0001$).

In 92.4% of ILPSg versus 88.9% of NPHg (ns), glycaemic control was reached with one basal-acting insulin \pm rapid analogues before meals. Among type 2 diabetic pregnancies, only two of 7 (28.6%) women being treated with ILPS and three of 18 (16.7%) being treated with NPH needed two basal insulins plus three short-acting insulin injections; the same therapy was used in two of 46 GDM (4.3%) of the ILPSg and one of 18 GDM (5.5%) of the NPHg.

As short-acting analogue, lispro was used in 51% of the ILPSg and in 50% the NPHg (ns).

6. Primary Endpoints

Maternal outcome was similar between ILPSg and NPHg in terms of mode and time of delivery (Table 2). The rate of hypertension, which was higher in all of NPHg, was found to be similar when patients with type 2 diabetes were split from those with GDM (hypertensive disorders in type 2 diabetes: 42.8% in ILPSg versus 44.4% in NPHg, ns; GDM: 17.4 ILPSg versus 33.3% NPHg, ns).

Neonatal outcomes did not statistically differ (Table 2), except when considering excessive ponderal index. Of three newborns whose ponderal index was $>2.85 \text{ g}/\text{cm}^3$, two were delivered by mothers affected by GDM and one by type 2 diabetes. Two of these mothers were treated with aspart and one with lispro as short-acting analogue. All these women had used NPH as basal insulin. Those women whose pregestational BMI was lower showed a lower weight gain, with higher capillary blood glucose levels and a higher insulin need. However, all these parameters did not reach significant levels.

Three newborns reported minor congenital malformations (two in the ILPSg and one in the NPHg, ns) consisting in heart defects (patent or persistent foramen ovale), not requiring surgery.

No newborn was SGA. There were no stillbirths or neonatal deaths nor neonatal complications needing intensive care (data not shown in table).

7. Secondary Endpoints

Fasting capillary blood glucose values and the number/severity of hypoglycaemic episodes were available for 58 women only (30 in the ILPSg and 28 in the NPHg).

FCBG were not different throughout pregnancy (Table 3). As we considered FCBG $< 95 \text{ mg}/\text{dL}$, this cutoff was obtained in 66.7% of the ILPSg versus 66.8% of the NPHg (ns) at the end of pregnancy.

Both groups of patients reported only mild and/or moderate hypoglycaemias, with no severe and serious episodes. The number of reported events was similar for both therapies (ILPSg 0.3 ± 0.3 versus NPHg 0.3 ± 0.7 , ns; type 2 ILPSg 0.2 ± 0.5 versus NPHg 0.5 ± 1.0 , ns; GDM ILPSg 0.1 ± 0.3 versus NPHg 0.1 ± 0.2 , ns).

TABLE 1: Baseline characteristics of pregnant women receiving ILPS or NPH insulin.

	ILPSg	NPHg	P
No.	53	36	
Type of diabetes (T2DM/GDM)	7/46	18/18	0.0002
Caucasian ethnicity (%)	90.6 (48/53)	91.7 (33/36)	ns
Age (yrs)			
Total	34.7 ± 5.8	34.2 ± 4.8	ns
T2DM	36.0 ± 4.3	33.4 ± 5.3	ns
GDM	34.5 ± 6.0	34.9 ± 4.3	ns
Prepregnancy BMI (kg/m ²)			
Total	28.6 ± 5.7	28.8 ± 7.2	ns
T2DM	30.9 ± 6.0	29.2 ± 7.2	ns
GDM	28.3 ± 5.7	28.4 ± 7.3	ns
Gestational week at 1st visit			
Total	22.2 ± 8.4	14.1 ± 8.9	<0.0001
T2DM	15.4 ± 9.6	9.2 ± 6.6	ns
GDM	23.3 ± 7.8	19.0 ± 8.3	ns
Gestational week at enrollment			
Total	27.8 ± 8.1	20.3 ± 11.1	0.0004
T2DM	15.4 ± 13.2	11.1 ± 7.1	ns
GDM	29.1 ± 6.3	29.5 ± 4.9	ns
Weight increase at enrollment (kg)			
Total	7.7 ± 6.7	4.4 ± 4.4	0.01
T2DM	4.7 ± 6.7	1.7 ± 2.4	ns
GDM	8.2 ± 6.7	7.1 ± 4.3	ns
Preenrollment short-acting insulin dose (units/kg/day)			
Total	0.18 ± 0.10	0.27 ± 0.16	0.01
T2DM	0.22 ± 0.13	0.30 ± 0.18	ns
GDM	0.18 ± 0.09	0.23 ± 0.14	ns

BMI: body mass index; T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus.

TABLE 2: Maternal and fetal outcomes in pregnant women receiving ILPS or NPH insulin.

	ILPSg			NPHg		
Number of subjects	Total	T2DM	GDM	Total	T2DM	GDM
	53	7	46	36	18	18
Maternal outcome						
Gestational week at delivery	38.3 ± 1.4	38.4 ± 2.1	38.3 ± 1.3	38.6 ± 1.0	38.9 ± 0.9	38.4 ± 1.1
Caesarean section (%)	67.9	100	65.2	80.5	100	61.1
Preterm delivery (%)	7.5	0	8.7	2.8	0	5.5
Hypertensive disorders (%)	20.7	42.8	17.4	38.9	44.4*	33.3
Neonatal outcome						
Newborn weight (g)	3328.5 ± 517.1	3104.3 ± 444.7	3361.3 ± 523.5	3376.3 ± 604.2	3338.5 ± 669.3	3409.4 ± 561.2
LGA (%)	15.1	14.3	15.2	22.2	22.2	22.2
PI (g/cm ³)	2.2 ± 0.3	2.1 ± 0.3	2.2 ± 0.3	2.3 ± 0.3	2.3 ± 0.4	2.3 ± 0.3
PI > 2.85 g/cm ³ (%)	0	0	0	8.3	5.5	11.1
APGAR at 5'	9.7 ± 0.5	9.8 ± 0.4	9.7 ± 0.5	9.5 ± 0.7	9.4 ± 0.8	9.6 ± 0.5
Congenital malformations (%)	3.8	0	4.3	2.8	0	5.5
Neonatal hypoglycemia (%)	7.5	0	8.7	8.3	11.1	5.5
Hyperbilirubinemia (%)	9.4	0	10.9	8.3	11.1	5.5

T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus; LGA: large for gestational age; PI: ponderal index.

*ILPS versus NPH $P = 0.02$.

TABLE 3: Glycaemic control and weight gain in pregnant women receiving ILPS or NPH insulin.

	ILPSg	No. pt	NPHg	No. pt	P
FCBG mg/dL (mmol/L)					
Before enrollment					
Total	100.7 ± 15.1 (5.6 ± 0.8)	21	100.6 ± 17.4 (5.6 ± 1.0)	25	ns
T2DM	110.0 ± 7.8 (6.1 ± 0.4)	4	109.8 ± 15.8 (6.1 ± 0.9)	12	ns
GDM	98.6 ± 15.8 (5.5 ± 0.9)	17	92.2 ± 14.5 (5.1 ± 0.8)	13	ns
At the end of pregnancy					
Total	93.6 ± 13.4** (5.2 ± 0.7)	30	95.7 ± 10.8* (5.3 ± 0.6)	28	ns
T2DM	89.2 ± 12.7* (4.9 ± 0.7)	4	95.5 ± 8.2° (5.3 ± 0.4)	13	ns
GDM	94.3 ± 13.5* (5.2 ± 0.7)	26	95.8 ± 12.8* (5.3 ± 0.7)	15	ns
HbA1c% (mmol/L)					
At the end of pregnancy					
Total	5.4 ± 1.1 (36.0 ± 12.2)	34	5.3 ± 0.7 (34.4 ± 8.1)	33	ns
T2DM	5.8 ± 1.1 (39.6 ± 12.6)	7	5.4 ± 0.7 (36.8 ± 8.2)	18	ns
GDM	5.3 ± 1.1 (35.0 ± 12.1)	27	5.0 ± 0.6 (31.5 ± 7.2)	15	ns
Weight gain (kg)					
At the end of pregnancy					
Total	10.4 ± 6.1	53	9.9 ± 4.2	36	ns
T2DM	12.0 ± 6.1	7	10.8 ± 4.9	18	ns
GDM	10.2 ± 6.1	46	9.1 ± 3.3	18	ns

FCBG: fasting capillary blood glucose; T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus.

Paired *t*-test's *P* between preenrollment versus the end of pregnancy: *ns; **0.02; °0.004.

Weight gain did not differ between the two treatment-groups, in type 2 diabetic pregnant women as well as in those with GDM (Table 3).

Insulin need was higher in the NPHg either as total daily insulin in both types of diabetes or as rapid-acting analogue in type 2 diabetic women only (Table 4).

8. Discussion

Taking into account the physiological changes of glycemic profiles in pregnancy [26] and the consolidated results [27] that adjustment of postprandial, rather than preprandial, blood glucose values improve pregnancy outcomes, it is generally known that insulin analogues may produce better glycaemic control with less hypoglycemia risk compared with the use of human insulin (level of evidence: E) [9]. However, only lispro and aspart are currently used in pregnancy, pending clinical trials definitively proving safety and efficacy of other analogues (such as glulisine and glargine) [9]. Recently, a randomized controlled trial in 310 pregnant women with type 1 diabetes demonstrated noninferiority of detemir versus

NPH insulin in terms of maternal efficacy (HbA1c) and safety (hypoglycemia) [14]. So, the FDA has reclassified insulin detemir from pregnancy category C to pregnancy category B (<http://www.fda.gov/drugs/drugsafety/>).

Gestational diabetes is a metabolically heterogeneous disorder; therefore, when high fasting blood glucose values are found, treatment with a basal insulin is compulsory. ILPS could be an “on label” alternative therapeutic option to NPH insulin. To this aim, we retrospectively evaluated pregnancy outcome in women with GDM or type 2 diabetes mellitus treated with ILPS or NPH insulin.

As the distribution of the two types of diabetes was different in the two treatment approaches, we divided the results into subgroups. Not surprisingly, the earlier gestational age at enrollment of type 2 diabetic patients could explain the difference in weight increase from prepregnancy when compared to those with GDM, even though this difference disappeared by the end of pregnancy. However, as GDM was diagnosed quite early, we cannot exclude the extent to which some of the women classified as GDM were affected by unknown type 2 pregestational diabetes.

TABLE 4: Insulin need (units/kg/day) at the end of pregnancy in women receiving ILPS or NPH insulin.

	ILPSg (<i>n</i> = 53)	NPHg (<i>n</i> = 36)	<i>P</i>
Total insulin			
Total	0.36 ± 0.27	0.58 ± 0.20	<0.0001
T2DM	0.50 ± 0.22	0.68 ± 0.18	<0.05
GDM	0.34 ± 0.28	0.48 ± 0.19	<0.05
Short-acting insulin analogue			
Total	0.30 ± 0.18	0.44 ± 0.17	0.001
T2DM	0.33 ± 0.13	0.50 ± 0.14	0.01
GDM	0.30 ± 0.19	0.38 ± 0.18	ns
Intermediate-acting insulin			
Total	0.12 ± 0.10	0.14 ± 0.07	ns
T2DM	0.18 ± 0.07	0.17 ± 0.07	ns
GDM	0.11 ± 0.09	0.11 ± 0.06	ns

T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus.

Overall, the maternal and neonatal outcomes of diabetic women treated with ILPS for about half of pregnancy were not different from those of women treated with NPH.

The high prevalence of hypertension found in both types of diabetes, not associated with any of the two basal insulins used, was similar to that reported by other studies [28, 29].

Birth weight, ponderal index, and prevalence of LGA were similar in the two groups. However, an excessive ponderal index, a more precise measure of obesity in pediatrics, was found in three newborns of women with a worse metabolic control and higher insulin requirement, all belonging to the NPHg. There are contrasting results on the birth weight of newborns of diabetic pregnancies under lispro [6, 30, 31]. Our data does not show an increased birth weight in the ILPSg when risk factors are similar (maternal BMI and weight gain, type of diabetes), taking into account that the percentage of aspart users was similar in the two groups. This result is important as an indirect effect of insulin analogues through placental action is hypothesized [30].

Metabolic control was similar and quite satisfactory as fasting blood glucose goals were reached in about two-thirds of each group. Moreover, the reduction of FCBG throughout pregnancy, even if not always significant, is the expression of stable metabolic control in a time span characterized by a progressive increase of insulin resistance [32].

We cannot draw any conclusion about the influence of either ILPS or NPH on hypoglycemia as the episodes were all mild or moderate and extremely rare.

In fact, although hypoglycemia is reported as a complication in type 1 diabetic women, with a higher incidence in early pregnancy [33, 34], this finding is less frequent in type 2 and gestational diabetes because of the different pathogenesis of the disease.

The low insulin requirement in both groups can be explained by our patients' BMI which was significantly less than described in other populations [35] as well as by the adherence to MNT, as indirectly shown by the weight gain, and the short duration of disease for type 2 diabetes.

Interestingly, total daily insulin need was higher both in type 2 diabetes and GDM treated with NPH while a higher

need for short-acting analogue was found in type 2 diabetic women only. Moreover, eight women with GDM were well controlled by ILPS at bedtime only. These data could be due to a longer duration of glucose-lowering activity of ILPS in this population [16].

Indeed, recent comparative studies examining the pharmacokinetic and pharmacodynamic profiles [36] or the efficacy and safety of ILPS with the other two long-acting insulin analogues [16] suggest that ILPS has a similar glycemic control to glargine and detemir in type 2 diabetes mellitus, out of pregnancy.

Today, caution in the use of most insulin analogues during pregnancy [9] is derived from available data that is incomplete and sometimes contradictory [37].

The placenta expresses both insulin-like growth factor 1 (IGF-1) and insulin receptors on both the maternal and fetal sides. As pregnancy progresses, insulin receptors are reduced while IGF-1 receptors do not suffer significant changes on the maternal side [38]. Insulin analogues may interact differently with insulin or IGF-1 receptors, activating metabolic and mitogenic pathways in a different way from native insulin [39] and opening the possibility of an indirect effect of maternal insulin on the fetus. Careful attention must be paid to the use of long-acting insulin analogues during pregnancy due to increased mitogenic activity observed *in vitro* with respect to insulin and short-acting analogues [40].

For this reason, the use of ILPS is a viable therapeutic option when pregnant women with type 2 or gestational diabetes need a basal insulin.

The limitations of this study are based on the retrospective nature as well as the limited size of the study group (mainly, of the type 2 diabetic women). However, despite the limited sample size, differences between ILPS and NPH were large enough to reach statistical significance.

Thus, the descriptive nature of our study cannot give firm conclusions, but it is more of a basis for hypotheses and new studies about the use of ILPS in pregnancy.

Very recently a retrospective study of pregnant women with type 1 diabetes using either insulin detemir (*n* = 67) or glargine (*n* = 46) demonstrated that pregnancy outcome

was comparable, except for a lower prevalence of large for gestational age infants in women on glargine [41].

Larger and prospective studies comparing the three long-acting analogues should be encouraged to evaluate their safety and efficacy in pregnancy.

In conclusion, this retrospective multicenter study demonstrates that pregnancy outcome in women with type 2 and gestational diabetes mellitus treated with insulin lispro protamine suspension was similar to those of those treated with NPH insulin, except for a lower insulin requirement.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

The ETS-Domain Transcription Factor Elk-1 Regulates COX-2 Gene Expression and Inhibits Glucose-Stimulated Insulin Secretion in the Pancreatic β -Cell Line INS-1

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Cyclooxygenase-2 (COX-2) expression is associated with many aspects of physiological and pathological conditions, including pancreatic β -cell dysfunction. Prostaglandin E2 (PGE2) production, as a consequence of COX-2 gene induction, has been reported to impair β -cell function. The molecular mechanisms involved in the regulation of COX-2 gene expression are not fully understood. We previously demonstrated that transcription factor Elk-1 significantly upregulated COX-2 gene promoter activity. In this report, we used pancreatic β -cell line (INS-1) to explore the relationships between Elk-1 and COX-2. We first investigated the effects of Elk-1 on COX-2 transcriptional regulation and expression in INS-1 cells. We thus undertook to study the binding of Elk-1 to its putative binding sites in the COX-2 promoter. We also analysed glucose-stimulated insulin secretion (GSIS) in INS-1 cells that overexpressed Elk-1. Our results demonstrate that Elk-1 efficiently upregulates COX-2 expression at least partly through directly binding to the -82/-69 region of COX-2 promoter. Overexpression of Elk-1 inhibits GSIS in INS-1 cells. These findings will be helpful for better understanding the transcriptional regulation of COX-2 in pancreatic β -cell. Moreover, Elk-1, the transcriptional regulator of COX-2 expression, will be a potential target for the prevention of β -cell dysfunction mediated by PGE2.

1. Introduction

Cyclooxygenase-2 (COX-2) is a key enzyme that catalyzes the production of prostaglandins (PGs) and other inflammatory substances from arachidonic acid. COX-2 catalytic product PGs participate in many physiological and pathological processes, such as inflammation, pain, angiogenesis, blood pressure regulation, and immune response [1]. COX-2, as an inducible cyclooxygenase, is normally undetectable in most tissues and organs but can be rapidly induced by cytokines, growth factors, bacterial endotoxins, carcinogenic factor stimulation, and other stimuli [2–5]. The aberrant expression of COX-2 is associated with many aspects of physiological and pathological conditions such as cell malignant transformation, inflammation, cell growth and apoptosis, tumor

angiogenesis, invasiveness, and metastasis [6–10]. Prostaglandin E2 (PGE2) production has been reported to impair β -cell function from studies in pancreatic β -cells and isolated islets [11–13]. Moreover, inhibition of COX-2 activity was shown to protect β -cell function in inflammatory factor stimulus and increased basal insulin secretion [12, 13].

In view of the important role of COX-2 in the occurrence and development of diabetes mellitus, it is necessary to progress in-depth studies on the molecular mechanisms involved in the regulation of COX-2 gene expression. At present, research about the COX-2 gene regulation mainly focused on the level of transcriptional regulation. The COX-2 promoter region contains a canonical TATA element and a number of cis-activating consensus sequences, including cAMP responsive element (CRE), E-box, NF-IL6

(CCAAT/enhancer-binding protein- β), AP-2, SP-1, NF- κ B, and STAT sites [14–21]. The specific transcription factors involved in COX-2 activation are dependent on both cell type and stimulus. For example, AP2, NF-IL-6, and CRE elements are essential for IL-1 β -induced activation of the COX-2 gene in human microvascular endothelial cell line, HMEC-1 [15]. Moreover, NF- κ B transcription factor mediates the induction of COX-2 by interleukin-1 in rheumatoid synoviocytes [16]. We previously demonstrated that transcription factor Elk-1 significantly upregulated COX-2 gene promoter activity and identified several putative binding sites for Elk-1 [22].

Elk-1 is a member of the Ets family of transcription factors. The Ets gene family conserves an 85-amino acid DNA-binding ETS domain that binds the consensus sequence 5'-GGA (A/T)-3' in the promoter region of the target genes [23] and has various biological functions, including control of cellular proliferation, differentiation, hematopoiesis, apoptosis, tissue remodeling, angiogenesis, and transformation [24–28]. Previous studies showed that most of the Ets family members including Elk-1 are important substrates of the MAPKs, the PI3 kinases, and Ca²⁺ specific signaling pathways, which can be activated by growth factors or cellular stress [29]. Other studies confirmed that inducible COX-2 expression is related to the activation of the MAPKs signaling pathway [30, 31]. Thus, transcription factor Elk-1 may be an important bridge between the external stimuli and the induction of COX-2 gene expression.

The aim of this study was to investigate the relationships between Elk-1 and COX-2. To check the relevance of our hypothesis, we first investigated the effects of Elk-1 on COX-2 transcriptional regulation and expression in the pancreatic β -cell line INS-1. We thus undertook to study the binding of Elk-1 to its putative binding sites in the COX-2 promoter. We also analysed glucose-stimulated insulin secretion (GSIS) in INS-1 cells that overexpressed Elk-1.

2. Materials and Methods

2.1. Cell Line and Cell Culture. INS-1 cells were grown in RPMI 1640 medium containing 11.1 mM glucose supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 100 IU/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere (5% CO₂, 95% air) at 37°C.

2.2. Plasmid Construction. The Elk-1 expression plasmid (pCMV3.0b-Elk-1) and luciferase reporter construct containing the rat COX-2 promoter (–2026/+44) were constructed in our previous study [22]. Two mutant constructs containing the sequence –2026/+44 in which nucleotides –82 to –69 were deleted or mutated from CGAGGCGGAAAGAC to CGAGGCAAGAAGAC were made by using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The constructs were named pCOX-2 (–2026/+44, del–82/–69) and pCOX-2 (–2026/+44, m–82/–69), respectively. All constructs were verified by DNA sequencing.

2.3. Cell Transient Transfection and Luciferase Assay. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For luciferase assay, INS-1 cells were plated into 12-well cell culture plates 1 day before transfection. Each transfection was performed using 0.8 μ g luciferase reporter construct, 0.8 μ g Elk-1 expression plasmid or pCMV3.0b empty vector as control, and 4 ng Renilla luciferase reporter vector, pRL-SV40, as an internal control (Promega). 48 h after transfection, cells were washed with PBS and lysed using 1 \times passive lysis buffer. Firefly and Renilla luciferase activities were measured with a GloMax-20/20 luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. Each experiment was performed in triplicate and independently repeated three times.

To explore the effect of Elk-1 on endogenous COX-2 expression and glucose-stimulated insulin secretion of INS-1 cells, cells were transiently transfected with Elk-1 expression plasmid pCMV3.0b-Elk-1 or empty vector pCMV3.0b as control. 48 h after transfection, the cells were harvested for quantitative real-time RT-PCR or Western blot analysis, or proceed to glucose-stimulated insulin secretion (GSIS) assay as follows.

2.4. Quantitative Real-Time RT-PCR. Total RNAs of INS-1 cells were prepared by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After spectrophotometry quantification, 1 μ g of total RNA was used for reverse transcription (RT) in a 20 μ L final volume with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems). The reactions were performed in a volume of 10 μ L containing 1 μ L diluted cDNA, 20 \times TaqMan Gene Expression Assay Mix, and 2 \times TaqMan Universal PCR Master Mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The TaqMan Gene Expression Assay Mix used for Elk-1 and COX-2 had the product number Rn01756649_g1 and Rn01483828_m1. Rat β -actin (product number Rn00667869_m1) was used to calibrate the original concentration of mRNA. Each quantification PCR was performed in triplicate and independently repeated three times. The mRNA concentration was defined as the ratio of target mRNA copies relative to GAPDH mRNA copies.

2.5. Western Blot Analysis. INS-1 cells were lysed in ice-cold lysis buffer containing the following reagents: 50 mM Tris-HCl pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; complete proteinase inhibitor mixture (1 tablet per 10 mL, Roche). Protein concentration in the cell lysate was quantified using the DC Protein Assay Kit (Bio-Rad). Protein aliquots were electrophoresed by 12% SDS-PAGE and transferred to PVDF membrane (Millipore). Nonspecific protein interactions were blocked by incubation in 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl,

and 0.1% Tween 20 (pH 7.6)) at room temperature for 1 h and then washed with TBST. Membranes were then incubated at 4°C overnight with anti-Elk-1 (Santa Cruz), anti-COX-2 (Santa Cruz), or anti- β -actin (Santa Cruz) antibodies in fresh blocking buffer. The blots were washed and then incubated with HRP-conjugated secondary antibodies (Amersham) for 1 h at room temperature. The bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore) using X-ray film (Kodak). Prestained markers (Thermo) were used as internal molecular weight standards. The densities of the bands on the Western blot were analyzed with Quantity One software (Bio-Rad).

2.6. Knockdown of Elk-1 by RNA Interference (RNAi). Elk-1 specific small interfering RNA (siRNA) and negative siRNA were synthesized by GenePharma. The sequences were as follows: Elk-1 siRNA-1, 5'-GGCCAGAAGUUUGUCUAC-Att-3'; siRNA-2, 5'-AGGCCAAGGUGGCUUAGCAtt-3'; siRNA-3, 5'-GCCAUCCUAAACAGAGAAUAtt-3'; negative siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'. INS-1 cells were transiently transfected with siRNA using NeoFx reagent (Ambion) according to the manufacturer's protocol. 48 h after transfection, the cells were harvested for real-time RT-PCR or Western blot analysis as described above.

2.7. Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were isolated from INS-1 cells with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instructions. Protein concentration was determined with DC Protein Assay Kit (Bio-Rad). EMSA was performed using DIG Gel Shift Kit (Roche) according to the manufacturer's protocol. The sense probe sequences for EMSA were as follows: wild-type probe 1, 5'-AAAGCCGAGGCGGAAAGACACAGT-3', which corresponds to nucleotide -87 to -64 of rat COX-2 promoter; wild-type probe 2, 5'-TTCGGTAGTTTCCGAAGGGCTGTT-3', which corresponds to nucleotide -1300 to -1277 of COX-2 promoter; wild-type probe 3, 5'-ACCACCCATTTCGACCCCCACC-3', which corresponds to nucleotide -1824 to -1801 of COX-2 promoter; mutant probe 1, 5'-AAAGCCGAGGCAAGAAGACACAGT-3'. Double-stranded probes were synthesized, and the 3'-end of wild-type probe was labelled with digoxigenin-11-ddUTP. Nuclear extracts (5 μ g protein) were incubated with 1 μ g poly[d (I-C)], the binding buffer attached to the kit, and DIG-labelled wild-type probe for 15 min at room temperature. Bound DNA complexes were separated on a 5% nondenaturing polyacrylamide gel electrophoresis and transferred to a nylon membrane (Roche). The nylon membranes were cross-linked, and chemiluminescent detection was performed using CSPD, and signals were recorded on X-ray film.

In supershift analyses, Elk-1 antibody (3 μ g; Santa Cruz) was added to nuclear extracts in gel shift buffer (above) for 1 h at 4°C, followed by addition of probe, and the subsequent protocol was the same as above.

2.8. GSIS Assay. One day before transfection, INS-1 cells (2×10^5) were seeded into 500 μ L RPMI 1640 medium with standard glucose concentration (11.1 mM) in 24-well cell culture plates. The cells were transfected with Elk-1 expression plasmid pCMV3.0b-Elk-1 or empty vector pCMV3.0b as control for 48 h as described above. After incubation for 1 h in glucose-free Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 20 mM NaHCO_3 , 16 mM HEPES, 2.56 mM CaCl_2 , and 0.2% BSA), the cells were treated for 1 h in KRB buffer with low (3.3 mM) and high (16.7 mM) glucose. The supernatants were obtained for insulin concentration determination using a rat/mouse insulin ELISA kit (Linco Research). Each experiment was performed in triplicate and independently repeated three times.

2.9. Statistical Analysis. Data were presented as mean \pm SEM. Differences in the mean of two samples were analysed by Student's *t*-test with differences $P < 0.05$ considered significant. Statistical analysis was performed with SPSS 17.0 software.

3. Results

3.1. Elk-1 Upregulated COX-2 Gene Expression. In the previous study, we demonstrated that transcription factor Elk-1 significantly upregulated COX-2 gene promoter activity [22]. To explore the effect of Elk-1 on endogenous COX-2 gene expression, INS-1 cells were transiently transfected with Elk-1 overexpression vector or control vector. Overexpression of Elk-1 significantly increased COX-2 mRNA and protein expression (Figure 1).

3.2. Elk-1 RNAi Downregulated COX-2 Gene Expression. INS-1 cells were transfected either with Elk-1 siRNAs (including 3 siRNAs) or control siRNA. We measured Elk-1 protein levels to select the siRNAs that can effectively silence Elk-1 expression. As shown in Figure 2(a), Elk-1 siRNA-1 effectively silenced Elk-1 gene expression; therefore, we used Elk-1 siRNA-1 in subsequent experiments. We silenced Elk-1 expression in INS-1 cells and then measured COX-2 mRNA and protein expression. COX-2 mRNA and protein expression levels were significantly decreased with Elk-1 RNAi (Figures 2(b), 2(c), and 2(d)).

3.3. Identification of Elk-1 Binding Site in COX-2 Promoter. Based on sequence analysis, rat COX-2 promoter region contains three predicted consensus binding sites for Elk-1, which correspond to promoter region of -82/-69, -1295/-1282, and -1819/-1806, respectively. To confirm whether Elk-1 can bind to these three sites, we synthesized and labelled the oligonucleotides spanning the three regions and additional five nucleotides on each side (i.e., -87/-64, -1300/-1277, and -1824/-1801) and used them as probes in EMSA experiments. As shown in Figure 3(a) (lane 2), a slower-migrating complex appeared when INS-1 nuclear extracts were incubated

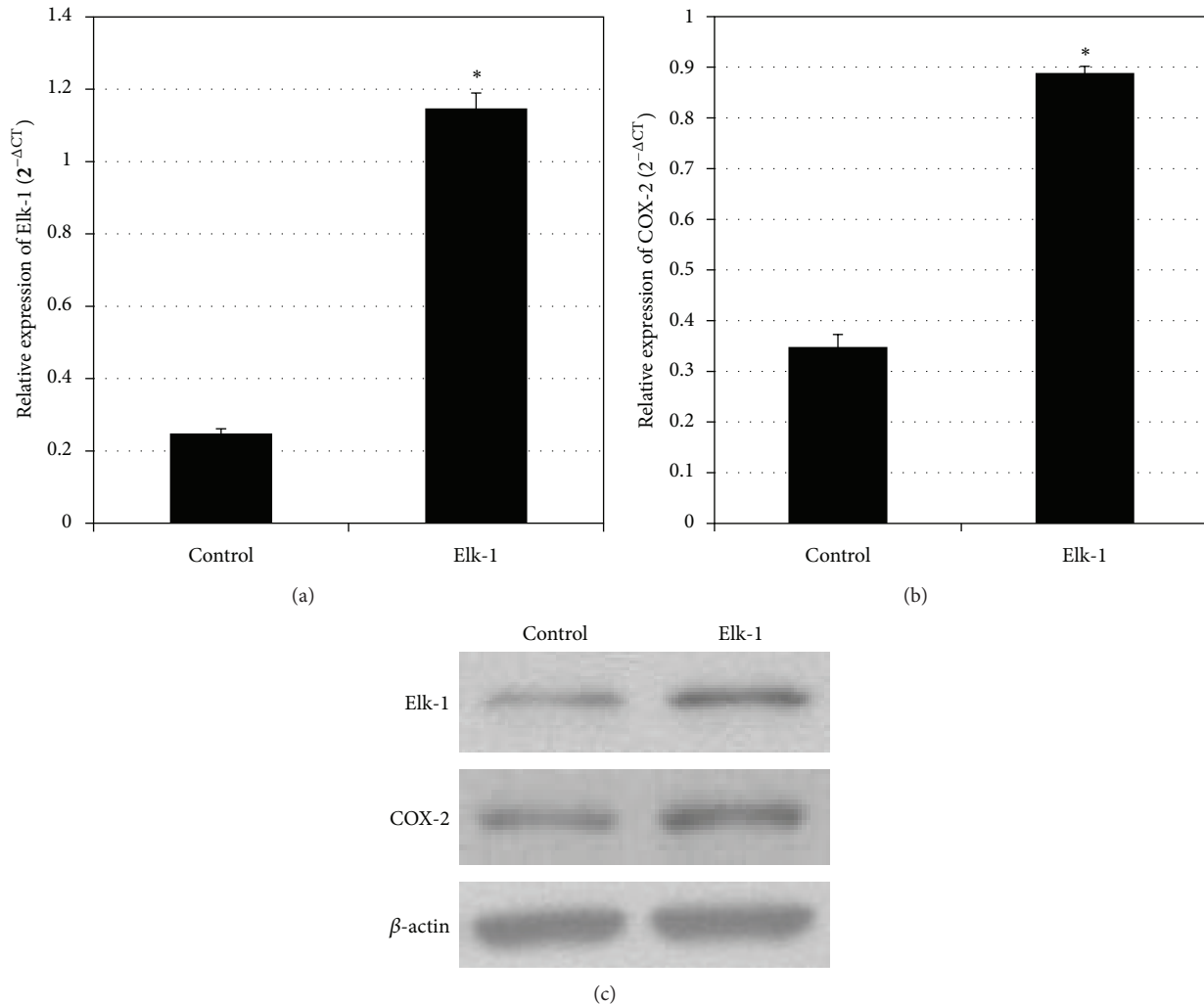


FIGURE 1: Elk-1 upregulated COX-2 gene expression. (a and b) Elk-1 and COX-2 mRNA levels were determined by quantitative real-time RT-PCR. Relative mRNA expression was expressed as mean \pm SEM. * $P < 0.001$ versus control. (c) Elk-1 and COX-2 protein levels were assayed by Western blot analysis. β -actin levels served as internal control.

with the digoxigenin-11-ddUTP-labelled wild-type probe 1 (−87/−64 of COX-2 promoter), but not probe 2 and probe 3 (−1300/−1277 and −1824/−1801), indicating that −82/−69 region is the binding site for Elk-1. The slower-migrating complex was significantly inhibited by a molar excess of unlabelled wild-type probe 1 (Figure 3(b), lanes 3 and 4). In contrast, the unlabelled mutant probe 1 reduced the inhibitory effect (Figure 3(b), lanes 5 and 6). To determine if Elk-1 is responsible for the shift seen in EMSA, Elk-1 antibody was added to the EMSA-binding reaction, and the complex can be supershifted by the addition of Elk-1 antibody (Figure 3(b), lane 7).

3.4. Elk-1 Upregulated COX-2 Promoter Activity through Elk-1 Binding Site. By transient cotransfections, we showed that overexpression of Elk-1 led to a significant increase in relative luciferase activity of COX-2 promoter (Figure 4). The contribution of the Elk-1 binding site was studied by site-directed

mutagenesis in INS-1 cells. When the −82/−69 Elk-1 binding site on the −2026/+44 region was deleted or mutated, the enhanced effect of Elk-1 was deeply reduced (Figure 4). This suggested that the −82/−69 Elk-1 binding site is implicated in the enhancement of COX-2 promoter by Elk-1.

3.5. Elk-1 Inhibited Glucose-Stimulated Insulin Secretion in INS-1 Cells. To determine the effect of Elk-1 on GSIS function in pancreatic β -cells, we performed experiments with Elk-1 overexpressing INS-1 cells. As shown in Figure 5, control cells secreted 87.00 ± 1.74 ng insulin·h^{−1}·mg protein^{−1} and demonstrated a 6.9-fold increase in insulin secretion with 16.7 mM glucose, whereas Elk-1 overexpressing cells secreted 45.37 ± 2.50 ng insulin·h^{−1}·mg protein^{−1} and demonstrated a 3.6-fold increase in insulin secretion ($P < 0.001$ versus control). Therefore, Elk-1 overexpressing group demonstrated a decrease of GSIS to 52% of the control value.

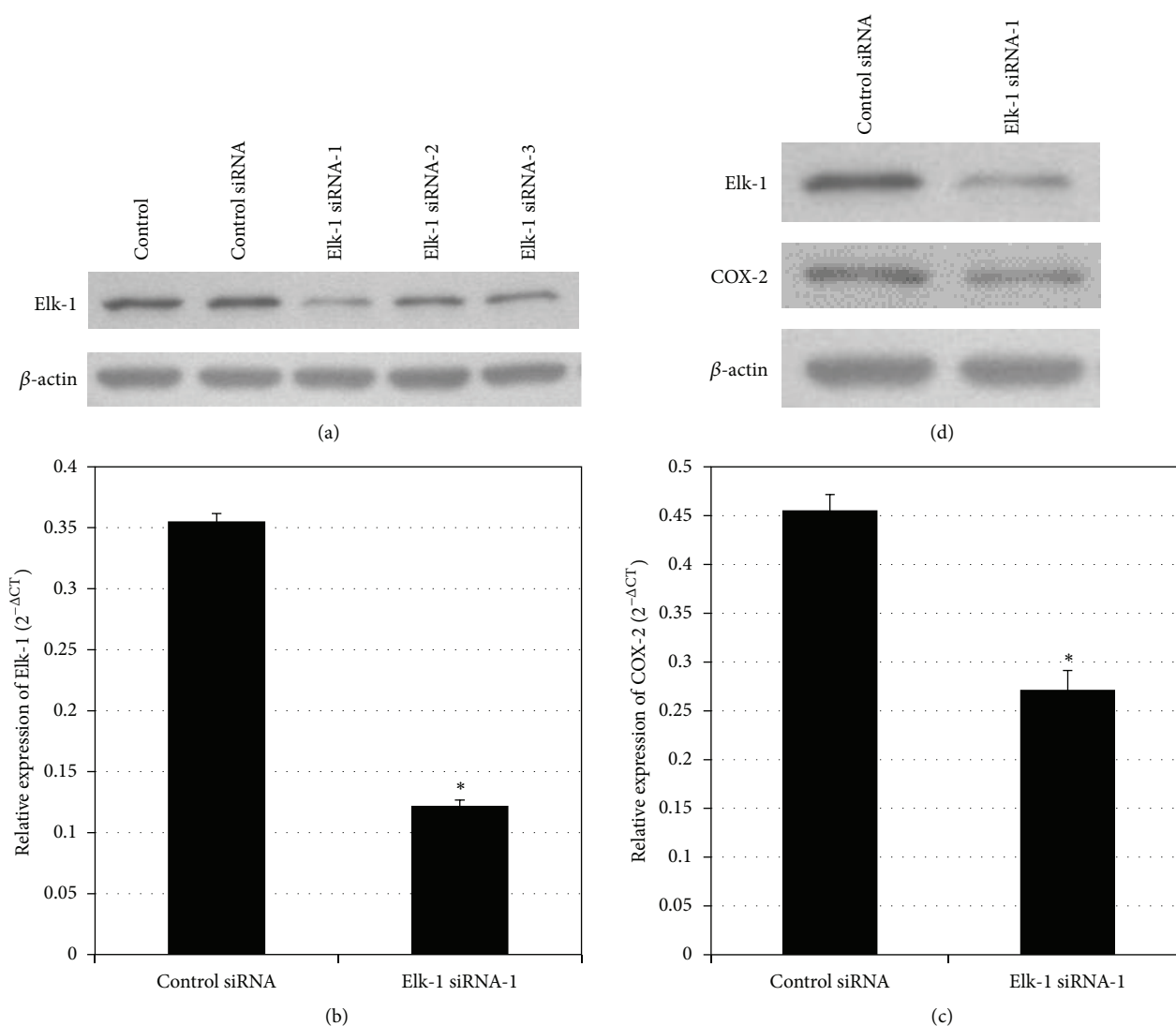


FIGURE 2: Elk-1 RNAi downregulated COX-2 gene expression. (a) INS-1 cells were transiently transfected with control siRNA and Elk-1 siRNAs (1, 2, and 3), respectively. Untransfected cells were used as control. 48 h after transfection, Elk-1 protein levels were assayed by Western blot analysis. β -actin levels served as internal control. (b and c) INS-1 cells were transiently transfected with control siRNA and Elk-1 siRNA-1, respectively. 48 h after transfection, Elk-1 and COX-2 mRNA levels were determined by quantitative real-time RT-PCR. Relative mRNA expression was expressed as mean \pm SEM. * $P < 0.001$ versus control siRNA transfected group. (d) Elk-1 and COX-2 protein levels were assayed by Western blot analysis. β -actin levels served as internal control.

4. Discussion

COX-2 is an immediate early gene. Depending on the cell type, it can be activated by a variety of stimuli. Upregulation of COX-2 expression is involved in various physiological and pathological conditions, including pancreatic β -cell dysfunction. Previous studies indicated that COX-2 activation might play a pathogenic role in diabetes [32–34], and COX-2 inhibition can protect rat islets from cytokine-induced inhibition of glucose-stimulated insulin secretion [13], implicating the important role of COX-2 in cytokine-mediated β -cell dysfunction and diabetes development. Thus, understanding the molecular mechanisms involved in the regulation of COX-2 gene expression in β -cells will help to better understand and restrain the dysfunction of pancreatic β -cell.

COX-2 expression is regulated by the binding of specific transcription factors to cis-acting elements on the COX-2 promoter [35]. Several studies showed that some stimuli could upregulate COX-2 expression, and in these studies, the expression and activity of Elk-1 were also increased [36–38], indicating the potential role of Elk-1 in COX-2 regulation. In our previous study, we demonstrated that Elk-1 significantly upregulated COX-2 promoter activity [22]. But how Elk-1 participates in the regulation of COX-2 expression has not been researched so far.

In this study, we investigated the effects of Elk-1 on COX-2 gene expression and GSIS function in INS-1 rat insulinoma cells and explored whether Elk-1 regulates COX-2 expression through its potential binding site in COX-2 promoter. Overexpression study demonstrated that Elk-1 overexpression

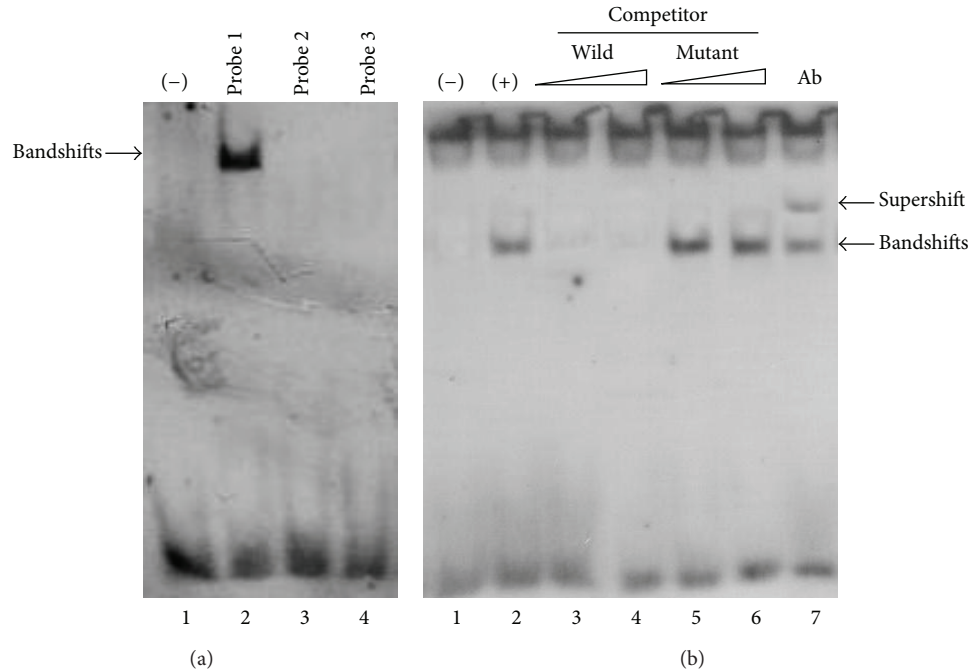


FIGURE 3: Identification of Elk-1 binding site in COX-2 promoter by electrophoretic mobility shift assay (EMSA). (a) Wild-type probe 1 (-87/-64 of COX-2 promoter) was incubated without (lane 1) or with (lane 2) INS-1 nuclear proteins. Wild-type probe 2 (-1300/-1277) (lane 3) or wild-type probe 3 (-1824/-1801) (lane 4) was incubated with INS-1 nuclear proteins. (b) Wild-type probe 1 was incubated without (lane 1) or with (lane 2) INS-1 nuclear proteins in the absence or presence of unlabelled probe 1 (lanes 3-6). Lanes 3 and 4 contain the wild-type probe 1, and lanes 5 and 6 contain the mutant probe 1, each at 50- and 100-fold molar excess, respectively. In lane 7, wild-type probe 1 was incubated with INS-1 nuclear proteins in the presence of Elk-1 antibody.

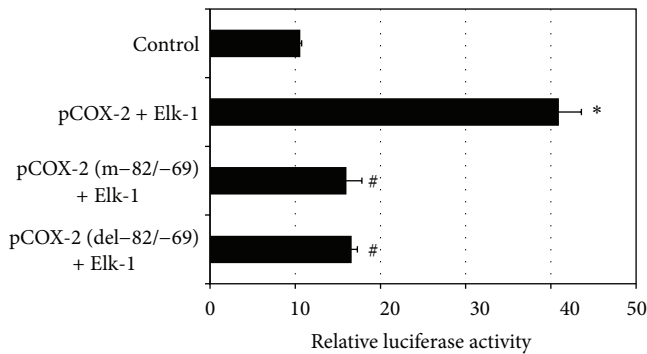


FIGURE 4: Elk-1 upregulated COX-2 promoter activity through Elk-1 binding site. The Elk-1 expression vector (pCMV3.0b-Elk-1) or control vector (pCMV3.0b) was transfected together with construct pCOX-2 (-2026/+44), pCOX-2 (-2026/+44, m-82/-69), or pCOX-2 (-2026/+44, del-82/-69). Relative luciferase activity was expressed as mean \pm SEM and represented three different experiments in triplicate for each fragment. * P < 0.001 versus control. # P < 0.001 versus pCOX-2 (-2026/+44) transfected with pCMV3.0b-Elk-1.

significantly increased COX-2 mRNA and protein expression. On the contrary, Elk-1 RNAi significantly decreased COX-2 expression. EMSA and site-directed mutagenesis experiments indicated that the effect of Elk-1 on COX-2 transcription involves one Elk-1 cis-element of COX-2 promoter located between nucleotides -82 and -69, but not the other two predicted consensus cis-elements (-1295/-1282

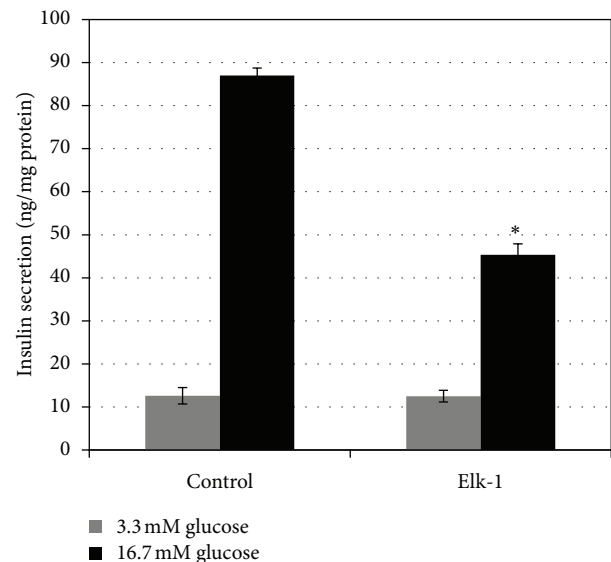


FIGURE 5: Elk-1 inhibited glucose-stimulated insulin secretion in INS-1 cells. INS-1 cells were transfected with Elk-1 expression plasmid pCMV3.0b-Elk-1 or empty vector pCMV3.0b as control for 48 h. Control cells demonstrated a 6.9-fold increase in insulin secretion with 16.7 mM glucose, whereas Elk-1 overexpressing cells had only a 3.6-fold increase. Elk-1 overexpressing group demonstrated a decrease of GSIS to 52% of the control value. Each experiment was done in triplicate and repeated three times. * P < 0.001 versus control.

and -1819/-1806). These results suggested that Elk-1 probably upregulates COX-2 gene expression at least partly through Elk-1 directly binding to the -82/-69 region of COX-2 promoter. This was the first report that characterized the Elk-1 cis-element in COX-2 promoter.

To further investigate the role of Elk-1 on pancreatic β -cells dysfunction, we assessed GSIS function in INS-1 cells that overexpressed Elk-1. As expected, Elk-1 overexpressing group demonstrated a decrease of GSIS to 52% of the control value. This result demonstrated that Elk-1 can inhibit GSIS in INS-1 cells. Because COX-2 plays an important role in β -cell dysfunction and Elk-1 can upregulate COX-2 gene expression, we presumed that Elk-1 inhibits GSIS in INS-1 cells at least partly through upregulating COX-2 gene expression. Other unknown mechanisms of Elk-1's inhibitory role on GSIS remain to be determined by further experiments such as chromatin immunoprecipitation (ChIP) sequencing, transcriptome sequencing, and gene array.

5. Conclusions

In conclusion, our study demonstrated that transcription factor Elk-1 efficiently upregulates the expression of COX-2 in INS-1 cells and that this may be a new explanation for the mechanism of β -cell insulin secretion impairments by some stimuli. Overexpression of Elk-1 may inhibit insulin secretion in β -cells by causing upregulation of COX-2. These findings will be helpful for better understanding the transcriptional regulation of COX-2 in pancreatic β -cell. Moreover, Elk-1, the transcriptional regulator of COX-2 expression, will be a potential target for the prevention of β -cell dysfunction mediated by PGE₂.

Authors' Contribution

X.-F. Zhang and Y. Zhu contributed equally to this work.

Acknowledgments

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Clinical Study

Comparison of Efficiencies of Michigan Neuropathy Screening Instrument, Neurothesiometer, and Electromyography for Diagnosis of Diabetic Neuropathy

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Aim. This study compares the effectiveness of Michigan Neuropathy Screening Instrument (MNSI), neurothesiometer, and electromyography (EMG) in detecting diabetic peripheral neuropathy in patients with diabetes type 2. **Materials and Methods.** 106 patients with diabetes type 2 treated at the outpatient clinic of Ankara Numune Education and Research Hospital Department of Endocrinology between September 2008 and May 2009 were included in this study. Patients were evaluated by glycemic regulation tests, MNSI (questionnaire and physical examination), EMG (for detecting sensorial and motor defects in right median, ulnar, posterior tibial, and bilateral sural nerves), and neurothesiometer (for detecting alterations in cold and warm sensations as well as vibratory sensations). **Results.** According to the MNSI score, there was diabetic peripheral neuropathy in 34 (32.1%) patients (score ≥ 2.5). However, when the patients were evaluated by EMG and neurothesiometer, neurological impairments were detected in 49 (46.2%) and 79 (74.5%) patients, respectively. **Conclusion.** According to our findings, questionnaires and physical examination often present lower diabetic peripheral neuropathy prevalence. Hence, we recommend that in the evaluation of diabetic patients neurological tests should be used for more accurate results and thus early treatment options to prevent neuropathic complications.

1. Introduction

Diabetic neuropathy is the most common microvascular complication of diabetes, and it is a major cause of morbidity and mortality. Neuropathy is estimated to be present in 10%–90% of the patients with diabetes although it changes according to diagnostic criteria and patient population. Diabetic peripheral neuropathy is the most common type of diabetic neuropathy, and it is frequently used synonymously with it [1].

Early diagnosis and appropriate treatment are important to prevent disease complications, especially diabetic foot and ulceration, but there is not a single and simple method that can be used to diagnose diabetic peripheral neuropathy. According to American Diabetes Association (ADA)

recommendations, diabetic peripheral neuropathy diagnosis in clinical practice is made in the presence of signs and symptoms of peripheral nervous system dysfunction after other causes of neuropathy are excluded in patients with diabetes. Considering that 50% of the patients with diabetic peripheral neuropathy have no symptoms consistent with neuropathy, neurological examination of the patients should be carefully performed. To confirm the diagnosis, quantitative electrophysiological tests and sensory and autonomic function tests can be performed [2, 3].

The aim of this study was to compare the effectiveness of Michigan Neuropathy Screening Instrument (MNSI), neurothesiometer, and electromyography (EMG) in detecting diabetic peripheral neuropathy in patients with diabetes type 2.

2. Patients and Methods

2.1. Patients. The study was conducted in Ankara Numune Education and Research Hospital Department of Endocrinology from September 2009 to February 2010. 106 type 2 diabetes patients with or without symptoms of neuropathy were enrolled in the study.

Patients who had conditions that could present with neuropathy such as hereditary sensory neuropathy, vitamin B12 or folate deficiency, paraneoplastic conditions, autoimmune diseases, uremia, hypothyroidism, and ethanol abuse were excluded.

Patients' height and weight were measured and used to calculate body mass index (BMI). Blood pressure was recorded in supine position after 5 minutes of rest using oscillometry. Hypertension was defined as $\geq 140/90$ mmHg at examination or presence of antihypertensive treatment.

Medication history (use of insulin, oral antidiabetic, antihypertensive, and lipid-lowering drugs) was noted. Retina evaluation was performed by an ophthalmologist. Retinopathy was classified into five categories using an international system of classification: absence of retinopathy, mild nonproliferative retinopathy, moderate nonproliferative retinopathy, severe nonproliferative retinopathy, and proliferative retinopathy [4].

The study complied with the declaration of Helsinki and was approved by the local research ethics committee. All the subjects gave written informed consent.

2.2. Laboratory Examinations. Blood samples were obtained at 8 a.m. after 12 hours of fasting. Lipid profile (total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C)) were measured with colorimetric enzymatic method (Aerost device, Abbott Diagnostics, USA). Low-density lipoprotein cholesterol (LDL-C) was calculated by Friedewald's formula. Hyperlipidemia was considered present when lipid-lowering drugs were in use or when samples at admission showed total cholesterol ≥ 200 mg/dL or triglycerides ≥ 150 mg/dL. Glycosylated hemoglobin (HbA1c) was measured by immunoturbidimetric method (C8000 device, Abbott Company, USA). For the diagnosis of nephropathy, the patients were asked to collect urine for 24 hours after urinating the first urine in the morning and not to do any exercise 24 hours before and during the collecting procedure. Urinary albumin excretion (UAE) was measured with Multigent microalbumin (μ Alb) turbidimetric immunoassay method (Aerost device, Abbott Diagnostics, USA). We considered <30 mg/day as normoalbuminuria, $30-300$ mg/day as microalbuminuria, and >300 mg/day as macroalbuminuria. Microalbuminuria is defined as a total of three positive 24-hour urine collections measured at different days to confirm the diagnosis. Plasma creatinine levels of the patients were measured, and glomerular filtration rate (GFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [5]. B12 levels of all patients were measured by chemiluminescence method on an ADVIA Centaur XP analyser.

2.3. Assessment of Diabetic Neuropathy. All patients were evaluated for diabetic peripheral neuropathy using MNSI, EMG, and neurothesiometer. All tests for neurological assessment were performed on the same day.

2.3.1. MNSI. A 15-item questionnaire form of MNSI consisting of yes/no questions was applied to all the patients. 13 items assess symptoms of diabetic peripheral neuropathy, 1 item assesses peripheral vascular disease, and 1 item assesses general asthenia [6].

Michigan Neuropathy Screening Instrument. Answer the following yes or no questions based on how you feel in your legs and feet.

- (1) Are your legs and/or feet numb?
- (2) Have you ever had burning sensation in your legs and/or feet?
- (3) Are your feet too sensitive to touch?
- (4) Do you get muscle cramps in your legs and/or feet?
- (5) Have you ever had any prickling feelings in your legs or feet?
- (6) Does it hurt when the bed covers touch your skin?
- (7) When you get into the tub or shower, are you able to distinguish the hot water from the cold water?
- (8) Have you ever had an open sore on your foot?
- (9) Has your doctor ever told you that you have diabetic neuropathy?
- (10) Do you feel weak all over most of the time?
- (11) Are your symptoms worse at night?
- (12) Do your legs hurt when you walk?
- (13) Are you able to sense your feet when you walk?
- (14) Is the skin on your feet so dry that it cracks open?
- (15) Have you ever had an amputation?

Source: a practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. *Diabetes Care* 1994; 17: 1281-9.

After the questionnaires, patients were evaluated neurologically.

- (i) In physical examination, feet were evaluated for deformity, dry skin, callus, infection, and ulceration. Foot deformities included prominent metatarsal heads, hallux valgus, joint subluxation, and Charcot joint. One point was given if any of these signs were present and an additional one point was given if ulceration was present.
- (ii) Vibration sense was evaluated using 128 Hz vibration fork. Vibrating fork was located on the interphalangeal joint of the right great toe. If the patient could not perceive vibration, two points were given. If the patient perceived vibration on the great toe, diaposone was located over ankle (inner malleolus) while it was still vibrating and the patient was asked to

compare vibrations from two locations. If vibration was perceived better in ankle, 1 point was given. If no difference could be found, no point was given. Zero point was accepted as normal, 1 point showed mild-moderate deficit, and 2 points showed a severe deficit.

- (iii) Achilles reflex was observed and reported as absent, decreased, or normal. Patients with normal Achilles reflex were given 0 point while patients with decreased Achilles reflex got 0.5 point and patients with no reflex got 1 point.

Positive responses and abnormal physical examination findings were recorded in the questionnaire form. In the questionnaire form risk of neuropathy was accepted to increase with higher number of positive responses. Diabetic peripheral neuropathy was diagnosed in patients with a physical examination score ≥ 2.5 . In this study MNSI was accepted as a diagnostic test according to ADA recommendations.

2.3.2. Neurothesiometer. For neurothesiometer evaluation a TSA II device (Neurosensory Analyzer Model TSA II, Medoc Ltd., Israel) was used. TSA II is a device used to quantitatively evaluate thin fiber dysfunction. Thermal tests quantitatively measure hot, cold sensations and pain sensation induced by these and compare them with corresponding age group. Deviance from normal values may show presence of peripheral nervous system disease. In vibratory tests, the same type measures are made and compared with vibration thresholds of the population.

Patients sat comfortably in a quiet room with a room temperature of 18–22°C. Before the test, the patient was informed about the procedure and a trial was made without recording. Thermocouple was fixed to the surface to be tested (in our study to right palmar thenar and to right foot 1. metatarsal regions), with metal surface contacting adequately with skin. For sensory measurements, patients were asked to push the button as soon as they perceived the heat changes. In thermal test mode, thermocouple was attached to patients' skin (first to the right palmar thenar surface, then to the right foot plantar 1. metatarsal region). Thermocouple included semiconductors that form temperature gradient between upper and lower stimulating surfaces. Test was started using 30–32°C adaptation temperature. After a few seconds patients could not perceive any difference in temperature. For threshold detection, a perceivable heat stimulus was produced by the device. Data were recorded by a computer when the patient pushed the button in her/his hand and each cycle of the measurement was completed after this. After the measurement, thermocouple was returned to adaptation temperature. A waiting period of a few seconds was allowed before the second stimulus.

TSA II measured threshold values for 4 sensory modalities.

- (i) Heat sensation conducted with C fibers was generally 1–2°C above adaptation temperature.
- (ii) Cold sensation perceived by A delta fibers was generally 1–2°C below adaptation temperature.

- (iii) The threshold value of pain sensation induced by heat which is generally conducted with C fibers and partially A delta fibers was approximately 45°C.
- (iv) The pain sensation induced by cold which is conducted both with C and A delta fibers had a threshold value of 10°C. For pain measurements patients were told that it was not a pain tolerance test and they should push the button as soon as they perceive pain.
- (v) Sense of vibration was measured from plantar side of the right foot, the 1st metatarsal region. Stimulus threshold values for vibration sense were 0.1–130 microns/s. Two types of stimuli were used in this method. One of them was increasing stimulus intensity till it was perceived and the other was decreasing stimulus intensity till it could not be perceived. Then printouts were obtained.

Threshold values for heat, cold, and vibration were measured in all the patients. Values were compared with the same age normal population using TSA II software [7].

2.3.3. EMG. EMG was performed in all the patients involved in the study. Nerve conduction studies were studied using Nihon Kohden MEB-9104K neuropack μ device (Tokyo, Japan). Nerve conduction studies were as follows: ulnar nerve sensory and motor conduction in upper extremity and deep peroneal motor, posterior tibial motor, and sural nerve conduction in both lower extremities. Based on the results of a study of the Turkish population, the normal limits for nerve conduction evaluations were determined as follows: distal latency for ulnar nerve motor conduction was 3.3 ms, Compound Muscle Action Potential (CMAP) amplitude was 7 mV, motor conduction velocity was 39.6 m/s; ulnar nerve distal sensory conduction speed was 37.3 m/s, amplitude was 7 μ V; deep peroneal nerve motor conduction distal latency was 5.8 ms, amplitude was 3.6 mV, conduction speed was 40.9 m/s, F response latency was 52 ms; sural nerve conduction speed was 33.8 m/s, amplitude was 5 μ V. At least two pathological nerve conduction studies, one of which was in the sural nerve, led to symmetric polyneuropathy diagnosis [8].

2.4. Statistical Analyses. Statistical evaluations of the results of this study were done using IBM SPSS (Statistical Package for Social Sciences) for Windows 20.0. In the assessment, in addition to descriptive statistical methods (frequency, mean, and standard deviation), *t*-test was used for the comparison of data and logistic regression analysis was used for diagnostic comparisons. We used a 95% confidence interval and considered a *P* value < 0.05 as statistically significant. Sensitivity and specificity of diagnostic tests were calculated according to the gold standard.

3. Results

Demographic data of 106 patients with diabetes type 2 included in this study are given in Table 1. Mean age of the patients was 49.55 ± 10.28 years. Forty-three patients were

TABLE 1: General characteristics of the study group.

	Total
Number of cases	106
Age (year)*	49.55 ± 10.28
Sex: male/female	43/63
BMI (kg/m ²)	30.3
DM disease length* (months)	99.48 ± 80.96
Recently diagnosed DM, N (%)	4 (3.77)
Not taking a treatment, N (%)	4 (3.8)
Taking treatment, N (%)	102 (96.22)
Oral antidiabetics	57 (55.88)
Insulin	24 (23.53)
Oral antidiabetics + insulin	21 (20.58)

DM: diabetes mellitus; * mean ± standard deviation.

male. Mean duration of diabetes was 99.48 ± 80.96 months (0–312 months). Four patients recently had diabetes diagnosis and they were not receiving diabetes treatment at the time of testing. Fifty-seven patients were using oral antidiabetics, 24 were using insulin, and 21 were using insulin and oral antidiabetic combination. 46 patients had hypertension and 41 of them were on antihypertensive treatment. Of the patients with hyperlipidemia, 21 patients were on statins, and 14 were on fenofibrate treatment. Mean values for LDL cholesterol and triglyceride levels were 108.1 mg/dL and 176.4 mg/dL, respectively. Mean BMI was 30.3 kg/m². 52 patients were obese. All patients had normal serum vitamin B12 levels.

Mean HgbA1c value of the patients was 8.4 ± 2.3 (5.3–15.9). Retinopathy was detected in 26 patients (16 mild nonproliferative, 4 severe nonproliferative, and 6 proliferative retinopathy) and nephropathy was detected in 28 patients (23 patients had microalbuminuria and 5 had macroalbuminuria). Mean glomerular filtration rate was 92 mL/min/1.73 m².

In the assessment for neuropathy, the mean score of the patients obtained in the MNSI questionnaire form was 6.7 ± 2.7 (maximum 12, minimum 3 points). After the questionnaire, physical examination part of MNSI was applied to the patients. According to MNSI, diabetic peripheral neuropathy (score ≥ 2.5) was detected in 34 patients (32.1%). Mean diabetic period for the 34 patients diagnosed with diabetic peripheral neuropathy by MNSI was 125.9 (0–300) months. The diabetic period was longer compared to the patients not diagnosed by MNSI, and the difference was statistically significant ($P = 0.04$). Mean HbA1c level was 8.6% (5.8–15.9). The difference between the groups was not statistically significant ($P = 0.63$). While 13 patients had hypertension, 25 patients were diagnosed with hyperlipidemia and 11 patients were on lipid-lowering treatment because of this. 16 patients had obesity. There was no statistically significant difference between the groups in terms of hypertension, hyperlipidemia, and obesity ($P = 0.72$, $P = 0.07$, and $P = 0.08$, resp.). Retina examination showed proliferative retinopathy in 6 patients, mild nonproliferative retinopathy in 4 patients, and severe nonproliferative retinopathy in 3 patients. Retinopathy was higher in the group diagnosed with diabetic peripheral neuropathy by MNSI compared to the group without diabetic

TABLE 2: Results of MNSI, EMG, and neurothesiometer.

	Total (n: 106)
MNSI questionnaire score*	6.7 ± 2.7
Mean examination score*	1.55 ± 0.75
Diabetic peripheral neuropathy with MNSI (MNSI ≥ 2.5), N (%)	34 (32.1)
Diabetic peripheral neuropathy with EMG N (%)	49 (46.2)
Diabetic peripheral neuropathy with neurothesiometer N (%)	79 (74.5)

* Mean ± standard deviation.

neuropathy ($P = 0.01$). In the neuropathic group, 12 patients had microalbuminuria and 3 had macroalbuminuria. There was no significant difference between the groups ($P = 0.089$, $P = 0.18$, resp.).

As a result of EMG evaluations, neuropathy was diagnosed in 54 patients (50.9%). 49 patients had diabetic polyneuropathy (46.2%) and 5 had mononeuropathy. Of these patients, 10 patients had sensory neuropathy, 9 patients had motor neuropathy, and 30 patients had both sensory and motor neuropathies.

Neurothesiometer evaluations revealed change in heat and/or vibration thresholds in 79 of 106 patients (74.5%). Increase in threshold was detected in cold sensation in 5 patients and in heat sensation in 10 patients. Eighteen patients had threshold increase both in cold and heat sensations, 13 patients had increase in vibration sense threshold, and 33 patients had increase in both thermal (cold and heat) and vibration sense thresholds.

When only MNSI score was used for diagnosis, diabetic peripheral neuropathy was detected in 34 of 106 patients (32.1%). Polyneuropathy findings were detected in 49 patients (46.2%) with EMG and in 79 patients (74.5%) with neurothesiometer (Table 2).

30 (91.2%) of the patients diagnosed with diabetic peripheral neuropathy by MNSI were diagnosed with thin fiber neuropathy by neurothesiometer and 20 (58.8%) had EMG consistent with diabetic peripheral neuropathy. In neurothesiometer evaluation, threshold increases were detected in both thermal and vibration senses in 12 patients, in thermal sense in 4 patients, in heat and vibration senses in 7 patients, only in vibration sense in 4 patients, in cold and vibration senses in 1 patient, and in heat sense in 2 patients. Neurothesiometer evaluation was normal in 4 patients. In EMG evaluation 14 patients had distal peripheral sensory and motor neuropathy, 4 patients had only motor, and 2 patients had only sensory neuropathy. EMG was normal in 14 patients who had neuropathy detected by MNSI.

In neurothesiometer evaluation increase in thermal and/or vibration senses consistent with diabetic peripheral neuropathy was detected in 79 patients included in this study. In 30 (39.2%) of these patients neuropathy was also detected by MNSI and in 45 (42%) patients polyneuropathy was detected by EMG. MNSI questionnaire form revealed findings consistent with neuropathic pain (number of positive responses ≥ 7) in 44 patients who were detected to have

diabetic peripheral neuropathy by neurothesiometer. These results showed that probability of neuropathy according to both MNSI questionnaire form and MNSI physical examination results was high in patients detected to have nerve conduction defects by neurothesiometer ($P < 0.01$). In 17 (37.5%) patients detected to have dysfunction by neurothesiometer, neuropathy could not be detected by MNSI or EMG.

In EMG evaluation 49 patients (46.2%) had diabetic polyneuropathy. In 20 (37%) of these patients, neuropathy was also present according to MNSI examination score. In patients detected to have nerve conduction deficit with EMG, probability of being symptomatic (questionnaire score ≥ 7) according to the questionnaire form was 58.5%. No significant difference could be detected between EMG and MNSI ($P > 0.05$). In neurothesiometer evaluation nerve conduction deficit was detected in 89.7% of the patients who had dysfunction consistent with neuropathy in EMG ($P < 0.001$). In 4 patients (11.8%) who had dysfunction in EMG both MNSI and neurothesiometer evaluations were normal.

EMG method had a sensitivity of 55% and specificity of 58% based on MNSI. Positive and negative predictive values were 38% and 73%, respectively. Neurothesiometer method had a sensitivity of 91% and specificity of 50% based on MNSI. Positive and negative predictive values were 39% and 88%, respectively. EMG and neurothesiometer together had a sensitivity of 53% and specificity of 62%. Positive and negative predictive values when two methods used together were 40% and 73%, respectively.

4. Discussion

Distal symmetric polyneuropathy is the most common form of diabetic neuropathy. Epidemiologic studies have identified the duration and severity of hyperglycemia as major risk factors for the development of diabetic neuropathy in patients with diabetes [9, 10]. In our study we identified a longer diabetic period in the group diagnosed with diabetic peripheral neuropathy than in the group without diabetic neuropathy. However, we did not find any difference in the glycemic regulations of the groups. A study on the role of glucose control on the neuropathy in patients with type 1 and type 2 diabetes suggests that in type 1 diabetes, glucose control has a large effect on the prevention of neuropathy; therefore, future efforts should continue to concentrate on this avenue of treatment. In contrast, patients with type 2 diabetes, glucose control does not play a significant role in the prevention of neuropathy [11]. Vascular risk factors also appear to be associated with the risk of developing diabetic neuropathy. Evidence of this association comes from the European Diabetes (EURO-DIAB) Prospective Complications Study [12]. In addition to duration of diabetes and glycosylated hemoglobin value, the incidence of neuropathy was significantly associated with increased triglyceride level, body mass index, smoking, and the presence of hypertension at baseline. Several of these risk factors are markers of insulin resistance. Similarly, in our study, we evaluated 106 patients with diabetes type 2 not only in terms of neuropathy prevalence but also for vascular risk factors. We found no significant difference between the 34

patients diagnosed with diabetic peripheral neuropathy by MNSI and those without a diagnosis of neuropathy in terms of hypertension, hyperlipidemia, and obesity prevalence. However, we observed an increased rate of retinopathy, another microvascular complication of diabetes, in patients diagnosed with neuropathy.

In this study we compared the effectiveness of three different methods that can be used in the diagnosis of diabetic polyneuropathy. MNSI was used as a diagnostic method based on symptoms and signs. EMG and neurothesiometer were used to confirm diagnosis and to evaluate the diagnostic efficiency of each of the methods used in this study.

MNSI score was ≥ 2.5 in 34 patients involved in our study and diagnosis of diabetic neuropathy was made. This test was developed by Neurology Department of Michigan University and had first been applied to 56 patients with diabetes. These patients had also been evaluated for diabetic neuropathy according to San Antonio Consensus Statement and Mayo Clinic protocol. Neuropathy had been detected in 28 of 29 patients that had an MNSI score > 2 . In questionnaire form of MNSI, 20 patients with diabetic neuropathy and 18 patients without diabetic neuropathy had given positive responses to ≤ 6 questions. But 2 patients that had not had neuropathy and 14 patients that had neuropathy had given positive responses to ≥ 7 questions. These results had shown that many patients without neuropathy gave positive responses to ≤ 6 questions [6]. Similarly, in our study of 34 patients who were diagnosed as diabetic neuropathy based on MNSI, 24 gave positive responses to ≥ 7 questions, and of the 72 patients who were not diagnosed as diabetic neuropathy, 28 gave positive responses to ≥ 7 questions. These results suggest that diagnosing neuropathy depending only on symptoms can be misleading.

In the second part of our study, EMG was performed to all the patients included in our study. Like our study nerve conduction studies were made in the second part of the test recommended by Michigan University to confirm the diagnosis and to grade neuropathy. In the section called Michigan Diabetic Neuropathy Score (MDNS), patients were evaluated with neurological examination, nerve conduction studies, Neuropathic Deficiency Score (NDS), vibration threshold, autonomic function tests, and Neuropathy Symptom Profile (NSP), and results were graded from 0 to 3. Abnormal nerve conduction results were seen in 69% of patients detected to have neuropathy with MNSI [6].

In our study, in 20 of the patients (58.8%) diagnosed with neuropathy by MNSI, nerve conduction defect was detected with EMG. In our study, apart from evaluation made by Michigan University, EMG was also applied to patients who could not be diagnosed with MNSI. In this group of patients MNSI failed to diagnose neuropathy, and peripheral nerve conduction deficits were observed in 29 patients. Although abnormal electrodiagnostic tests are not considered as diagnostic by themselves, this result has shown that nerve conduction deficits developed at a high rate even in patients who were not diagnosed with neuropathic based on signs and symptoms. These patients have an increased risk for complications based on neuropathy and they should be followed up.

In this multicenter cross-sectional study done in Turkey, neurologic examinations and nerve conduction studies along with clinical diabetic neuropathy score and Leeds Assessment of Neuropathic Symptoms and Signs pain scale were performed on 1113 patients with diabetes to determine the prevalence of diabetic peripheral neuropathy. Prevalence of diabetic peripheral neuropathy determined only by clinical examination was 40.4% and it rose to 62.2%, when nerve conduction studies were combined with clinical examination [13]. Similarly, in our study, we observed that using clinical examinations and nerve conduction studies together is important for accurate diagnosis of DPN.

If neuropathy mainly affects thin, unmyelinated nerve fibers, electrophysiological tests are frequently normal. Neurothesiometer device was developed in recent years for quantitative sensory testing (QST) (CASE IV device was developed by Peter Dyck and colleagues). It allows appropriate evaluation of threshold values for vibration, heat, and pain senses. Neurothesiometer allows evaluations in which intensity and features of the stimulus are controlled well (e.g., tests applied to the same patient at different times and in different centers give the same results). QST is a valuable device to follow progression of neuropathy in patients with diabetic neuropathy [14].

In our study, neurothesiometer evaluation was applied to all the patients as a QST and threshold values for vibration, heat, and pain were evaluated. In neurothesiometer evaluation increased threshold was detected in 79 patients. In 30 (39.2%) of these patients neuropathy was present in MNSI. According to MNSI questionnaire form positive response number was ≥ 7 in 44 (57.7%) patients. In 30 of 34 patients diagnosed with neuropathy by MNSI, neurothesiometer showed nerve conduction deficit. In 89.7% of patients detected to have a dysfunction consistent with neuropathy in EMG, nerve conduction deficit was also detected with neurothesiometer ($P < 0.001$).

This test is a noninvasive screening test, but it cannot be diagnostic by itself because of its subjective nature. It was used to evaluate neuropathy incidence in 1011 patients who had a diagnosis of diabetes for more than 10 years in Spain to evaluate neuropathy prevalence. Diagnosis of neuropathy was confirmed by DN4 questionnaire form developed by French Neuropathic Pain Group which includes both history and physical examinations. After the study neuropathy was detected in 39.6% and subclinic neuropathy was detected in 36.8% of the patients. This study suggests that polyneuropathy is underdiagnosed and quantitative evaluation will be helpful [15].

In a study by Kincaid et al., clinical evaluation, nerve conduction study, and neurothesiometer were compared in the diagnosis of diabetic neuropathy. In a multicenter study involving 227 patients with diabetes mellitus, vibration threshold was measured with neurothesiometer, and peroneal, tibial, and sural nerves were evaluated with nerve conduction studies. Results of this study showed that these tests cannot be used interchangeably, but they could be complementary [16].

A study of 152 patients with diabetic peripheral neuropathy gave the patients electrodiagnostic evaluation and

quantitative vibration perception thresholds testing with the Vibratron II and neurothesiometer and concluded that vibration perception thresholds determined with the neurothesiometer are less variable than the thresholds determined with the vibratron and they are more reflective of peripheral nerve function. The results of this study indicate that the neurothesiometer can be used reliably in clinical research trials [17].

In a study with 2022 diabetic patients, peripheral polyneuropathy was diagnosed by vibration perception threshold at the tip of both great toes using a 128-Hz tuning fork and a neurothesiometer. Vibration perception threshold was also measured in 175 nondiabetic control subjects to define normal values. Finally, the vibration perception threshold measured by the neurothesiometer was 2.5 times higher in patients with an abnormal tuning fork test. The plot of the difference of both methods against their mean yielded a good agreement of the two VPT measurements [18].

5. Conclusions

In conclusion, this study evaluated 106 diabetic patients and found dysfunction consistent with neuropathy in 34 patients with MNSI, in 54 patients with EMG, and in 79 patients with neurothesiometer. Although electrodiagnostic evaluations do not have diagnostic value themselves according to ADA, they are thought to confirm the diagnoses. However in our study a higher rate of neuropathy was observed in electrophysiologic tests than in anamnesis and physical examination. Symptoms consistent with neuropathy were also detected in patients who were diagnosed with neuropathy by EMG and neurothesiometer but were not diagnosed neuropathy by MNSI. According to these data, diagnosing with diabetic neuropathy based only on anamnesis and physical examination will cause underdiagnosis of the problem.

MNSI can be performed easily by a physician at office conditions although EMG requires an experienced neurologist and neurology laboratory. To perform neurothesiometer, help is needed from trained staff. But if early diagnosis of diabetic peripheral neuropathy and thus prevention of complications with high morbidity are taken into account, common use of electrodiagnostic methods can be considered to be cost-effective.

Conflict of Interests

None of the authors have any potential conflict of interests associated with this research.

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Research Article

Analysis of Inflammatory Mediators in Type 2 Diabetes Patients

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The main aim of this study is to assess the inflammatory markers in type 2 diabetes mellitus (T2DM) by measuring some cytokines concentrations and lymphocytes subset and correlate them with other laboratory investigations. Fifty-seven patients with type-2 diabetes and 30 healthy volunteers were enrolled in this study. Data for the C-reactive protein (CRP), haemoglobin, HbA1c, and autoantibody levels were obtained from the patients files. The cytokine concentrations were measured in patient's serum using commercially available ELISA assays. Lymphocytes subsets were measured by flow cytometric methods. The levels of IL-1 β , IL-6, IL-15, and TNF- α were found to be decreased in T2DM patients, whereas the levels of IL-10, IFN- γ , and caspase-1 were increased, compared to normal controls. T2DM patients with hypertension show significantly decreased levels of IL-1 β and caspase-1 compared to patients without hypertension. No significant differences in lymphocytes subset between cases and normal control were observed. Significant correlations were found between HbA1c and IL-6; body mass index (BMI) was significantly correlated with CRP, TNF- α , and phosphate; the weight (Wt) was associated with CRP and IFN- γ . In conclusion, an alteration in the function of the immune system was observed in T2DM patient.

1. Introduction

Type 2 diabetes mellitus (T2DM) represents a significant global health problem. The burden of diabetes has increased sharply in Oman over the last decade, rising from 8.3% in 1991 to 11.6% in 2000, and 12.3% in 2008 among adults aged 20 years and older [1, 2].

Inflammation is considered to be a key regulator of the pathogenesis of T2DM, but what triggers this inflammation still unknown [3]. However, it may be related to obesity. Obesity is associated with enlargement of adipose tissue and consequently increases the number of adipose tissue macrophages [4, 5]. These macrophages are responsible for almost all adipose tissue tumor necrosis factor- α (TNF- α) expression, significant amounts of interleukin-6 (IL-6), and other acute-phase response markers and mediators of inflammation [5–7].

Many proinflammatory cytokines play a central role in inflammatory reaction and were shown to increase the risk of T2DM [8, 9]. These pro-Inflammatory cytokines can enhance insulin resistance directly in adipocytes, muscle and hepatic cells, leading to systemic disruption of insulin sensitivity and impaired glucose homeostasis. Increased levels of these pro-inflammatory cytokines lead to hepatic production and secretion of acute-phase proteins such as C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), amyloid-A, α 1-acid glycoprotein, and haptoglobin. These proteins appear in the early stages of T2DM, and their circulating concentrations increase as the disease progresses [3, 8–10].

It has been reported that normal individuals with detectable levels of IL-1 β and elevated levels of IL-6 had an independently increased risk to develop T2DM, whereas those with increased concentrations of IL-6 but undetectable levels of IL-1 β had no significantly increased risk [11, 12].

Another study showed that levels of IL-6, TNF- α , and TNF-receptor were elevated in insulin-treated, but not in sulfonylurea-treated patients [13]. Moreover, levels of serum glucose, pro-inflammatory cytokines (IL-6, IL-12, and TNF- α), endothelial dysfunction markers (vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and nitric oxide), and lipid abnormality were highest in T2DM with cardiovascular complications [14]. Recently, it has been shown that T2DM patients had a significantly higher CD14 (+) CD16 (+) fluorescence intensity, TLR4 expression, and serum IL-6 and C-reactive protein (CRP) levels, compared to normal controls [15].

Here in this study, we extend the analysis of the role of inflammation in T2DM by measuring the levels of several cytokines, including IL-1, IL-6, IL-10, IL-15, IFN- γ , TNF- α , and caspase-1 in T2DM and correlate them with other laboratory investigations. We will also examine whether there is an alteration in lymphocytes subsets in T2DM compared to the normal controls.

2. Patients and Methods

Fifty-seven patients (28 male, and 29 females, mean age 52 ± 11.5) with type 2 diabetes and 30 healthy volunteers (20 male, and 10 females, mean age 35 ± 7) were enrolled in this study (Table 1). Patients attended the outpatients clinic at Sultan Qaboos University Hospital (SQUH), Muscat, Oman. Confirmed T2DM patients were selected randomly; however, cases associated with other inflammatory diseases such as cancer or autoimmunity were excluded from this study. Informed consent was obtained from each subject. The Medical Research and Ethics Committee (MREC) at the College of Medicine, Sultan Qaboos University (SQU), approved this study.

Data for the C-reactive protein (CRP), hemoglobin, HbA1c and autoantibody levels were obtained from the patients files. The cytokine concentrations were measured in patients sera using commercially available ELISA assays (R&D Systems), performed according to the manufacturer's instructions. The lymphocytes subset was measured using monoclonal antibodies available as a Multiset panel (BD, USA), and analyzed using FACSCalibur flow cytometry (BD, USA), equipped with automated program.

2.1. Statistical Analysis. Statistical analyses were performed using SPSS software (20.0 version). Data normality was tested using KS test. Quantitative data were presented as mean \pm SD. The statistical significance between means was estimated by Student's *t*-test (independent samples) when appropriate. Pearson's correlation coefficient (*r*) was used to measure the strength of the association between the two variables. Differences were considered statistically significant at $P < 0.05$.

3. The Results

3.1. Cytokines Levels. The levels of IL-1 β , IL-6, and IL-15 TNF- α were decreased in T2DM patients compared to

TABLE 1: Demographic information and laboratory investigations of the T2DM patients.

Characteristics	Mean \pm SD/ <i>n</i> (%)	
	T2DM patients	Healthy controls
Age	52.3 ± 11.5	35 ± 7
Sex		
Male	28 (49)	20 (67%)
Female	29 (51)	10 (33%)
Disease duration		
≥ 5 years	51 (89.4)	NA
< 5 years	6 (10.6)	
Height	158.8 ± 7.8	165 ± 10
Weight	76.8 ± 13.8	73 ± 10.2
BMI	30.4 ± 5.2	NA
HbA1c	8.2 ± 1.4	NA
CRP	11.1 ± 24.6	NA
Vitamin D	44.2 ± 20.2	NA
Hypertension	38 (67)	0
TC	5.1 ± 1.1	NA
LDL	3.1 ± 1.0	NA
HDL	1.2 ± 0.4	NA
TG	1.6 ± 1.1	NA
Current smokers	2 (3.5)	0
Treatment (%)		NA
Insulin	12 (21)	
Oral		
(Metformin/sulfonylurea)	27 (47)	
(Metformin only)	4 (7)	
Oral + insulin	9 (16)	
Diet + exercise	5 (9)	

T2DM: type 2 diabetes mellitus; BMI: body mass index; HbA1c: glycated haemoglobin; CRP: C-reactive protein; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglycerides. NA: not available.

normal controls (Figure 1). Mean baseline level of IL-1 (211 ± 132 pg/mL) was lower among T2DM compared with control subjects (672 ± 385 pg/mL) but statistically insignificant (P value = 0.157).

However, the mean value of IL-6 was significantly (P value of 0.0001) lower in T2DM cases (1.76 ± 2.5 pg/mL), compared to control subjects (43 ± 20 pg/mL). The mean value of IL-15 was 4 ± 2.9 pg/mL, which is also significantly lower (P value = 0.004) than the mean value for the controls (10.2 ± 10.3 pg/mL). Similar significant difference (P value = 0.00002) was observed for TNF- α ; the mean baseline levels were 13 ± 11 pg/mL for T2DM patients and 147 ± 160 pg/mL for the control subjects.

When the patients' data was sorted according the treatment (insulin or sulfonylurea), no significant difference was observed between those patients treated with insulin only or those patients treated with sulfonylurea (data not shown).

The levels of IL-10, IFN- γ , and caspase-1 were increased in T2DM patients compared to normal controls (Figure 2).

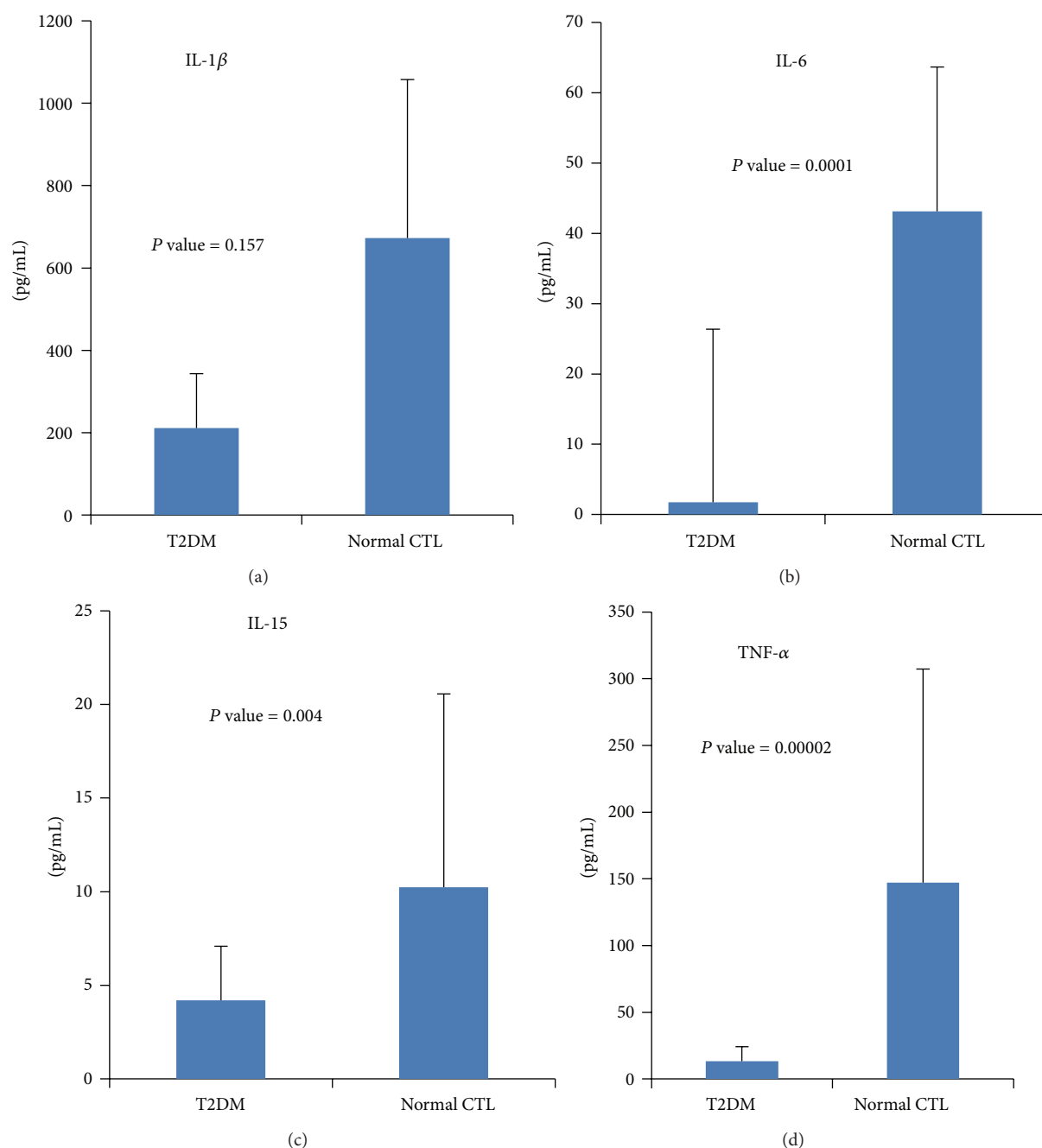


FIGURE 1: Decreased cytokine profile in T2DM.

Mean baseline levels of IL-10 (6.95 ± 6 pg/mL) were significantly higher (P value = 0.012) among T2DM patients compared to control subjects (2.9 ± 5.15 pg/mL). Likewise, the mean value of caspase-1 was 95.9 ± 116 pg/mL, which is significantly higher (P value = 0.0005) than the mean value for the control (20.2 ± 15 pg/mL). However, no significant differences were observed in the levels of IFN- γ between patients (2.97 ± 4 pg/mL) and controls (1.8 ± 4.1 pg/mL).

When the patient's data was distributed according to the treatment, no significant difference was observed between patients in insulin only or patients treated with sulfonylurea;

this is obviously because of the very small number in both groups; a large cohort should be used in the future.

3.2. Effect of Hypertension on Cytokine Production. T2DM Patients with hypertension show decreased levels of pro-inflammatory cytokines (Figure 3). Interestingly; T2DM patients with hypertension shows significantly decreased levels of IL-1 β and caspase-1 (P value = 0.024, 0.028, resp.). TNF- α and IL-15 levels were also decreased but statistically insignificant. IL-6 and IL-10 levels were slightly increased in T2DM patients with hypertension.

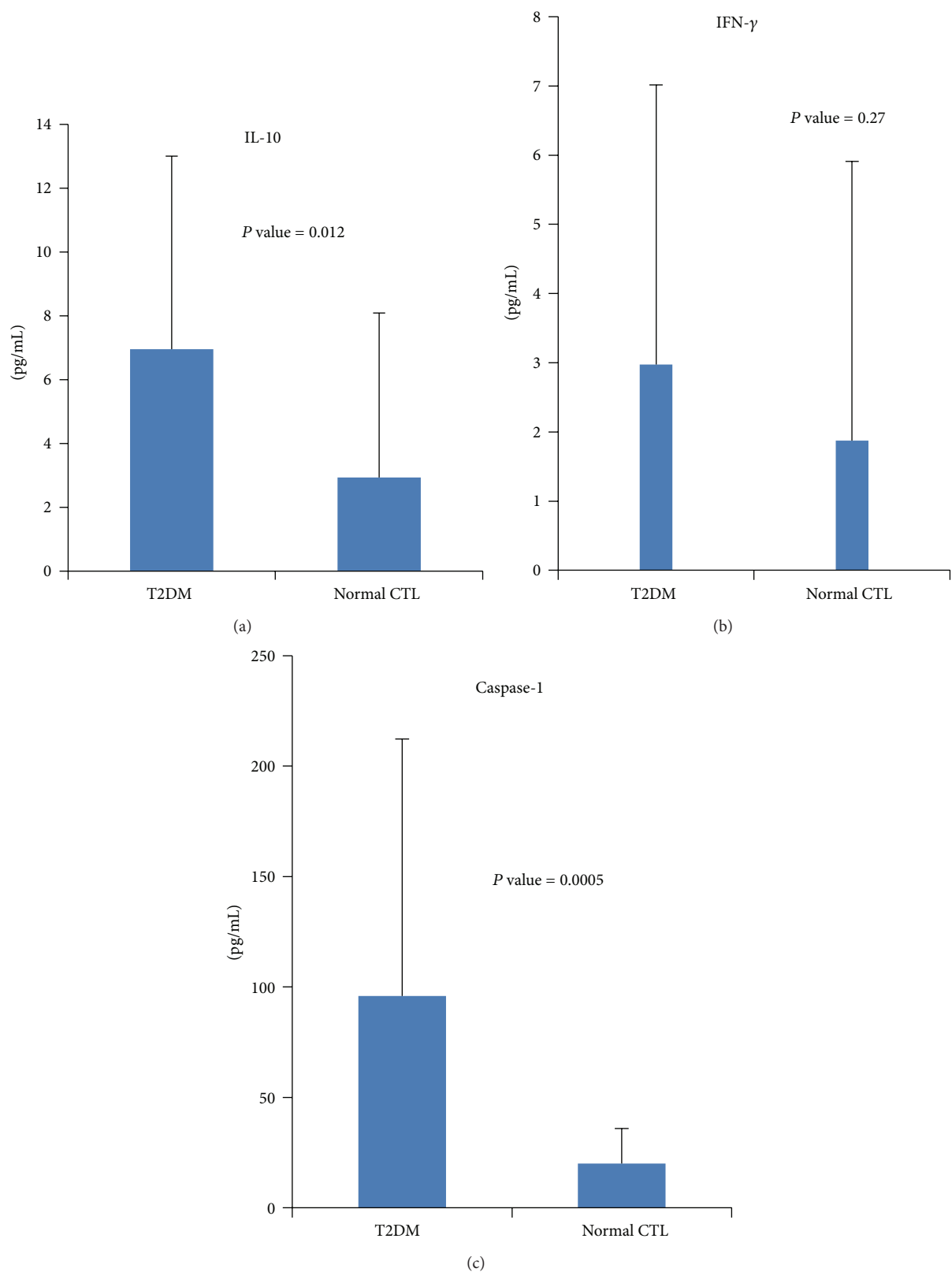


FIGURE 2: Increased cytokine profile in T2DM.

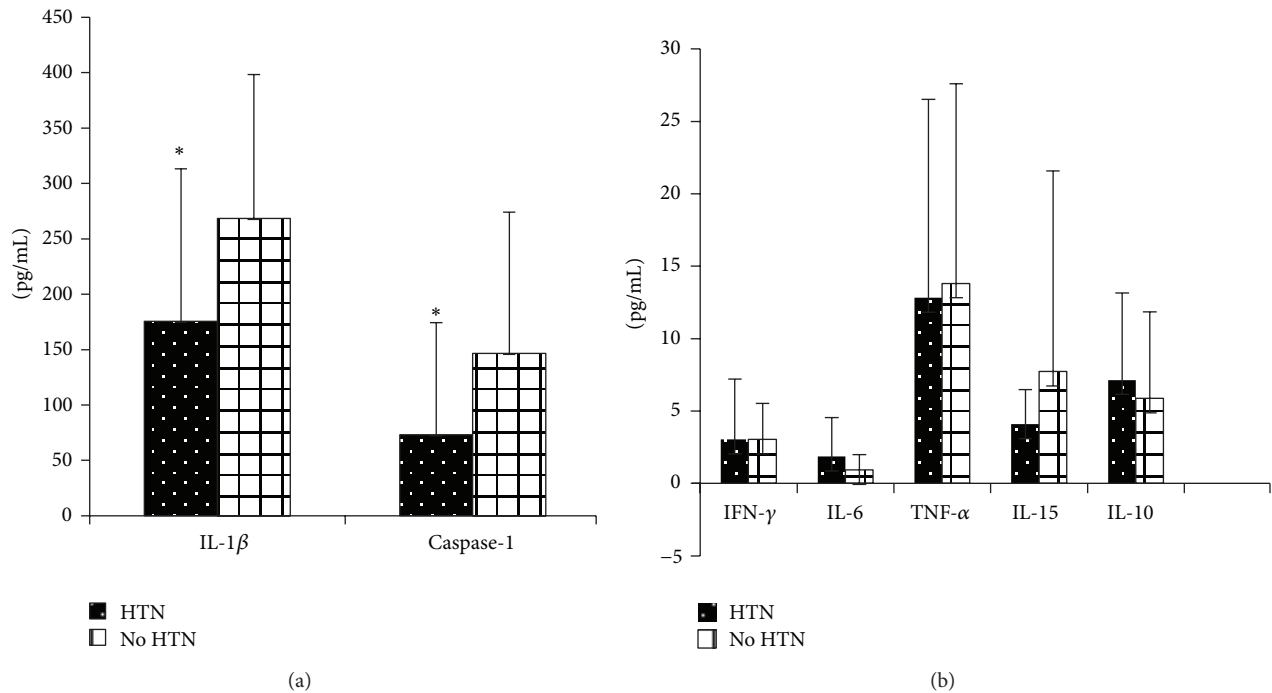


FIGURE 3: Cytokine profile in T2DM with or without hypertension. *Significant difference (P value < 0.05).

TABLE 2: Lymphocytes subset in T2DM and normal controls.

	CD3+	CD8+	CD4+	CD4+/CD8+	CD56+	CD19+
Normal control, $n = 30$	69 ± 8.14	25 ± 5	38 ± 8	0.7 ± 1	12 ± 5	14 ± 4.7
T2DM, $n = 59$	71 ± 8.9	27 ± 8.2	43 ± 7.5	0.75 ± 1	14 ± 7.5	13 ± 4.7
P value	NS	NS	NS	NS	NS	NS

NS: not significant.

3.3. Lymphocyte Subsets. Lymphocytes subsets in T2DM and normal controls are shown in Table 2; several immune cells were measured, including lymphocytes (CD3+), T helper cells (CD4+), T-cytotoxic cells (CD8+), double positive CD4/CD8, natural killer cells (CD56+), and B cells (CD19+), and no significant differences between T2DM patients and normal controls were observed, Table 2.

3.4. Correlation Analysis. HbA1c was positively correlated with IL-6 with a significant P -value of 0.005, and BMI was positively and significantly correlated with CRP (P value = 0.001), TNF- α (P value = 0.013) and phosphate (P value = 0.008). The weight (Wt.) of the patients was significantly correlated with CRP and IFN- γ , P values of 0.11 and 0.044, respectively. In addition, calcium level was correlated with phosphate (P value = 0.004), hypertension (P value = 0.032), LDL (P value = 0.03) and dyslipidemia (P value = 0.011), Table 3.

4. Discussion

Our data showed decreased levels of IL-1 β , IL-6, IL-15, and TNF- α and increased levels of IL-10, IFN- γ , and caspase-1

in T2DM compared to healthy controls. Decreased levels of inflammatory cytokines in our study were in disagreement with previous findings. A recent study by Marques-Vidal et al. (2013) found that subjects with T2DM had increased levels of IL-6, TNF- α , and hs-CRP, while no association was found with IL-1 β [16]. Moreover, it was reported that, high levels of inflammatory cytokines appear in early stage of T2DM and capable of predicting the development of type 2 diabetes through diminishing insulin sensitivity [9, 10]. This discrepancy can be attributed to the (I) duration of the diseases; the majority of patients included in our study have a long disease duration (greater than 5 years), (II) small sample size, and (III) the differences in age and sex of the studied groups; the age of the normal controls was lower than that of T2DM patients. Moreover, there were only 10 females on controls versus 29 females on T2DM group. These factors may have played essential roles in the cytokine production among these two study groups.

Mavridis et al. investigated inflammatory cytokines in insulin-treated T2DM patients and showed increased levels of IL-6, TNF- α in insulin-treated T2DM patients compared to sulfonylurea-treated patients. Also, they found a positive association between waist circumference and IL-6 and a significant correlation between HbA1c and IL-6 [13]. When

TABLE 3: Correlation analysis; data shows significant *P* value.

	IL-6	CRP	IFN- γ	TNF- α	Phosphate	HTN	LDL	Dyslipidemia
HbA1c	0.005	—	—	—	—	—	—	—
BMI	—	0.001	—	0.013	0.008	—	—	—
Wt.	—	0.011	0.044	—	—	—	—	—
Calcium	—	—	—	—	0.004	0.032	0.030	0.011

HbA1c: glycated haemoglobin; BMI: body mass index, Wt.: weight; CRP: C-reactive protein, HbA1c: glycated haemoglobin; TG: triglycerides; LDL: low-density lipoprotein; HTN: hypertension; TNF- α : tumor necrosis factor; IL-6: interleukin-6; IFN- γ : interferon.

we sorted our data of cytokines levels, according to the treatment (insulin or sulfonylurea) of the patients, we did not find any significant differences; this may be due to a small sample size in our study. However, our results showed that HbA1c levels were correlated with IL-6 levels, which is in accordance with previous findings [11, 12]. Nevertheless, from all these data, we cannot conclude whether poor glycaemic control leads to inflammation or whether inflammation leads to higher glucose levels; further studies are needed to assess such questions.

Moreover, our study showed that BMI was positively correlated with CRP, TNF- α , and phosphate levels; and the weight was positively correlated with CRP and IFN- γ , which is in accordance with previous findings [16–18]. This correlation can be explained as follows: obesity is associated with enlargement of adipose tissue and consequently increases the number of adipose tissues macrophages [4–6]. These macrophages are responsible for almost all adipose tissue TNF- α expressions and other acute-phase response markers and mediators of inflammation [5–7]. TNF- α , secreted by adipose tissue, may play a critical role in insulin resistance and the pathogenesis of type 2 diabetes. Several studies indicated that increased levels of cytokines and acute-phase proteins can participate in maintaining the insulin-resistant state [16, 19].

In T2DM patients with hypertension, the pathophysiology of cardiovascular disease is multifactorial; for example angiotensin II may be to a large degree responsible for triggering vascular inflammation by inducing oxidative stress [20]. However, our data shows that T2DM patients with hypertension had significantly lower levels of IL- β and Caspase-1 and slightly higher levels of IL-6 and IL-10, compared to patients with no hypertension. This can be attributed to the treatment of hypertension, such that drugs that target the renin-angiotensin system may reduce blood pressure and inflammation in T2DM patients with hypertension [20].

In conclusion, we found that, patients with established T2DM, had different cytokine profile than healthy controls, without a significant change in lymphocyte subsets; this indicates that, there is an alteration in the function of the immune system in T2DM patient.

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Clinical Study

Relation of Epicardial Fat Thickness with Carotid Intima-Media Thickness in Patients with Type 2 Diabetes Mellitus

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Aims. The aim of this study was to investigate the relationship of echocardiographic epicardial fat thickness (EFT) with carotid intima-media thickness (CIMT), in patients with type 2 diabetes mellitus (T2DM). **Methods and Results.** A total of 139 patients with T2DM (mean age 54.3 ± 9.2 and 49.6% male) and 40 age and sex-matched control subjects were evaluated. Echocardiographic EFT and ultrasonographic CIMT were measured in all subjects. Patients with T2DM had significantly increased EFT and CIMT than those of the controls (6.0 ± 1.5 mm versus 4.42 ± 1.0 mm, $P < 0.001$ and 0.76 ± 0.17 mm versus 0.57 ± 0.14 mm, $P < 0.001$, resp.). EFT was correlated with CIMT, waist circumference, BMI, age, duration of T2DM, HbA1c in the type 2 diabetic patients. Linear regression analysis showed that CIMT ($\beta = 3.52$, $t = 3.72$, $P < 0.001$) and waist circumference ($\beta = 0.36$, $t = 2.26$, $P = 0.03$) were found to be independent predictors of EFT. A cutoff high risk EFT value of 6.3 mm showed a sensitivity and specificity of 72.5% and 71.7%, respectively, for the prediction of subclinical atherosclerosis. **Conclusion.** We found that echocardiographic EFT was significantly higher in patients with T2DM. Our study also showed that EFT was strongly correlated with waist circumference and CIMT as being independent of sex.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common chronic diseases in the worldwide, the incidence of which tends to grow steadily. It is associated with a high risk of cardiovascular disease (CVD) which is the leading cause of death in patients with type 2 diabetes mellitus [1].

Obesity, insulin resistance, and diabetes have identified a proinflammatory state associated with increased adiposity [2]. Epicardial adipose tissue (EAT) is a visceral fat depot of the heart located along the large coronary arteries and on the surface of the ventricles and apex [3]. The embryological origin of EAT is similar to intra-abdominal visceral adipose tissue [4]. Several studies have shown that EAT is not only an anatomic depot of fat but also may serve as a local source of proinflammatory cytokines related to coronary artery disease (CAD) [5]. Therefore, EAT thickness has been considered to be a possible cardiovascular risk indicator [6, 7]. Transthoracic echocardiography (TTE), magnetic resonance imag-

ing (MRI), and multislice computed tomography (MSCT) scanning have been conventional methods for quantifying EAT [8]. Assessment of EAT by TTE could be a simple and practical tool for cardiovascular risk stratification in clinical practice [3].

Carotid intima-media thickness (CIMT) is a simple and inexpensive tool to assess the cumulative effect of atherosclerotic risk factors and is an independent predictor of future cardiovascular (CV) risk [9]. The ultrasound-based measurement of CIMT has become a standard for assessing atherosclerosis and is recommended by the American Heart Association for the noninvasive assessment of cardiovascular risk [10, 11].

Previous studies have reported that increased EAT is associated with CAD, metabolic syndrome (MetS) and obesity [12–16]. In the present study, we evaluated type 2 diabetic patients to investigate epicardial fat thickness by TTE and investigate its relationship with CIMT.

2. Methods

2.1. Patient Population. In this observational, cross-sectional study, 139 type 2 diabetic patients, having this diagnosis for at least 1 year, were consecutively included in the study. The control group consisted of 40 sex and age-matched healthy people. T2DM was diagnosed according to the American Diabetes Association criteria [17]. The study protocol was approved by our local ethics committee, and all patients gave their written informed consent to participate in the study.

Exclusion criteria of the study were subjects with known ischemic heart disease, cerebrovascular disease, peripheral vascular disease, congestive heart failure, valvular heart disease, and chronic kidney disease.

Medical history was obtained and physical examination was performed in all patients and controls. Blood pressure was measured three times—5 min apart—in a sitting position, on the right arm, and the mean value was calculated. Weight and height of the patients were measured without heavy outer garments and shoes, after a 12 h fasting period. Body-mass index (BMI) was calculated as body weight divided by the square of the height. Waist circumference was measured at the level of midway between the lower rib margin and iliac crest after removal of the clothes. Blood samples were withdrawn by venipuncture from all subjects following 12 h of fasting. Fasting blood glucose, serum creatinine, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and triglyceride levels were recorded. Glucose, creatinine, and lipid profile were determined using standard methods. Hemoglobin A1c (HbA1c) levels were measured by high pressure liquid chromatography with a thermo system. Serum CRP levels were evaluated using the nephelometric method.

2.2. Measurements of Epicardial Adipose Tissue Thickness. Each patient underwent a complete transthoracic echocardiography using the American Society of Echocardiography guidelines of measurement [18]. Echocardiogram was performed using a Vivid 7 (General Electronic, Wauke-sha, Wisconsin, USA) with a 2.5–3.5 MHz transducer, placed on the III–IV left intercostal space along the parasternal line, with patients being supine in left lateral decubitus and the head of the bed kept at 30°. All examinations were performed by an experienced cardiologist, blind to the patient's clinical information. Epicardial fat was identified as the space or layer anterior to the right ventricle with decreased echoreflexivity compared with the myocardium and pericardium. Epicardial fat thickness (EFT) was measured in end diastole on the free wall of the right ventricle from the parasternal long- and short-axis views, as previously described [19, 20]. The maximum values at any site were measured, and the average value was considered. Imaging constraints were used to ensure that the epicardial fat thickness was not measured obliquely. The intraobserver correlation coefficient was 0.96. Parasternal long- and short-axis views allow the most accurate measurement of EAT on the right ventricle, with optimal cursor beam orientation in each view.

2.3. Carotid Ultrasonography. Carotid arteries were evaluated using a Logiq 7 (General Electronic, Wauke-sha, Wisconsin,

USA) with a 7.5 MHz transducer. All examinations were performed by an experienced radiologist, blind to the patient's clinical information. Measurements involved a primary transverse and longitudinal scanning of the common carotid artery, bifurcation, and internal carotid. The CIMT was measured on the far wall at 1 cm from bifurcation of the common carotid artery as the distance between the lumen-intima interface and the media-adventitia interface. At least three measurements were performed on both sides, and the average measurement was taken as the CIMT. All measurements were made at a plaque-free site. The intraobserver correlation coefficient was 0.97.

2.4. Statistical Analysis. SPSS statistical software (SPSS for Windows, version 17.0, Inc., Chicago, IL, USA) was used for all statistical calculations. Categorical variables were expressed as number and proportions, while continuous variables were expressed as mean \pm standard deviation. Chi-square (χ^2) test was used to compare groups regarding categorical variables. Continuous variables were compared with Student *t*-test (while comparing parametric variables between diabetic patients and controls) or Mann-Whitney *U* test (while comparing nonparametric variables between diabetic patients and controls). Correlation analysis was performed using Pearson or Spearman tests. Linear regression analysis was used to explore the independent determinants of EFT. Receiver operating characteristic (ROC) curve analysis was performed to determine cutoff high risk value of EFT when patients are divided into two groups according to CIMT ($<$ or ≥ 0.9 mm). Levels of statistical significance were set at a *P* value < 0.05 .

3. Results

There were 139 patients (mean age 54.3 ± 9.2 and 49.6% male) in the T2DM group and 40 patients (mean age 52.1 ± 7.3 and 50% male) in the control group. The demographic findings and laboratory values of the study groups are presented on Table 1. The mean duration of disease in patients with T2DM was 6.5 ± 3.9 years. The Age, frequencies of sex distribution, hypertension, hyperlipidaemia, smoking, family history of the CAD, and waist circumference were similar between patients with T2DM and the controls. BMI was higher in the control group compared with the value of the type 2 diabetic patients (29.1 ± 4.2 versus 27.6 ± 3.1 , $P = 0.03$, resp.). Patients with T2DM had significantly increased EFT and CIMT than those of the controls (6.0 ± 1.5 mm versus 4.42 ± 1.0 mm, $P < 0.001$ and 0.76 ± 0.17 mm versus 0.57 ± 0.14 mm, $P < 0.001$, resp.). When laboratory findings were compared, type 2 diabetic patients had significantly higher fasting blood glucose, creatinine, C-reactive protein levels, and HbA1c, but total cholesterol, LDL, HDL and triglyceride levels did not differ between the two groups.

The variables correlated with EFT in the type 2 diabetic patients were CIMT ($r = 0.479$, $P < 0.001$) (Figure 1), waist circumference ($r = 0.371$, $P < 0.001$), BMI ($r = 0.315$, $P < 0.001$), age ($r = 0.260$, $P = 0.002$), duration of DM ($r = 0.258$, $P = 0.003$) (Figure 2), and HbA1c ($r = 0.200$, $P = 0.032$) (Table 2), and those in the controls were; CIMT ($r = 0.690$,

TABLE 1: Clinical and biochemical characteristics of the patients with type 2 diabetic patients and healthy controls.

Parameters	DM (<i>n</i> = 139)	Controls (<i>n</i> = 40)	<i>P</i> value
Age (years)	54.3 ± 9.2	52.1 ± 7.3	0.109
Gender (male, %)	49.6%	50%	0.968
Disease duration (yrs)	6.5 ± 3.9	—	—
Hypertension (%)	41%	35%	0.494
Smoking (%)	20.9%	15%	0.410
Hyperlipidaemia (%)	35.3%	32.5%	0.747
Family history of CAD (%)	16.5%	15%	0.815
Epicardial fat thickness (mm)	6.0 ± 1.5	4.42 ± 1.0	<0.001
Carotid artery intima-media thickness (mm)	0.76 ± 0.17	0.57 ± 0.14	<0.001
Body mass index (kg/m ²)	27.6 ± 3.1	29.1 ± 4.2	0.03
Waist circumference (cm)	90.8 ± 9.3	89.1 ± 11.2	0.392
Systolic blood pressure (mm Hg)	133.9 ± 17.6	129.4 ± 12.4	0.209
Diastolic blood pressure (mm Hg)*	78 (40–100)	80 (60–90)	0.440
Fasting glucose (mg/dL)	147 ± 38.8	94.1 ± 6.3	<0.001
Creatinine (mg/dL)	0.88 ± 0.15	0.77 ± 0.17	0.03
Total cholesterol (mg/dL)	199.1 ± 28.1	198.4 ± 23.9	0.933
LDL (mg/dL)	116.1 ± 25.0	118.3 ± 18.0	0.734
HDL (mg/dL)	43.5 ± 9.4	46.6 ± 10.0	0.247
Triglyceride (mg/dL)	172 ± 72.7	163.2 ± 85.2	0.681
C-reactive protein (mg/dL)	3.0 (2–13.1)	1.9 (0.93–7.6)	0.002
HbA1c (%)	7.4 ± 1.2	5.1 ± 0.41	<0.001
Hemoglobin (g/dL)	14.2 ± 1.6	14.3 ± 1.7	0.741
Platelets (×10 ⁹ /L)	276.2 ± 70.4	251.5 ± 42.9	0.167

Data are presented as mean ± s.d. or *n* (%). CAD: coronary artery disease; HDL: high-density lipoprotein; LDL: low-density lipoprotein. * Diastolic blood pressure is presented as the median (min–max).

TABLE 2: The univariate correlations of the epicardial fat thickness.

Variable	<i>r</i>	<i>P</i>
Carotid artery intima-media thickness	0.479	<0.001
Waist circumference	0.371	<0.001
Body mass index	0.315	<0.001
Age	0.260	0.002
Disease duration	0.258	0.003
HbA1c	0.200	0.032
Weight	0.165	0.054

The correlations with a *P* > 0.10 are not shown.

P < 0.001), waist circumference (*r* = 0.420, *P* = 0.02), and age (*r* = 0.365, *P* = 0.02). When we performed subgroup analysis in the patients with T2DM according to sex, EFT was correlated with CIMT (*r* = 0.481, *P* < 0.001), waist circumference (*r* = 0.429, *P* < 0.001), age (0.348, *P* = 0.003), BMI (*r* = 0.391, *P* < 0.001), duration of DM (*r* = 0.293, *P* = 0.014), and weight (*r* = 0.285, *P* = 0.018) in female patients and CIMT (*r* = 0.481, *P* < 0.001) and waist circumference (*r* = 0.263, *P* < 0.03) in male patients. After multivariate stepwise linear regression analysis, CIMT (β = 3.52, *t* = 3.72, *P* < 0.001) and waist circumference (β = 0.36, *t* = 2.26, *P* = 0.03) were found to be independent relevant factors of EFT in patients with T2DM (Table 3).

The patients with T2DM are divided into two groups according to the values of CIMT (< or ≥0.9 mm), which is

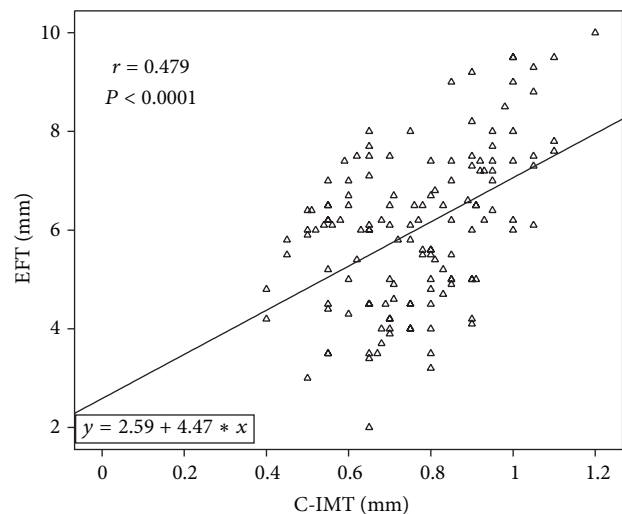


FIGURE 1: Epicardial fat thickness (EFT) is positively correlated with carotid intima-media thickness (CIMT) in diabetic patients.

an indicator level of subclinical organ damage as proposed by ESC hypertension guideline [21], to determine cutoff high risk value of EFT in patients T2DM by receiver operating characteristic (ROC) curve analysis. In the ROC curve analysis, the area under the curve (AUC, Figure 3) was found statistically significant (AUC = 0.797, 95% CI: 0.709–0.884, *P* < 0.001). As an optimal cutoff point, high risk EFT value

TABLE 3: Independent predictors for epicardial fat thickness by multivariate stepwise linear regression analysis.

Model	Unstandardized coefficients		Standardized coefficients	<i>t</i>	<i>P</i> value	95.0% confidence interval for <i>B</i>	
	<i>B</i>	Std. error	Beta			Lower bound	Upper bound
Carotid artery intima-media thickness	3.52	0.946	0.362	3.719	<0.001	1.64	5.40
Waist circumference	0.36	0.016	0.220	2.263	0.026	0.004	0.068

The model including CIMT, age, weight, HbA1c, waist circumference, duration of DM and BMI. *B*: Coefficient of regression.

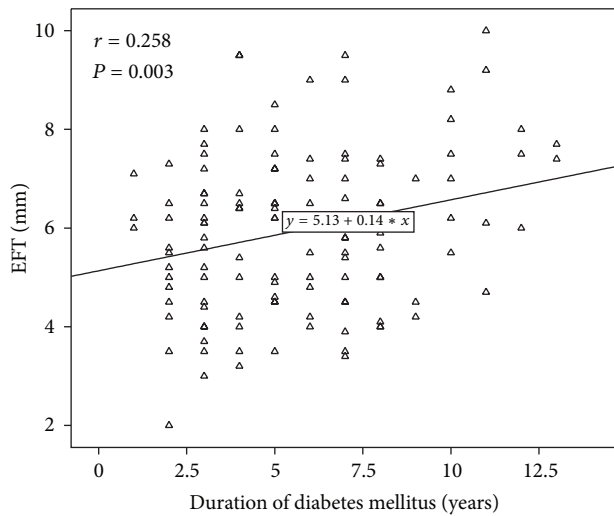


FIGURE 2: Duration of diabetes mellitus is linear and positively correlated with epicardial fat thickness (EFT).

of 6.3 mm was determined with a 72.5% sensitivity and 71.7% specificity.

4. Discussion

The major findings of the present study were first, patients with T2DM had increased EFT and CIMT compared with age- and sex-matched controls. Second, EFT was correlated with CIMT, waist circumference, BMI, age, duration of DM, and HbA1c in patients with T2DM. Third, CIMT and waist circumference were found to be independent predictors of EFT. Finally, EFT of 6.3 mm was determined as a high risk value for subclinical atherosclerosis with a 72.5% sensitivity and 71.7% specificity in ROC curve analysis.

Epicardial, mesenteric, and omental fats share the same origin from the splanchnopleuric mesoderm [4]. It has been shown that EAT produces inflammatory mediators such as interleukin (IL)-6, IL-1b, tumor necrosis factor (TNF)-a, and monocyte chemotactic protein (MCP-1) in patients with significant coronary artery disease [22] and expresses mRNAs of adiponectin, resistin, leptin, IL-6, TNF-a, and CD-45 [23]. Several studies have shown that EAT can play a role in the development and aggravation of CAD [8, 22–24]. In addition, EFT has been shown to be related to MetS, abdominal visceral adiposity, subclinical atherosclerosis, nonalcoholic fatty liver disease, type 1 DM, impaired fasting glucose, and hypertension [19, 20, 25–29].

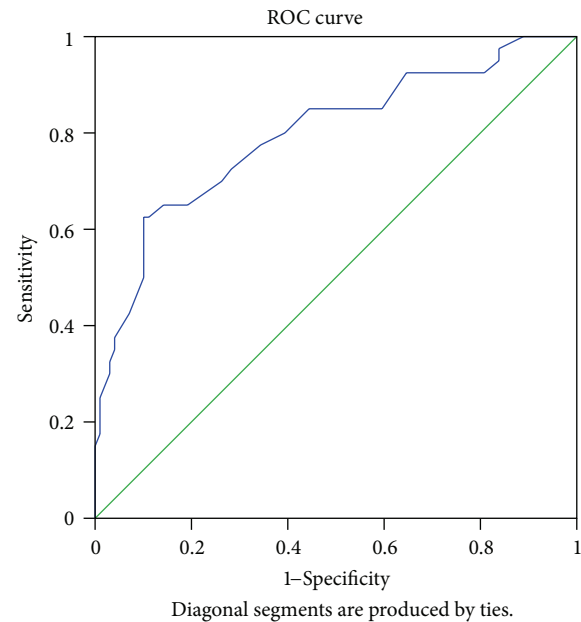


FIGURE 3: Receiver operating characteristics (ROC) curve (showing sensitivity and specificity of the epicardial fat thickness to determine cutoff high risk value). The area under the ROC curve of 0.797 indicates a very good accuracy of the test.

To our knowledge, as a main difference when compared to previously published data in patients with MetS, this is the first study in the literature focusing on the relationship between EFT and CIMT in patients with T2DM. It is also important that a positive linear and significant relationship between EFT and duration of diabetes mellitus was found in our study. According to these results, EFT may be used as a marker of subclinical atherosclerosis and disease progression in patients with T2DM. Further studies are required to support this hypothesis.

There is very limited study investigating the relationship between T2DM and EFT. Recently, in a study performed by Kim et al. [30], increased EAT thickness assessed by cardiovascular magnetic resonance (CMR) was an independent risk factor for significant coronary artery stenosis in asymptomatic type 2 diabetes. There were 100 patients and no control group in that trial. In another study reported in a series of 49 type 2 diabetic patients by Wang et al. [31], EAT volume assessed by cardiac multislice computed tomography was shown to be increased and was associated with unfavourable components of MetS and coronary atherosclerosis. In our study, similar to these trials, we found

that EFT was increased in patients with T2DM. In contrast to these trials, we evaluated EFT by TTE in a larger population with a control group and sought relation between EFT and CIMT, which is increasingly used as a surrogate marker for atherosclerosis. Although epicardial fat is readily visualized on high-speed CT and MRI, widespread use of these methods for its assessment is not practical. Echocardiographic EFT measurement in the current practice appears to be feasible, as well as reliable due to good reproducibility which has been shown both in previous studies [19, 20, 32] and in this study.

In our study, patients with T2DM had lower BMI. However, EFT and CIMT were higher in the diabetic patients compared to the controls. Additionally, BMI correlated with EFT in female diabetic patients, but it did not correlate with EFT in male diabetic patients and did not find independent predictor for EFT. Waist circumference not only was correlated with EFT but also was independent predictor for EFT in patients with T2DM and also in both sex. It has been reported that BMI is not a good measure of body adiposity [33]. Similarly, we also found that waist circumference was more reliable parameter than BMI to predict EFT in this study.

Iacobellis et al. [34] have reported that threshold values of high risk EFT to predict MetS are median values of 9.5 mm (85% sensitivity and 63% specificity) and 7.5 mm (82% sensitivity and 62% specificity) in white men and women, respectively. In another study performed by Natale et al. [35] in a large population of hypertensives, Patients with EFT > 7 mm showed a significantly increase in CIMT (0.84 ± 0.2 mm). In a large study performed on patients presenting for cardiovascular preventive care, Nelson et al. [36] have reported that EAT thickness ≥ 5.0 mm may identify an individual with a higher likelihood of having detectable carotid atherosclerosis. In our study, we found that EFT of 6.3 mm was determined as a high risk value for subclinical atherosclerosis with a 72.5% sensitivity and 71.7% specificity. Different race and patient population in those studies may have created these different threshold values of EFT. Even if different threshold values of EFT have been found in all these studies, increased EFT seems to be related to atherosclerotic process.

Our study had some limitations. This is a case control study, and prospective studies are necessary to show relation between EFT with CIMT and waist circumference. All data were based on a single measurement and may not reflect the association of EFT and CIMT regarding changes with time. In order to establish EFT as a high risk criteria of atherosclerosis in type 2 diabetic patients, further studies are necessary on larger series.

In conclusion, we found that EFT measure by TTE was increased in patients with T2DM. Our study also demonstrated that EFT was strongly correlated with waist circumference and CIMT as being independent of sex. Threshold value of high risk EFT was determined to be 6.3 mm. These results from our study population may suggest that the echocardiographic assessment of EFT is the reliable marker of atherosclerosis and increased CV risk in patients with T2DM. Further studies are needed to confirm these findings.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Clinical Study

Cigarette Smoking Is Associated with Prolongation of the QTc Interval Duration in Patients with Type 2 Diabetes Mellitus

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Aims. Aim of the study was to evaluate the effect of smoking on autonomic nervous system (ANS) activity and QTc interval duration in patients with Type 2 diabetes mellitus (T2DM). **Methods.** A total of 70 patients with T2DM (35 chronic smokers, 35 nonsmokers) treated with oral antidiabetic medications underwent continuous ECG Holter monitoring for 24 hours and analysis of time- and frequency-domain measures of heart rate variability (HRV). HRV over short time was also assessed using the deep breathing test. In addition, baroreflex sensitivity (BRS) was evaluated using the spontaneous sequence method. The mean QTc interval was measured from the 24-hour ECG recordings. **Results.** Smokers had lower body mass index (BMI) and exhibited higher 24-hour mean heart rate. There was no difference regarding all measures of ANS activity between the two groups. Smokers showed increased mean QTc duration during the 24 hours (439.25 ± 26.95 versus 425.05 ± 23.03 ms, $P = 0.021$) as well as in both day (439.14 ± 24.31 ms, $P = 0.042$) and night periods (440.91 ± 32.30 versus 425.51 ± 24.98 ms, $P = 0.033$). The association between smoking status and mean QTc interval persisted after adjusting for BMI. **Conclusions.** Cigarette smoking is associated with prolongation of the QTc interval in patients with T2DM by a mechanism independent of ANS dysfunction.

1. Introduction

Patients with Type 2 diabetes mellitus (T2DM) who smoke are at increased risk of developing cardiovascular disease, including cardiac arrhythmias [1]. Prolongation of the QT interval corrected for heart rate (QTc) is associated with a lowered ventricular fibrillation threshold and other potentially lethal arrhythmias, as polymorphic ventricular tachycardia (torsades de pointes), and has been proven to be an independent risk factor for sudden cardiac death (especially QTc values > 440 ms), both in the general population [2] as well as in patients with T2DM [3].

Autonomic nervous system (ANS) function has been consistently shown to be associated with QTc interval duration in patients with diabetes and in healthy individuals [4]. Furthermore, in patients with diabetes, QTc interval prolongation is one of the main manifestations of cardiac autonomic neuropathy (CAN) [5]. There is no widely accepted single

approach to the diagnosis of CAN in diabetes. Assessment of heart rate variability (HRV), orthostatic hypotension, and 24 h blood pressure profiles provides indices of both parasympathetic and sympathetic autonomic function and can be used in clinical settings. The analysis of HRV and measurement of arterial baroreflex sensitivity (BRS) have been proven to detect ANS dysfunction at a very early stage in patients with diabetes [5]. Furthermore, the evaluation of HRV during deep breathing is a highly sensitive and reliable test for early detection of parasympathetic dysfunction in a wide range of autonomic disorders [6]. Other methods, such as the classic battery of the tests of Ewing, cardiac sympathetic imaging, microneurography, and occlusion plethysmography, may also be used, especially in research settings [5].

It is well known that cigarette smoking alters autonomic cardiac control [7] and arterial baroreceptor function [8] and has also been demonstrated to prolong QT interval in healthy individuals in some [9–11] but not all studies [12]. However,

there is relatively little information in the literature as regards to the impact of smoking on ANS function in general [13], and particularly on QTc interval duration in patients with T2DM, since both diabetes and cigarette smoking are considered as important modifiable risk factors for heart disease [1].

The aim of the present study was to evaluate QTc interval duration in smokers compared to nonsmokers with Type 2 diabetes mellitus, in relation to ANS activity.

2. Materials and Methods

2.1. Study Sample. A total of 35 consecutive chronic smokers with T2DM, matched one-to-one for age, sex, and duration of diabetes with 35 never-smokers with T2DM, were included in the study. To decide about the required sample size and since there is lack of relevant data in the literature, we, a priori, hypothesised that a QTc difference of 10 ± 5 ms between the two studied groups would be clinically significant and meaningful. Thus, and in order to achieve statistical power greater than or equal to 90%, at 5% Type I error rate of two sided hypotheses, a total of 35 consecutive chronic smokers with T2DM, matched one-to-one for age, sex, and duration of diabetes with 35 never-smokers with T2DM, was deemed adequate. Smoking status was expressed using the Brinkman index (BI), which was calculated by multiplying the number of cigarettes smoked per day by the duration of smoking in years [14]. None of the patients was taking insulin, β -blockers, antiarrhythmic drugs, and medications known to affect ANS activity or increase QT interval duration. In addition, patients who had coronary heart disease, cardiac arrhythmias, or any history of other chronic disease were excluded from the study. All patients had normal thyroid function and serum electrolytes. Patients who reported any hypoglycaemic event during the 24 hours prior to the study were also excluded. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or the use of antihypertensive medication. The study was approved by the participating Hospital's Ethics Committee and carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2008 [15]. The patients who took part in the study were attending the outpatient diabetes clinic of the Laiko General Hospital in Athens, Greece, and gave their written informed consent for participation. All smokers were encouraged to quit smoking.

2.2. Analysis of the 24-Hour Ambulatory ECG Recordings. All patients underwent continuous ECG Holter monitoring for 24 hours. For this study the digital ECG Holter recorder Spider View (ELA Medical, France) with seven electrodes was used to record three-channel ECGs. The 24-hour recordings were analysed using the SyneScope Holter analysis software (version 3.00, ELA Medical, France). Artefacts and ectopic beats were automatically edited from analysis. In addition, the QRS complex classification was reviewed by an experienced cardiologist blinded to the patients' clinical characteristics.

All the time- and frequency-domain parameters of HRV recommended by the Task Force of the European Society of Cardiology and the North American Society of

Pacing and Electrophysiology were calculated from the 24-hour ECG recordings [16]. The values of the time-domain parameters of HRV were expressed in milliseconds (ms). HRV in the frequency-domain was computed by SyneScope using fast Fourier transformation analysis. The total power and, respectively, the power in very low frequency ((VLF) ≤ 0.04 Hz), low frequency ((LF), 0.04–0.15 Hz), and the high frequency ((HF), 0.15–0.40 Hz) bands were evaluated. All the frequency-domain parameters of HRV were calculated as absolute values and expressed in ms^2 . Furthermore, the LF and HF powers were expressed in normalised units (nu). The LF and HF powers expressed in nu represent the relative value of each power component in proportion to the total power minus the VLF component [16].

QT intervals were measured from the 24-hour ECG recordings using the semiautomated method and corrected for heart rate using Bazett's formula ($\text{QTc} = \text{QT}/\sqrt{\text{RR}}$) [17]. The QTc interval during the day and night periods was separately analysed (day period was defined as 9.00 am–9.00 pm; night period: 11.00 pm–6.00 am).

2.3. Evaluation of HRV during Deep Breathing and BRS Estimation. All measurements took place between 8:00 and 10:00 am in a quiet room, with stable temperature ($20\text{--}24^\circ\text{C}$). Both tests were performed following an adequate rest period of 20 minutes. All patients were fasted for 8 hours and studied in the supine position.

BRS estimation was carried out by the spontaneous sequence method using the BaroCor System (AtCor Medical, Sydney, Australia). The BaroCor System enables the calculation of BRS from the estimated central blood pressure changes on heart rate using a radial artery tonometer (CBM 7000; Colins Medical Instruments Corp., San Antonio, TX, USA). Central blood pressure values were estimated from radial measurements using the mathematical transfer function proposed by Chen et al. [18]. Continuous ECG and blood pressure measurements were performed simultaneously for 20 minutes. Baroreflex effectiveness index (BEI) was also assessed through BaroCor System software. BEI quantifies the number of times in which the baroreflex is effective in driving the sinus node [19].

HRV during deep breathing was evaluated in all patients using the VariaCardio TF4-System (Medical Research Limited, Leeds, UK). The result provided by the software was assessed by calculating the ratio of the maximum and minimum heart rates during six cycles of paced deep breathing and expressed as the Expiration-Inspiration ratio (E/I ratio) [5].

2.4. Statistical Analysis. Continuous variables are presented as mean \pm one-standard deviation. Normality of distributions was evaluated with the Kolmogorov-Smirnov test, and a significance level <0.05 was used to reject the null hypothesis of normal distribution. Nonnormally distributed variables were log-transformed for analysis. Comparisons between normally distributed continuous variables were performed with the calculation of the Student's *t*-test, while nonparametric variables with the Wilcoxon Mann-Whitney *U* Test. Associations between categorical variables were tested with

TABLE 1: Patients' demographic and clinical characteristics (data are expressed as mean \pm SD).

Variable	Chronic smokers	Nonsmokers	P
N	35	35	
Gender F-M	15-20	15-20	
Age (years)	55.1 \pm 9.0	56.9 \pm 8.2	0.379
Body mass index (kg/m ²)	29.8 \pm 5.1	32.6 \pm 5.2	0.026
Heart rate (beats/min)	80.4 \pm 10.8	73.1 \pm 10.1	0.006
SBP (mmHg)	125.3 \pm 14.6	128.1 \pm 15.6	0.442
DBP (mmHg)	74.2 \pm 10.3	77.1 \pm 8.5	0.205
Total cholesterol (mmol/L)	5.0 \pm 0.9	4.7 \pm 1	0.302
Triglycerides (mmol/L)	2.0 \pm 1.9	1.8 \pm 0.8	0.700
HDL-C (mmol/L)	1.1 \pm 0.3	1.2 \pm 0.3	0.366
LDL-C (mmol/L)	3.1 \pm 0.9	2.7 \pm 0.9	0.066
Fasting glucose (mmol/L)	7.4 \pm 2.2	7.3 \pm 1.5	0.788
HbA1c (mmol/mol)	55.2 \pm 11.5	51.3 \pm 10.3	0.145
Duration of diabetes (yrs)	5.3 \pm 4.5	5.3 \pm 4.3	0.936
Brinkman index (cigarettes/day \times years)	927 \pm 735	—	
Number of patients with arterial hypertension	12	17	0.225
Number of patients on ACE inhibitors	7	10	0.403
Number of patients on AT1 antagonists	7	9	ns
Number of patients on ACE + AT1	1	1	ns
Number of patients on Ca-blockers	3	5	ns
Number of patients on diuretics	7	7	ns
Number of patients on metformin	31	28	ns
Number of patients on sitagliptin	14	8	0.122
Number of patients on sulfonylureas	10	16	0.138
Number of patients on glitazones	6	10	ns
Number of patients on meglitinides	2	0	ns
Number of patients on statins	14	19	0.231

SBP: systolic blood pressure; DBP: diastolic blood pressure; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; ACE: angiotensin-converting enzyme.

Hypertension was defined as systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg or the use of antihypertensive medication.

the use of contingency tables and the calculation of the Chi-square test. Multivariate linear regression analysis was performed to assess the possible combined influence of the different variables on QTc interval duration. All statistical tests were performed by using SPSS v18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

The demographic and clinical characteristics of the study participants are summarised in Table 1. Smokers and nonsmokers were not different regarding age, diabetes duration, HbA1c, fasting glucose, total cholesterol, triglycerides HDL-cholesterol, LDL-cholesterol, and blood pressure, but smokers had lower BMI (mean \pm SD) (29.8 \pm 5.1 versus 32.6 \pm 5.2 kg/m², P = 0.026). In addition there was no difference between the two groups regarding the use of medications for treatment of T2DM, hypertension, and dyslipidaemia. As expected, smokers had significantly higher mean 24-hour heart rate (80.4 \pm 10.8 versus 73.1 \pm 10.1 beats/min, P = 0.006), due to smoking-induced adrenergic stimulation.

All values of HRV parameters are presented in detail in Tables 2 and 3. As noted, there was no difference regarding

all the time- and frequency-domain HRV measurements between the two groups. Furthermore, the BRS measurements and BEI did not differ between smokers and nonsmokers, and the E/I ratio was similar in both groups (Table 4).

Smokers showed increased mean QTc duration during the 24 hours (439.25 \pm 26.95 versus 425.05 \pm 23.03 ms, P = 0.021), as well as in both day (439.14 \pm 24.31 versus 427.17 \pm 23.99 ms, P = 0.042) and night periods (440.91 \pm 32.3 versus 425.51 \pm 24.98 ms, P = 0.033) (Figure 1, Table 4). Moreover, the association between smoking status and mean QTc interval during 24 hours and day and night periods remained significant after adjusting for BMI. Specifically, after adjusting for BMI, cigarette smoking was positively correlated with QTc during the 24 hours (β = 0.30, P = 0.015), as well as during the day (β = 0.27, P = 0.034) and night periods (β = 0.32, P = 0.015). However, there was no statistically significant correlation between mean QTc interval duration and the Brinkman index.

Furthermore, during the 24-hour period, 17/35 (48.5%) chronic smokers versus 10/35 (28.5%) nonsmokers (P = 0.086) exhibited QTc values longer than 440 ms (and, resp., 16/35 (45.7%) versus 11/35 (31%) (P = 0.22) during the day

TABLE 2: Mean values (\pm SD) of frequency-domain parameters of HRV during 24-hour, day, and night periods.

Variable	Chronic smokers	Nonsmokers	<i>P</i>
Total power 24 hours (ms^2)	2339 \pm 1864	2242 \pm 1212	0.800
Total power day (ms^2)	1984 \pm 1570	1768 \pm 894	0.497
Total power night (ms^2)	2734 \pm 2733	2800 \pm 1877	0.908
Low frequency (LF) power 24 hours (ms^2)	585 \pm 684	561 \pm 436	0.862
LF power 24 hours in normalised units (nu)	64.36 \pm 11.79	65.87 \pm 11.02	0.590
LF power day (ms^2)	482 \pm 531	428 \pm 307	0.616
LF power day (nu)	63.46 \pm 9.45	65.74 \pm 11.57	0.389
LF power night (ms^2)	768 \pm 1186	754 \pm 762	0.955
LF power night (nu)	66 \pm 14.01	66.76 \pm 11.40	0.809
High frequency (HF) power 24 hours (ms^2)	208 \pm 374	181 \pm 200	0.707
HF power 24 hours (nu)	19.39 \pm 7.79	19.28 \pm 8.37	0.956
HF power day (ms^2)	170 \pm 295	114 \pm 140	0.331
HF power day (nu)	17.89 \pm 7.74	16.27 \pm 6.78	0.374
HF power night (ms^2)	284 \pm 576	257 \pm 309	0.815
HF power night (nu)	21.26 \pm 10.17	21.1 \pm 9.17	0.946
LF/HF ratio 24 hours	4.15 \pm 2.64	4.24 \pm 2.31	0.771
LF/HF ratio day	4.6 \pm 3.03	5.01 \pm 2.78	0.566
LF/HF ratio night	4.55 \pm 3.95	3.91 \pm 2.17	0.423

Day period: 09:00–21:00; night period: 23:00–06:00.

TABLE 3: Mean values (\pm SD) of time-domain parameters of HRV during 24-hour, day, and night periods.

Variable (ms)	Chronic smokers	Nonsmokers	<i>P</i>
SDNN 24 hours	114.98 \pm 33.05	123.32 \pm 39.04	0.348
SDNN day	92.64 \pm 30.64	97.91 \pm 26.15	0.452
SDNN night	83.43 \pm 27.55	90.41 \pm 30.68	0.331
PNN30 24 hours	13.79 \pm 11.79	13.58 \pm 10.99	0.94
PNN30 day	11.70 \pm 11.45	10.06 \pm 9.3	0.523
PNN30 night	17.03 \pm 14.71	20 \pm 15.81	0.43
RMSSD 24 hours	28.41 \pm 17.28	28.51 \pm 14.18	0.979
RMSSD day	26.51 \pm 16.07	24.41 \pm 12.36	0.553
RMSSD night	30.61 \pm 22.3	34.01 \pm 18.77	0.502
SDANN 5 min 24 hours	104.34 \pm 31.65	112.5 \pm 40.04	0.358
SDANN 5 min day	82.04 \pm 30.4	86.97 \pm 27.95	0.493
SDANN 5 min night	62.19 \pm 20.94	67.69 \pm 31.97	0.407

SDNN: standard deviation of all normal-to-normal RR intervals; PNN30: number of pairs of adjacent NN intervals differing >30 ms in the entire recording divided by the total number of all NN intervals; RMSSD: the square root of the mean of the sum of the squares of differences between adjacent NN intervals; SDANN: 5 min, standard deviation of the averages of NN intervals in all 5-minute segments of the entire recording.

and 19/35 (54%) versus 11/35 (31%) ($P = 0.064$) during the night periods).

4. Discussion

The present study exhibited a positive association between mean QTc interval duration and cigarette smoking in patients with Type 2 diabetes, which was evident also separately during both the day and night periods. To the best of our knowledge this is the first study in the literature to

TABLE 4: Mean values (\pm SD) of E/I index, BRS measurements, and QTc interval duration during 24-hour, day, and night periods.

Variable	Chronic smokers	Nonsmokers	<i>P</i>
E/I index	1.29 \pm 0.15	1.23 \pm 0.1	0.085
RBRS (ms mmHg^{-1})	9.35 \pm 5.97	8.07 \pm 3.73	0.287
CBRS (ms mmHg^{-1})	8.09 \pm 4.57	7.19 \pm 4.08	0.388
SE RBRS	0.99 \pm 0.78	0.81 \pm 0.64	0.308
SE CBRS	0.92 \pm 0.87	0.73 \pm 0.68	0.313
RBEI	0.294 \pm 0.157	0.331 \pm 0.121	0.283
CBEI	0.287 \pm 0.162	0.327 \pm 0.128	0.271
QTc 24 hours (ms)	439.25 \pm 26.95	425.05 \pm 23.03	0.021
QTc day (ms)	439.14 \pm 24.31	427.17 \pm 23.99	0.042
QTc night (ms)	440.91 \pm 32.3	425.51 \pm 24.98	0.033

E/I index: expiration/inspiration index; RBRS: radial baroreflex sensitivity; CBRS: central baroreflex sensitivity; RBEI: radial baroreflex effectiveness index; CBEI: central baroreflex effectiveness index.

CBRS was assessed through the evaluation of the estimated central blood pressure changes on heart rate.

RBRS was assessed through the evaluation of the radial artery blood pressure changes on heart rate.

demonstrate such an association between cigarette smoking and QTc interval prolongation specifically in patients with T2DM.

Previous reports have provided conflicting data on the effect of smoking on QTc interval duration in healthy individuals. This may be due to numerous uncontrolled variables in the few published studies, such as the number of cigarettes smoked per day, tar and nicotine content of the cigarettes, personality factors, baseline sympathetic values, and the like [20]. Some studies showed prolongation of the QTc interval in smokers [9–11], while others did not [12].

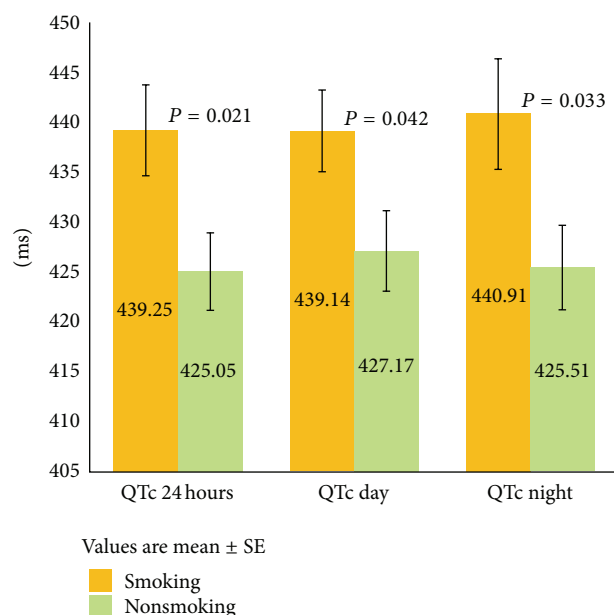


FIGURE 1: Results of mean QTc interval during the 24-hour, day, and night period in relation to smoking status.

Specifically, Ileri et al. [9] in a sample of 60 healthy volunteers (50% heavy smokers) reported that QTc was significantly longer in smokers compared to nonsmokers. This study was criticised [20] for possibly showing more acute than chronic effects of smoking on the results. Furthermore, Dilaveris et al. [10] disclosed in a sample of 1394 healthy subjects that only marginally prolonged QTc in smokers was seen, and Fauchier et al. [11] found that, among men who smoked, the number of cigarettes smoked per day was positively related to the corrected QT duration after adjustment for age. Contrary to these results, Mestre et al. [12], in a group of 37 smokers and 60 nonsmokers, found no significant differences in the duration of the QTc interval between smokers and nonsmokers.

Although there are many reports suggesting that smoking reduces HRV and attenuates BRS in normal individuals [7], there is only one study focusing on the effect of smoking on ANS activity in patients with diabetes, albeit without assessing QTc interval duration [13]. In that study, performed on 52 Japanese patients with T2DM, smokers exhibited lower BRS, lower myocardial uptake, and enhanced clearance of ^{123}I -metaiodobenzylguanidine (^{123}I -MIBG) compared to never-smokers, suggesting impaired ANS activity in the former group. HRV indices (namely, HF power and LF:HF ratio) were not different between the two groups. The distinction however of the present study was that BRS was assessed by the phenylephrine method, and HRV analysis was performed on 5 min ECG recordings. In the present study, BRS was assessed by the spontaneous sequence method, and HRV was measured by 24-hour ECG recordings, which are considered as more reliable techniques [16]. The association of cigarette smoking with QTc prolongation was found to be independent of ANS function in the current study. All cardiovascular ANS tests performed on the two groups did not reveal

any association between smoking status and ANS measures. Specifically, the results of time- and frequency-domain analyses of HRV over a 24-hour period, heart rate response to deep breathing and BRS, were not significantly different in chronic smokers compared to nonsmokers. Furthermore, despite the fact that chronic smokers exhibited a significant increase in mean 24-hour heart rate, there was no difference in baseline blood pressure between the two groups, possibly due to the antihypertensive medications used.

There is evidence that, in patients with diabetes, QTc interval prolongation could be associated with an increased risk of unexpected death, while at the same time QTc has been shown to be an accurate predictor of cardiac death in newly diagnosed patients with T2DM [3]. In the Rotterdam QT Project, studying 6,693 patients, it was shown that prolonged mean QTc duration of greater than 440 ms over 24 hours was related to a 2.3-time higher risk for sudden death compared with a QTc of 440 msec or less [21]. The MONICA/KORA Augsburg Cohort Study [22], evaluating the predictive role of prolonged QTc on mortality in 160 patients with diabetes, concluded that QTc prolongation > 440 ms is associated with a threefold increased mortality risk over 9 years but was weakly associated with cardiac autonomic neuropathy. Furthermore, prolongation of the QTc interval, even within the normal range, has been linked to increased cardiovascular risk [23]. In the present study, during the 24-hour period, there was a tendency for chronic smokers versus nonsmokers ($P = 0.086$) to exhibit QTc > 440 ms and, respectively, during the day ($P = 0.22$) and the night periods ($P = 0.064$).

There is no widely accepted single approach to the evaluation of ANS function in diabetes. Many methods have been proposed [5], a multitude of which were used in the current study. HRV assessment is considered a very valuable tool for the investigation of the sympathetic and parasympathetic functions of ANS [16]. In patients with diabetes, HRV evaluation derived from 24-hour ECG recordings has been proven to be more sensitive in detecting autonomic neuropathy than traditional autonomic reflex tests [5]. BRS assessment through the evaluation of the estimated central blood pressure changes on heart rate has been described to be more accurate in comparison to the peripheral blood pressure measurement [24]. The deep breathing test (expressed by the E/I ratio) is very easy to use and is considered as the most reproducible of the cardiac autonomic function tests [25]. A decreased heart rate variation in response to deep breathing has been suggested as a primary indicator of parasympathetic dysfunction [26]. Cigarette smoking stimulates the sympathetic nervous system mainly through the release of catecholamines by the adrenal cortex, and it has been demonstrated that in regular smokers the sympathetic nervous system is activated during the whole 24-hour period [27]. The increased sympathoadrenal activity has been shown to influence QT interval [4] and can potentially trigger malignant arrhythmias. In patients with T2DM, BRS is found to be negatively correlated with the QTc interval [28].

Strength of the present study is the fact that ANS function was assessed by a multitude of methods (HRV during 24 hours with continuous ECG recording, short-term HRV during deep breathing, and BRS) and thus could provide an

accurate assessment of it. In addition, smokers were matched one-to-one to nonsmokers in terms of age, gender, and diabetes duration and thus could eliminate potential bias due to these confounding factors.

4.1. Potential Limitations. Cigarette smoking, especially in combination with T2DM, is well known to increase the risk for accelerated atherosclerosis, which leads to coronary artery disease [1]. Endothelial damage, increased oxidative stress, exposure to chronic inflammation, impaired endogenous fibrinolysis, increased thrombosis susceptibility, and formation of advanced glycation end products (AGEs) mediated by smoking may all have an additional role to the QTc interval prolongation associated with T2DM [1]. The present study, being a retrospective cohort one, was not able to investigate this possible effect of cigarette smoking on CVD risk through QTc prolongation in diabetic patients, which should be assessed in a different study.

Furthermore, it is very difficult to deduce that there is a cause and effect connection between QTc interval prolongation and smoking from the present data. Subclinical coronary heart disease, known to prolong QTc interval [29], cannot be definitely ruled out, since coronary heart disease was excluded only on the premises of ECG findings and clinical history. It also remains controversial whether the effect of smoking on QTc interval prolongation is mostly an acute or a chronic event, since we have observed a greater mean QTc interval in smokers during the night (440.91 ± 32.3) compared to the day periods (439.14 ± 24.31) when participants were apparently more likely to smoke. Maybe this finding could be explained by the fact that QTc interval shows diurnal variation, since it has been shown that, in people with normally innervated hearts, QTc intervals are longer during sleep than during waking hours [30]. Finally, the present study could not provide any answer to the question of whether or not there is a linear dose effect of smoking on QTc interval duration (the BI was not associated with QTc duration). This finding can be explained by the fact that the number of patients studied was relatively small in order to evaluate this effect of the amount of smoking on QTc duration.

5. Conclusion

In conclusion, the present study showed that cigarette smoking was associated with prolongation of the QTc interval in patients with T2DM by a mechanism independent of ANS dysfunction, and this effect may be implicated with an increased cardiovascular risk in this population. Confirmation of the current findings in a larger prospective cohort study is definitely needed in order to validate these results and assess their generalizability in the general diabetic population.

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Clinical Study

Ketosis-Onset Diabetes and Ketosis-Prone Diabetes: Same or Not?

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Objective. To compare clinical characteristics, immunological markers, and β -cell functions of 4 subgroups (“A β ” classification system) of ketosis-onset diabetes and ketosis prone diabetes patients without known diabetes, presenting with ketosis or diabetic ketoacidosis (DKA) and admitted to our department from March 2011 to December 2011 in China, with 50 healthy persons as control group. **Results.** β -cell functional reserve was preserved in 63.52% of patients. In almost each subgroup (except A- β - subgroup of ketosis prone group), male patients were more than female ones. The age of the majority of patients in ketosis prone group was older than that of ketosis-onset group, except A- β - subgroup of ketosis prone group. The durations from the patient first time ketosis or DKA onset to admitting to the hospital have significant difference, which were much longer for the ketosis prone group except the A+ β + subgroup. BMI has no significant difference among subgroups. FPG of ketosis prone group was lower than that of A- β + subgroup and A+ β + subgroup in ketosis-onset group. A- β - subgroup and A+ β + subgroup of ketosis prone group have lower HbA1c than ketosis-onset group. **Conclusions.** Ketosis-onset diabetes and ketosis prone diabetes do not absolutely have the same clinical characteristics. Each subgroup shows different specialty.

1. Introduction

Atypical or ketosis-prone type 2 diabetes was first reported by Winter et al. in 1987 in black Americans [1] and is distinguishable from other subtypes of diabetes by clinical, immunological, and biological features. Till now, there are two kinds of phrases describe this type of disease: ketosis-prone diabetes [2–4] and ketosis-onset diabetes [5, 6]. One objective for classification of a disease is the opportunity to study its epidemiology, etiology, and pathogenesis to provide various effective interventions for its prevention and treatment. What is the connection between ketosis-onset diabetes and ketosis-prone diabetes? Do they mean the same kind of diseases? Or are they just different names that change as time passing by? Ketosis-prone diabetes (KPD) is defined as a widespread, emerging, heterogeneous syndrome characterized by patients who present with DKA or unprovoked ketosis but do not necessarily have the typical phenotype of autoimmune type 1 diabetes [7, 8]. While ketosis-onset diabetes patients present with ketosis or ketoacidosis without

known diabetes [9, 10], some investigators defined ketosis-onset diabetes as diabetes with the presence of diabetic ketosis and in the absence of glutamic acid decarboxylase (GAD) and tyrosin phosphatase (IA-2) autoantibodies. We choose patients separately fit for each of the above conditions to study. We mean to find if patients described by the two phases have the same range and feature. The aim of our test and comparison is to find a more accurate classification and denomination, which help the clinicians to make therapeutic regimen and judge the prognosis.

Maldonado et al. 2003 [2] evaluated patients with diabetic ketoacidosis for β -cell autoimmunity and human leukocyte antigen (HLA) class II alleles, with longitudinal measurements of β -cell function and biochemical and clinical parameters. They were classified into four A β groups, based on the presence of glutamic acid decarboxylase (GAD) 65, GAD 67, or IA-2 autoantibodies (A+ or A-) and β -cell functional reserve (β + or β -). The group distribution was A+ β -, A- β +, A- β -, and A+ β +. This “A β ” classification was cited in this study.

2. Method

All northeast Chinese patients with newly diagnosed diabetes presenting with an acute-onset ketosis admitted to our department between March 2011 and December 2011. There were 3 groups in our study: ketosis-onset group, ketosis-prone group, and healthy control group. Ketosis-onset diabetes was defined as follows: (1) patients were admitted to the hospital with ketosis (or DKA as the first symptom), (2) newly onset diabetes, diagnosed diabetes by clinical symptoms and the laboratory test after admission [11, 12]. Ketosis-prone diabetes was defined as follows: (1) newly occurred diabetes, having typical polydipsia, polyuria, polyphagia, and extenuation within 6 months; (2) ketosis (urine ketone body above 2+) or ketoacidosis no more than 6 months after onset. Both of the two groups should match the condition that (3) there are no obvious incentives such as infection, surgery, and trauma; (4) pregnancy diabetes, drug and pancreatic exocrinosis diseases, and endocrine diseases caused secondary diabetes were excluded [13–15]. A total of 159 consecutive, unrelated Chinese patients were investigated. The data included clinical characteristics, immunological markers, and β -cell function. During the study, recombinant human regular insulin was given by continuous intravenous injection, until the urinalysis is negative for 3 continuous days. The insulin therapy was stopped 1 h before study measurements. The target plasma glucose level during the infusion was 4.4–5.6 mmol/L. Participants received a weight-maintaining diet of 30 kcal/kg/24 h, during their hospitalization.

Data were collected on clinical characteristics (age, gender, symptoms, family history of diabetes, anthropometric features: height, weight, and so forth), biological parameters (fasting plasma glucose (FPG), urine ketone body (above 2+), total and high-density lipoprotein (HDL) cholesterol, and triglycerides were performed once at the time of the study in a fasting state), glycosylated hemoglobin (HbA1c), immunological markers, and β -cell function. HbA1c was detected by HLC-723G7 automatic HbA1c analyzers (Japan Tosoh Corporation). Glutamic acid decarboxylase (GADA) and tyrosin phosphatase antibodies (IA-2A) were detected by radioimmunoprecipitation (Euroimmun, Germany). Serum was analyzed for the presence of glutamic acid decarboxylase (GAD) 65 and IA-2 autoantibodies by highly sensitive and specific quantitative radioligand binding assay methods, as reported previously [16, 17], with recent modifications [18].

One of the autoimmune antibodies positive considered as A+, either antibody negative considered as A-. β -cell function was assessed at least 1 week after the acute episode, when patients were in a nearly normoglycaemic state. C-peptide levels were assessed at fasting state and 6 minutes after intravenous administration of 1 mg glucagons by the radioimmunological method using a commercial kit (Immunotech, France). β -cell functional reserve was considered preserved if the fasting C-peptide level was $>0.56 \mu\text{g/L}$.

According to test results of, respectively, 79 and 80 cases, patients were divided into 4 subgroups: group 1 as A+ β -, group 2 as A- β -, group 3 as A- β +, group 4 as A+ β +

Statistical analysis was performed using SPSS 11.5 statistical software. Results are expressed as mean and standard

deviation (SD), nonnormal data using nonparametric analysis. Comparison between the two groups was made using *t*-test, multiple comparison using analysis of variance between groups.

3. Result

A total of 159 patients (64 women) aged from 7 to 72 years presenting with ketosis or DKA were investigated. Among them, 6 (3.77%) reported a family history of diabetes. 45.9% patients complained of polyuria and polydipsia, with a median duration of 76 days (range: 1 day to 6 months), and 32.7% reported weight loss. BMI was $\geq 25 \text{ kg/m}^2$ in 27% of cases. GADA were detected in 31 (19.5%) and IA-2A were positive in 23 (14.5%) of patients. At least one immunological marker was detected in 33 cases (20.8%). β -cell functional reserve was preserved in 63.52% of patients.

According to immunological markers (present A+ or absent A-) and β -cell functional reserve (present β + or absent β -), patients of the two groups were divided into 4 subgroups: ketosis-onset diabetes group: 14 patients (17.72%) were A+ β -, 14 (17.72%) were A- β -, 40 (50.63%) were A- β +, and 11 (13.92%) were A+ β ++; ketosis-prone diabetes group: 20 patients (25%) were A+ β -, 10 (12.5%) were A- β -, 41 (51.25%) were A- β +, and 9 (11.25%) were A+ β +

The data of each subgroup was compared, respectively, to the subgroup of another group. In almost each subgroup (except A- β - subgroup of ketosis-prone group), male patients were more than female ones. The age of the majority of patients in ketosis-prone group was older than that of ketosis-onset group, except A- β - subgroup of ketosis-prone group. The onset of ketosis-prone diabetes group is much later than that of ketosis-onset diabetes group. All the patients received intravenous insulin treatment until urinalysis showed negative for 3 continuous days. The duration means the time from the first ketosis or ketoacidosis onset to the patient admitting to the hospital. The durations have significant difference, which were much longer for the ketosis-prone group except for the A+ β + subgroup. BMI has no significant difference among each subgroup. FPG of ketosis-prone group was lower than that of A- β + subgroup and A+ β + subgroup in ketosis-onset group. A- β - subgroup and A+ β + subgroup of ketosis-prone group have lower HbA1c than ketosis-onset group (Tables 1, 2, and 3). Triglycerides have significant difference in β + subgroups. The A+ β + subgroups in both groups are older and more like patients with Latent Adult onset autoimmune diabetes cases. Comparing to healthy control group, both ketosis-onset and ketosis-prone groups have higher FPG, HbA1c, and triglycerides (Table 4).

4. Discussion

In recent decades, there is a form of diabetes identified because of ketosis or DKA as the first symptom, which does not necessarily fit the typical characteristics of autoimmune type 1 diabetes mellitus. Because the etiology of this form of diabetes is unclear and the classic markers of autoimmune

TABLE 1: Ketosis-onset diabetes clinical feature.

Group	A+ β - (n = 14)	A- β - (n = 14)	A- β + (n = 40)	A+ β + (n = 11)
Gender (male : female)	4 : 3	11 : 3	3 : 2	8 : 3
Age (year)	23 \pm 8.43	38.5 \pm 13.43	45.73 \pm 8.15	38.64 \pm 11.24
Age range (year)	7–31	25–50	37–55	27–51
Duration (days)	29.64 \pm 22.26	39.79 \pm 35.27	60.2 \pm 58.38	57.91 \pm 51.36
BMI (kg/m ²)	20.21 \pm 2.16	21.91 \pm 3.95	24.19 \pm 2.67	22.73 \pm 2.53
FPG (mmol/L)	16.85 \pm 4.89	17.83 \pm 4.57	14.13 \pm 2.86	18.92 \pm 7.10
HbA1c (%)	13.44 \pm 2.89	12.89 \pm 2.59	11.08 \pm 2	12.45 \pm 2.92
Triglycerides (mmol/L)	2.70 \pm 2.23	2.74 \pm 2.03	2.87 \pm 1.73	2.63 \pm 1.94

TABLE 2: Ketosis prone diabetes clinical feature.

Group	A+ β - (n = 20)	A- β - (n = 10)	A- β + (n = 41)	A+ β + (n = 9)
Gender (male : female)	11 : 9	2 : 3	22 : 19	7 : 2
Age (year)	33.85 \pm 11.24	35.7 \pm 12.44	50.12 \pm 9.34	43.44 \pm 8.59
Age range (year)	22–45	23–48	40–72	35–52
Duration (days)	95.8 \pm 84.9	101.9 \pm 78.7	100.39 \pm 81.68	112.11 \pm 75.88
BMI (kg/m ²)	21.07 \pm 2.37	23.23 \pm 3.08	23.49 \pm 2.71	23.14 \pm 3.31
FPG (mmol/L)	18.57 \pm 5.92	16.57 \pm 3.65	12.26 \pm 2.96	11.76 \pm 2.6
HbA1c (%)	12.46 \pm 2.93	10.87 \pm 2.0	10.34 \pm 2.0	9.64 \pm 1.17
Triglycerides (mmol/L)	2.82 \pm 2.53	2.78 \pm 1.76	3.13 \pm 1.98	2.91 \pm 2.04

destruction of islet beta cells are also absent, the World Health Organization and the American Diabetes Association have classified this subtype as “idiopathic type 1 diabetes” or “type 1B” [19]. In present articles, there are main two phrases to name this form of disease: ketosis-onset diabetes and ketosis-prone diabetes. The result showed that ketosis-onset diabetes patients and ketosis-prone diabetes ones do not have absolutely the same clinical characters, and each subgroup showed different specialty. A- β + subgroup composes the main part of each group (both over 50 percent). There is ambiguously some kind of trend that ketosis-prone group had a later onset and milder clinical manifestations compared to ketosis-onset diabetes group. It seems that ketosis-onset group is more like type 1 diabetes while ketosis-prone group is more close to type 2 diabetes. The growing evidence, including older age at onset, higher rates of obesity, and genetic predisposition among affected individuals, suggests that ketosis-prone diabetes should be considered as a subset of type 2 diabetes or ketosis-prone type 2 diabetes [20, 21].

Although the experimental indexes in this study are not complete, but they are representatively enough to illustrate the problem. Physicians have kept trying to find a more scientific and accurate way to classify diabetes to understand mechanism and prognosis of this kind disease more better.

Ketosis-prone diabetes (KPD) is a widespread, emerging, heterogeneous syndrome characterized by patients who present with diabetic ketoacidosis or unprovoked ketosis but do not necessarily have the typical phenotype of autoimmune type 1 diabetes [22–24]. Multiple, severe forms of β -cell dysfunction appear to underlie the pathophysiology of KPD. The resulting “A β ” classification system of KPD has proven to

be highly accurate and predictive of such clinically important outcomes as glycemic control and insulin dependence, as well as being an aid to biochemical and molecular investigations into novel causes of β -cell dysfunction [25, 26]. These results demonstrate that patients with ketosis-onset diabetes are a heterogeneous group in which type 1 diabetes maybe more frequent cause. Ketosis-prone diabetes with remission is a well-known subtype of type 2 diabetes rather than type 1 [27, 28]. The acute presentation at diagnosis or sometimes later is explained by a functional and partially reversible β -cell deficiency [29]. There were no significant group differences in the BMI, which illustrated that the patients’ posture of the two groups showed nearly the same feature. The phrase “ketosis-onset diabetes” is used more often in Japanese and Chinese articles, while “ketosis-prone diabetes” is used more frequently in other countries’ articles [30]. The difference may be caused by racial differences. Recently, the term “ketosis-prone type 2 diabetes” has entered the literature. In general, this term refers to the A- β + KPD subgroup or, in some instances, is even further restricted to those A- β + patients who present with “unprovoked” DKA or ketosis and new onset diabetes [31].

A- β + patients comprise the largest subgroup of KPD patients and are the ones who most commonly come to the notice of physicians because they present with DKA and yet have all the clinical features and subsequent behavior of type 2 diabetes; hence, ketosis-prone type 2 diabetes is certainly a fitting description for them. A+ β - KPD is synonymous with classic, early onset autoimmune type 1 diabetes; A+ β + KPD may overlap with LADA. However, there are differences between LADA, as recently defined by the Immunology of

TABLE 3: Comparison between ketosis-onset and ketosis prone groups (*P* value).

Group	A+ β -	A- β -	A- β +	A+ β +
Gender (male : female)	NS	NS	<i>P</i> < 0.05	NS
Age	<i>P</i> < 0.05	NS	<i>P</i> < 0.05	NS
Duration	<i>P</i> < 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05	NS
BMI	NS	NS	NS	NS
FPG	NS	NS	<i>P</i> < 0.05	<i>P</i> < 0.05
HbA1c	NS	<i>P</i> < 0.05	NS	<i>P</i> < 0.05
Triglycerides	NS	NS	<i>P</i> < 0.05	<i>P</i> < 0.05

P < 0.05 means significant differences; NS: not significant.

TABLE 4: Healthy group compared with ketosis-onset and ketosis prone groups.

Group	Healthy group (<i>n</i> = 50)	Ketosis-onset group (<i>n</i> = 79)	Ketosis prone group (<i>n</i> = 80)
Gender (male : female)	13 : 12	51 : 28	11 : 9
Age (year)	38.37 \pm 25.74	27.35 \pm 20.82	44.85 \pm 23.12
Age range (year)	12–65	7–55	22–72
BMI (kg/m ²)	22.07 \pm 5.23	22.18 \pm 8.95	23.78 \pm 9.27
FPG (mmol/L)	6.57 \pm 1.12	16.75 \pm 4.65	14.57 \pm 3.23
HbA1c (%)	6.46 \pm 0.93	11.45 \pm 1.58	10.94 \pm 2.08
Triglycerides (mmol/L)	1.24 \pm 0.57	2.72 \pm 2.63	2.72 \pm 1.56

Diabetes Society, and A+ β + KPD patients; most importantly, the definition of LADA excludes patients who require insulin within the first 6 months after diagnosis, whereas the majority (90%) of A+ β + KPD patients present with DKA as the first manifestation of diabetes and therefore require insulin at the start [32, 33].

Extensive HLA typing has found that the frequencies of major class II alleles associated with susceptibility to autoimmune type 1 diabetes are not significantly higher in A- β - KPD patients than in ethnic matched population controls, whereas they are significantly higher in A+ β - KPD patients [34–36].

In summary, the classification of diabetes using insulin secretion evaluation and immunological markers, which seems to be the most effective scheme, allowed us to distinguish 4 subgroups of patients. In more than half of the cases (autoimmune type 1 and ketosis-prone type 2 diabetes), patients may be diagnosed by classical characteristics [37]. However, the other subgroups may require further investigations, such as repeat testing of β -cell function or genetic studies.

We suggest a coincident name to address this kind of diabetes which means they may have the same clinical characteristics, biochemical parameters, prognosis, mechanism, and so forth. Maybe we can even classify diabetes into ketosis-onset diabetes and non-ketosis-onset diabetes; regardless of the causes of diabetes, the onset displays ketosis or DKA or neither of them. If we keep on exploring the mechanism, perhaps we will find that there are only two prognoses of this form of diabetes: insulin-dependent and non-insulin-dependent diabetes which may be related to the initial onset.

Someone suggest that the four groups can be considered as type 1 DM, idiopathic type 1 DM, latent autoimmune diabetes in adults (LADA), and type 2 DM, respectively, according to their clinical characteristics, biochemical parameters, and therapeutic consequences. We consider that maybe we will find that only few cases are idiopathic, without motivation, as the study is undertaken more deeply. Most of these kinds of diabetes can be classified in to typical groups such as type 1 diabetes. We propose that the phrase “ketosis-onset diabetes” to may be used to describe the patients onset with unprovoked ketosis or DKA, and the phrase “ketosis-prone diabetes” to call those with causes. In this way, the readers could understand it better, whether the symptoms have reasons or it is a new case. Our research on race and the number of cases may have certain limitations. We will do further study in the future to search for the ignored causes of ketosis or DKA and the nature and mechanism of this of form diabetes.

Conflict of Interests

No conflict of interests was declared.

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Research Article

Subetta Treatment Increases Adiponectin Secretion by Mature Human Adipocytes *In Vitro*

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Purpose. To investigate the mechanism of action in peripheral tissues of novel complex drug containing release-active dilutions of antibodies to the beta subunit of the insulin receptor and antibodies to endothelial nitric oxide synthase (Subetta), which has shown efficacy in animal models of diabetes. **Methods.** Human mature adipocytes were incubated either with Subetta, with one of negative controls (placebo or vehicle), with one of nonspecific controls (release-active dilutions of antibodies to cannabinoid receptor type I or release-active dilutions of rabbit nonimmune serum), or with dimethyl sulfoxide (DMSO) at 37°C in a humidified incubator at 5% CO₂ for three days. Rosiglitazone was used as reference drug. Secretion of adiponectin was measured by quantitative enzyme-linked immunosorbent assay (ELISA). **Results.** Only Subetta significantly stimulates adiponectin production by mature human adipocytes. Nonspecific controls did not significantly affect adiponectin secretion, resulting in adiponectin levels comparable to background values of the negative controls and DMSO. **Conclusion.** Increasing adiponectin production in absence of insulin by Subetta probably via modulating effect on the beta subunit of the insulin receptor might serve as one of the mechanisms of the antidiabetic effect of this drug. These *in vitro* results give first insight on possible mechanism of action of Subetta and serve as a background for further studies.

1. Introduction

According to World Health Organization (WHO) (2012) more than 347 million people worldwide suffer from diabetes mellitus (DM), of which 90% have type 2 diabetes (T2D) [1]. Both the prevalence and morbidity, especially of T2D, continue to grow globally, especially in developing countries. This growth has led to strains on healthcare systems worldwide as T2D remains one of the leading causes of cardiovascular disorders, blindness, renal failure, amputations, and hospitalizations [2].

Adiponectin is a circulating protein produced by adipose tissue that is a versatile regulator of energy homeostasis, insulin sensitisation, inflammation/atherosclerotic processes, and anti-ischemic cardioprotection [3]. It is well established that adiponectin plays an important role in T2D, hypertension, multiple sclerosis, and dyslipidaemias [4]. Particularly, there is a strong association between hypoadiponectinemia and T2D [3]: both adipose adiponectin mRNA expression

and circulating adiponectin levels are significantly reduced in most rodent models of T2D, as well as T2D patients; the degree of glycosylated adiponectin and “high-molecular weight/total adiponectin ratio,” which correlates with insulin sensitivity, were significantly decreased in T2D patients compared to healthy controls; high adiponectin levels are associated with reduced risk of developing diabetes.

The most significant role adiponectin may play is that of sensitizing the liver and muscles to the action of insulin in both humans and rodents [5]. Adiponectin appears to increase insulin sensitivity by improving glucose and lipid metabolism. Adiponectin has an ameliorating function on glucose metabolism apart from insulin signalling [6]. In animal models of obesity and diabetes, adiponectin affected both skeletal muscle and liver, promoting fatty acid oxidation in muscle and inhibiting glucose production from the liver, thereby leading to decreases in circulating free fatty acids (FFAs), triglyceride, and glucose levels [7, 8]. FFAs as well as tumor necrosis factor alpha (TNF α) are factors released

from adipose tissue that contribute to insulin resistance. In addition, lipid accumulation in beta-cells might lead to reductions in insulin secretion [7]. In individuals with visceral obesity, there is a decrease in the insulin-mediated suppression of lipolysis, leading to increases in circulating FFAs concentrations that contribute to both peripheral and hepatic insulin resistance either by impairing insulin signalling pathways, by competitive inhibition of muscle glucose uptake, by stimulation of endogenous glucose production, thus contributing to hepatic insulin resistance, or by combination of mentioned mechanisms [6, 7, 9, 10].

Also it was shown that adiponectin regulates the expression of several pro- and anti-inflammatory cytokines. Its main anti-inflammatory function might be related to its capacity to suppress the synthesis of TNF α and interferon gamma (IFN γ) and to induce the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1RA) [11]. TNF α is a key modulator of adipocyte metabolism, with a direct role in several insulin-mediated processes, including glucose homeostasis and lipid metabolism [6, 7, 12]. The net effect of TNF α is to decrease lipogenesis (FFA uptake and triglyceride synthesis) and to increase lipolysis. TNF α is a major contributor to the development of adipose tissue insulin resistance [7]. Finally, there is substantial evidence of adiponectin effects on beta-cell function and survival, which are well known as key factors in the development of T2D along with insulin resistance [12]. Taking into consideration the above mentioned it could be concluded that adiponectin-targeted pharmaceutical strategies increasing circulating adiponectin levels may be therapeutic against T2D.

Subetta is drug containing release-active dilutions of antibodies to the beta subunit of the insulin receptor and antibodies to endothelial nitric oxide (NO) synthase. This novel class of drug was demonstrated to have a fundamentally new proantigen (cotargeted with antigen) targeted activity [13]. For the first time in the study of the repeated dilution process, it was found that the resulting ultra-dilution generates a novel activity absent in the starting material, called a release-activity. A distinctive feature of the release-active antibodies is their ability not to suppress, and to modify the activity of the antigen against which they were raised.

In two DM animal models, streptozotocin-induced DM [14] and spontaneous T2D in Goto Kakizaki rats [15], Subetta showed antidiabetic effects similar to that of rosiglitazone. Additionally, Subetta has shown promising results in its first clinical use in both type 1 diabetes (T1D) patients with poor glycemic control receiving intensive insulinotherapy and T2D patients receiving metformin/combination of basal insulin with metformin (unpublished data). Subetta treatment led to a significant decrease in fasting plasma glucose and glycated haemoglobin (HbA_{1C}) levels, exerted a normalizing effect on the daily glycemic levels (self-control of blood glucose and daily glucose monitoring), significantly lowered insulin resistance and plasma insulin levels, as well as lowering total cholesterol and low-density lipoproteins.

The aim of this work is to investigate the ability of Subetta to affect adiponectin secretion by mature human adipocytes.

2. Materials and Methods

Human preadipocytes (lot SL0047) were provided by Zen-Bio, Inc. (USA). Human preadipocytes were mixture of subcutaneous depots (abdomen, thigh, hip, flank, and breast) obtained from 5 healthy donors (sex: female; age: 47.0 ± 5.4) undergoing elective surgery that have signed informed consent, under existing institutional review board (IRB). The cells were pooled and grown together, then harvested and cryopreserved as the superlot (multidonor) that was used in the current study. Prior to use the cells undergo quality control in a number of functional assays (differentiation (measured by total triglyceride), lipolysis, insulin-induced glucose uptake) and also the results of the quality control have shown that there were not any differences between the depots. Preadipocyte medium (catalog number PM-1), adipocyte differentiation medium (catalog number DM-2), and adipocyte maintenance medium (catalog number AM-1) were provided by Zen-Bio, Inc. (USA). Enzyme-linked immunosorbent assay (ELISA) kit (catalog number ADIP-1) was the product of Zen-Bio, Inc. (USA). Rosiglitazone (catalog number R2408) was purchased from Sigma Aldrich (USA). Subetta, release-active dilutions (RAD) of antibodies (Abs) to the beta subunit of insulin receptor and Abs to endothelial NO synthase, RAD of Abs to cannabinoid receptor type I (R-CBI), RAD of rabbit nonimmune serum (RbS), RAD of purified water (placebo), and purified water were manufactured and supplied by OOO "NPF "MATERIA MEDICA HOLDING" (Russia). All of OOO "NPF "MATERIA MEDICA HOLDING" compounds, except for purified water, were manufactured using the method described previously [13] using routine methods described in the European Pharmacopoeia (6th Edition, 2007). All ultrahigh dilutions were prepared in glass vials. Starting substances were mixed with a solvent (ethanol-water solution) and shaken for 1 min to produce C1 dilution. All subsequent dilutions consisted of one part of the previous dilution to 99 parts of solvent (ethanol-water solution for intermediate dilutions and distilled water for preparation of the final dilution), with succession between each dilution. Solutions were prepared in sterile conditions, avoiding direct intense light, and were stored at room temperature. Rabbit polyclonal antibodies to the beta subunit of insulin receptor and to endothelial NO synthase were used as starting substances for Subetta, rabbit polyclonal antibodies to cannabinoid receptor type I—for RAD of Abs to R-CBI, rabbit nonimmune serum—for RAD of RbS. In case of placebo, purified water was used to prepare ultrahigh dilutions instead of starting substance.

The study was carried out by contract research organization Zen-Bio, Inc. (USA) in cooperation with OOO "NPF "MATERIA MEDICA HOLDING" (Russia). Human preadipocytes were plated at 40625 cells/cm^2 in $150 \mu\text{L}$ of preadipocyte medium. The cells were allowed to attach overnight in a 37°C humidified incubator at $5\% \text{ CO}_2$. The following day, the plating medium was removed and replaced with $150 \mu\text{L}$ of adipocyte differentiation medium and the cells allowed to incubate in a 37°C humidified incubator at $5\% \text{ CO}_2$ for 1 week. After 1 week, $90 \mu\text{L}$ of the differentiation medium was removed from each well and $120 \mu\text{L}$ of adipocyte maintenance

TABLE 1: Effect of Subetta and rosiglitazone on human adipocyte adiponectin secretion.

Sample	Adiponectin concentration (ng/mL)	<i>P</i> values (Subetta versus rest of the samples)	<i>P</i> values (Rosiglitazone versus rest of the samples)	Number of replicas
Subetta	27.97 ± 7.54	—	<i>P</i> = 0.028657	6
RAD of Abs to R-CBI	4.66 ± 1.40	<i>P</i> = 0.000134	<i>P</i> = 0.878582	5
RAD of RbS	5.18 ± 1.42	<i>P</i> = 0.000123	<i>P</i> = 0.433495	6
Placebo	5.52 ± 0.69	<i>P</i> = 0.000123	<i>P</i> = 0.491881	6
Purified water	8.73 ± 3.17	<i>P</i> = 0.000422	<i>P</i> = 0.972188	6
DMSO (0.10%)	4.44 ± 1.27	<i>P</i> = 0.000126	<i>P</i> = 0.452867	4
Rosiglitazone (1 μM)	14.22 ± 2.94	<i>P</i> = 0.028657	—	4

One-way analysis of variance (ANOVA) followed by Tukey HSD test was used for statistical analysis. ANOVA shows the following results: $F_{11/51} = 16.4840$, $P = 0.00000$, observed power = 1.0.

medium was added. The cells were then allowed to incubate in a 37°C humidified incubator at 5% CO₂ for 7 more days. After this additional week, mature adipocytes were ready for assay.

Prior to the assay, the cells were incubated in the absence of serum and hormones for three days. After starvation, test compounds (75 μL) were added and the cells were incubated at 37°C in a humidified incubator at 5% CO₂ for three days. Conditioned media was collected after 72 hours and either frozen for later evaluation or measured immediately. For the ELISA, 20 μL of the conditioned medium was added to 80 μL of pretreatment solution and heated to 100°C for 5 minutes. Once the samples were cooled to room temperature, 50 μL of each sample was added to 200 μL diluent buffer to generate a 5-fold dilution. Samples were mixed and 100 μL was transferred to the coated wells of the ELISA plate. Samples were incubated at room temperature for 1 hour after which the plate was washed and secondary antibody applied and incubated at room temperature for 1 hour. The plate was washed again and detection antibody was added and allowed to incubate for 1 hour at room temperature. Detection reagents were added and absorbance at 450 nm (SpectraMax 250: Molecular Devices) was determined.

The data are presented as mean value per group (M) ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey HSD test was used for statistical analysis and *P* values less than 0.05 were regarded as significant (STATISTICA 6.1 software).

3. Results and Discussion

Incubation of mature human adipocytes with Subetta for 72 hours resulted in a statistically significant increase in adiponectin concentration in the culture medium (Table 1). Nonspecific controls, RAD of Abs to R-CBI and RAD of RbS, did not significantly affect adiponectin secretion, resulting in adiponectin levels comparable to background values of the negative controls, placebo, purified water, and 0.1% dimethyl sulfoxide (DMSO). The reference drug rosiglitazone stimulated adiponectin secretion but to a lower level compared to that of Subetta and its effect was not significant in comparison with DMSO value.

Previously, Subetta demonstrated the ability to reduce hyperglycemia and improve glucose tolerance in various experimental models of diabetes mellitus and its effects were similar to that of rosiglitazone [14, 15]. Subetta was given by oral gavage, when it was studied *in vivo* but in the current study the direct action of Subetta on mature human adipocytes was estimated *in vitro*. Based on the activity demonstrated for the whole class of novel drugs [13] and on data from previous studies, we may conclude that the complex drug mainly exerts a modulating effect on the beta subunit of the insulin receptor regulating the insulin receptor's kinase activity and consequently activating receptor-associated signal pathways [16]. The ability of direct activation of insulin receptor and activate receptor-associated signal pathways in the absence of insulin was shown for L7 (Merck) [17, 18].

According to Shehzad et al. [4], insulin directly stimulates adiponectin gene expression. Specifically, insulin enhances adiponectin regulation and secretion selectively in adipocytes. While the exact mechanism of action of insulin on adiponectin biosynthesis remains unclear, there are several possible pathways to account for its activity. It has been suggested that insulin binding to its receptor activates a cell cascade inhibiting the activity of FoxO1, a suppressor of peroxisome proliferator-activated receptor gamma (PPAR gamma), which in turn results in the induction of adiponectin biosynthesis [4]. It is important that adiponectin has an ameliorating function on glucose metabolism apart from insulin signalling [6].

Taking into consideration the above mentioned, it could be assumed that Subetta via direct effect on beta subunit of the insulin receptor of mature human adipocytes activates insulin receptor in the absence of insulin in culture medium, which in its turn exerts activating receptor-associated signal pathways. As a result activity of FoxO1 is inhibited and thus adiponectin biosynthesis is induced. To confirm this suggestion, further studies (both *in vitro* and *in vivo*) should be conducted in order to provide a detailed investigation of the mechanism of Subetta influence on the production of adiponectin and also to assess the involvement of this process and its role in the mechanism of drug action.

4. Conclusions

In summary, the novel complex drug Subetta, containing RAD of antibodies to the beta subunit of insulin receptor and antibodies to endothelial NO synthase, stimulates adiponectin production by mature human adipocytes. This effect on adiponectin production is specific; neither RAD of Abs to R-CBI nor RAD of RbS affected adiponectin secretion by mature human adipocytes. Therefore, the ability of Subetta to exert its modulating effect on the insulin receptor even in absence of insulin might be the basis for stimulating adiponectin secretion and might serve as one of the mechanisms of the antidiabetic effect of this drug. The results of the current *in vitro* study give first insight on possible mechanism of action of Subetta and serve as a background for further studies.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Glucose Lowering Therapeutic Strategies for Type 2 Diabetic Patients with Chronic Kidney Disease in Primary Care Setting in France: A Cross-Sectional Study

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Aim. To understand glucose lowering therapeutic strategies of French general practitioners (GPs) in the management of type 2 diabetes mellitus (T2DM) patients with chronic kidney disease (CKD). **Methods.** A multicenter cross-sectional study was conducted from March to June 2011 among a sample of French GPs who contribute to the IMS Lifelink Disease Analyzer database. Eligible patients were those with T2DM and moderate-to-severe CKD who visited their GPs at least once during the study period. Data were collected through electronic medical records and an additional questionnaire. **Results.** 116 GPs included 297 patients: 86 with stage 3a (Group 1, GFR = 45–60 mL/min/1.73 m²) and 211 with stages 3b, 4, or 5 (Group 2, GFR < 45 mL/min/1.73 m²). Patients' mean age was approximately 75 years. Insulin was used in 19% of patients, and was predominant in those with severe CKD. More than two-thirds of patients were treated with glucose lowering agents which were either contraindicated or not recommended for CKD. **Conclusion** Physicians most commonly considered the severity of diabetes and not CKD in their therapeutic decision making, exposing patients to potential iatrogenic risks. The recent patient oriented approach and individualization of glycemic objectives according to patient profile rather than standard HbA1c would improve this situation.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a major health problem with a steady increase of prevalence worldwide. Data from the 2011 National Diabetes Statistics (National Institutes of Health) report that 8.3% of the US population had diabetes [1]. In France, the prevalence of pharmacologically treated diabetes increased from 4.39% in 2009 to 4.64% in 2011 according to the data collected by the French National Sickness Fund [2, 3]. Around one-third of patients with T2DM manifest some form of clinical kidney damage and

patients have a progressively increasing risk of developing chronic kidney disease (CKD) [4, 5]. Conversely, for 45% of patients who receive dialysis, diabetes is the primary cause of kidney failure [6–8]. In France, diabetes along with hypertension constitute the primary cause of kidney failure [9].

CKD is a common disease with an increasing prevalence, partially as a consequence of increasing prevalence of diabetes. According to the American National Kidney Foundation (NKF), renal function is chronically altered when anomalies in markers of renal disease (clinical proteinuria,

haematuria, leukocyturia, morphological and histological anomalies, or markers of tubule dysfunction) persist for more than three months or glomerular filtration rate (GFR) is less than 60 mL/min/1.73 m² for more than three months [10]. The disease is progressive and the definition of its staging differs in the French (HAS) and the American (Kidney/Disease Outcomes Quality Initiative, NKF 2007) guidelines [10, 11]. The French Nephrology Society (FNS) has tried to harmonize the different recommendations by proposing the classification in 6 stages, grouped by kidney function as described by the GFR. Patients with stage 1 or 2 CKD (GFR > 90 mL/min/1.73 m² and GFR = 60–90 mL/min/1.73 m², resp.) have normal or mildly reduced kidney function, those with stages 3a and 3b (GFR = 45–60 mL/min/1.73 m² and GFR = 30–45 mL/min/1.73 m²) have moderately reduced kidney function, and those with stage 4 or 5 (GFR = 15–30 mL/min/1.73 m² and GFR ≤ 15 mL/min/1.73 m²), respectively, have severe or end-stage renal disease.

Moderate-to-severe CKD (GFR < 60 mL/min/1.73 m²) is observed in 20%–30% of patients with T2DM [12, 13]. The presence of both diabetes and CKD obliges clinicians to take into account several clinical factors in the management of these diseases in order to (1) achieve glycaemic control, (2) avoid progression and/or complications of renal disease, and (3) control the risk of cardiovascular events and premature mortality which is extremely high in this category of patients [14].

The epidemiology of CKD among diabetic patients in France is relatively well known. For example, data from the 2007 ENTRED cohort [15] showed that two-thirds of T2DM patients had low GFR values: 14% with CKD stage 3a and 8% with CKD stages 3b, 4, or 5. Among this latter group, the proportion of people aged over 75 years and of women is significantly higher than that in the populations of T2DM patients with lower stages of CKD.

The management of diabetic patients with CKD in primary care is less well known. Understanding of therapeutic strategies used by general practitioners for these patients is important and allows shaping of appropriate educational messages towards physicians for a better management of T2DM patients with CKD.

The aim of BEMEDIR (medical need, diabetes and renal failure (BEsoin MEDical, Diabète et Insuffisance Rénale)) study was to describe and analyze how T2DM patients with moderate-to-severe CKD are managed by GPs in France.

2. Materials and Methods

We conducted a multicenter cross-sectional study in primary care setting in France. GPs contributing to the panel of IMS Lifelink EMR Disease Analyzer (DA) were invited to participate in the study. DA is a database of longitudinal electronic medical records (EMRs) of about 5 million patients collected from a panel of about 1200 physicians since 2000. Its validity and representativeness have been analyzed and published previously [16]. The study population consisted of patients diagnosed with T2DM and moderate-to-severe CKD whose

EMR data were available in DA and whose GPs accepted to complete an additional questionnaire on their diabetes care. In our study, each participating physician was asked to include up to four T2DM patients with CKD, two with CKD at stage 3a (Group 1, with GFR = 45–60 mL/min/1.73 m²), and two with CKD at stage 3b, 4, or 5 (Group 2, with GFR < 45 mL/min/1.73 m²). In addition to the information available through the patients' EMRs, an additional questionnaire was completed by physicians for each patient to provide more details on the management of diabetes and renal disease, their satisfaction with glycaemic control of the patients, the reasons for their treatment choices, and so forth. This additional questionnaire was then linked to the EMRs using GPs' unique national number, patient's date of birth and sex, and the date of the visit. An independent scientific committee validated the study protocol and questionnaire.

Once the data collection was complete, the database was locked for analysis. The GFR of each patient was calculated post hoc using MDRD formula to validate the classification of patients into Group 1 and 2 provided by the GPs.

3. Results

3.1. Patients Characteristics. A total of 116 GPs participated in the study (participation rate 10%). They included a total of 375 patients from 1 March 2011 to 15 June, 2011. Among these, 45 patients were excluded from the study because their EMR did not contain sufficient data to allow patient category control with GFR calculation. Of the remaining 330 patients, GPs had included 167 in Group 1, 152 in Group 2, and 11 without mentioning the group. After post hoc control of GFR value for each patient, 86 patients (29%) were included in Group 1 and 211 (71%) in Group 2. Finally, 33 patients were excluded from analysis as their GFR was more than 60 mL/min/1.73 m² and therefore failed to meet the inclusion criteria. The remaining 297 patients were used for further analysis.

Characteristics of each group of patients are shown in Table 1. Patients were generally of older age with a mean age of approximately 75 years, are overweight with a mean BMI of 29 kg/m², and had a relatively long (mean 12–14 years) history of diabetes. The mean HbA1c was approximately 7.1% in both groups. Group 1 patients were predominantly males, while Group 2 patients were predominantly females.

3.2. Glucose Lowering Treatments. Analysis of the type of glucose lowering therapy was performed in 195 of the 297 included patients who had available data on drug prescriptions. As shown in Figure 1(a), a large proportion of diabetic patients with CKD were treated with oral glucose lowering agents. In patients with moderate CKD (Group 1), 81% were treated with oral therapy, while 7% received insulin alone and 12% received insulin in association with oral therapy. Among patients with more severe kidney dysfunction (Group 2), a higher number were treated with insulin (24% alone and 11% in combination with oral agents). With regard to the choice of glucose lowering drugs (Figure 1(b)), 47% were treated with sulfonylurea and 58% were treated with metformin in Group 1, while these proportions were 30% and 39%,

TABLE 1: Patient demographics and disease characteristics.

Characteristics	Group 1	Group 2
Age in years	N = 86	N = 211
Mean (SD)	76.2 (± 11.3)	75.8 (± 10.5)
Gender ratio (M/F)	N = 86 1.9	N = 211 0.7
BMI (kg/m ²)	N = 86	N = 207
Mean (SD)	28.2 (± 4.8)	29.3 (± 5.5)
BMI (kg/m ²): N patients (%) with	N = 86	N = 211
BMI < 25	25 (29%)	44 (21%)
25 \leq BMI < 30	35 (41%)	81 (39%)
BMI \geq 30	26 (30%)	82 (40%)
GFR (mL/min/1.73 m ²)	N = 86	N = 211
Mean (SD)	51.9 (± 4.3)	29.41 (± 13.0)
Time since CKD diagnosis: N patients (%)		
≥ 1 year	N = 85 66 (77%),	N = 207 175 (83%)
≤ 1 year	N = 84 20 (23%)	N = 203 36 (17%)
Proteinuria: N patients (%) with		
Microproteinuria	39 (46%)	124 (60%)
Macroproteinuria	7 (8%)	38 (19%)
Time since diabetes diagnosis (years)	N = 86	N = 2011
Mean (SD)	12.6 (± 8.4)	14.2 (± 9.0)
HbA1c	N = 76	N = 197
Percentage (SD)	7.2 (± 1.1)	7.1 (± 1.3)
HbA1c < 7%	N = 76	N = 197
N patients (%)	36 (47%)	106 (54%)

N: number of patients, BMI: body mass index; CKD: chronic kidney disease; GFR: glomerular filtration rate; SD: standard deviation.

respectively, in Group 2. Correspondingly, a minority of patients were prescribed glitazones, alpha glucosidase inhibitors, or glinides (2, to 12% in Group 1 and 5, to 24% in Group 2).

3.3. Factors Taken into Account by GPs in the Choice of Glucose Lowering Strategy. GPs were asked to select the top five factors influencing their choices when determining a glucose lowering treatment strategy in their CKD patients. Factors the GPs have taken into consideration are reported in Figure 2. Severity of diabetes was the most frequently mentioned factor (78%), while only one GP out of two mentioned CKD (51%) among the first five factors considered. It is worth noting that only one GP out of three (30%) mentioned the risk of hypoglycemic episodes, even though this category of patients is at high risk of this event.

3.4. Adequacy of Glucose Lowering Therapy for CKD and Glycaemic Control. GPs were asked whether they were satisfied

with glycaemic control achieved for each patient. For 73% of patients, GPs declared being satisfied with the level of glycaemic control achieved. Mean HbA1c level was lower among patients whose GPs were satisfied with their glycaemic control (Table 2). The mean HbA1c level for patients with satisfactory glycaemic control (as declared by the GPs) was 6.7% (6.9% and 6.7% in Group 1 and 2, resp.) and ranged from 6.6% for patients receiving oral monotherapy to 7.4% for patients receiving insulin. Mean HbA1c in patients for whom GPs were not satisfied was approximately 8.2% in the two groups.

A 63% of patients were treated with glucose lowering treatments which were either contraindicated or not recommended for CKD patients based on the current French guidelines and summaries of product characteristics of prescribed drugs. A 45% of patients had satisfactory glycemic control, while 18% did not have satisfactory glycemic control based on the opinion of the GP. Only 37% of patients were treated with safe glucose lowering drugs for CKD. A 25% of them had satisfactory glycemic control based on the GPs opinion (Table 3).

3.5. Changes in Glucose Lowering Therapy Choice. In 57% of patients, therapeutic strategies had not been changed by GPs during the previous year, with no difference between the two groups (Table 4). The achievement of successful glycemic control was considered the main reason. Other reasons included, insufficient follow-up time to check for the effects of lifestyle and dietary measures (18.4%), waiting for specialist advice (13.5%), risk of hypoglycemia (6.7%), of side effects (6.1%) or of CKD worsening (6.7%), and taking into account patient insulin injection acceptance (7.4%). However, when GPs did introduce changes in glucose lowering therapies, they did so in relation to the presence of CKD in 55% of patients in Group 1 and 62% in Group 2. The most frequent change concerned the replacement of the oral glucose lowering drug with another (28% in Group 1 and 19% in Group 2). Other common changes were withdrawal of a drug (14% and 22%, resp.), dose reduction (21% and 17%, resp.), and a switch to insulin (21% and 22%, resp.).

When GPs were asked to provide their opinion on the best therapeutic strategy they would recommend in order to meet glycaemic control objectives in T2DM patients with CKD, most of them favored adopting a strict compliance with lifestyle and dietary measures (protein restriction, smoking cessation, physical activity, etc.) as the most fundamental aspects which should be taken into consideration. One GP out of three mentioned the need for drugs that could be used without restriction among patients with CKD (Table 5).

4. Discussion

GPs participating in this study had difficulty in appropriately identifying and classifying patients with CKD. This could lead to suboptimal therapeutic strategy, that is, lack of consideration of the severity of renal impairment in glucose lowering treatment strategy.

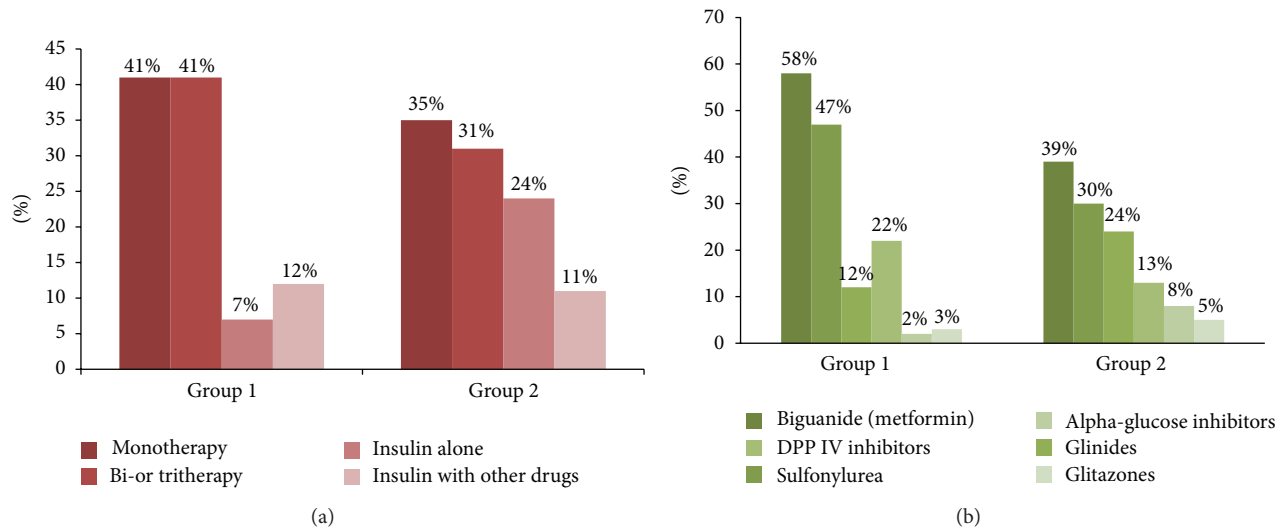


FIGURE 1: Glucose lowering therapy strategies adopted in the 195 patients with available prescription data. A large proportion of patients were treated with oral mono, dual, or triple therapies while the percentage of patients treated with insulin alone or in association with other drugs was lower and was higher in patients with severe CKD compared to moderate ones.

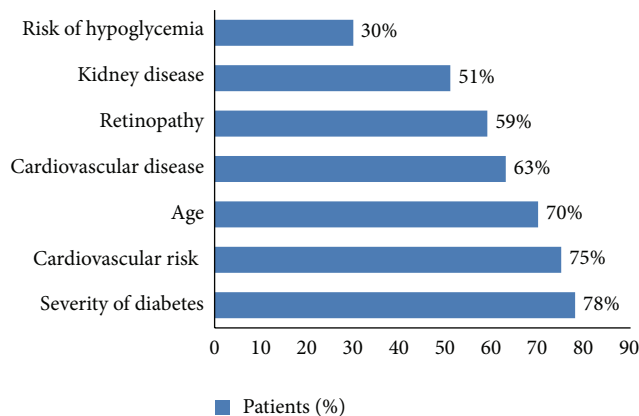


FIGURE 2: Factors considered by GPs when selecting glucose lowering therapy in CKD patients. Among the top five factors that GPs took into account, severity of diabetes was the most frequently mentioned while only one GP out of three mentioned the risk of hypoglycaemic episodes.

However, GPs' therapeutic management of T2DM patients with CKD was guided by the general glycaemic control achievement (HbA1c threshold), and they often ignored the severity of renal dysfunction (i.e., in selecting more appropriate treatments and HbA1c targets). GPs' satisfaction with patient glycaemic objectives, as well as their therapeutic strategy, was closely associated with HbA1c value of ~7%. Only half of the GPs mentioned the presence of CKD as one of the five most important factors influencing their strategy, and even fewer mentioned the risk of hypoglycemic episodes which is particularly important in patients with renal impairment.

For over half of patients, GPs did not change their glucose lowering therapy during the last year, thus potentially

TABLE 2: Association of HbA1c values with GP satisfaction of glycaemic control and with treatment types among all included patients ($N = 268$).

	Mean HbA1c % (SE)*
GP satisfied with glycaemic control	6.7 (0.9)
Treatment prescribed	
Monotherapy	6.6 (0.6)
Bitherapy	6.9 (0.8)
Tritherapy	7.0 (1.4)
Insulin + oral anti-diabetic	7.2 (1.2)
Insulin	7.4 (2.7)
Group	
Group 1 ($N = 75$)	6.9 (0.9)
Group 2 ($N = 193$)	6.7 (0.9)
GP not satisfied with glycaemic control	8.2 (1.4)
Group	
Group 1 ($N = 75$)	8.3 (1.3)
Group 2 ($N = 193$)	8.2 (1.5)

* Based on last measure of HbA1c. SE: standard error.

TABLE 3: GP's satisfaction with glycaemic control and its association with indication or recommendation of glucose lowering therapy in CKD patients.

	Treatment with contraindicated or not recommended drugs		
GP satisfied with glycaemic control	Yes (%)	No (%)	All (%)
Yes (%)	81 (45)	44 (24)	125 (69)
No (%)	33 (18)	22 (12)	55 (30)
All (%)	114 (63)	66 (36)	180 (100)

exposing CKD patients to risk of adverse drug reactions. However, when such change was conducted, they declared that renal disease was the main motivation for it.

TABLE 4: Change of treatment over the past year by stage of CKD among all included patients.

Change of treatment over the past year by stage of CKD	Group 1 (%)	Group 2 (%)	All (%)
Yes	36 (42)	93 (44)	129 (43)
No	50 (58)	118 (56)	168 (57)
All	86 (100)	211 (100)	297 (100)

TABLE 5: GP's optimal solution to meet glycaemic control objectives.

GP's optimal solution to meet glycaemic control objectives	Patients % N = 78
Patients' strict respect of lifestyle and dietary measures (including smoking cessation)	67
Regular physical exercise	46
New drug without contraindications for CKD	29
Patient acceptance to switch to insulin	28
Improvement in treatment observance	21
Strengthening of therapeutic education programs	17

Like any other observational study, our study was limited by the availability of data provided by its investigators. The 10% participation rate of GPs was similar to other studies conducted by the same team using a randomized list of physicians. However, we did not compare characteristics of participating and nonparticipating physicians. We had to exclude about 15% of patients because of unavailability of appropriate variables allowing the post hoc calculation of GFR. However, we consider that this choice was crucial in avoiding classification and inclusion bias. In other patients where these data were available, the calculation of GFR allowed reclassification of patients in Groups 1 and 2 and the exclusion of about 10% of noneligible patients.

The observed difficulties of GPs in managing T2DM patients with CKD may be linked to the lack of specific French guidelines on the management of diabetic patients with renal disease at the time of the study. Also, for some glucose lowering drugs, such as metformin, there is no consensus on the acceptable renal threshold for continuing the same therapy in case of renal dysfunction [17].

The presence of kidney disease brings an additional layer of complexity to the management of T2DM patients, compared to those with diabetes alone. As the kidneys play an important role in the elimination of insulin and some oral glucose lowering drugs, impaired renal function makes CKD patients exposed to drugs or their metabolites for a longer period of time, potentially resulting in adverse side effects [18]. This includes a higher risk of hypoglycemia to which the reduction of renal neoglucogenesis associated with CKD largely contributes. Therefore, glucose levels of diabetic patients with CKD must be closely monitored. This often results in the adjustment of glucose lowering therapy. Moreover, T2DM patients with CKD are often older in age compared to the diabetic patients without CKD, often have other cardiovascular risk factors [19] and are at higher risk of

developing cardiovascular disease, polypharmacy, and drug interactions.

Our study provides new qualitative information on physician's priorities and decision-making process in the management of T2DM patients with CKD. It also confirms the results of another published research on glucose lowering strategies used by GPs for these patients [15], showing that metformin and sulfonylurea were the most prescribed glucose lowering agents. The authors of the latter study explained that the presence of kidney disease which, in some cases, remained unidentified did not significantly influence the prescribing of glucose lowering agents.

In France, a group of experts from the French Nephrology Society and the Diabetes Society developed recommendations to guide professionals in the management of diabetic patients with impaired renal function [20]. According to these recommendations, metformin remains the first-line drug for the management of T2DM patients, but its dosage must be divided by two when GFR is between 60 and 30 mL/min/1.73 m² and it must be withdrawn in patients with GFR below 30 mL/min/1.73 m². The use of sulfonylurea is possible but the predominant renal elimination of this class should prompt the clinicians to favor products with short half lives and inactive metabolites and to adopt a strict dosage adjustment. Glucose lowering therapy with insulin is recommended in cases of renal impairment and the importance of the dose adjustment should be emphasized. These recommendations are in line with the most recent American and European guidelines (ADA/EASD) on the management of T2DM patients [21–23] which suggest individualization of glycaemic objectives based on patient's conditions, with more ambitious individual HbA1c goals in younger patients among those with CKD. Metformin should be used with caution among patients with mild-to-moderate CKD because of its renal elimination. Although there is debate on the threshold of serum creatinine or GFR, it should be avoided in severe CKD patients (GFR < 30 mL/min/1.73 m²). Most insulin secretagogues, especially glibenclamide, should be avoided in patients with CKD, because of the risk of hypoglycaemia. Most dipeptidyl peptidase-4 (DPP-4) inhibitors have prominent renal elimination, and thus dose reduction is necessary among patients with CKD for sitagliptin, vildagliptin, and saxagliptin. The exception is linagliptin which is mainly eliminated unchanged in bile and intestine. Therefore, for this product, no dosage regimen adjustment is necessary based on its summary of product characteristics for a renal impaired patient, regardless of the severity of the renal impairment.

5. Conclusions

We identified a number of issues regarding the management of T2DM patients with moderate-to-severe CKD by French GPs. Our study suggests that GPs are mainly focused on managing the glycaemic control of their patients, and they do not always consider CKD as a coexisting condition in the management of these patients. These findings reinforce the need for more accurate information for GPs about T2DM and CKD.

Implementation of specific guidelines for T2DM patients with CKD would allow GPs to be better informed and to adapt their therapeutic strategy to each clinical situation. As CKD patients are generally of older age, at high risk of developing cardiovascular disease and have other comorbid conditions, the patient centered approach and individualization of therapeutic strategy based on comorbidities, as introduced by the new ADA/EASD guidelines would improve this situation.

Conflict of Interests

C. Attali has worked as member of the scientific board for studies sponsored by AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, and Novartis. B. Detournay works for CEMKA-EVAL, a company providing consultancy services for most pharmaceutical companies and public institutions involved in health care in France. N. Grandfils and M. Toussi work for IMS Health, a global company providing data acquisition, analysis and consultancy services in health care. D. Joly received honorary for expertise missions and advisory board participation from Amgen, Boehringer Ingelheim, Genzyme, and Novartis. D. Simon has worked as a speaker's bureau (or has given lectures) for GlaxoSmithKline, Johnson & Johnson, Sanofi-Aventis, and Servier, on advisory panels for AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Glaxo-Smith Kline, Janssen, and Novartis, and has participated as an investigator in studies conducted by Lilly, France, Novartis, and Novo Nordisk. B. Vergès received honorary for expertise missions or conferences for AstraZeneca, Bristol-Myers Squibb, Boehringer-Ingelheim, Bayer, Lilly, MSD, Novartis, Novo-Nordisk, Sanofi-Aventis, Servier and Takeda. O. Delaitre and Y. Briand work for Boehringer Ingelheim France, a pharmaceutical company.

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Review Article

Nonalcoholic Fatty Liver Disease, Diabetes Mellitus and Cardiovascular Disease: Newer Data

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Nonalcoholic fatty liver disease (NAFLD) is the most common, chronic liver disease worldwide. Within this spectrum, steatosis alone is apparently benign, while nonalcoholic steatohepatitis may progress to cirrhosis and hepatocellular carcinoma. NAFLD is strongly associated with obesity, dyslipidemia, type 2 diabetes mellitus, and cardiovascular disease. The pathogenesis of hepatic steatosis is not clearly known, but its main characteristics are considered insulin resistance, mitochondrial dysfunction, increased free fatty acids reflux from adipose tissue to the liver, hepatocyte lipotoxicity, stimulation of chronic necroinflammation, and fibrogenic response. With recent advances in technology, advanced imaging techniques provide important information for diagnosis. There is a significant research effort in developing noninvasive monitoring of disease progression to fibrosis and response to therapy with potential novel biomarkers, in order to facilitate diagnosis for the detection of advanced cirrhosis and to minimize the need of liver biopsy. The identification of NAFLD should be sought as part of the routine assessment of type 2 diabetics, as sought the microvascular complications and cardiovascular disease, because it is essential for the early diagnosis and proper intervention. Diet, exercise training, and weight loss provide significant clinical benefits and must be considered of first line for treating NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver clinicopathologic conditions, ranging from pure fatty steatosis (fatty infiltration in >5% of hepatocytes) which is apparently a benign condition to nonalcoholic steatohepatitis (NASH), which may progress to cirrhosis, liver failure, and hepatocellular carcinoma (HCC). It is characterized by excessive fat accumulation in the liver parenchyma of patients who have no history of alcohol abuse (<20 g per day in men and <10 g in women). NASH is characterized by biochemical evidence of hepatocellular damage (elevation of aminotransferase levels), histological findings of the type of alcoholic hepatitis (steatosis, lobular inflammatory cell infiltration, Mallory's hyaline, and fibrosis), and no other cause of liver damage.

NAFLD is the most common liver disease worldwide. Its reported prevalence varies, depending on the population of the study and using diagnostic criteria. In the general population exceeds 15%, but it is far greater in the overweight,

obese, and in subjects with type 2 diabetes (T2DM) as well type 1 diabetics [1–3]. Recent data indicate high prevalence in adolescents. The overall prevalence of NAFLD is reported 12.5%, increasing to 23.0% in overweight/obese, higher in boys than in girls [4]. NAFLD is described in the 60% of the subjects with mixed hyperlipidemia, and in 83% of those with both mixed hyperlipidemia and an elevated serum alanine aminotransferase (ALT) [5].

NAFLD is strongly associated with T2DM and cardiovascular disease (CVD). It is characterized by insulin resistance and mitochondrial dysfunction [6]. Indeed, there is a gradual increase in the severity of insulin resistance in the range of NAFLD which may contribute to the evolution of liver damage. Also, is associated with an increased risk of kidney disease in subjects with multiple CVD risk factors and tends to be considered as an independent CVD marker [7]. Diabetes, dyslipidemia, hypertension and CVD coexist more frequently in individuals with NAFLD [8].

Health cost appears to be greater in NAFLD individuals than in general population. When were used data from the

“Study of Health in Pomerania,” Germany, to assess the relation of fatty liver disease to self-reported health care utilization and costs at baseline and 5 years, in a general population cohort study of 4310 adults aged 20 to 79 years at baseline, subjects with NAFLD and increased serum ALT levels, after controlling for comorbid condition, had 26% higher overall health care costs [9].

2. NAFLD and T2DM

From various studies, the prevalence of NAFLD seems higher in type 2 diabetics than in general population, independent of glycemic control [10]. Type 2 diabetics have approximately 80% more fatty liver compared with nondiabetics matched for age and sex [11]. In a study of 2589 individuals from the community-based Framingham Heart Study, after multivariate adjustment for other fat depots (visceral adipose tissue, waist circumference, and body mass index (BMI)), fatty liver remained associated with diabetes, impaired fasting glucose, hypertension, metabolic syndrome, HDL cholesterol, triglycerides, and adiponectin levels (all $P < 0.001$), whereas associations with systolic (SBP) and diastolic (DBP) blood pressure were attenuated ($P > 0.05$) [12].

There are studies which highlight diabetes as risk marker for NAFLD/NASH appearance. In a study of 458 Italian patients with histological proven NASH, diabetes was the most significant marker of NASH and fibrosis and in those with normal ALT [13]. Severe fibrosis was independently predicted by diabetes (OR = 1.8; 95% CI, 1.4–2.3) in the overall series and in those with normal ALT and insulin resistance according to homeostasis model assessment (HOMA-IR) (OR = 1.97; 95% CI, 1.2–3.7) in patients with normal ALT [13]. In a cohort of 827 patients with NAFLD, advanced fibrosis was associated with insulin resistance [14].

Several studies have shown that NAFLD predicts the appearance of diabetes independently of conventional risk factors, as obesity, insulin resistance, and metabolic syndrome, suggesting that NAFLD could have a direct causal relationship with the appearance of diabetes, probably by promoting the insulin resistance [15]. It has been shown that increased liver enzymes predict T2DM independent of obesity [16]. In another study, metabolic changes (lipid, liver enzymes, blood pressure, and body weight) potentially associated with conversion to diabetes were investigated, it was found that in subjects who converted to new-onset diabetes, ALT ($P = 0.0005$) and triglycerides' ($P = 0.030$) concentrations are increased in absence of changes in body weight up to 18 months before the diabetes manifestation, but neither parameters increased significantly in nonconverters with high baseline glucose concentrations (>6.1 mmol/L) [17].

The poor controlled diabetes, also, promotes or worsens hepatic steatosis, thus feeding a vicious cycle that binds the two situations.

Therefore, hepatic steatosis, diabetes, and metabolic syndrome are part of the same disease process ultimately leading to increased cardiovascular morbidity and mortality risk.

3. NAFLD and CVD

Several prospective, epidemiological studies have shown that elevation of liver enzymes and ultrasonographic appearance of hepatic steatosis are predictors of CVD independent of conventional risk factors [18, 19]. Indeed, among 1221 apparently healthy subjects who were recruited from a health check-up program, NAFLD was a predictor of CVD independent of conventional risk factors (odds ratio 4.12, 95% CI, 1.58 to 10.75, $P = 0.004$) and had central role in the cardiovascular risk of metabolic syndrome [19]. Metabolic syndrome was also independently associated with cardiovascular events, but simultaneous inclusion of NAFLD and metabolic syndrome in a multivariate model revealed that NAFLD but not metabolic syndrome retained a statistically significant correlation with CVD [19]. In a study of subjects with elevated ALT levels was found NAFLD and increased coronary heart disease (CHD), as assessed by Framingham risk score [20]. In another study, NAFLD subjects' survival was found lower compared with matched controls after a mean followup of 13.7 years [21]. Mortality was not increased in patients with steatosis, but it was found higher in NASH patients. These subjects more often died from cardiovascular ($P = 0.04$) and liver-related ($P = 0.04$) causes [21].

Subjects with NAFLD have significantly higher carotid artery intima-media thickness (IMT), a marker of subclinical atherosclerosis, comparing with those without fatty liver disease (mean IMT = 0.417 mm versus 0.395 mm, $P < 0.001$), impaired endothelial function, and lower concentrations of adiponectin [4, 22]. IMT is strongly associated with degree of hepatic steatosis, necroinflammation, and fibrosis among NAFLD patients ($P < 0.001$ for all) [23]. Similarly, the severity of histological features of NAFLD independently predicted carotid IMT ($P < 0.001$) after adjustment for all confounders associated with the presence and severity of coronary atherosclerosis and cardiovascular disease [23]. These results suggest that the severity of liver histopathology among NAFLD patients is strongly associated with early carotid atherosclerosis, independent of classical risk factors, insulin resistance, and the presence of metabolic syndrome [23]. When the vasodilatory response of the brachial artery was assessed in response to ischemia (a test of endothelial function) as well as cardiovascular profile (10-year risk of coronary events) in 52 NAFLD cases and 82 age- and sex-matched controls, were endothelial dysfunction and increased risk of cardiovascular events in NAFLD subjects compared with controls observed [24].

It seems that NASH can predict a more atherogenic risk profile in a manner that is partly independent of the contribution of visceral adiposity. In a study, the differential contribution of NASH and visceral adiposity to nontraditional cardiovascular risk biomarkers in adult men was assessed [25]. 45 consecutive, overweight male patients with biopsy-proven NASH, 45 overweight men without ultrasound-diagnosed hepatic steatosis and 45 healthy male volunteers were included. All participants were matched for age; NASH and overweight patients were also matched for BMI and visceral adiposity (as estimated by abdominal ultrasonography) [25]. Plasma concentrations of high-sensitivity C-reactive

protein (hs-CRP), fibrinogen, and plasminogen activator inhibitor-1 (PAI-1) activity were found markedly lower in nonobese healthy volunteers, intermediate in overweight nonsteatotic patients, and the highest in overweight subjects with biopsy-proven NASH, after adjustment for age, BMI, smoking, plasma triglycerides, and insulin resistance [25]. Also, the highest concentrations of adiponectin were found in nonobese healthy subjects and the lower in those with biopsy-proven NASH [25].

The relationship between hypertension and NAFLD has also been investigated. Higher prevalence of NAFLD in non-obese nondiabetic hypertensive patients with normal liver enzymes compared with adjusted nonhypertensive subjects. It has been found [26].

Abnormal left ventricular energy metabolism, fat accumulated in the epicardial area despite normal left ventricular morphological features, and systolic and diastolic functions, in nondiabetic young with fatty liver compared with nondiabetic matched for anthropometric features without fatty liver. It has also been reported [27]. It is interesting that, the addition of pioglitazone to insulin therapy, in type 2 diabetics, reduced myocardial and hepatic steatosis [28].

NAFLD is significantly associated with an increased CVD risk (odds ratio (OR) 1.84, 95% CI, 1.4–2.1, $P < 0.001$) among type 2 diabetics independent of classical risk factors, liver enzymes, or metabolic syndrome [29]. The independent association among NAFLD and CVD is supported by another large study following type 2 diabetics for 6.5 years [30]. Significant association of NAFLD with incident CVD (hazard ratio 1.96 (1.4–2.7), $P < 0.001$) by adjustment for sex, age, smoking, diabetes duration, HbA1c, LDL-cholesterol and medications was found [30].

NAFLD in type 1 diabetics is associated with higher prevalence of CVD compared with non-NAFLD type 1 diabetics independently of age, sex, smoking, diabetes duration, HbA1c, LDL-cholesterol, HDL-cholesterol, triglycerides, SBP, and medication use (adjusted OR 7.36, 95% CI, 1.60–34.3, $P < 0.01$) [3].

4. Pathogenesis of NAFLD

The pathogenesis of hepatic steatosis remains poorly understood but its main characteristics are considered insulin resistance and mitochondrial dysfunction [6]. If insulin resistance precedes NAFLD or the opposite remains unanswered. The source of stored hepatic fat results from dietary carbohydrates and fatty acids and the release of fat from adipocytes, by lipolysis or de novo hepatic lipogenesis [6]. An imbalance between the mechanisms may modify the rate of fat oxidation and fat removal from the liver. Defects in multiple levels may tip the metabolic balance towards hepatic fat accumulation: excessive substrate supply to the liver (i.e., glucose and fatty acids), intrahepatic imbalance between lipid synthesis and oxidation; inadequate export to peripheral tissues; and a combination of the above [31]. Many molecular defects at these different steps have been described in NAFLD.

4.1. Hepatic Insulin Resistance and NAFLD. Insulin resistance leads to hepatocyte steatosis by stimulation of insulin

secretion and by increased lipolysis in adipose tissue, which increases circulating fatty acids. Increased uptake of circulating free fatty acids (FFA) by hepatocytes leads to mitochondrial beta-oxidation overload, with the consequent accumulation of fatty acids within hepatocytes. Fatty acids are substrates and inducers of the microsomal lipooxygenases cytochrome P-450 2E1 and 4A, resulting in the production of free oxygen radicals capable of inducing lipid peroxidation of hepatocyte membranes [32]. Hyperinsulinemia resulting from insulin resistance increases the synthesis of fatty acids in hepatocytes by increasing glycolysis and favors the accumulation of triglycerides within hepatocytes by decreasing hepatic production of apolipoprotein B-100. Reactive oxygen species (ROS) trigger lipid peroxidation, which causes cell death and releases malondialdehyde (MDA) and 4-hydroxynonenal (HNE). MDA and HNE cause cell death; cross-link proteins, leading to the formation of Mallory's hyaline, and activate stellate cells promoting collagen synthesis. HNE has chemotactic activity for neutrophils, promoting tissue inflammation. ROS also induce the formation of cytokines tumor necrosis factor- α (TNF- α), transforming growth factor β (TGF- β) and interleukin-8. TNF- α and TGF- β cause caspase activation and hepatocyte death. TGF- β activates collagen synthesis by stellate cells and activates tissue transglutaminases, promoting the formation of Mallory's hyaline. The TNF- α induced by ROS further impairs the flow of electrons along the respiratory chain in mitochondria. Mitochondrial ROS cause expression of the Fas ligand (a death receptor member of TNFR family) in hepatocytes, which normally express the membrane receptor Fas. The Fas ligand on one hepatocyte can interact with Fas on another hepatocyte, causing fractional killing [32].

Elevated levels of triacylglycerol (TAG), diacylglycerol (DAG) and free cholesterol, NF- κ B signaling activation, mitochondrial dysfunction, and FFA toxic role it have reported [6, 31].

4.2. Adipose Tissue Insulin Resistance and NAFLD. Adipose tissue insulin resistance is associated with increased hepatic fat synthesis, regardless of obesity [15]. Adipocytes account for approximately 60%–70% of the FFA used for hepatic triglyceride synthesis and very low-density lipoprotein (VLDL) secretion [31]. Many factors that regulate VLDL metabolism may promote steatosis.

The adipocyte is a dynamic endocrine organ and nutrient sensor that tightly regulates energy supply. When nutrient supply exceeds adipose tissue adaptation, adipocyte hypertrophy and other poorly understood factors set off a pathologic adipocyte-macrophage crosstalk. The final result is adipocyte insulin resistance and the chronic release of FFA with toxic effects in distant tissues such as muscle, liver, and pancreatic β -cells as well as on the heart and vascular bed [15]. The precise series of events occurring is incompletely understood. Although the mechanisms by which the hepatic fat aggregation is associated with adipose tissue inflammation were not elucidated, macrophages infiltration may contribute to adipose tissue insulin resistance.

Excessive FFA availability promotes the accumulation of intramyocellular lipids and the formation of a variety of

fat-derived potentially toxic lipid metabolites such as ceramide and DAG that activate the IKK/NF- κ B pathway and cause insulin resistance. Inhibition of muscle insulin signaling and insulin resistance inhibits insulin action on adipose glucose uptake and lipid synthesis, further increasing the rate of FFA release into the circulation, and contributes to the development of insulin resistance and type 2 diabetes [15].

There is ample evidence that increases of plasma FFA concentrations cause hepatic insulin resistance, impair insulin signaling, and stimulate hepatic glucose production by driving both hepatic gluconeogenesis and glycogenolysis [15]. Induction of hepatic insulin resistance is following lipid infusion and correlates closely with increase in plasma FFA [15].

4.3. From Insulin Resistance to NASH. It is known that only 10%–25% of NAFLD subjects develop NASH [33]. Factors responsible for this evolution have been subject to extensive research but still remain incompletely understood. Still, data are fragmented and arise largely from rodents given the natural difficulties of assessing human liver tissue. One must keep in mind that there are significant metabolic/molecular differences between livers from humans and rodents and even between rodent species.

With these limitations in mind, in an effort to organize the current understanding of NAFLD and NASH, a framework is proposed on the progression from adipose tissue insulin resistance to NAFLD and NASH [31]. A prerequisite or “first step” for NASH appears to be adipose tissue insulin resistance, providing the necessary “lipotoxic environment” that ensures ample substrate supply to the liver (i.e., high FFA flux) and compensatory hyperinsulinemia that stimulates excessive hepatic triglyceride synthesis and the formation of toxic saturated fatty acids. The “second step” towards NASH is the development of hepatic steatosis and of a lipid pool from where lipid-derived toxic metabolites may activate inflammatory pathways. Dietary and genetic factors may condition the metabolic adaptation of the liver to this harmful environment. Compensated steatosis exacerbates hepatic insulin resistance, stimulates VLDL secretion, and increases mitochondrial beta-oxidation. If a new steady state is achieved, only benign steatosis and/or dyslipidemia (high triglyceride, low HDL-cholesterol) takes place. The “third step” for the progression from simple “bland” steatosis to active necroinflammation depends on the ability of the liver to adapt to longstanding triglyceride accumulation. If mitochondrial function cannot adapt to the increased FFA flux and respiratory oxidation collapses, lipid-derived toxic metabolites activate inflammatory pathways and hepatocyte lipotoxicity with stimulation of chronic necrosis and inflammation. Fibrosis is the final “fourth step,” involving chronic activation of hepatic stellate cells in a yet poorly understood cross-talk of Kupffer cells with hepatocytes. The magnitude of the crosstalk between hepatocytes, macrophages, and hepatic stellate cells determines the degree of the fibrogenic response and potential progression to cirrhosis. In this setting, low plasma adiponectin levels are believed to promote steatosis and fibrosis by allowing unchecked triglycerides synthesis and activation of hepatic stellate cells, respectively.

The association between NAFLD, insulin resistance, and T2DM seems to be strong and partly due to genetic and environmental factors. The polymorphism of certain genes has been shown both in animal model and human studies that plays a significant role [34–36]. Diets rich in saturated fats, soft drinks, and meat and low in antioxidants, fish, and omega-3 fat are associated with development of NAFLD [37, 38].

5. Recent Advances in Diagnosis of NAFLD

The diagnosis of NAFLD/NASH is usually suspected in subjects with asymptomatic elevation of aminotransferase level, radiologic findings of fatty liver, or unexplained persistent hepatomegaly. The clinical diagnosis and liver tests have a poor predictive value with respect to histologic involvement. Imaging studies, although being of help in to determining the presence and amount of fatty infiltration of liver, cannot be used accurately determine the severity of liver damage. NAFLD is often diagnosed by a combination of clinical, laboratory and imaging data, but the clinical suspicion of NAFLD and its severity can only be confirmed with a liver biopsy [39]. Liver biopsy remains the best diagnostic tool for confirming NAFLD and evaluating necroinflammation/fibrosis, as well as the most sensitive and specific means of providing important prognostic information. Although liver biopsy is a relatively safe procedure when, performed by experienced clinicians, it has poor patient acceptance, it is not risk free and is difficult to be repeated.

Reliable and reproducible noninvasive methods for evaluating hepatocellular fat accumulation as well as the variable degree of hepatocyte necroinflammation (activity or grade of disease) and fibrosis (stage of disease), for frequent monitoring of disease progression, of treatment efficacy, and for prognosis assessment are strongly needed. With recent advances in technology, advanced imaging techniques (sonographic and magnetic elastography, magnetic spectroscopy) provide important information for diagnosis and usually diagnosis is based on these [40]. Several laboratory investigators try to identify potential novel biomarkers based on the current knowledge of the pathophysiologic mechanisms involved in NAFLD progression [41–43]. An ideal biomarker should be simple, reproducible, inexpensive, readily available, and accurate for a particular disease process. Potential rational targets for biomarkers development in NAFLD/NASH are based on the central role of inflammatory cytokines in the development of NAFLD, on the different oxidation products of several oxidation pathways, on mediators of fibrogenesis/fibrosis, on mediators/receptors involved in the hepatocyte apoptosis, and on breath biomarkers. Different mechanisms have been proposed including an increased production of reactive oxygen species and mitochondrial outer permeabilization, resulting in a cascade of events leading to inflammation (TNF- α , adiponectin, C-reactive protein, IL-6, Resistin, and visfatin), hepatocellular apoptosis (Fas, circulating active caspase 3), fibrogenesis, and fibrosis (TGF- β , tissue elasticity) [41]. It has evaluated the use of breath biomarkers in the study of NAFLD, such breath ethanol, ethane, and breath acetone [42]. Also, efforts attempts are

made to identify noninvasive indicators of liver fibrosis by using routinely determined and easily available clinical and biochemical variables [44]. All these markers are under investigation and their clinical utility remains to be determined [41–44].

It is noted that with all above, diagnosis of significant fibrosis may be satisfactory, but that of steatosis and NASH without fibrosis is problematic.

6. Therapy of NAFLD

There is currently no established treatment for NAFLD or NASH, although weight loss is recommended [39]. Several pharmaceutical interventions have been evaluated but none has been approved for general use. Most treatment studies have focused on subjects with NASH because of their potential to progress to fibrosis and cirrhosis; however, the findings have been limited by variations in treatment endpoints and a paucity of randomized, placebo-controlled, powerful and of sufficient duration trials.

Lifestyle changes, mostly focused on weight loss, have been demonstrated to improve liver aminotransferases and histological findings in obese with fatty liver [45]. In overweight or obese individuals with biopsy-proven NASH, weight reduction achieved through lifestyle intervention leads to improvements in liver histology [46]. The content of diet slimming is of no particular importance (advising to avoid alcohol), if lead to weight loss. However, although weight loss appears to be beneficial, rapid weight loss after gastroplasty has been associated with increased hepatitis despite reductions in steatosis on liver biopsy [47]. Weight loss agents had no significant effects compared with weight loss only [48]. In obese patients undergoing bariatric surgery, hepatic steatosis is decreased from 53% to 32%, three months after surgery as overall mortality [49, 50]. Cross-sectional investigations have shown an independent association between physical fitness and hepatic triglyceride concentration [51]. Regular exercise reduces hepatic and visceral lipids in previously sedentary obese individuals even in absence of weight loss [52].

Several pharmaceutical interventions have attempted to NAFLD/NASH, with limited benefit overall. Some studies have been performed with drugs acting cytoprotective or antioxidants or tumor necrosis factor antagonists or decreasing cytokines production, inhibitors of TGF- β , and semisynthetic agonists of receptor Farnesoid X with unsuccessful or moderate results [53–58]. In recent years, drugs that inhibit the system renin-angiotensin and α -receptors with moderate biochemical and histological response are studied [59]. Sought treatment options for NASH, especially when is accompanied by fibrosis, but there are not large randomized trials.

Administration of lipid-lowering drugs has been evaluated in patients with NAFLD/NASH, but not in large prospective controlled trials and it has been associated with biochemical and histological improvement, but not all studies. The use of statins appears to be safe in patients monitored closely, to treat hyperlipidemia [39, 60]. The long-term ezetimibe therapy can lead to improvement in metabolic,

biochemical, and histological abnormalities of NAFLD [61]. In small uncontrolled studies moderate or no benefit from use of fibrates has reported [62, 63].

The common metabolic disorders of T2DM and NAFLD can explain the greater success of drugs used to treat diabetes, with the most studies to target the use of drugs that improve insulin resistance. Use of metformin in NAFLD patients does not appear to help and is not recommended in nondiabetic NAFLD/NASH patients [39, 58]. Clinical trials of pioglitazone have shown promising results (partial biochemical and histological efficacy) but the short-term effect and side effects may limit widespread acceptance [56]. At present, they are used only in clinical trials, while they can be used in type 2 diabetic patients with NAFLD/NASH. Intensive insulin therapy in type 2 diabetic patients with NAFLD/NASH appears to significantly decrease on steatosis [64]. GLP-1 receptors were detected on human hepatocytes and treatment with exendin-4 quantitatively reduced triglyceride stores compared with control-treated cells [65]. The current preclinical evidence shows that GLP-1 analogs and DPP-4 inhibitors can improve hepatic steatosis independent of weight loss but is controversial whether the pancreatic-type GLP-1 receptor is present or responsible for conferring the GLP-1 signal in the hepatocyte [66].

In patients with NAFLD/NASH, all cardiovascular risk factors (obesity, hypertension, hyperglycemia, and hyperlipidemia) are treated [39, 67]. The identification of NAFLD should be sought as part of assessment of diabetic patients for proper implementation of lifestyle and pharmaceutical interventions [68].

Liver transplantation is recommended in patients with decompensate cirrhosis due NASH as long as underlying comorbidities permit [32].

7. Natural History: Prognosis

NAFLD often follows a benign course but may leads to fibrosis, cirrhosis due NASH and HCC. The evolution of fibrosis in NASH has been found in 25%–33% of the cases [69]. Factors favoring the evolution to cirrhosis are fibrosis, obesity (visceral), diabetes, and hypertension [70]. There is epidemiological evidence that NASH and cirrhosis are associated with increased HCC risk [71]. Mild steatosis is not associated with an increased risk mortality compared with general population [72]. In type 2 diabetics, presence of NAFLD is associated with increased total mortality, regardless of classic risk factors [73]. Diabetics with NAFLD have twice risk mortality compared with nondiabetics without NAFLD, with more common causes of death malignancy (33% of death) and liver related complications (19% of death) [73].

8. Conclusions

NAFLD is strongly associated with T2DM and CVD. Within this spectrum, steatosis alone is apparently benign, while nonalcoholic steatohepatitis may progress to cirrhosis and hepatocellular carcinoma. Its pathogenesis is complex and involves insulin resistance and mitochondrial dysfunction

with increased FFA reflux from adipose tissue to the liver which play a key role in the chronic activation of inflammatory pathways and hepatocyte lipotoxicity with stimulation of chronic inflammation and necrosis. There is significant research effort in developing noninvasive monitoring of disease progression to fibrosis and response to therapy with potential novel biomarkers, which promise to facilitate diagnosis for the detection of advanced cirrhosis in order to minimize the need of liver biopsy, which are not used in clinical practice. The identification of NAFLD should be sought as part of routine assessment of diabetic patients, as sought the microvascular complications and CVD, because it is essential for the early diagnosis and proper implementation of lifestyle and pharmaceutical interventions. Diet, exercise, and weight loss provide significant clinical benefits and must be considered of first line for treating NAFLD/NASH.

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Research Article

Assessment of Carotid Atherosclerosis in Type 2 Diabetes Mellitus Patients with Microalbuminuria by High-Frequency Ultrasonography

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The aim of this study is to evaluate carotid atherosclerosis in patients of type 2 diabetes mellitus with microalbuminuria (MA) by high-frequency ultrasonography. Two hundred and fifty patients of type 2 diabetes mellitus were divided into two groups according to urinary albumin excretion rate (UAER): normoalbuminuria group (130 cases) and microalbuminuria group (120 cases). The intimal-medial thickness (IMT) and the atherosclerotic plaques of carotid artery were observed in both groups by high-frequency ultrasound. Fasting blood glucose (FBG), hemoglobin A1c, and lipid profiles were measured. The values of IMT of microalbuminuria group were significantly higher than those of normoalbuminuria group ($P < 0.05$). In univariate analysis, IMT was positively and significantly associated with age ($r = 0.265$, $P < 0.05$), waist circumference ($r = 0.263$, $P < 0.05$), body mass index ($r = 0.285$, $P < 0.05$), systolic blood pressure ($r = 0.276$, $P < 0.05$), UAER ($r = 0.359$, $P < 0.05$), HbA1c ($r = 0.462$, $P < 0.05$) and, duration of diabetes ($r = 0.370$, $P < 0.05$). In multivariate linear regression analysis, UAER and HbA1c were independent predictors of IMT ($P < 0.05$ for all). In the two groups, the rate of soft plaques was higher than that of dense plaques and calcified plaques. In conclusion, there is a significant association between microalbuminuria and IMT which is regarded as the early sign of carotid atherosclerosis in type 2 diabetic patients.

1. Introduction

Diabetes mellitus is associated with aggressive vascular abnormalities in human subjects, and atherosclerosis is regarded as the leading cause of morbidity and mortality in diabetic patients [1]. Microalbuminuria has a strong prediction of both the development of diabetic nephropathy and subsequent atherosclerotic vascular dysfunction [2, 3]. The previous epidemiologic study has proved the predictive value of microalbuminuria for atherosclerotic vascular disease in the patients of type 2 diabetes [4]. Several biochemical parameters including soluble vascular cell adhesion molecule1, sialic acid, C-reactive protein, and fibrinogen have been proved to be significantly associated with microalbuminuria [5–7]. And those parameters were believed to

indicate endothelial dysfunction and chronic inflammation. These findings may support a hypothesis that microalbuminuria reflects generalized vascular damage which may promote atherosclerosis [8, 9]. But the underlying mechanism is still unclear. High-frequency B-mode ultrasonography is a noninvasive method of detecting carotid artery wall and provides measurement of intima-media thickness (IMT) and presence of plaques [10, 11]. The IMT is significantly higher in diabetic patients than that in nondiabetic patients [12]. And the increased IMT can predict future events of silent brain infarction and coronary heart disease in the patients of type 2 diabetes mellitus [13, 14]. Carotid artery plaque is another marker of systemic subclinical atherosclerosis. But the previous reports showed the inconsistent associations among IMT, plaque, risk factors, and clinical disease

[15–18]. And which one is a more powerful predictor of vascular outcomes, IMT or plaque, is still in controversy [18, 19]. In addition, the reported results of the relationship between microalbuminuria and carotid IMT is also different [20, 21]. Therefore, in this study we sought to clarify the relationship between microalbuminuria and markers of carotid atherosclerosis including IMT and plaque in type 2 diabetic patients.

2. Materials and Methods

2.1. Participants. The study included 250 patients of type 2 diabetes mellitus at the Department of Endocrinology of the second Affiliated Hospital of Dalian Medical University. Type 2 diabetes mellitus was diagnosed according to the 1999 World Health Organization criteria. The Ethics Committee of the second Affiliated Hospital of Dalian Medical University approved the study. All patients gave their informed consent to participate in the study. According to the level of urinary albumin excretion rate (UAER), 250 patients of type 2 diabetes mellitus were divided into two groups: normoalbuminuria group (UAER < 30 mg/24 h; 130 cases, 66 males and 64 females; mean age, 56.45 ± 9.35 years; age range, 29–76 years; diabetes duration, 7.57 ± 5.53 years; treatment with diet or oral drugs) and microalbuminuria group (30 mg/24 h < UAER < 300 mg/24 h; 120 cases, 62 males and 58 females; mean age, 57.67 ± 11.12 years; age range, 41–80 years; diabetes duration, 8.00 ± 5.12 years; treatment with diet or oral drugs). Medical history was obtained and physical examination was performed in all patients. Blood was withdrawn from all subjects following 12 h of fasting. Type 1 diabetes mellitus, hypertension, history of ischemic heart disease, renal impairment (serum creatinine > 150 $\mu\text{mol/L}$), and valvular heart diseases were excluded. The clinical conditions that could cause transient elevations in urinary albumin excretion, such as exercise, urinary tract infection, febrile illness, were also excluded.

2.2. Carotid Artery Ultrasonography. Carotid artery ultrasonography was performed by an experienced specialist physician who was specifically trained for the vascular ultrasonography. A real-time ultrasound scanner was used: Hitachi EUB 7500 with a linear 3–15 MHz probe (Hitachi Medical Systems, Tokyo, Japan). The patients were examined in the supine position with the head turned 45° contralateral to the side of scanning. B-mode images were obtained in longitudinal section. IMT was defined as the distance between the lumen-intima and the media-adventitia ultrasound interfaces. The IMT on the far wall of the bilateral common carotid artery about 10 mm proximal to the bifurcation of the carotid artery was measured manually as previously described [22, 23]. Three measurements on both sides were performed for each patient and the mean value was obtained for analysis. A high degree of reproducibility (a mean difference in CIMT: 0.020 mm) was shown in paired CIMT measurements in the same arteries. And an intraclass correlation coefficient was 0.93. The presence of plaque was defined as an area of focal wall thickening >50% greater than surrounding

wall thickness confirmed by marking and comparing plaque thickness with the thickness of the surrounding wall during scanning by electronic calipers. Furthermore, the plaques were classified into three types: calcified plaques (hyperechogenic), dense plaques (less hyperechogenic than calcified lesions), and soft plaques (isoechogenic in comparison with blood), based on their echogenic properties according to the criteria established by Johnson et al. [24].

2.3. Laboratory Assays. The following laboratory parameters were obtained: total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), hemoglobin A1c (HbA1c), fasting plasma glucose (FBG), and urinary albumin excretion rate (UAER). Serum concentrations of TC, TG, LDL, HDL, and FBG were measured by enzymatic method. HbA1c was measured by high performance liquid chromatography (BRO-RAD Company, USA). UAER was obtained by a 24-hour urine collection. Body mass index (BMI) was calculated as weight in kilograms divided by height in meter squared. All the measurements were performed 3 times.

2.4. Statistical Analysis. The software of SPSS version 13.0 for Windows (SPSS Inc., IL, USA) was used for statistical analysis. Statistical significance between two groups was determined by the Wilcoxon rank-sum test. Continuous variables were expressed as median and range. Pearson's chi-square (χ^2) test was used to compare groups regarding categorical variables. Correlation analysis including Pearson's for continuous and Spearman's for discrete variables and multiple linear stepwise regression analysis was used to show the influences of variables on IMT. All tests were performed with $P < 0.05$ considered statistically significant.

3. Results

250 type 2 diabetic patients with or without microalbuminuria were screened. The characteristics of the 250 enrolled patients are presented in Table 1.

Patients of the microalbuminuria group with elevated UAER had higher FBG, BMI, waist and hip circumference, triglycerides, HbA1c, and IMT than those of the normoalbuminuria group with normal UAER ($P < 0.05$ for all; Table 1, Figure 1).

The plaque incidence rate and plaque type rate (soft, dense, and calcified plaques) between two groups were shown in Table 2. The plaque type rate was also compared within the group presented in Table 2. There were no significant difference in plaque incidence rate and plaque type rate between normoalbuminuria and microalbuminuria groups ($P < 0.05$ for all). In both normoalbuminuria and microalbuminuria groups, the rate of soft plaques was the highest. And the rate of soft plaque was higher than that of dense and calcified plaques. The rate of dense plaques was higher than that of calcified plaques. In detail, in normoalbuminuria groups, soft plaques rate (66.67) > dense plaques rate (25) > calcified plaques rate (8.33), and in microalbuminuria group, soft

TABLE 1: Characteristics of patients of the study groups (M P₂₅–P₇₅).

Characteristic	Normoalbuminuria group N = 130		Microalbuminuria group N = 120		P value
Sex (F/M)	66/64		62/58		NS
Smoker (%)	21.5		22.5		NS
Age (yrs)	50.70	48.82–52.60	51.94	49.71–54.25	NS
Duration of diabetes (yrs)	6.79	5.69–8.03	7.46	6.32–8.38	NS
Waist circumference (cm)	79.88	78.25–81.49	83.25	81.55–84.88	<0.05
Hip circumference (cm)	85.16	83.84–86.23	89.6	88.2–102.5	<0.05
BMI (kg/m ²)	23.34	22.65–24.25	25.55	25.02–26.37	<0.05
Systolic BP (mmHg)	116.48	13.38–119.77	117.14	113.65–120.34	NS
Diastolic BP (mmHg)	74.92	73.5–76.7	73.65	71.76–75.45	NS
FBG (mmol/L)	8.38	7.55–9.25	11.65	10.62–12.64	<0.05
HbA1c (%)	6.54	6.31–6.82	7.92	7.72–8.15	<0.05
Total cholesterol (mmol/L)	4.46	4.22–4.70	4.66	4.52–4.88	NS
Triglycerides (mmol/L)	1.52	1.26–1.78	2.12	1.85–2.39	<0.05
LDL cholesterol (mmol/L)	3.05	2.79–3.29	3.15	2.97–3.30	NS
HDL cholesterol (mmol/L)	0.92	0.84–0.97	0.92	0.86–0.96	NS
UAER (mg/24 hr)	11.25	9.49–12.8	87.55	74.56–100.58	<0.05
IMT (mm)	0.75	0.72–0.77	0.92	0.87–0.95	<0.05

NS: not significant; F/M: female/male; BP: blood pressure; BMI: body mass index; FBG: fasting blood glucose; LDL: low density lipoprotein; HDL: high density lipoprotein; UAER: urinary albumin excretion rate.

TABLE 2: Comparisons of plaques' types and rates of two groups.

Groups	Presence of plaques (cases)			Plaques' types (number and rate)		
	Cases	+	–	Soft plaques	Calcified plaques	Dense plaques
Normoalbuminuria group	130	69	61	72 (66.67)	9 (8.33)	27 (25)
Microalbuminuria group	120	62	58	65 (68.42)	7 (7.37)	23 (24.21)

Comparison of plaques' rates between two groups: $\chi^2 = 0.209$, $P > 0.05$; comparison of plaques' types and numbers between two groups: $\chi^2 = 0.210$, $P > 0.05$.

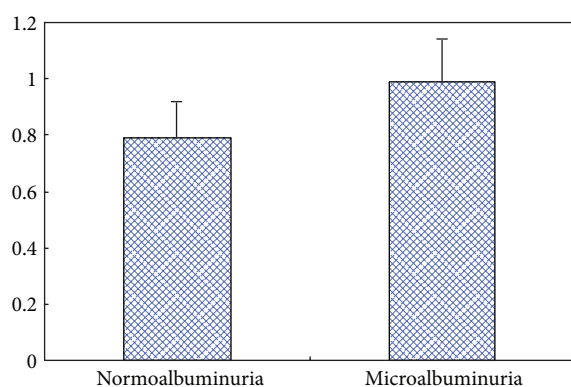


FIGURE 1: Comparison of IMT between the two study groups. Continuous variables were expressed as median and range. $P < 0.05$ from the Wilcoxon rank-sum test for the difference between normoalbuminuria group and microalbuminuria group. IMT: intima-media thickness.

plaques rate (68.42) > dense plaques rate (24.21) > calcified plaques rate (7.37) (Table 2).

In univariate analysis, IMT was positively and significantly associated with age ($r = 0.265$, $P < 0.05$), waist circumference ($r = 0.263$, $P < 0.05$), body mass index ($r = 0.285$, $P < 0.05$), systolic blood pressure ($r = 0.276$, $P < 0.05$), UAER ($r = 0.359$, $P < 0.05$), HbA1c ($r = 0.462$, $P < 0.05$), and duration of diabetes ($r = 0.370$, $P < 0.05$). In multiple stepwise regression analyses, age, body mass index, systolic and diastolic blood pressure, waist and hip circumference, UAER, FBG, plasma HbA1C concentration, serum concentrations of triglycerides and total, HDL and LDL cholesterol, current smoking, and duration of diabetes mellitus were included in the model as independent variables. UAER and HbA1c were appeared to be significantly associated with IMT ($P < 0.05$ for all) (Table 3).

4. Discussion

In the present study, we found that the values of IMT of type 2 diabetic patients with microalbuminuria was significantly higher than those without microalbuminuria. And UAER was an independent predictor of IMT. The results indicated

TABLE 3: Multiple stepwise regression analyses.

Characteristics	IMT	
	Standardized coefficient beta	P value
UAER (mg/24 hr)	0.269	<0.05
HbA1c (%)	0.458	<0.05

The model including age, body mass index, systolic and diastolic blood pressure, waist and hip circumference, UAER, FBG, plasma HbA1C concentration, serum concentrations of triglycerides and total, HDL and LDL cholesterol, current smoking, and the duration of diabetes mellitus.

that microalbuminuria was related to atherosclerosis in the early stage of diabetic nephropathy. Maybe there is a close relationship between atherosclerosis and diabetic nephropathy. But the mechanism underlying the relationship between microalbuminuria and atherosclerosis in type 2 diabetic patients is still unknown. Nand et al. [25] results showed that microalbuminuria was found to be associated with carotid atherosclerosis in middle aged individuals. But the subjects enrolled in this study were not only limited to the diabetic patients. There was a hypothesis that increased UAER could reflect a generalized vascular dysfunction which was caused by structural alterations, such as a reduction in the density of heparan sulfate-proteoglycan (HS-PG) and/or the sulphation of HS within the extracellular matrix of the glomerular basement membrane and vascular wall [8, 9]. HS-PG is synthesized in endothelial and myomedial cells. It is a normal component of glomerular basement membrane, endothelial vascular surface, and basement membrane of vascular smooth muscle cells. Furthermore, many proteins, such as lipoprotein lipase, tissue factor pathway inhibitor, platelet factor 4, and antithrombin III, are anchored to the vascular wall through interaction with the chains of HS-PG, which may enhance albuminuria and processes involved in atherogenesis [9, 26–28].

Stehouwer et al. [29] found that microalbuminuria was linearly associated with impaired endothelium-dependent, flow-mediated vasodilation in elderly individuals without and with diabetes. It is possible that endothelial leakiness, as reflected by UAE, is in part a primary and possibly genetically determined vascular risk factor, or that it mirrors the endothelial dysfunction featuring the atherosclerotic process or arises from the action of yet unknown risk factors [30]. Also, the previous study results showed that endothelial dysfunction assessed by brachial artery flow-mediated dilation (FMD) was associated with urinary albumin excretion (UAE) and was interrelated with carotid IMT in type 2 diabetic patients with microalbuminuria [31].

In the same time, our study showed that the value of HbA1c in type 2 diabetic patients with microalbuminuria was significantly higher than that in patients with normoalbuminuria. And IMT was positively and significantly associated with HbA1c. The result indicated that HbA1c maybe played an important role in the relationship between carotid atherosclerosis and microalbuminuria. HbA1c can accurately reflect longer-term glycemia. Clinically, HbA1c is now used to assess glycemic control in patients of diabetes mellitus, and it is regarded as a useful method of screening and diagnosing

diabetes. And HbA1c has been accepted as the best marker for diabetic microvascular complications [30]. Moreover, HbA1c is associated closely with advanced glycation end products (AGEs) [32]. The previous study showed that AGEs are widespread in the diabetic vascular system and contribute to the development of atherosclerosis [33]. AGEs contribute to many microvascular and macrovascular complications through the formation of bridging between molecules in the basement membrane of the extracellular matrix by joining the receptor for advanced glycation end products (RAGE). Concerning microalbuminuria, it was reported that the accumulation of AGEs in the glomerular and tubulointerstitial spaces correlates with the severity of diabetic nephropathy [34].

In addition, the results showed that there were no significant difference in plaque incidence rate between normoalbuminuria and microalbuminuria groups. This result was in line with the previous studies [21]. But there was no studies which concerned the plaque types' differences. Our results showed that there were significant differences of plaque types in both normoalbuminuria and microalbuminuria groups. And the incidence rate of soft plaques was the most compared with dense plaques and calcified plaques. It is known that the presence of carotid plaques correlates with an increase in the risk of stroke and cerebral infarction, and softer plaques are more likely to be unstable or vulnerable plaques when compared to calcified plaques [35–38].

Our study has its limitations. First, the study population is small. Second, some type 2 diabetic patients have already been treated for diabetes and hyperlipidemia which may lead to inaccuracy of the results.

5. Conclusion

Our data show that there is a significant association between microalbuminuria and IMT which is regarded as the early sign of carotid atherosclerosis in type 2 diabetic patients. Routine screening of carotid artery IMT and plaque presence in type 2 diabetic patients with microalbuminuria is necessary. It helps us not only to detect early atherosclerosis but to prevent further development of diabetic nephropathy and cardiovascular events by applying more intensive therapy. However, larger and further studies are needed to confirm our results.

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