Journal of Diabetes Research

Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications 2017

Lead Guest Editor: Joseph F. Ndisang
Guest Editors: Sharad Rastogi and Alfredo Vannacci

Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications 2017

Journal of Diabetes Research

Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications 2017

Lead Guest Editor: Joseph F. Ndisang
Guest Editors: Sharad Rastogi and Alfredo Vannacci

Editorial Board

Contents

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 1478294, 3 pages
https://doi.org/10.1155/2017/1478294

Editorial

Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications 2017

Joseph Fomusi Ndisang,[1] Alfredo Vannacci,[2] and Sharad Rastogi[3]

[1]*Department of Physiology, University of Saskatchewan College of Medicine, 107 Wiggins Road, Saskatoon, SK, Canada S7N 5E5*
[2]*Department of Neurosciences, Psychology, Drug Research and Child Health Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy*
[3]*The Medical Affairs Company, Cardiovascular, Troy, MI 48085, USA*

Correspondence should be addressed to Joseph Fomusi Ndisang; joseph.ndisang@usask.ca

Received 13 August 2017; Accepted 13 August 2017; Published 15 November 2017

The global escalation of obesity and diabetes in developed and developing nations poses a great health challenge. Obesity is one of the major causes of type 2 diabetes. Type 1 diabetes is primarily due to the autoimmune-mediated destruction of pancreatic beta cell leading to insulin deficiency [1–3]. This is usually accompanied by alterations in lipid metabolism, enhanced hyperglycemia-mediated oxidative stress, endothelial cell dysfunction, and apoptosis [1–3]. Similarly, in type 2 diabetes, increased glucotoxicity, lipotoxicity, endoplasmic reticulum-induced stress, and apoptosis lead to the progressive loss of beta cells [1–5]. While type 1 diabetes is characterized by the presence of beta cell autoantibodies, a combination of peripheral insulin resistance and dysfunctional insulin secretion by pancreatic beta cells is implicated in the pathogenesis of type 2 diabetes [1–3]. However, both forms of diabetes are associated by a wide variety of complications such as cardiomyopathy, nephropathy, and neuropathy. Although insulin resistance has traditionally been associated with type 2 diabetes, mounting evidence indicates that the incidence of insulin resistance in type 1 diabetes is increasing [6]; therefore, novel mechanistic approaches deciphering insulin resistance are needed. Many pathophysiological factors are implicated in insulin resistance [5, 7]. Although the exact natures of these factors are not completely understood, it is widely accepted that oxidative stress, inflammation, and genetic, habitual, environmental, and other epigenetic factors play a significant role.

In the past two decades, significant strides have been made in elucidating important mechanisms associated with insulin resistance, overt diabetes, and related cardiometabolic diseases. However, more intense research is still needed for a more comprehensive understanding of the pathophysiological profile of insulin resistance in diabetes, especially in situations where diabetes is comorbid with other chronic diseases. Therefore, this special issue is a collection of research and review papers that address a broad range of mechanisms associated with insulin resistance, type 1 diabetes, type 2 diabetes, and related cardiometabolic complications. A common pathophysiological destructive force in type 1 and type 2 diabetes is the high levels of advanced glycation end products generated by hyperglycaemia [1–3]. To unveil further insights on advanced glycation end products, T. Okura et al. wrote an article on the putative pathophysiological role of advanced glycation end products on deregulation of insulin signalling in type2 diabetes. To further expatiate on dysfunctional insulin signalling, F. De C. Cartolano et al. investigated the impact of insulin resistance on lipid metabolism at preclinical level and found that insulin resistance and diabetes are powerful predictors of quantitative and qualitative features of lipoprotein dysfunction and are directly associated with increased atherogenic risk.

Dyslipidaemia, obesity, and visceral adiposity are common risk factors for insulin resistance, type 2 diabetes, and

cardiovascular complications [8, 9]. To shed more insight on this theme, C. Fujii et al. investigated the relationships between the composition of free fatty acids and metabolic parameters and found that serum linoleic acid is negatively correlated with the accumulation of visceral fat and insulin resistance. On the other hand, Y. Long et al. investigated the effects of overexpressing gamma-glutamyltransferase on insulin sensitivity and found the short-term overexpression of liver-specific gamma-glutamyltransferase ameliorates insulin sensitivity. Although liver disease commonly occurs in diabetes, other organ complications including cardiomyopathy, neuropathy, and nephropathy are highly documented [1, 2, 4, 5]. To underscore the role of the kidney in diabetes and hypertension, U. Kampmann et al. gave their insights on effects of renal denervation on insulin sensitivity in nondiabetic patients with treatment for resistant hypertension in an article featuring in this special issue. On the other hand, to give further insight on diabetic neuropathy, L. Luo et al. wrote an article underscoring gene profiles of neurotrophin-mitogen-activated protein kinase (MAPK) signalling in patients with diabetic peripheral neuropathy.

Besides obesity, another important habitual factor that affects the development of insulin resistance and type2 diabetes is sedentary lifestyle. Moreover, sedentary lifestyle is one of the modifiable risk factors of type 2 diabetes and the value of exercise to improve insulin signalling and glucose metabolism cannot be overemphasized [10]. Accordingly, in a research article, C. J. Gaffney et al. investigated how acute restoration of normoglycemia affected energy metabolism during exercise in nonobese patients with type 2 diabetes. The authors reported that insulin-induced normoglycemia increased blood glucose during subsequent exercise without altering overall substrate utilization. Similarly, in another article on exercise, S. Benedini et al. underscored the benefits of sports in enhancing insulin sensitivity and glucose metabolism and suggested a putative role of the exercise-induced myokine and irisin in the beneficial effects of exercise on glucose metabolism. Irisin is a protein produced in the muscles during exercise [11]. Generally, small increments of irisin levels in the blood trigger a parallel increment in energy expenditure in mammals with no changes in movement or food intake [11].

Besides exercise, weight-loss surgeries are referred to as bariatric surgery is adopted to reduce health risks associated with obesity [12]. Among the different types of bariatric surgeries is Roux-en-Y gastric bypass surgery [12]. In a related article featuring in this special issue, X. Zhao et al. explored the impact of Roux-en-Y gastric bypass surgery on cardiovascular risk in diabetic patient comorbid with obesity. The authors showed that the Roux-en-Y gastric bypass surgery is an effective strategy to mitigate cardiovascular risk in diabetic patients with comorbid of obesity. In addition to surgery, another emerging therapeutic pathway featuring in this special issue is a review article by J. A. David et al. where the authors highlighted the role of the nuclear-factor-E2-related-factor-2 (Nrf2)/kelchlike ECH-associated-protein-1 (Keap1)/antioxidant response element (ARE) pathway as a target for prevention, prognosis, and treatment of type 2 diabetes. The Nrf2/Keap1/ARE has been well documented for its cytoprotective effects, with potential therapeutic applications [13].

Collectively, the articles featuring in this special issue cover a wide breadth of topics of great interest and would benefit a wide audience.

Joseph Fomusi Ndisang
Alfredo Vannacci
Sharad Rastogi

References

[1] M. Mishra and J. F. Ndisang, "A critical and comprehensive insight on heme oxygenase and related products including carbon monoxide, bilirubin, biliverdin and ferritin in type-1 and type-2 diabetes," *Current Pharmaceutical Design*, vol. 20, no. 9, pp. 1370–1391, 2014.

[2] J. F. Ndisang and A. Jadhav, "Hemin therapy improves kidney function in male streptozotocin-induced diabetic rats: role of the heme oxygenase/atrial natriuretic peptide/adiponectin axis," *Endocrinology*, vol. 155, no. 1, pp. 215–229, 2014.

[3] M. C. Petersen, D. F. Vatner, and G. I. Shulman, "Regulation of hepatic glucose metabolism in health and disease," *Nature Reviews Endocrinology*, vol. 13, no. 10, pp. 572–587, 2017.

[4] J. F. Ndisang, A. Jadhav, and M. Mishra, "The heme oxygenase system suppresses perirenal visceral adiposity, abates renal inflammation and ameliorates diabetic nephropathy in Zucker diabetic fatty rats," *PLoS One*, vol. 9, no. 1, article e87936, 2014.

[5] P. Hossain, B. Kawar, and M. El Nahas, "Obesity and diabetes in the developing world—a growing challenge," *The New England Journal of Medicine*, vol. 356, no. 3, pp. 213–215, 2007.

[6] M. Vladu, D. Clenciu, I. C. Efrem et al., "Insulin resistance and chronic kidney disease in patients with type 1 diabetes mellitus," *Journal of Nutrition and Metabolism*, vol. 2017, Article ID 6425359, 5 pages, 2017.

[7] J. F. Ndisang and A. Jadhav, "Heme arginate therapy enhanced adiponectin and atrial natriuretic peptide, but abated endothelin-1 with attenuation of kidney histopathological lesions in mineralocorticoid-induced hypertension," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 334, no. 1, pp. 87–98, 2010.

[8] L. Duvnjak and M. Duvnjak, "The metabolic syndrome: an ongoing story," *Journal of Physiology and Pharmacology*, vol. 60, Supplement 7, pp. 19–24, 2009.

[9] J. F. Ndisang and M. Mishra, "The heme oxygenase system selectively suppresses the proinflammatory macrophage m1 phenotype and potentiates insulin signaling in spontaneously hypertensive rats," *American Journal of Hypertension*, vol. 26, no. 9, pp. 1123–1131, 2013.

[10] B. K. Pedersen, "Anti-inflammatory effects of exercise: role in diabetes and cardiovascular disease," *European Journal of Clinical Investigation*, vol. 47, no. 8, pp. 600–611, 2017.

[11] P. Bostrom, J. Wu, M. P. Jedrychowski et al., "A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis," *Nature*, vol. 481, no. 7382, pp. 463–468, 2012.

[12] G. K. Dimitriadis, M. S. Randeva, and A. D. Miras, "Potential hormone mechanisms of bariatric surgery," *Current Obesity Reports*, vol. 6, no. 3, pp. 253–265, 2017.

[13] Z. Zhang, S. Zhou, X. Jiang et al., "The role of the Nrf2/Keap1 pathway in obesity and metabolic syndrome," *Reviews in Endocrine & Metabolic Disorders*, vol. 16, no. 1, pp. 35–45, 2015.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 6915310, 9 pages
https://doi.org/10.1155/2017/6915310

Research Article

Effects of Renal Denervation on Insulin Sensitivity and Inflammatory Markers in Nondiabetic Patients with Treatment-Resistant Hypertension

Ulla Kampmann,[1] Ole N. Mathiassen,[2] Kent L. Christensen,[2] Niels H. Buus,[3] Mette Bjerre,[4] Henrik Vase,[2] Niels Møller,[1,4] Anne Kaltoft,[2] and Per L. Poulsen[1]

[1]*Department of Endocrinology and Internal Medicine, Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark*
[2]*Department of Cardiology, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, 8200 Aarhus, Denmark*
[3]*Department of Nephrology, Aalborg University Hospital, Hobrovej 18-22, 9000 Aalborg, Denmark*
[4]*Medical Research Laboratory, Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark*

Correspondence should be addressed to Ulla Kampmann; ulla@opstrup.dk

Received 28 March 2017; Revised 9 July 2017; Accepted 25 July 2017; Published 7 September 2017

Academic Editor: Joseph F. Ndisang

Increased sympathetic activity is important in the pathogenesis of hypertension and insulin resistance. Afferent signaling from the kidneys elevates the central sympathetic drive. We investigated the effect of catheter-based renal sympathetic denervation (RDN) on glucose metabolism, inflammatory markers, and blood pressure in nondiabetic patients with treatment-resistant hypertension. Eight subjects were included in an open-labelled study. Each patient was studied before and 6 months after RDN. Endogenous glucose production was assessed by a 3-^{3}H glucose tracer, insulin sensitivity was examined by hyperinsulinemic euglycemic clamp, hormones and inflammatory markers were analyzed, and blood pressure was measured by office blood pressure readings and 24-hour ambulatory blood pressure monitoring. Insulin sensitivity (M-value) increased nonsignificantly from 2.68 ± 0.28 to 3.07 ± 0.41 ($p = 0.12$). A significant inverse correlation between the increase in M-value and BMI 6 months after RDN ($p = 0.03$) was found, suggesting beneficial effects on leaner subjects. Blood pressure decreased significantly, but there were no changes in hormones, inflammatory markers, or endogenous glucose production. Our results indicate that RDN may improve insulin sensitivity in some patients with treatment-resistant hypertension, albeit confirmation of these indications of beneficial effects on leaner subjects awaits the outcome of larger randomized controlled studies.

1. Introduction

Hypertension is associated with impaired glucose metabolism and insulin resistance [1], and an increased central sympathetic activity plays an important role in both conditions. Afferent signaling from the kidneys is an important contributor to the elevated central sympathetic drive, leading to hypertension, insulin resistance, heart failure, and potentially chronic kidney disease [2]. Despite the availability of many safe and effective antihypertensive drugs, blood pressure in many patients remains poorly controlled and there has been an increasing interest in the use of catheter-based renal denervation (RDN) to treat resistant hypertension [3]. The clinical results on RDN for the treatment of resistant hypertension are however diverse [3–6]. The overall effect of RDN on blood pressure is questionable, but it seems that there might be responders and nonresponders. Further studies have also been suggested to assess the effectiveness of RDN treatment for heart failure, insulin resistance, obstructive sleep apnea, atrial fibrillation, and end-stage renal disease [3]. In the current study, we investigated the effect of RDN on glucose metabolism, using gold standard methods, that is, hyperinsulinemic euglycemic clamp (HEC) and glucose isotope dilution, to assess hepatic and peripheral insulin sensitivity. As increased inflammation is associated with both hypertension and insulin resistance [7, 8], we also evaluated the effect of RDN on inflammatory markers. Accordingly, our study was designed to test

whether an attenuation of the sympathetic nervous system by RDN would improve both insulin sensitivity and blood pressure and reduce inflammation.

2. Material and Methods

2.1. Study Participants. Eight patients with treatment-resistant hypertension (6 males and 2 females), defined as daytime 24-hour ambulatory blood pressure monitoring (ABPM) systolic blood pressure ≥ 145 mmHg (preceded by 1 month of scheduled drug intake showing at least 85% adherence), were included in the study. The inclusion criteria for treatment-resistant hypertension did not strictly follow AHA guidelines, as the patients in the current study were recruited from the Reset Study [9]. The study criteria are presented in Table 1. The participants were healthy, except for having treatment-resistant hypertension, as assessed by self-report, previous medical history, a physical examination, and a broad biochemical profile. Secondary forms of hypertension were excluded by means of computed axial tomography (CT) imaging of renal arteries, echocardiography, and hormone analysis. Prior to the inclusion of the current study, the 8 subjects had participated in the Reset Study [9] as part of the sham group, ensuring that drug intake was sufficient the preceding 6 months before inclusion in our study.

The participants were asked to fill in a questionnaire at baseline and at follow-up 6 months after RDN, concerning medical history, medication, smoking habits, physical activity, diet, and sleeping patterns, and they were asked not to implement changes in lifestyle during the intervention period. Changes in antihypertensive medication during follow-up were only allowed if requested by the patient or if potentially harmful changes in blood pressure arose. Study subjects were told to refrain from major physical exercise 48 hours prior to both study days.

The participants gave a written informed consent prior to the study participation. The study was conducted in accordance with the Helsinki Declaration, and the study protocol was reviewed and approved by the Regional Ethical Committee. The protocol was registered at Clinicaltrials.gov. NCT01631370.

2.2. Design. The study was open-labelled, where each patient was studied on two occasions, shortly before RDN and 6 months after RDN.

On the day of the study, the subjects rested on bed from 0730 h in a quiet, thermoneutral environment. The subjects fasted from the night before and during the experiments. An intravenous cannula was inserted into an antecubital vein for infusions, and another intravenous cannula was inserted into a dorsal hand vein for blood sampling. The latter was maintained heated, allowing for arterialized blood samples to be drawn. Plasma glucose levels were determined every 10 min during the clamp period, and blood samples were drawn at $t = 0$, 100, 110, 120, 220, 230, and 240 min.

At first, a 2-hour basal period ($t = 0$–120 min) was performed with infusion of a 3-^{3}H glucose tracer to assess endogenous glucose production. The basal period was followed by a 2-hour clamp period ($t = 120$–240 min) with

Table 1: Study criteria.

Inclusion
(i) Age 30 to 70 years
(ii) One month of stable antihypertensive treatment with at least three antihypertensive agents including a diuretic (or in case of diuretic intolerance, a minimum of three nondiuretic antihypertensive drugs)
(iii) Daytime ABPM systolic blood pressure ≥ 145 mmHg (preceded by 1 month of scheduled drug intake showing at least 85% adherence)
Exclusion
General
(i) Noncompliant personality (abuse, mental illness)
(ii) Pregnancy/inadequate contraception in fertile women
(iii) Known allergy to iodine-containing X-ray contrast agent
Comorbidity
(i) Diabetes
(ii) Secondary hypertension
(iii) Malignant disease
(iv) Congestive heart failure NYHA 3-4
(v) Chronic renal failure stages 4-5 (eGFR ≤ 30 ml/min/1.73m^2)
(vi) Stable angina pectoris (CCS classes 2–4)
(vii) Unstable angina pectoris
(viii) Coronary artery disease with indication for coronary intervention
(ix) Recent myocardial infarction or coronary intervention (<6 months)
(x) Permanent atrial fibrillation
(xi) Orthostatic syncope (<6 months)
(xii) Symptomatic peripheral artery disease
Paraclinical
(i) Clinically significant abnormal electrolytes and liver function tests.
(ii) Hemoglobin < 7.0 mmol/l
(iii) Abnormal thyroid function
(iv) Macroscopic haematuria
(v) ECG: AV-block grades 2 and 3 or AV-block grades 1 + branch block
Echocardiography
(i) Left ventricular ejection fraction < 50%
(ii) Significant valvular disease
CT-angiography and selective angiography of renal arteries
(i) Pronounced calcification in iliaco-aortic or renal arteries
(ii) Multiple renal arteries: accessory renal arteries estimated to carry more than 10% of the kidney's blood supply (small polar arteries accepted) and being undersized (see below) for ablation procedure
(iii) Renal artery diameter < 4 mm
(iv) Renal artery length (from ostium to first major side branch) < 20 mm
(v) Renal artery disease (stenosis, fibromuscular dysplasia; prior intervention, dissection)

a HEC to assess insulin sensitivity. The 3-^{3}H glucose tracer infusion continued during the clamp period. Indirect calorimetry was performed during the last 30 minutes of both the basal period and the clamp period.

2.3. Blood Pressure Measurements. Office blood pressure readings were taken in a seated position 30 minutes after the end of the study day. Averages of the triplicate measures were used. ABPM was done using either the SpaceLab 90207 or 90217 ABPM monitor with BP readings every 20 minutes. Nighttime and daytime periods were defined as 23:00–07:00 (night) and 07:00–23:00 (day). A minimum of 50% successful readings during nighttime and daytime was demanded for each ABPM to qualify for analysis.

2.4. Hyperinsulinemic Euglycemic Clamp. The HEC was performed to assess insulin sensitivity. Insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) was given from $t = 120$ min as a continuous infusion at 0.8 mU $\times$ kg^{-1} $\times$ min^{-1} for 120 minutes. Insulin infusates were prepared in 19 ml of isotonic saline and 1 ml of the subject's blood to prevent adsorption of insulin to plastic surfaces and infused at 5 ml/hour. Plasma glucose was clamped at 5 mmol/l by adjusting the infusion rate of 20% glucose according to plasma glucose measurements. Plasma glucose was measured every 10 min immediately after sampling. Glucose was given with a carrier infusion of 0.9% NaCl. To prevent hypokalemia, 0.22 meq/l KCl was added the glucose infusate. Steady-state plasma glucose infusion rates during the last 30 minutes of the clamp were used for estimating insulin sensitivity (M-value) [10, 11].

2.5. Tracer. For the assessment of endogenous glucose production (EGP) during both the basal and the clamp period, the 3-^{3}H glucose tracer (New England Nuclear Life Science Products, Boston, MA, USA) was infused from $t = 0$–40 min (0.12 μCi/min). A priming dose of 3-^{3}H glucose (12 μCi) was given as a bolus of 1.5 ml prior to the constant infusion [12]. The tracer (3–^{3} H glucose) was added to the glucose infusion during the clamp period. Glucose flux rates were calculated at 10 min intervals from $t = 100$–120 and $t = 220$–240 min [13]. Glucose rate of appearance (Ra) was calculated from Steele's equation for a nonsteady state [14, 15]. During the clamp period, EGP was calculated by subtracting the rate of exogenous glucose infusion from the rate of appearance of 3–^{3}H glucose.

2.6. Indirect Calorimetry. The respiratory quotient (RQ) and resting energy expenditure (REE) were estimated by indirect calorimetry using a computerized flow through a canopy gas analyzer system (Deltatrac; Datex Instruments, Helsinki, Finland). Indirect calorimetry was performed during the last 30 minutes of the basal period and during the last 30 minutes of the clamp period. Mean values of the last 25 min were used for calculations. Lipid oxidation and glucose oxidation were estimated after correction for protein oxidation, which were calculated on the basis of urea nitrogen excretion [16].

2.7. Blood Analyses and Assays. Plasma levels of creatinine, electrolytes, lipids, HbA1c, TSH, and liver enzymes were determined by standard laboratory measures. Plasma glucose was measured in duplicate immediately after sampling on Beckman Glucoanalyzers (Beckman Instruments, Palo Alto, CA). Serum and EDTA plasma samples were frozen immediately after collection and stored at −20°C until the time of analyses.

The specific activity of 3-^{3}H glucose was measured as described [17]. Insulin (DAKO, Glostrup, Denmark), C-peptide (ALPCO, Salem, NH, USA), and cortisol (EIA-1887, AH-Diagnostic) were analyzed using ELISA-based kits. Free fatty acids (FFA) were analyzed with a commercial kit (Wako Chemicals, Neuss, Germany). Plasma cytokines (interleukin- (IL-) 1β, IL6, IL-10, IFN-γ, and TNF-α) were analyzed by Luminex Performance Human High Sensitivity Cytokine Magnetic Panel A (Bio-Techne, Abingdon, UK). Detection limit for the analytes was between 0.3 and 1.5 pg/ml. High-sensitive C-reactive protein (hsCRP, BAM 17072, and MAB 17071, R&D Systems Europe Ltd., Abingdon, UK) was analyzed using an in-house assay. Adiponectin was analyzed by a validated in-house time-resolved immunofluorometric assay (TRIFMA) as previously described [18]. The detection limit was 1.5 μg/l, and intra- and interassay coefficients of variation were <5% and <7%, respectively. Glucagon was analyzed by an in-house radioimmunoassay [19]. Total IGF-I was measured in acid ethanol-extracted serum, using an in-house TRIFMA as previously described [20].

2.8. Renal Denervation. RDN was carried out at one single invasive cardiovascular center and performed by one single experienced invasive cardiologist with a record of 45 procedures before initiating the current protocol. Patients were admitted in the morning and prepared for femoral artery catheterization with a 6F diagnostic catheter. Pretreatment included oral acetaminophen and 10 mg oral morphine. Unless previously examined, a coronary angiography was performed at first, in order to exclude possible asymptomatic severe proximal coronary stenosis. Thereafter, renal angiography was performed to confirm the findings from the renal CT angiography that renal artery anatomy was suitable for RDN therapy (Table 1). At this moment, sedation was administered (fentanyl, midazolam) and the Symplicity renal denervation catheter (Medtronic) was advanced, and four-to-six discrete, low-power radio frequency treatments were applied circumferentially along the length of each main renal artery, aiming at covering the entire lumen. After the procedure, patients were submitted to the ward for routine observation and were discharged in the evening or the next morning.

2.9. Safety. All adverse events and complication were systematically recorded. Specific interventional-related safety data included bleeding or femoral pseudoaneurysm requiring intervention, renal artery dissection, myocardial infarction, stroke, and death. Specific follow-up-related safety record concerned blood pressure, renal function, electrolyte disarrangement, stroke, transitory ischemic attack, myocardial infarction, and symptomatic hypotension.

Table 2: Anthropometric and biochemical measures.

Characteristics	Baseline	6 months after RDN	p value
Men/women	6/2		
Age (years)	62.5 ± 2.55		
BMI (kg/m^2)	28.9 (26.6; 34.0)	29.9 (26.4; 34.2)	0.94
Fasting plasma glucose (mmol/l)	6.31 ± 0.35	6.14 ± 0.31	0.29
HbA1c (mmol/mol)	37.0 (35.0; 39.0)	37.5 (36.0; 40.5)	0.58
P-sodium (mmol/l)	142.4 ± 0.68	141.9 ± 0.74	0.52
P-potassium (mmol/l)	3.45 (3.15; 3.60)	3.55 (3.40; 3.65)	0.06
P-total cholesterol (mmol/l)	5.26 ± 0.41	5.25 ± 0.36	0.91
P-LDL cholesterol (mmol/l)	3.18 ± 0.34	3.19 ± 0.31	0.94
P-HDL cholesterol (mmol/l)	1.33 ± 0.11	1.34 ± 0.14	0.74
P-triglycerides (mmol/l)	1.45 (1.05; 2.25)	1.35 (1.10; 1.95)	0.94
P-TSH ($\times 10^{-3}$ ie/l)	1.95 (1.36; 2.81)	1.80 (1.40; 2.73)	0.38
P-ALAT (I/U)	23.50 ± 3.82	23.63 ± 4.83	0.93
P-creatinine (μmol/l)	86.88 ± 7.52	84.38 ± 7.62	0.53
eGFR (ml/min)	72.38 ± 4.80	76.00 ± 4.67	0.31
B-leucocytes ($\times 10^9$/l)	5.36 ± 0.39	5.49 ± 0.46	0.76
B-thrombocytes ($\times 10^9$/l)	194.4 ± 15.9	188.1 ± 14.6	0.40
B-hemoglobin (mmol/l)	9.04 ± 0.26	8.93 ± 0.24	0.41

All data are presented as means with SE or median (range).

2.10. Statistical Analysis. Results are expressed as mean ± SE (parametric data) or median (range) (nonparametric data). Data were tested for normal distribution using the Shapiro-Wilk test. Differences between baseline and endpoint measures and between basal and clamp data were assessed using a one-sample t-test or Wilcoxon signed-rank test, as appropriate. A p value <0.05 was considered statistically significant. Pearson correlation was used to test for correlations. Power calculations were based on the primary endpoint, namely, M-values, and a detectable difference in M-values was 14%. It could be calculated that eight patients would be required to demonstrate a significant difference at 80% power and 5% significance. All calculations were carried out using Sigma Plot version 11.0.

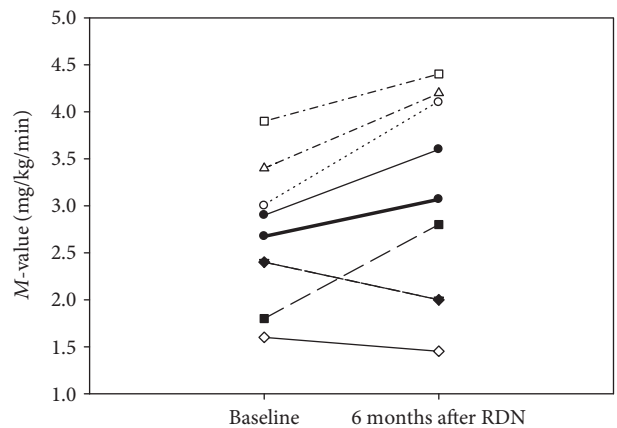

Figure 1: M-values for the 8 subjects at baseline and at 6 months after RDN. Two subjects have the same M-values and cannot be separated from each other in the graph (from 2.4 to 2.0 after 6 months). The thick black line depicts mean values of the M-values (2.86 versus 3.07; $p = 0.12$).

3. Results

3.1. Patient Characteristics and Medication. Clinical characteristics of the study subjects are presented in Table 2. Six men and 2 postmenopausal women were included. Subjects were nondiabetic with a HbA1C at 37.0 (35.0; 39.0) mmol/mol. There were no significant changes in BMI, HbA1c, fasting plasma glucose, or lipid profile before and 6 months after renal denervation. There were no changes in prescription during the six-month follow-up. Medication for cardiovascular disease and hypertension comprised the following: ace inhibitor ($n = 2$), angiotensin receptor blocker ($n = 6$), calcium channel blocker ($n = 7$), beta blocker ($n = 3$), thiazide diuretic ($n = 3$), aldosterone inhibitor ($n = 1$), alpha adrenergic blocker ($n = 4$), centrally acting sympatholytic agent ($n = 1$), acetylsalicylic acid ($n = 5$), and lipid-lowering treatment ($n = 2$).

3.2. Insulin Sensitivity, Endogenous Glucose Production, and Hormones. Insulin sensitivity expressed as M-value improved nonsignificantly from 2.68 ± 0.28 to 3.07 ± 0.41 mg/kg/min ($p = 0.12$) (Figure 1). However, a significant inverse correlation between the increase in M-value and BMI 6 months after RDN ($p = 0.03$) (Figure 2) was found. EGP decreased insignificantly both during the basal period (1.73 ± 0.16 versus 1.36 ± 0.19 mg/kg/min ($p = 0.27$)) and during the clamp period (0.62 ± 0.14 versus 0.36 ± 0.28 mg/kg/min ($p = 0.52$)) 6 months after RDN. There were no significant changes in C-peptide, glucagon, free

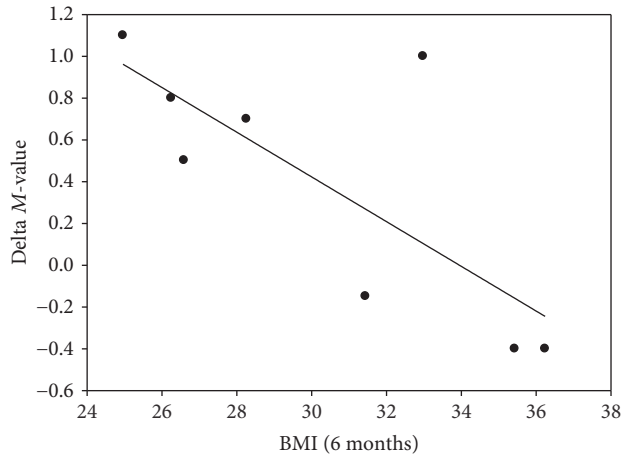

Figure 2: Correlation between BMI at 6 months after RDN and the difference in M-value before RDN and 6 months after RDN. $R = 0.75$; $p = 0.03$.

fatty acids (FFA), insulin, cortisol, or IGF-I after 6 months (Table 3).

3.3. Systemic Inflammation. Interferon-γ, IL-1β, IL-6, IL-10, and TNF-α were analyzed, but as cytokine levels were very low, only IL-1β, IL-6, and TNF-α were detectable and were not affected significantly 6 months after RDN (Table 3).

3.4. Energy Expenditure. Fasting and insulin-stimulated resting energy expenditure (EE) and respiratory quotient (RQ) were obtained by indirect calorimetry and did not change significantly 6 months after RDN. Glucose oxidation, however, decreased significantly ($p = 0.018$) during the clamp period after RDN. Concurrently, lipid oxidation increased the borderline significantly ($p = 0.06$), whereas protein oxidation was practically unchanged (Table 3).

3.5. Blood Pressure Data. Six months after RDN, office systolic blood pressure and office diastolic blood pressure decreased significantly from 180.4 ± 6.79 to 164 ± 8.06 ($p = 0.01$) and 94.88 ± 4.72 to 87.88 ± 3.63 ($p = 0.04$), respectively. There were a significant borderline decrease in nighttime ABPM systolic blood pressure ($p = 0.06$) and a significant decrease in nighttime ABPM diastolic blood pressure ($p = 0.03$) (Table 4).

4. Discussion

This study, based on a small sample size, showed no overall effect of RDN on insulin sensitivity, assessed by the HEC. The significant inverse correlation between the increase in M-value and BMI 6 months after RDN could indicate that RDN could have a potential effect on nonobese subjects, without severe insulin resistance. This is in a way a puzzling finding as obesity contributes to an increase in sympathetic nervous system activity and obesity itself contributes to insulin resistance. However, one could speculate that the most obese subjects in the current study are so insulin resistant due to their obesity that the renal denervation is relatively less effective compared to the leaner subjects that are less insulin resistant. Our findings are in line with the recently published study by Miroslawka et al. [21] where 23 patients with treatment-resistant hypertension underwent RDN and a two-step HEC with glucose tracer infusion was performed before and 6 months after RDN. The authors found no improvement in insulin sensitivity, but 18 subjects had the metabolic syndrome and the mean BMI for all 23 patients was $32\,kg/m^2$, compared to only $29\,kg/m^2$ in our study. In addition, Miroslawka et al. found an insignificant decrease in EGP, whereas we found an insignificant decrease in EGP both during the basal and the clamp period 6 months after RDN, compatible with a modest improvement in hepatic insulin sensitivity. This is supported by a recent study, in which it was found that in a nonhypertensive obese canine model, RDN completely normalized hepatic insulin sensitivity (EGP), assessed by the HEC, in high-fat diet-fed animals, compared to sham animals. The authors accordingly suggested that the renal nerves play a role in the regulation of insulin action specifically as regards EGP [22]. In our study, glucose oxidation also decreased significantly during the clamp period after RDN whereas lipid oxidation concurrently increased the borderline significantly, possibly as a result of a lower EGP after RDN.

In parallel with the Symplicity HTN-2 trial [4], we found a significant decrease in office-based blood pressure measurements after RDN. However, the decrease in blood pressure could relate to inadequacies of the study design, as subsequent more rigorously designed clinical trials using a sham procedure and ABPM have failed to demonstrate a BP-lowering effect [5, 6, 9]. In the current study, it is plausible that systolic office blood pressure falls significantly as the subjects are more used to the procedures on the second day of examination. Therefore, the 24-hour ambulatory blood pressure measurements are much more valid.

Hypertension is associated with the infiltration of T cells into the kidney and vasculature, with the release of cytokines, including IFN-γ and TNF-α, which promote sodium retention, vasoconstriction, and oxidative injury [7]. Obesity causes lipid accumulation in adipocytes that can increase the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, and this obesity-associated chronic low-grade inflammation leads to insulin resistance [23]. Moreover, increased levels of inflammatory markers such as CRP and white cell blood count correlate with incident type 2 diabetes [8]. In our study, we could not demonstrate an improvement in inflammatory markers 6 months after RDN; it should however be noted that the levels of some cytokines before RDN were below detection limit, making detection of any change analytically problematic.

One of the limitations of the study is the small sample size, although 8 subjects should suffice for fulfilling the purpose of the study. Power calculations were based on the primary endpoint, namely, the M-values found in the hyperinsulinemic euglycemic clamp. Clamp studies are labour intensive and technically demanding and are therefore most commonly conducted in a restricted number of subjects, often including around 8–10 subjects. Moreover, the method had only been applied to 2 subjects treated with RDN [24] when our study was designed, and therefore, there was no

Table 3: Circulating hormones, cytokines, and metabolic parameters.

	Baseline values		6 months after RDN		p value	p value	p value	p value
	Basal*	Clamp**	Basal	Clamp	Difference between basal and clamp values at baseline	Difference between basal and clamp values 6 months after RDN	Difference between basal values at baseline and 6 months after RDN	Difference between clamp values at baseline and 6 months after RDN
M-value (mg/kg/min)		2.68 ± 0.28		3.07 ± 0.41				0.12
P-C-peptide (pmol/l)	795.0 ± 121.8	466.0 ± 89.86	920.4 ± 209.7	529.3 ± 124.7	0.001	0.009	0.32	0.18
P-glucagon (pg/ml)	67.13 ± 5.81	48.63 ± 4.35	65.50 ± 6.99	45.88 ± 4.36	<0.001	<0.001	0.73	0.47
P-FFA (mmol/l)	0.52 ± 0.05	0.06 ± 0.009	0.53 ± 0.07	0.05 ± 0.012	<0.001	<0.001	0.88	0.14
P-insulin (pmol/l)	81.88 ± 13.41	500.0 ± 63.53	92.50 ± 11.56	495.8 ± 75.51	<0.001	0.008	0.20	0.88
P-cortisol (ng/ml)	137.3 ± 15.72		145.6 ± 15.53				0.46	
IGF-I (ng/ml)	124.3 ± 9.79		132.8 ± 12.21				0.14	
Adiponectin (mg/l)	12.94 ± 0.79		12.72 ± 1.16				0.64	
hsCRP (mg/l)	4.60 ± 1.28		4.99 ± 1.14				0.65	
TNF-α (pg/ml)	4.62 ± 0.93		5.21 ± 0.81				0.21	
IL-6 (pg/ml)	0.64 ± 0.11		0.67 ± 0.09				0.78	
IL-1β (pg/ml)	0.04 ± 0.006		0.05 ± 0.01				0.15	
EGP (mg/kg/min)	1.73 ± 0.16	0.62 ± 0.14	1.36 ± 0.19	0.36 ± 0.28	0.002	0.004	0.27	0.52
EE (kcal)	1950 ± 108	1917 ± 114	1901 ± 105	1881 ± 96	0.19	0.48	0.43	0.53
RQ	0.82 ± 0.01	0.88 ± 0.01	0.84 ± 0.01	0.88 ± 0.02	0.007	0.03	0.43	1.00
Glucose oxidation. (mg/kg/min)	1.11 ± 0.21	1.58 ± 0.13	1.31 ± 0.28	0.92 ± 0.21	0.06	0.22	0.62	0.018
Protein oxidation (mg/kg/min)	0.69 ± 0.05	0.67 ± 0.05	0.74 (0.65;1.09)	0.86 (0.59;0.94)	0.98	1.0	0.26	0.36
Lipid oxidation (mg/kg/min)	0.65 ± 0.06	0.42 ± 0.08	0.55 ± 0.05	0.78 ± 0.12	0.002	0.19	0.29	0.06

*Basal is at time = 0 min except for EGP (endogenous glucose production), where basal is at time = 120 min; **clamp is at time = 240 min. All data are presented as means with SE or median (range).

Table 4: Blood pressure measurements.

	Baseline	6 months after RDN	p value
Office systolic blood pressure (mmHg)	180.4 ± 6.79	164.8 ± 8.06	0.01
Office diastolic blood pressure (mmHg)	94.88 ± 4.72	87.88 ± 3.63	0.04
Daytime ABPM systolic (mmHg)	158.3 ± 4.72	154.4 ± 5.53	0.49
Daytime ABPM diastolic (mmHg)	89.43 ± 2.84	88.29 ± 4.32	0.74
Nighttime ABPM systolic (mmHg)	151.3 ± 5.78	138.1 ± 8.00	0.06
Nighttime ABPM diastolic (mmHg)	83.71 ± 2.80	75.57 ± 4.64	0.03

All data are presented as means with SE or median (range). ABMP: 24-hour blood pressure measurements ($n = 7$).

precedent that allowed us to compare our results or power calculations with those of other studies. Later, Miroslawka et al. [21] published a study including 23 subjects with treatment-resistant hypertension who were examined by an HEC before and 6 months after RDN. However, as mentioned previously, most of the participants in the study suffered from the metabolic syndrome and were more obese than the participants in our study.

Another limitation is that we used the unipolar Symplicity Flex catheter and not a multipolar catheter in our study. Accordingly, we cannot exclude that the failure of RDN to improve insulin resistance in all 8 subjects is due to insufficient ablation or recurrence due to nerve regeneration after 6 months.

Using an open-labelled study design, the significant reduction in blood pressure and the partial improvement in insulin sensitivity could be explained by the positive "placebo" effects of being included in a study, potentially leading to a greater awareness of a healthier lifestyle and a better compliance to medication. The observed reductions in blood pressure should therefore be interpreted cautiously as previously mentioned.

Another point of criticism could also be that both males and females were included in the study. However, as we only included postmenopausal women, we considered them to be comparable to the men in our study group. Moreover, we performed a subanalysis only including the men, and the data on the most important outcomes (M-value and blood pressure) only showed a significant change in office diastolic blood pressure, but as 24-hour ambulatory blood pressure measurements are more precise, we do not find the change highly relevant.

On the other hand, our study design also has strengths. One single, highly skilled, and experienced invasive cardiologist performed the RDN, potentially increasing the rate of success. In addition, to assess insulin sensitivity, we used the gold standard method, HEC, and to assess hepatic insulin sensitivity, we used a glucose tracer. Until recently, all studies focusing on the effects of RDN on glucose metabolism have used less suited measures like HOMA to assess insulin sensitivity. Thus, in the study by Mahfoud et al., 50 patients with treatment-resistant hypertension were included in a study where 37 patients underwent RDN and 13 patients comprised a control group. Forty percent of the participants had type 2 diabetes before inclusion in the study. Fasting glucose, insulin, C-peptide levels, and insulin resistance assessed by HOMA-IR decreased significantly 3 months after RDN, whereas there were no significant changes in the control group [25]. In the DREAMS study by Verloop et al., 29 patients with the metabolic syndrome were treated with RDN. Insulin sensitivity was assessed by the simple index assessing insulin sensitivity through the oral glucose tolerance test and did not change 6 and 12 months after RDN [26].

Also, in the studies just mentioned, most of the participants had diabetes or prediabetes, making the study groups more heterogeneous compared to subjects in our study.

To our knowledge, no other study has assessed the effects of RDN on inflammatory markers, a parameter that could be a contributor to the association between hypertension and insulin resistance. In addition, the finding that RDN may improve insulin sensitivity in leaner subjects is also of novelty.

5. Conclusions

In this relatively small, nonrandomized study, using gold standard methods to assess insulin sensitivity, there were no significant changes in basal and insulin-stimulated glucose disposal and EGP after RDN. We did however find an overall trend towards improved insulin sensitivity largely driven by more pronounced changes in leaner subjects, suggesting that RDN may be specifically beneficial in this subgroup.

Ideally, reducing sympathetic tone by RDN could improve both blood pressure and insulin sensitivity, thus targeting two important risk factors of cardiovascular disease. Currently, the challenge lies in identifying subgroups that could benefit from RDN. For now, there is not enough evidence to expand the indication for RDN to include insulin resistance, as further and larger studies are needed.

6. Limitations

The most important limitation of the current study is the small sample size, but as previously mentioned, there was no precedent that allowed us to compare our results or power calculations with those of other studies when our study was designed. Power calculations were based on the primary endpoint, the M-values found in the hyperinsulinemic euglycemic clamp. The SD of the M-value has been shown to be 10% in previous studies in our laboratory, and λ is the

difference that we wished to detect, in this case 14%. The power calculation was therefore $N = 2 \times 7.9 \times (10/14)^2 = 8$. Consequently, eight patients would be required to demonstrate a significant difference at 80% power and 5% significance.

Another important limitation of the study is the fact that we included both men and postmenopausal women in our study, but as described in the study by Wassertheil-Smoller et al. [27], women aged <55 years tend to have lower prevalence rates of hypertension, compared with men, but women aged 55 to 74 years have similar rates. The women in our study were aged between 55 and 74, making them comparable to the men in our study group. We, however, performed subanalyses only including the men, but the results did overall not change significantly. We therefore decided to pool male and female patients in order to get a larger "*n*."

Abbreviations

RDN: Renal denervation
HEC: Hyperinsulinemic euglycemic clamp
ABPM: 24-hour ambulatory blood pressure monitoring
EGP: Endogenous glucose production
CT: Computed axial tomography
Ra: Rate of appearance
RQ: Respiratory quotient
REE: Resting energy expenditure
FFA: Free fatty acids.

Conflicts of Interest

The contributing authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Ulla Kampmann collected, analyzed, and interpreted the data and wrote the manuscript. Ole N. Mathiassen, Ulla Kampmann, Henrik Vase, and Per L. Poulsen designed the study, interpreted the data, and wrote the manuscript. Kent L. Christensen, Niels H. Buus, and Mette Bjerre collected and analyzed the data. Niels Møller interpreted the data and wrote the manuscript. Anne Kaltoft performed the renal denervation and collected and interpreted the data. All authors have approved the final article.

Acknowledgments

The authors wish to acknowledge the laboratory technician Annette Mengel for the invaluable assistance with biochemical analyses and assistance during the study day. The study was supported by a grant from the Danish Heart Foundation, and Anne Kaltoft has received speaker honoraria from Medtronic.

References

[1] E. Ferrannini and W. Cushman, "Diabetes and hypertension: the bad companions," *Lancet*, vol. 380, pp. 601–610, 2012.

[2] M. P. Schlaich, D. Hering, P. Sobotka et al., "Effects of renal denervation on sympathetic activation, blood pressure, and glucose metabolism in patients with resistant hypertension," *Frontiers in Physiology*, vol. 3, pp. 1–7, 2012.

[3] C. V. S. Ram and A. S. Kumar, "Renal denervation therapy for resistant hypertension: a clinical update," *Journal of Human Hypertension*, vol. 12, pp. 699–704, 2014.

[4] Simplicity HTN-2 Investigators, "Renal sympathetic denervation in patients with treatment-resistant hypertension (the simplicity HTN-2 trial): a randomised controlled trial," *Lancet*, vol. 376, pp. 1903–1909, 2010.

[5] D. L. Bhatt, D. E. Kandzari, W. W. O'Neill et al., "A controlled trial of renal denervation for resistant hypertension," *The New England Journal of Medicine*, vol. 370, pp. 1393–1401, 2014.

[6] S. Desch, T. Okon, D. Heinemann et al., "Randomized sham-controlled trial of renal sympathetic denervation in mild resistant hypertension," *Hypertension*, vol. 65, pp. 1202–1208, 2015.

[7] D. Harrison, "The immune system in hypertension," *Transactions of the American Clinical and Climatological Association*, vol. 125, pp. 130–138, 2014.

[8] S. Shoelson, J. Lee, and A. B. Goldfine, "Inflammation and insulin resistance," *The Journal of Clinical Investigation*, vol. 116, pp. 1793–1801, 2006.

[9] O. N. Mathiassen, H. Vase, J. N. Bech et al., "Renal denervation in treatment-resistant essential hypertension. A randomized, SHAM-controlled, double-blinded 24-h blood pressure-based trial," *Journal of Hypertension*, vol. 34, pp. 1639–1647, 2016.

[10] R. A. DeFronzo, J. D. Tobin, and R. Andres, "Glucose clamp technique: a method for quantifying insulin secretion and resistance," *The American Journal of Physiology*, vol. 237, pp. 214–223, 1979.

[11] U. Kampmann, P. Hoeyem, A. Mengel et al., "Insulin dose-response studies in severely insulin-resistant type 2 diabetes—evidence for effectiveness of very high insulin doses," *Diabetes, Obesity & Metabolism*, vol. 13, pp. 511–516, 2011.

[12] O. Hother-Nielsen and H. Beck-Nielsen, "On the determination of basal glucose production rate in patients with type 2 (non-insulin-dependent) diabetes mellitus using primed-continuous 3-^{3}H-glucose infusion," *Diabetologia*, vol. 33, pp. 603–610, 1990.

[13] R. Steele, "Influences of glucose loading and of injected insulin on hepatic glucose output," *Annals of the New York Academy of Sciences*, vol. 82, pp. 420–430, 1959.

[14] R. C. Debodo, R. Steele, N. Altszuler, A. Dunn, and J. S. Bishop, "On the hormonal regulation of carbohydrate metabolism; studies with C14 glucose," *Recent Progress in Hormone Research*, vol. 19, pp. 445–488, 1963.

[15] J. Radziuk, K. H. Norwich, and M. Vranic, "Experimental validation of measurements of glucose turnover in non-steady state," *The American Journal of Physiology*, vol. 234, pp. 84–93, 1978.

[16] E. Ferrannini, "The theoretical bases of indirect calorimetry: a review," *Metabolism*, vol. 37, pp. 287–301, 1988.

[17] N. Moller, P. C. Butler, M. A. Antsiferov, and K. G. Alberti, "Effects of growth hormone on insulin sensitivity and forearm metabolism in normal man," *Diabetologia*, vol. 32, pp. 105–110, 1989.

[18] J. Frystyk, L. Tarnow, T. K. Hansen, H. H. Parving, and A. Flyvbjerg, "Increased serum adiponectin levels in type 1

diabetic patients with microvascular complications," *Diabetologia*, vol. 48, pp. 1911–1918, 2005.

[19] H. Orskov, H. G. Thomsen, and H. Yde, "Wick chromatography for rapid and reliable immunoassay of insulin, glucagon and growth hormone," *Nature*, vol. 219, pp. 193–195, 1968.

[20] J. Frystyk, B. Dinesen, and H. Orskov, "Non-competitive time-resolved immunofluorometric assays for determination of human insulin-like growth factor I and II," *Growth Regulation*, vol. 5, pp. 169–176, 1995.

[21] A. K. Miroslawka, P. F. Gjessing, M. D. Solbu, O. M. Fuskevåg, T. G. Jenssen, and T. K. Steigen, "Renal denervation for resistant hypertension fails to improve insulin resistance as assessed by hyperinsulinemic euglycemic step clamp," *Diabetes*, vol. 65, pp. 2164–2168, 2016.

[22] M. S. Iyer, R. N. Bergman, J. E. Korman et al., "Renal denervation reverses hepatic insulin resistance induced by high-fat diet," *Diabetes*, vol. 65, pp. 3453–3463, 2016.

[23] L. Chen, R. Chen, H. Wang, and F. Liang, "Mechanisms linking inflammation to insulin resistance," *International Journal of Endocrinology*, vol. 2015, Article ID 508409, 9 pages, 2015.

[24] M. P. Schlaich, N. Straznicky, M. Grima et al., "Renal denervation: a potential new treatment modality for polycystic ovary syndrome?," *Journal of Hypertension*, vol. 29, pp. 991–996, 2011.

[25] F. Mahfoud, M. Schlaich, I. Kindermann et al., "Effect of renal sympathetic denervation on glucose metabolism in patients with resistant hypertension: a pilot study," *Circulation*, vol. 123, pp. 1940–1946, 2011.

[26] W. L. Verloop, W. Spiering, E. E. Vink et al., "Denervation of the renal arteries in metabolic syndrome: the DREAMS-study," *Hypertension*, vol. 65, pp. 751–757, 2015.

[27] S. Wassertheil-Smoller, G. Anderson, B. M. Psaty et al., "Hypertension and its treatment in postmenopausal women baseline data from the women's health initiative," *Hypertension*, vol. 36, pp. 780–789, 2000.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 1018796, 8 pages
https://doi.org/10.1155/2017/1018796

Research Article

Insulin Resistance Predicts Atherogenic Lipoprotein Profile in Nondiabetic Subjects

Flávia De C. Cartolano,[1] Gabriela D. Dias,[1] Maria C. P. de Freitas,[1] Antônio M. Figueiredo Neto,[2] and Nágila R. T. Damasceno[1]

[1]*Department of Nutrition, School of Public Health, University of Sao Paulo, São Paulo, SP, Brazil*
[2]*Department of Experimental Physics, Institute of Physics, University of Sao Paulo, São Paulo, SP, Brazil*

Correspondence should be addressed to Nágila R. T. Damasceno; nagila@usp.br

Received 20 March 2017; Revised 31 May 2017; Accepted 13 June 2017; Published 22 August 2017

Academic Editor: Joseph F. Ndisang

Background. Atherogenic diabetes is associated with an increased cardiovascular risk and mortality in diabetic individuals; however, the impact of insulin resistance (IR) in lipid metabolism in preclinical stages is generally underreported. For that, we evaluated the capacity of IR to predict an atherogenic lipid subfraction profile. *Methods*. Complete clinical evaluation and biochemical analysis (lipid, glucose profile, LDL, and HDL subfractions and LDL phenotype and size) were performed in 181 patients. The impact of IR as a predictor of atherogenic lipoproteins was tested by logistic regression analysis in raw and adjusted models. *Results*. HDL-C and Apo AI were significantly lower in individuals with IR. Individuals with IR had a higher percentage of small HDL particles, lower percentage in the larger ones, and reduced frequency of phenotype A (IR = 62%; non-IR = 83%). IR individuals had reduced probability to have large HDL (OR = 0.213; CI = 0.999–0.457) and had twice more chances to show increased small HDL (OR = 2.486; CI = 1.341–7.051). IR was a significant predictor of small LDL (OR = 3.075; CI = 1.341–7.051) and atherogenic phenotype (OR = 3.176; CI = 1.469–6.867). *Conclusion*. IR, previously DM2 diagnosis, is a strong predictor of quantitative and qualitative features of lipoproteins directly associated with an increased atherogenic risk.

1. Introduction

The negative impact of diabetes in cardiovascular risk factors, atherogenesis, and cardiovascular events is well stablished in literature; however, the role of insulin resistance (IR) previously DM2 diagnosis is not totally clear. Clinically, it was defined as the inability of glucose uptake and utilization by insulin-dependent tissues and reduced insulin sensitivity, being the basis of type 2 diabetes mellitus (DM2) [1–3]. Almost 415 million people around the world are suffering from DM2, and recent data estimated that 318 million adults have impaired glucose tolerance and IR is prevalent in 20 to 30% of general population and in 90% of patients with DM2 [4]. In 2014, 11.9 million of Brazilian lived with DM2 and, by 2035, it is estimated that this prevalence will increase to 19.2 million [5].

IR is linked with hypertriglyceridemia and high-density lipoprotein cholesterol (HDL-C) [6–8]; however, the relationship with low-density lipoprotein cholesterol (LDL-C) is contradictory [3]. These findings can be explained by compensatory hyperinsulinemia due to IR, which induces increased free fatty acid (FFA) efflux from adipose tissue, thus raising VLDL production in the liver and, consequently, plasma triacylglycerol (TAG) and also reducing HDL-C by activation of cholesterol ester transfer protein (CETP) and increased clearance by the kidneys [9, 10].

Recently, Li et al. [10] described the complex bidirectional relationship of lipoprotein homeostasis and IR. HDL acts in both, IR and β-cell function, improving insulin secretion, increasing insulin sensitivity in the target tissues (adipose and muscle cells), and promoting positive effects on β-cell survival. This relation was confirmed by

the association of qualitative and quantitative parameters of lipoprotein and DM2 [11, 12]. Up to now, few studies had analyzed lipoprotein subfractions in IR individuals before DM2 development [13, 14].

Regarding this background, the aim of this study was to compare the impact of IR effect on lipid metabolism and to evaluate if IR is a predictor of atherogenic lipoprotein profile in Brazilian individuals with IR and without DM2.

2. Methods

2.1. Subjects and Study Design. One hundred eighty-one adults of both genders were selected. Individuals were recruited from the University Hospital of the University of Sao Paulo. Subjects included in the study were 30 to 74 years old, without cardiovascular events (assessed by ECG and clinical evaluation) and without diagnosis of DM1 and DM2. Presence of DM was firstly evaluated by a direct interview using a structured questionnaire in which medical diagnosis of DM2 and current use of insulin and/or hypoglycemic drugs were self-reported by individuals. After, fasting glucose and insulin were analyzed to confirm DM2 diagnosis. If the fasting glucose level was close to the cut-off point (≥7.0 mmol/L), a second analysis was performed to confirm DM2, as recommended by Brazilian Diabetes Society [15]. Pregnant or lactating women, individuals who participate in other studies, illicit drug users, and alcoholics were not enrolled in this protocol. This study was approved by the Ethic Committee in Research of the University Hospital (n° 1126/11) and the School of Public Health, University of Sao Paulo (n° 2264). All subjects gave their written informed consent to participate and have their data published.

2.2. Demographic, Clinical, and Anthropometric Features. Demographic and clinical profile was evaluated using a structured questionnaire addressing gender, age, clinical information, family history of chronic diseases (father and mother), smoking status, blood pressure, and regular medication use.

From weight and height measures, body mass index (BMI) was calculated as weight (kg) divided by the square of the standing height (m^2). The waist circumference (WC) and body composition (BIA) (Analyzer® model Quantum II, RJL Systems, Michigan, USA) were also evaluated.

2.3. Biochemical Analysis. After 12 h of fasting, blood samples were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (1.0 μg/mL). The protease inhibitors aprotinin (10.0 μg/mL), benzamidine (10.0 μM), and phenylmethylsulfonyl fluoride (PMSF) (5.0 μM) plus the antioxidant butylated hydroxytoluene (BHT) (100.0 μM) were added to the samples. Plasma and serum were separated by centrifugation at 3000 rpm for 10 minutes at 4°C, and samples were kept frozen at −80°C until analysis.

Plasma triacylglycerol (TAG), total cholesterol (TC), and HDL-C levels were measured using commercial kits from Labtest® (Minas Gerais, Brazil). Low-density lipoprotein cholesterol (LDL-C) level was calculated using the Friedewald equation [16]. Non-HDL was calculated from TC minus HDL-C. The apolipoprotein B (APO B) and apolipoprotein AI (APO AI) were determined by standard methods, using the autokit APO A1 and APO B® (Wako Chemicals USA Inc., Richmond, VA, USA). Glucose level was analyzed by an enzymatic and colorimetric kit (Glucose PAP Liquiform®, Labtest, Minas Gerais, Brazil). The insulin was performed by a commercial kit Insulin Human Direct ELISA Kit® (Life Technologies, Grand Island, NY). Insulin resistance (IR) was calculated with the homeostasis model assessment-insulin resistance (HOMA-IR) as follows: [fasting insulin concentration (μU/mL) × fasting glucose (mmol/L)/22.5] [17]. IR classification was performed according to Stern et al. [18] that takes account HOMA-IR and BMI values: HOMA-IR > 4.65 or BMI > 28.90 kg/m^2 or HOMA > 3.60 and BMI > 27.50 kg/m^2. Based in these criteria, individuals were divided into the IR group and non-IR group.

The lipoprotein fractions (VLDL and IDL) and subfractions (HDL and LDL) were determined by the Lipoprint® system. The LDL1 and LDL2 subfractions were classified as LDL_{LARGE}, and subfractions LDL3 to LDL7 as smaller and denser particles (LDL_{SMALL}). For HDL, ten subfractions were identified: HDL_{LARGE} (HDL1 to HDL3), $HDL_{INTERMEDIATE}$ (HDL4 to HDL7), and HDL_{SMALL} (HDL8 to HDL10). The LDL phenotypes were based in cut-off points (phenotype A ≥ 268 Å and phenotype non-A < 268 Å). The mean LDL size was determined. All analyses were conducted in duplicate, and coefficients of variance intra- and interassay were 1–15%.

2.4. Statistical Analysis. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS®), version 20.0. A two-sided p value <0.05 was considered statistically significant. The Kolmogorov-Smirnov test ($p > 0.05$) was applied to assess normality of data. Continuous variables with normally distributed data are presented as mean values and standard deviations (SD) while nonnormally distributed data are presented as medians and 25th and 75th percentiles. Categorical variables are presented as absolute values (n) and percentage (%). The comparison between groups was performed using Student's t-test for normally distributed data. Nonnormally distributed data were analyzed using the nonparametric Mann–Whitney U test. Categorical variables were compared using Pearson's chi-square test. To identify the effect of IR on lipoprotein subfraction profile, univariate logistic regression analysis was performed using the IR as an independent factor. Afterward, variables that showed correlations with IR values ($p < 0.20$) have been included into a multivariate logistic regression analysis. HDL1, HDL4, and HDL10 did not match this criterion. Model A which included age and gender as covariate and additional adjustment, respectively, was made for smoking, statin, and/or fibrate use in model B. Adjusted odds ratio (AOR) and 95% confidence interval (CI) were determined.

3. Results

The average age was 51.3 (12.0) years old for the non-IR group and 52.5 (9.3) years old for the IR group. About 60% of the subjects were women, and both groups showed similar frequency of smoking ($p = 0.113$). As expected, the IR group presented higher values for weight, BMI, and WC (Table 1).

Table 1: Demographic, clinical profile, and anthropometry of the subjects according to the presence of insulin resistance.

Variables	Total ($n = 181$)	Non-IR group ($n = 64$)	IR group ($n = 117$)	p
Women (n, %)	110.0 (60.8)	40.0 (62.5)	70.0 (59.8)	0.725
Age, years (mean, SD)	52.1 (10.3)	51.3 (12.0)	52.5 (9.3)	0.476
Weight, kg (mean, SD)	80.1 (16.9)	67.4 (10.6)	87.0 (15.7)	**<0.001**
BMI, % (mean, SD)	30.1 (5.5)	25.0 (2.6)	32.9 (4.6)	**<0.001**
WC, cm (mean, SD)	98.3 (13.0)	86.1 (7.4)	105.0 (10.2)	**<0.001**
FM, % (mean, SD)	35.2 (11.9)	29.5 (9.8)	38.3 (11.9)	**<0.001**
SBP, mmHg (mean, SD)	134.3 (19.5)	130.2 (21.5)	136.6 (18.0)	**0.004**
DBP, mmHg (mean, SD)	82.0 (10.2)	78.5 (10.2)	83.9 (9.7)	**<0.001**
Smoking (n, %)	34.0 (18.8)	16.0 (25.0)	18.0 (15.4)	0.113
Statin (n, %)	45.0 (24.9)	15.0 (23.4)	30.0 (25.6)	0.743
Fibrate (n, %)	4.0 (2.2)	1.0 (1.6)	3.0 (2.6)	0.661

Data presented as mean (standard deviation) or absolute value (frequency). Comparative analysis for categorical variables was performed by Pearson's chi-square test ($p < 0.05$), and continuous variables were performed by Student's t-test ($p < 0.05$). BMI: body mass index; WC: waist circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; non-IR group: individuals without insulin resistance; IR group: individuals with insulin resistance.

Table 2: Biochemical profile of the subject according presence of insulin resistance.

Variables	Total ($n = 181$)	Non-IR group ($n = 64$)	IR group ($n = 117$)	p
TC (mmol/L)	5.38 (1.01)	5.43 (0.91)	5.35 (1.06)	0.591
HDL-C (mmol/L)	0.98 (0.28)	1.09 (0.26)	0.93 (0.28)	**0.001**
LDL-C (mmol/L)	3.67 (0.91)	3.72 (0.80)	3.62 (1.04)	0.495
TAG (mmol/L)	1.37 (1.03; 1.99)	1.15 (0.97; 1.50)	1.52 (1.14; 2.16)	**<0.001**
Glucose (mmol/L)	5.33 (8; 5.7)	5.1 (4.8; 5.3)	5.4 (5.1; 5.8)	**<0.001**
Insulin (μU/mL)	17.0 (7.0)	12.0 (3.0)	20.0 (7.0)	**<0.001**
HOMA-IR	4.2 (1.9)	2.8 (0.7)	5.0 (2.0)	**<0.001**
TAG/HDL-C	1.5 (1.0; 2.3)	1.9 (0.8; 1.5)	1.6 (1.2; 2.6)	**<0.001**
Non-HDL-C (mmol/L)	4.42 (0.95)	4.38 (0.91)	4.42 (0.98)	0.686
APO AI (g/L)	1.33 (0.27)	1.42 (0.24)	1.29 (0.28)	**0.001**
APO B (g/L)	1.05 (0.23)	1.04 (0.23)	1.05 (0.23)	0.752
LDL size (Å)	270.0 (267.0; 272.0)	271.0 (269.0; 272.0)	270.0 (265.0; 272.0)	**0.045**
Phenotype A (n, %)	125.0 (69.0)	53.0 (83.0)	72.0 (62.0)	**0.003**

Data presented as mean (standard deviation) and median (p25-p75). Comparative analysis was performed by Student's t-tests or Mann–Whitney U test ($p < 0.05$). TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TAG: triacylglycerol; TAG/HDL-C: ratio between TAG and HDL-C; APO AI: apolipoprotein AI; APO B: apolipoprotein B; non-IR group: individuals without insulin resistance; IR group: individuals with insulin resistance.

HDL-C, APO AI, and LDL size levels were significantly lower in the IR group, which showed a higher TAG/HDL ratio. Over 80% of non-IR subjects exhibited phenotype A versus 62% in the IR group ($p = 0.003$) (Table 2).

The distribution of lipoprotein subfractions (Table 3) shows that the IR group had a higher percentage of intermediate (HDL5, HDL6, and HDL7) and small HDL particles (HDL 8) and lower percentage of large particles (HDL2 and HDL3), contributing to decrease in HDL_{LARGE} in the IR group. VLDL and LDL2 subfractions in the IR group showed higher percentages than those observed in non-IR subjects.

Presence of IR was associated with reduced chances to have HDL_{LARGE} (HDL2 and HDL3) and increased small particles (HDL8) and HDL_{SMALL}. Regarding LDL subfractions, IR was a predictor of significant chance of increased LDL_{SMALL} (OR = 2.826; CI = 1.263–6.324) and phenotype non-A (OR = 3.011; CI = 1.424–6.366) (Table 4).

4. Discussion

This study showed that IR, without established DM2, is already a predictor of more atherogenic lipoprotein profile, contributing for a worse cardiovascular risk of Brazilian individuals.

IR is a key factor for the development of atherosclerosis and DM2, and the most common associated metabolic abnormality is high TAG and low HDL-C levels, whereas TC and LDL-C are not consistently altered [19].

Lipoproteins are heterogeneous structures, which vary in their size, density, and chemical composition and confer additional value to cholesterol content [20–22]. There are few studies that sought to analyze IR effects on lipid metabolism and lipoprotein subfractions in individuals without DM already clinically diagnosed [13, 14]. Recently, Shah et al. [23] described lipoprotein subclasses as a better

Table 3: Distribution of lipoprotein subfractions of the subjects according to the presence of insulin resistance.

Variables (%)	Total ($n = 181$)	Non-IR group ($n = 64$)	IR group ($n = 117$)	p
VLDL	17.85 (3.93)	16.80 (3.29)	18.42 (4.15)	**0.017**
IDL	28.64 (4.09)	29.08 (3.54)	28.39 (4.36)	0.277
LDL1	16.88 (3.99)	17.56 (3.61)	16.51 (4.16)	0.089
LDL2	9.84 (4.01)	8.84 (3.65)	10.39 (4.10)	**0.013**
LDL3	2.48 (2.75)	1.78 (2.00)	2.87 (3.02)	0.062
LDL4	0.42 (1.09)	0.28 (1.09)	0.50 (0.36)	0.063
LDL5	0.06 (0.40)	0.07 (0.46)	0.05 (0.36)	0.931
LDL6	0.01 (0.14)	0.03 (0.24)	0.00 (0.00)	0.176
LDL7	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.000
LDL_{LARGE}	2.97 (3.83)	2.16 (3.20)	3.42 (4.07)	0.081
LDL_{SMALL}	26.72 (4.81)	26.40 (4.92)	26.89 (4.77)	0.515
HDL1	10.66 (3.61)	11.00 (3.57)	10.47 (3.64)	0.346
HDL2	12.32 (4.36)	14.33 (4.15)	11.23 (4.08)	**<0.001**
HDL3	7.34 (2.11)	8.32 (2.08)	6.81 (1.94)	**<0.001**
HDL4	9.37 (1.60)	9.45 (1.39)	9.33 (1.70)	0.619
HDL5	11.06 (1.61)	10.58 (1.55)	11.32 (1.59)	**0.003**
HDL6	21.66 (3.01)	20.31 (2.76)	22.40 (2.88)	**<0.001**
HDL7	7.72 (1.43)	7.37 (1.33)	7.92 (1.45)	**0.013**
HDL8	7.64 (1.73)	7.23 (1.58)	7.87 (1.78)	**0.017**
HDL9	5.98 (1.77)	5.64 (1.61)	6.16 (1.83)	0.056
HDL10	6.22 (3.98)	5.75 (3.62)	6.48 (4.15)	0.239
HDL_{LARGE}	30.32 (8.45)	33.64 (8.66)	28.51 (7.80)	**<0.001**
$HDL_{INTERMEDIATE}$	49.81 (4.87)	47.72 (4.72)	50.95 (4.58)	**<0.001**
HDL_{SMALL}	19.84 (6.50)	18.63 (6.10)	20.51 (6.64)	0.063

Data presented as mean (standard deviation). Comparative analysis was performed by Student's t-tests or Mann–Whitney U test ($p < 0.05$). VLDL: very low-density lipoprotein; IDL: intermediate density lipoprotein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; non-IR group: individuals without insulin resistance; IR group: individuals with insulin resistance.

marker to detect lipoprotein abnormalities in normoglycemic and prediabetic subjects.

Hypertriglyceridemia is considered the principal lipid abnormality in IR and plays a pivotal role in diabetic dyslipidemia [24–27]. This causal and strict relationship between IR and dyslipidemia was recently revised by Li et al. [10]. Our results confirmed that IR has negative effects on lipid metabolism and reinforces the connection between DM and CVD. Lower values of HDL-C, APO AI, higher TAG levels, TAG/HDL ratio, and percentage of VLDL observed in the IR group characterize a typical basis for atherogenic DM. This scenario evidences that changes in physical structure of lipoproteins start earlier to DM2 and reaffirms the negative impact of IR, highlighting the relevance of strategies focused in its prevention.

Elevated TAG levels are results of increased production and decreased clearance of TAG-rich lipoproteins in both fasting and nonfasting states [28]. These events favor increased production of VLDL, a prominent IR feature [29]. In agreement, our data showed that VLDL percentage in the non-IR group was lower than that in the IR group, confirming that individuals with IR were four times more likely to have lower HDL_{LARGE} percentage. This result was mainly due to differences in HDL2 and HDL3. Garvey et al. [12] using nuclear magnetic resonance (NMR) observed that progressive IR was associated with a decrease in HDL size, depletion of large HDL particles, and a modest rise in small HDL. They also described the increase in VLDL size and in large VLDL levels before and after multiple adjustment (age, BMI, and gender). Similarly, MacLean et al. [13] described in a small sample of obese insulin-resistance women that concentration of large HDL was lower, while HDL size was negatively correlated with plasma insulin. Our results expand this previous study, because it was based on a bigger sample and included both genders. In addition, our data show the concordance of results obtained by Lipoprint system and NMR [30].

The relationship between HDL size and cardiovascular risk is still a controversial issue. Clinical and epidemiological studies have shown that low HDL-C content is strongly and independently associated with cardiovascular diseases (CVD) [31]. However, some studies have questioned this association, hypothesizing that it requires a more specific analysis of this lipoprotein [32]. There is an important heterogeneity among HDL subfractions, comprising from small to large HDL, and different HDL subfractions appear to be associated with different functions. The HDL analysis performed on the JUPITER study

Table 4: Multivariate logistic regression for effect of insulin resistance on lipoprotein subfraction profile.

Variables (%)	Raw data		Model A		Model B	
	OR	95% CI	AOR	95% CI	AOR	95% CI
VLDL						
Non-IR group	1.000		1.000		1.000	
IR group	1.974	[0.921; 4.230]	2.205	[0.965; 5.038]	2.332	[1.007; 5.404]
HDL2						
Non-IR group	1.000		1.000		1.000	
IR group	0.283	[0.141; 0.571]	0.267	[0.129; 0.552]	0.230	[0.108; 0.493]
HDL3						
Non-IR group	1.000		1.000		1.000	
IR group	0.322	[0.160; 0.645]	0.302	[0.146; 0.625]	0.298	[0.142; 0.625]
HDL5						
Non-IR group	1.000		1.000		1.000	
IR group	1.850	[0.881; 3.884]	1.843	[0.873; 3.890]	1.897	[0.890; 4.044]
HDL6						
Non-IR group	1.000		1.000		1.000	
IR group	3.367	[1.460; 7.766]	3.726	[1.579; 8.793]	3.936	[1.631; 9.498]
HDL7						
Non-IR group	1.000		1.000		1.000	
IR group	1.926	[0.919; 4.038]	1.927	[0.917; 4.047]	1.980	[0.930; 4.216]
HDL8						
Non-IR group	1.000		1.000		1.000	
IR group	2.305	[1.504; 5.039]	2.305	[1.042; 5.098]	2.363	[1.025; 5.445]
HDL9						
Non-IR group	1.000		1.000		1.000	
IR group	1.631	[0.772; 3.446]	1.367	[0.771; 3.474]	1.755	[0.806; 3.822]
HDL_{LARGE}						
Non-IR group	1.000		1.000		1.000	
IR group	0.249	[0.123; 0.505]	0.232	[0.111; 0.484]	0.213	[0.099; 0.457]
$HDL_{INTERMETDIATE}$						
Non-IR group	1.000		1.000		1.000	
IR group	3.367	[1.460; 7.766]	3.843	[1.606; 9.197]	4.698	[1.855; 11.901]
HDL_{SMALL}						
Non-IR group	1.000		1.000		1.000	
IR group	2.400	[1.099; 5.239]	2.429	[1.110; 5.319]	2.486	[1.115; 5.543]
LDL_{SMALL}						
Non-IR group	1.000		1.000		1.000	
IR group	2.826	[1.263; 6.324]	2. 794	[1.237; 6.310]	3.075	[1.341; 7.051]
Phenotype non-A						
Non-IR group	1.000		1.000		1.000	
IR group	3.011	[1.424; 6.366]	3.001	[1.404; 6.416]	3.176	[1.469; 6.867]

$n = 181$. Model A: adjusted for gender and age. Model B: adjusted for gender, age, smoking, statin, and fibrate. VLDL: very low-density lipoprotein; HDL-C: high-density lipoprotein cholesterol; non-IR group: individuals without insulin resistance; IR group: with insulin resistance.

suggested that concentration of HDL particles, rather than cholesterol content in the lipoprotein, was a more robust predictor of CVD events and a more appropriate target for therapeutic interventions [33].

According to Pirillo et al. [34], large HDL particles are more competent in reverse cholesterol transport (RCT), classically described as the primary physiological function of HDL [35], which represents the capacity to transfer excess cholesterol from peripheral cells to the liver for excretion, contributing to attenuate the atherosclerotic stimulus. However, recent studies have shown that small HDL particles have greater antioxidant and anti-inflammatory properties, preventing LDL oxidation in the subendothelial layer through reactive oxygen species (ROS) action [36].

Although there were controversial results in the literature, the majority of studies have associated lower concentrations of large HDL with cardiovascular events, dyslipidemia, obesity, metabolic syndrome, and diabetes [37].

Plausible biological mechanisms supporting the role of IR in HDL have been recently reviewed [26, 27]. The decrease in HDL-C induced by IR is associated with increased catabolism of this lipoprotein. This event involves the elevated transfer of TAG to HDL owing to hypertriglyceridemia caused by increased VLDL hepatic synthesis and decreased lipoprotein lipase (LPL) activity. This process is modulated by CETP, which leads formation of HDL rich in TAG, turning this lipoprotein a good substrate for the hepatic lipase enzyme (HL), responsible for HDL catabolism [26]. Our results corroborate with the negative influence of IR in the connection between TAG and HDL-C levels. In addition, the odds values also confirmed that IR was a predictor for higher VLDL and small HDL and lower large HDL particles. Possibly, these changes in HDL subfractions modify its functionality, reducing its antiatherogenic role.

In addition of the significant effect of IR on classical lipid profile, VLDL, and HDL subfractions, our data also showed that IR was related with increased chances to have smaller LDL. However, these structural changes were not related with modifications in total APO B and LDL-C levels, confirming that distribution of LDL and particle size adds information to classical evaluation of cholesterol and APO B content in this lipoprotein [38]. Small LDL particles are cited in several studies for their link with atherosclerosis [39].

Our results disclose that before DM2 diagnosis, the presence of IR can already cause multiple changes in lipids and structure of lipoproteins and it is supported by the worse LDL phenotype in the IR-group. Classically, two LDL phenotypes, named A and B, based on LDL dense and size are described [20, 21]. In our study, phenotype non-A (more atherogenic profile) was present in 17% of non-IR subjects, whereas this percentage was 38% in the IR group. Previous studies did not describe the negative impact of IR in these phenotypes. Thus, the higher prevalence of phenotype non-A observed in our study contributes to add more information regarding a more atherogenic lipoprotein profile when associated with higher TAG levels and small HDL particles. These results are particularly relevant because phenotypes obtained by Lipoprint system showed high concordance with other validated methods such as NMR (phenotype A = 100% and phenotype B = 75%) and Zaxis (phenotype A = 100% and phenotype B = 95%) [40].

Assessment of IR using the HOMA-IR equation is a simple and nonexpensive tool able to be used in clinical trials and in clinical practice routine. It has a high concordance level with hyperinsulinemic-euglycemic clamp technique, accepted as the gold standard method for IR diagnosis [41, 42]. However, some studies had demonstrated that HOMA-IR cut-off points are gender and age specific [43], while Gayoso-Diz et al. showed that metabolic syndrome components should be considered for IR classification [44]. These aspects suggest that HOMA-IR data should be analyzed with caution.

Based in these interactions, our odds ratios were adjusted by age and sex; however, few influences were detected by these confounders. The cut-off point used in our study was based on a previous large-population study that included Caucasian (European subjects from 17 cities), Mexican American, and Pima Indian [18]. Stern et al. [18] demonstrated that HOMA-IR isolated and/or combined with BMI could identify individuals with IR, previously DM2 diagnosis. This model is adequate for clinical trials due its high capacity to identify properly individuals with IR (specificity of 92% for HOMA-IR>4.65) but can also be used in clinical practice routine when BMI could be included (HOMA-IR > 3.6 and BMI > 27.5 kg/m^2) or analyzed alone (BMI > 28.9 kg/m^2). Additional advantage of this model is the ability to identify IR based only on one measure of BMI. This aspect is particularly relevant for individuals and population in developing countries where health systems are unable to diagnose early dysglycemia, and the incidence of DM shows accelerated growth. Though previous studies have proposed lesser cut-off points (<2.0), values adopted in our study were due to similar ethnic between individuals (Caucasian and Mexican American), and opposite to other studies, cut-off points proposed by Stern et al. [18] were validated by euglycemic insulin clamp technique in a large population-based study. Indeed, many studies described cut-off points ranging from 1.55 to 3.8 when different populations are analyzed, with different health status and distinct statistical approaches (ROC and percentiles), frequently based on a cross-sectional design. Recently, Lee et al. based on a large Chinese transversal and prospective study with 15 years of follow-up proposed cut-off points for dysglycemia (1.4) and DM2 (2.0) [45]. Despite the relevant results described, these cut-off points were not validated by euglycemia clamp technique and were based only on one glucose analysis. In addition to these aspects, the ethnicity exerts influence in IR as previously described [46, 47], explaining, in part, the different cut-off points described in literature [44]. In our study, most individuals were Caucasian and only 1.7% ($n = 3$) were Japanese Brazilian. Despite that, we decided not to exclude these individuals because they did not change our results' profile and this ethnic distribution is a representative of Brazilian population.

Altogether, clinical evaluation of IR can predict future changes in lipid metabolism and its impact in the development of CVD. These results are particularly relevant because they highlight the negative impact of IR, previously DM2 diagnosis, in qualitative aspects of lipid metabolism and cardiovascular risk not described previously in literature.

Finally, despite of the robust predictive role of IR on atherogenic lipoprotein profile observed in our study, we assume potential limitations of these results. First, criteria used to define DM could include additional analysis such as glucose tolerance test; however, in order to avoid this costly and time-consuming technique, we performed a rigorous and direct interview addressing specific questions about DM. The diagnosis was confirmed by fasting glucose, repeating it in cases where diagnosis was not clear. Second, some individuals included in our study were under statin/fibrate treatment. In this case, all groups were matched and the

individuals enrolled should be under the same drug protocol at least 30 days prior to data collection. Third, the criteria used to determine IR were not a gold standard accepted as the hyperinsulinemic-euglycemic clamp, tolerance test glucose minimal model of Bergman, hyperglycemic clamp, or oral tolerance glucose/meal test. However, our goal was to identify the capacity of IR to predict atherogenic lipoprotein using a fast, simple, and low-cost tool applicable to clinical practice such as HOMA-IR.

In conclusion, our results showed that IR is associated with significant changes in quantitative and qualitative aspects of lipoproteins and it is a robust predictor of atherogenic lipoprotein profile in nondiabetic subjects.

Abbreviations

Apo B: Apolipoprotein B
BHT: Butylated hydroxytoluene
BIA: Bioimpedance electric
BMI: Body mass index
CETP: Cholesterol ester transfer protein
CVD: Cardiovascular disease
DM2: Type 2 diabetes mellitus
ECG: Electrocardiogram
EDTA: Ethylenediaminetetraacetic acid
FFA: Free fatty acid
HDL: High-density lipoprotein
HDL-C: High-density lipoprotein cholesterol
HOMA-IR: Homeostasis model assessment-insulin resistance
IDL: Intermediate density lipoprotein
IR: Insulin resistance
LDL: Low-density lipoprotein
LDL-C: Low-density lipoprotein cholesterol
LPL: Lipoprotein lipase
NMR: Nuclear magnetic resonance
PMSF: Phenylmethylsulfonyl fluoride
RCT: Reverse cholesterol transport
TAG: Triacylglycerol
WC: Waist circumference.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Flávia De C. Cartolano and Gabriela D. Dias wrote the manuscript. Flávia De C. Cartolano and Maria C. P. de Freitas performed the data analysis. Nágila R. T. Damasceno designed, drafted, and critically reviewed the manuscript. Antônio M. Figueiredo Neto reviewed critically the manuscript. All authors approved the final version of the manuscript.

Acknowledgments

This study was granted by the National Institute of Science and Technology for Complex Fluids (INCT-FCx 2008/57685-7), NAP-FCx-USP, Sao Paulo Research Foundation (FAPESP 2011/12523-2, 2011/13616-4, 2015/06222-0, 2015/06565-0), and National Council for Scientific and Technological Development (CNPq).

References

[1] H. Himsworth, "Diabetes mellitus: its differentiation into insulin sensitive and insulin insensitive types," *The Lancet*, vol. 227, pp. 127–130, 1936.

[2] C. R. Kahn, "Insulin action, diabetogenes, and the cause of type II diabetes," *Diabetes*, vol. 43, pp. 1066–1084, 1994.

[3] G. M. Reaven, "Insulin resistance: the link between obesity and cardiovascular disease," *The Medical Clinics of North America*, vol. 95, pp. 875–892, 2011.

[4] E. Ferrannini, "The insulin resistance syndrome," *Current Opinion in Nephrology & Hypertension*, vol. 1, pp. 291–298, 1992.

[5] International Diabetes Federation, *IDF Diabetes Atlas*, International Diabetes Federation, Brussels, 2014, June 2016, http://www.idf.org/diabetesatlas.

[6] E. Ferrannini, S. M. Haffner, B. D. Mitchell, and M. P. Stern, "Hyperinsulinaemia: the key feature of a cardiovascular and metabolic syndrome," *Diabetologia*, vol. 34, pp. 416–422, 1991.

[7] G. M. Reaven, "Pathophysiology of insulin resistance in human disease," *Physiological Reviews*, vol. 75, pp. 473–486, 1995.

[8] R. H. Unger, "Reinventing type 2 diabetes: pathogenesis, treatment, and prevention," *The Journal of the American Medical Association*, vol. 299, pp. 1185–1187, 2008.

[9] G. Reaven, "Insulin resistance and coronary heart disease in nondiabetic individuals," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, pp. 1754–1759, 2012.

[10] N. Li, J. Fu, D. P. Koonen, J. A. Kuivenhoven, H. Snieder, and M. H. Hofker, "Are hypertriglyceridemia and low HDL causal factors in the development of insulin resistance?," *Atherosclerosis*, vol. 233, pp. 130–138, 2014.

[11] R. H. Mackey, S. Mora, A. G. Bertoni et al., "Lipoprotein particles and incident type 2 diabetes in the multi-ethnic study of atherosclerosis," *Diabetes Care*, vol. 38, pp. 628–636, 2015.

[12] W. T. Garvey, S. Kwon, D. Zheng et al., "Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance," *Diabetes*, vol. 52, pp. 453–462, 2003.

[13] P. S. MacLean, S. Vadlamudi, K. G. MacDonald, W. J. Pories, J. A. Houmard, and H. A. Barakat, "Impact of insulin resistance on lipoprotein subpopulation distribution in lean and morbidly obese nondiabetic women," *Metabolism*, vol. 49, pp. 285–292, 2000.

[14] A. Festa, K. Williams, A. J. Hanley et al., "Nuclear magnetic resonance lipoprotein abnormalities in prediabetic subjects in the Insulin Resistance Atherosclerosis Study," *Circulation*, vol. 111, pp. 3465–3472, 2005.

[15] A. Milech, A. P. Angelucci, A. Golbert, A. J. Carrilho, A. C. Ramalho, and A. C. Aguiar, *Diretrizes da Sociedade Brasileira de Diabetes (2015-2016)*, A.C. Farmacêutica, São Paulo, 2016, orgnization José Egidio Paulo de Oliveira, Sérgio Vencio.

[16] W. T. Friedewald, R. I. Levy, and D. S. Fredrickson, "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clinical Chemistry*, vol. 18, pp. 499–402, 1972.

[17] D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner, "Homeostasis model

assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, pp. 412–419, 1985.

[18] S. E. Stern, K. Williams, E. Ferrannini, R. A. DeFronzo, C. Bogardus, and M. P. Stern, "Identification of individuals with insulin resistance using routine clinical measurements," *Diabetes*, vol. 54, pp. 333–339, 2005.

[19] R. A. DeFronzo and E. Ferrannini, "Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease," *Diabetes Care*, vol. 14, pp. 173–194, 1991.

[20] C. Packard, M. Caslake, and J. Shepherd, "The role of small, dense low density lipoprotein (LDL): a new look," *International Journal of Cardiology*, vol. 74, Supplement 1, pp. S17–S22, 2000.

[21] K. K. Berneis and R. M. Krauss, "Metabolic origins and clinical significance of LDL heterogeneity," *Journal of Lipid Research*, vol. 43, pp. 1363–1379, 2002.

[22] M. R. Diffenderfer and E. J. Schaefer, "The composition and metabolism of large and small LDL," *Current Opinion in Lipidology*, vol. 25, pp. 221–226, 2014.

[23] A. S. Shah, W. S. Davidson, Z. Gao, L. M. Dolan, T. R. Kimball, and E. M. Urbina, "Superiority of lipoprotein particle number to detect associations with arterial thickness and stiffness in obese youth with and without prediabetes," *Journal of Clinical Lipidology*, vol. 10, pp. 610–618, 2016.

[24] M. Arca, G. Pigna, and C. Favoccia, "Mechanisms of diabetic dyslipidemia: relevance for atherogenesis," *Current Vascular Pharmacology*, vol. 10, pp. 684–686, 2012.

[25] G. Steiner and M. Vranic, "Hyperinsulinemia and hypertriglyceridemia, a vicious cycle with atherogenic potential," *International Journal of Obesity*, vol. 6, Supplement 1, pp. 117–124, 1982.

[26] A. von Eckardstein and R. A. Sibler, "Possible contributions of lipoproteins and cholesterol to the pathogenesis of diabetes mellitus type 2," *Current Opinion in Lipidology*, vol. 22, pp. 26–32, 2011.

[27] B. G. Drew, K. A. Rye, S. J. Duffy, P. Barter, and B. A. Kingwell, "The emerging role of HDL in glucose metabolism," *Nature Reviews Endocrinology*, vol. 8, pp. 237–245, 2012.

[28] L. Wu and K. G. Parhofer, "Diabetic dyslipidemia," *Metabolism*, vol. 63, pp. 1469–1479, 2014.

[29] M. Adiels, S. O. Olofsson, M. R. Taskinen, and J. Borén, "Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, pp. 1225–1236, 2008.

[30] J. D. Otvos, E. J. Jeyarajah, D. W. Bennett, and R. M. Krauss, "Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement," *Clinical Chemistry*, vol. 38, no. 9, pp. 1632–1638, 1992.

[31] P. J. Barter, R. Puranik, and K. A. Rye, "New insights into the role of HDL as an anti-inflammatory agent in the prevention of cardiovascular disease," *Current Cardiology Reports*, vol. 9, pp. 493–498, 2007.

[32] C. R. Sirtori, L. Calabresi, G. Franceschini et al., "Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study," *Circulation*, vol. 103, pp. 1949–1954, 2001.

[33] R. S. Rosenson, H. B. Brewer, B. Ansell et al., "Translation of high-density lipoprotein function into clinical practice: current prospects and future challenges," *Circulation*, vol. 128, pp. 1256–1267, 2013.

[34] A. Pirillo, G. D. Norata, and A. L. Catapano, "High-density lipoprotein subfractions—what the clinicians need to know," *Cardiology*, vol. 124, pp. 116–125, 2013.

[35] J. A. Glomset, "The plasma lecithins:cholesterol acyltransferase reaction," *Journal of Lipid Research*, vol. 9, pp. 155–167, 1968.

[36] A. Kontush and M. J. Chapman, "Antiatherogenic function of HDL particle subpopulations: focus on antioxidative activities," *Current Opinion in Lipidology*, vol. 21, pp. 312–318, 2010.

[37] L. Tian, L. Jia, F. Mingde et al., "Alterations of high density lipoprotein subclasses in obese subjects," *Lipids*, vol. 41, pp. 789–796, 2006.

[38] F. M. Sacks and H. Campos, "Clinical review 163: cardiovascular endocrinology: low-density lipoprotein size and cardiovascular disease: a reappraisal," *The Journal of Clinical Endocrinology and Metabolism*, vol. 88, pp. 4525–4532, 2003.

[39] B. Lamarche, I. Lemieux, and J. P. Després, "The small, dense LDL phenotype and the risk of coronary heart disease: epidemiology, pathophysiology and therapeutic aspects," *Diabetes & Metabolism*, vol. 25, pp. 199–211, 1999.

[40] D. M. Hoefner, D. S. Hodel, J. F. O'Brien et al., "Development of a rapid, quantitative method for LDL subfractionation with use of the quantimetrix lipoprint LDL system," *Clinical Chemistry*, vol. 47, pp. 266–274, 2001.

[41] E. Bonora, G. Targher, M. Alberiche et al., "Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity," *Diabetes Care*, vol. 23, pp. 57–63, 2000.

[42] R. A. DeFronzo, J. D. Tobin, and R. Andres, "The glucose clamp technique: a method for quantifying insulin secretion and resistance," *The American Journal of Physiology*, vol. 237, pp. E214–E223, 1979.

[43] P. Gayoso-Diz, A. Otero-González, M. X. Rodriguez-Alvarez et al., "Insulin resistance index (HOMA-IR) levels in a general adult population: curves percentile by gender and age. The EPIRCE study," *Diabetes Research and Clinical Practice*, vol. 94, pp. 146–155, 2011.

[44] P. Gayoso-Diz, A. Otero-González, M. X. Rodriguez-Alvarez et al., "Insulin resistance (HOMA-IR) cut-off values and the metabolic syndrome in a general adult population: effect of gender and age: EPIRCE cross-sectional study," *BMC Endocrine Disorders*, vol. 13, p. 47, 2013.

[45] C. H. Lee, A. Z. L. Shih, Y. C. Woo et al., "Optimal cut-offs of homeostasis model assessment of insulin resistance (HOMA-IR) to identify dysglycemia and type 2 diabetes mellitus: a 15-year prospective study in Chinese," *PLoS One*, vol. 11, article e0163424, 2016.

[46] P. M. McKeigue, B. Shah, and M. G. Marmot, "Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians," *Lancet*, vol. 337, pp. 382–386, 1991.

[47] M. A. Banerji, N. Faridi, R. Atluri, R. L. Chaiken, and H. E. Lebovitz, "Body composition, visceral fat, leptin, and insulin resistance in Asian Indian men," *The Journal of Clinical Endocrinology and Metabolism*, vol. 84, pp. 137–144, 1999.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 4826724, 15 pages
https://doi.org/10.1155/2017/4826724

Review Article

The Nrf2/Keap1/ARE Pathway and Oxidative Stress as a Therapeutic Target in Type II Diabetes Mellitus

Joshua A. David, William J. Rifkin, Piul S. Rabbani, and Daniel J. Ceradini

Hansjörg Wyss Department of Plastic and Reconstructive Surgery, New York University School of Medicine, 430 East 29th Street, New York, NY 10016, USA

Correspondence should be addressed to Daniel J. Ceradini; daniel.ceradini@nyumc.org

Received 12 April 2017; Revised 3 July 2017; Accepted 20 July 2017; Published 20 August 2017

Academic Editor: Joseph F. Ndisang

Despite improvements in awareness and treatment of type II diabetes mellitus (TIIDM), this disease remains a major source of morbidity and mortality worldwide, and prevalence continues to rise. Oxidative damage caused by free radicals has long been known to contribute to the pathogenesis and progression of TIIDM and its complications. Only recently, however, has the role of the Nrf2/Keap1/ARE master antioxidant pathway in diabetic dysfunction begun to be elucidated. There is accumulating evidence that this pathway is implicated in diabetic damage to the pancreas, heart, and skin, among other cell types and tissues. Animal studies and clinical trials have shown promising results suggesting that activation of this pathway can delay or reverse some of these impairments in TIIDM. In this review, we outline the role of oxidative damage and the Nrf2/Keap1/ARE pathway in TIIDM, focusing on current and future efforts to utilize this relationship as a therapeutic target for prevention, prognosis, and treatment of TIID.

1. Introduction

The worldwide prevalence of diabetes mellitus (DM) was estimated at 8.5% in 2014, and the morbidity resulting from the microvascular and macrovascular complications of this disease is enormous [1]. Costs attributable to DM in 2013 were $101.4 billion in the United States alone, making it the most expensive medical condition by a significant margin [2–4]. The chronic hyperglycemia and impairments in insulin secretion and action that characterize type II diabetes mellitus (TIIDM) are associated with long-term damage, dysfunction, and failure of many organs, including the eyes, kidneys, nerves, heart, and blood vessels [5]. Decades of scientific research, randomized human trials, and clinical experience have demonstrated that a combination of lifestyle modifications and pharmaceutical interventions has the capacity to prevent or delay the onset of TIIDM and many of its devastating complications [6]. Consequently, major therapeutic advances, coupled with increased diabetes awareness and the implementation of national programs and guidelines for diabetes prevention, have helped alleviate disease-related morbidity and mortality [7]. Despite these improvements, this disease continues to pose a tremendous burden in the US, and the prevalence, deaths, and costs attributable to TIIDM are expected to continue increasing drastically [8]. As we gain further insight into the molecular underpinnings of this disease and its destructive sequelae, we advance the opportunity to develop novel, targeted approaches for diabetes treatment, prognosis, and, ultimately, prevention. In recent years, the Nrf2/Keap1/ARE antioxidant pathway has emerged as one such promising avenue of research. In this review, we summarize the roles of oxidative stress and the Nrf2/Keap1/ARE pathway in TIIDM, as well as the current state of efforts aimed at exploiting this relationship in order to minimize the devastating impact of this disease across the globe.

2. Oxidative Stress and Diabetes Mellitus

Oxidative stress occurs when free radical production overwhelms the endogenous antioxidant ability to neutralize these highly reactive chemical compounds. The ensuing cellular damage, such as DNA cross-linking and apoptosis, is a hallmark of oxidative stress and is a fundamental

pathological process in cancer, aging, and a variety of chronic diseases [9–12]. In TIIDM, dysfunctional redox homeostasis has long been known to play a role in the pathogenesis of the disease and its complications through a variety of mechanisms, and diabetic patients have been shown to possess increased cellular levels of reactive oxygen species (ROS) and ROS-induced DNA damage [13–15]. Landmark studies by Giacco and Brownlee showed that the increased glycemic load in diabetes overwhelms the Krebs cycle, resulting in the inhibition of election transfer within the mitochondrial membrane and the accumulation of free radicals [14]. In particular, these free radicals include the highly reactive superoxide and hydroxyl compounds [16]. As ROS production increases, upregulation of four biochemical pathways occurs: polyol flux, intracellular advanced glycosylation end product (AGE) formation, protein kinase C activation, and hexosamine pathway flux [14]. These perturbations result in a variety of downstream effects known to underlie the pathogenesis and progression of TIIDM, including the depletion of natural antioxidant molecules and damage to vascular cells, as well as alterations in gene and protein expression, blood flow, and endothelial cell permeability [13, 17, 18].

3. The Nrf2/Keap1/ARE Pathway

Knowledge of the relationship between oxidative stress and TIIDM has precipitated intense investigation into the failure of the diabetic system to appropriately respond to increased oxidative loads. Regulation of cellular redox homeostasis under both stressed and nonstressed conditions occurs primarily at the transcriptional level, and the Nrf2/Keap1/ARE pathway is the primary mediator of this response. This signaling pathway regulates the expression of over 100 genes and functions related to oxidative stress and cell survival, including direct antioxidant proteins, phase I and II electrophile detoxification enzymes, the transport of toxic solutes, free radical metabolism, inhibition of inflammation, glutathione homeostasis, proteasome function, and the recognition of DNA damage, as well as the expression of various related growth factors and transcription factors [19] (Figure 1).

The principal mediator of this response is nuclear factor E2-related factor 2 (Nrf2), a master transcription factor. Upon binding the upstream cis-regulatory antioxidant response element (ARE) sequence located in the promoter regions of cytoprotective genes, Nrf2 triggers the transcriptional induction of multiple detoxifying enzymes [20]. Under nonstressed conditions, Nrf2 activity is suppressed by its native repressor Kelch-like ECH-associated protein 1 (Keap1), through interactions with a hairpin motif in the C terminus of the Nrf2-ECH homologous domain (Neh2) phosphorylation site on Nrf2. Keap1 is a cytoplasmic, actin cytoskeleton-associated adapter protein of the Cullin3-(Cul3-) based E3-ligase complex, which tags Nrf2 for ubiquitination and subsequent proteosomal degradation within the cytoplasm [21]. This signaling pathway has been established as the major mechanism of cellular defense against oxidative stress both physiologically and in a wide array of disease models [19]. First isolated in cloning studies in 1994 [22], the critical role of Nrf2, a member of the cap'n'collar family of basic leucine zipper transcription factors, in both constitutive and inducible ARE gene expression was soon elucidated both *in vitro* [23] and *in vivo* [20, 21, 24]. The precise molecular mechanisms of Nrf2 and Keap1 interaction are a topic of debate, particularly given the distinct subcellular locations of these two molecules [25]. It is nonetheless understood that modification of cysteine residues in the primary structure of Keap1, which act as cellular sensors for inducers of environmental stress, by thiol-reactive chemical species during states of excess oxidative or electrophilic stress results in the disruption of the Nrf2-Keap1 dimer and stabilization of Nrf2 [26]. Nrf2, once stabilized, is no longer repressed by Keap1 and becomes free to heterodimerize with members of the Maf family of transcription factors. With the assistance of a nuclear localization sequence, the Nrf2 heterodimer can rapidly translocate into the nucleus and bind to the ARE, resulting in the recruitment of elements required for the transcriptional activation of a variety of genes such as glutathione S-transferase A2 (GSTA2), NADPH quinone oxidoreductase (NQO-1), superoxide dismutase (SOD1), and heme oxygenase-1 (Ho-1) [19, 20, 27]. These antioxidant enzymes function to transform free radicals into less toxic substances through four primary mechanisms: (1) oxidation and reduction reactions, in which functional groups on hydrophobic molecules are exposed, (2) nucleophilic trapping processes, (3) transporter efflux of toxic metabolites, and (4) maintenance of reduced conditions by thiol-containing molecules [19, 28]. This protective stress recognition mechanism by Keap1 dually ensures suppression of Nrf2 during nonstressed conditions and an appropriate antioxidant response during periods of excessive cellular stress.

Given the intimate relationship between TIIDM and oxidative damage, the involvement of the Nrf2/Keap1/ARE pathway in this unsolved clinical problem has become a topic of great interest. We now know that dysfunction of this master antioxidant pathway is associated with the pathophysiology of diabetes and a wide range of its complications, such as diabetic nephropathy and impaired cutaneous wound healing, in both animal and human models [29–31] (Figure 2).

While the mechanism or mechanisms of this dysfunction in diabetes have only begun to be elucidated, therapies targeting the Nrf2/Keap1/ARE pathway represent a promising avenue in current research. As a critical upstream mediator of not only the global antioxidant response but also of anti-inflammatory genes and transcription factors involved in mitochondrial function, the Nrf2/Keap1/ARE pathway represents an ideal target in treating the widespread oxidative damage implicated in pancreatic damage, insulin resistance and sensitivity, and the progression of a broad spectrum of diabetic complications. Additionally, the inducible nature of this signaling pathway allows Keap1 to uniquely both sense the cellular redox state and responsively modify the degree of Nrf2 degradation via ubiquitination in response to this oxidative stress. This allows for modulation of cellular redox homeostasis via highly specific transcriptional activation of only those genes containing an ARE in the promoter region [32]. Furthermore, the Nrf2-mediated oxidative response may also possess aspects that are specific to diabetes. The activation of the aforementioned pathways underlying the

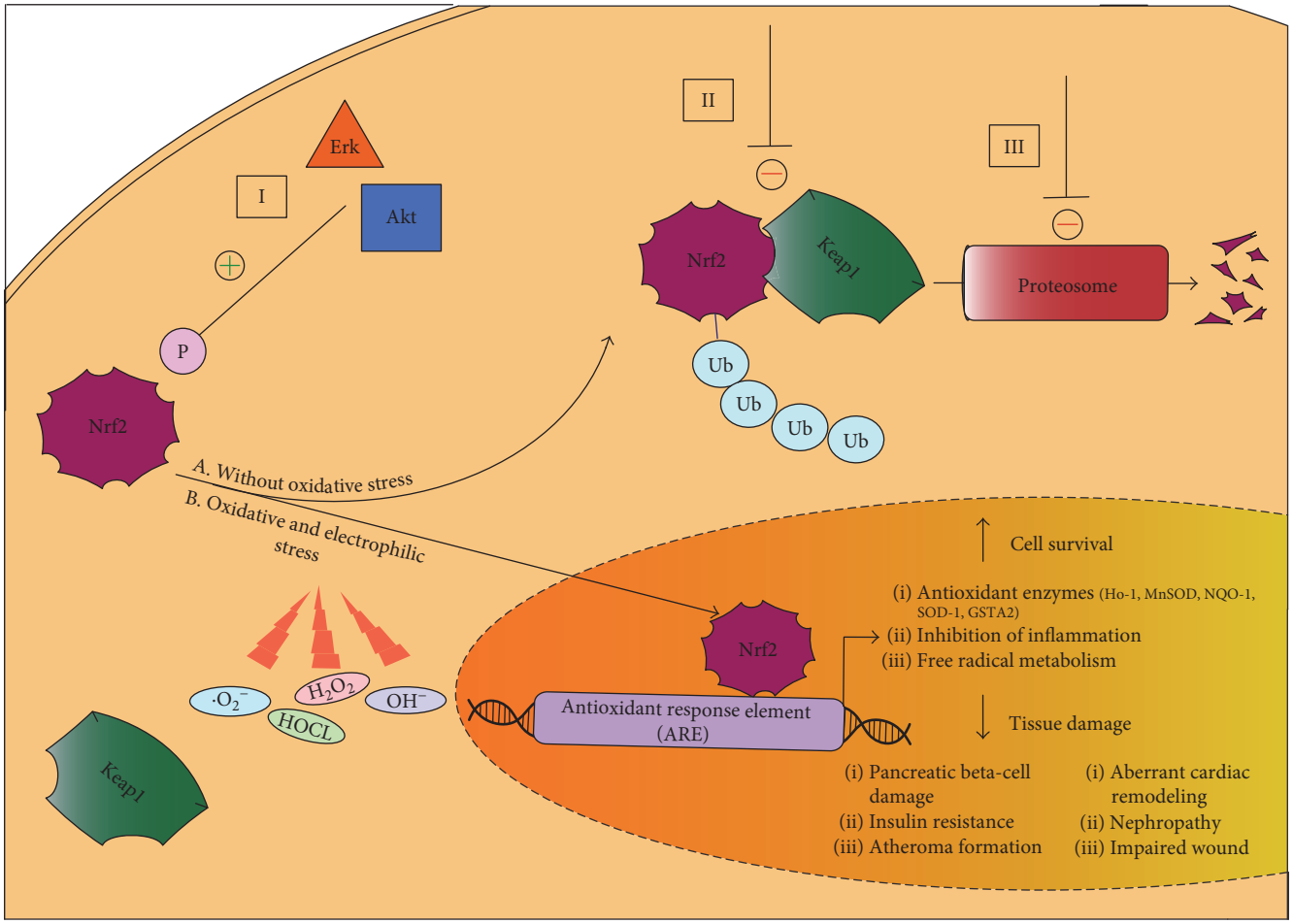

Figure 1: The Nrf2/Keap1/ARE pathway in type II diabetes mellitus. (A) Under nonstressed conditions, the Nrf2 transcription factor is covalently bound to cysteine residues on its native repressor Keap1 in the cytoplasm. This results in the constitutive ubiquitination and degradation of Nrf2 in the proteasome and inhibition of the anti-oxidant response. (B) Under conditions of electrophilic or oxidative stress, cysteine residues on Keap1 are modified, resulting in the stabilization and translocation of Nrf2 into the nucleus, where it can bind to the promoter region of the ARE and initiate the transcription of various cytoprotective enzymes which function to promote cellular survival through a variety of mechanisms, including the upregulation of antioxidant function, inflammatory inhibition, and the transport of toxic metabolites. These cellular adaptations have been shown to improve a wide array of tissue damage underlying the pathogenesis and progression of diabetes. (B) There are three major mechanisms of Nrf2 induction by small molecule activators. (I) Upstream kinases such as Akt and Erk phosphorylate Nrf2 at specific sites, favoring its release by Keap1 and nuclear translocation. (II) Modification of Keap1 cysteine residues disrupts the Nrf2-Keap1 complex, favoring dissociation of Nrf2 and subsequent nuclear translocation. (III) Inhibition of Nrf2 ubiquitination by Keap1 and degradation by the proteasome augments Nrf2 availability, thus favoring nuclear translocation of Nrf2. Ub: ubiquitination; P: phosphorylation.

pathogenesis of diabetic complications has been tied to a singular hyperglycemia-induced event in the mitochondria, overproduction of superoxide by the electron-transport chain [13]. Furthermore, studies have shown that the absence of Nrf2 may exacerbate both type I and type II diabetes [33, 34]. This hyperglycemia-specific increase in ROS overproduction by the mitochondria may explain why classic antioxidants, low molecular-weight compounds that can scavenge reactive oxygen intermediates, have not been proven beneficial in the treatment of diabetic complications. In contrast to these classic or direct antioxidants, the battery of cytoprotective agents that are upregulated by the Nrf2/Keap1/ARE pathway have been termed "ultimate antioxidants," which possess long half-lives, are not depleted throughout the course of their wide range of chemical detoxification reactions, and can even accelerate regeneration of other antioxidants, such as glutathione [35]. Lastly, the ability to target this pathway at a variety of locations, as will be discussed later, grants an incredibly rich degree of flexibility and diversity as the search for rational and clinically relevant therapeutics evolves.

4. Nrf2/Keap1/ARE and the β-Cell

Pancreatic β-cell dysfunction and the resulting impairments in insulin sensitivity and production are a critical component in the development and progression of both type I and type II DM [36], and oxidative stress is one important mechanism whereby this damage occurs [37]. Despite overexpression of Nrf2 downstream endogenous antioxidant genes by the

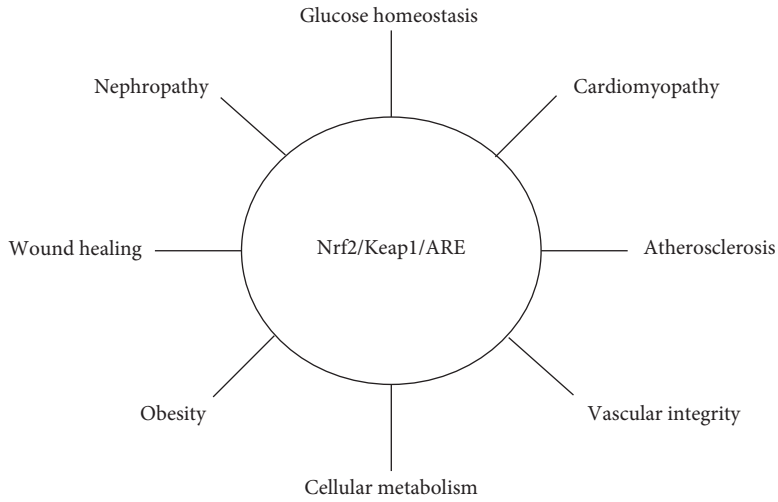

Figure 2: The Nrf2/Keap1/ARE pathway is involved in multiple tissue types.

pancreas in order to curtail cellular damage and salvage insulin-secreting ability, oxidative damage ultimately overloads this protective response in TIIDM [38–40]. In humans, these deleterious effects manifest as reduced β-cell mass and DNA damage in the pancreatic islets of patients with TIIDM [41]. Reversing the decline and eventual failure of pancreatic β-cells is critical for preventing TIIDM and its progression [42].

Animal studies have shown that the Nrf2/Keap1/ARE system is a crucial defensive pathway in the physiological and pathological protection of pancreatic β-cells. In β-cell-specific transgenic mice, Nrf2 depletion depressed the expression of cytoprotective antioxidant genes in pancreatic islets and exacerbated oxidative β-cell damage, while Nrf2 induction suppressed the accumulation of intracellular ROS, the formation of ROS-induced DNA adducts, and pancreatic β-cell apoptosis within the islets [43]. Further studies showed the preservation of β-cell mass and function in diabetic mice with genetically modified upregulation of Nrf2 via Keap1 knockout [44]. Pancreatic β-cell protection by the Nrf2/Keap1/ARE system is not limited to free radical scavenge but includes reduction of inflammation via the NF-kappaB pathway [45] and maintenance of critical cellular degradation systems such as apoptosis, autophagy, and proteosomal degradation [46, 47].

In addition to β-cell injury, oxidative stress also affects pancreatic insulin secretion, although this relationship is less clear. While some studies show that ROS impairs insulin release through mechanisms such as a reduction in ATP production [48] and increased glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity [49], there is a growing body of evidence indicating that oxidative and electrophilic stress can actually augment, and may even be necessary for, insulin release [50, 51]. The role of the Nrf2/Keap1/ARE pathway in insulin secretion is similarly controversial. While insulin content and secretion are decreased in the pancreatic islets of Nrf2 knockout mice and upregulation of Nrf2 appears to improve the insulin-releasing potential of β-cells [44, 52, 53], Nrf2 deficiency has also been associated with decreased blood glucose, enhanced insulin signaling, and decreased fat and body weight in Nrf2 knockout mouse models [54–57]. Clearly, a great deal of work remains before we completely understand the role of oxidative stress and Nrf2 with regard to glucose and insulin homeostasis. Of note, while most studies have focused on the role of Nrf2/Keap1/ARE on glucose and insulin handling within β-cells, emerging evidence suggests that this pathway may also play a dynamic role in other pancreatic islet cells (α-cells, δ-cells, and PP-cells), possibly by preventing differentiation of β-cells into these insulin-negative cell types under conditions of oxidative stress [58].

5. Nrf2 and Insulin Resistance

In addition to β-cell dysfunction, insulin resistance in a wide range of tissues is a hallmark of TIIDM, resulting in elevated blood glucose levels and exacerbation of pancreatic damage as it attempts to compensate for perceived hypoglycemia. Studies by Uruno et al. in murine models of Nrf2 overexpression via both genetic Keap1 knockdown and pharmacological induction suggest that Nrf2 activation can improve insulin sensitivity in diabetes and abrogate diabetes and obesity in mice [44]. Body weight and blood glucose levels were decreased in diabetic mice with Keap1 knockout. These findings were attributed to Nrf2-mediated stimulation of energy consumption in skeletal muscle and brown adipose tissue. Further studies showed that Nrf2 induction in mice also suppressed gluconeogenesis, owing to transcriptional repression of a variety of enzymes including the gluconeogenic enzyme glucose-6-phosphatase (G-6-P). *In vitro* studies using murine hepatocytes confirmed that Nrf2 attenuates G-6-P expression in these cells, despite stimulation of gluconeogenesis using a cAMP analog. In addition to its inhibitory effect on G-6-P, Nrf2 was shown to decrease expression of other genes related to gluconeogenesis, as well as augment insulin sensitivity in an insulin tolerance test. A more recent study in a murine model suggests that increased Nrf2 signaling may also improve insulin resistance via suppression of oxidative stress

Table 1: Nrf2/Keap1/ARE and diabetic complications.

Diabetic complication	Pathogenesis	Nrf2- (or downstream-) mediated effects
Atherosclerosis	(i) oxLDL formation	(i) Protection from oxLDL transformation of phagocytic cells [77, 78]
	(ii) Proinflammatory response in endothelial cells	(ii) Inhibition of proinflammatory response at atherosusceptible sites [71, 79]
Heart failure	(i) Aberrant cardiac and ECM remodeling	(i) Blood pressure regulation [72]
	(ii) Insulin resistance of myocytes	(ii) Protection of myocardium following ischemia
	(iii) Impaired regulation of intracellular calcium	(iii) Diminishes ROS and myocardial hypertrophy [99]
	(iv) Accumulation of AGE products	
Diabetic nephropathy	(i) Renal oxidative and nitrosative stress	(i) Improvement of metabolic indices (e.g., polydipsia and polyuria) [110]
	(ii) Mesangial cell proliferation, inflammation, fibrosis	(ii) Reversal in dysfunction of key growth factors and ECM proteins [111–113]
Wound healing	(i) Keap1 overexpression	(i) Impairments in angiogenesis and reepithelialization [120]
	(ii) Loss of wound redox homeostasis	
	(iii) Chronic inflammatory microenvironment	

in the hypothalamus, a phenomenon that may affect systemic metabolic regulation [59]. Furthermore, obesity is associated with an increased risk of developing insulin resistance and TIIDM, and murine studies have likewise shown that Nrf2 induction can suppress weight gain and increase skeletal muscle oxygen consumption, mitochondrial redox homeostasis, and ATP production, as well as augment cellular glucose uptake [44, 60, 61].

6. Diabetic Complications (Table 1)

6.1. Cardiovascular Disease. The absolute risk of cardiovascular disease (CVD) is 2-fold greater in patients with TIIDM versus those without, and at least 65% of people with DM die of heart disease or stroke [3, 62]. Given this close link between TIIDM and CVD, it is not surprising that oxidative stress has been implicated in the pathogenesis of many CVD disorders, including hypertension [63], heart failure (HF) [64], atherosclerosis [65], and ischemia-reperfusion injury [66]. Free radical-induced endothelial damage is thought to be the initiating step in CVD [67], and hyperglycemia-induced ROS in TIIDM exacerbates impairments in angiogenesis and neovascularization through means such as disruption of endothelial progenitor cell function and vascular homeostasis [68, 69]. The importance of Nrf2 and its downstream elements to vascular integrity has also become increasingly apparent, and studies have illuminated their role in functions such as augmentation of blood vessel branching [70], preservation of endothelial cell function [71], blood pressure regulation [72], and protection of the myocardium following ischemia [73, 74]. In particular, evidence of Ho-1, the Nrf2 downstream enzyme that catalyzes the degradation of heme into biliverdin, ferrous iron, and carbon monoxide, as an important mediator against vascular dysfunction in diabetes has recently emerged. Upregulation of Ho-1 levels was shown to improve left ventricular ejection fraction and inhibit remodeling in diabetic rats with myocardial infarction, and *in vivo* and *in vitro* studies demonstrated that Ho-1 overexpression attenuated angiotensin II-mediated cardiac hypertrophy in these mice [74]. Studies in streptozotocin-induced diabetic mice suggest that these effects are due to reductions in oxidative stress, inflammation, and apoptosis [75]. Therefore, the likely link among ROS and the Nrf2/Keap1/ARE pathway to the plethora of CVD-related complications that afflict diabetic patients has emerged as a subject of intense investigation.

6.2. Atherosclerosis. Nrf2 has been implicated in a variety of processes intrinsic to the formation of atherosclerotic plaque. As an indispensable component of the antioxidant response within macrophages [76], Nrf2 protects these phagocytic cells from oxidized low-density lipoproteins (oxLDL) and foam cell transformation, fundamental steps in atheroma formation [77, 78]. Additionally, Nrf2 appears to inhibit the proinflammatory response in endothelial cells located at atherosusceptible sites, conferring them a protective advantage in response to diabetic hyperglycemia [71, 79]. The significance of these effects is largely attributed to the downstream activation of the Nrf2 downstream antioxidant enzyme HO-1, which has been independently found to play a significant defensive role against atherosclerosis [80]. However, there are also studies suggesting that Nrf2 can promote atheroma formation, possibly due to interactions with a variety of well-described proatherogenic factors such as vascular cell adhesion molecule 1 (VCAM-1) and interleukin-1 (IL-1) [81–83]. For instance, overexpression of Ho-1 was found to be associated with worsening coronary atherosclerosis in an autopsy study of Japanese patients with diabetes mellitus [84]. This seeming contradiction might be explained by a differential response of Nrf2 to laminar versus oscillatory blood flow, as atheroma formation is not uniform throughout the vascular system but rather disposed to bifurcation and branch points [85]. Regardless, mounting evidence of the

importance of Nrf2 for vascular integrity and long-term endothelial function suggests that the Nrf2/Keap1/ARE pathway is influential in atherosclerotic resistance and may be a useful target for protection against coronary artery disease (CAD), peripheral vascular disease, and cerebrovascular disease in diabetic populations.

6.3. Heart Failure. Not only is there a well-established association between TIIDM and the development of HF, but this relationship also persists even in the absence of other risk factors such as CAD or hypertension, suggesting that TIIDM may mediate an exclusive form of cardiomyopathy [86]. Multiple mechanisms, such as impaired regulation of intracellular calcium and accumulation of AGE products, have been suggested to underlie this dysfunction, all of which ultimately result in oxidative stress and myocardial toxicity [87]. Not surprisingly, ROS and mitochondrial dysfunction are increased in the diabetic heart [64]. The resulting cardiac cellular necrosis and apoptosis impair contractile and electrical function, two contributing features of HF [88–90]. In addition to causing cellular damage, ROS can modify proteins essential in excitation-contraction coupling [91], activate hypertrophy-signaling kinases [92], and stimulate cardiac extracellular matrix (ECM) remodeling [93]. Furthermore, ROS exacerbates insulin resistance of myocytes, a key element of diabetes-induced cardiac dysfunction [94].

Evidence that aberrant cardiac remodeling is attenuated by a variety of Nrf2 target genes such as SOD [95], HO-1 [96] and glutathione peroxidase (GPx) [97] has motivated investigation into a possible protective role of the Nrf2/Keap1/ARE pathway against cardiomyopathy in DM. In murine models of HF and TIIDM, oxidative stress attenuates the expression of Nrf2 in cardiomyocytes and downregulates glucose utilization, resulting in insulin resistance [98]. Furthermore, Nrf2 overexpression diminishes ROS and myocardial hypertrophy, an effect that was facilitated by extracellular signal-related kinase (Erk), which normally acts to activate Nrf2 during oxidative stress [99]. It has therefore may be that Erk-mediated Nrf2 downregulation may underlie individual susceptibilities to CVD-related diabetic complications.

A number of studies utilizing pharmacologic Nrf2 activators have implicated the antioxidant properties of the Nrf2/Keap1/ARE pathway in cardioprotection [100, 101]. Consequently, there is a great deal of interest in therapeutically targeting this pathway as a means of preventing or reversing pathological cardiac remodeling [102] or delaying ventricular failure, the hemodynamic hallmark of HF in TIIDM [103, 104]. However, chronic overactivation of the Nrf2/Keap1/ARE signaling pathway may actually contribute to cardiomyopathy, which undermines the encouraging results of acute Nrf2 induction [105]. A long-term phase III clinical study in TIID patients with stage 4 chronic kidney disease (CKD) (BEACON trial) was terminated early due to a higher rate of cardiovascular events in the treatment group [106]. It is unclear as to why these adverse events were observed in this study and not in an earlier clinical trial in patients with stage 3 CKD (BEAM trial), but possibilities include significantly longer length of drug exposure or the use of a 20 mg fixed dose as opposed to an adjustable dosage [107]. However, whether this truly reflects a cardiomyopathic tendency of Nrf2 or is alternatively the result of other factors, such as an inherently increased rate of cardiovascular events in patients with more severe CKD, remains unknown.

6.4. Diabetic Nephropathy. Diabetic nephropathy is a well-known microvascular complication of chronic hyperglycemia, and both oxidative stress and an impaired response by the Nrf2/Keap1/ARE system have been implicated in its progression via renal cell apoptosis, fibrosis, and deficiencies in cellular regeneration [11, 108, 109]. In a streptozocin- (STZ-) induced mouse diabetes model, Nrf2 activation with sulforaphane suppressed nephropathy and significantly improved metabolic indices associated with TIIDM, such as hyperglycemia, polydipsia, polyuria, and weight loss [110]. These benefits can be largely attributed to decreases in renal oxidative and nitrosative stress, which act to reverse dysfunction in multiple known mediators of diabetic nephropathy such as transforming growth factor beta (TGF-B), ECM proteins such as fibronectin and collagen IV, and p21, a cell-cycle regulator [111]. Similar findings with other known activators of the Nrf2/Keap1/ARE pathway, such as resveratrol and MG-132, support the therapeutic targeting of this system to ameliorate the oxidative damage and glucose-induced mesangial cell proliferation, inflammation, and fibrosis which underlies diabetic nephropathy [112, 113]. In humans, decreased levels of Nrf2 and expression of target genes in the peripheral blood of patients with CKD further support the contribution of an impaired Nrf2 antioxidant signaling pathway to systemic oxidative overload and inflammation in diabetic nephropathy [114].

6.5. Wound Healing. Impaired wound healing is a well-known and devastating complication of TIIDM and represents the leading cause of chronic wounds and lower extremity amputations in the US [115]. However, despite adherence to tight control of blood glucose levels and advances in synthetic and biologic healing modalities, chronic wounds persist in diabetic patients, suggesting a more fundamental pathology in the diabetic regenerative milieu [116]. As in other diabetic complications, oxidative stress is important for the development of chronic wounds, and AGE in the diabetic wound microenvironment appear to impair wound contraction and remodeling, the inflammatory response, and ECM proliferation. [117] Several natural Nrf2 activators have shown a promise in treating diabetic wounds, and early induction of the Nrf2 pathway through the rhomboid family protein RHBDF2 accelerated cutaneous wound healing in mice [118, 119]. Interestingly, recent work in tissue regeneration models has demonstrated that hyperglycemia in diabetes is associated with Keap1 dysfunction, which prevents nuclear localization of Nrf2 and thus is an appropriate stress response [120]. Utilizing a cutaneous gene therapy model, these studies showed that small-interfering RNA (siRNA) targeted at Keap1 restored wound redox homeostasis, accelerated healing, and counteracted impairments in angiogenesis and reepithelialization, two critical functions of wound healing disrupted in diabetes, by restoring Nrf2 localization. This seems to support the notion that aberrant overexpression of Keap1 and resulting

Nrf2 repression is a possible mechanism of the redox homeostasis dysfunction and impaired wound healing in diabetes. Whether this relationship extends to other aspects of the disease, and the relative contribution of Nrf2 to specific wound healing functions, remains to be seen.

7. The Nrf2/Keap1/ARE Pathway as a Therapeutic Target

Given the broad accountability of oxidative stress for many pathological processes, the Nrf2/Keap1/ARE system has emerged as a logical therapeutic target for the prevention or treatment of disease. This pathway has been studied most intensively in cancer [19] but also in chronic obstructive pulmonary disease (COPD) [121], neurodegenerative disorders [122], and autoimmune diseases such as inflammatory bowel disease (IBD) [123] and rheumatoid arthritis [124]. A multitude of clinical trials has also been pursued in order to assess the efficacy of targeting or modifying elements of the pathway in order to diminish ROS-induced damage in human disease [125–128]. As a critical upstream mediator of the pathway, Nrf2 induction has formed the basis of most of this research. There are three primary mechanisms by which current pharmacological activators increase Nrf2 expression (Figure 2). These consist of (1) activation of upstream kinases such as protein kinase B (Akt) and extracellular signal-regulated kinases (Erk), which phosphorylate specific sites favoring the release of Nrf2 from Keap1; (2) modification of Keap1 cysteine residues, which disrupts the Nrf2-Keap1 complex and favors Nrf2 dissociation; and (3) blockage of ubiquitination and/or proteosomal degradation of Nrf2 [129]. The end result of all of these mechanisms is Nrf2 stabilization and subsequent translocation into the nucleus, where it can exert its transcriptional effects and commence an antioxidant cascade. Of note, Nrf2 activators have already made their way into clinical practice; in 2013, dimethyl fumarate (BG-12, brand name Tecfidera®) was approved by the FDA for the treatment of multiple sclerosis and is thought to exert its therapeutic effects via augmentation of Nrf2's downstream cytoprotective, anti-inflammatory, and antioxidant properties [130, 131]. These achievements represent a promise that the Nrf2 pathway can be effectively used in other diseases in which oxidative stress plays a major role, such as TIIDM.

With regard to TIIDM, knowledge of the pivotal role of oxidative stress in the pathogenesis and progression of the disease originally precipitated investigation into natural antioxidants, such as vitamin E, vitamin C, and coenzyme Q10, as logical initial approaches [132–134]. However, results of these studies have generally been disappointing, and human clinical trials have not shown any benefit of organic molecules as adjunct therapies in preventing or treating diabetic complications [135–137]. Therefore, over the past decade, a significant amount of research, predominantly utilizing high throughput cell-based screening assays, has been devoted to identifying clinically applicable small molecule activators or inducers of endogenous antioxidant mechanisms, such as the Nrf2/Keap1/ARE pathway [138]. These "new mechanism-based antioxidants" have emerged as the new frontier of defense against oxidative stress and inflammation in TIIDM.

An array of Nrf2 small molecule activators, both natural and synthetic, has been identified and studied extensively (Table 2).

These include sulforaphane, curcumin, cinnamaldehyde, pterostilbene, oltipraz, and resveratrol. Some of the most encouraging candidates fall under the category of synthetic triterpenoids; triterpenoid, 2-cyano-3,12-dioxooleane-,1,9(11)-dien-28-oic acid (CDDO), and its derivatives have yielded highly promising results in animal models of heart failure [102], insulin resistance [52], and obesity [155]. Bardoxolone methyl (CDDO-Me), one such derivative originally developed as an anticancer drug, was incidentally found to exhibit renoprotective effects and has made its way into human trials. A phase II clinical trial (BEAM) in adults with TIIDM and advanced CKD showed significant improvements in glomerular filtration rate (GFR) with only mild side effects such as muscle spasms, hypomagnesemia, and gastrointestinal distress [107]. Unfortunately, a subsequent phase III trial (BEACON) in patients with TIIDM and stage IV CKD was terminated early due to serious adverse events [106].

In addition to systemic administration, targeted delivery systems represent a potential approach to treatment of localized diabetes complications. For example, an engineered lipid-protein system (lipoproteoplex) demonstrated safe and efficient delivery of si*Keap1* to diabetic wounds and resulted in accelerated wound healing [156]. Such novel delivery systems could also potentially circumvent the known toxicities resulting from covalent modifications (e.g., alkylation) of Keap1 cysteine residues that form the basis of function for many reported Nrf2 stabilizers [157]. Therefore, Nrf2 pathway modulation via direct, noncovalent inhibition of Nrf2-Keap1 protein-protein complexes is emphasized in current research. Additionally, strategies aimed at adjacent or downstream elements in the Nrf2 pathway have also gained traction as an alternative or combinatorial approaches to treatment. For instance, repression of BTB domain and CNC homolog (Bach1), a nuclear inhibitor of Nrf2, in combination with traditional Nrf2 activators, has shown promising results in neurodegenerative disease models for safely increasing the efficiency and biological activity of these agents [158].

In addition to the pathogenesis of TIIDM and its major complications, treatment of the metabolic alterations in TIIDM has also become a focus of intense investigation. Shin et al. found that Nrf2 regulates fibroblast growth factor 21 (FGF21), a key mediator of glucose and lipid metabolism, in mice [159]. This may have implications in guiding treatment of obesity in TIIDM, which is itself regulated, at least in part, by Nrf2 action on lipogenic gene expression and fatty acid synthesis [156]. Studies have shown that Nrf2/Keap1/ARE activators can exert potent reductions in body weight and hepatic fat accumulation in mice with an excellent safety profile and tolerance, representing potential novel, noninvasive options for managing obesogenesis in TIIDM [156, 160].

As we learn more about the protective aspects of the Nrf2 pathway, we must also appreciate the potential hazards of

Table 2: Small molecule activators of Nrf2 in TIIDM.

Molecule	Source	Mechanism of Nrf2 activation	Evidence
Sulforaphane (SFN)	Natural (cruciferous vegetables such as broccoli, brussel sprouts, and cabbage)	Modification of Keap1 cysteine residues	(i) Pancreatic β-cell protection [45] (ii) Prevented cardiac oxidative damage, inflammation, and hyperglycemic-induced fibrosis [139] (iii) Renal protection in db mice [110]
Curcumin (CUR)	Natural (turmeric)	Modification of Keap1 cysteine residues	(i) Reduced number of prediabetic individuals who progressed to type II DM [140] (ii) Activates liver enzymes involved in glycolysis, gluconeogenesis, and lipid metabolism [141]
Bardoxolone methyl (CDDO-Me/RTA 402)	Synthetic (derivative of oleanolic acid)	Modification of Keap1 cysteine residues	(i) Efficacy in short-term clinical trials in patients with type II DM and CKD [107] (ii) Did not reduce risk of end-stage renal disease (ESRD) or death from cardiovascular failure in patients with DM and stage IV CKD and was terminated early due to side effects [106]
Tert-butylhydro-quinone (tBHQ)	Synthetic (preservative in unsaturated vegetable oils and edible animal fats)	Modification of Keap1 cysteine residues/activation of upstream kinases	(i) Prevented glucose-induced impairments in diabetic retinopathy [142]
Cinnamic aldehyde (CA)	Natural (found in bark of cinnamon tree)	Activation of upstream kinases	(i) Lowered blood glucose, total cholesterol, triglycerides, and increased HDL* in diabetic rats and mice [143] (ii) Prevented development of hypertension in conditions of insulin resistance [144] (iii) Improved renal and glomerular function [110]
Resveratrol (RES)	Natural (polyphenol, found in the skin of red grapes, peanuts, and berries)	Activation of upstream kinases	(i) Reduced blood glucose and HbA1c** levels [145] (ii) Restored secretory function of β-cells in response to toxicity [146] (iii) Renoprotective effects in DM [147]
Magnesium lithospermate B (MLB)	Natural (active polyphenol acid of *Radix Salviae miltiorrhizae* herb)	Activation of upstream kinases	(i) Suppressed progression of renal injury in diabetic rats [148] (ii) Protection against DM-related atherosclerosis [149]
MG132	Synthetic peptide aldehyde	Proteosome inhibitor	(i) Renoprotective against DM-induced oxidative damage, inflammation, and fibrosis [113]
Pterostilbene	Natural (blueberries, grapes)	Mechanism unclear	(i) Protective against β-cell apoptosis [150]
Catechins	Natural (flavenols, found in red wine, berries, grapes)	Likely activation of upstream kinases	(i) Reduced hepatic glucose production and enhanced pancreatic function [151, 152] (ii) Decreased cytokine-induced β-cell damage *in vitro* [153] (iii) Prevented reduction in islet mass *in vivo* [154] (iv) Protected against nephrotoxicity [155]

*HDL: high-density lipoprotein; **HbA1c: a marker of chronic hyperglycemia.

targeting Nrf2 as a therapeutic means. Accruing evidence points to a "dark side" of Nrf2, which also regulates cell proliferation [161]. Nrf2 and its downstream genes are over-expressed in many cancer cell lines and human cancer tissues, and ove-activation of this pathway appears to contribute to the evolution of cancer and chemoresistance of cancer cells [162, 163]. Furthermore, mutations in Nrf2 and Keap1 have been found in a large percentage of malignancies [164, 165]. These findings should not impede our pursuit of targeting the Nrf2/Keap1/ARE pathway in the treatment of diabetes but should rather encourage a cautious and vigilant approach.

8. Nrf2 as a Potential Biomarker

Although biomarkers as a means of noninvasive disease prediction or prevention have been a topic of intense investigation for many years, very few have made their way into the clinical setting, and utility is largely limited to ad hoc corroboration of pathologic events, such as myocardial ischemia and heart failure. An increasing number of studies have been published on biomarkers of oxidative stress in a wide array of human disease [166], and AGE [167], nitrotyrosine [168], preoxiredoxin [169], and 8-hydroxy-2′-deoxyguanosine (8-OhdG) [170] levels in the skin, plasma, and urine

samples have been investigated for both type I and type II DM. However, lack of validation for these markers remains a major obstacle to clinical utility [171]. The adoption of Nrf2, or elements of the Nrf2/Keap1/ARE pathway, as potential biomarkers has been proposed for prognostic purposes in cancer and neurodegeneration [172, 173]. The pursuit of using the Nrf2 pathway as a biomarker for TIIDM and its complications remains in its infancy, but existing evidence suggests that there may indeed be utility in prediabetic and diabetic patients, and research is likely to accelerate in this field as the medical landscape continues to shift towards a front-end, preventative approach with regard to chronic disease management [31, 174].

9. Conclusion

We have answered many questions regarding the role of the Nrf2/Keap1/ARE pathway and oxidative stress in TIIDM, but more remain before we can capitalize on this relationship to attain widespread, clinical significance in humans. For instance, much of what we know is derived from animal studies, and it is unclear as to what extent the murine antioxidant system reflects that of humans. Additionally, despite clear evidence of dysfunction in the Nrf2/Keap1/ARE antioxidant response across a wide range of tissue types and disease stages in TIIDM, the specific mechanisms underlying Nrf2 dysfunction have yet to be fully elucidated. We must also be cognizant of alternative, potentially confounding effects that independent players within Nrf2/Keap1/ARE pathway may exert. For instance, in addition to its cytoprotective role against oxidative stress as part of the Nrf2/Keap1/ARE pathway, accumulating research indicates that the Nrf2 molecule can also independently control the expression of genes responsible for many aspects of cellular metabolism. These studies, largely dependent on transgenic diabetic and Nrf2 knockout murine models, have implicated this molecule in TIIDM pathogenesis, prevention, and progression, via means that are wholly distinct from its role in oxidative protection. For instance, Nrf2 regulates blood glucose homeostasis and metabolic reprogramming by redirecting anabolic pathways [175], inhibiting lipogenesis [176], and promoting insulin sensitization, thereby ameliorating insulin resistance [177]. A more robust understanding of these varied roles may help explain seeming paradoxes in the role of the Nrf2/Keap1/ARE pathway such as what we see in insulin homeostasis and atheroma formation. These examples highlight not only the pleotropic effects of Nrf2 throughout tissues but also the variety of ways in which it responds and interacts, often contradictorily, with different types of stress.

Looking towards the future, we must continue to validate the Nrf2/Keap1/ARE pathway as a mediator of the oxidative stress underlying TIIDM and further explore this role in less morbid complications such as retinopathy and neuropathy. As we continue to screen for and develop ways to target the Nrf2/Keap1/ARE pathway, the importance of identifying novel delivery systems and nontoxic mechanisms of Nrf2 activation will accelerate translation of these therapeutics into human trials. Finally, encouraging evidence for the use of the Nrf2/Keap1/ARE as a biomarker should catalyze efforts to validate its use in the clinical setting. The intertwining roles of Nrf2, oxidative stress, and TIIDM will continue to provoke interest for a time to come, but it is becoming increasingly clear that further understanding this intimate relationship has preventative, prognostic, and therapeutic value in combatting this devastating disease.

Conflicts of Interest

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

Acknowledgments

This work was supported by the American Diabetes Association "Pathway to Stop Diabetes" to Daniel J. Ceradini [Grant no. 1-16-ACE-08].

References

[1] G. Roglic, *Global Report on Diabetes*, World Health Organization, 2016.

[2] J. A. O'Brien, A. R. Patrick, and J. Caro, "Estimates of direct medical costs for microvascular and macrovascular complications resulting from type 2 diabetes mellitus in the United States in 2000," *Clinical Therapeutics*, vol. 25, pp. 1017–1038, 2003.

[3] D. Lloyd-Jones, R. J. Adams, T. M. Brown et al., "Heart disease and stroke statistics—2010 update: a report from the American Heart Association," *Circulation*, vol. 121, pp. e46–e215, 2010.

[4] J. L. Dieleman, R. Baral, M. Birger et al., "US spending on personal health care and public health, 1996-2013," *The Journal of the American Medical Association*, vol. 316, pp. 2627–2646, 2016.

[5] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 37, Supplement 1, pp. S81–S90, 2014.

[6] W. C. Knowler, E. Barrett-Connor, S. E. Fowler et al., "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin," *New England Journal of Medicine*, vol. 346, no. 6, pp. 393–403, 2002.

[7] E. W. Gregg, Y. Li, J. Wang et al., "Changes in diabetes-related complications in the United States, 1990-2010," *The New England Journal of Medicine*, vol. 370, pp. 1514–1523, 2014.

[8] W. R. Rowley, C. Bezold, Y. Arikan, E. Byrne, and S. Krohe, "Diabetes 2030: insights from yesterday, today, and future trends," *Population Health Management*, vol. 20, pp. 6–12, 2017.

[9] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, "Oxidative stress, inflammation, and cancer: how are they linked?," *Free Radical Biology and Medicine*, vol. 49, pp. 1603–1616, 2010.

[10] P. Soysal, A. T. Isik, A. F. Carvalho et al., "Oxidative stress and frailty: a systematic review and synthesis of the best evidence," *Maturitas*, vol. 99, pp. 66–72, 2017.

[11] D. M. Small, J. S. Coombes, N. Bennett, D. W. Johnson, and G. C. Gobe, "Oxidative stress, antioxidant therapies and

chronic kidney disease," *Nephrology*, vol. 17, pp. 311–321, 2012.

[12] A. J. McGuinness and E. Sapey, "Oxidative stress in COPD: sources, markers, and potential mechanisms," *Journal of Clinical Medicine*, vol. 6, no. 2, 2017.

[13] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[14] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, pp. 1058–1070, 2010.

[15] P. Dandona, K. Thusu, S. Cook et al., "Oxidative damage to DNA in diabetes mellitus," *Lancet*, vol. 347, pp. 444-445, 1996.

[16] T. Nishikawa, D. Edelstein, X. L. Du et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, pp. 787–790, 2000.

[17] B. Williams, B. Gallacher, H. Patel, and C. Orme, "Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro," *Diabetes*, vol. 46, pp. 1497–1503, 1997.

[18] H. Ishii, M. R. Jirousek, D. Koya et al., "Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor," *Science*, vol. 272, pp. 728–731, 1996.

[19] T. W. Kensler, N. Wakabayashi, and S. Biswal, "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway," *Annual Review of Pharmacology and Toxicology*, vol. 47, pp. 89–116, 2007.

[20] K. Itoh, T. Chiba, S. Takahashi et al., "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements," *Biochemical and Biophysical Research Communications*, vol. 236, pp. 313–322, 1997.

[21] M. McMahon, K. Itoh, M. Yamamoto, and J. D. Hayes, "Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression," *The Journal of Biological Chemistry*, vol. 278, pp. 21592–21600, 2003.

[22] P. Moi, K. Chan, I. Asunis, A. Cao, and Y. W. Kan, "Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, pp. 9926–9930, 1994.

[23] R. Venugopal and A. K. Jaiswal, "Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:-quinone oxidoreductase1 gene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, pp. 14960–14965, 1996.

[24] T. Nguyen, P. J. Sherratt, P. Nioi, C. S. Yang, and C. B. Pickett, "Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1," *The Journal of Biological Chemistry*, vol. 280, pp. 32485–32492, 2005.

[25] T. Nguyen, P. Nioi, and C. B. Pickett, "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress," *The Journal of Biological Chemistry*, vol. 284, pp. 13291–13295, 2009.

[26] D. D. Zhang and M. Hannink, "Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress," *Molecular and Cellular Biology*, vol. 23, pp. 8137–8151, 2003.

[27] J. Alam, D. Stewart, C. Touchard, S. Boinapally, A. M. Choi, and J. L. Cook, "Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene," *The Journal of Biological Chemistry*, vol. 274, pp. 26071–26078, 1999.

[28] L. F. Chasseaud, "The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents," *Advances in Cancer Research*, vol. 29, pp. 175–274, 1979.

[29] A. Uruno, Y. Yagishita, and M. Yamamoto, "The Keap1-Nrf2 system and diabetes mellitus," *Archives of Biochemistry and Biophysics*, vol. 566, pp. 76–84, 2015.

[30] M. Long, M. Rojo de la Vega, Q. Wen et al., "An essential role of NRF2 in diabetic wound healing," *Diabetes*, vol. 65, pp. 780–793, 2016.

[31] A. S. Jiménez-Osorio, A. Picazo, S. González-Reyes, D. Barrera-Oviedo, M. E. Rodríguez-Arellano, and J. Pedraza-Chaverri, "Nrf2 and redox status in prediabetic and diabetic patients," *International Journal of Molecular Sciences*, vol. 15, pp. 20290–20305, 2014.

[32] M. C. Lu, J. A. Ji, Z. Y. Jiang, and Q. D. You, "The Keap1-Nrf2-ARE pathway as a potential preventive and therapeutic target: an update," *Medicinal Research Review*, vol. 36, pp. 924–963, 2016.

[33] L. M. Aleksunes, S. A. Reisman, R. L. Yeager, M. J. Goedken, and C. D. Klaassen, "Nuclear factor erythroid 2-related factor 2 deletion impairs glucose tolerance and exacerbates hyperglycemia in type 1 diabetic mice," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 333, no. 1, pp. 140–151, 2010.

[34] M. S. Bitar and F. Al-Mulla, "A defect in Nrf2 signaling constitutes a mechanism for cellular stress hypersensitivity in a genetic rat model of type 2 diabetes," *American Journal of Physiology Endocrinology and Metabolism*, vol. 301, no. 6, pp. 1119–1129, 2011.

[35] A. T. Dinkova-Kostova and P. Talalay, "Direct and indirect antioxidant properties of inducers of cytoprotective proteins," *Molecular Nutrition & Food Research*, vol. 52, no. 1, pp. 128–138, 2008.

[36] M. Cnop, N. Welsh, J. C. Jonas, A. Jörns, S. Lenzen, and D. L. Eizirik, "Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities," *Diabetes*, vol. 54, Supplement 2, pp. S97–107, 2005.

[37] S. Fujimoto, E. Mukai, and N. Inagaki, "Role of endogenous ROS production in impaired metabolism-secretion coupling of diabetic pancreatic beta cells," *Progress in Biophysics and Molecular Biology*, vol. 107, pp. 304–310, 2011.

[38] J. S. Harmon, M. Bogdani, S. D. Parazzoli et al., "Beta-cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice," *Endocrinology*, vol. 150, pp. 4855–4862, 2009.

[39] H. M. Kubisch, J. Wang, T. M. Bray, and J. P. Phillips, "Targeted overexpression of cu/Zn superoxide dismutase protects pancreatic beta-cells against oxidative stress," *Diabetes*, vol. 46, pp. 1563–1566, 1997.

[40] M. Sasaki, S. Fujimoto, Y. Sato et al., "Reduction of reactive oxygen species ameliorates metabolism-secretion coupling

in islets of diabetic GK rats by suppressing lactate overproduction," *Diabetes*, vol. 62, pp. 1996–2003, 2013.

[41] H. Sakuraba, H. Mizukami, N. Yagihashi, R. Wada, C. Hanyu, and S. Yagihashi, "Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients," *Diabetologia*, vol. 45, pp. 85–96, 2002.

[42] A. Vetere, A. Choudhary, S. M. Burns, and B. K. Wagner, "Targeting the pancreatic beta-cell to treat diabetes," *Nature Reviews. Drug Discovery*, vol. 13, pp. 278–289, 2014.

[43] Y. Yagishita, T. Fukutomi, A. Sugawara et al., "Nrf2 protects pancreatic beta-cells from oxidative and nitrosative stress in diabetic model mice," *Diabetes*, vol. 63, pp. 605–618, 2014.

[44] A. Uruno, Y. Furusawa, Y. Yagishita et al., "The Keap1-Nrf2 system prevents onset of diabetes mellitus," *Molecular and Cellular Biology*, vol. 33, pp. 2996–3010, 2013.

[45] M. Y. Song, E. K. Kim, W. S. Moon et al., "Sulforaphane protects against cytokine- and streptozotocin-induced beta-cell damage by suppressing the NF-kappaB pathway," *Toxicology and Applied Pharmacology*, vol. 235, pp. 57–67, 2009.

[46] W. Li, W. Wu, H. Song et al., "Targeting Nrf2 by dihydro-CDDO-trifluoroethyl amide enhances autophagic clearance and viability of beta-cells in a setting of oxidative stress," *FEBS Letters*, vol. 588, pp. 2115–2124, 2014.

[47] S. Lee, E. G. Hur, I. G. Ryoo, K. A. Jung, J. Kwak, and M. K. Kwak, "Involvement of the Nrf2-proteasome pathway in the endoplasmic reticulum stress response in pancreatic beta-cells," *Toxicology and Applied Pharmacology*, vol. 264, pp. 431–438, 2012.

[48] B. McEvoy, R. Sumayao, C. Slattery, T. McMorrow, and P. Newsholme, "Cystine accumulation attenuates insulin release from the pancreatic beta-cell due to elevated oxidative stress and decreased ATP levels," *The Journal of Physiology*, vol. 593, pp. 5167–5182, 2015.

[49] K. Sakai, K. Matsumoto, T. Nishikawa et al., "Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells," *Biochemical and Biophysical Research Communications*, vol. 300, pp. 216–222, 2003.

[50] M. Saadeh, T. C. Ferrante, A. Kane, O. Shirihai, B. E. Corkey, and J. T. Deeney, "Reactive oxygen species stimulate insulin secretion in rat pancreatic islets: studies using mono-oleoylglycerol," *PLoS One*, vol. 7, article e30200, 2012.

[51] C. Leloup, C. Tourrel-Cuzin, C. Magnan et al., "Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion," *Diabetes*, vol. 58, pp. 673–681, 2009.

[52] P. K. Saha, V. T. Reddy, M. Konopleva, M. Andreeff, and L. Chan, "The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid methyl ester has potent anti-diabetic effects in diet-induced diabetic mice and Lepr(db/db) mice," *The Journal of Biological Chemistry*, vol. 285, pp. 40581–40592, 2010.

[53] Z. Yu, W. Shao, Y. Chiang et al., "Oltipraz upregulates the nuclear factor (erythroid-derived 2)-like 2 (NRF2) antioxidant system and prevents insulin resistance and obesity induced by a high-fat diet in C57BL/6J mice," *Diabetologia*, vol. 54, pp. 922–934, 2011.

[54] Y. K. Zhang, K. C. Wu, J. Liu, and C. D. Klaassen, "Nrf2 deficiency improves glucose tolerance in mice fed a high-fat diet," *Toxicology and Applied Pharmacology*, vol. 264, pp. 305–314, 2012.

[55] P. J. Meakin, S. Chowdhry, R. S. Sharma et al., "Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance," *Molecular and Cellular Biology*, vol. 34, pp. 3305–3320, 2014.

[56] A. K. Meher, P. R. Sharma, V. A. Lira et al., "Nrf2 deficiency in myeloid cells is not sufficient to protect mice from high-fat diet-induced adipose tissue inflammation and insulin resistance," *Free Radical Biology and Medicine*, vol. 52, pp. 1708–1715, 2012.

[57] J. Pi, L. Leung, P. Xue et al., "Deficiency in the nuclear factor E2-related factor-2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity," *The Journal of Biological Chemistry*, vol. 285, pp. 9292–9300, 2010.

[58] C. Talchai, S. Xuan, H. V. Lin, L. Sussel, and D. Accili, "Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure," *Cell*, vol. 150, pp. 1223–1234, 2012.

[59] Y. Yagishita, A. Uruno, T. Fukutomi et al., "Nrf2 improves leptin and insulin resistance provoked by hypothalamic oxidative stress," *Cell Reports*, vol. 18, no. 8, pp. 2030–2044, 2017.

[60] S. E. Kahn, R. L. Hull, and K. M. Utzschneider, "Mechanisms linking obesity to insulin resistance and type 2 diabetes," *Nature*, vol. 444, pp. 840–846, 2006.

[61] K. M. Holmström, L. Baird, Y. Zhang et al., "Nrf2 impacts cellular bioenergetics by controlling substrate availability for mitochondrial respiration," *Biology Open*, vol. 2, no. 8, pp. 761–770, 2013.

[62] C. S. Fox, S. Coady, P. D. Sorlie et al., "Trends in cardiovascular complications of diabetes," *The Journal of the American Medical Association*, vol. 292, pp. 2495–2499, 2004.

[63] D. G. Harrison, M. C. Gongora, T. J. Guzik, and J. Widder, "Oxidative stress and hypertension," *Journal of the American Society of Hypertension*, vol. 1, pp. 30–44, 2007.

[64] H. Tsutsui, S. Kinugawa, and S. Matsushima, "Oxidative stress and heart failure," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 301, pp. H2181–H2190, 2011.

[65] D. Harrison, K. K. Griendling, U. Landmesser, B. Hornig, and H. Drexler, "Role of oxidative stress in atherosclerosis," *The American Journal of Cardiology*, vol. 91, pp. 7a–11a, 2003.

[66] J. L. Zweier, "Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury," *The Journal of Biological Chemistry*, vol. 263, pp. 1353–1357, 1988.

[67] I. M. Fearon and S. P. Faux, "Oxidative stress and cardiovascular disease: novel tools give (free) radical insight," *Journal of Molecular and Cellular Cardiology*, vol. 47, pp. 372–381, 2009.

[68] M. J. Callaghan, D. J. Ceradini, and G. C. Gurtner, "Hyperglycemia-induced reactive oxygen species and impaired endothelial progenitor cell function," *Antioxidants & Redox Signaling*, vol. 7, pp. 1476–1482, 2005.

[69] A. Martin, M. R. Komada, and D. C. Sane, "Abnormal angiogenesis in diabetes mellitus," *Medicinal Research Reviews*, vol. 23, pp. 117–145, 2003.

[70] Y. Wei, J. Gong, R. K. Thimmulappa, B. Kosmider, S. Biswal, and E. J. Duh, "Nrf2 acts cell-autonomously in endothelium

to regulate tip cell formation and vascular branching," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, pp. E3910–E3918, 2013.

[71] Z. Ungvari, L. Bailey-Downs, T. Gautam et al., "Adaptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 300, pp. H1133–H1140, 2011.

[72] T. M. Chen, J. Li, L. Liu et al., "Effects of heme oxygenase-1 upregulation on blood pressure and cardiac function in an animal model of hypertensive myocardial infarction," *International Journal of Molecular Sciences*, vol. 14, pp. 2684–2706, 2013.

[73] J. L. Zweier, P. Kuppusamy, and G. A. Lutty, "Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 11, pp. 4046–4050, 1988.

[74] J. Li, T. M. Chen, J. Cao et al., "Long-term effects of heme oxygenase 1 overexpression on post-infarction heart function in diabetic rats," *Chinese Journal of Applied Physiology*, vol. 30, no. 5, pp. 421–426, 2014.

[75] Y. Zhao, L. Zhang, Y. Qiao et al., "Heme oxygenase-1 prevents cardiac dysfunction in streptozotocin-diabetic mice by reducing inflammation, oxidative stress, apoptosis and enhancing autophagy," *PLoS One*, vol. 8, no. 9, article e75927, 2013.

[76] H. Zhu, Z. Jia, L. Zhang et al., "Antioxidants and phase 2 enzymes in macrophages: regulation by Nrf2 signaling and protection against oxidative and electrophilic stress," *Experimental Biology and Medicine*, vol. 233, pp. 463–474, 2008.

[77] H. K. Jyrkkänen, E. Kansanen, M. Inkala et al., "Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo," *Circulation Research*, vol. 103, pp. e1–e9, 2008.

[78] A. K. Ruotsalainen, M. Inkala, M. E. Partanen et al., "The absence of macrophage Nrf2 promotes early atherogenesis," *Cardiovascular Research*, vol. 98, pp. 107–115, 2013.

[79] M. Zakkar, K. HeidenVan der, A. Luong le et al., "Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, pp. 1851–1857, 2009.

[80] K. Ishikawa and Y. Maruyama, "Heme oxygenase as an intrinsic defense system in vascular wall: implication against atherogenesis," *Journal of Atherosclerosis and Thrombosis*, vol. 8, pp. 63–70, 2001.

[81] T. E. Sussan, J. Jun, R. Thimmulappa et al., "Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice," *PLoS One*, vol. 3, article e3791, 2008.

[82] X. L. Chen, S. E. Varner, A. S. Rao et al., "Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism," *The Journal of Biological Chemistry*, vol. 278, pp. 703–711, 2003.

[83] S. Freigang, F. Ampenberger, G. Spohn et al., "Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis," *European Journal of Immunology*, vol. 41, pp. 2040–2051, 2011.

[84] J. Song, S. Sumiyoshi, Y. Nakashima et al., "Overexpression of heme oxygenase-1 in coronary atherosclerosis of Japanese autopsies with diabetes mellitus: Hisayama study," *Atherosclerosis*, vol. 202, no. 2, pp. 573–581, 2009.

[85] T. Hosoya, A. Maruyama, M. I. Kang et al., "Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells," *The Journal of Biological Chemistry*, vol. 280, pp. 27244–27250, 2005.

[86] W. B. Kannel and D. L. McGee, "Diabetes and cardiovascular disease. The Framingham study," *The Journal of the American Medical Association*, vol. 241, pp. 2035–2038, 1979.

[87] H. Mönkemann, A. S. VrieseDe, H. J. Blom et al., "Early molecular events in the development of the diabetic cardiomyopathy," *Amino Acids*, vol. 23, pp. 331–336, 2002.

[88] A. Chesley, M. S. Lundberg, T. Asai et al., "The β_2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G_i-dependent coupling to phosphatidylinositol 3′-kinase," *Circulation Research*, vol. 87, pp. 1172–1179, 2000.

[89] R. Bolli, W. X. Zhu, C. J. Hartley et al., "Attenuation of dysfunction in the postischemic 'stunned' myocardium by dimethylthiourea," *Circulation*, vol. 76, pp. 458–468, 1987.

[90] A. C. Kohler, C. M. Sag, and L. S. Maier, "Reactive oxygen species and excitation-contraction coupling in the context of cardiac pathology," *Journal of Molecular and Cellular Cardiology*, vol. 73, pp. 92–102, 2014.

[91] A. Beresewicz and M. Horackova, "Alterations in electrical and contractile behavior of isolated cardiomyocytes by hydrogen peroxide: possible ionic mechanisms," *Journal of Molecular and Cellular Cardiology*, vol. 23, pp. 899–918, 1991.

[92] A. Sabri, H. H. Hughie, and P. A. Lucchesi, "Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes," *Antioxidants & Redox Signaling*, vol. 5, pp. 731–740, 2003.

[93] S. Rajagopalan, X. P. Meng, S. Ramasamy, D. G. Harrison, and Z. S. Galis, "Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability," *The Journal of Clinical Investigation*, vol. 98, pp. 2572–2579, 1996.

[94] E. Ingelsson, J. Sundström, J. Arnlöv, B. Zethelius, and L. Lind, "Insulin resistance and risk of congestive heart failure," *The Journal of the American Medical Association*, vol. 294, pp. 334–341, 2005.

[95] Z. Lu, X. Xu, X. Hu et al., "Extracellular superoxide dismutase deficiency exacerbates pressure overload-induced left ventricular hypertrophy and dysfunction," *Hypertension*, vol. 51, pp. 19–25, 2008.

[96] C. M. Hu, Y. H. Chen, M. T. Chiang, and L. Y. Chau, "Heme oxygenase-1 inhibits angiotensin II-induced cardiac hypertrophy in vitro and in vivo," *Circulation*, vol. 110, pp. 309–316, 2004.

[97] S. Matsushima, S. Kinugawa, T. Ide et al., "Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 291, pp. H2237–H2245, 2006.

[98] Y. Tan, T. Ichikawa, J. Li et al., "Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo," *Diabetes*, vol. 60, pp. 625–633, 2011.

[99] J. Li, T. Ichikawa, L. Villacorta et al., "Nrf2 protects against maladaptive cardiac responses to hemodynamic stress,"

Arteriosclerosis, Thrombosis, and Vascular Biology, vol. 29, pp. 1843–1850, 2009.

[100] T. Ichikawa, J. Li, C. J. Meyer, J. S. Janicki, M. Hannink, and T. Cui, "Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes," *PLoS One*, vol. 4, article e8391, 2009.

[101] H. Ashrafian, G. Czibik, M. Bellahcene et al., "Fumarate is cardioprotective via activation of the Nrf2 antioxidant pathway," *Cell Metabolism*, vol. 15, pp. 361–371, 2012.

[102] Y. Xing, T. Niu, W. Wang et al., "Triterpenoid dihydro-CDDO-trifluoroethyl amide protects against maladaptive cardiac remodeling and dysfunction in mice: a critical role of Nrf2," *PLoS One*, vol. 7, article e44899, 2012.

[103] T. E. Sussan, T. Rangasamy, D. J. Blake et al., "Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, pp. 250–255, 2009.

[104] T. Abe, Y. Ohga, N. Tabayashi et al., "Left ventricular diastolic dysfunction in type 2 diabetes mellitus model rats," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 282, pp. H138–H148, 2002.

[105] N. S. Rajasekaran, S. Varadharaj, G. D. Khanderao et al., "Sustained activation of nuclear erythroid 2-related factor 2/antioxidant response element signaling promotes reductive stress in the human mutant protein aggregation cardiomyopathy in mice," *Antioxidants & Redox Signaling*, vol. 14, pp. 957–971, 2011.

[106] D. Zeeuwde, T. Akizawa, P. Audhya et al., "Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease," *The New England Journal of Medicine*, vol. 369, pp. 2492–2503, 2013.

[107] P. E. Pergola, P. Raskin, R. D. Toto et al., "Bardoxolone methyl and kidney function in CKD with type 2 diabetes," *The New England Journal of Medicine*, vol. 365, pp. 327–336, 2011.

[108] L. M. Pedruzzi, M. B. Stockler-Pinto, M. Leite Jr., and D. Mafra, "Nrf2-keap1 system versus NF-kappaB: the good and the evil in chronic kidney disease?," *Biochimie*, vol. 94, pp. 2461–2466, 2012.

[109] H. J. Kim and N. D. Vaziri, "Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure," *American Journal of Physiology Renal Physiology*, vol. 298, pp. F662–F671, 2010.

[110] H. Zheng, S. A. Whitman, W. Wu et al., "Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy," *Diabetes*, vol. 60, pp. 3055–3066, 2011.

[111] K. Yoh, A. Hirayama, K. Ishizaki et al., "Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in Nrf2-deficient mice," *Genes to Cells*, vol. 13, pp. 1159–1170, 2008.

[112] B. Jiang, L. Guo, B. Y. Li et al., "Resveratrol attenuates early diabetic nephropathy by down-regulating glutathione S-transferases mu in diabetic rats," *Journal of Medicinal Food*, vol. 16, pp. 481–486, 2013.

[113] W. Cui, B. Li, Y. Bai et al., "Potential role for Nrf2 activation in the therapeutic effect of MG132 on diabetic nephropathy in OVE26 diabetic mice," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 304, pp. E87–E99, 2013.

[114] L. M. Pedruzzi, L. F. Cardozo, J. B. Daleprane et al., "Systemic inflammation and oxidative stress in hemodialysis patients are associated with down-regulation of Nrf2," *Journal of Nephrology*, vol. 28, pp. 495–501, 2015.

[115] American Diabetes Association, "Economic costs of diabetes in the U.S. in 2012," *Diabetes Care*, vol. 36, pp. 1033–1046, 2013.

[116] W. Duckworth, C. Abraira, T. Moritz et al., "Glucose control and vascular complications in veterans with type 2 diabetes," *The New England Journal of Medicine*, vol. 360, pp. 129–139, 2009.

[117] M. Peppa, P. Stavroulakis, and S. A. Raptis, "Advanced glycoxidation products and impaired diabetic wound healing," *Wound Repair and Regeneration*, vol. 17, pp. 461–472, 2009.

[118] D. R. Senger and S. Cao, "Diabetic wound healing and activation of Nrf2 by herbal medicine," *Journal of Nature and Science*, vol. 2, 2016.

[119] V. Hosur, L. M. Burzenski, T. M. Stearns et al., "Early induction of NRF2 antioxidant pathway by RHBDF2 mediates rapid cutaneous wound healing," *Experimental and Molecular Pathology*, vol. 102, no. 2, pp. 337–346, 2017.

[120] M. A. Soares, O. D. Cohen, Y. C. Low et al., "Restoration of Nrf2 signaling normalizes the regenerative niche," *Diabetes*, vol. 65, pp. 633–646, 2016.

[121] A. Boutten, D. Goven, E. Artaud-Macari, J. Boczkowski, and M. Bonay, "NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease," *Trends in Molecular Medicine*, vol. 17, pp. 363–371, 2011.

[122] C. P. Ramsey, C. A. Glass, M. B. Montgomery et al., "Expression of Nrf2 in neurodegenerative diseases," *Journal of Neuropathology and Experimental Neurology*, vol. 66, pp. 75–85, 2007.

[123] L. M. Aleksunes and J. E. Manautou, "Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease," *Toxicologic Pathology*, vol. 35, pp. 459–473, 2007.

[124] C. J. Wruck, A. Fragoulis, A. Gurzynski et al., "Role of oxidative stress in rheumatoid arthritis: insights from the Nrf2-knockout mice," *Annals of the Rheumatic Diseases*, vol. 70, pp. 844–850, 2011.

[125] T. A. Shapiro, J. W. Fahey, A. T. Dinkova-Kostova et al., "Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study," *Nutrition and Cancer*, vol. 55, pp. 53–62, 2006.

[126] B. S. Cornblatt, L. Ye, A. T. Dinkova-Kostova et al., "Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast," *Carcinogenesis*, vol. 28, pp. 1485–1490, 2007.

[127] H. Hatcher, R. Planalp, J. Cho, F. M. Torti, and S. V. Torti, "Curcumin: from ancient medicine to current clinical trials," *Cellular and Molecular Life Sciences*, vol. 65, pp. 1631–1652, 2008.

[128] Y. Zhang and R. Munday, "Dithiolethiones for cancer chemoprevention: where do we stand?," *Molecular Cancer Therapeutics*, vol. 7, pp. 3470–3479, 2008.

[129] E. Bhakkiyalakshmi, D. Sireesh, P. Rajaguru, R. Paulmurugan, and K. M. Ramkumar, "The emerging role of redox-sensitive Nrf2-Keap1 pathway in diabetes," *Pharmacological Research*, vol. 91, pp. 104–114, 2015.

[130] R. J. Fox, M. Kita, S. L. Cohan et al., "BG-12 (dimethyl fumarate): a review of mechanism of action, efficacy, and safety," *Current Medical Research and Opinion*, vol. 30, pp. 251–262, 2014.

[131] R. J. Fox, D. H. Miller, J. T. Phillips et al., "Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis," *The New England Journal of Medicine*, vol. 367, pp. 1087–1097, 2012.

[132] H. Kaneto, Y. Kajimoto, J. Miyagawa et al., "Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity," *Diabetes*, vol. 48, pp. 28–2406, 1999.

[133] K. M. Ramkumar, R. S. Vijayakumar, P. Vanitha et al., "Protective effect of gallic acid on alloxan-induced oxidative stress and osmotic fragility in rats," *Human & Experimental Toxicology*, vol. 33, pp. 638–649, 2014.

[134] M. Latha, L. Pari, K. M. Ramkumar et al., "Antidiabetic effects of scoparic acid D isolated from Scoparia dulcis in rats with streptozotocin-induced diabetes," *Natural Product Research*, vol. 23, pp. 1528–1540, 2009.

[135] S. Golbidi, S. A. Ebadi, and I. Laher, "Antioxidants in the treatment of diabetes," *Current Diabetes Reviews*, vol. 7, pp. 106–125, 2011.

[136] E. Lonn, S. Yusuf, B. Hoogwerf et al., "Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and MICRO-HOPE substudy," *Diabetes Care*, vol. 25, pp. 1919–1927, 2002.

[137] J. Belch, A. MacCuish, I. Campbell et al., "The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease," *BMJ*, vol. 337, article a1840, 2008.

[138] K. C. Wu, P. R. McDonald, J. J. Liu, R. Chaguturu, and C. D. Klaassen, "Implementation of a high-throughput screen for identifying small molecules to activate the Keap1-Nrf2-ARE pathway," *PLoS One*, vol. 7, article e44686, 2012.

[139] Y. Bai, W. Cui, Y. Xin et al., "Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation," *Journal of Molecular and Cellular Cardiology*, vol. 57, pp. 82–95, 2013.

[140] S. Chuengsamarn, S. Rattanamongkolgul, R. Luechapudiporn, C. Phisalaphong, and S. Jirawatnotai, "Curcumin extract for prevention of type 2 diabetes," *Diabetes Care*, vol. 35, pp. 2121–2127, 2012.

[141] K. I. Seo, M. S. Choi, U. J. Jung et al., "Effect of curcumin supplementation on blood glucose, plasma insulin, and glucose homeostasis related enzyme activities in diabetic db/db mice," *Molecular Nutrition & Food Research*, vol. 52, pp. 995–1004, 2008.

[142] Q. Zhong, M. Mishra, and R. A. Kowluru, "Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy," *Investigative Ophthalmology & Visual Science*, vol. 54, pp. 3941–3948, 2013.

[143] P. Subash Babu, S. Prabuseenivasan, and S. Ignacimuthu, "Cinnamaldehyde—a potential antidiabetic agent," *Phytomedicine*, vol. 14, pp. 15–22, 2007.

[144] H. M. El-Bassossy, A. Fahmy, and D. Badawy, "Cinnamaldehyde protects from the hypertension associated with diabetes," *Food and Chemical Toxicology*, vol. 49, pp. 3007–3012, 2011.

[145] A. Movahed, I. Nabipour, X. Lieben Louis et al., "Antihyperglycemic effects of short term resveratrol supplementation in type 2 diabetic patients," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 851267, 11 pages, 2013.

[146] J. H. Lee, M. Y. Song, E. K. Song et al., "Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway," *Diabetes*, vol. 58, pp. 344–351, 2009.

[147] P. Palsamy and S. Subramanian, "Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1812, pp. 719–731, 2011.

[148] G. T. Lee, H. Ha, M. Jung et al., "Delayed treatment with lithospermate B attenuates experimental diabetic renal injury," *Journal of the American Society of Nephrology*, vol. 14, pp. 709–720, 2003.

[149] K. Y. Hur, S. H. Kim, M. A. Choi et al., "Protective effects of magnesium lithospermate B against diabetic atherosclerosis via Nrf2-ARE-NQO1 transcriptional pathway," *Atherosclerosis*, vol. 211, pp. 69–76, 2010.

[150] E. Bhakkiyalakshmi, D. Shalini, T. V. Sekar, P. Rajaguru, R. Paulmurugan, and K. M. Ramkumar, "Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2," *British Journal of Pharmacology*, vol. 171, pp. 1747–1757, 2014.

[151] S. Wolfram, D. Raederstorff, M. Preller et al., "Epigallocatechin gallate supplementation alleviates diabetes in rodents," *Journal of Nutrition*, vol. 136, pp. 2512–2518, 2006.

[152] M. E. Waltner-Law, X. L. Wang, B. K. Law, R. K. Hall, M. Nawano, and D. K. Granner, "Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production," *Journal of Biological Chemistry*, vol. 277, pp. 34933–34940, 2002.

[153] M. K. Han, "Epigallocatechin gallate, a constituent of green tea, suppresses cytokine-induced pancreatic beta-cell damage," *Experimental & Molecular Medicine*, vol. 35, pp. 136–139, 2003.

[154] E. K. Song, H. Hur, and M. K. Han, "Epigallocatechin gallate prevents autoimmune diabetes induced by multiple low doses of streptozotocin in mice," *Archives of Pharmacal Research*, vol. 26, pp. 559–563, 2003.

[155] K. Sahin, M. Tuzcu, H. Gencoglu et al., "Epigallocatechin-3-gallate activates Nrf2/HO-1 signaling pathway in cisplatin-induced nephrotoxicity in rats," *Life Sciences*, vol. 87, pp. 240–245, 2010.

[156] S. Shin, J. Wakabayashi, M. S. Yates et al., "Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide," *European Journal of Pharmacology*, vol. 620, pp. 138–144, 2009.

[157] P. S. Rabbani, A. Zhou, Z. M. Borab et al., "Novel lipoproteoplex delivers Keap1 siRNA-based gene therapy to accelerate diabetic wound healing," *Biomaterials*, vol. 132, pp. 1–15, 2017.

[158] I. G. Gazaryan and B. Thomas, "The status of Nrf2-based therapeutics: current perspectives and future prospects," *Neural Regeneration Research*, vol. 11, pp. 1708–1711, 2016.

[159] Y. Furusawa, A. Uruno, Y. Yagishita, C. Higashi, and M. Yamamoto, "Nrf2 induces fibroblast growth factor 21 in diabetic mice," *Genes to Cells*, vol. 19, pp. 864–878, 2014.

[160] M. Chin, C. Y. Lee, J. C. Chuang et al., "Bardoxolone methyl analogs RTA 405 and dh404 are well tolerated and exhibit efficacy in rodent models of type 2 diabetes and obesity,"

American Journal of Physiology. Renal Physiology, vol. 304, pp. F1438–F1446, 2013.

[161] H. M. Leinonen, E. Kansanen, P. Pölönen, M. Heinäniemi, and A. L. Levonen, "Dysregulation of the Keap1-Nrf2 pathway in cancer," *Biochemical Society Transactions*, vol. 43, pp. 645–649, 2015.

[162] A. Lau, N. F. Villeneuve, Z. Sun, P. K. Wong, and D. D. Zhang, "Dual roles of Nrf2 in cancer," *Pharmacology Research*, vol. 58, pp. 262–270, 2008.

[163] J. D. Hayes and M. McMahon, "The double-edged sword of Nrf2: subversion of redox homeostasis during the evolution of cancer," *Molecular Cell*, vol. 21, pp. 732–734, 2006.

[164] T. Shibata, T. Ohta, K. I. Tong et al., "Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, pp. 13568–13573, 2008.

[165] T. Ohta, K. Iijima, M. Miyamoto et al., "Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth," *Cancer Research*, vol. 68, pp. 1303–1309, 2008.

[166] J. Frijhoff, P. G. Winyard, N. Zarkovic et al., "Clinical relevance of biomarkers of oxidative stress," *Antioxidants & Redox Signaling*, vol. 23, pp. 1144–1170, 2015.

[167] J. M. Ashraf, M. Y. Arfat, Z. Arif, J. Ahmad, and K. Alam, "A clinical correlation of anti-DNA-AGE autoantibodies in type 2 diabetes mellitus with disease duration," *Cellular Immunology*, vol. 293, pp. 74–79, 2015.

[168] A. Ceriello, F. Mercuri, L. Quagliaro et al., "Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress," *Diabetologia*, vol. 44, pp. 834–838, 2001.

[169] A. Abbasi, E. Corpeleijn, R. T. Gansevoort et al., "Circulating peroxiredoxin 4 and type 2 diabetes risk: the Prevention of Renal and Vascular Endstage Disease (PREVEND) study," *Diabetologia*, vol. 57, pp. 1842–1849, 2014.

[170] M. Serdar, E. Sertoglu, M. Uyanik et al., "Comparison of 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels using mass spectrometer and urine albumin creatinine ratio as a predictor of development of diabetic nephropathy," *Free Radical Research*, vol. 46, pp. 1291–1295, 2012.

[171] I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, and A. Milzani, "Biomarkers of oxidative damage in human disease," *Clinical Chemistry*, vol. 52, pp. 601–623, 2006.

[172] W. C. Tsai, D. Y. Hueng, C. R. Lin, T. C. Yang, and H. W. Gao, "Nrf2 expressions correlate with WHO grades in gliomas and meningiomas," *International Journal of Molecular Sciences*, vol. 17, 2016.

[173] J. A. Johnson, D. A. Johnson, A. D. Kraft et al., "The Nrf2-ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration," *Annals of the New York Academy of Sciences*, vol. 1147, pp. 61–69, 2008.

[174] A. S. Jiménez-Osorio, S. González-Reyes, W. R. García-Niño et al., "Association of nuclear factor-erythroid 2-related factor 2, thioredoxin interacting protein, and heme oxygenase-1 gene polymorphisms with diabetes and obesity in Mexican patients," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 7367641, 8 pages, 2016.

[175] Y. Mitsuishi, K. Taguchi, Y. Kawatani et al., "Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming," *Cancer Cell*, vol. 22, pp. 66–79, 2012.

[176] J. D. Hayes and A. T. Dinkova-Kostova, "The Nrf2 regulatory network provides an interface between redox and intermediary metabolism," *Trends in Biochemical Sciences*, vol. 39, pp. 199–218, 2014.

[177] Z. W. Yu, D. Li, W. H. Ling, and T. R. Jin, "Role of nuclear factor (erythroid-derived 2)-like 2 in metabolic homeostasis and insulin action: a novel opportunity for diabetes treatment?," *World Journal of Diabetes*, vol. 3, pp. 19–28, 2012.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 8103904, 11 pages
https://doi.org/10.1155/2017/8103904

Research Article

Gene Expression Profiling Identifies Downregulation of the Neurotrophin-MAPK Signaling Pathway in Female Diabetic Peripheral Neuropathy Patients

Lin Luo,[1] Wen-Hua Zhou,[2] Jiang-Jia Cai,[1] Mei Feng,[3] Mi Zhou,[3] Su-Pei Hu,[4] Jin Xu,[1,3] and Lin-Dan Ji[1,5]

[1]*Zhejiang Key Laboratory of Pathophysiology, Medical School of Ningbo University, Ningbo 315211, China*
[2]*Laboratory of Behavioral Neuroscience, Ningbo Addiction Research and Treatment Center, Medical School of Ningbo University, Ningbo 315211, China*
[3]*Department of Preventive Medicine, Medical School of Ningbo University, Ningbo 315211, China*
[4]*Department of Research and Teaching, Ningbo No. 2 Hospital, Ningbo 315010, China*
[5]*Department of Biochemistry, Medical School of Ningbo University, Ningbo 315211, China*

Correspondence should be addressed to Jin Xu; xujin1@nbu.edu.cn and Lin-Dan Ji; jilindan@nbu.edu.cn

Received 14 April 2017; Revised 14 June 2017; Accepted 2 July 2017; Published 16 August 2017

Academic Editor: Joseph F. Ndisang

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes mellitus (DM). It is not diagnosed or managed properly in the majority of patients because its pathogenesis remains controversial. In this study, human whole genome microarrays identified 2898 and 4493 differentially expressed genes (DEGs) in DM and DPN patients, respectively. A further KEGG pathway analysis indicated that DPN and DM share four pathways, including apoptosis, B cell receptor signaling pathway, endocytosis, and Toll-like receptor signaling pathway. The DEGs identified through comparison of DPN and DM were significantly enriched in MAPK signaling pathway, NOD-like receptor signaling pathway, and neurotrophin signaling pathway, while the "neurotrophin-MAPK signaling pathway" was notably downregulated. Seven DEGs from the neurotrophin-MAPK signaling pathway were validated in additional 78 samples, and the results confirmed the initial microarray findings. These findings demonstrated that downregulation of the neurotrophin-MAPK signaling pathway may be the major mechanism of DPN pathogenesis, thus providing a potential approach for DPN treatment.

1. Introduction

Diabetes mellitus (DM) has become one of the largest global public health problems of this century. According to the World Health Organization global report on diabetes, over 8.5% of the global population, approximately 422 million adults, have diabetes [1]. Diabetes of all types can lead to complications in many parts of the body and can increase the overall risk of premature death. Potential complications include cardiomyopathy, vasculopathy, neuropathy, retinopathy, nephropathy, dermatopathy, and encephalopathy [2, 3]. Diabetic peripheral neuropathy (DPN) is characterized as a chronic symmetrical, progressive disorder with early symptoms of pain, allodynia, and paresthesia, and it affects approximately 50% of people with considerable morbidity, mortality, and diminished quality of life [4]. Despite the development of diagnostic methods and therapeutic modalities, DPN is still not diagnosed or managed properly in most patients [5].

Over the past decade, there have been many achievements in understanding the pathogenesis of DPN. However, controversy remains because of a multifactorial etiology involving both metabolic and vascular factors [6]. Chronic hyperglycemia used to be regarded as the major factor in the initiation of various metabolic events underlying DPN. Results from the Diabetes Control and Complication Trial

(DCCT) support the hypothesis that DPN develops as a result of increased blood glucose and have suggested that treating hyperglycemia in type 1 DM can markedly decrease the incidence of DPN, by up to 60 to 70% [7]. However, more recent studies have reported that intensive glucose control in patients with type 2 DM has no significant effect on the development of DPN [8]. Over 40% of type 2 DM patients develop DPN despite good glucose control, thus suggesting that hyperglycemia is only one of the many key events that cause nerve and microvascular injury [9]. Nevertheless, hyperglycemia has effects on several major, well-characterized biochemical pathways that include activation of the polyol pathway [10], formation of advanced glycation end products (AGEs) and their receptors [11], activation of protein kinase C (PKC) [12] and inducible nitric oxide synthase [13], increased poly(ADP-ribose) polymerase (PARP) activity [14], and elevated inflammation [15]. Furthermore, oxidative stress and mitochondrial dysfunction [16], hypoxia and ischemia [17], elevated cytokines [18], and deficiencies in neurotrophic factors [19] also play significant etiologic roles in DPN. Despite advances in delineating the etiology of DPN, few effective therapies exist to manage, delay, or prevent the development of painful DPN. Therefore, identifying precise mechanisms and related therapeutic drugs remains paramount.

Systems biology approaches, such as whole genome expression profiling, may provide new insights into the molecular mechanisms of DPN without preset bias. However, previous studies have identified global transcriptomic changes only in animal models [20]. In the present study, we used high-throughput genome-wide expression microarrays to identify alterations in the transcriptome, both common and distinct, between type 2 DM and DPN. The results may provide relevant information for the future development of new mechanism-based diagnostics and therapies.

2. Material and Methods

2.1. Study Design. A workflow was designed to identify candidate genes whose expression levels may differentiate among healthy controls (CN), DM patients, and DPN patients (Figure 1). After obtaining three individual differentially expressed gene (DEG) datasets (DM versus CN, DPN versus CN, and DPN versus DM), we used a Venn diagram to identify the shared and distinct gene expression changes between DPN and DM. Subsequently, several gene annotation databases were used to identify the potential biological functions and signaling pathways involved in DPN. Finally, DEGs of interest were further validated in an independent sample set.

2.2. Participants. The participants were recruited from hospitals affiliated with Ningbo University from April 2015 to June 2016. The healthy controls had no family history of diabetes or neurologic disorders, exhibited normal glucose tolerance, and were free of any major chronic diseases. Type 2 DM cases were defined as meeting at least one of the following criteria: fasting plasma glucose $\geq$7 mmol/L, 2 h plasma glucose after oral glucose tolerance test (OGTT) $\geq$ 11.1 mmol/L, and use of glucose-lowering drugs or physician-diagnosed diabetes. Diabetic peripheral neuropathy was diagnosed by a positive assessment through neurologic examinations and nerve conduction studies, as previously described [20]. In brief, the criteria for DPN were as follows: (1) confirmed type 2 DM patients; (2) decreased sensation and positive neuropathic sensory symptoms (including pricking, burning, stabbing, or aching pain) in the toes, feet, or legs; (3) decreased distal sensation and unequivocally decreased or absent ankle reflexes; (4) and abnormal motor and sensory nerve conduction. Patients with type 1 DM, cardiovascular diseases, previous history of neurologic disorders, peripheral vascular occlusive disease, autoimmune disease, or any other possible causes of peripheral neuropathy were excluded from all groups. Patients with any clinically observable diabetic complications were excluded from the DM group. The DPN patients involved in the present study were all new cases without any antineuropathy medication. Patients with any other major acute or chronic complication associated with diabetes or vitamin B12/folic acid deficiencies were excluded from the DPN group. The protocol of this study was approved by the medical ethics committee of Ningbo University. The health records and blood samples (2 mL) of the participants were collected after informed written consents were provided by the subjects. A total of 6 healthy controls, 6 DM patients, and 6 DPN patients were recruited for the microarray analysis. In the validation stage, another 26 healthy controls, 26 DM patients, and 26 DPN patients were included. An overview of the clinical and demographic characteristics of the participants can be found in Table 1.

2.3. RNA Preparation. Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) by using a TaKaRa MiniBEST Universal RNA Extraction Kit according to the manufacturer's instructions. The RNA quality was determined on the basis of an optical density (OD) 260/280 ratio $\geq$ 1.8 and OD260/230 ratio $\geq$ 2.0 using a NanoDrop ND-1000 spectrophotometer. RNA integrity was determined using an Agilent 2100 Bioanalyzer. The intensity of the 18S and 28S rRNA bands was examined on a 1% formaldehyde-agarose gel. RNA samples with an RNA integrity number (RIN) of $\geq$6.0 and 28S/18S > 1.5 were subjected to microarray analysis.

2.4. Microarray Analysis. The microarray analysis was performed by BGI Inc., including RNA amplification, probe labeling, hybridization, and data extraction. Briefly, aliquots (100 ng) of total RNA were amplified and transcribed into fluorescent Cy5-labeled antisense RNAs (aRNAs) by using a OneArray Amino Allyl aRNA Amplification Kit (Phalanx Biotech, San Diego, CA) according to the manufacturer's instructions. The Cy5-labeled aRNAs were fragmented and then hybridized to the Human Whole Genome OneArray Version 6.0 (Phalanx Biotech, San Diego, CA). Nonspecific binding targets were washed out three times. The arrays were scanned by an Agilent G2505C Microarray Scanner (Agilent Technologies, Wilmington, DE). The fluorescence intensities of each spot were analyzed with Feature Extraction software

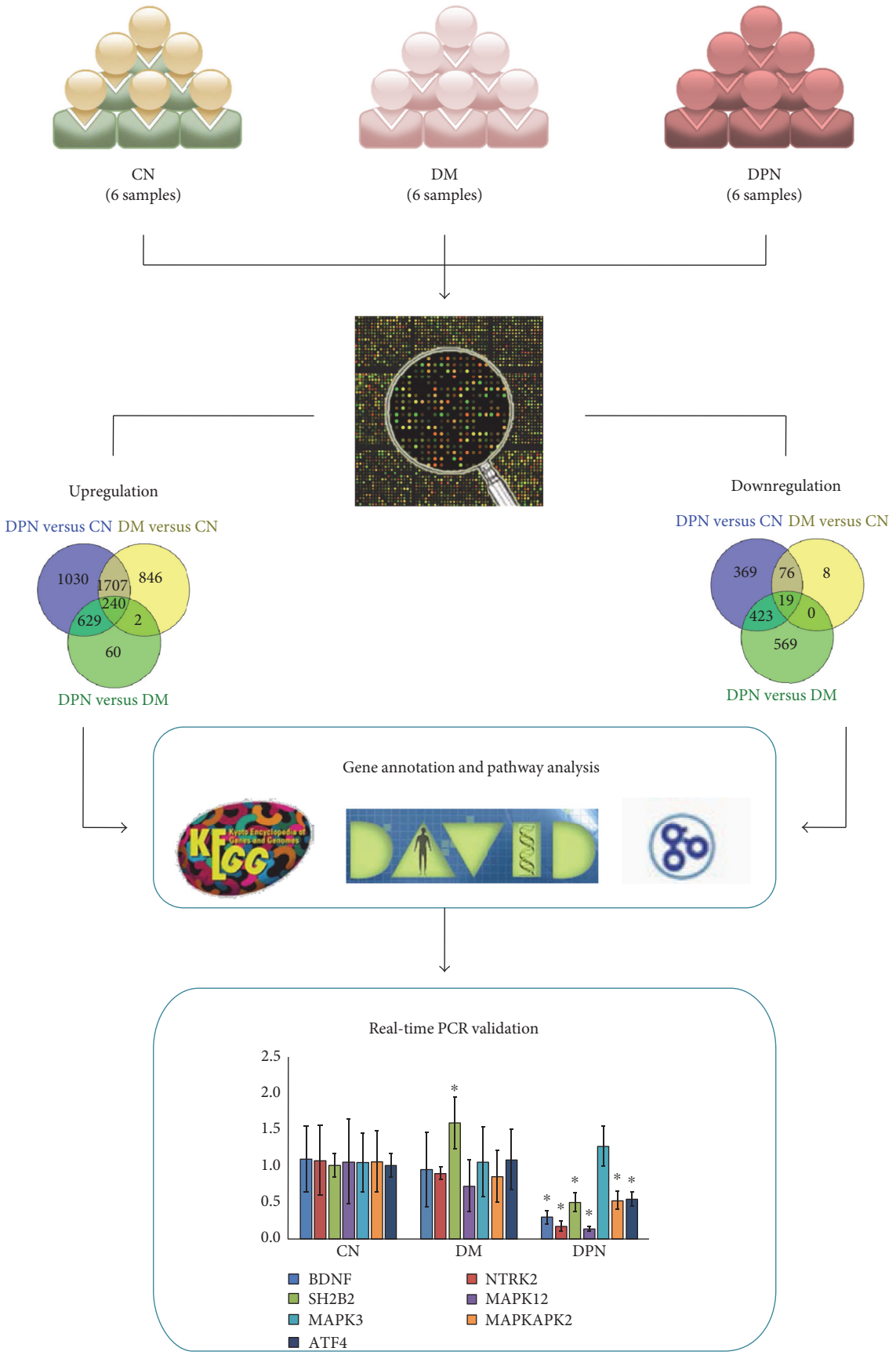

Figure 1: The workflow of the study. The asterisks represent statistical significance ($P < 0.05$) compared with the control group.

Table 1: Clinical and demographic characteristics of the participants.

	Control	T2DM	DPN
Discovery panel			
Number	6	6	6
Gender	Female	Female	Female
Age (y)	51.17 ± 6.08	53.83 ± 10.80	57.33 ± 7.92
Fasting glucose (mmol/L)	5.10 ± 0.21	9.23 ± 2.02*	9.95 ± 2.11*
Validation panel			
Number	26	26	26
Gender	Female	Female	Female
Age (y)	50.91 ± 5.60	51.25 ± 8.13	53.21 ± 11.15
Fasting glucose (mmol/L)	5.27 ± 0.59	8.96 ± 1.58*	9.72 ± 1.81*

The asterisks represent statistical significance ($P < 0.05$) compared with the control group.

(Agilent Technologies, Wilmington, DE). Overall, 18 microarray chips were analyzed in this study.

2.5. Statistical Analysis of the Microarray Data. The microarray data were normalized using the R/Bioconductor Limma package [21]. An empirical Bayes model was used to compare DEGs between groups, and the criteria for DEGs were FDR < 0.05 and $|\log_2 (\text{ratio})| \geq 1$. Hierarchical clustering was performed to visualize distinguishable gene expression profiles among the samples. The gene annotation and biological interpretation of the identified DEGs were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 [22]. Biological functions, represented by Gene Ontology terms (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes pathways (http://genome.jp/kegg/), were deemed significant at a Benjamini-Hochberg-corrected $P < 0.05$. The R/Bioconductor Pathview package was used for pathway-based gene data integration and visualization [23].

2.6. Quantitative Real-Time PCR (qRT-PCR). Seven representative genes selected from DEGs involved in the neurotrophin-MAPK signaling pathway were validated by qRT-PCR in another 26 independent samples from each group (CN, DM, and DPN). Total RNA was extracted as described above, and double-stranded cDNA was synthesized using a PrimeScript RT Reagent Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using LightCycler 480 SYBR Green I Master mix (Roche, Mannheim, Germany). The $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression of each gene, using GAPDH expression to normalize each sample. All experiments were run in triplicate and repeated three times. Differences in expression among groups were evaluated with a one-way analysis of variance (ANOVA) using SPSS 16.0 software. A significant difference was considered to be indicated by $P < 0.05$.

Table 2: Number of differentially expressed genes in different groups.

	DM versus CN	DPN versus CN	DPN versus DM
Upregulated	2795	3606	931
Downregulated	103	887	1011

3. Results

3.1. Identification of DEGs among DM Patients, DPN Patients, and Healthy Controls. The gene expression data from this study are available in the Gene Expression Omnibus (GEO, accession number GSE95849). Compared with healthy individuals, patients with DM and DPN exhibited 2795 and 3606 upregulated genes, respectively, whereas 103 genes and 887 genes were expressed at a lower level in DM and DPN patients compared with the healthy controls (Table 2). Among these DEGs, DM and DPN patients shared 1947 upregulated and 95 downregulated genes. In DPN patients, compared with DM patients, 1942 genes were differentially expressed, including 931 upregulated and 1011 downregulated genes (Table 2).

3.2. Functional Annotation and Enrichment Analysis of the DEGs. According to the GO analysis, the DEGs of DM and DPN patients were enriched in many similar GO terms as well as distinct GO terms (Figure 2). Compared with the profile of healthy controls, the DM-associated gene expression profile primarily included DEGs related to intracellular transport, immune response, cell activation, protein localization, and inflammatory response (Figure 2(a)). The DPN patients exhibited differential expression of genes involved in protein transport, protein localization, leukocyte activation, immune response, protein kinase cascades, and cell death (Figure 2(b)). Between the DPN and DM group, the differentially expressed genes were enriched in cell activation, cellular response to stress, and cell death as well as in the regulation of transcription and translation (Figure 2(c)).

KEGG pathway analysis revealed that, in comparison with healthy controls, DPN and DM shared four pathways (Table 2), including apoptosis (hsa04210), B cell receptor signaling pathway (hsa04662), endocytosis (hsa04144), and Toll-like receptor signaling pathway (hsa04620). Moreover, Fc gamma R-mediated phagocytosis (hsa04666), chemokine signaling pathway (hsa04062), and insulin signaling pathway (hsa04910) were enriched in the DM group, and NOD-like receptor signaling pathway (hsa04621), lysosome (hsa04142), valine, leucine and isoleucine degradation (hsa00280), and amino sugar and nucleotide sugar metabolism (hsa00520) were enriched in the DPN group. In contrast, for DEGs between DPN and DM patients, the most enriched pathway was MAPK signaling pathway (hsa04010), followed by NOD-like receptor signaling pathway (hsa04621) and neurotrophin signaling pathway (hsa04722). For the gene set lists of these three pathways, see electronic Supplementary Table 1 available online at https://doi.org/10.1155/2017/8103904. When the DEGs identified between DPN and DM patients were further

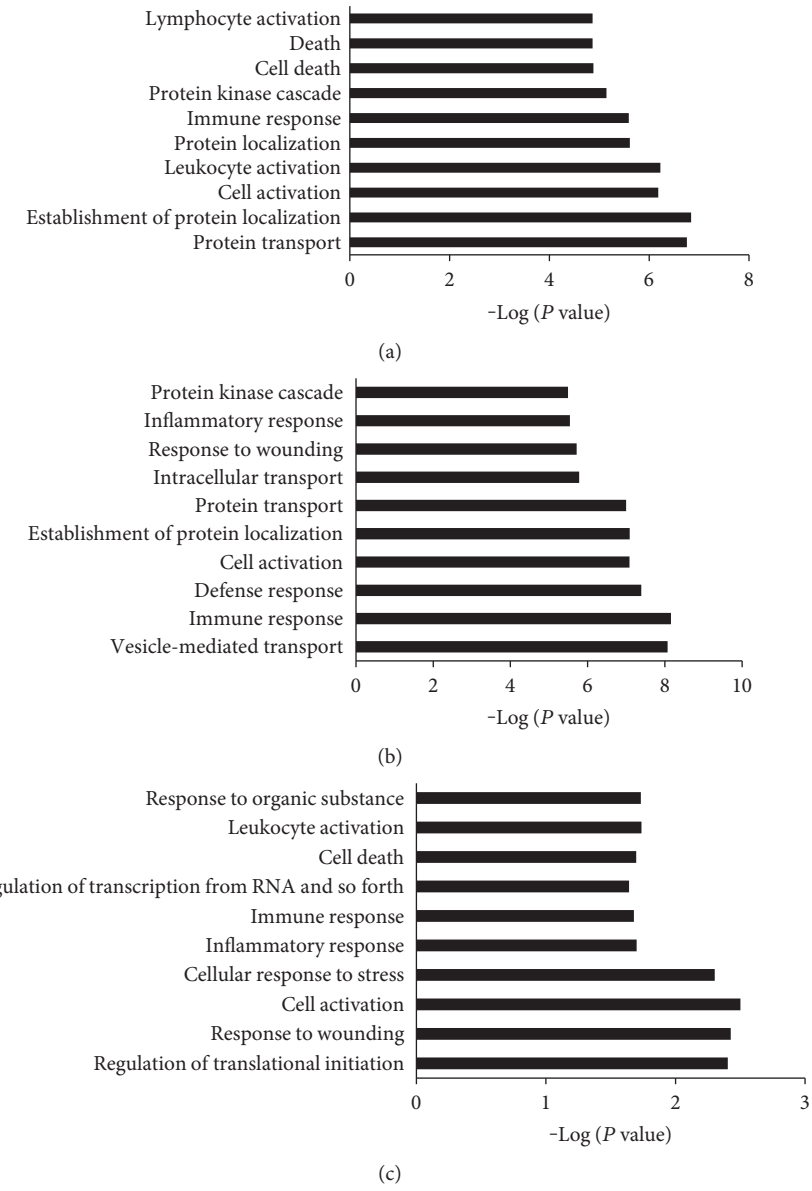

Figure 2: The top 10 biological processes identified by GO analysis of the DEGs: (a) DPN versus CN, (b) DM versus CN, and (c) DPN versus DM. Bars represent −log (*P* value).

analyzed with regard to upregulation and downregulation, the upregulated genes were enriched in the infectious disease category, whereas the downregulated genes were enriched in the "neurotrophin signaling pathway" and "MAPK signaling pathway" (Table 3). A KEGG Pathview analysis also showed that downregulation of the neurotrophin-MAPK signaling pathway was the major pathogenesic pathway in diabetic peripheral neuropathy patients (Figure 3).

3.3. Validation of DEGs in the Neurotrophin-MAPK Signaling Pathway. Seven DEGs selected from the "neurotrophin-MAPK signaling pathway" were further validated in an additional 78 participants by qRT-PCR. These genes are *BDNF* (brain-derived neurotrophic factor), *NTRK2* (neurotrophic tyrosine kinase type 2 receptor, also known as TrkB), *SH2B2* (SH2B adaptor protein 2, also known as rAPS), *MAPK3* (mitogen-activated protein kinase 3, also known as Erk), *MAPK12* (mitogen-activated protein kinase 12, also known as p38), *MAPKAPK2* (mitogen-activated protein kinase-activated protein kinase 2), and *ATF4* (activating transcription factor 4, also known as CREB). The results confirmed the initial microarray findings for all of the genes except *MAPK3*, whose expression was not changed in DM patients or in DPN patients (Figure 4).

4. Discussion

DPN is the most common complication of diabetes, affecting up to 50% of patients, and it contributes significantly to pain, loss of sensation, numbness, injury, and lower extremity amputation [4]. Because complex pathways are implicated in the pathophysiology of DPN, there are still no specific

Table 3: The significant KEGG pathways for the DEGs of DPN versus DM.

	KEGG pathway	Count	Benjamini q value
Upregulated	hsa05164: influenza A	25	0.001
	hsa05162: measles	21	0.001
	hsa00020: citrate cycle (TCA cycle)	9	0.008
	hsa05168: herpes simplex infection	23	0.009
Downregulated	hsa04722: neurotrophin signaling pathway	21	0.001
	hsa04010: MAPK signaling pathway	31	0.003
	hsa04668: TNF signaling pathway	18	0.003
	hsa04380: osteoclast differentiation	20	0.003
	hsa05169: Epstein-Barr virus infection	25	0.003
	hsa05166: HTLV-I infection	30	0.003
	hsa04660: T cell receptor signaling pathway	16	0.010
	hsa05215: prostate cancer	14	0.020
	hsa05142: Chagas disease (American trypanosomiasis)	15	0.029
	hsa05145: toxoplasmosis	16	0.031
	hsa04068: FoxO signaling pathway	17	0.039
	hsa04064: NF-kappa B signaling pathway	13	0.039
	hsa04621: NOD-like receptor signaling pathway	10	0.040

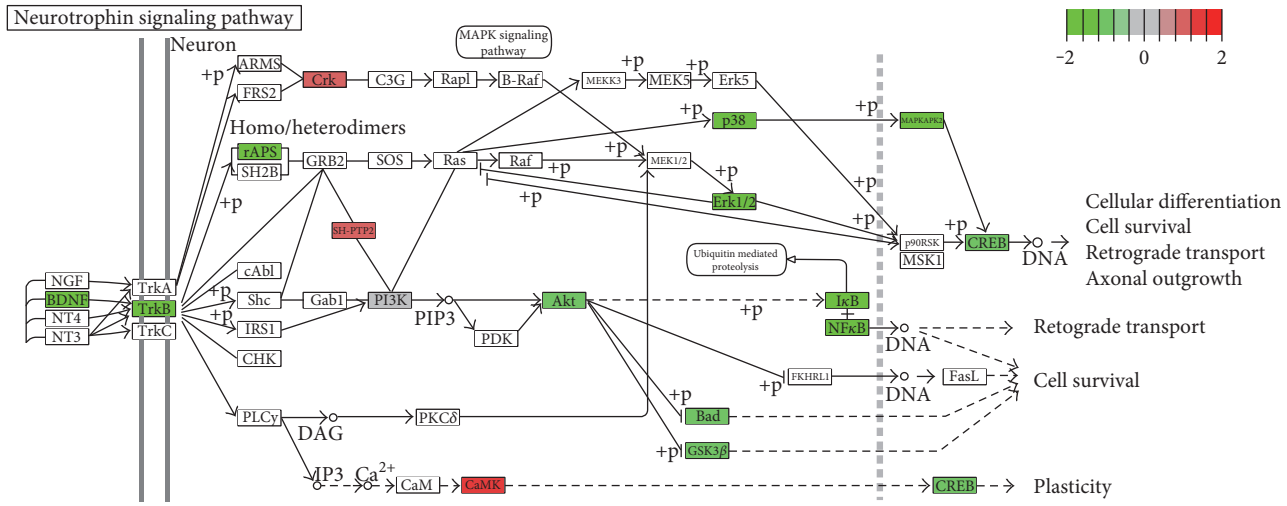

Figure 3: Downregulation of the neurotrophin-MAPK signaling pathway in DPN.

treatments and no means of predicting or preventing DPN onset or progression. Gene expression microarrays have been widely used in diabetic studies, because alterations in transcriptional profiles provide a robust and sensitive way to better understand the mechanisms of the disease and its complications. Microarray analyses have previously been used to investigate the mechanisms underlying diabetic cardiomyopathy [24], diabetic nephropathy [25], diabetic bone disease [26], and diabetic periodontitis [27] in diabetic patients or animal models. However, only one study has been conducted in patients with diabetic neuropathy to identify the DEGs and the related biological pathways responsible for the progression of diabetic neuropathy [28]. That study comprised a microarray experiment performed on human sural nerves collected from 50 patients with diabetic neuropathy during a 52-week clinical trial. Hyperglycemia may cause damage to the majority of the peripheral nerve system, not just the sural nerve. Moreover, as mentioned in that article, it is highly unlikely that future studies of diabetic neuropathy in patients will include the collection of sural nerve biopsies [28]. Therefore, performing new transcriptional microarray analyses with commonly used human sample material, such as PBMCs, to identify the gene expression signatures of DPN is necessary. Actually, transcriptome studies in PBMCs have been widely used in diabetic studies. Gene expression profiles in PBMCs can clinically stratify patients with recent-onset type 1 DM [29] and reflect the pathophysiology of type 2 DM [30]. In PBMCs from children with diabetes, the gene expression microarrays identified that type 1 and type 2 DM likely shared a common pathway for β-cell

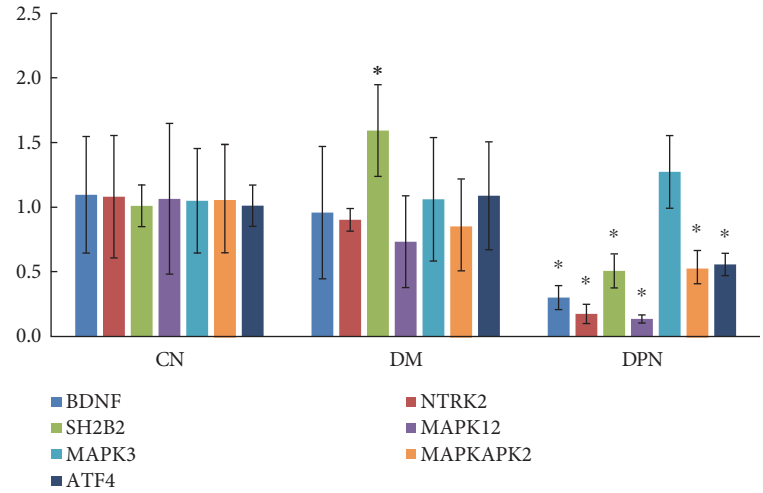

Figure 4: Microarray data were validated by qRT-PCR. The results are expressed as the mean ± SD. The asterisks represent statistical significance ($P < 0.05$) compared with the control group.

dysfunction that includes secretion of IL-1β and prostaglandins by immune effector cells [31]. Furthermore, a microarray study of gene expression in PBMCs identified that *THBS1* and *COX1* genes were upregulated, while *MMP9* and *COX2* genes were downregulated in patients with diabetic nephropathy [32].

The abovementioned microarray study on DPN identified DEGs that are functionally enriched in inflammatory responses and defense response pathways and may potentially be responsible for the progression of diabetic neuropathy [28]. In the current study, we found that DPN and DM shared four pathways, at least two of which were directly associated with immune-related functions, the "B cell receptor signaling pathway" and "Toll-like receptor signaling pathway." It is widely accepted that inflammation and immunity are crucially involved in diabetes and a majority of diabetic complications [33, 34]; therefore, changes in the immune response may not be a distinct mechanism of DPN. We further compared the expression profiles of DPN and DM patients and identified that DPN-specific DEGs were significantly enriched in "MAPK signaling pathway", "NOD-like receptor signaling pathway", and "neurotrophin signaling pathway". A stratification analysis further indicated that "neurotrophin signaling pathway" and "MAPK signaling pathway" were the top two pathways with downregulated DEGs. Although the DEGs in neurotrophin-MAPK signaling pathway are all located in autosome, caution should still be taken since only female subjects were involved in the present study, and more experiments are required to confirm it in male patients.

Neurotrophic factors are essential molecules that develop and maintain the nervous system by promoting the growth and survival of neurons. The neurotrophin family of growth factors includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin- (NT-) 3/4/5. Glial cell-derived neurotrophic factors (GDNF) form a second family and include GDNF, neurturin, artemin, and persephin [35, 36]. Deficiencies in NGF and NT-3 have long been reported in both the tissue and sera of DPN patients. Diabetes also decreases the anterograde and retrograde axonal transport of BDNF, NGF, and NT-3 in peripheral nerves [37–39]. In the current study, the expression levels of NGF, NT-3, and NT-4 remained unchanged. However, the BDNF level decreased significantly. BDNF plays important roles in regulating the survival and growth of neurons and influences synaptic efficiency and plasticity. The human BDNF gene consists of 11 exons, and the majority of the BDNF transcripts are detected not only in the brain but also in blood cells [40]. BDNF mediates neuronal differentiation and survival by binding and activating tropomyosin receptor kinase B (TrkB), an important member of the larger Trk family [41]. In the current study, the *NTRK2* gene, which encodes TrkB in humans, was specifically downregulated in DPN patients but not in DM patients, thus strongly suggesting that downregulation of the BDNF-TrkB signaling pathway is associated with DPN.

The binding of BDNF to TrkB leads to the dimerization and autophosphorylation of tyrosine residues in the intracellular domain of the receptor [42], which in turn leads to phosphorylation of tyrosine residues in the juxtamembrane domain or the C-terminus of the receptor [43]. These tyrosine residues serve as docking sites for multiple adaptor molecules, including Shc adaptor proteins, fibroblast growth factor receptor substrate 2 (FRS2), phospholipase Cγ (PLCγ), SH2B adaptor proteins, ankyrin repeat-rich membrane spanning (ARMS), Csk homology kinase (CHK), insulin receptor substrate 1 (IRS1), and c-ABL1 [43]. Among these adaptor molecules, only the expression level of *SH2B2* (also known as rAPS) decreased in DPN patients, probably due to the downregulation of TrkB. TrkB activates three major intracellular signaling cascades: the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and the PLCγ-Ca^{2+} pathway [44]. However, in the current study, only the MAPK pathway was affected in DPN patients. Among the three major MAPK family members, the Erk1/2 and p38 pathways were found to be downregulated in our original microarray study. However, after

Table 4: Expression of TLR family genes.

Gene symbol	Log_2 (ratio)			P value		
	DM/CN	DPN/CN	DPN/DM	DM/CN	DPN/CN	DPN/DM
TLR1	1.744	2.033	0.289	4.40*E*−04	5.22*E*−05	0.516
TLR2	2.175	2.056	−0.119	9.34*E*−04	1.05*E*−05	0.818
TLR4	2.861	4.254	1.393	1.73*E*−05	5.71*E*−09	1.38*E*−02
TLR5	2.213	3.682	1.469	6.31*E*−03	2.55*E*−11	0.054
TLR6	2.908	2.169	−0.738	4.24*E*−05	5.15*E*−06	0.213
TLR7	1.677	3.475	1.799	1.44*E*−02	2.72*E*−09	6.67*E*−03
TLR8	2.041	2.785	0.745	1.31*E*−04	2.61*E*−06	0.109
TLR9	1.623	1.135	−0.488	3.37*E*−04	3.82*E*−04	0.232

TLR3 and *TLR10* were removed after data normalization because of the low fluorescent intensity of probes.

Table 5: The significant KEGG pathways for the DEGs of different groups.

	KEGG pathway	Count	Benjamini q value
DM	hsa04210: apoptosis	32	0.009
	hsa04620: Toll-like receptor signaling pathway	35	0.007
	hsa04144: endocytosis	53	0.014
	hsa04666: Fc gamma R-mediated phagocytosis	32	0.014
	hsa04062: chemokine signaling pathway	53	0.013
	hsa04662: B cell receptor signaling pathway	26	0.024
	hsa04910: insulin signaling pathway	39	0.044
DPN	hsa04662: B cell receptor signaling pathway	39	0.000
	hsa04210: apoptosis	40	0.004
	hsa04621: NOD-like receptor signaling pathway	31	0.004
	hsa04144: endocytosis	69	0.009
	hsa04620: Toll-like receptor signaling pathway	41	0.032
	hsa04142: lysosome	46	0.028
	hsa00280: valine, leucine, and isoleucine degradation	22	0.026
	hsa00520: amino sugar and nucleotide sugar metabolism	22	0.026
DPN versus DM	hsa04010: MAPK signaling pathway	53	0.001
	hsa04621: NOD-like receptor signaling pathway	18	0.016
	hsa04722: neurotrophin signaling pathway	27	0.029

qRT-PCR validation, only the expression level of p38 decreased. This result is actually reasonable, because the direct downstream target of p38, MAPKAPK2, was also downregulated, whereas the downstream targets of Erk1/2, MSK1, and p90RSK were unchanged.

In neuronal development and function, neurotrophins activate the p38 MAPK/MAPKAPK2 pathway through Trk receptor-mediated signaling mechanisms [45]. Subsequently, MAPKAPK2 phosphorylates CREB (cAMP response element binding protein) and other transcription factors [46]. These transcription factors in turn regulate the expression of genes whose products are involved in many aspects of neural development and function, including cell fate decisions, axon growth, dendrite pruning, synaptic function, and plasticity [45]. In the present study, 2898 genes exhibited altered expression in DM patients, whereas this number in DPN patients was 4493. Moreover, in DPN patients, compared with DM patients, the number of downregulated genes increased 7 times, possibly because of decreased CREB expression mediated by the downregulation of the neurotrophin-MAPK signaling pathway.

In addition to the "neurotrophin signaling pathway" and "MAPK signaling pathway," the present study also showed that the "NOD-like receptor signaling pathway" was affected in DPN patients. Nucleotide-binding oligomerization domain containing 2 (NOD2), a member of the NOD-like receptor family, has been found to be one of the critical components of a signal transduction pathway linking renal injury to inflammation and podocyte insulin resistance in diabetic nephropathy [47]. A recent study has also suggested that the formation and activation of the NOD-like receptor protein 1 (NLRP1) inflammasome induces neuroinflammation and neuron injury during hyperglycemia, thus representing a novel mechanism of diabetes-associated neuron injury [48]. Interestingly, our microarray data indicated that the expression of *NOD2* and *NLRP1* remained

unchanged in DM patients but increased significantly in DPN patients (3.76-fold for *NOD2* and 2.07-fold for *NLRP1*). Therefore, the NOD-like receptor signaling pathway may be an important mechanism in the pathogenesis of diabetic complications.

It is now widely accepted that increased inflammation is a key etiological factor in the development of many chronic diseases, including diabetes [49]. Similar to Nod-like receptors, Toll-like receptors (TLRs) belong to another major family of pattern recognition receptors, which have been demonstrated to play a critical role in the innate immune system [50]. Among these TLRs, TLR4 plays an important role in many inflammatory disorders, and system inflammation facilitated by TLR4 is involved in the pathophysiological process of diabetes [51]. A recent study has further demonstrated that TLR4 may be a potential diagnostic biomarker for DPN [52]. In the current microarray study, *TLR4* expression increased in both DM and DPN patients, and there was even higher expression in the DPN patients (Table 4), in accordance with the results of the abovementioned study [52]. Moreover, it has been suggested that the inflammatory effects of high glucose may be mediated through the modulation of inflammatory responses resulting from TLR activation in diabetes [53]. The present microarray study showed that the "Toll-like receptor signaling pathway" was significantly enriched, and all TLR family genes were upregulated in both DPN and DM patients (Tables 4 and 5). These results strongly suggest that although some TLR genes, such as *TLR4* and *TLR7*, were expressed at much higher levels in DPN patients, they may not be sensitive biomarkers for DPN, because the TLR signaling pathway is involved in diabetes and multiple diabetic complications.

5. Conclusions

In summary, these findings provide the first demonstration that downregulation of the neurotrophin-MAPK signaling pathway may be the major pathogenesis of DPN. The use of growth factors in treating DPN has been extensively explored [54], and NGF, BDNF, and NT-3 have been assessed in various levels of clinical trials of DPN, with limited success [35]. The current study suggests that pharmacological targeting of both the neurotrophin signaling pathway and the MAPK signaling pathway at multiple levels may provide a potential approach for the treatment of DPN.

Abbreviations

BDNF: Brain-derived neurotrophic factor
CREB: cAMP response element binding protein
DAVID: Database for Annotation, Visualization and Integrated Discovery
DEG: Differentially expressed gene
DM: Diabetes mellitus
DPN: Diabetic peripheral neuropathy
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
NGF: Nerve growth factor
OGTT: Oral glucose tolerance test
qRT-PCR: Quantitative real-time PCR
RIN: RNA integrity number
TLR: Toll-like receptor.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors thank all the study participants and the clinical staffs from the affiliated hospitals of Ningbo University for their contribution to this research study. This work was supported by the Applied Research Project on Nonprofit Technology of Zhejiang Province (2015C33309), the National Natural Science Foundation of China (81541039, 81402747), the Natural Science Foundation of Ningbo (2016A610085, 2017A610199), and the K.C. Wong Magna Fund of Ningbo University.

References

[1] World Health Organization, *Global Report on Diabetes*, Geneva, 2016.

[2] R. Singh, L. Kishore, and N. Kaur, "Diabetic peripheral neuropathy: current perspective and future directions," *Pharmacological Research*, vol. 80, pp. 21–35, 2014.

[3] J. M. Forbes and M. E. Cooper, "Mechanisms of diabetic complications," *Physiological Reviews*, vol. 93, no. 1, pp. 137–188, 2013.

[4] J. L. Edwards, A. M. Vincent, H. T. Cheng, and E. L. Feldman, "Diabetic neuropathy: mechanisms to management," *Pharmacology & Therapeutics*, vol. 120, no. 1, pp. 1–34, 2008.

[5] S. Tesfaye and D. Selvarajah, "Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy," *Diabetes/Metabolism Research and Reviews*, vol. 28, Supplement 1, pp. 8–14, 2012.

[6] N. E. Cameron, S. E. Eaton, M. A. Cotter, and S. Tesfaye, "Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy," *Diabetologia*, vol. 44, no. 11, pp. 1973–1988, 2001.

[7] DCCT Research Group, "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[8] W. Duckworth, C. Abraira, T. Moritz et al., "Glucose control and vascular complications in veterans with type 2 diabetes," *The New England Journal of Medicine*, vol. 360, no. 2, pp. 129–139, 2009.

[9] K. Juster-Switlyk and A. G. Smith, "Updates in diabetic peripheral neuropathy," *F1000Res*, vol. 5, 2016.

[10] P. J. Oates, "Polyol pathway and diabetic peripheral neuropathy," *International Review of Neurobiology*, vol. 50, pp. 325–392, 2002.

[11] S. Y. Goh and M. E. Cooper, "Clinical review: the role of advanced glycation end products in progression and complications of diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 4, pp. 1143–1152, 2008.

[12] I. G. Obrosova, "Diabetes and the peripheral nerve," *Biochimica et Biophysica Acta*, vol. 1792, no. 10, pp. 931–940, 2009.

[13] I. Vareniuk, I. A. Pavlov, and I. G. Obrosova, "Inducible nitric oxide synthase gene deficiency counteracts multiple manifestations of peripheral neuropathy in a streptozotocin-induced mouse model of diabetes," *Diabetologia*, vol. 51, no. 11, pp. 2126–2133, 2008.

[14] I. G. Obrosova, W. Xu, V. V. Lyzogubov et al., "PARP inhibition or gene deficiency counteracts intraepidermal nerve fiber loss and neuropathic pain in advanced diabetic neuropathy," *Free Radical Biology & Medicine*, vol. 44, no. 6, pp. 972–981, 2008.

[15] N. E. Cameron and M. A. Cotter, "Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway," *Current Drug Targets*, vol. 9, no. 1, pp. 60–67, 2008.

[16] A. M. Vincent, J. W. Russell, P. Low, and E. L. Feldman, "Oxidative stress in the pathogenesis of diabetic neuropathy," *Endocrine Reviews*, vol. 25, no. 4, pp. 612–628, 2004.

[17] P. J. Dyck and C. Giannini, "Pathologic alterations in the diabetic neuropathies of humans: a review," *Journal of Neuropathology and Experimental Neurology*, vol. 55, no. 12, pp. 1181–1193, 1996.

[18] J. Doupis, T. E. Lyons, S. Wu, C. Gnardellis, T. Dinh, and A. Veves, "Microvascular reactivity and inflammatory cytokines in painful and painless peripheral diabetic neuropathy," *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 6, pp. 2157–2163, 2009.

[19] S. C. Apfel, "Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold?," *International Review of Neurobiology*, vol. 50, pp. 393–413, 2002.

[20] J. Hur, P. D. O'Brien, V. Nair et al., "Transcriptional networks of murine diabetic peripheral neuropathy and nephropathy: common and distinct gene expression patterns," *Diabetologia*, vol. 59, no. 6, pp. 1297–1306, 2016.

[21] G. K. Smyth, *Limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, Springer, 2005.

[22] W. Huang da, B. T. Sherman, and R. A. Lempicki, "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources," *Nature Protocols*, vol. 4, no. 1, pp. 44–57, 2009.

[23] W. Luo and C. Brouwer, "Pathview: an R/Bioconductor package for pathway-based data integration and visualization," *Bioinformatics*, vol. 29, no. 14, pp. 1830-1831, 2013.

[24] J. Lasheras, M. Vila, M. Zamora et al., "Gene expression profiling in hearts of diabetic mice uncovers a potential role of estrogen-related receptor gamma in diabetic cardiomyopathy," *Molecular and Cellular Endocrinology*, vol. 430, pp. 77–88, 2016.

[25] M. L. Caramori, Y. Kim, A. B. Goldfine et al., "Differential gene expression in diabetic nephropathy in individuals with type 1 diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 100, no. 6, pp. E876–E882, 2015.

[26] A. T. Haug, K. F. Braun, S. Ehnert et al., "Gene expression changes in cancellous bone of type 2 diabetics: a biomolecular basis for diabetic bone disease," *Langenbeck's Archives of Surgery*, vol. 399, no. 5, pp. 639–647, 2014.

[27] O. M. Andriankaja, J. Galicia, G. Dong, W. Xiao, F. Alawi, and D. T. Graves, "Gene expression dynamics during diabetic periodontitis," *Journal of Dental Research*, vol. 91, no. 12, pp. 1160–1165, 2012.

[28] J. Hur, K. A. Sullivan, M. Pande et al., "The identification of gene expression profiles associated with progression of human diabetic neuropathy," *Brain*, vol. 134, Part 11, pp. 3222–3235, 2011.

[29] K. M. Irvine, P. Gallego, X. An et al., "Peripheral blood monocyte gene expression profile clinically stratifies patients with recent-onset type 1 diabetes," *Diabetes*, vol. 61, no. 5, pp. 1281–1290, 2012.

[30] T. Takamura, M. Honda, Y. Sakai et al., "Gene expression profiles in peripheral blood mononuclear cells reflect the pathophysiology of type 2 diabetes," *Biochemical and Biophysical Research Communications*, vol. 361, no. 2, pp. 379–384, 2007.

[31] E. C. Kaizer, C. L. Glaser, D. Chaussabel, J. Banchereau, V. Pascual, and P. C. White, "Gene expression in peripheral blood mononuclear cells from children with diabetes," *The Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 9, pp. 3705–3711, 2007.

[32] D. K. Moczulski, H. Fojcik, A. Wielgorecki et al., "Expression pattern of genes in peripheral blood mononuclear cells in diabetic nephropathy," *Diabetic Medicine*, vol. 24, no. 3, pp. 266–271, 2007.

[33] J. Wada and H. Makino, "Innate immunity in diabetes and diabetic nephropathy," *Nature Reviews Nephrology*, vol. 12, no. 1, pp. 13–26, 2016.

[34] J. Nilsson, E. Bengtsson, G. N. Fredrikson, and H. Bjorkbacka, "Inflammation and immunity in diabetic vascular complications," *Current Opinion in Lipidology*, vol. 19, no. 5, pp. 519–524, 2008.

[35] K. L. Farmer, C. Li, and R. T. Dobrowsky, "Diabetic peripheral neuropathy: should a chaperone accompany our therapeutic approach?," *Pharmacological Reviews*, vol. 64, no. 4, pp. 880–900, 2012.

[36] T. J. Boucher and S. B. McMahon, "Neurotrophic factors and neuropathic pain," *Current Opinion in Pharmacology*, vol. 1, no. 1, pp. 66–72, 2001.

[37] R. Hellweg and H. D. Hartung, "Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy," *Journal of Neuroscience Research*, vol. 26, no. 2, pp. 258–267, 1990.

[38] R. Hellweg, G. Raivich, H. D. Hartung, C. Hock, and G. W. Kreutzberg, "Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy," *Experimental Neurology*, vol. 130, no. 1, pp. 24–30, 1994.

[39] A. P. Mizisin, N. A. Calcutt, D. R. Tomlinson, A. Gallagher, and P. Fernyhough, "Neurotrophin-3 reverses nerve conduction velocity deficits in streptozotocin-diabetic rats," *Journal of the Peripheral Nervous System*, vol. 4, no. 3-4, pp. 211–221, 1999.

[40] A. Cattaneo, N. Cattane, V. Begni, C. M. Pariante, and M. A. Riva, "The human BDNF gene: peripheral gene expression and protein levels as biomarkers for psychiatric disorders," *Translational Psychiatry*, vol. 6, no. 11, article e958, 2016.

[41] B. Xu, W. Gottschalk, A. Chow et al., "The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB," *The Journal of Neuroscience*, vol. 20, no. 18, pp. 6888–6897, 2000.

[42] M. E. Cunningham and L. A. Greene, "A function-structure model for NGF-activated TRK," *The EMBO Journal*, vol. 17, no. 24, pp. 7282–7293, 1998.

[43] L. Minichiello, "TrkB signalling pathways in LTP and learning," *Nature Reviews Neuroscience*, vol. 10, no. 12, pp. 850–860, 2009.

[44] E. J. Huang and L. F. Reichardt, "Trk receptors: roles in neuronal signal transduction," *Annual Review of Biochemistry*, vol. 72, pp. 609–642, 2003.

[45] E. J. Huang and L. F. Reichardt, "Neurotrophins: roles in neuronal development and function," *Annual Review of Neuroscience*, vol. 24, pp. 677–736, 2001.

[46] B. Mayr and M. Montminy, "Transcriptional regulation by the phosphorylation-dependent factor CREB," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 8, pp. 599–609, 2001.

[47] P. Du, B. Fan, H. Han et al., "NOD2 promotes renal injury by exacerbating inflammation and podocyte insulin resistance in diabetic nephropathy," *Kidney International*, vol. 84, no. 2, pp. 265–276, 2013.

[48] X. F. Meng, X. L. Wang, X. J. Tian et al., "Nod-like receptor protein 1 inflammasome mediates neuron injury under high glucose," *Molecular Neurobiology*, vol. 49, no. 2, pp. 673–684, 2014.

[49] M. Y. Donath, "Multiple benefits of targeting inflammation in the treatment of type 2 diabetes," *Diabetologia*, vol. 59, no. 4, pp. 679–682, 2016.

[50] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 4, no. 7, pp. 499–511, 2004.

[51] M. R. Dasu, S. Devaraj, S. Park, and I. Jialal, "Increased Toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects," *Diabetes Care*, vol. 33, no. 4, pp. 861–868, 2010.

[52] T. Zhu, Q. Meng, J. Ji, X. Lou, and L. Zhang, "Toll-like receptor 4 and tumor necrosis factor-alpha as diagnostic biomarkers for diabetic peripheral neuropathy," *Neuroscience Letters*, vol. 585, pp. 28–32, 2015.

[53] M. R. Dasu, S. Devaraj, L. Zhao, D. H. Hwang, and I. Jialal, "High glucose induces Toll-like receptor expression in human monocytes: mechanism of activation," *Diabetes*, vol. 57, no. 11, pp. 3090–3098, 2008.

[54] N. A. Calcutt, C. G. Jolivalt, and P. Fernyhough, "Growth factors as therapeutics for diabetic neuropathy," *Current Drug Targets*, vol. 9, no. 1, pp. 47–59, 2008.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 5612049, 8 pages
https://doi.org/10.1155/2017/5612049

Research Article

Decreased Cardiovascular Risk after Roux-en-Y Gastric Bypass Surgery in Chinese Diabetic Patients with Obesity

Xue Zhao,[1] Wenyan Duan,[1,2] Chenglin Sun,[1] Zhuo Li,[1] Yujia Liu,[1] Xianchao Xiao,[1] Gang Wang,[1] Xiaokun Gang,[1] and Guixia Wang[1]

[1]*Department of Endocrinology and Metabolism, The First Hospital of Jilin University, Changchun, Jilin Province 130021, China*
[2]*Jilin Province People's Hospital, Changchun, Jilin Province 130021, China*

Correspondence should be addressed to Xiaokun Gang; insightful@126.com and Guixia Wang; gwang168@jlu.edu.cn

Received 18 December 2016; Accepted 5 June 2017; Published 4 July 2017

Academic Editor: Alfredo Vannacci

Background. The influence of bariatric surgery on cardiovascular risks in Chinese diabetic patients remains unclear. Here, we aimed to explore the impact of Roux-en-Y gastric bypass surgery (RYGB) on cardiovascular risks in Chinese diabetic patients with obesity. *Methods*. Twenty Chinese patients with T2DM and obesity undergoing RYGB surgery were included in this study. Cardiovascular risk factors were measured before and 18 months after surgery. A 10-year cardiovascular risk was calculated by the UKPDS risk engine. Linear regression analysis was performed on CHD risk, stroke risk, and baseline metabolic parameters. *Results*. The complete remission rate of diabetes was 90% after RYGB surgery, with significant improvements in blood pressure, BMI, glucose, and lipid metabolism ($P < 0.05$). The 10-year cardiovascular risk of coronary heart disease reduced from 13.05% to 3.81% ($P = 0.001$) and the 10-year risk of stroke reduced from 19.66% to 14.22% ($P = 0.002$). In subgroup analysis, Chinese diabetic patients who were women, <45 years old, with BMI < 35 kg/m^2, and DM duration > 5 years, using noninsulin therapy presented more obvious improvements in the 10-year cardiovascular risk after RYGB surgery. WHR, age, LDL-C, and HbA1c were the most important factors influencing CHD or stroke risk after RYGB surgery ($P < 0.01$). *Conclusion*. RYGB surgery is an effective treatment to reduce cardiovascular risk in Chinese diabetic patients with obesity.

1. Introduction

Diabetes mellitus has been considered as a global burden of public health with striking prevalence in the past few years [1]. Emerging evidence has shown the close relationship between diabetes and cardiovascular diseases (CVD) [2]. Also, CVD is known to make great contribution to the high mortality rate of patients with type 2 diabetes (T2DM) [3]. Thus, it becomes extremely important to perform effective prevention strategies related to CVD during management of patients with T2DM.

Cardiovascular risk factors mainly refer to hypertension, hyperlipidemia, hyperglycemia, hyperinsulinemia, and so on. These risk factors play critical roles in the incidence and development of CVD. Among available methods to reduce cardiovascular risks, losing body weight seems to be important for a full benefit [4]. Studies have shown that a 10% reduction in body weight in people with obesity can lead to significant improvements in glucose, insulin, lipid profiles, and inflammatory markers [5]. Bariatric surgery, known as one of the most effective methods to lose weight, has shown to be beneficial in decreasing cardiovascular risk factors and preventing CVD events in studies on Hispanic, Mediterranean, and Swedish patients with T2DM [6] [7]. However, there was very little available evidence in Chinese patients with T2DM and obesity on cardiovascular risk after bariatric surgery. Different from other ethnical subjects, Chinese obese patients with T2DM usually hold a smaller BMI (<35 kg/m^2) and more obvious islet dysfunction at the early stage of T2DM. Thus, it is of great value to explore the effect of bariatric surgery on CV risk in Chinese.

Most previous studies focusing on cardiovascular risks in diabetic patients or obese patients applied Framingham risk scores to predict 10-year cardiovascular risk. Different from the traditional Framingham score [8], the UKPDS risk equations are specific for patients with T2DM, which incorporates glycemic parameters (HbA1c and duration of DM) into a model to calculate the 10-year risk of fatal and nonfatal CHD. Thus, the UKPDS risk engine tool might provide more accurate estimates and confidential power in the 10-year CHD risk for Chinese diabetic patients than traditional Framingham risk scores [9]. However, little evidence was available related to the cardiovascular risk calculated by the UKPDS risk engine tool in diabetic patients.

Based on abovementioned, we aimed to explore the impact of Roux-en-Y gastric bypass surgery on cardiovascular risks calculated by the UKPDS risk engine in Chinese diabetic patients with obesity and to observe its long-term effect at 18 months after surgery.

2. Methods

2.1. Subjects and Study Design. Twenty diabetic subjects with obesity who underwent laparoscopic RYGB surgery (LRYGB) in Jiahe Surgical Hospital were enrolled in this study. Medical history, age, body weight, body mass index (BMI), chest circumference (CC), hip circumference (HC), waist circumference (WC), waist-hip ratio (WHR), blood pressure (BP), and current medications were recorded before and after surgery. Fasting plasma glucose (FPG), fasting C-peptide (FCP), fasting insulin (FINS), 2-hour postprandial C-peptide (PCP), 2-hour postprandial glucose (PPG), 2-hour postprandial insulin (PINS) followed by oral glucose tolerance test (OGTT), HbA1c, and lipid profiles were measured preoperation and postoperation (1, 3, 6, 12, and 18 months). At the same time, we calculated the homeostasis model assessment insulin resistance (HOMA-IR) and the homeostasis model assessment β-cell (HOMA-β).

Patients with following diseases or medical histories were excluded: acute complications of T2DM, type 1 diabetes (T1DM), or latent autoimmune diabetes in adult (LADA); a mental disorder or unstable psychiatric illness; and severe alcohol or drug dependency, with a history of coronary heart disease, cerebral infarction, renal failure, heart failure, and severe hypertension, with high surgical risk (such as active ulcer), or with a medical history of gallstones and/or cholecystectomy.

According to the World Medical Association's Declaration of Helsinki, the approval from the Ethics Committee of our institution was achieved at the beginning. Also, informed consent was obtained from all participants.

2.2. Definitions of Diabetes, Obesity, Elevated BP, and Diabetes Remission. According to the 1999 World Health Organization criteria, patients were diagnosed with T2DM if they achieve the following values: fasting plasma glucose ≥ 7.0 mmol/L and/or 2 h plasma glucose ≥ 11.1 mmol/L.

For the diagnosis of obesity, BMI was applied for classification to complying with Working Group on Obesity in China (WGOC) standards [10]: (1) normal weight: $18.5\,\text{kg/m}^2 < \text{BMI} < 24\,\text{kg/m}^2$; (2) overweight: $24\,\text{kg/m}^2 \leq \text{BMI} < 28\,\text{kg/m}^2$; and (3) obesity: $\text{BMI} \geq 28\,\text{kg/m}^2$.

Elevated blood pressure was defined as systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg or current treatment for hypertension based on criteria established by JCDCG [11].

The postoperative effect on T2DM was characterized as complete remission, partial remission, and no remission [12]: (1) complete remission: FPG < 7.0 mmol/L, PPG ≤ 10.0 mmol/L, and HbA1c $< 6.5\%$ for 1 year without extra medication; (2) partial remission: FPG ≥ 7.0 mmol/L (but lower than before), PPG > 10.0 mmol/L (but lower than before), or HbA1c $\geq 6.5\%$ (but lower than before) or a decreased dosage of medication; and (3) no remission: FPG ≥ 7.0 mmol/L (higher than before), PPG > 10.0 mmol/L (higher than before), or HbA1c $\geq 6.5\%$ (higher than before) or an increased dosage of medication.

2.3. Anthropometric Measurements and Laboratory Assays. Standard methods were used to measure weight, BMI, waist circumference, chest circumference, hip circumference, waist-hip ratio, and blood pressure before and after RYGB surgery. BP was measured for 3 times by the same person using a mercury sphygmomanometer (Riva-Rocci System, ERKA, Chemnitz, Germany). All biomedical examinations were applied after receipt blood samples from patients with an overnight fast (>10 hours). Glucose oxidase method was applied to measure plasma glucose concentration. We tested HbA1c and lipid profiles including serum total cholesterol (TC), serum triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) using a ci16200 Architect automatic analyzer (Architect, Illinois, USA). C-peptide and serum insulin were measured using radioimmunoassay (RIA) (Linco Research, St Charles, MO, USA). To evaluate the insulin resistant state and β-cell function, we applied the following formulas: $\text{HOMA} - \text{IR} = \text{fasting insulin (mIU/L)} \times \text{fasting glucose (mmol/L)}/22.5$ [13] and $\text{HOMA} - \beta = 20 \times \text{fasting insulin (mIU/L)}/[\text{fasting glucose (mmol/L)} - 3.5]$ [13].

2.4. Cardiovascular Risk Assessment. Using the UKPDS risk engine tool, we calculated the 10-year cardiovascular risk of enrolled patients at baseline and at different postoperative time points (1, 3, 6, 12, and 18 months). This tool mainly evaluates an individual's 10-year risk of coronary heart disease (CHD), fatal CHD, stroke, and fatal stroke based on categorical values, including age, gender, HbA1c, DM duration, HDL cholesterol, total cholesterol, smoking status, blood pressure, and atrial fibrillation. The risk engine is available from the Diabetes Trials Unit, Oxford University Centre for Diabetes, Endocrinology, and Metabolism (from www.dtu.ox.ac.uk/index.php?maindoc/riskengine/).

2.5. Statistical Analysis. Continuous variables were presented as mean $\pm$ SD or mean $\pm$ SEM. Categorical variables are

presented as numbers and percentages. Continuous variables in the different groups were tested for normal distribution using the Kruskal-Wallis normality test. For the comparison of baseline and postoperative parameters, we applied a paired t-test for the data subject to normal distribution and the related sample Wilcoxon signed rank test for the data subject to abnormal distribution. It was considered as statistical significance if $P < 0.05$. All statistics were calculated with SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Among all the 20 diabetic patients with obesity undergoing RYGB surgery, 7 were males (35%) and 13 were females (65%), with a total mean age of 42.70 ± 12.60 years old. The mean duration from the diagnosis of T2DM was 5.35 ± 2.72 years. And detailed population characteristics and medical history are shown in Table 1. The complete remission rate of T2DM was 90% (18/20) which refers to patients who can reach a target glucose level without oral hypoglycemic drug intervention after surgery for one year. The partial remission rate was 10% (2/20); these patients continued oral hypoglycemic drug treatment with a decreased dosage. Among 16 (80%) patients with elevated blood pressure at baseline, 11 of 16 (69%) patients had normalized BP after RYGB surgery and 5 of 16 patients (31%) still need antihypertension treatment, but with a decreased dosage of medication.

As shown in Table 2, clinical metabolic parameters related to adiposity, glucose metabolism, and lipid metabolism were improved significantly. BMI changed from $34.20 \pm 6.22\,kg/m^2$ to $25.89 \pm 3.89\,kg/m^2$ (18 months) with 23.41% reduction ($P < 0.001$). WHR reduced significantly from the first month to the third month after surgery ($P = 0.048$), with the final reduction of 8.08% at 18 months comparing to baseline. Besides above, SBP decreased significantly ($P = 0.001$) with 9.20% reduction while DBP performed a slighter reduction after RYGB surgery with 6.25% reduction ($P = 0.015$). Fasting glucose, insulin, C-peptide, and PPG, PINS, and PCP as well as HbA1c presented significant decreases after surgery (18 months) comparing with baseline levels ($P < 0.01$). At 18 months after surgery, there were 47.08% decrease in fasting plasma glucose ($P < 0.001$), 48.45% decrease in 2-hour postprandial glucose ($P < 0.001$), 30.53% decrease in HbA1c ($P < 0.001$), 64.56% decrease in fasting insulin ($P < 0.001$), 53.73% decrease in 2-hour postprandial insulin ($P < 0.001$), 37.30% decrease in fasting C-peptide ($P < 0.001$), and 19.59% decrease in 2-hour postprandial C-peptide ($P = 0.002$). HOMA-IR reduced significantly after RYGB surgery with 79.67% reduction ($P < 0.001$). HOMA-β significantly increased at the first 3 months after surgery ($P < 0.001$), then decreased subsequently to the baseline level ($P = 0.391$).

In lipid metabolism, TG and TC levels were significantly decreased, from 2.52 mmol/L (1.67, 3.88) to 1.14 mmol/L (0.81, 1.62) ($P < 0.001$) and from 6.59 mmol/L (5.17, 8.04) to 4.77 mmol/L (4.23, 5.21) ($P < 0.001$), respectively, while HDL-C increased after the surgery from 1.21 mmol/L (0.79, 1.70) to 2.25 mmol/L (1.71, 2.37) ($P < 0.001$). The decrease of TG and TC reached to 60.36% and 34.53%, respectively, and the increase of HDL-C was 70.29% after surgery (18 months). No statistical difference was found in the LDL-C level before and after surgery (18 month) ($P = 0.642$), but LDL-C showed a decrease at first 3 months and an increase thereafter.

Table 1: Study population characteristics in T2DM patients with obesity at baseline ($n = 20$).

Variables	Before RYGB	After RYGB
Sex (M/F)	7/13	
Age (years)	42.70 ± 12.60	
Duration (year)	5.35 ± 2.72	
Smoking	9 (45%)	
OHA	12 (60%)	2 (10%)
Insulin therapy	7 (35%)	0
OHA + insulin	1 (5%)	0
Antihypertension	16 (80%)	5 (25%)

Note. OHA: oral hypoglycemic agent. Data represent means ± SD.

The results on estimated 10-year cardiovascular risks calculated by the UKPDS risk engine presented significant reductions after RYGB surgery (18 months), which are shown in Table 3. The absolute and relative risks after surgery are also shown in Table 3. The risks of CHD, fatal CHD, stroke, and fatal stroke reduced 71% ($P < 0.001$), 74% ($P < 0.001$), 28% ($P = 0.002$), and 38% ($P = 0.001$), respectively.

Although cardiovascular risk was significantly decreased, the role of age, gender, BMI, DM duration, smoking, and insulin usage in reducing CV risk after RYGB surgery was not known. Thus, we divided the patients into different subgroups, which were classified by age (>45 years and ≤45 years), DM duration (≥5 years and <5 years), BMI ($>35\,kg/m^2$ and $\leq 35\,kg/m^2$), smoking status, gender, and insulin therapy as shown in Figure 1. For CHD risk, the results showed that females could be more favorable from RYGB surgery than males ($P = 0.001$ versus $P = 0.028$). Patients who were younger than 45 years old can benefit more than people aged > 45 years old ($P = 0.003$ versus $P = 0.011$). As for BMI, patients with mild obesity ($25\,kg/m^2 < BMI \leq 35\,kg/m^2$) presented more CHD risk reduced after surgery comparing those with morbid obesity ($P = 0.003$ versus $P = 0.015$). Patients with T2DM duration > 5 years can benefit more than those < 5 years through surgery ($P = 0.003$ versus $P = 0.012$). Furthermore, patients who used noninsulin therapy (oral hypoglycemic agents, exercise, and life modification) reached larger improvements than those who used insulin therapy ($P = 0.003$ versus $P = 0.012$). However, no obvious difference was found in patients with smoking habits and without smoking habits ($P = 0.004$ versus $P = 0.008$), although they both presented marked decreases in cardiovascular risk after RYGB surgery.

To find the most important factors influencing CHD and stroke risks after RYGB surgery in Chinese diabetic patients with obesity, linear regression analysis was performed (Table 4). The results which presented baseline WHR (β-coefficient: 0.407; $P < 0.001$), age (β-coefficient: 0.003; $P < 0.001$), LDL-C (β-coefficient: 0.017; $P = 0.002$), and

Table 2: Changes of cardiometabolic factors before and after RYGB ($n = 20$).

Parameters	Before RYGB	After RYGB (18 months)	P value
Weight (kg)	94.70 ± 21.95	70.66 ± 11.48	<0.001
BMI (kg/m^2)	34.20 ± 6.22	25.89 ± 3.89	<0.001
WHR	1.00 ± 0.10	0.91 ± 0.09	0.002
SBP (mmHg)	136.65 ± 21.56	122.20 ± 10.18	0.001
DBP (mmHg)	85.50 ± 10.56	79.15 ± 1.16	0.015
FPG (mmol/L)	10.57 ± 3.46	5.59 ± 0.84	<0.001
PPG (mmol/L)	16.59 ± 4.31	8.55 ± 1.80	<0.001
HbA1c (%)	8.48 ± 1.49	5.89 ± 0.62	<0.001
FINS (mU/L)	19.17 (15.7, 26.8)	6.61 (5.1, 12.2)	<0.001
PINS (mU/L)	44.48 (27.2, 63.4)	24.76 (15.4, 36.6)	0.003
Fasting C-peptide (nmol/L)	1.21 ± 0.37	0.76 ± 0.23	<0.001
PCP (nmol/L)	2.52 ± 1.14	2.03 ± 0.88	0.002
HOMA-IR	8.41 (5.7, 14.3)	1.82 (1.14, 3.11)	<0.001
HOMA-beta (%)	54.83 (39.3, 100.4)	80.7 (146.8, 119.1)	0.391
TC (mmol/L)	6.59 (5.2, 8.0)	4.77 (4.2, 5.2)	<0.001
TG (mmol/L)	2.52 (1.7, 3.9)	1.14 (0.8, 1.6)	<0.001
LDL-C (mmol/L)	3.08 ± 1.15	2.94 ± 0.91	0.642
HDL-C (mmol/L)	1.21 (0.8, 1.7)	2.25 (1.7, 2.4)	<0.001

Note. WHR: waist-hip ratio; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; PPG: 2-hour postprandial glucose; FINS: fasting insulin; PINS: 2-hour postprandial insulin; FCP: fasting C-peptide; PCP: 2-hour postprandial C-peptide; HbA1c: hemoglobin A1c; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model of assessment for insulin resistance index; HOMA-β: HOMA of beta cell function. Data represent means ± SD or M (P25, P75). $P < 0.05$: significant difference.

Table 3: Estimated 10-year cardiovascular risk before versus after RYGB surgery ($n = 20$).

Cardiovascular event	Presurgery mean risk (%)	Postsurgery mean risk (%)	Absolute risk reduction (%)	95% CI	Relative risk reduction (%)	P value
n	20	20				
CHD	13.05 ± 2.71	3.81 ± 1.07	9.24	(5.02, 13.46)	71	<0.001
Fatal CHD	7.09 ± 1.92	1.83 ± 0.66	5.26	(2.37, 8.14)	74	<0.001
Stroke	19.66 ± 4.46	14.22 ± 3.44	5.43	(1.72, 9.13)	28	0.002
Fatal stroke	2.86 ± 0.61	1.78 ± 0.42	1.08	(0.49, 1.66)	38	0.001

Note. UKPDS: United Kingdom Prospective Diabetes Study; RYGB: Roux-en-Y gastric bypass; CI: confidence interval; CHD: coronary heart disease. Data represent means ± SEM. $P < 0.05$: significant difference.

HbA1c (β-coefficient: −0.009; $P = 0.007$) variables were related to the final CHD risk. Age (β-coefficient: 0.009; $P < 0.001$) and WHR (β-coefficient: 0.552; $P = 0.004$) were the most important factors influencing final stroke risk after surgery.

4. Discussion

4.1. Effects of RYGB Surgery on Obesity and Blood Pressure. We found twenty obese patients with T2DM who showed significant improvement in nearly all metabolic parameters related to cardiovascular risks after RYGB surgery. And eighteen of them got diabetes remission with a rate of 90%. Comparing with other studies [14, 15], our research seems to be more effective on the remission rate. It can be explained by several reasons: the lower BMI for inclusion, longer follow-up time, and different judging criteria. Most studies performed in Western countries usually apply RYGB surgery on obese T2DM patients with BMI > 35 kg/m^2 according to standards of medical care in diabetes in 2011 from American Diabetes Association [16]. Since Chinese patients with T2DM present smaller BMI and progressing β-cell dysfunction at earlier time, the criteria for bariatric surgery in China are 28 kg/m^2. Thus, it might be easier to correct metabolic disturbances for those patients with less BMI and T2DM duration. Furthermore, a longer follow-up time is necessary to detect the sustained effect of RYGB surgery on T2DM patients. However, current evidence in T2DM patients is limited and most available studies focusing on RYGB in Chinese population performed a 1-year follow-up or less. Thus, we

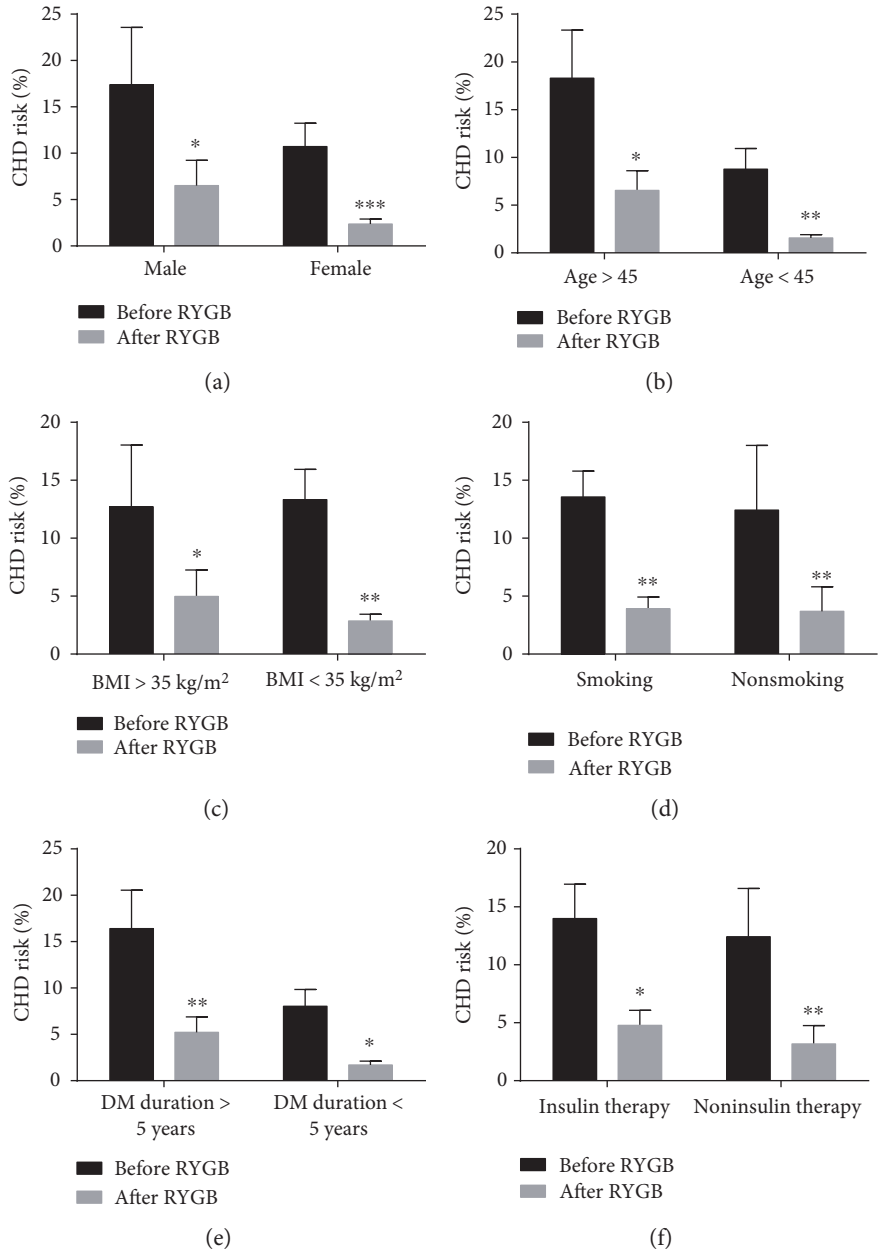

Figure 1: Changes of 10-year coronary heart disease (CHD) risk before and after RYGB surgery in different subgroups divided by gender, age, BMI, smoking status, DM duration, and insulin usage (baseline versus 18 months). (a) Male versus female; (b) age ≥ 45 years old versus <45 years old; (c) BMI < 35 kg/m^2 versus BMI ≥ 35 kg/m^2; (d) smoking versus nonsmoking; (e) DM duration ≥ 5 years versus DM duration < 5 years; and (f) insulin usage versus noninsulin usage. Data represent means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

finally analyzed a longer observation time until 18 months in order to reflect the real long-term benefits of RYGB surgery.

It is known that more than 80% of patients with T2DM are overweight or obese, and weight loss remains the hallmark in their management. RYGB surgery can help patients lose their weight quickly and sustained for a long time. In our study, patients got nearly 8.3% weight loss in the first month postsurgery ($P < 0.001$), and a long-term weight loss was also kept for 18 months reaching 25.31% weight loss finally. BMI and WHR were the known indices to evaluate adiposity and cardiovascular risks. Our results presented that RYGB surgery had obvious impacts on these variables. The possible mechanisms were unclear, mainly including decreased food intake [17], reduced absorption area after the surgery, altered gut hormones (GLP-1, PYY), gut-brain-liver axis stimulation, gut microbiota changes, and elevated bile acids [18, 19]. Although various evidences are emerging to uncover the mechanism of weight loss after RYGB surgery, it is still not clear.

Blood pressure is an important risk factor of CVD. Our results showed that RYGB surgery can significantly decrease both SBP and DBP. Moreover, SBP presented a larger reduction comparing to DBP. Similar results were also reported by Zhang et al. [20] and van Schinkel et al.

Table 4: Regression analysis on CHD or stroke risk (18 months) and basal clinical metabolic variables in patients with diabetes and obesity ($n = 20$).

Risks	Variables	β-coefficients	P value
CHD	WHR	0.407	<0.001
	Age	0.003	<0.001
	LDL-C	0.017	0.002
Stroke	HbA1c	−0.009	0.007
	Age	0.009	<0.001
	WHR	0.552	0.004

Note. CHD: coronary heart disease. $P < 0.05$: significant difference.

[21] Thus, RYGB surgery is favorable to diabetic patients with hypertension.

4.2. RYGB Surgery and Glucose Metabolism as well as Lipid Metabolism. Studies have that shown bariatric surgery not only reduces the mortality related to obesity but also leads to the obviously effective glycemic control. Thus, some studies recommend this operation to be viewed as "metabolic" rather than "bariatric" surgery. In the present study, all enrolled T2DM patients had a problem in controlling their hyperglycemia at baseline. 60% patients were under treatment of oral hypoglycemic drugs, 35% patients were using insulin therapy, and the rest used the combined plan of the above two strategies. After surgery, nearly 90% patients got diabetes remission without a dosage of diabetic medications and achieved significant improvements in plasma glucose and HbA1c. Moreover, 45% patients presented hyperinsulinemia at baseline with an average level of insulin of 19.17 mU/L. Haffner et al. [22] reported that hyperinsulinemia or insulin resistance kept the central role in pathogenesis of metabolic syndrome, T2DM, and CVD. A higher HOMA-IR (≥2.6) confers higher cardiovascular risk, reported by the studies on 667 adolescents (16-17 years old) [23] and 185 Greek children [24]. Consistently, mean HOMA-IR in our patients changed from 12.19 to 2.48 with significant reduction after RYGB surgery. At the same time, the HOMA-β increased especially in the first 3 months after RYGB surgery indicating the marked improvement in β-cell function at an early stage. Similar results were also presented by Lin et al. and Camastra et al. [25, 26]. They found enhanced insulin sensitivity in specific tissues such as liver, adipose, and skeleton muscle after RYGB surgery which could sustain a long-term effect. However, the improved HOMA-β did not continue increasing after the first 3 months and went back to the baseline level at 18 months. This indicated that RYGB surgery could not improve the final β-cell function, and the initial improvement might be the result of decreased BMI.

Disturbances in lipid profiles have been shown to make great contribution in metabolic disorders in a growing body of studies and have always been linked to insulin resistance and CVD. In our study, we found significant decreases in TG and TC and increase in HDL-C after RYGB surgery with statistical difference ($P < 0.05$). These changes might be the results of improved insulin resistance and adiposity to some extent. However, there was no difference in the LDL-C level before and after surgery ($P = 0.642$). Comparing with other studies, we found that most patients in our study presented not obvious elevation in LDL-C at the baseline level which was actually at a high level of normal range. Thus, the change of LDL-C was minimal with no statistical difference after surgery.

4.3. RYGB and Estimated 10-Year Cardiovascular Risks. A growing body of evidence has revealed the significant improvements in cardiovascular events after bariatric surgery [27, 28]. Adams et al. [27] showed in their retrospective cohort study of 7925 matched surgical patients and obese controls that the long-term mortality was significantly reduced, especially the decrease in coronary heart disease of 56% (2.6 versus 5.9 per 10,000 person-years, $P = 0.006$). Studies have also presented the definite advantages of bariatric surgery in weight control and ameliorating cardiovascular risk factors, comparing with lifestyle modification or conventional medical therapy [7, 29]. Thus, bariatric surgery can effectively reach the goal to improve life expectancy by lowering CVD risk, which was known as the greatest threat to obese patients.

However, it usually takes too much time to monitor the end-point cardiac events, and the loss rate is another tricky problem. Thus, a better tool to predict the long-term CVD risk is required and convenient. The Framingham risk score based on Framingham Heart Study incorporates the levels of many risk factors into a single equation to produce a likelihood of CHD event in the subsequent 10 years [30]. Thereafter, in order to estimate the CVD risk specially for T2DM patients, Stevens and his colleagues presented the UKPDS risk engine tool which was suitable for T2DM patients after adding HbA1c and DM duration [8]. For a long time, the RYGB surgery was only performed in patients with morbid obesity, and the outcomes related to CVD risk in patients with mild obesity were not clear. Also, it stayed unexplored in Chinese patients with T2DM and obesity.

Using the UKPDS risk engine tool, we targeted Chinese diabetic patients undergoing RYGB surgery and found the obvious decrease in risk of CHD and stroke from 13.05% to 3.81% and 19.66% to 14.22%, respectively. The relative risk reduction reached 71% and 28%, which were consistent with another two previous studies on patients with T2DM [31, 32]. Shah et al. found that in 15 Indian diabetic patients with mild obesity, RYGB was beneficial for the glucose control and CVD risk after the 9-month follow-up [32]. In their study, the 10-year CHD risk changed from 14.9% to 4.7%, with a decrease of 69% ($P = 0.001$), and the 10-year stroke risk changed from 3.7% to 2.8%, with a decrease of 32% ($P = 0.03$). Thereafter, a larger and longer study from Cohen et al. evaluated the effects of gastric bypass surgery on 66 American patients with diabetes with encouraging results after nearly a 5-year follow-up [31]. Cohen et al. presented the significant change in the 10-year CHD risk from 35.3% to 10.3%, resolving 71% decrease ($P = 0.001$). Thus, we can believe that RYGB surgery effectively reduce the cardiovascular risk in patients with T2DM and mild obesity (BMI < 35 kg/m^2), especially in Chinese population.

Previous studies showed that patients with diabetes had greater reduction of CHD risk than nondiabetic patients ($P < 0.01$) [33] [34]. And people who were female or >45 years old were more favorable after RYGB surgery [33, 34]. Based on above results, we further applied subgroup analysis to explore the specific effects of age, gender, BMI, DM duration, smoking status, and insulin therapy on magnitude of postoperative changes of CHD risk in Chinese population. In the present study, we found that females got a larger degree of reduce in CHD risk than males, consistent with previous studies. Different from previous studies, we found that diabetic patients who were <45 years old presented more decrease in CHD risk than older patients. The explanation for this inconsistency with a previous study might be the differences in targeted population and an estimating tool. The previous studies were mainly based on obese people > 35 kg/m^2 with prevalence of T2DM of 28% and 38%, respectively. Furthermore, they predicted the 10-year CHD risk by the Framingham risk score, rather than the UKPDS risk engine tool. We targeted the T2DM patients with the application of the UKPDS risk engine. Thus, these differences in the study design might influence the results to some extent. Besides these, we also found diabetic patients with BMI ≤ 35 kg/m^2 and with DM duration > 5 years using non-insulin therapy were more favorable in the 10-year CHD risk than the others. These data indicated, in Chinese population with diabetes, that BMI together with T2DM duration and diabetic therapy were also important factors to influence the final CHD risk. Also, more effective preventing strategy should be put into practice especially on diabetic patients with BMI ≤ 35 kg/m^2 in Asian countries. And RYGB surgery seemed to present greater advantages in this population. Definitely, future studies are required to validate our findings in a larger population.

As for the factors related to CHD and stroke risks after RYGB surgery, we explored regression analysis. According to results shown in Table 4, we can conclude that WHR, age, LDL-C, and HbA1c were the most important factors. These demonstrated that adiposity, age, lipid metabolism, and glucose metabolism were related to the effect of RYGB on cardiovascular risks. On the other hand, these revealed that RYGB surgery was a more effective treatment in decreasing cardiovascular risks in diabetic Chinese by reducing weight, glucose, and lipid level at the same time comparing oral hypoglycemic medications or exercise.

5. Conclusions

This study presented that RYGB surgery could lead to significant decreases in cardiovascular risk factors in Chinese diabetic patients with obesity. The estimated 10-year cardiovascular risks decreased significantly at 18 months after RYGB surgery. And WHR, age, LDL-C, and HbA1c were the most important influencing factors for the reduced cardiovascular risk after RYGB surgery. In conclusion, our findings suggest that bariatric surgery is an effective treatment to reduce cardiovascular risk in Chinese diabetic patients with obesity.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Xue Zhao and Wenyan Duan designed this study. Xianchao Xiao, Zhuo Li, and Gang Wang recruited the patients. Chenglin Sun and Yujia Liu performed the data extraction and analysis. Xue Zhao, Xiaokun Gang, and Guixia Wang wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank all the study participants. They acknowledge Dr. Mingxing Ding for his kind assistance. This work was supported by grants (belonging to Guixia Wang) from National Natural Science Foundation of China (81670732) and the grants (belonging to Xue Zhao) from Graduate Innovation Fund of Jilin University (2016042).

References

[1] W. Yang, J. Lu, J. Weng et al., "Prevalence of diabetes among men and women in China," *New England Journal of Medicine*, vol. 362, no. 12, pp. 1090–1101, 2010.

[2] G. Kranenburg, Y. van der Graaf, J. van der Leeuw et al., "The relation between HbA1c and cardiovascular events in patients with type 2 diabetes with and without vascular disease," *Diabetes Care*, vol. 38, no. 10, pp. 1930–1936, 2015.

[3] W. D. Strain and C. Smith, "Cardiovascular outcome studies in diabetes: how do we make sense of these new data?" *Diabetes Therapy*, vol. 7, no. 2, pp. 175–185, 2016.

[4] P. Sanchis, C. Frances, J. Nicolau et al., "Cardiovascular risk profile in Mediterranean patients submitted to bariatric surgery and intensive lifestyle intervention: impact of both interventions after 1 year of follow-up," *Obesity Surgery*, vol. 25, no. 1, pp. 97–108, 2014.

[5] C. A. Dow, C. A. Thomson, S. W. Flatt, N. E. Sherwood, B. Pakiz, and C. L. Rock, "Predictors of improvement in cardiometabolic risk factors with weight loss in women," *Journal of the American Heart Association*, vol. 2, no. 6, article e000152, 2013.

[6] N. De La Cruz-Muñoz, G. Lopez-Mitnik, K. L. Arheart et al., "Reduction in cardiometabolic disease risk following gastric bypass surgery among Hispanic adults," *Metabolic Syndrome and Related Disorders*, vol. 11, no. 4, pp. 262–266, 2013.

[7] L. Sjöström, A.-K. Lindroos, M. Peltonen et al., "Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery," *New England Journal of Medicine*, vol. 351, no. 26, pp. 2683–2693, 2004.

[8] R. J. Stevens, V. Kothari, A. I. Adler, I. M. Stratton, and R. R. Holman, "The UKPDS risk engine: a model for the risk of coronary heart disease in type II diabetes (UKPDS 56)," *Clinical Science*, vol. 101, no. 6, pp. 671–679, 2001.

[9] P. W. Wilson, R. B. D'Agostino, D. Levy, A. M. Belanger, H. Silbershatz, and W. B. Kannel, "Prediction of coronary heart disease using risk factor categories," *Circulation*, vol. 97, no. 18, pp. 1837–1847, 1998.

[10] B. F. Zhou, "Effect of body mass index on all-cause mortality and incidence of cardiovascular diseases—report for meta-analysis of prospective studies on optimal cut-off points of body mass index in Chinese adults," *Biomedical & Environmental Sciences*, vol. 15, no. 3, pp. 245–252, 2002.

[11] Joint Committee for Developing Chinese guidelines on Prevention and Treatment of Dyslipidemia in Adults, "Chinese guidelines on prevention and treatment of dyslipidemia in adults," *Zhonghua Xin Xue Guan Bing Za Zhi*, vol. 35, no. 5, pp. 390–419, 2007.

[12] J. B. Buse, S. Caprio, W. T. Cefalu et al., "How do we define cure of diabetes?" *Diabetes Care*, vol. 32, no. 11, pp. 2133–2135, 2009.

[13] Y. Song, J. E. Manson, L. Tinker et al., "Insulin sensitivity and insulin secretion determined by homeostasis model assessment and risk of diabetes in a multiethnic cohort of women: the Women's Health Initiative Observational Study," *Diabetes Care*, vol. 30, no. 7, pp. 1747–1752, 2007.

[14] M. Nora, M. Guimaraes, R. Almeida et al., "Metabolic laparoscopic gastric bypass for obese patients with type 2 diabetes," *Obesity Surgery*, vol. 21, no. 11, pp. 1643–1649, 2011.

[15] M. DiGiorgi, D. J. Rosen, J. J. Choi et al., "Re-emergence of diabetes after gastric bypass in patients with mid- to long-term follow-up," *Surgery for Obesity and Related Diseases : Official Journal of the American Society for Bariatric Surgery*, vol. 6, no. 3, pp. 249–253, 2010.

[16] American Diabetes A, "Standards of medical care in diabetes—2011," *Diabetes Care*, vol. 34, Supplement 1, pp. S11–S61, 2011.

[17] N. Hamza, M. H. Abbas, A. Darwish, Z. Shafeek, J. New, and B. J. Ammori, "Predictors of remission of type 2 diabetes mellitus after laparoscopic gastric banding and bypass," *Surgery for Obesity and Related Diseases*, vol. 7, no. 6, pp. 691–696, 2011.

[18] C. W. Roux, A. SJB, R. L. Batterham et al., "Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters," *Annals of Surgery*, vol. 243, no. 1, pp. 108–114, 2006.

[19] K. K. Ryan, V. Tremaroli, C. Clemmensen et al., "FXR is a molecular target for the effects of vertical sleeve gastrectomy," *Nature*, vol. 509, no. 7499, pp. 183–188, 2014.

[20] H. Zhang, Y. Pu, J. Chen et al., "Gastrointestinal intervention ameliorates high blood pressure through antagonizing overdrive of the sympathetic nerve in hypertensive patients and rats," *Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease*, vol. 3, no. 5, article e000929, 2014.

[21] L. D. van Schinkel, M. A. Sleddering, M. A. Lips et al., "Effects of bariatric surgery on pericardial ectopic fat depositions and cardiovascular function," *Clinical Endocrinology*, vol. 81, no. 5, pp. 689–695, 2014.

[22] S. M. Haffner, R. A. Valdez, H. P. Hazuda, B. D. Mitchell, P. A. Morales, and M. P. Stern, "Prospective analysis of the insulin-resistance syndrome (syndrome X)," *Diabetes*, vol. 41, no. 6, pp. 715–722, 1992.

[23] R. Burrows, P. Correa-Burrows, M. Reyes, E. Blanco, C. Albala, and S. Gahagan, "Healthy Chilean adolescents with HOMA-IR $\geq$ 2.6 have increased cardiometabolic risk: association with genetic, biological, and environmental factors," *Journal of Diabetes Research*, vol. 2015, p. 783296, 2015.

[24] I. I. Sakou, "Insulin resistance and cardiometabolic risk factors in obese children and adolescents: a hierarchical approach," *Journal of Pediatric Endocrinology & Metabolism*, vol. 28, pp. 589–596, 2015.

[25] E. Lin, Z. Liang, J. Frediani et al., "Improvement in β-cell function in patients with normal and hyperglycemia following Roux-en-Y gastric bypass surgery," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 299, no. 5, pp. E706–E712, 2010.

[26] S. Camastra, A. Gastaldelli, A. Mari et al., "Early and longer term effects of gastric bypass surgery on tissue-specific insulin sensitivity and beta cell function in morbidly obese patients with and without type 2 diabetes," *Diabetologia*, vol. 54, no. 8, pp. 2093–2102, 2011.

[27] T. D. Adams, R. E. Gress, S. C. Smith et al., "Long-term mortality after gastric bypass surgery," *New England Journal of Medicine*, vol. 357, no. 8, pp. 753–761, 2007.

[28] L. Sjöström, M. Peltonen, P. Jacobson et al., "Bariatric surgery and long-term cardiovascular events," *Jama*, vol. 307, no. 1, 2012.

[29] P. R. Schauer, D. L. Bhatt, J. P. Kirwan et al., "Bariatric surgery versus intensive medical therapy for diabetes—3-year outcomes," *New England Journal of Medicine*, vol. 370, no. 21, pp. 2002–2013, 2014.

[30] P. Brindle, A. Beswick, T. Fahey, and S. Ebrahim, "Accuracy and impact of risk assessment in the primary prevention of cardiovascular disease: a systematic review," *Heart*, vol. 92, no. 12, pp. 1752–1759, 2006.

[31] R. V. Cohen, J. C. Pinheiro, C. A. Schiavon, J. E. Salles, B. L. Wajchenberg, and D. E. Cummings, "Effects of gastric bypass surgery in patients with type 2 diabetes and only mild obesity," *Diabetes Care*, vol. 35, no. 7, pp. 1420–1428, 2012.

[32] S. S. Shah, J. S. Todkar, P. S. Shah, and D. E. Cummings, "Diabetes remission and reduced cardiovascular risk after gastric bypass in Asian Indians with body mass index <35 kg/m^2," *Surgery for Obesity and Related Diseases : Official Journal of the American Society for Bariatric Surgery*, vol. 6, no. 4, pp. 332–338, 2010.

[33] A. Torquati, K. Wright, W. Melvin, and W. Richards, "Effect of gastric bypass operation on Framingham and actual risk of cardiovascular events in class II to III obesity," *Journal of the American College of Surgeons*, vol. 204, no. 5, pp. 776–782, 2007, discussion 782-773.

[34] D. Arterburn, D. P. Schauer, R. E. Wise et al., "Change in predicted 10-year cardiovascular risk following laparoscopic Roux-en-Y gastric bypass surgery," *Obesity Surgery*, vol. 19, no. 2, pp. 184–189, 2009.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 5139750, 7 pages
https://doi.org/10.1155/2017/5139750

Research Article

High Serum Advanced Glycation End Products Are Associated with Decreased Insulin Secretion in Patients with Type 2 Diabetes: A Brief Report

Tsuyoshi Okura,[1] Etsuko Ueta,[2] Risa Nakamura,[1] Yohei Fujioka,[1] Keisuke Sumi,[1] Kazuhisa Matsumoto,[1] Kyoko Shoji,[1] Kazuhiko Matsuzawa,[3] Shoichiro Izawa,[1] Yuri Nomi,[4] Hitomi Mihara,[5] Yuzuru Otsuka,[5] Masahiko Kato,[1] Shin-ichi Taniguchi,[3] and Kazuhiro Yamamoto[1]

[1]*Division of Cardiovascular Medicine, Endocrinology and Metabolism, Department of Molecular Medicine and Therapeutics, Tottori University Faculty of Medicine, Yonago, Tottori, Japan*
[2]*School of Health Science, Tottori University Faculty of Medicine, Yonago, Tottori, Japan*
[3]*Department of Regional Medicine, Tottori University Faculty of Medicine, Yonago, Tottori, Japan*
[4]*Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan*
[5]*Department of Food and Nutrition, Toita Women's College, Tokyo, Japan*

Correspondence should be addressed to Tsuyoshi Okura; ohkura@med.tottori-u.ac.jp

Received 15 February 2017; Revised 24 March 2017; Accepted 12 April 2017; Published 12 June 2017

Academic Editor: Joseph F. Ndisang

Objective. Advanced glycation end products (AGEs) are important in the pathophysiology of type 2 diabetes mellitus (T2DM). They directly cause insulin secretory defects in animal and cell culture models and may promote insulin resistance in nondiabetic subjects. We have developed a highly sensitive liquid chromatography-tandem mass spectrometry method for measuring AGEs in human serum. Here, we use this method to investigate the relationship between AGEs and insulin secretion and resistance in patients with T2DM. *Methods*. Our study involved 15 participants with T2DM not on medication and 20 nondiabetic healthy participants. We measured the AGE carboxyethyllysine (CEL), carboxymethyllysine (CML), and methylglyoxal-hydro-imidazolone (MG-H1). Plasma glucose and insulin were measured in these participants during a meal tolerance test, and the glucose disposal rate was measured during a euglycemic-hyperinsulinemic clamp. *Results*. CML and CEL levels were significantly higher in T2DM than non-DM participants. CML showed a significant negative correlation with insulin secretion, HOMA-%B, and a significant positive correlation with the insulin sensitivity index in T2DM participants. There was no correlation between any of the AGEs measured and glucose disposal rate. *Conclusions*. These results suggest that AGE might play a role in the development or prediction of insulin secretory defects in type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus is a heterogeneous disease characterized by insulin resistance and defective insulin secretion [1]. Advanced glycation end products (AGEs) are produced by a nonenzymatic reaction between amino and carbonyl groups [2]. This reaction, called the Maillard or amino-carbonyl reaction, is accelerated in the state of hyperglycemia in diabetes. AGEs have been reported to be correlated with the progression of diabetes and aging [3]. An animal study demonstrated that exposure to excess AGEs activates pathways of β-cell damage which, via mitochondrial superoxide generation, can impair insulin secretion [4]. A human study also showed a cross-sectional association between AGEs and acute insulin secretion during glucose tolerance testing in healthy humans [5]. Another human study demonstrated that the circulating level of AGEs is associated with insulin resistance as evaluated by the homeostasis model assessment

Table 1: Participant characteristics.

	T2DM	Non-DM	*P* value
n	15	20	
Sex (male/female)	9/6	12/8	
Age (years)	56.1 ± 12.0	33.9 ± 9.5	<0.001
BMI (kg/m^2)	27.46 ± 4.1	21.8 ± 2.9	<0.001
Waist circumference (cm)	95.7 ± 12.3	77.2 ± 9.9	<0.001
Fasting plasma glucose (mmol/L)	6.83 ± 0.75	4.80 ± 0.43	<0.001
HbA1c (%)	7.33 ± 0.83	5.33 ± 0.27	<0.001
HbA1c (mmol/mol)	(56.6)	(35.0)	
Insulinogenic index (IGI)	0.83 ± 1.07	1.44 ± 1.34	0.14
IRI-AUC	861.8 ± 429.2	616.3 ± 293.7	0.07
HOMA-%B (%)	73.7 ± 37.9	124.1 ± 68.8	<0.05
HOMA-IR	3.94 ± 2.50	1.70 ± 1.13	<0.001
Insulin sensitivity index (ISI)	4.11 ± 3.16	7.31 ± 3.32	<0.001
GDR	5.44 ± 2.34	9.52 ± 2.61	<0.001

Data are mean ± standard deviation. GDR: glucose disposal rate; HOMA-%B: homeostasis model assessment of beta cell function; HOMA-IR: homeostasis model assessment for insulin resistance; IRI-AUC: immunoreactive insulin area under the curve; Non-DM: nondiabetic study participants; T2DM: study participants with type 2 diabetes mellitus.

for insulin resistance (HOMA-IR), even in nonobese, nondiabetic subjects [6]. These results suggest that AGEs may affect insulin secretion as well as insulin resistance. However, there are few studies on the relationship between AGEs and insulin secretion ability or insulin resistance in patients with type 2 diabetes. Moreover, the most precise method for assessing insulin resistance is the glucose clamp technique, but this method is very complicated [7]. Instead, the HOMA-IR index is widely used in clinical practice and in clinical studies [8]. However, the validity of HOMA-IR may be limited in some patients, particularly those with a low BMI, reduced β-cell function, and high fasting glucose levels [9]. Since Asian and Japanese patients often show reduced β-cell function [10], a clamp study is required for evaluating insulin resistance in these populations.

Nε-(carboxymethyl)lysine (CML), Nε-(carboxyethyl)lysine (CEL), glyoxal-derived hydroimidazolone (G-H1), and methylglyoxal-derived hydroimidazolone (MG-H1) are known as representative AGEs generated in vivo [2]. Some AGEs such as CML are formed by an oxidative process and are called glycoxidation products. Several methods have been developed to determine the AGE content in biological samples. CEL, CML, and pentosidine have been measured using an immunological method with antibodies like rabbit-anti-CML-IgG and D12 antibody for CML [2]. Although ELISA is rapid, specific antibodies for each compound are required, the results are expressed in arbitrary units instead of actual concentrations, and the sample matrix, which can lead to incorrect estimation of AGE levels, affects the specificity of the assay significantly [11]. Recently, we developed a method for the simultaneous quantitation of several AGEs in brown-colored food using liquid chromatography-tandem mass spectrometry without ion-pair reagents and derivatization (LC-MS/MS method) [2]. We also developed a method for the simultaneous quantitation of several AGEs in human serum using LC-MS/MS method in this study.

According to past reports, there were some reports about the relationship between AGEs and insulin secretion and insulin resistance in the human healthy subjects; however, there were few reports about type 2 DM subjects. Furthermore, there were some reports about the relationship between AGEs and insulin secretion and insulin resistance by using the ELISA method; however, there were few reports about the exact quantitation of AGEs by using LC-MS/MS methods. Therefore, the aim of this study is to investigate the relationship between AGEs and insulin secretion and insulin resistance in the type 2 DM subjects by using LC-MS/MS methods. Based on previous studies, we hypothesized that patients with type 2 diabetes mellitus would show a correlation between AGE content and both insulin secretion ability and insulin resistance. In this study, we performed a meal tolerance test (MTT) and a glucose clamp in Japanese patients with type 2 diabetes mellitus and nondiabetic healthy volunteers and measured serum AGEs by the LC-MS/MS method.

2. Research Design and Methods

2.1. Subjects. Nine males and six females with type 2 diabetes mellitus (T2DM participants) participated in this study at Tottori University Hospital between 2014 and 2016. Type 2 diabetes mellitus was diagnosed using the criteria of the World Health Organization [12]. Patients with pancreatic disease, liver disease, or renal failure or those taking diabetogenic medications such as corticosteroids were excluded from this study. All T2DM participants were on diet therapy alone. Twelve male and eight female nondiabetic healthy volunteers (non-DM participants) were also recruited for this study. None of the non-DM participants had type 2 diabetes mellitus or were taking diabetic medications. Participant characteristics from the T2DM and non-DM groups are given in Table 1. The mean age, BMI, waist circumstance,

fasting plasma glucose (FPG), HbA1c, and HOMA-IR of the DM group were significantly higher than those of the non-DM group, and the mean insulin sensitivity index (ISI) and glucose disposal rate (GDR) of the DM group were significantly lower than those of the non-DM group. There was no significant difference in insulin AUC and insulinogenic index (IGI) between the DM group and non-DM group. All participants were examined using the protocols reported in our previous study [13].

This study was approved by the Ethics Committee of the Faculty of Medicine, Tottori University (approval number G161). Informed consent was obtained from all of the participants using a procedure approved by the Ethics Committee.

2.2. Meal Tolerance Test. After fasting for at least 12 h, participants visited the clinic in the morning and consumed a test meal prepared by the Japan Diabetes Society (460 kcal/1882 kJ; 15% protein, 35% fat, and 50% carbohydrate; 1.6 g salt) [14]. Plasma glucose and insulin were measured at 0 (fasting), 30, 60, 120, and 180 min after the test meal. Plasma glucose was measured using the glucose oxidase method. Plasma insulin levels were measured using chemiluminescent immunoassays. Plasma insulin was defined as immunoreactive insulin (IRI). HbA1c was measured by high-performance liquid chromatography. HbA1c percentage values were converted to International Federation of Clinical Chemistry values (mmol/mol) using the HbA1c converter developed by the National Institutes of Diabetes and Digestive and Kidney Diseases [15].

2.3. Euglycemic-Hyperinsulinemic Clamp. Glucose clamps were performed 2 days after the MTT. We examined the participants in the morning after an overnight fast. We cannulated an antecubital vein to administer the infusate, and we also cannulated a dorsal vein and kept warm to facilitate venous sampling and provide arterialized venous blood. We performed the euglycemic-hyperinsulinemic clamp to determine insulin sensitivity in the peripheral tissues by using an artificial endocrine pancreas (STG 55; Nikkiso, Shizuoka, Japan) [7]. We used a primed constant infusion of insulin (100 mU/m^2/min) and computer-controlled exogenous infusion of a glucose solution to achieve steady-state plasma insulin levels and maintain plasma glucose levels at 5.2 mmol/L (95 mg/dL). The previous studies reported that the steady-state plasma insulin level was 1200 pmol/L in patients with type 2 diabetes mellitus, by using this insulin infusion protocol [16, 17]. We calculated the steady-state glucose infusion rate between 90 and 120 min, and we defined the mean glucose infusion rate during this time as GDR (glucose disposal rate), which was used as a marker of peripheral insulin sensitivity. The glucose clamp method is a well-established procedure at our hospital [13, 18].

In a previous report, a GDR > 10.0 mg·kg^{-1}·min^{-1} at an insulin infusion rate of 100 mU/m^2/min was considered normal [19], and a GDR < 5.0 mg·kg^{-1}·min^{-1} was considered to be obviously insulin resistant [20].

2.4. Calculation of Insulin Resistance and Secretion Indexes. HOMA-IR [8] = [fasting plasma glucose (mmol/L)] × [fasting plasma insulin (pmol/L)]/135. The normal range for HOMA-IR is <2.5 [21].

HOMA-%B (homeostasis model assessment of beta cell function) [8] = {20 × [fasting plasma insulin (pmol/L)]}/{[fasting plasma glucose (mmol/L)] − 3.5} (%).

Insulin sensitivity index (ISI) [22] = 10,000/$\sqrt{}${[fasting plasma glucose (mmol/L) × fasting plasma insulin (pmol/L)] × [mean glucose × mean insulin during the MTT]}. The normal range for ISI is >2.5 [23].

Insulinogenic index (IGI) [24] = {[insulin (pmol/L) at 30 min] − [insulin (pmol/L) at 0 min]}/{[glucose (mmol/L) at 30 min] − [glucose (mmol/L) at 0 min]}.

2.5. Measurement of AGEs [2]

2.5.1. Chemicals and Reagents. Standards CML, CEL, and MG-H1 and internal standards CML-d_4, CEL-d_4, and MG-H1-d_3 were purchased from PolyPeptide Group (Strasbourg, France). All other reagents were of the highest grade available and were purchased from Wako (Osaka, Japan).

Each stock solution of CML, CEL, and MG-H1 at 0.1 mg/mL was prepared in purified water and stored at −20°C. Stock solutions of internal standards CML-d_4, CEL-d_4, and MG-H1-d_3, at 0.1 mg/mL, were prepared in the same manner. Before analysis, we prepared working standard solutions (final concentrations 0–20 ng/mL) and internal standard working solutions (final concentration 10 ng/mL) by diluting the stock solutions using 1% aqueous formic acid.

2.5.2. Instruments. High-performance liquid chromatography experiments were performed on a prominence series liquid chromatograph system (Shimadzu, Kyoto, Japan) consisting of a binary pump, in-line degasser, autosampler, and column oven. Chromatographic separation was achieved with an Intrada Amino Acid column (2.0 mm I.D. × 150 mm, Imtakt Co. Ltd., Kyoto, Japan) at 40°C with an injection volume of 10 μL. The mobile phase consisted of solvent A, containing 100 mM ammonium formate in water, and solvent B, containing 0.5% formic acid in acetonitrile. The separation conditions were a linear gradient from 75 to 50% of solvent B from 0 to 7 min, from 50 to 0% of solvent B from 7 to 9 min, and 0% of solvent B from 9 to 20 min. The flow rate was 0.3 mL/min. The column was equilibrated for 6 min under the initial conditions before each injection.

An AB Sciex QTRAP 5500 mass spectrometer (AB SCIEX, Tokyo, Japan) equipped with an electrospray ion source in the positive ion mode was used under the following operating conditions: curtain gas 10 psi; ion spray voltage 5000 V; temperature of ion source 700°C; ion source gas 1 50 psi; ion source gas 2 70 psi; collision gas 8.0 psi; and entrance potential 10 V. Seven glycation free adducts were detected individually in the postcolumn by MS/MS with multiple reaction monitoring (MRM) for transition of the parent ions to the product ions. LC-MS/MS data were acquired and processed using Analyst version 1.5 software (Applied Biosystems/MDS Analytical Technologies, Tokyo, Japan).

2.5.3. Preparation of Serum Extracts. Whole blood was collected into vacuum blood collection tubes containing a serum separating agent and a procoagulant film (VENOJECT II, Terumo Co., Tokyo, Japan) and refrigerated at 4°C. After standing for 30 min, serum was obtained by centrifugation at 3000 ×g for 10 min and stored at −80°C until deproteinization. For deproteinization, an aliquot of serum (50 μL) was mixed with 6% aqueous sulfosalicylic acid (50 μL) and centrifuged at 13000 rpm for 5 min. The supernatant was transferred to a filter unit (ULTRAFREE-C3LCR, 0.2 μm, Merck Millipore, Darmstadt, Germany) and centrifuged below 12000 ×g for 5 min. The supernatant was transferred to a microtube and then diluted three times with 1% aqueous formic acid, forming the serum extract.

2.5.4. Method Validation. Matrix effects of the serum extracts were determined by preparing mixtures containing 25, 100, and 200 ng/mL of each AGE in 1% aqueous formic acid. Next, 5 μL of each mixture was added to 45 μL of a serum extract or 1% aqueous formic acid. For the blank sample (0 ng/mL), 5 μL of water was added to 45 μL of a serum extract or 1% aqueous formic acid. The final concentrations of the AGEs were 0, 2.5, 10, and 20 ng/mL. These standard solutions dissolved in serum extracts or 1% aqueous formic acid were stored at −30°C until LC-MS/MS analysis.

An internal standard method using isotopic AGEs was also performed. Aliquots of the serum extracts (45 μL) were dispensed and spiked with a mixture of three internal standards (CML-d_4, CEL-d_4, and MG-H1-d_3) at a final concentration of 10 ng/mL (5 μL) and stored at −30°C until LC-MS/MS analysis. Calibration curves of analyte/internal standard peak area ratio versus AGE concentration were constructed for each of the three AGEs.

Samples were prepared for a recovery test by mixing 45 μL of the serum extract with 5 μL of the respective stock solutions (CEL, CML, and MG-H1; final concentration: 2.5, 10, and 20 ng/mL, resp.) containing a mixture of three internal standards (final concentration 10 ng/mL). Percent recovery was calculated according to the formula:

$$\text{Rec}\,(\%) = \left\{\frac{[C(\text{a}) - C(\text{b})]}{C(\text{c})}\right\} \times 100, \quad (1)$$

where Rec is the recovery, $C(\text{a})$ is the concentration in spiked sample, $C(\text{b})$ is the initial concentration, and $C(\text{c})$ is the concentration of standard mixture of three AGEs.

Our methods about meal tolerance test, glucose clamp test, and AGE measurements were already described in the past reports [2, 13, 18].

2.5.5. Statistical Analysis. Data are expressed as mean ± standard deviation of the mean. The area under the curve was calculated according to the trapezoidal rule. Differences in the mean value of AGEs between T2DM and non-DM participants were assessed using an unpaired *t*-test. Correlations between parametric clinical variables and AGEs were determined using Pearson's correlation analysis. Values of $P < 0.05$ were considered significant. SPSS software version 24.0 (SPSS, Chicago, IL, USA) was used for all analyses.

Table 2: Operating parameters for serum AGE measurement using a QTRAP 5500 mass spectrometer equipped with an electrospray ion source operating in the positive ion mode.

Compound	Retention time (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	DP (V)	CE (V)	CXP (V)
CEL	5.64	219.1	84.1	111	27	6
CML	6.01	205.1	84.1	76	25	10
MG-H1	9.09	229.1	70.1	46	30	8
CEL-d_4	5.65	223.1	88.1	111	27	6
CML-d_4	5.99	209.1	88.1	76	25	10
MG-H1-d_3	9.07	232.1	70.1	46	30	8

DP: declustering potential; CE: collision energy; CXP: collision cell exit potential; CEL: carboxyethyllysine; CML: carboxymethyllysine; MG-H1: methyl-glyoxal-hydro-imidazolone.

3. Results

When standard CML was analyzed by MS/MS with flow injection, CML showed an intense molecular ion at *m*/*z* 205.10 [M+H]+. Therefore, product ion scanning was conducted for the ion, and CML-specific fragment ions at *m*/*z* 84.10 [M+H−121]+ were identified. Similarly, MS/MS analysis was performed for CEL and MG-H1. The identified ions (*m*/*z* 219.10 and 84.10 for CEL, *m*/*z* 229.10 and 70.10 for MG-H1) allowed selective detection of analytes using electrospray ionization- (ESI-) MS with MRM. All of the settings for MRM are summarized in Table 2. Although the detailed information of the separation mode and sample preparation for the Intrada Amino Acid column has not been released by the supplier, the sample preparation method was suggested by the supplier, and separation and quantitation were very good for human serum samples the same as food samples [2]. Ion enhancement was found in CEL, CML, and MG-H1 when the serum was diluted to 6.67 times. However, it showed good sensitivity and linearity ($R^2 > 0.97$) in the range of 0–20 ng/mL. Therefore, the concentration of CML, CEL, and MG-H1 was calculated from the peak area of AGE with the peak area of internal standard of CML-d4, CEL-d4, and MG-H1-d3.

The mean values of CEL and CML were significantly higher in T2DM participants than non-DM participants (Table 3). MG-H1 was also higher in T2DM participants than non-DM participants, but this difference was not statistically significant.

Across all T2DM participants, there were no correlations between the AGEs and HbA1c (Table 4). CML was strongly negatively correlated with the HOMA-%B and IRI area under the curve and showed a significant positive correlation with ISI. However, the AGEs did not correlate with GDR or HOMA-IR. CEL and did not correlate with any insulin secretion or resistance indexes.

Across all non-DM participants, CML, CEL, and MG-H1 did not correlate with HbA1c or any insulin secretion or resistance indexes (Table 4).

Table 3: Serum AGEs levels.

	AGE		
	CEL (ng/mL)	CML (ng/mL)	MG-H1 (ng/mL)
T2DM ($n = 15$)	15.4 ± 6.6	21.0 ± 7.0	27.2 ± 20.7
Non-DM ($n = 20$)	9.8 ± 2.2	15.1 ± 4.2	17.5 ± 8.2
P value	<0.001	<0.01	NS

Data are mean ± standard deviation. AGEs: advanced glycation end products; CEL: carboxyethyllysine; CML: carboxymethyllysine; MG-H1: methyl-glyoxal-hydro-imidazolone; T2DM: study participants with type 2 diabetes mellitus; Non-DM: nondiabetic study participants; NS: not significant.

Table 4: Correlation coefficients for the associations between AGEs and clinical parameters.

	T2DM ($n = 15$)					
Index	CEL		CML		MG-H1	
	r	P	r	P	r	P
HbA1c	−0.43	NS	−0.10	NS	−0.11	NS
IGI	−0.31	NS	−0.39	NS	−0.30	NS
IRI-AUC	−0.27	NS	−0.53	<0.05	−0.20	NS
HOMA-%B	−0.14	NS	−0.63	<0.05	0.01	NS
HOMA-IR	−0.13	NS	−0.47	NS	−0.04	NS
ISI	0.26	NS	0.72	<0.01	−0.04	NS
GDR	0.31	NS	0.13	NS	0.15	NS
	Non-DM ($n = 20$)					
Index	CEL		CML		MG-H1	
	r	P	r	P	r	P
HbA1c	−0.03	NS	−0.10	NS	−0.05	NS
IGI	0.14	NS	0.26	NS	−0.03	NS
IRI-AUC	0.10	NS	0.24	NS	−0.18	NS
HOMA-%B	0.11	NS	0.05	NS	−0.29	NS
HOMA-IR	0.04	NS	−0.03	NS	−0.12	NS
ISI	−0.12	NS	−0.24	NS	−0.12	NS
GDR	0.16	NS	0.12	NS	0.03	NS

Correlation coefficients were determined using Pearson's product moment correlation coefficient test. AGEs: advanced glycation end products; CEL: carboxyethyllysine; CML: carboxymethyllysine; GDR: glucose disposal rate; HOMA-%B: homeostasis model assessment of beta cell function; HOMA-IR: homeostasis model assessment for insulin resistance; IGI: insulinogenic index; IRI-AUC: immunoreactive insulin area under the curve; ISI: insulin sensitivity index; MG-H1: methyl-glyoxal-hydro-imidazolone; Non-DM: nondiabetic study participants; T2DM: study participants with type 2 diabetes mellitus; NS: not significant.

4. Discussion

This study shows that CML and CEL levels were significantly higher in T2DM than non-DM participants. CML was significantly negatively correlated with insulin secretion, HOMA-%B and IRI-AUC, and positively correlated with ISI, but was not correlated with GDR in T2DM. A previous study using cell culture and animal models demonstrated that exposure to excess AGEs activates pathways of β-cell damage, which can impair insulin secretion [4]. β-Cells exposed to AGEs displayed acute glucose-stimulated insulin secretory defects, mitochondrial abnormalities including excess superoxide generation, and reduced calcium flux. Another study suggested that AGE injections can initiate β-cell dysfunction in vivo [25]. A recent in vitro study also indicated that CML caused mitochondrial dysfunction and mitophagy in β-cells and that high levels of AGEs may induce β-cell dysfunction and impair insulin secretion ability [26]. A recent epidemiological study also showed that increased fasting CML levels may be predictive of type 2 diabetes development [27]. These results suggest that CML decreases insulin secretion and is important in the pathophysiology of impaired glucose metabolism.

In our study, CML correlated with ISI, an index which is greatly affected by insulin secretion ability. Conversely, CML and the other AGEs did not correlate with the GDR. It may be difficult for the findings to explain the reasons, we consider that HOMA-IR mainly reflects the insulin resistance of liver and ISI mainly reflects the insulin resistance of muscle [28]. However, ISI is greatly affected by the insulin secretion ability, and if the insulin secretion ability is decreased, ISI shows low insulin resistance [29]. We measured GDR using the glucose clamp method, which is a precise method for assessing insulin resistance of muscle [28]. Therefore, we consider the results that CML correlates with ACU-IRI and ISI, but not GDR and HOMA-IR, which means CML mainly affects insulin secretion ability rather than insulin resistance. Thus, we suggest that CML has a greater effect on insulin secretion ability than on insulin resistance. A recent study reported that a diet low in AGEs increased insulin sensitivity in healthy, overweight individuals [30]. Insulin sensitivity as evaluated by the glucose clamp method increased after a low-AGE diet and showed a tendency to decrease after a high-AGE diet. There was no difference in body weight or insulin secretion between these diet groups. The authors suggested restricting dietary AGE content as an effective strategy to decrease diabetes and cardiovascular disease risks in overweight individuals. Another study reported that a low-AGE diet ameliorates insulin resistance in obese people with the metabolic syndrome without necessitating a major reduction in adiposity [31]. However, these studies differ from our study in that their subjects did not have type 2 diabetes. We suggest that the results of animal and cell culture studies on AGE content and diabetes [4] are likely to be more relevant to the present study; however, further study is needed.

Our study had several limitations. The relatively small number of participants (total 35, T2DM 15, non-DM 20) and the difference in age and BMI between the T2DM and non-DM groups indicate that our results require confirmation with a larger study. Therefore, we decided to add "A Brief Report" in the title. Furthermore, the DM group was aged and obese compared to the control group; therefore, the elevated levels of CML and CEL may be due to aging and obesity. However, glucose clamp test is a very complicated method, and it is difficult to recruit the patients with poorly controlled diabetes without medications and older nondiabetic participants with obesity. We are currently conducting a larger study, the results of which we plan

to publish in the future. MTT was used in our study as OGTTs are best avoided in patients with severe diabetes because of the risk of hyperglycemia. As IGI and ISI were developed from OGTTs, we propose that differences between consuming a test meal and a pure glucose load may also affect glucose and insulin levels. Despite these limitations, we think our study contributes to our understanding of the pathophysiology of type 2 diabetes.

In summary, CML and CEL levels were significantly higher in T2DM than non-DM participants. CML was significantly negatively correlated with insulin secretion, HOMA-%B, and IRI-AUC and positively correlated with ISI in T2DM participants but was not correlated with insulin resistance as evaluated by the glucose clamp method. In conclusion, these results suggest that AGE might play a role in the development or prediction of insulin secretory defects in type 2 diabetes.

Abbreviations

AGEs: Advanced glycation end products
AUC: Area under the curve
CEL: Carboxyethyllysine
CML: Carboxymethyllysine
GDR: Glucose disposal rate
HOMA-%B: Homeostasis model assessment of beta cell function
HOMA-IR: Homeostasis model assessment for insulin resistance
IGI: Insulinogenic index
IRI: Immunoreactive insulin
ISI: Insulin sensitivity index
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
MG-H1: Methyl-glyoxal-hydro-imidazolone
MTT: Meal tolerance test
Non-DM: Nondiabetic study participants
OGTT: Oral glucose tolerance test
T2DM: Study participants with type 2 diabetes mellitus.

Disclosure

Tsuyoshi Okura is the guarantor of this work and takes responsibility for the integrity of the data and the accuracy of the data analyses.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Tsuyoshi Okura participated in the design of the study and performed the statistical analysis. Etsuko Ueta, Risa Nakamura, Yohei Fujioka, Keisuke Sumi, Kazuhisa Matsumoto, Kyoko Shoji, Kazuhiko Matsuzawa, Shoichiro Izawa, Yuri Nomi, and Hitomi Mihara collected the data. Yuzuru Otsuka, Masahiko Kato, Shin-ichi Taniguchi, and Kazuhiro Yamamoto conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

This work was supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research (C) Grant no. 16K08935 (2016–2018), a JSPS KAKENHI Grant-in-Aid for Young Scientists (B) Grant no. 26870373 (2014–2015), and grants for young researchers from the Japan Association for Diabetes Education and Care (2014). This work was also supported by an MSD research grant (2015). The authors thank Ms. Megumi Endo, Ms. Kayo Kato, Ms. Yoshiko Oda, and Katusmi Nagata for their excellent technical assistance. This work was carried out at the Tottori University Faculty of Medicine, Tottori, Japan.

References

[1] R. A. DeFronzo, "Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM," *Diabetes*, vol. 37, no. 6, pp. 667–687, 1988.

[2] Y. Nomi, H. Annaka, S. Sato et al., "Simultaneous quantitation of advanced glycation end products in soy sauce and beer by liquid chromatography-tandem mass spectrometry without ion-pair reagents and derivatization," *Journal of Agricultural and Food Chemistry*, vol. 64, no. 44, pp. 8397–8405, 2016.

[3] S. R. Thorpe and J. W. Baynes, "Maillard reaction products in tissue proteins: new products and new perspectives," *Amino Acids*, vol. 25, no. 3-4, pp. 275–281, 2003.

[4] M. T. Coughlan, F. Y. Yap, D. C. Tong et al., "Advanced glycation end products are direct modulators of β-cell function," *Diabetes*, vol. 60, no. 10, pp. 2523–2532, 2011.

[5] J. M. Forbes, K. C. Sourris, M. P. de Courten et al., "Advanced glycation end products (AGEs) are cross-sectionally associated with insulin secretion in healthy subjects," *Amino Acids*, vol. 46, no. 10, pp. 321–326, 2014.

[6] K. C. Tan, S. W. Shiu, Y. Wong, and X. Tam, "Serum advanced glycation end products (AGEs) are associated with insulin resistance," *Diabetes/Metabolism Research and Reviews*, vol. 27, no. 5, pp. 488–492, 2011.

[7] R. A. DeFronzo, J. D. Tobin, and R. Andres, "Glucose clamp technique: a method for quantifying insulin secretion and resistance," *The American Journal of Physiology*, vol. 237, no. 3, pp. 214–223, 1979.

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

[9] E. S. Kang, Y. S. Yun, S. W. Park et al., "Limitation of the validity of the homeostasis model assessment as an index of insulin resistance in Korea," *Metabolism*, vol. 54, no. 2, pp. 206–211, 2005.

[10] K. Kodama, D. Tojjar, S. Yamada, K. Toda, C. J. Patel, and A. J. Butte, "Ethnic differences in the relationship between insulin sensitivity and insulin response: a systematic review

and meta-analysis," *Diabetes Care*, vol. 36, no. 6, pp. 1789–1796, 2013.

[11] A. Charissou, L. Ait-Ameur, and I. Birlouez-Aragon, "Evaluation of a gas chromatography/mass spectrometry method for the quantification of carboxymethyllysine in food samples," *Journal of Chromatography*, vol. 1140, no. 1-2, pp. 189–194, 2007.

[12] K. G. Alberti and P. Z. Zimmet, "Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation," *Diabetic Medicine*, vol. 15, no. 7, pp. 539–553, 1998.

[13] T. Ohkura, H. Shiochi, Y. Fujioka et al., "20/(fasting C-peptide x fasting plasma glucose) is a simple and effective index of insulin resistance in patients with type 2 diabetes mellitus: a preliminary report," *Cardiovascular Diabetology*, vol. 12, no. 1, p. 21, 2013.

[14] G. Yoshino, M. Tominaga, T. Hirano et al., "The test meal A:A pilot model for the international standard of test meal for an assessment of both postprandial hyperglycemia and hyperlipidemia," *Journal of the Japan Diabetes Society*, vol. 49, no. 5, pp. 361–371, 2006.

[15] National Institutes of Diabetes and Digestive and Kidney Diseases, "The HbA1c converter," 1999, http://www.ngsp.org/convert1.asp.

[16] R. Kawamori, M. Matsuhisa, J. Kinoshita et al., "Pioglitazone enhances splanchnic glucose uptake as well as peripheral glucose uptake in non-insulin-dependent diabetes mellitus. AD-4833 Clamp-OGL Study Group," *Diabetes Research and Clinical Practice*, vol. 41, no. 1, pp. 35–43, 1998.

[17] Y. Tamura, Y. Tanaka, F. Sato et al., "Effects of diet and exercise on muscle and liver intracellular lipid contents and insulin sensitivity in type 2 diabetic patients," *The Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 6, pp. 3191–3196, 2005.

[18] H. Shiochi, T. Ohkura, Y. Fujioka et al., "Bezafibrate improves insulin resistance evaluated using the glucose clamp technique in patients with type 2 diabetes mellitus: a small-scale clinical study," *Diabetology and Metabolic Syndrome*, vol. 6, no. 1, p. 113, 2014.

[19] Y. Sakurai, Y. Tamura, K. Takeno et al., "Determinants of intramyocellular lipid accumulation after dietary fat loading in non-obese men," *Journal of Diabetes Investigation*, vol. 4, no. 4, pp. 310–317, 2011.

[20] F. Sato, Y. Tamura, H. Watada et al., "Effects of diet-induced moderate weight reduction on intrahepatic and intramyocellular triglycerides and glucose metabolism in obese subjects," *The Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 8, pp. 3326–3329, 2007.

[21] E. Bonora, S. Kiechl, J. Willeit, and M. Muggeo, "Prevalence of insulin resistance in metabolic disorders: the Bruneck study," *Diabetes*, vol. 47, no. 10, pp. 1643–1649, 1998.

[22] M. Matsuda and R. A. DeFronzo, "Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp," *Diabetes Care*, vol. 22, no. 9, pp. 1462–1470, 1999.

[23] W. N. Kernan, S. E. Inzucchi, C. M. Viscoli et al., "Pioglitazone improves insulin sensitivity among nondiabetic patients with a recent transient ischemic attack or ischemic stroke," *Stroke*, vol. 34, no. 6, pp. 1431–1436, 2003.

[24] H. S. Seltzer, E. W. Allen, A. L. Herron Jr, and M. T. Brennan, "Insulin secretion in response to glycemic stimulus: relation of delayed initial release to carbohydrate intolerance in mild diabetes mellitus," *Journal of Clinical Investigation*, vol. 46, no. 3, pp. 323–335, 1967.

[25] Z. Zhao, C. Zhao, and X. H. Zhang, "Advanced glycation end products inhibit glucose-stimulated insulin secretion through nitric oxide-dependent inhibition of cytochrome c oxidase and adenosine triphosphate synthesis," *Endocrinology*, vol. 150, no. 6, pp. 2569–2576, 2009.

[26] M. C. Lo, M. H. Chen, W. S. Lee et al., "Nε-(carboxymethyl) lysine-induced mitochondrial fission and mitophagy cause decreased insulin secretion from β-cells," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 309, no. 10, p. E829-39, 2015.

[27] V. C. Luft, B. B. Duncan, M. I. Schmidt et al., "Carboxymethyl lysine, an advanced glycation end product, and incident diabetes: a case-cohort analysis of the ARIC study," *Diabetic Medicine*, vol. 33, no. 10, p. 1392, 2015.

[28] R. Muniyappa, S. Lee, H. Chen, and M. J. Quon, "Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 294, no. 1, pp. E15–E26, 2008, Review.

[29] M. Kanauchi, N. Tsujimoto, and T. Hashimoto, "Validation of simple indices to assess insulin sensitivity based on the oral glucose tolerance test in the Japanese population," *Diabetes Research and Clinical Practice*, vol. 55, no. 3, pp. 229–235, 2002.

[30] B. de Courten, M. P. de Courten, G. Soldatos et al., "Diet low in advanced glycation end products increases insulin sensitivity in healthy overweight individuals: a double-blind, randomized, crossover trial," *The American Journal of Clinical Nutrition*, vol. 103, no. 6, pp. 1426–1433, 2016.

[31] H. Vlassara, W. Cai, E. Tripp et al., "Oral AGE restriction ameliorates insulin resistance in obese individuals with the metabolic syndrome: a randomised controlled trial," *Diabetologia*, vol. 59, no. 10, pp. 2181–2192, 2016.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 2654520, 9 pages
https://doi.org/10.1155/2017/2654520

Research Article

Liver-Specific Overexpression of Gamma-Glutamyltransferase Ameliorates Insulin Sensitivity of Male C57BL/6 Mice

Yang Long,[1,2] Dan Jia,[3] Libin Wei,[4] Yumei Yang,[1,5] Haoming Tian,[1] and Tao Chen[1]

[1]*Department of Endocrinology and Metabolism, West China Hospital of Sichuan University, Chengdu 610041, China*
[2]*Laboratory of Endocrinology, Experimental Medicine Center, The Affiliated Hospital of Southwest Medical University Luzhou 646000, China*
[3]*Division of General Practice, West China Hospital of Sichuan University, Chengdu 610041, China*
[4]*Department of Stomatology, Hebei Medical University Affiliated North China Petroleum Bureau General Hospital, Renqiu 062552, China*
[5]*Health Examination Management Center, Sichuan Province People's Hospital, Chengdu 610072, China*

Correspondence should be addressed to Haoming Tian; hmtian999@126.com and Tao Chen; dr.chentao@qq.com

Received 8 February 2017; Revised 18 April 2017; Accepted 4 May 2017; Published 4 June 2017

Academic Editor: Joseph F. Ndisang

In the current study, we developed a liver-specific GGT-overexpressing mice model by rapid injection pLIVE-GGT vector through tail vein and investigated the effects of GGT elevation on glucose metabolism and insulin sensitivity. The serum GGT activity was significantly increased after 7 days of pLIVE-GGT1 vector delivery and lasted for about 3 weeks. GGT overexpression reduced the levels of GSSG and GSH in the liver and serum and had no effects on total antioxidative capacity in the liver, kidney, and skeletal muscle except for the pancreas. Increased GGT activity had no effect on the glucose tolerance but could facilitate blood glucose lowering after intraperitoneal insulin administration. The results of Western blotting showed that increased GGT activity enhanced insulin-induced AKT phosphorylation at Ser473. Furthermore, GGT inhibitor could attenuate the changes of insulin-induced blood glucose uptake and AKT phosphorylation in the liver. In summary, the present study developed a liver-specific GGT-overexpressing mice model and found that GGT elevation in short term had no effects on glucose metabolism but could increase insulin sensitivity through enhancing the activity of insulin signaling pathway.

1. Introduction

The prevalence of type 2 diabetes (T2D) is increasing worldwide, while the underlining mechanism is not fully elucidated. Recently, several prospective studies and meta-analyses suggested that gamma-glutamyltransferase (GGT), a previously recognized marker of alcoholic drinking and fatty liver, could predict the risk of T2D [1–3]. Such an association existed even when GGT was at physiologic level [4], in nonalcoholic drinkers and subjects without nonalcoholic fatty liver disease (NAFLD) [5, 6]. One study suggested that BMI could predict T2D only when GGT was at physiologic high levels [7].

GGT exists on the surface of nearly all kinds of epithelial cells and plays a critical role in regulating reactive oxygen species (ROS) level through balancing reduced glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) and oxidized form glutathione disulfide (GSSG). The nature substrate of GGT is GSH, and the gamma-glutamyl of GSH can only be cleaved by GGT. GGT broke down GSH in extracellular fluids [5]. This process demands the cooperation of Fe(III) and will lead to the production of the superoxide anion and hydrogen peroxide [8]. Theoretically, increased GGT activity would result in altered levels of GSSG/GSH and overproduction of ROS, thus changing the oxidative status. Our previous study found that increased GGT activity combined with ferritin levels was linked to increased risk of T2D, and the mechanism might be related to increased oxidative stress [9]. Furthermore, other studies showed that elevated serum GGT concentration could be associated with islet beta-cell function and/or insulin resistance [10, 11].

However, the associations between elevated serum GGT and T2D, insulin resistance, and islet beta-cell function were built on epidemiological observational studies. In these studies, GGT elevation usually was accompanied by NALFD, ferritin, and other markers of oxidative stress and chronic inflammation [9, 12, 13]. Therefore, it is difficult to deduce whether causative relationship existed between GGT and T2D in such complicated clinical settings. To better understand their relationship, the present study developed a liver-specific GGT1-overexpressing mice model to control confounding factors and tested the effects of isolated GGT elevation on GSSG/GSH metabolism, glucose metabolism, and insulin sensitivity.

2. Method

2.1. Construction of GGT1 Systemic and Liver-Specific Overexpression Vector. For systemic expression, pcDNA3.1-Zeo(+) vector was used. The encoding region of mouse GGT1 was amplified with primers listed below by RT-PCR. For more effective expression of GGT1, two different Kozak sequences were selected and added in different primers (GGT-F1-KOZg: 5′-ACGGGATCCAAGCGCCATGAAGAATCG -GT-3′; GGT-F1-KOZa: 5′-ACGGGATCCAAGCACCATGAAGAATCGGT-3′). Then, the GGT1 cDNA was cloned into the BamHI and XhoI sites of pcDNA3.1-Zeo(+) to generate two different recombinant vectors (pcDNA3.1-ggt1-KOZg and pcDNA3.1-ggt1-KOZa).

pLIVE™ vector, which is designed for liver-specific expression and utilizes a chimeric promoter composed of the mouse minimal albumin promoter and the mouse alpha fetoprotein enhancer II(Mirus Bio Corporation), was selected to construct the liver-specific GGT1 overexpression vector. The pcDNA3.1-ggt1-KOZa was excised with BamHI and XhoI endonucleases and purified by using standard techniques; then, the GGT1 cDNA with Kozak sequence (ACCATGA) was cloned into the BamHI and XhoI sites of pLIVE vector to generate pLIVE-ggt1-KOZa vector. The vector DNAs were prepared by an AxyPrep™ Endo-Free plasma Maxiprep kit.

2.2. In Vitro Expression and Enzyme Activity Assays. COS7 cells were cultured in high-glucose DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FBS in 5% CO2, in a 37°C incubator. Approximately 1×10^4 cells were plated and transiently transfected with 0.5 μg of pcDNA3.1-ggt1-KOZg or pcDNA3.1-ggt1-KOZa using Lipofectamine® LTX Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 hours, the cell culture mediums were collected. Then, the cells were scraped off the plate and lysed immediately with PBS followed by determination of protein concentration with a Thermo Scientific Pierce BCA Protein Assay Kit. The culture mediums and the cell lysates were subjected to detect GGT activity using a γ-Glutamyl transferase Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.3. Generation of a Liver-Specific GGT1-Overexpressing Mouse Model. Male C57BL/6 mice were purchased from Chongqing Tengxin Biotechnology Co. Ltd (Chongqing, China). All animals were maintained in a constant 12 h light/12 h dark cycle and fed with a standard rodent chow and water ad libitum. The constructs of pLIVE as a control and pLIVE-ggt1-KOZa vector DNA (100μg/mice) were delivered to the mouse liver using the hydrodynamic tail vein injection procedure according to the instruction provided by manufacturer. To confirm whether the changes in insulin sensitivity in GGT1-L-OE mice were induced by liver-specific overexpression of GGT, GGT1-L-OE mice were administered with 2.5 mg/kg/d GGsTop™ (GGT1-L-OE-sTop mice). For serum GGT activity measurement, mice serum samples were collected at 1, 2, and 3 weeks after injection. For tissue GGT activity measurement, mice were sacrificed at 14 days and the liver, kidney, pancreas, epididymal adipose, and skeletal muscle were quickly frozen in liquid nitrogen and stored at −80°C. Serum and tissue total glutathione (T-GSSG), GSSG, and GSH and total oxidization capacity were assayed at 14 days after vector DNA injection.

To investigate the effects of GGT overexpression on the glucose tolerance and insulin sensitivity, intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were performed at 14 days after vector DNA injection. For measurement of AKT phosphorylation, 14 days after vector DNA injection, mice were fasted for 6 h and were injected intraperitoneally with or without 0.75 U insulin/kg body weight. Immediately after insulin stimulation, the mouse liver was quickly frozen in liquid nitrogen and stored at −80°C.

2.4. IPGTT and ITT. In brief, IPGTT was performed by intraperitoneal injection with 1 g glucose/kg body weight after a 6 h fast, and the blood glucose was measured before and at 15, 30, 60, and 120 minutes after glucose injection. For ITT, mice were injected intraperitoneally with 0.75 U insulin/kg body weight after a 6 h fast and the blood glucose was measured before and at 15, 30, 60, and 120 minutes after insulin injection. Blood glucose concentrations were measured by the Roche Accu-Chek active glucose meter (Roche Diagnostics GmbH, Mannheim, Germany).

2.5. Measurement of GGT and Oxidative Stress Markers. Serum and tissue GGT activities were tested by the γ-Glutamyl transferase Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of T-GSSG, GSSG, and GSH in serum and tissue were measured with the GSH/GSSG assay kit (Beyotime, Beijing, China). As for measurement in tissue lysates, the concentrations of T-GSSG, GSSG, and GSH were normalized by the protein concentrations of tissue lysates. The serum and tissue total oxidization capacities were assayed with a Total Antioxidant Capacity Assay Kit (Beyotime, Beijing, China).

2.6. Western Blotting. Tissue lysates were prepared in lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice in 1.5 mL microtubes for 15 min and centrifuged for 5 min at 12,000*g* at 4°C. The supernatant was collected and protein concentrations were measured using the Thermo Scientific Pierce BCA Protein Assay Kit

(Pierce Biotechnology, Rockford, USA); then, the protein samples were stored at −80°C until further examination.

For the Western blot, tissue lysates were subjected to SDS-PAGE and immunoblotting was performed using specific antibodies against AKT (Cell Signaling Technology Inc., Boston, USA), phospho-AKT (Ser473) (Cell Signaling Technology Inc., Boston, USA), and GAPDH (ZSGB-Bio Inc., Beijing, China).

2.7. Statistics. Data were expressed as mean ± SEM. Data were analyzed by 1-way ANOVA. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Evaluation of the Translation Efficiency of GGT1 and Generation of Liver-Specific GGT1-L-OE Mice. As shown in Figure 1, the GGT activities were significantly increased in the culture mediums and the cell lysates from COS7 cells which were transiently transfected with pcDNA3.1-ggt1-KOZa but not with pcDNA3.1-ggt1-KOZg. These data indicated that the Kozak sequence ACCATGA, which was added in the recombinant vector pcDNA3.1-ggt1-KOZa, promoted more effective translation than the consensus GCCATGA in the vector pcDNA3.1-ggt1-KOZg. Then, the Kozak sequence, ACCATGA, was selected to generate liver-specific GGT1 overexpression vector, pLIVE-ggt1-KOZa.

As shown in Figure 2(a), the serum GGT activities were significantly increased after 7 days of pLIVE-GGT1 vector delivery. The serum GGT activities were decreased by 50% after 14 days of plasmid delivery and by 70% after 21 days of plasmid delivery. So, we designed to explore the effect of liver-specific GGT1 overexpression for a short period of 14 days on the glucose tolerance and insulin sensitivity.

The liver, pancreas, kidney, epididymal adipose, and skeletal muscle of GGT1-L-OE mice were lysed and subjected to test GGT1 activities on the 14 days after plasmid delivery. As expected, the GGT enzymatic activities were significantly increased in the liver but not in the kidney, pancreas, epididymal adipose, or skeletal muscle (Figure 2(b)).

The levels of T-GSSG, GSSG, and GSH were reduced in serum and liver homogenates. The total GSSG levels were significantly reduced by 50% in serum and by 30% in liver homogenates from GGT1-L-OE mice compared with those from control mice. Compared with those in control mice, serum GSH levels were decreased by 30% in GGT1-L-OE mice. But there was no significant decrease in liver homogenates from GGT1-L-OE mice compared with those from control mice. Similarly, no significant changes in the total antioxidant capacity were observed in the liver from GGT1-L-OE mice compared with that from control mice.

3.2. Liver-Specific Overexpression of GGT Had No Effect on the Glucose Tolerance. As shown in Figures 3(a) and 3(b), the levels of blood glucose were slightly increased before and at 30 and 60 minutes after glucose challenge in GGT1-L-OE mice than those in the control mice, but the differences of the levels of blood glucose and the AUC of IPGTT curve between the two groups were not statistically significant. Furthermore, the levels of blood glucose were slightly decreased before and at 30, 60, and 120 minutes after glucose challenge in GGT1-L-sTop mice which were treated with GGsTop, than those in the control mice, but there were no statistical significances between the two groups. These results indicated that liver-specific overexpression of GGT in two weeks had no effect on the glucose tolerance in mice without metabolic diseases.

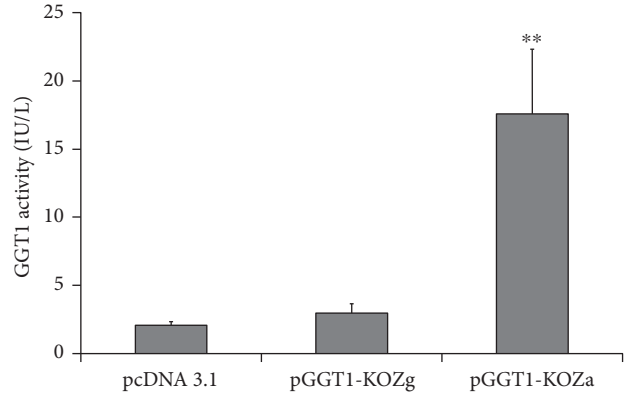

Figure 1: Expression of GGT1 in COS7 cell lines. $^{**}P < 0.001$.

3.3. Liver-Specific Overexpression of GGT Increased Insulin Sensitivity in C57BL/6 Mice. Furthermore, we investigated whether specific overexpression of GGT in the liver affected whole-body insulin sensitivity by ITT. As shown in Figures 4(a) and 4(b), blood glucose concentrations were decreased more in GGT1-L-OE mice during ITT when compared with those in the control mice. The levels of blood glucose were decreased significantly at 15 and 30 minutes after insulin administration. Moreover, the AUC of ITT curve in GGT1-L-OE mice was significantly decreased by 15% when compared with that in the controls (Figure 4(b)). Similarly, the levels of phosphorylated AKT at Ser473, induced by insulin, were significantly increased in the liver lysates of GGT1-L-OE mice compared with those in the liver lysates of the controls (Figures 4(c) and 4(d)).

To confirm whether the increased insulin sensitivity in GGT1-L-OE mice was induced by specific overexpression of GGT in the liver, GGsTop was used. As expected, blood glucose concentrations decreased less in the GGT1-L-OE-sTop mice during ITT compared with those in the GGT1-L-OE mice. The differences of blood glucose levels were significant at 30, 60, and 120 minutes after insulin administration. Also, the AUC of ITT curve in GGT1-L-OE-sTop mice was significantly increased by approximately 30% when compared with that in the GGT1-L-OE mice (Figure 4(b)). The levels of phosphorylated AKT at Ser473, induced by insulin, were decreased by about 20% in the liver lysates of the GGT1-L-OE-sTop mice compared with those in the liver lysates of the GGT1-L-OE mice, although no statistical significance was reached ($P = 0.075$). These results suggested that specific overexpression of GGT in the liver might increase insulin sensitivity in C57BL/6 mice.

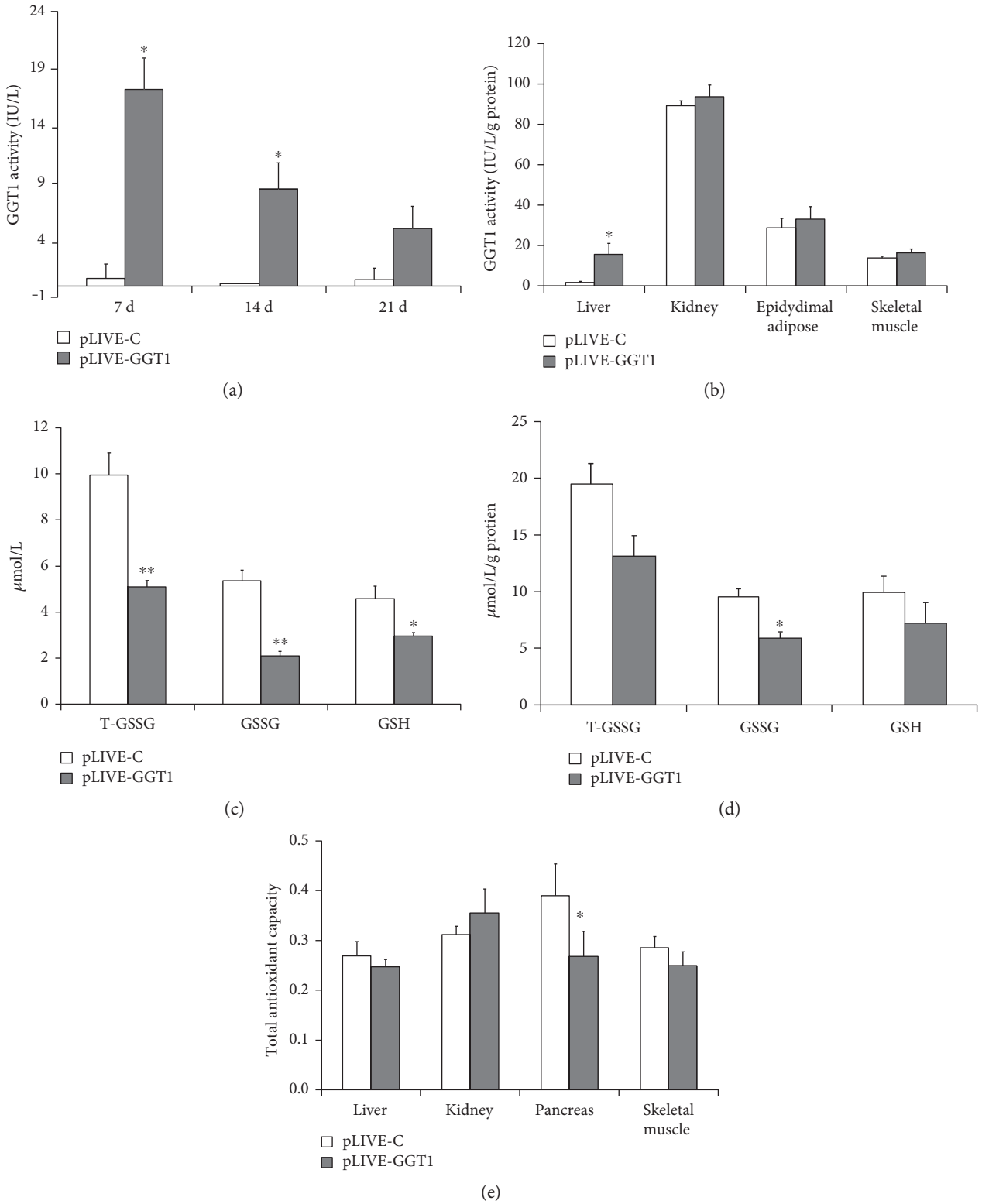

Figure 2: Generation of liver-specific GGT1-overexpressing mice. (a) Serum GGT activities on 7, 14, and 21 days after plasmid delivery through tail vein injection. (b) GGT activities in the liver, kidney, skeletal muscle, and pancreas on the 14 days after plasmid delivery. (c) The concentrations of T-GSSG, GSSG, and GSH in serum. (d) The concentrations of T-GSSG, GSSG, and GSH in liver homogenates. (e) The levels of total antioxidant capacity in the liver, kidney, pancreas, and skeletal muscle. $^{*}P < 0.05$, $^{**}P < 0.001$.

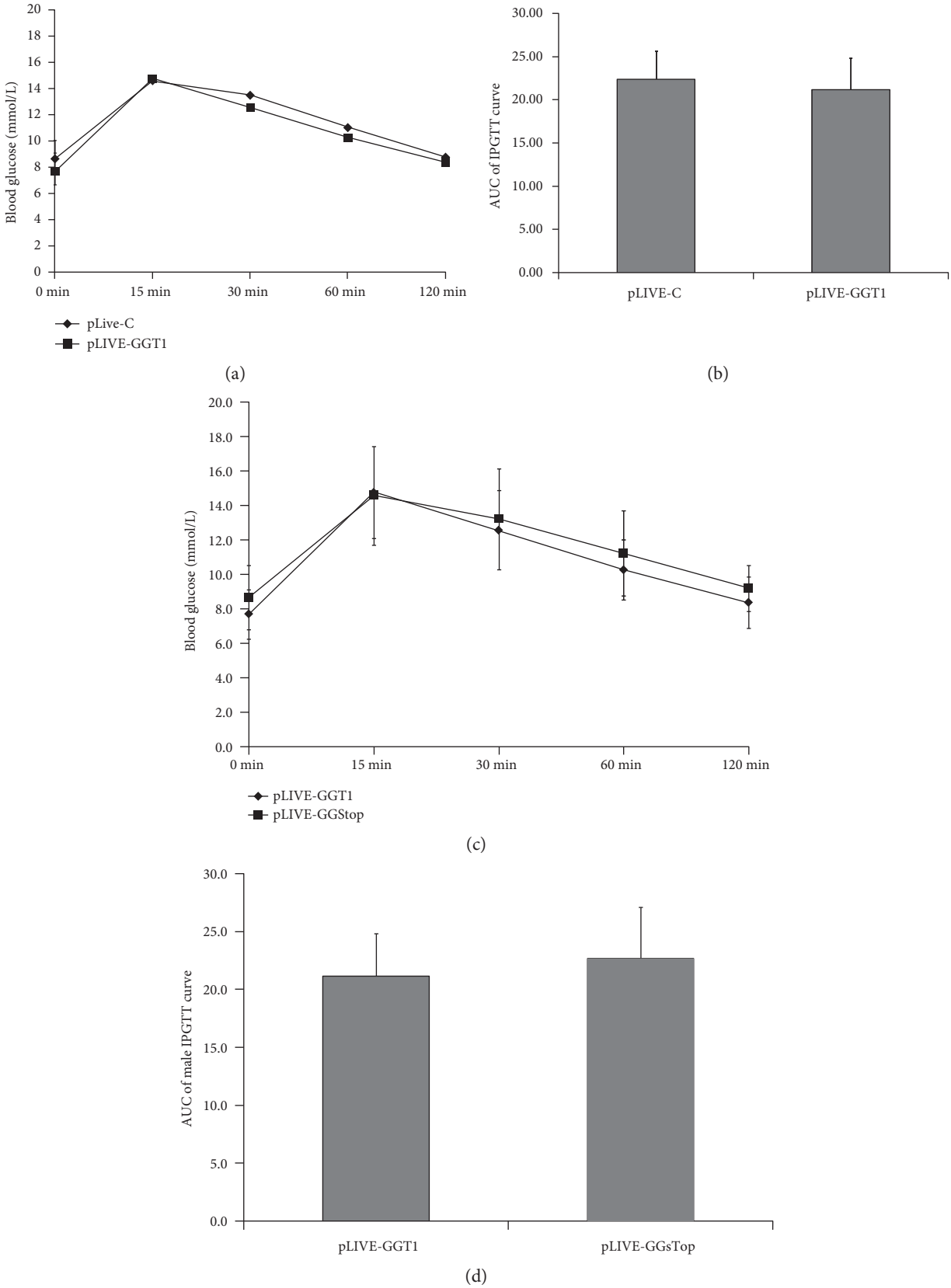

Figure 3: Overexpression of GGT1 in the liver had no effect on glucose tolerance. (a) IPGTT was performed and the blood glucose was measured at the indicated time points ($n = 13$–17); (b) AUC of the IPGTT curve. (c) IPGTT was performed in GGT1-L-OE mice and GGT1-L-OE-sTop mice which are treated with GGsTop, a highly selective GGT inhibitor, and the AUC was evaluated (d).

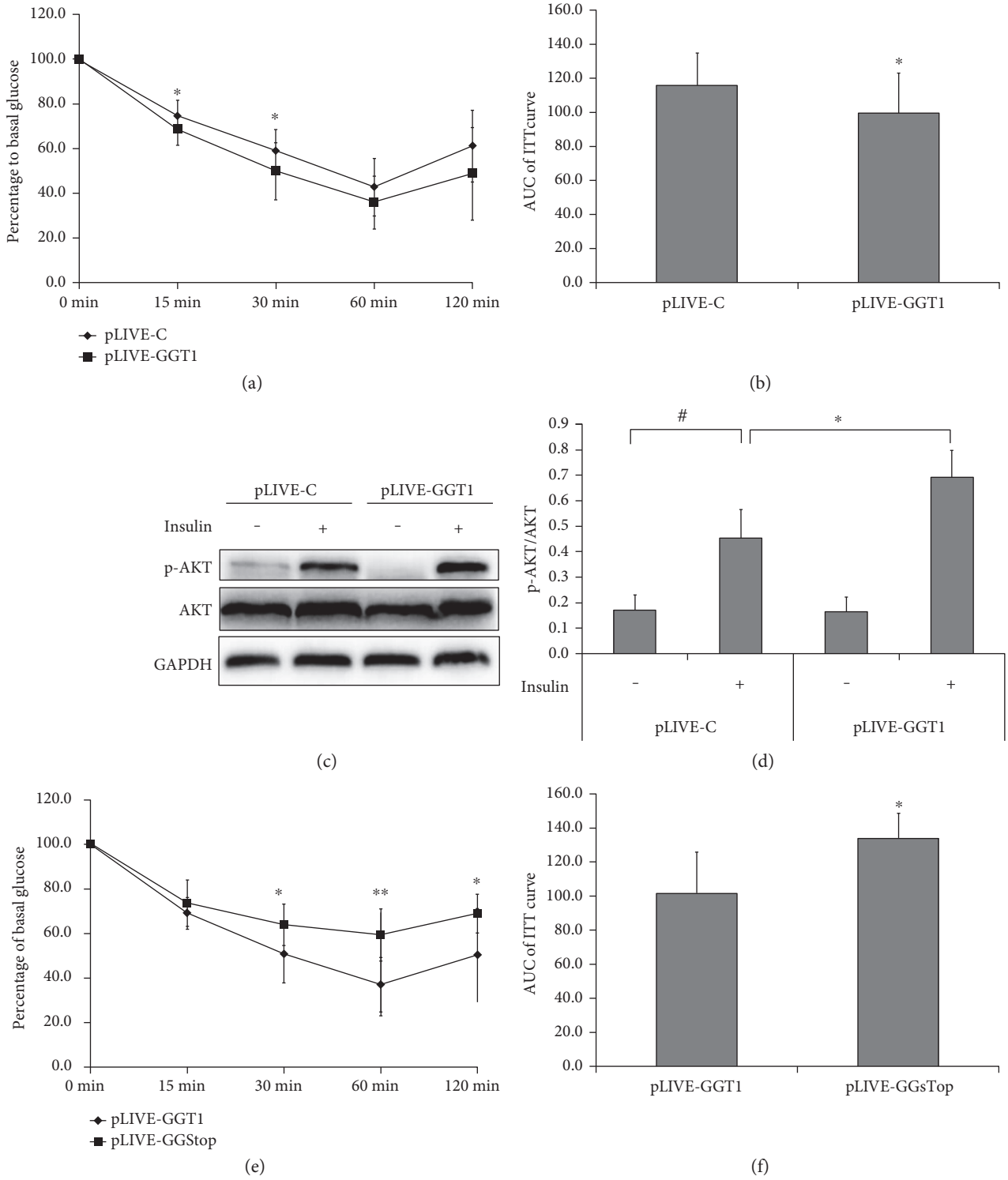

Figure 4: Continued.

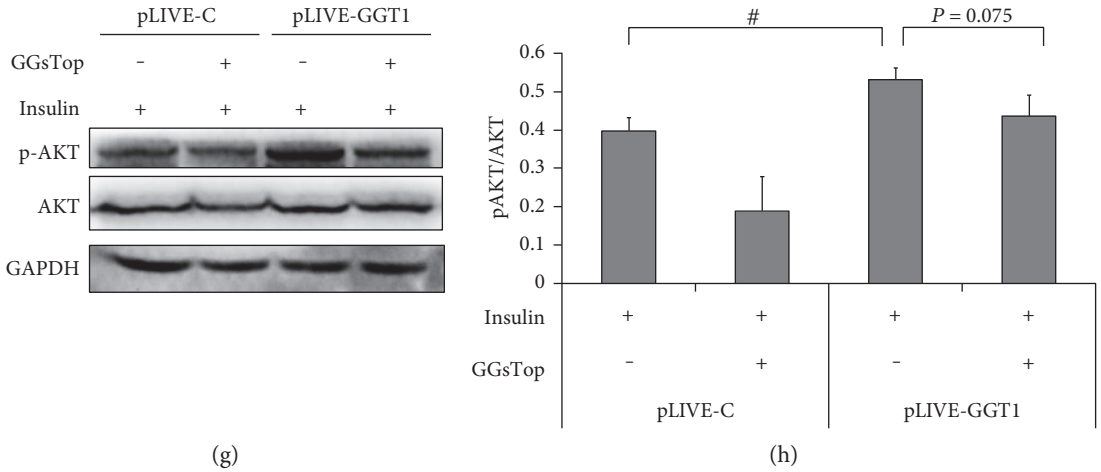

Figure 4: Overexpression of GGT1 in the liver increased insulin sensitivity. (a) ITT was performed and the blood glucose was measured at the indicated time points ($n = 13–17$); (b) AUC of blood glucose after ITT. (c) The liver from fasted C57BL/6 mice injected with or without 0.75 U insulin/kg body weight for 15 min was subjected to Western blotting using a rabbit anti-phosphor-AKT (Ser473) polyclonal antibody or rabbit anti-total AKT polyclonal antibody. (d) Fold changes of phosphor-AKT versus total AKT relative to the basal levels in GGT1-L-OE and control mice were quantified by densitometry. (e) ITT was performed in GGT1-L-OE mice and GGT1-L-OE-sTop mice which were treated with GGsTop, a highly selective GGT inhibitor, and the AUC was counted (f). (g) Liver homogenates were immunoblotted with anti-phosphor-AKT (Ser473) antibody or anti-AKT antibody. (h) The relative expression of phosphor-AKT was presented by setting the mean density of blots. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{\#} < 0.05$.

4. Discussion

GGT transgenic mice models had been used to study osteoporosis and tumor metastasis [14, 15], but regrettably, no study focused on glucose metabolism. In the present study, we successfully developed a short-term liver-specific GGT1-overexpressing mice model and found that increased activity of GGT in the liver and circulation enhanced the catabolism of GSSG/GSH. GGT elevation, in two weeks, had no effects on the glucose metabolism but surprisingly might increase insulin sensitivity in the liver of the transgenic mice. Such results were quite different from those observed in epidemiologic studies that elevated GGT activity increased the risk of T2D.

GGT elevation was common in T2D and other clinical conditions such as obesity, NAFLD, prediabetes, and metabolic syndrome [5]. In such conditions, GGT elevation usually was accompanied by increased levels of ferritin [9, 12], uric acid [16], and markers of chronic inflammation such as CRP [12] and IL-6 [13]. Besides GGT, all of the above factors have been reported to be associated with increased risk of T2D [17–19]. Iron overload initiated pro-oxidant reaction and was involved in multiple pathological mechanisms [17, 20]. GGT might exert effects in cooperation with iron. For example, our previous study observed that GGT might affect T2D risks by synergetic action with increased serum ferritin rather than GGT alone [9]. Interestingly, another study of gestational diabetes (GDM) among a large cohort of mothers in California also showed that GGT only predicted GDM in women with top tertile of HOMA-IR before pregnancy, suggesting interaction between GGT and HOMA-IR [21]. The latter was a well-established correlate of serum ferritin. In this study, we purely increased GGT activity and neglected any potential interactive effects with other aforementioned factors. This could be an explanation for the conflicting conclusions between previous epidemiologic studies and this experimental study.

This study observed that GGT overexpression reduced the levels of GSSG/GSH but had little effects on total antioxidant capacity, suggesting its influence on ROS being mild. To our surprise, this study showed that increased GGT activity enhanced insulin-induced AKT phosphorylation at Ser473 and insulin-induced glucose uptake. GGT is critical to maintain cysteine homeostasis. It has been reported that L-cysteine increased phosphatidylinositol 3,4,5-trisphosphate (PIP3), a positive regulator of phosphorylation of AKT, in 3T3-L1 adipocytes, and subsequently enhances glucose utilization by the activation of phosphoinositide 3-kinase and phosphorylation of AKT [22, 23]. So, tt is plausible to deduce that liver-specific GGT overexpression increased AKT phosphorylation through cysteine-mediated mechanisms. Published studies already suggested that GGT elevation could just be one of the corresponding changes to other offending factors such as oxidative stress. GGT elevation in T2D could be a compensation mechanism but not strong enough to ameliorate increased oxidative stress [20, 24]. GGT might actually play a protective role against the harmful impact by the aforementioned factors. When GGT elevation is sufficient, like in the GGT1-L-OE mice from this study, it might exert beneficial effects on insulin sensitivity via AKT-mediated mechanisms.

There were some limitations in the present study. The major one was the short-term duration of GGT overexpression. Hydrodynamic delivery has been proved to be an effective method for targeted gene transfection in animals. It works by a rapid injection of DNA solutions via the tail vein, leading to significant transgene expression in multiple organs, especially in the liver. The expression of some

transfected genes by this method could last for several months [25]. While in this study, GGT elevation could only persisted for less than 3 weeks. We had tried different buffer solutions and plasmid concentrations but failed to extend the expression time. A future study should employ other transgenic methods to evaluate the effects of prolonged GGT overactivity on insulin sensitivity. Secondly, the GGT-mediated insulin sensitivity improvement was observed only in normal C57BL/6 mice but not in mice with metabolic diseases such as obesity or T2D. Thus, it is not known whether elevated GGT activity influences glucose metabolism and/or insulin sensitivity in T2D or metabolic syndrome mice models. Further studies are needed to confirm such relationship in those mice models.

In conclusion, the presented study demonstrated that GGT elevation in a short term had no effects on glucose tolerance but could promote insulin sensitivity in male C57BL/6 mice. These results supported the notion that GGT elevation could be a protective factor rather than an offending factor to T2D.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yang Long and Dan Jia contributed equally to this work.

Acknowledgments

This study was supported by the Chinese National Nature Science Foundation for the Youth (81100572) "The effects of gamma-glutamyl transpeptidase overexpression on insulin resistance and islet beta-cell function in mice and its related mechanisms."

References

[1] D.-H. Lee, D. Jacobs, M. Gross et al., "Gamma-glutamyltransferase is a predictor of incident diabetes and hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) Study," *Clinical Chemistry*, vol. 49, no. 8, pp. 1358–1366, 2003.

[2] N. Nakanishi, K. Suzuki, and K. Tatara, "Serum gamma-glutamyltransferase and risk of metabolic syndrome and type 2 diabetes in middle-aged Japanese men," *Diabetes Care*, vol. 27, no. 6, pp. 1427–1432, 2004.

[3] S. K. Kunutsor, A. Abbasi, and A. I. Adler, "Gamma-glutamyl transferase and risk of type II diabetes: an updated systematic review and dose-response meta-analysis," *Annals of Epidemiology*, vol. 24, no. 11, pp. 809–816, 2014.

[4] P. Andre, B. Balkau, C. Born, M. A. Charles, E. Eschwege, and Group DESIRs, "Three-year increase of gamma-glutamyltransferase level and development of type 2 diabetes in middle-aged men and women: the D.E.S.I.R. cohort," *Diabetologia*, vol. 49, no. 11, pp. 2599–2603, 2006.

[5] J. B. Whitfield, "Gamma glutamyl transferase," *Critical Reviews in Clinical Laboratory Sciences*, vol. 38, no. 4, pp. 263–355, 2001.

[6] C. Sabanayagam, A. Shankar, J. Li, C. Pollard, and A. Ducatman, "Serum gamma-glutamyl transferase level and diabetes mellitus among US adults," *European Journal of Epidemiology*, vol. 24, no. 7, pp. 369–373, 2009.

[7] D. A. Lawlor, N. Sattar, G. D. Smith, and S. Ebrahim, "The associations of physical activity and adiposity with alanine aminotransferase and gamma-glutamyltransferase," *American Journal of Epidemiology*, vol. 161, no. 11, pp. 1081–1088, 2005.

[8] M. Iciek, G. Chwatko, H. Rokita, E. Bald, and L. Wlodek, "The effects of modulation of gamma-glutamyl transpeptidase activity in HepG2 cells on thiol homeostasis and caspase-3-activity," *Biochimica et Biophysica Acta*, vol. 1773, no. 2, pp. 201–208, 2007.

[9] T. Chen, Y. Ren, Y. Liu et al., "Serum gamma-glutamyl transferase, ferritin and the risk of type 2 diabetes in women from a Chinese minority," *Diabetes Research and Clinical Practice*, vol. 90, no. 3, pp. 352–357, 2010.

[10] C. Thamer, O. Tschritter, M. Haap et al., "Elevated serum GGT concentrations predict reduced insulin sensitivity and increased intrahepatic lipids," *Hormone and Metabolic Research = Hormon- und Stoffwechselforschung = Hormones et Metabolisme*, vol. 37, no. 4, pp. 246–251, 2005.

[11] L. Wang, J. Zhang, B. Wang et al., "New evidence for an association between liver enzymes and pancreatic islet beta-cell dysfunction in young obese patients," *Endocrine*, vol. 44, no. 3, pp. 688–695, 2013.

[12] D. H. Lee and D. R. Jacobs Jr., "Association between serum gamma-glutamyltransferase and C-reactive protein," *Atherosclerosis*, vol. 178, no. 2, pp. 327–330, 2005.

[13] R. D. Bradley, A. L. Fitzpatrick, D. R. Jacobs Jr., D. H. Lee, N. Swords Jenny, and D. Herrington, "Associations between gamma-glutamyltransferase (GGT) and biomarkers of atherosclerosis: the Multi-ethnic Study of Atherosclerosis (MESA)," *Atherosclerosis*, vol. 233, no. 2, pp. 387–393, 2014.

[14] K. Hiramatsu, Y. Asaba, S. Takeshita et al., "Overexpression of gamma-glutamyltransferase in transgenic mice accelerates bone resorption and causes osteoporosis," *Endocrinology*, vol. 148, no. 6, pp. 2708–2715, 2007.

[15] E. Obrador, J. Carretero, A. Ortega et al., "Gamma-glutamyl transpeptidase overexpression increases metastatic growth of B16 melanoma cells in the mouse liver," *Hepatology*, vol. 35, no. 1, pp. 74–81, 2002.

[16] J. X. Zhang, G. D. Xiang, L. Xiang, and J. Dong, "Serum gamma-glutamyl transferase is associated with the elevated uric acid levels in normotensive Chinese adults," *Clinica Chimica Acta; International Journal of Clinical Chemistry*, vol. 441, pp. 122–126, 2015.

[17] Z. Zhao, S. Li, G. Liu et al., "Body iron stores and heme-iron intake in relation to risk of type 2 diabetes: a systematic review and meta-analysis," *PLoS One*, vol. 7, no. 7, article e41641, 2012.

[18] I. Sluijs, J. W. Beulens, A. D. van der, A. M. Spijkerman, M. B. Schulze, and Y. T. van der Schouw, "Plasma uric acid is associated with increased risk of type 2 diabetes independent of diet and metabolic risk factors," *The Journal of Nutrition*, vol. 143, no. 1, pp. 80–85, 2013.

[19] X. Wang, W. Bao, J. Liu et al., "Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis," *Diabetes Care*, vol. 36, no. 1, pp. 166–175, 2013.

[20] G. Koenig and S. Seneff, "Gamma-glutamyltransferase: a predictive biomarker of cellular antioxidant inadequacy and

disease risk," *Disease Markers*, vol. 2015, Article ID 818570, p. 18, 2015.

[21] S. B. Sridhar, F. Xu, J. Darbinian, C. P. Quesenberry, A. Ferrara, and M. M. Hedderson, "Pregravid liver enzyme levels and risk of gestational diabetes mellitus during a subsequent pregnancy," *Diabetes Care*, vol. 37, no. 7, pp. 1878–1884, 2014.

[22] P. Manna and S. K. Jain, "Hydrogen sulfide and L-cysteine increase phosphatidylinositol 3,4,5-trisphosphate (PIP3) and glucose utilization by inhibiting phosphatase and tensin homolog (PTEN) protein and activating phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase (AKT)/protein kinase Czeta/lambda (PKCzeta/lambda) in 3T3l1 adipocytes," *The Journal of Biological Chemistry*, vol. 286, no. 46, pp. 39848–39859, 2011.

[23] P. Manna and S. K. Jain, "L-cysteine and hydrogen sulfide increase PIP3 and AMPK/PPARgamma expression and decrease ROS and vascular inflammation markers in high glucose treated human U937 monocytes," *Journal of Cellular Biochemistry*, vol. 114, no. 10, pp. 2334–2345, 2013.

[24] K. Gawlik, J. W. Naskalski, D. Fedak et al., "Markers of antioxidant defense in patients with type 2 diabetes," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 2352361, p. 6, 2016.

[25] M. S. Al-Dosari, J. E. Knapp, and D. Liu, "Hydrodynamic delivery," *Advances in Genetics*, vol. 54, pp. 65–82, 2005.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 8248725, 8 pages
https://doi.org/10.1155/2017/8248725

Research Article

Exercise Metabolism in Nonobese Patients with Type 2 Diabetes Following the Acute Restoration of Normoglycaemia

Christopher J. Gaffney,[1,2] Peter Mansell,[1] Francis B. Stephens,[2] Ian A. Macdonald,[1] and Kostas Tsintzas[1]

[1]*Medical Research Council/Arthritis Research UK Centre for Musculoskeletal Ageing Research, School of Life Sciences, The University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK*
[2]*School of Sport and Health Sciences, University of Exeter, St Luke's Campus, Exeter EX1 2LU, UK*

Correspondence should be addressed to Kostas Tsintzas; kostas.tsintzas@nottingham.ac.uk

Received 1 January 2017; Revised 9 February 2017; Accepted 14 February 2017; Published 29 May 2017

Academic Editor: Joseph F. Ndisang

This study investigated how acute restoration of normoglycaemia affected energy metabolism during exercise in nonobese patients with type 2 diabetes. Six subjects (mean ± SEM) aged 56.2 ± 2.7 years, with a BMI of 24.5 ± 1.5 kg/m^2 and a $VO_{2\ peak}$ of 28.7 ml/kg/min, attended the lab on two randomised occasions for a four-hour resting infusion of insulin or saline, followed by 30 minutes cycling at 50% $VO_{2\ peak}$. During the 4 h resting infusion, there was a greater ($P < 0.0001$) reduction in blood glucose in insulin treatment (INS) (from 11.2 ± 0.6 to 5.6 ± 0.1 mmol/l) than in saline treatment/control (CON) (from 11.5 ± 0.7 to 8.5 ± 0.6 mmol/l). This was associated with a lower ($P < 0.05$) resting metabolic rate in INS (3.87 ± 0.17) than in CON (4.39 ± 0.30 kJ/min). During subsequent exercise, blood glucose increased significantly in INS from 5.6 ± 0.1 at 0 min to 6.3 ± 0.3 mmol/l at 30 min ($P < 0.01$), which was accompanied by a lower blood lactate response ($P < 0.05$). Oxygen uptake, rates of substrate utilization, heart rate, and ratings of perceived exertion were not different between trials. Insulin-induced normoglycaemia increased blood glucose during subsequent exercise without altering overall substrate utilization.

1. Introduction

Around 90% of adults with type 2 diabetes (T2D) are overweight or obese [1], and a previous research has shown that glycaemic control can be improved in these individuals by weight loss through a structured exercise program or a nutritional intervention [2, 3]. Little research is conducted on the c. 360,000 UK-based nonobese patients with T2D (BMI < 25 kg/m^2) (based on the data in [4]), which still possess a significant risk of secondary complications [5] and have an equal risk of cardiovascular disease to their obese counterparts [6].

Exercise is an important component in the management of T2D as it can help control weight and improve cardiovascular fitness [7], which are important to mitigate secondary complications. Exercise is also crucial to improving insulin sensitivity [8] and overall glycaemic control [9]. Exercise does, however, present potential challenges in patients with T2D, and this includes the risk of hypoglycaemic episodes, particularly in those taking sulfonylureas and insulin [10]. During exercise, patients with T2D have normal or elevated rates of skeletal muscle glucose disposal but impaired hepatic glucose output [11], increasing the risk of hypoglycaemia. Moreover, exercise increases insulin sensitivity for up to 72 hours postexercise [12], which presents a risk of hypoglycaemia during recovery from exercise. Overweight and obese patients with T2D not taking insulin are able to reduce their blood glucose levels during exercise [13–15], and insulin and exercise synergistically increase muscle glucose uptake [16]. Reductions in blood glucose have also been observed in patients with T2D with both mild [13] and substantial [14, 17] preexercise hyperglycaemia. Both moderate intensity continuous exercise and particularly high intensity exercise are able to reduce nocturnal/fasting glycaemia [15], potentially predisposing to the risk of hypoglycaemia in the fasted state. In addition, plasma glucose utilization is increased

Table 1: Subject demographics and inclusion and exclusion criteria for subjects.

Subject demographics	
Age	56.2 ± 2.7 years
Gender distribution	5 male and 1 female participants
Height (m)	1.64 ± 0.04
Weight (kg)	66.7 ± 6.0
BMI (kg/m^2)	24.5 ± 1.5
Time since diagnosis (years)	7.8 ± 1.4
Diabetes treatment	Metformin ($n = 6$)
HbA1c (%)/mmol/mol	9.4 ± 0.3/78.9 ± 3.8
Fasting blood glucose (mmol/l)	11.3 ± 0.6
Predicted RMR (Schofield equations) [21] (MJ/d)	6.36 ± 0.31
Calculated RMR (MJ/d)	5.31 ± 1.15
$VO_{2\ peak}$ (ml/kg/min)	28.7 (82 ± 4% of the predicted values for healthy individuals of similar age)
Inclusion criteria	Exclusion criteria
Type 2 diabetes diagnosed >2 yrs before consent	History of cardiac disease
Age 40–69 years inclusive	History of cerebrovascular events or transient ischemic episodes
BMI: <30 kg/m^2	History of intermittent claudication
Suboptimal glycaemic control: HbA1c > 8% (64 mmol/mol)	Significant hypertension defined as a systolic BP > 170 mmHg and/or diastolic BP > 95 mmHg
Evidence of recent, regular moderate physical activity (PAL of 1.6–1.7) [22]	Any other disease likely to affect the ability to exercise including arthritis and respiratory disease
Normal resting 12-lead ECG	Any cardiorespiratory drugs other than thiazide diuretics, aspirin, ACE-inhibitors, and statins
No significant ECG changes or chest pain during a Bruce protocol exercise ECG to stage III with a normal physiological response to exercise	Secondary complications: any diabetic retinopathy other than mild background retinopathy, nephropathy (proteinuria on >1 occasion or raised creatinine), and sensory neuropathy

Data are presented as mean ± SEM.

during exercise in nonobese [18] and obese [19] patients with T2D. Therefore, patients taking oral glucose-lowering medication prior to exercise are at a heightened risk of hypoglycaemia.

The aim of this study was to determine if exercise in normoglycaemic nonobese patients with T2D was possible without significant reductions in blood glucose levels. Indeed, we sought to determine whether restoration of preexercise normoglycaemia in nonobese patients with T2D was associated with changes in perceived exertion, substrate utilization, or hypoglycemic episodes during subsequent moderate exercise.

2. Research Design and Methods

2.1. Subjects. Six nonobese and nonsmoking participants (five male) with a diagnosis of type 2 diabetes gave written informed consent to participate in this study, which was approved by Nottingham University Hospital Ethics Committee and conformed to the Declaration of Helsinki. All participants had type 2 diabetes for at least three years (mean duration of 7.8 yrs and range of 3 to 13 years) and had suboptimally controlled diabetes [HbA1c > 8% (64 mmol/mol)]. Subjects were recruited from Nottingham University Hospital Diabetes Register and from participating General Practitioner Surgeries. Subjects were 56.2 ± 2.7 years of age, were 66.7 ± 6.0 kg in weight, and had a BMI of 24.5 ± 1.5 kg/m^2 and a $VO_{2\ peak}$ of 28.7 ml/kg/min. Subjects were excluded if they had significant complications of diabetes, vascular disease, abnormal renal or hepatic function, or other disorders that prevented exercise, including respiratory diseases and arthritis (Table 1). Furthermore, participants were excluded if they produced a recent abnormal Bruce protocol treadmill test to stage III [20]. Subjects were not taking medications that may alter the response to exercise including beta blockers and calcium channel blockers.

2.2. Preliminary Measurements. Subjects attended the laboratory prior to the main experimental visits where they were familiarized with the ventilated canopy system (GEM Indirect Calorimetry, GEMNutrition Ltd., Daresbury, UK) that was used for the measurement of a resting metabolic rate [23]. Subjects then completed an incremental exercise test to the maximum predicted heart rate (220 minus age in years) using an electrically braked cycle ergometer (Lode, Groningen, Netherlands) for the determination of maximum oxygen consumption [20]. Before each test, subjects were allowed to warm up at a workload of 20 W for 5 minutes whilst pedaling at 50 rpm. The test used involved a continuous

incremental test to exhaustion, with the workload being increased progressively every 3 min by 15–30 W from an initial workload of 40 W. The oxygen uptake during the last minute of the test was taken as the $VO_{2\ peak}$ value of the individual. Maximal effort was determined by achievement of a maximal predicted heart rate (±10%), a respiratory exchange ratio (RER) exceeding 1.1, a VE/VCO_2 ratio exceeding 30, and a plateau of O_2 consumption, although this was not always present (thus, we report $VO_{2\ peak}$). During all experimental visits, an online gas analysis system (Vmax 29, SensorMedics, USA) was used to measure O_2 consumption, CO_2 production, and the RER. The measurements during this test were used to calculate the workload required (% of $VO_{2\ peak}$) during experimental visits.

2.3. Experimental Design. Subjects attended the laboratory on two randomized occasions (morning visits), which were separated by at least two weeks. One experimental visit comprised of an insulin infusion (INS trial), and the other a saline infusion (CON trial) whilst resting for four hours. Subjects were asked to maintain habitual levels of physical activity and a typical isocaloric food intake in the 48 hours before experimental visits. Compliance with the isocaloric diet and typical food intake was assessed through the completion of food diaries. Subjects stopped taking medication for the control of T2D 24 hours before each trial and did not take any habitual morning medication on the day of the trial. Subjects attended the laboratory after an overnight fast, which was defined as the cessation of food and drink other than water from 10.00 pm on the previous evening.

At the beginning of each visit, a resting metabolic rate was measured for 20 minutes by indirect calorimetry using a ventilated canopy connected to an online gas analysis system. Substrate oxidation rates were calculated from the measurements of O_2 consumption and CO_2 production using stoichiometric equations [23]. Two retrograde cannulae were then inserted in an antecubital vein and a dorsal hand vein for the infusion of insulin/saline and for blood sampling, respectively. The subject's hand and wrist was kept in a hot air box maintained at 55–60°C throughout the trial to arterialize the blood as previously described [24]. Having arterialized the blood for 10 minutes, a blood sample was then taken for the baseline measurement of whole blood glucose and lactate (determined immediately on the YSI, 2300 Stat autoanalyzer), serum insulin, sodium and potassium, and plasma free fatty acid (FFA) concentrations.

Subjects then rested in a semisupine position for 4 hours whilst receiving an infusion of insulin or 0.9% saline. The infusion of human soluble insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) was started at a rate of 0.05 mU/kg/hr and varied between 0.05 in hour one and 0.01 mU/kg/hr in hour four of the resting phase (average infusion rate at 1.5 ± 0.2 ml/h) to maintain blood glucose levels at approximately 6 mmol/l. Insulin infusion was stopped before the exercise started. Saline was infused at 2.3 ± 0.1 ml/h. Insulin and saline were administered in a single-blind design. For safety monitoring, arterialized venous blood samples were taken every 15 minutes to measure glucose and lactate concentrations using the glucose oxidase and L-lactate oxidase methods, respectively (YSI, 2300 Stat autoanalyzer, Yellow Springs Instruments, Yellow Springs, USA). Data presented are every one hour for blood glucose and insulin during the resting phase. Additional blood samples were taken every 60 minutes to measure serum insulin using a radioimmunoassay (Diagnostics Products Corporation, Llanberis, Wales, UK), serum sodium and potassium by flame photometry, and plasma FFA using a commercially available kit (NEFA-C test, Wako, Osaka, Japan). The resting metabolic rate was further measured during the last 20 minutes of each hour and after the removal of the ventilated canopy. Brachial artery blood pressure and the heart rate were also determined using an automated Dinamap blood pressure monitor (Dinamap vital signs monitor, GE Healthcare, Chicago, IL). Both insulin and saline infusions were stopped at the end of the four-hour rest period.

Following the four-hour rest period of insulin or saline infusion, subjects then completed the exercise portion of the visit, which comprised of 30 minutes cycling at 50% $VO_{2\ peak}$. This exercise intensity and duration is recommended to improve glycaemic control by the American College of Sports Medicine and the American Diabetes Association [11] and therefore represents a suitably challenging exercise stimulus for patients with type 2 diabetes. Every ten minutes throughout exercise, expired air and arterialized venous blood samples were collected, and heart rate and blood pressure were measured. At the same time intervals, the subject's rating of perceived exertion was determined using the Borg scale [25]. Following the completion of exercise, subjects were provided with a meal of mixed macronutrient composition and were observed for 1 hour, after which they were permitted to leave the lab once blood glucose concentrations were stabilized.

2.4. Statistics. Data met the criteria for normality (a Gaussian distribution) when tested using a Kolmogorov-Smirnov test with Dallal-Wilkinson-Lille. A two-way repeated-measure analysis of variance (ANOVA) was used to determine if there were statistically significant differences in blood metabolites, metabolic rate and rates of substrate utilization between the insulin and control trials, and over time. Separate two-way ANOVA were completed for the resting and exercise phases, respectively. When a significant main effect was observed, a Holm-Sidak test was used to correct for multiple comparisons and locate differences. Data were analyzed using GraphPad Prism (GraphPad Prism 7.0, GraphPad Software Inc.). Data are presented as means ± SEM, and statistical significance was set at $P < 0.05$.

3. Results

3.1. Resting Phase. In the resting phase, the insulin infusion (INS) significantly increased serum insulin levels in comparison to CON ($P < 0.0001$), peaking after 60 minutes of infusion (Figure 1(a)) and then fell as the insulin infusion rate was lowered in response to the lowering of blood glucose. This higher level of insulin in the INS trial promoted a fall in blood glucose from 11.2 ± 0.6 to 5.6 ± 0.1 mmol/l after 4 h rest in the INS trial, which was lower ($P < 0.0001$) compared

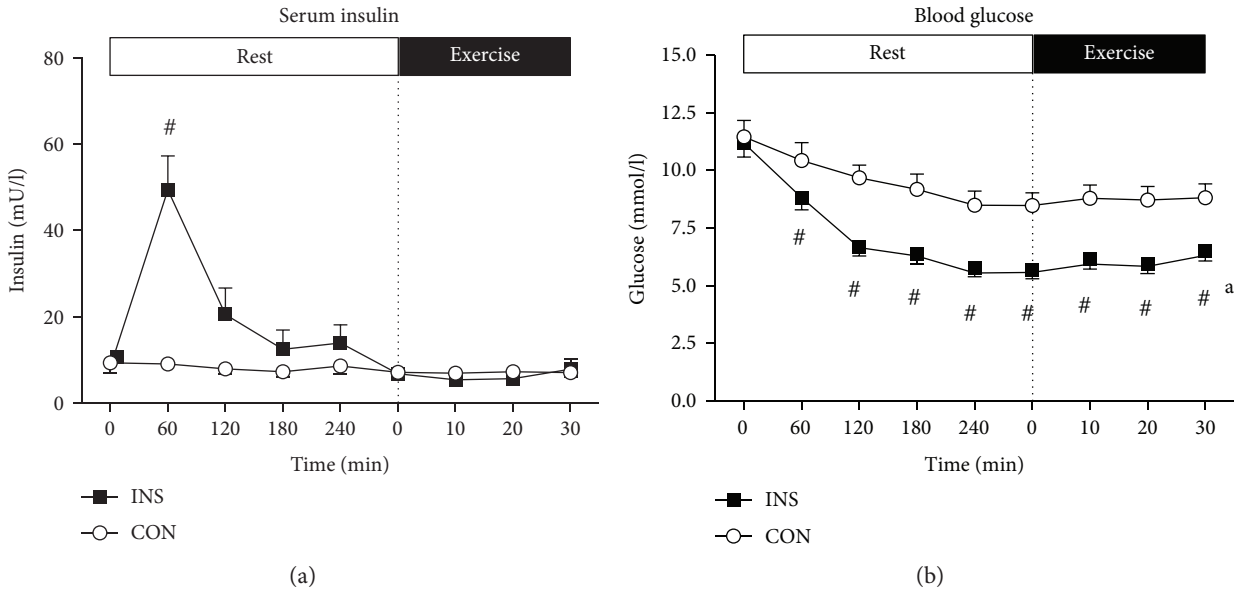

Figure 1: Serum insulin (a) and blood glucose (b) concentrations during the INS and CON trials. Data represent $n = 6$, mean ± SEM. (a) During the resting phase, the infusion significantly increased serum insulin in INS compared to CON (main effect of infusion, $P < 0.01$); there were also significant interaction ($P < 0.0001$) and time ($P < 0.0001$) effects. During exercise, there was no effect of infusion, time, or an interaction. (b) At rest, the infusion significantly lowered blood glucose in INS compared to CON (main effect of infusion, $P < 0.01$); there were also significant interaction ($P < 0.0001$) and time ($P < 0.0001$) effects. During exercise, blood glucose increased significantly in INS ($P < 0.01$) but not in CON. $^{\#}P < 0.0001$ from CON; $^{a}P < 0.01$ from immediately before exercise (0 min).

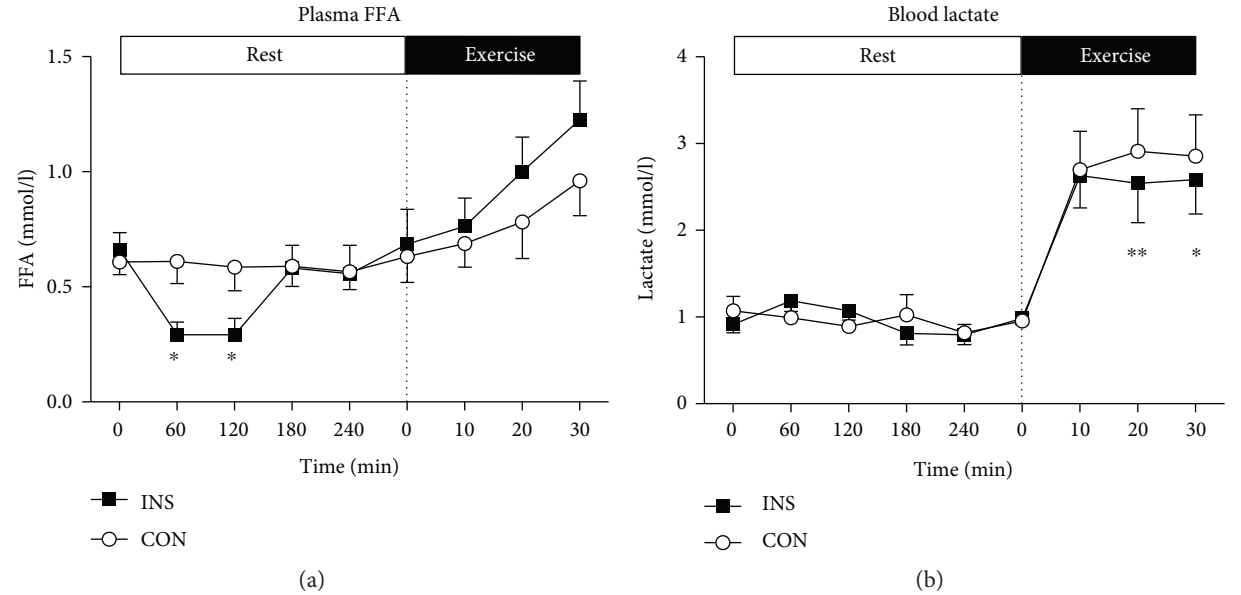

Figure 2: Plasma FFA (a) and blood lactate (b) concentrations during the INS and CON trials. Data represent $n = 6$, mean ± SEM. (a) At rest, there were significant time ($P < 0.001$) and interaction ($P < 0.05$) effects, where plasma FFA were suppressed in INS. During exercise, there was a significant time effect ($P < 0.001$) and a trend ($P = 0.085$) for an interaction effect, where plasma FFA in INS increased to greater extent than that in CON. (b) At rest, there was no significant effect of the insulin infusion or time on blood lactate, although there was a significant interaction effect ($P < 0.01$). During exercise, there were significant time ($P < 0.001$) and interaction ($P < 0.05$) effects, where the blood lactate response was lower in INS than that in CON. $^{*}P < 0.05$ from CON; $^{**}P < 0.01$ from CON.

with the fall in the CON trial (from 11.5 ± 0.7 to 8.5 ± 0.6 mmol/l, Figure 1(b)). The infusion of insulin suppressed FFA concentrations in the INS trial during the first 2 h of rest compared to CON ($P < 0.05$, Figure 2(a)). The infusion of insulin (INS) was also associated with an increase in average resting RER in comparison to CON (0.93 ± 0.01 versus 0.83 ± 0.02, $P < 0.05$), an increase in average CHO oxidation by 16.6 ± 4.7 g ($P < 0.05$), and a decrease in average fat

Table 2: Physiological and metabolic responses to the infusion of insulin (INS) and saline (CON) in patients with T2D whilst resting for four hours.

		Baseline	60 mins	120 mins	180 mins	240 mins
EE	CON	4.29 ± 0.28	4.57 ± 0.41	4.28 ± 0.26	4.29 ± 0.35	4.42 ± 0.33
	INS	4.01 ± 0.24	3.88 ± 0.14	3.91 ± 0.21*	3.78 ± 0.24	3.90 ± 0.16
RER	CON	0.89 ± 0.04	0.80 ± 0.05	0.83 ± 0.02	0.82 ± 0.02	0.85 ± 0.02
	INS	0.89 ± 0.04	0.94 ± 0.02*	0.94 ± 0.02**	0.92 ± 0.04	0.91 ± 0.02
CHO ox	CON	0.17 ± 0.03	0.09 ± 0.03	0.12 ± 0.01	0.10 ± 0.02	0.13 ± 0.02
	INS	0.16 ± 0.03	0.20 ± 0.02*	0.19 ± 0.02**	0.16 ± 0.03	0.17 ± 0.02
FAT ox	CON	0.04 ± 0.01	0.09 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
	INS	0.04 ± 0.01	0.02 ± 0.01*	0.02 ± 0.01**	0.03 ± 0.01	0.03 ± 0.01

Data represent $n = 6$, and values are means ± SEM for energy expenditure (EE) in kJ/min, respiratory exchange ratio (RER), carbohydrate oxidation (CHO ox), and fat oxidation (FAT ox) in g/min. Star symbols denote a significant difference between CON and INS ($^*P < 0.05$, $^{**}P < 0.01$).

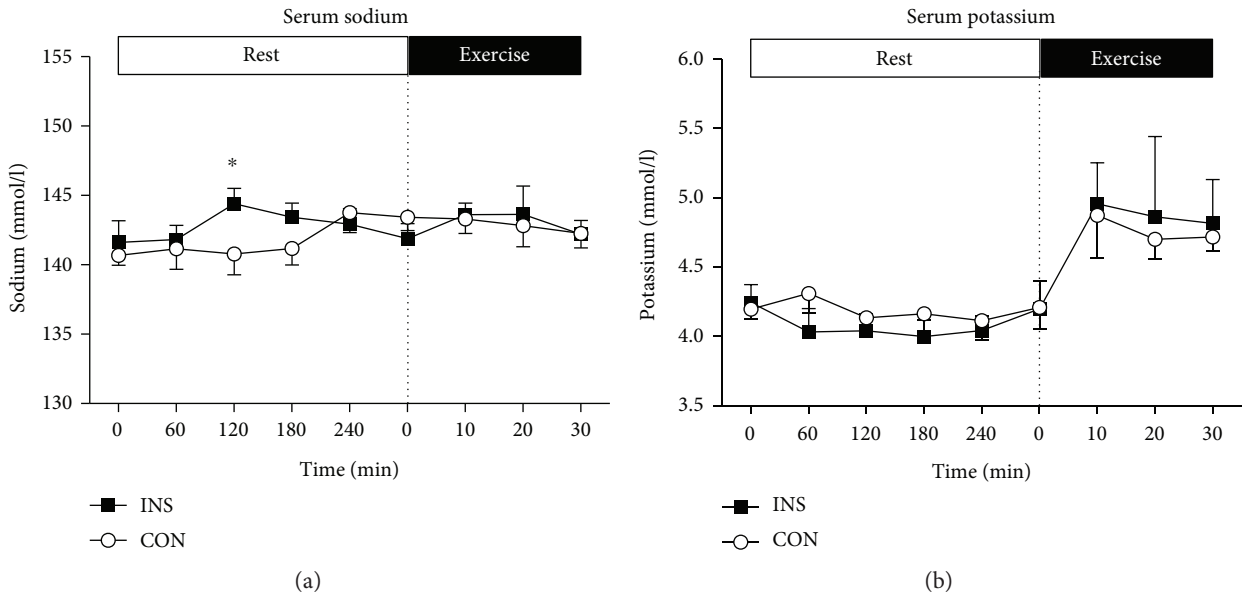

Figure 3: Serum sodium (a) and potassium (b) concentrations during the INS and CON trials. Data represent $n = 6$, mean ± SEM. (a) At rest, the infusion significantly increased serum sodium in INS compared to that in CON ($P < 0.05$) but there were no significant interaction or time effects. During exercise, there were no significant main effects for infusion, time, or interaction. (b) At rest, there was a trend ($P = 0.060$) for lower potassium in INS than in CON and a trend ($P = 0.063$) for time effect. During exercise, there was a significant increase in serum potassium (time effect $P < 0.05$) but there were no significant differences between INS and CON. $^*P < 0.05$ from CON.

oxidation by 9.9 ± 2.9 g over four hours ($P < 0.05$, Table 2). During the four-hour infusion period, the average resting metabolic rate was lower in the INS trial (3.87 ± 0.17 kJ/min) when compared to that in the CON trial (4.39 ± 0.30 kJ/min) ($P < 0.05$, Table 2).

3.2. Exercise Phase. Immediately before and during the 30 min of moderate intensity exercise, there were no significant differences in serum insulin levels between INS and CON (Figure 1(a)). Moreover, serum insulin concentrations were maintained at preexercise levels in both groups throughout the exercise phase (INS: 6.3 ± 0.9 mU/l versus CON: 7.0 ± 1.2 mU/l; Figure 1(a)). At the start of exercise, blood glucose was significantly lower ($P < 0.0001$) in the INS trial (5.6 ± 0.1 mmol/l) than in the CON trial (8.5 ± 0.6 mmol/l). During exercise, there was a small but significant increase ($P < 0.01$) in blood glucose in the INS trial (from 5.6 ± 0.1 mmol/l immediately before commencing exercise to 6.3 ± 0.03 mmol/l at 30 min of exercise) (Figure 1(b)). During the same period in the CON trial, blood glucose levels were maintained at 8.8 ± 0.6 mmol/l and were lower than those in INS at all time points ($P < 0.0001$). During exercise, there was a trend ($P = 0.085$) for a greater increase in FFA concentration in the INS trial (from 0.69 ± 0.15 to 1.23 ± 0.17 mmol/l, $P < 0.0001$), when compared with the CON trial (from 0.63 ± 0.11 to 0.96 ± 0.15 mmol/l, $P < 0.001$; Figure 2(a)). There was a greater increase in blood lactate in the CON than in the INS trial (interaction, $P < 0.05$), with higher rates at 20 min ($P < 0.01$) and 30 min ($P < 0.05$) of exercise (Figure 2(b)). There was an increase in serum potassium throughout exercise (Figure 3(b)), with no significant differences between INS

Table 3: Physiological and metabolic responses to submaximal cycling following infusion with insulin (INS) or saline (CON) in patients with T2D.

	O_2 uptake (ml/kg/min)	RER	CHO ox (g/min)	Fat ox (g/min)	Heart rate (beats/min)	RPE (6–20)
CON	14.1 ± 1.1	0.86 ± 0.02	0.80 ± 0.11	0.17 ± 0.04	124 ± 6	11.6 ± 0.6
INS	14.1 ± 1.5	0.74 ± 0.07	0.74 ± 0.07	0.18 ± 0.03	118 ± 7	11.2 ± 0.9

Values represent $n = 6$ and are displayed as mean ± SEM for 30 min of exercise. RER denotes respiratory exchange ratio; CHO ox denotes carbohydrate oxidation rate; fat ox denotes fat oxidation.

and CON trials. There were no significant differences in serum sodium (Figure 3(a)), oxygen uptake, RER, rates of CHO and fat oxidation, heart rate, or ratings of perceived exertion (Table 3) between INS and CON trials.

4. Discussion

These data showed for the first time that nonobese patients with T2D display similar metabolic responses to moderate exercise performed in the normoglycaemic and hyperglycaemic states. All subjects in the INS trial completed 30 min exercise without biochemical or symptomatic hypoglycaemia during exercise or the one-hour recovery after exercise, despite starting the exercise phase with a mean blood glucose of 5.6 ± 0.1 mmol/l. As a matter of fact, blood glucose increased during exercise following insulin infusion. Also, following saline infusion, blood glucose did not fall during exercise, suggesting impairment in exercise-mediated glucose disposal in lean subjects with T2D. Blood glucose levels in T2D typically fall due to an increase in skeletal muscle glucose uptake that surpasses any increase in hepatic glucose production [26]. Moreover, since the liver is sensitive to circulating insulin concentrations, the peripheral levels of which were similar between trials, the liver was not likely responsible for this increase in blood glucose. A reduced glucose uptake as a result of reduced mass action effect of glucose was therefore more likely responsible for the absence of lowered blood glucose during exercise. Indeed, blood glucose was lower in INS than in CON throughout the resting phase, which may have led to a lower mass action effect of glucose [27] during exercise. Hepatic glucose production and muscle glucose uptake were not directly measured in this study, however, and therefore, future studies to directly test this hypothesis are warranted. Subcutaneous insulin poses a risk of hypoglycaemia during subsequent exercise, and data in this study are not sufficient to mitigate this concern. Patients on insulin therapy display higher levels of serum insulin [28] than those displayed in this study at the start of exercise. Despite the higher blood glucose in the CON trial during both the resting and exercise phases, RER values were not significantly different between INS and CON during the exercise phase. This suggests a similar contribution of CHO and fat oxidation to energy metabolism.

Patients with T2D and high preexercise blood glucose levels display an increase in peripheral glucose uptake compared to euglycaemic, insulin-sensitive controls [16]. As a result, previous studies with both mild [13] and high [14, 17] pre-exercise hyperglycaemia have shown reductions in blood glucose during exercise. Other studies, however, similar to the present study, have not shown a reduction in blood glucose levels during exercise in the fasted state [19], perhaps due to the insulin resistance associated with an overnight fast [29]. As far as we are aware, the present study is the first in which no lowering in blood glucose was observed in nonobese patients with type 2 diabetes during exercise performed under normoglycaemic conditions.

There was a tendency for a greater increase in plasma FFA concentration during exercise in INS compared with that in CON. Greater levels of plasma FFA prevent glucose uptake by muscle [30, 31] and could therefore explain the increase in blood glucose levels in the INS trial. In support of this suggestion, reductions in plasma FFA through treatment with acipimox have been shown to decrease fat oxidation, increase carbohydrate oxidation, and lower blood glucose during moderate intensity exercise in T2D patients [32]. Patients with T2D have impaired ability to oxidize muscle glycogen during exercise [19], and the lower plasma glucose availability in the INS trial perhaps explains the greater increase in FFA in that trial.

Individuals with untreated T2D have increased resting energy expenditure, that is, associated with greater gluconeogenesis [33], but a lower thermic response to food intake [34] in comparison to insulin-sensitive controls. Short-term (8 days) subcutaneous insulin injection is sufficient to lower the resting metabolic rate and increase the thermic response to food intake [35]. Furthermore, 12 months of subcutaneous insulin is also sufficient to reduce the resting metabolic rate and lower hepatic glucose production [36]. The present study extends those findings by reporting a lower resting metabolic rate in response to acute insulin infusion (4 hours).

This study has shown that the infusion of insulin to achieve normoglycaemia in resting T2D subjects maintains blood glucose concentrations at euglycaemic levels during subsequent moderate intensity exercise. Subject numbers were limited due to difficulties of identifying and recruiting patients with nonobese T2D, a cohort that represent 10% of T2D patients, who were able and willing to exercise safely. Further studies of larger cohorts are warranted to determine if these results are extended to larger populations. Glucose-lowering medication was stopped 24 hours before study visits. Although this study shows that hypoglycaemia was not present during exercise, the risk would be greater following habitual T2D medication, particularly sulfonylureas and insulin. Further studies are required to elucidate the mechanisms underlying changes in metabolic physiology following normalization of blood glucose in T2D by glucose-lowering medications.

5. Conclusions

These data demonstrate that short-duration continuous exercise of moderate intensity is safe when nonobese patients with T2D are exercising following insulin-induced restoration of normoglycaemia. Furthermore, patients with T2D display similar metabolic responses to exercise in the normoglycaemic or hyperglycaemic state. Given the wealth of studies investigating the metabolic responses to exercise and health benefits in obese type 2 diabetes patients (for review see [37]), the data from the present study highlight the need for further research to investigate the potential differences between obese and nonobese patients with T2D in the efficacy of exercise in disease management.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendment or comparable ethical standards.

Conflicts of Interest

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

Authors' Contributions

Christopher J. Gaffney, Peter Mansell, Ian A. Macdonald, Francis B. Stephens, and Kostas Tsintzas drafted the manuscript. Figures were constructed by Christopher J. Gaffney and Kostas Tsintzas. All of the authors researched data and reviewed and edited the manuscript. Statistics were completed by Christopher J. Gaffney and Kostas Tsintzas.

Acknowledgments

All experiments completed as part of this study comply with the laws at the time of experimentation in the United Kingdom. This work was supported by the Arthritis Research UK (ARUK) and the Medical Research Council (MRC). The authors thank the members of the Human Physiology Group, MRC/ARUK Centre for Musculoskeletal Ageing Research, the University of Nottingham for technical assistance, and Dr Catherine J Wills for clinical input on study days. Christopher J. Gaffney, Peter Mansell, Ian A. Macdonald, and Kostas Tsintzas are part of the MRC/ARUK Centre for Musculoskeletal Ageing Research, The University of Nottingham.

References

[1] M. Gatineau, C. Hancock, and N. Holman, "Adult obesity and type 2 diabetes," *Public Health England*, 2014.

[2] E. L. Lim, K. G. Hollingsworth, B. S. Aribisala, M. J. Chen, J. C. Mathers, and R. Taylor, "Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol," *Diabetologia*, vol. 54, no. 10, pp. 2506–2514, 2011.

[3] E. P. Weiss, S. G. Albert, D. N. Reeds et al., "Calorie restriction and matched weight loss from exercise: independent and additive effects on glucoregulation and the incretin system in overweight women and men," *Diabetes Care*, vol. 38, no. 7, pp. 1253–1262, 2015.

[4] Diabetes UK, "Diabetes facts and stats: 2015," 2015.

[5] P. D. Barma, S. Ranabir, L. Prasad, and T. P. Singh, "Clinical and biochemical profile of lean type 2 diabetes mellitus," *Indian Journal of Endocrinology and Metabolism*, vol. 15, Supplement 1, pp. S40–S43, 2011.

[6] A. Vaag and S. S. Lund, "Non-obese patients with type 2 diabetes and prediabetic subjects: distinct phenotypes requiring special diabetes treatment and (or) prevention?" *Applied Physiology, Nutrition, and Metabolism*, vol. 32, no. 5, pp. 912–920, 2007.

[7] M. D. Hordern, D. W. Dunstan, J. B. Prins, M. K. Baker, M. A. Singh, and J. S. Coombes, "Exercise prescription for patients with type 2 diabetes and pre-diabetes: a position statement from Exercise and Sport Science Australia," *Journal of Science and Medicine in Sport*, vol. 15, no. 1, pp. 25–31, 2012.

[8] V. B. O'Leary, C. M. Marchetti, R. K. Krishnan, B. P. Stetzer, F. Gonzalez, and J. P. Kirwan, "Exercise-induced reversal of insulin resistance in obese elderly is associated with reduced visceral fat," *Journal of Applied Physiology*, vol. 100, no. 5, pp. 1584–1589, 2006.

[9] D. Umpierre, P. A. B. Ribeiro, B. D. Schaan, and J. P. Ribeiro, "Volume of supervised exercise training impacts glycaemic control in patients with type 2 diabetes: a systematic review with meta-regression analysis," *Diabetologia*, vol. 56, no. 2, pp. 242–251, 2013.

[10] N. N. Zammitt and B. M. Frier, "Hypoglycemia in type 2 diabetes," *Diabetes Care*, vol. 28, no. 12, pp. 2948–2961, 2005.

[11] S. R. Colberg, R. J. Sigal, B. Fernhall et al., "Exercise and type 2 diabetes: the American College of Sports Medicine and the American Diabetes Association: joint position statement," *Diabetes Care*, vol. 33, no. 12, pp. e147–e167, 2010.

[12] K. L. Way, D. A. Hackett, M. K. Baker, and N. A. Johnson, "The effect of regular exercise on insulin sensitivity in type 2 diabetes mellitus: a systematic review and meta-analysis," *Diabetes and Metabolism Journal*, vol. 40, no. 4, pp. 253–271, 2016.

[13] A. Giacca, Y. Groenewoud, E. Tsui, P. McClean, and B. Zinman, "Glucose production, utilization, and cycling in response to moderate exercise in obese subjects with type 2 diabetes and mild hyperglycemia," *Diabetes*, vol. 47, no. 11, pp. 1763–1770, 1998.

[14] J.-W. Van Dijk, R. J. F. Manders, K. Tummers et al., "Both resistance-and endurance-type exercise reduce the prevalence of hyperglycaemia in individuals with impaired glucose tolerance and in insulin-treated and non-insulin-treated type 2 diabetic patients," *Diabetologia*, vol. 55, no. 5, pp. 1273–1282, 2012.

[15] T. Terada, B. J. Wilson, E. Myette-Côté et al., "Targeting specific interstitial glycemic parameters with high-intensity interval exercise and fasted-state exercise in type 2 diabetes," *Metabolism*, vol. 65, no. 5, pp. 599–608, 2016.

[16] R. A. DeFronzo, E. Ferrannini, Y. Sato, P. Felig, and J. Wahren, "Synergistic interaction between exercise and insulin on

peripheral glucose uptake," *The Journal of Clinical Investigation*, vol. 68, no. 6, pp. 1468–1474, 1981.

[17] I. K. Martin, A. Katz, and J. Wahren, "Splanchnic and muscle metabolism during exercise in NIDDM patients," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 269, no. 3 Part 1, pp. E583–E590, 1995.

[18] L. B. Borghouts, A. J. M. Wagenmakers, P. L. L. Goyens, and H. A. Keizer, "Substrate utilization in non-obese type II diabetic patients at rest and during exercise," *Clinical Science*, vol. 103, no. 6, pp. 559–566, 2002.

[19] S. R. Colberg, J. M. Hagberg, S. D. McCole, J. M. Zmuda, P. D. Thompson, and D. E. Kelley, "Utilization of glycogen but not plasma glucose is reduced in individuals with NIDDM during mild-intensity exercise," *Journal of Applied Physiology*, vol. 81, no. 5, pp. 2027–2033, 1996.

[20] G. F. Fletcher, P. A. Ades, P. Kligfield et al., "Exercise standards for testing and training," *Circulation*, vol. 128, no. 8, pp. 873–934, 2013.

[21] W. N. Schofield, "Predicting basal metabolic rate, new standards and review of previous work," *Human Nutrition. Clinical Nutrition*, vol. 39, Supplement 1, pp. 5–41, 1985.

[22] (DoH) Department of Health, "Report on Health and Social Subjects No 41, "Dietary reference values for food energy and nutrients for the United Kingdom."," *Committee on Medical Aspects of Food Policy*, 1991.

[23] K. N. Frayn, "Calculation of substrate oxidation rates in vivo from gaseous exchange," *Journal of Applied Physiology*, vol. 55, no. 2, pp. 628–634, 1983.

[24] N. Abumrad, D. Rabin, M. Diamond, and W. Lacy, "Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man," *Metabolism*, vol. 30, no. 30, pp. 936–940, 1981.

[25] G. A. Borg, "Perceived exertion: a note on "history" and methods," *Medicine and Science in Sports*, vol. 5, no. 2, pp. 90–93, 1973.

[26] H. L. Minuk, M. Vranic, E. B. Marliss, A. K. Hanna, A. M. Albisser, and B. Zinman, "Glucoregulatory and metabolic response to exercise in obese noninsulin-dependent diabetes," *The American Journal of Physiology*, vol. 240, no. 5, pp. E458–E464, 1981.

[27] S. Del Prato, M. Matsuda, D. C. Simonson et al., "Studies on the mass action effect of glucose in NIDDM and IDDM: evidence for glucose resistance," *Diabetologia*, vol. 40, no. 6, pp. 687–697, 1997.

[28] K. Hermansen, M. Colombo, H. Storgaard, A. ØStergaard, K. Kølendorf, and S. Madsbad, "Improved postprandial glycemic control with biphasic insulin aspart relative to biphasic insulin lispro and biphasic human insulin in patients with type 2 diabetes," *Diabetes Care*, vol. 25, no. 5, pp. 883–888, 2002.

[29] H. R. Farshchi, M. A. Taylor, and I. A. Macdonald, "Deleterious effects of omitting breakfast on insulin sensitivity and fasting lipid profiles in healthy lean women," *The American Journal of Clinical Nutrition*, vol. 81, no. 2, pp. 388–396, 2005.

[30] P. J. Randle, P. B. Garland, C. N. Hales, and E. A. Newsholme, "The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus," *Lancet (London, England)*, vol. 1, no. 7285, pp. 785–789, 1963.

[31] G. Boden, "Obesity, insulin resistance and free fatty acids," *Current Opinion in Endocrinology, Diabetes, and Obesity*, vol. 18, no. 2, pp. 139–143, 2011.

[32] A. O. Akanji, E. Osifo, M. Kirk, and T. D. Hockaday, "The effects of changes in plasma nonesterified fatty acid levels on oxidative metabolism during moderate exercise in patients with non-insulin-dependent diabetes mellitus," *Metabolism*, vol. 42, no. 4, pp. 426–434, 1993.

[33] S. Buscemi, M. Donatelli, G. Grosso et al., "Resting energy expenditure in type 2 diabetic patients and the effect of insulin bolus," *Diabetes Research and Clinical Practice*, vol. 106, no. 3, pp. 605–610, 2014.

[34] Y. L. Muller, P. Piaggi, D. Hoffman et al., "Common genetic variation in the glucokinase gene (GCK) is associated with type 2 diabetes and rates of carbohydrate oxidation and energy expenditure," *Diabetologia*, vol. 57, no. 7, pp. 1382–1390, 2014.

[35] R. Gougeon, "Thermic and metabolic responses to oral glucose in obese subjects with non-insulin-dependent diabetes mellitus treated with insulin or a very-low-energy diet," *The American Journal of Clinical Nutrition*, vol. 64, no. 1, pp. 78–86, 1996.

[36] A. Franssila-Kallunki and L. Groop, "Factors associated with basal metabolic rate in patients with type 2 (non-insulin-dependent) diabetes mellitus," *Diabetologia*, vol. 35, no. 10, pp. 962–966, 1992.

[37] Z. Yang, C. A. Scott, C. Mao, J. Tang, and A. J. Farmer, "Resistance exercise versus aerobic exercise for type 2 diabetes: a systematic review and meta-analysis," *Sports Medicine*, vol. 44, no. 4, pp. 487–499, 2014.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 1567467, 9 pages
https://doi.org/10.1155/2017/1567467

Research Article

Relationships between Composition of Major Fatty Acids and Fat Distribution and Insulin Resistance in Japanese

Chikako Fujii,[1] Toshihide Kawai,[1,2,3] Koichiro Azuma,[2] Yuko Oguma,[2] Fuminori Katsukawa,[2] Hiroshi Hirose,[1] Kumiko Tanaka,[1] Shu Meguro,[1] Hideo Matsumoto,[2] and Hiroshi Itoh[1]

[1]*Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan*
[2]*Institute for Integrated Sports Medicine, Keio University School of Medicine, Tokyo, Japan*
[3]*Department of Internal Medicine, Tokyo Saiseikai Central Hospital, Tokyo, Japan*

Correspondence should be addressed to Toshihide Kawai; tkawai@keio.jp

Received 5 January 2017; Revised 7 March 2017; Accepted 12 March 2017; Published 30 April 2017

Academic Editor: Joseph F. Ndisang

Objective. The aim of this study was to evaluate the relationships between the composition of free fatty acids (FFAs) and metabolic parameters, including body fat distribution, in Japanese. *Methods*. The study subjects were 111 Japanese patients (54 males, 57 females). Metabolic parameters and visceral and subcutaneous fat areas as determined by CT scanning at the umbilical level were measured. Glucose tolerance test (GTT) was performed by administering 75 g glucose orally. *Results*. The percentage of linoleic acid (C18:2), the greatest constituent among FFAs, was negatively correlated with visceral fat area ($r = -0.411$, $p < 0.0001$), fasting glucose ($r = -0.330$, $p < 0.0001$), HbA1c ($r = -0.231$, $p = 0.0146$), and systolic blood pressure ($r = -0.224$, $p = 0.0184$). Linoleic acid percentage was also significantly negatively correlated with HOMA-IR ($r = -0.416$, $p < 0.0001$) by simple correlation. Based on the findings of OGTT, the 111 subjects were classified into three groups: 33 with normal glucose tolerance, 71 with impaired glucose tolerance (IGT), and 7 diabetic subjects. The percentage of serum linoleic acid in diabetic subjects was significantly lower than that in normal subjects. *Conclusion*. We conclude that serum linoleic acid level is negatively correlated with the accumulation of visceral fat in relation to a reduction of insulin resistance in Japanese subjects.

1. Introduction

Approximately 415 million people worldwide have diabetes mellitus [1], and the number is expected to increase to 642 million by the year 2040 [1, 2]. Diabetes mellitus is associated with a markedly increased risk of coronary heart disease, stroke, and renal failure, as well as disability [3]. Free fatty acids (FFAs), which are absorbed from ingested food and also liberated by adipocytes and reassembled into triglycerides, are often elevated in obese individuals. The accumulation of visceral fat induces insulin resistance and worsens glucose and lipid metabolism and is important in the development of type 2 diabetes [4]. Recently, the effects of FFAs on insulin's action have been investigated intensively [5, 6]. We have previously shown that FFAs cause β cell damage mainly by apoptosis in vitro [7] and by ER stress in mice [8]. The serum composition of FFAs in humans differs from race to race, and from environment to environment, because it reflects the average fat intake in the preceding days [9–12]. Despite intensive in vitro and in vivo studies to elucidate the impairment of insulin's action induced by chronic elevation of FFAs, however, the physiological interactions whereby the chronic effects of fatty acids on pancreatic beta cells affect insulin's action on glucose and insulin resistance remain unclear. To clarify these issues, firstly we evaluated the relationships between serum FFA composition and body fat area as determined by computed tomographic (CT) scanning at the umbilical level. Second, we evaluated the relationships between three major FFAs and the presence of diabetes, in humans.

Table 1: Baseline characteristics of subjects.

Parameters	Values
N (male/female)	111 (54/57)
Age (years)	51.5 ± 1.1
Height (cm)	162.5 ± 0.8
Body mass index (kg/m^2)	26.4 ± 0.5
Waist circumference (cm)	91.3 ± 1.4
Systolic blood pressure (mmHg)	128.4 ± 2.3
Diastolic blood pressure (mmHg)	78.2 ± 1.5
Biochemical markers	
Fasting plasma glucose (mg/dL)	107.0 ± 1.5
Hemoglobin A1c (%)	5.7 ± 0.9
Immunoreactive insulin (μU/mL)	9.4 ± 0.7
Total cholesterol (mg/dL)	211.0 ± 5.9
High-density lipoprotein cholesterol (mg/dL)	57.1 ± 1.8
Low-density lipoprotein cholesterol (mg/dL)	123.8 ± 4.4
Triglyceride (mg/dL)	131.4 ± 8.0
Free fatty acids (mEq/L)	0.6 ± 0.0
Aspartate aminotransferase (IU/L)	26.2 ± 1.1
Alanine aminotransferase (IU/L)	31.9 ± 2.5
Creatinine (mg/dL)	0.7 ± 0.0
Uric acid (mg/dL)	5.8 ± 0.1
High molecular weight adiponectin (μg/mL) ($n = 63$)	2.9 ± 0.2
Leptin (ng/mL) ($n = 63$)	12.4 ± 1.5
75 g OGTT	
30 min glucose (mg/dL)	180.1 ± 3.4
60 min glucose (mg/dL)	199.9 ± 5.3
120 min glucose (mg/dL)	161.9 ± 5.1
30 min IRI (μU/mL)	50.4 ± 3.7
60 min IRI (μU/mL)	70.1 ± 5.3
120 min IRI (μU/mL)	68.7 ± 4.7
HOMA-IR	2.4 ± 0.2
Insulinogenic index	0.7 ± 0.1
CT scan	
Visceral fat (cm^2)	122.6 ± 5.4
Subcutaneous fat (cm^2)	209.0 ± 12.9
V/S ratio	0.7 ± 0.0

Data are mean ± SEM. IRI: immunoreactive insulin; HOMA-IR: homeostasis model assessment insulin resistance index.

Table 2: Component ratio analysis of twenty-four FFA fractions.

Free fatty acid	Symbol	%
Lauric acid	C12:0	0.098 ± 0.91
Myristic acid	C14:0	1.04 ± 0.40
Myristoleic acid	C14:1	0.098 ± 0.04
Palmitic acid	C16:0	22.37 ± 0.23
Palmitoleic acid	C16:1	2.57 ± 0.10
Stearic acid	C18:0	6.86 ± 0.10
Oleic acid	C18:1	20.06 ± 0.32
Linoleic acid	C18:2	29.73 ± 0.45
Gamma-linolenic acid	C18:3(6)	0.31 ± 0.16
Linolenic acid	C18:3(3)	0.88 ± 0.26
Arachidic acid	C20:0	0.051 ± 0.002
Eicosenoic acid	C20:1	0.19 ± 0.01
Eicosadienoic acid	C20:2	0.19 ± 0.01
5-8-11-Eicosatrienoic acid	C20:3(9)	0.054 ± 0.003
Dihomo-gamma-linolenic acid	C20:3(6)	1.19 ± 0.03
Arachidonic acid	C20:4	5.64 ± 0.13
Eicosapentaenoic acid	C20:5	2.87 ± 0.19
Behenic acid	C22:0	0.11 ± 0.01
Erucic acid	C22:1	0.026 ± 0.003
Docosatetraenoic acid	C22:4	0.13 ± 0.01
Docosapentaenoic acid	C22:5	0.82 ± 0.03
Lignoceric acid	C24:0	0.044 ± 0.003
Docosahexaenoic acid	C22:6	4.61 ± 0.17
Nervonic acid	C24:1	0.11 ± 0.05

Data are mean ± SEM.

2. Materials and Methods

This cross-sectional study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Review Board of Keio University.

2.1. Participants. We enrolled 111 Japanese patients (54 men and 57 women) who attended our outpatient sports clinic at Keio University Hospital in Tokyo (Table 1). In men, mean ± standard error of the mean (SEM) age was 50.1 ± 1.4 years; body mass index (BMI), 26.7 ± 0.7 kg/m^2; fasting plasma glucose (FPG), 109.2 ± 2.4 mg/dL; and hemoglobin A1c (HbA1c), 5.6 ± 0.2%. In women, mean ± SEM age was 52.7 ± 1.8 years; BMI, 26.2 ± 0.8 kg/m^2; FPG, 104.9 ± 2.0 mg/dL; and HbA1c, 5.9 ± 0.1%. All subjects received dietary instructions for using a meal-exchange plan by nutritionists. The ideal dietary caloric intake for each patient was calculated as the ideal body weight (kilograms) × 25–30 kcal/kg. We recommended them to take 50–60% total calories as carbohydrate, 20–25% as fat, and 15–20% as protein. The subjects' meal preference did not deviate from that of the standard Japanese population. The physical activity level for each subject was determined by a questionnaire. None of the subjects was taking any medication or was a smoker. The nature of the procedure was explained to the subjects, and written informed consent was obtained from all participants.

2.2. Measurements and OGTT. The body weight of each patient was measured at the sports clinic. BMI was determined as weight corrected for height: weight (kg)/height (m^2). Blood pressure (BP) was determined in the sitting position after a 10-minute rest.

A fasting blood sample was drawn from each subject before breakfast in the early morning, after an overnight fast. Glucose tolerance test (GTT) was performed by administering 75 g glucose orally, and the results were classified as

Table 3: Stepwise regression analysis of relationships between dependent variables and the most significant independent FFA variables.

Dependent variable	Contributory cause	Correlation coefficient	F value	p value
BMI (kg/m^2)	Palmitic acid	$r = 0.304$	14.707	<0.0001
sBP (mmHg)	Linoleic acid	$r = -0.224$	5.732	0.0184
dBP (mmHg)	—	—	—	—
Biomarkers				
0 min glu (mg/dL)	Linoleic acid	$r = -0.330$	9.383	<0.0001
120 min glu (mg/dL)	Linoleic acid	$r = -0.389$	14.041	<0.0001
0 min IRI (μU/mL)	Myristic acid	$r = 0.430$	14.014	<0.0001
120 min IRI (μU/mL)	Palmitoleic acid	$r = 0.342$	12.237	0.0006
HbA1c (*N*) (%)	Linoleic acid	$r = -0.231$	6.165	0.0146
HOMA-IR	Myristic acid	$r = 0.520$	13.792	<0.0001
I.I.	—	—	—	—
TC (mg/dL)	Linolenic acid	$r = 0.282$	5.554	0.0216
TG (mg/dL)	Oleic acid	$r = 0.510$	26.853	<0.0001
HDL-C (mg/dL)	Oleic acid	$r = -0.470$	18.722	<0.0001
LDL-C (mg/dL)	Behenic acid	$r = -0.344$	6.584	0.0134
AST (IU/L)	Myristic acid	$r = 0.270$	8.467	0.0044
ALT (IU/L)	Palmitic acid	$r = 0.289$	9.86	0.0022
Cre (mg/dL)	Gamma linolenic acid	$r = -0.215$	6.022	0.0337
UA (mg/dL)	Eicosenoic acid	$r = 0.247$	6.002	0.0162
HMW-ADPN (μg/mL) ($n = 63$)	Linoleic acid	$r = 0.327$	12.917	0.0108
Leptin (ng/mL) ($n = 63$)	Lauric acid	$r = 0.303$	6.157	0.0159
CT scan				
V-fat (cm^2)	Linoleic acid	$r = -0.411$	17.73	<0.0001
S-fat (cm^2)	Dihomo-γ-linolenic acid	$r = 0.272$	9.221	0.0002

BMI: body mass index; sBP: systolic blood pressure; dBP: diastolic blood pressure; 0 min glu: 0 min glucose; 120 min glu: 120 min glucose; 0 min IRI: 0 min immunoreactive insulin; 120 min IRI: 120 min-immunoreactive insulin; HbA1c: hemoglobin A1c; HOMA-IR: homeostasis model assessment insulin resistance index; I.I.: insulinogenic index; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Cre: creatinine; UA: uric acid; HMW-ADPN: high molecular weight adiponectin; V-fat: visceral fat area; S-fat: subcutaneous fat area.

normal glucose tolerance, impaired glucose tolerance (IGT), or diabetes on the basis of the World Health Organization criteria [13]. Plasma glucose (PG) was determined by the glucose oxidase method. Plasma immunoreactive insulin (IRI) level was measured by radioimmunoassay (Shionogi, Tokyo, Japan). Twenty-four fractions of FFAs (lauric, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, gamma-linolenic, linolenic, arachidic, eicosenoic, eicosadienoic, 5-8-11-eicosatrienoic, dihomo-gamma-linolenic, arachidonic, eicosapentaenoic, behenic, erucic, docosatetraenoic, docosapentaenoic, lignoceric, docosahexaenoic, and nervonic acid) (Table 2) were determined by capillary gas chromatography (HP 6890, Hitachi, 30 m capillary column, inner diameter 0.32 mm, phase layer 0.25 μm) [14]. The n6/n3 polyunsaturated fatty acid (PUFA) ratio was calculated as (linoleic + gamma linolenic + eicosadienoic + dihomo-gamma-linolenic + arachidonic + docosatetraenoic acid) / (linolenic + eicosapentaenoic + docosapentaenoic + docosahexaenoic acids). Glycosylated hemoglobin (HbA1c) was determined by high-performance liquid chromatography (HPLC) (Toso Co. Ltd., Tokyo, Japan). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) were measured enzymatically (Hitachi autoanalyzer). Insulin resistance was assessed using the homeostasis model assessment (HOMA) system described by Matthews et al. [15] with the formula $(\text{insulin } (\mu\text{U/mL}) \times \text{plasma glucose } (\text{mg/dL})/405)$. High molecular weight (HMW) adiponectin was determined by sensitive latex particle-enhanced turbidimetric immunoassay (LTIA) (Hitachi H7170, Japan). Leptin was determined by radioimmunoassay (Hitachi ARC-950, Japan).

2.3. Computed Tomography. Subcutaneous and visceral fat distributions were determined by measuring a −150 to −50 Hounsfield unit (HU) area using CT scanning at the umbilical level as described previously [16]. V/S ratio was calculated as visceral fat area/subcutaneous fat area.

2.4. Statistical Analysis. All results are expressed as mean ± SEM. Relationships between variables were analyzed by simple correlation and by linear stepwise regression analysis with calculation of Pearson product correlation coefficients. The Kruskal-Wallis test was used to evaluate

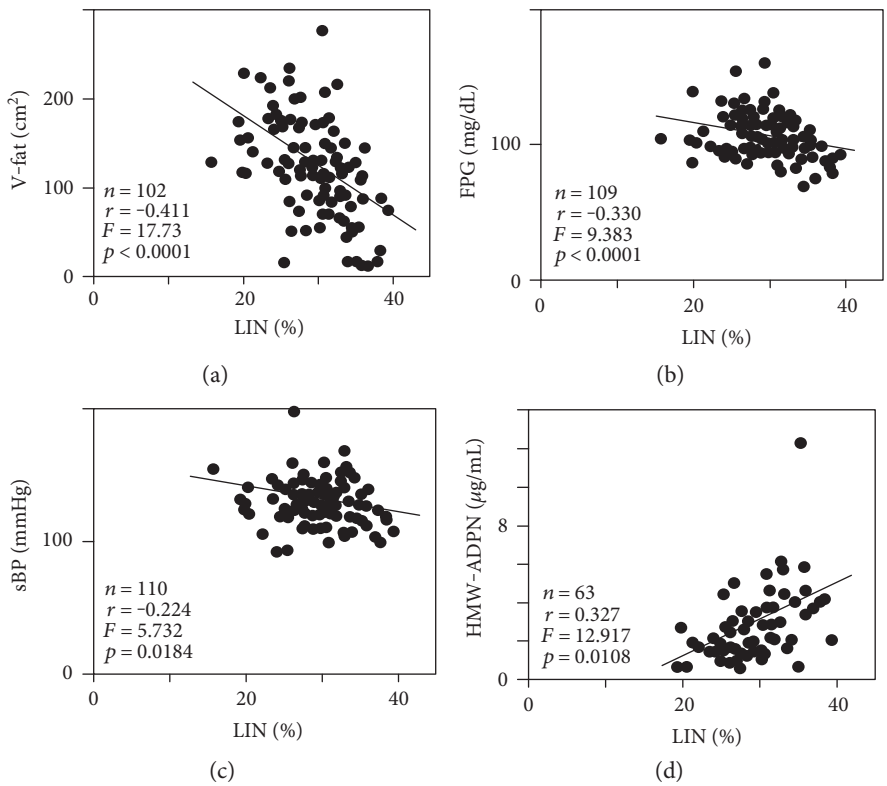

Figure 1: Relationships between ratio of serum linoleic acid (LIN, C18:2) and that of visceral fat area (V-fat) as determined by computed tomographic (CT) scanning at the umbilical level (a), that of fasting plasma glucose (FPG) (b), that of systolic blood pressure (sBP) (c), and that of high molecular weight (HMW) adiponectin (ADPN) (d) by linear stepwise regression analysis.

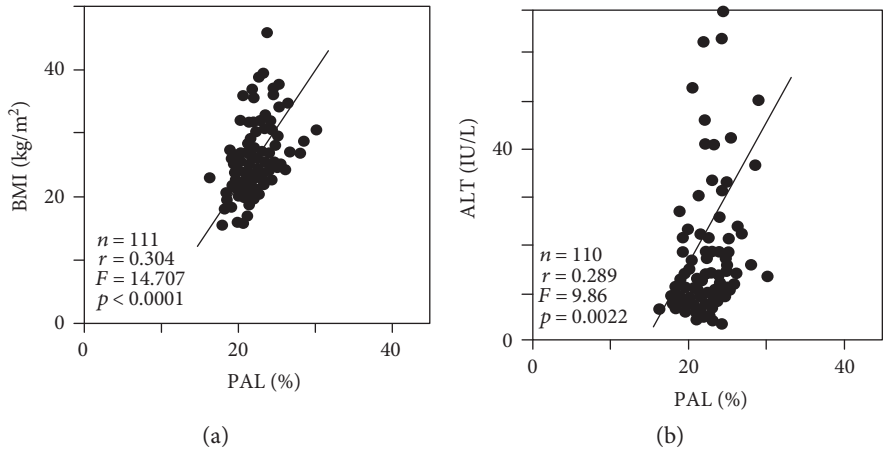

Figure 2: Relationships between ratio of serum palmitic acid (PAL, C16:0) and that of body mass index (BMI) (a) and that of alanine aminotransferase (ALT) (b).

comparisons among groups. A p value less than 0.05 was considered statistically significant. Statistical analyses were carried out using SPSS Statistics 23 software (IBM, Armonk, NY, USA).

3. Results

3.1. Relationships between FFA Composition and Metabolic Parameters. We measured twenty-four fractions of FFAs in

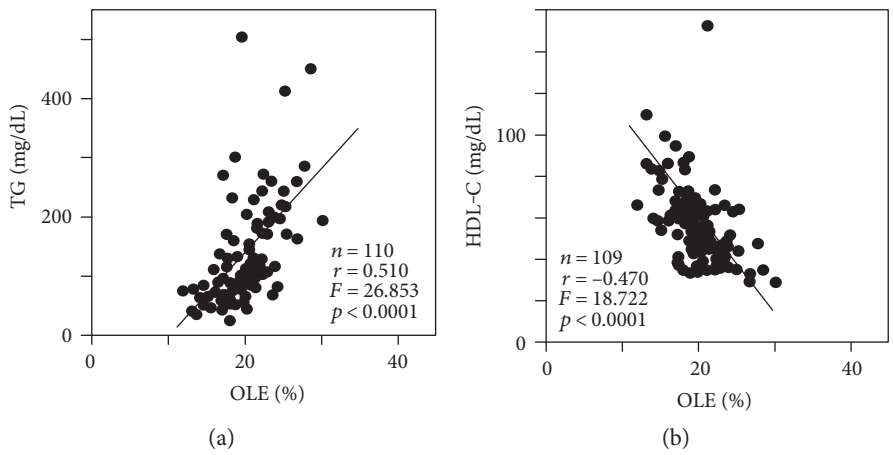

Figure 3: Relationships between ratio of serum oleic acid (OLE, C18:1) and that of triglyceride (TG) (a) and that of high-density lipoprotein cholesterol (HDL-C) (b).

Table 4: Subjects classified as normal glucose tolerant, impaired glucose tolerant (IGT), and diabetic based on OGTT.

Parameters	Normal	IGT	Diabetes	p value
N	33	71	7	—
(Male/female)	(13/20)	(43/28)	(2/5)	—
Age (years)	47.9 ± 2.2	52.5 ± 1.4	58.4 ± 3.6	0.0601
Body mass index (kg/m^2)	26.2 ± 1.1	26.5 ± 0.7	26.4 ± 0.9	0.808
Fasting glucose (mg/dL)	93.8 ± 1.6	109.4 ± 1.5	141.6 ± 4.8	<0.0001
Hemoglobin A1c (%)	5.2 ± 0.2	6.0 ± 0.1	6.4 ± 0.1	<0.0001
HOMA-IR	1.5 ± 0.2	2.7 ± 0.3	4.1 ± 1.5	0.0011
Visceral fat area (cm^2)	115.6 ± 11.5	124.0 ± 6.3	141.3 ± 17.3	0.712

Kruskal-Wallis test was used for comparisons among groups. Data are mean ± SEM. HOMA-IR: homeostasis model assessment insulin resistance index.

all subjects, as shown in Table 2. Mean ± SEM proportions of constituents were 29.7 ± 0.5% linoleic acid, 22.4 ± 0.2% palmitic acid, 20.1 ± 0.3% oleic acid, 6.9 ± 0.1% stearic acid, 5.6 ± 0.1% arachidonic acid, and 4.6 ± 0.2% docosahexaenoic acid, and the others were below 3.0% each. First, we analyzed the relationships between twenty-four FFA fractions and twenty-two metabolic parameters (BMI, sBP, dBP, 0 min glucose, 120 min glucose, 0 min IRI, 120 min IRI, HbA1c, HOMA-IR, I.I, TC, TG, HDL-C, LDL-C, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, uric acid, HMW adiponectin, leptin, visceral fat area, and subcutaneous fat area) by simple correlation analysis (Supplementary Table available online at https://doi.org/10.1155/2017/1567467). Second, multivariate analysis models were developed by stepwise regression with twenty-two metabolic parameters as dependent variables and by simple correlation with significant fractions among all twenty-four FFA fractions as independent variables. Table 3 shows the relationships between dependent metabolic variables and the most significant independent FFA variables.

As the most significant contributory cause, linoleic acid (%), the greatest constituent among the twenty-four FFA fractions, was negatively correlated with visceral fat area ($r = -0.411$, $F = 17.73$, $p < 0.0001$) (Figure 1(a)), whereas it was not correlated with subcutaneous fat area. Linoleic acid was negatively correlated with FPG ($r = -0.330$, $F = 9.383$, $p < 0.0001$) (Figure 1(b)) and sBP ($r = -0.224$, $F = 5.732$, $p = 0.0184$) (Figure 1(c)) and positively correlated with HMW adiponectin ($n = 63$, $r = 0.327$, $F = 12.917$, $p = 0.0108$) (Figure 1(d)). In addition, linoleic acid was also significantly negatively correlated with 120 min IRI ($r = -0.383$, $p < 0.0001$) and HOMA-IR ($r = -0.416$, $p < 0.0001$) by simple correlation analysis (Supplementary Table). Palmitic acid (%) was positively correlated with BMI ($r = 0.304$, $F = 14.707$, $p < 0.0001$) (Figure 2(a)) and ALT ($r = 0.289$, $F = 9.86$, $p = 0.0022$) (Figure 2(b)). Oleic acid (%) was positively correlated with TG ($r = 0.510$, $F = 26.853$, $p < 0.0001$) (Figure 3(a)) and negatively correlated with HDL-C ($r = -0.470$, $F = 18.722$, $p < 0.0001$) (Figure 3(b)).

3.2. OGTT and Fraction of Three Major FFAs. Based on the findings of OGTT, the 111 subjects were classified into three groups: 33 with normal glucose tolerance, 71 with IGT, and 7 diabetic subjects (Table 4). There were no significant differences in macronutrient balance and physical activity level among the three groups. Figure 4 shows the

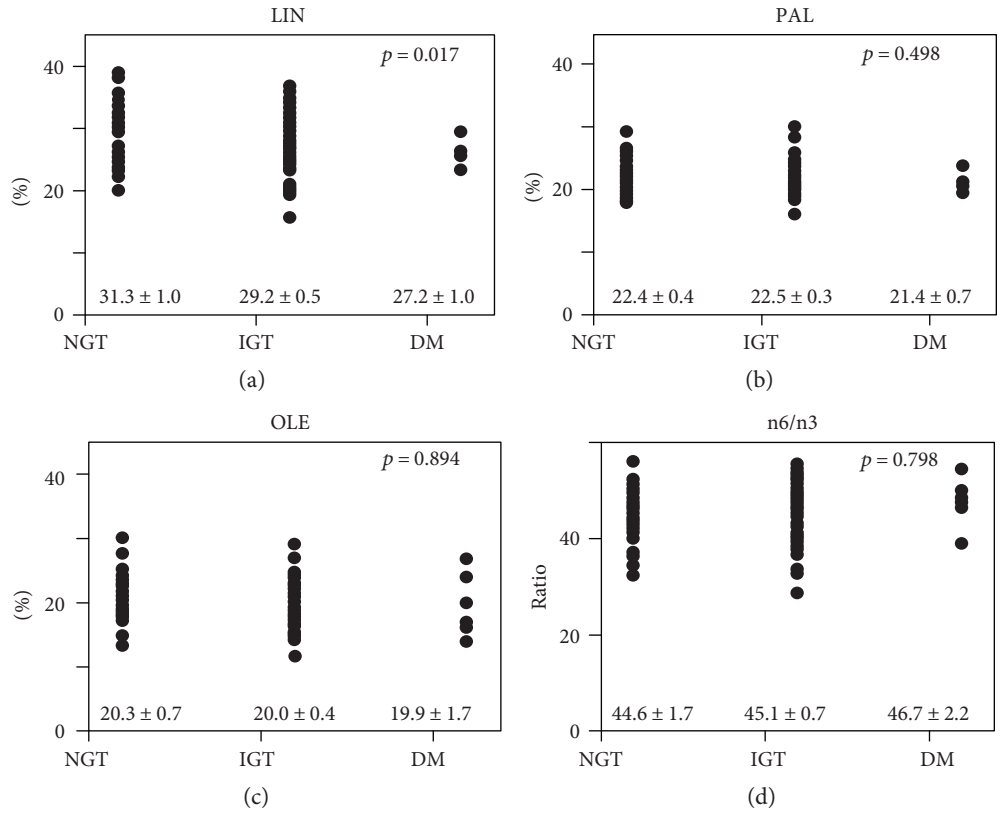

Figure 4: Percentages of linoleic acid (C18:2) (a), palmitic acid (C16:0) (b), and oleic acid (C18:1) (c) and n6/n3 PUFA ratio (d) in serum in normal glucose tolerant (NGT, $n = 33$), impaired glucose tolerant (IGT, $n = 71$), and diabetic (DM, $n = 7$) Japanese subjects by a cross-sectional evaluation. Kruskal-Wallis test was performed. Data are mean ± SEM.

relationships between the major three FFAs and classification by OGTT. The percentage of linoleic acid in diabetic subjects was significantly lower than that in normal subjects (Figure 4(a)). On the other hand, the percentages of palmitic acid (Figure 4(b)) and oleic acid (Figure 4(c)) showed no significant differences among the groups. The n6/n3 PUFA ratio was not significantly different among the groups (Figure 4(d)).

Linoleic acid was significantly negatively correlated with visceral fat area ($n = 30$, $r = -0.556$, $p = 0.0014$) (Figure 5(a)) and HOMA-IR ($n = 31$, $r = -0.704$, $p < 0.0001$) (Figure 5(b)) by simple correlation analysis in normal subjects. In IGT subjects, linoleic acid was also significantly negatively correlated with visceral fat area ($n = 66$, $r = -0.444$, $p < 0.0001$) (Figure 5(c)) and HOMA-IR ($n = 69$, $r = -0.393$, $p = 0.0008$) (Figure 5(d)), as well as in normal subjects. The percentage of linoleic acid in IGT subjects with insulin resistance, which was defined as HOMA-IR > 2.5, was significantly lower than that in normal subjects (linoleic acid (%); 31.32 ± 0.97 in NGT ($n = 30$) versus 27.58 ± 0.76 in IGT with HOMA-IR > 2.5 ($n = 30$), $p = 0.0036$). In addition, the percentages of linoleic acid in IGT subjects with HOMA-IR > 2.5 were significantly lower than those in IGT subjects with HOMA-IR < 2.5 (linoleic acid (%); 30.45 ± 0.93 in IGT with HOMA-IR < 2.5 ($n = 41$) versus 27.58 ± 0.76 in IGT with HOMA-IR > 2.5 ($n = 30$), $p = 0.0051$).

4. Discussion

To investigate which FFA fraction is beneficial or deleterious for the development of type 2 diabetes in a race is important. In this study, we demonstrated that serum linoleic acid (%) was negatively correlated with the accumulation of visceral fat in Japanese subjects by a cross-sectional evaluation. Linoleic acid, which is an essential fatty acid, is the greatest fatty acid component in serum in Japanese subjects. The percentage of linoleic acid was negatively correlated with visceral fat area and was significantly lower in diabetic subjects than in normal subjects in our study.

This observation in our study is consistent with a report that compared the risk of developing type 2 diabetes in relation to FFA fractions in serum in a longitudinal observation of Finnish subjects [17]. In addition, the percentage of linoleic acid was negatively correlated with visceral fat area in our study. Because the accumulation of visceral fat worsens glucose and lipid metabolism and is important in the development of type 2 diabetes [4], a low level of linoleic acid in serum might be related to the development of type 2 diabetes. However, our cross-sectional evaluation in humans raises concern that the causal relation between linoleic acid and visceral fat remains unknown.

It is reported that dietary safflower oil, which is rich in the linoleic acid, reduced trunk adipose mass and increased total

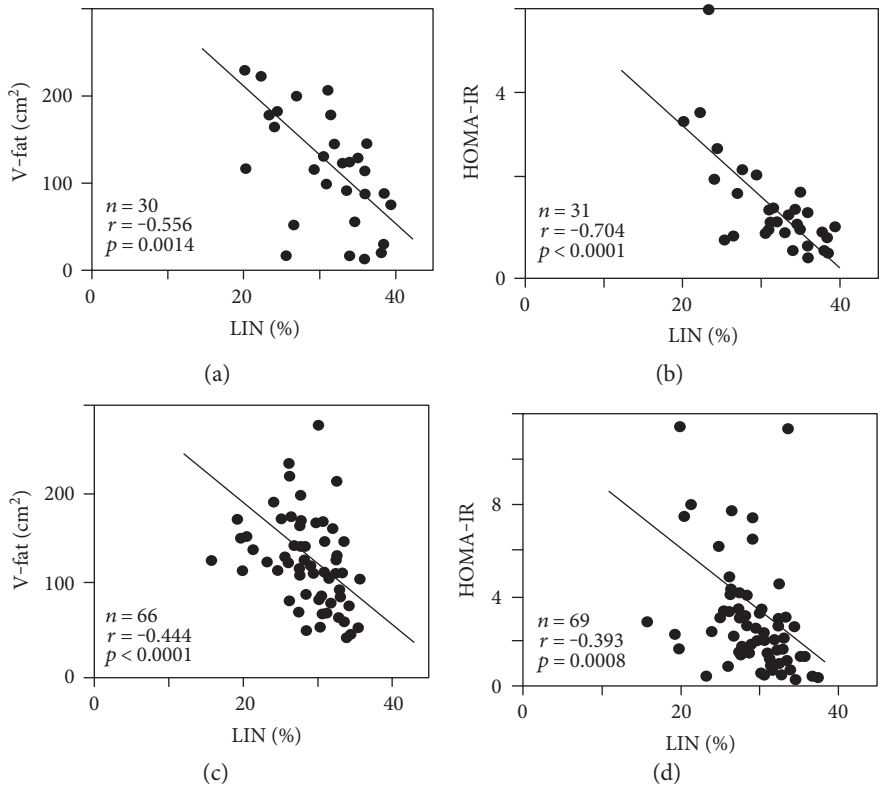

Figure 5: Relationships between ratio of serum linoleic acid (LIN, C18:2) and that of visceral fat area (V-fat) (a) and that of homeostasis model assessment insulin resistance index (HOMA-IR) (b) in subjects with normal glucose tolerance and ratio of serum linoleic acid and that of V-fat (c) and that of HOMA-IR (d) in subjects with impaired glucose tolerance, by simple correlation analysis.

body lean mass in obese women with type 2 diabetes and was associated with gene expression of uncoupling protein (UCP) 1 and UCP content in the adipose tissue of rats [18, 19]. Park et al. [20] showed that a diet supplemented with conjugated linoleic acid decreased body weight in mice and enhanced fatty acid β-oxidation, supported by increased carnitine palmitoyltransferase activity in the skeletal muscle and fat pad, and also that linoleic acid reduced lipoprotein lipase activity while apparently enhancing lipolysis in a study of 3T3-L1 adipocytes treated with conjugated linoleic acid. In the present study, including subjects without diabetes, the percentage of linoleic acid was significantly negatively correlated with visceral fat area. Although we cannot exclude that the reduced body weight by linoleic acid in an animal study was due to a toxic effect on adipocytes, it is rational that visceral fat does not cause a decrease in linoleic acid level in serum, but linoleic acid decreases visceral fat in humans.

Adiponectin is generally present in plasma at a high concentration and is inversely associated with visceral fat accumulation [21]. High molecular weight form of adiponectin is considered an active form of adiponectin in vitro [22, 23]. Clinical studies [24, 25] also suggest that HMW adiponectin is more useful as an indicator than is total adiponectin, particularly in type 2 diabetic patients. We also conducted a cross-sectional study in healthy Japanese male subjects without any medication and reported that HMW adiponectin measured by ELISA was as effective as the HMW/total adiponectin ratio for predicting insulin resistance and/or metabolic syndrome [26]. Serum linoleic acid was correlated with HMW adiponectin in our study. It is suggested that linoleic acid and linoleic acid-derived fatty acids might increase serum adiponectin level through Keto A, which is a linoleic acid-derived fatty acid produced by gut lactic acid bacteria, and induce adipocyte differentiation via the activation of peroxisome proliferator-activated receptor γ (PPAR-γ) [27, 28]. This mechanism might be associated with the finding that pioglitazone, a PPAR-γ agonist, induced an increase in serum adiponectin level in humans [29].

Palmitic acid, which is the second major component in Japanese subjects, was correlated with BMI and ALT (Figure 2). Previously, we have shown that a palmitic acid-supplemented diet might cause deterioration of glucose tolerance by suppression of insulin secretion from pancreatic β cells in mice [8]. However, there was no difference in the level of palmitic acid among the normal, IGT, and diabetic groups in the current human study (Figure 4). It is reported that a palmitic acid-rich diet might change the

gut microbiota and induce weight gain and hepatic lipid accumulation compared to an unsaturated fat diet in mice [30–32]. The discrepancies in the reported findings might be due to species differences.

Oleic acid, which is the third major FFA component in Japanese subjects, was correlated with serum TG and negatively correlated with HDL-C (Figure 3), while it was not correlated with parameters associated with insulin. In addition, there was no difference in oleic acid level among the normal, IGT, and diabetic groups (Figure 4). It is widely regarded that a Mediterranean diet, which is rich in oleic acid (olive oil), might be beneficial for the prevention and treatment of type 2 diabetes [33, 34]. On the other hand, in a recent study, oleic acid plasma level was shown to be a selective biomarker of impaired glucose tolerance in several cohorts [35]. These discrepancies might be related to racial differences. Investigation of the appropriate oleic acid intake in different races might be important.

The n6/n3 PUFA ratio is suggested to be important in disease development, for example, cardiovascular disease in rodent models fed with a high-fat diet [36]. However, the relationships of n6/n3 PUFA ratio and type 2 diabetes are not clear. In the present study, n6/n3 PUFA ratio was not significantly different among the normal, IGT, and diabetes groups (Figure 4(d)).

This study has several limitations. First, the sample size was small. Second, the study design did not allow for correlations between the dietary components of FFAs and the serum components of FFAs. Third, this cross-sectional study design raises concern that the causal relation between fatty acids and parameters remains unknown, as we discussed above. Further longitudinal studies are needed with a greater number of subjects. Lastly, the precise mechanisms for the results in this cross-sectional study should be elucidated. Recently, several studies have suggested that differences in the dietary composition might change the gut microbiota in obesity and type 2 diabetes [37–39]. de Wit et al. [32] showed that a diet rich in unsaturated fat induced changes in gut microbiota composition and mucosal PPARα target gene expression. Dietary *trans*-10, *cis*-12-conjugated linoleic acid (*t*10*c*12-CLA) significantly decreased visceral fat mass in mice. Analysis of the microbiota composition under *t*10*c*12-CLA supplementation revealed a lower proportion of Firmicutes and a higher proportion of Bacteroidetes compared with that under no supplementation [40]. Linoleic acid might change the gut microbiota composition and reduce visceral fat.

5. Conclusion

In conclusion, we demonstrated that linoleic acid percentage was negatively correlated with the accumulation of visceral fat in relation to a reduction of insulin resistance in Japanese subjects.

Disclosure

The authors alone are responsible for the content and writing of this article. An earlier version of this work was presented as an abstract at the 71st Annual Meeting of the American Diabetes Association, 2011.

Conflicts of Interest

The authors report no conflict of interest.

Acknowledgments

This work was supported by JSPS KAKENHI, Grant nos. 23500854 and 26350905. The authors thank Dr. Wendy Gray for editing the paper.

References

[1] International Diabetes Federation, *Diabetes Atlas 7th edition*, International Diabetes Federation, Brussels, Belgium, 2015.

[2] P. Hossain, B. Kawar, and M. El Nahas, "Obesity and diabetes in the developing world – a growing challenge," *The New England Journal of Medicine*, vol. 356, no. 3, pp. 213–215, 2007.

[3] S. M. Haffner, S. Lehto, T. Rënnemaa, K. Pyërala, and M. Laakso, "Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction," *The New England Journal of Medicine*, vol. 339, no. 4, pp. 229–234, 1998.

[4] S. Fujioka, Y. Matsuzawa, K. Tokunaga, and S. Tarui, "Contribution of intraabdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity," *Metabolism*, vol. 36, no. 1, pp. 54–59, 1987.

[5] H. Hirose, Y. H. Lee, L. R. Inman, Y. Nagasawa, J. H. Johnson, and R. H. Unger, "Defective fatty acid-mediated β-cell compensation in Zucker diabetic fatty rats," *The Journal of Biological Chemistry*, vol. 271, no. 10, pp. 5633–5637, 1996.

[6] G. Boden, "Role of fatty acids in the pathogenesis of insulin resistance and NIDDM," *Diabetes*, vol. 46, no. 1, pp. 3–10, 1997.

[7] T. Kawai, H. Hirose, Y. Seto, H. Fujita, and T. Saruta, "Chronic effects of different fatty acids and leptin in INS-1 cells," *Diabetes Research and Clinical Practice*, vol. 51, no. 1, pp. 1–8, 2001.

[8] T. Hirata, T. Kawai, H. Hirose et al., "Palmitic acid-rich diet suppresses glucose-stimulated insulin secretion (GSIS) and induces endoplasmic reticulum (ER) stress in pancreatic islets in mice," *Endocrine Research*, vol. 41, no. 1, pp. 8–15, 2016.

[9] S. M. Lopes, S. L. Trimbo, E. A. Mascioli, and F. L. Blackburn, "Human plasma fatty acid variations and how they are related to dietary intake," *The American Journal of Clinical Nutrition*, vol. 53, no. 3, pp. 628–637, 1991.

[10] E. J. Feskens, S. M. Virtanen, L. Räsänen et al., "Dietary factors determining diabetes and impaired glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study," *Diabetes Care*, vol. 18, no. 8, pp. 1104–1112, 1995.

[11] S. G. Anderson, T. A. B. Sanders, and J. K. Cruickshank, "Plasma fatty acid composition as a predictor of arterial stiffness and mortality," *Hypertension*, vol. 53, no. 5, pp. 839–845, 2009.

[12] K. Li, B. A. McNulty, A. M. Tiernery et al., "Dietary fat intakes in Irish adults in 2011: how much has changed in 10 years?" *British Journal of Nutrition*, vol. 115, no. 10, pp. 1798–1809, 2016.

[13] K. G. M. M. Alberti and P. Z. Zimmet, "Definition, diagnosis and classification of diabetes mellitus and its complications. Provisional report of a WHO consultation," *Diabetic Medicine*, vol. 15, no. 7, pp. 539–553, 1998.

[14] M. Haug, I. Dieterich, C. Laubach, D. Reinhardt, and G. Harzer, "Capillary gas chromatography of fatty acid methyl esters from human milk lipid subclasses," *Journal of Chromatography*, vol. 279, pp. 549–553, 1983.

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

[16] T. Kawai, I. Takei, Y. Oguma et al., "Effects of troglitazone on fat distribution in the treatment of male type 2 diabetes," *Metabolism*, vol. 48, no. 9, pp. 1102–1107, 1999.

[17] B. Vessby, A. Aro, E. Skarfors, L. Berqlund, I. Salminen, and H. Lithell, "The risk to develop NIDDM is related to the fatty acid composition of the serum cholesterol esters," *Diabetes*, vol. 43, no. 11, pp. 1353–1357, 1994.

[18] T. Matsuo, H. Takeuchi, H. Suzuki, and M. Suzuki, "Body fat accumulation is greater in rats fed a beef tallow diet than in rats fed a safflower or soybean oil diet," *Asia Pacific Journal of Clinical Nutrition*, vol. 11, no. 4, pp. 302–308, 2002.

[19] L. E. Norris, A. L. Collene, M. L. Asp et al., "Comparison of dietary conjugated linoleic acid with safflower oil on body composition in obese postmenopausal woman with type 2 diabetes mellitus," *The American Journal of Clinical Nutrition*, vol. 90, no. 3, pp. 468–476, 2009.

[20] Y. Park, K. J. Albright, W. Liu, J. M. Storkson, M. E. Cook, and M. W. Pariza, "Effect of conjugated linoleic acid on body composition in mice," *Lipids*, vol. 32, no. 8, pp. 853–858, 1997.

[21] Y. Matsuzawa, "Establishment of a concept of visceral fat syndrome and discovery of adiponectin," *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, vol. 86, no. 2, pp. 131–141, 2010.

[22] N. Ouchi, H. Kobayashi, S. Kihara et al., "Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells," *The Journal of Biological Chemistry*, vol. 279, no. 2, pp. 1304–1309, 2004.

[23] Y. Wang, K. S. Lam, J. Y. Xu et al., "Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner," *The Journal of Biological Chemistry*, vol. 280, no. 18, pp. 18341–18347, 2005.

[24] H. Kobayashi, N. Ouchi, S. Kihara et al., "Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin," *Circulation Research*, vol. 94, no. 4, pp. e27–e31, 2004.

[25] U. B. Pajvani, M. Hawkins, T. P. Combs et al., "Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity," *The Journal of Biological Chemistry*, vol. 279, no. 13, pp. 12152–12162, 2004.

[26] Y. Seino, H. Hirose, I. Saito, and H. Itoh, "High molecular weight multimer form of adiponectin as a useful marker to evaluate insulin resistance and metabolic syndrome in Japanese men," *Metabolism*, vol. 56, no. 11, pp. 1493–1499, 2007.

[27] S. A. Kliewer, S. S. Sundseth, S. A. Jones et al., "Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptor alpha and gamma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 9, pp. 4318–4323, 1997.

[28] T. Goto, Y. Kim, T. Furuzono et al., "10-oxo-12(Z)-octadecenoic acid, a linoleic acid metabolite produced by gut lactic acid bacteria, potently activates PPARγ and stimulates adipogenesis," *Biochemical and Biophysical Research Communications*, vol. 459, no. 4, pp. 597–603, 2015.

[29] H. Hirose, T. Kawai, Y. Yamamoto et al., "Effects of pioglitazone on metabolic parameters, body fat distribution, and serum adiponectin levels in Japanese male patients with type 2 diabetes," *Metabolism*, vol. 51, no. 3, pp. 314–317, 2002.

[30] R. E. Ley, F. Backhed, P. Turnbaugh, C. A. Lozaupone, R. D. Knight, and J. I. Gordon, "Obesity alters gut microbial ecology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 31, pp. 11070–11075, 2005.

[31] G. Musso, R. Gambino, and M. Cassader, "Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes," *Annual Review of Medicine*, vol. 62, pp. 361–380, 2011.

[32] N. de Wit, M. Derrien, H. Bosch-Vermeulen et al., "Saturated fat simulates obesity and hepatic steatosis and affects gut microbiota composition by an enhanced overflow of dietary fat to the distal intestine," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 303, no. 5, pp. G589–G599, 2012.

[33] K. Esposito, M. I. Maiorino, A. Ceriello, and D. Giugliano, "Prevention and control of type 2 diabetes by Mediterranean diet: a systematic review," *Diabetes Research and Clinical Practice*, vol. 89, no. 2, pp. 97–102, 2010.

[34] D. Romaguera, M. Guevara, T. Norat et al., "Mediterranean diet and type 2 diabetes risk in the European Prospective Investigation into Cancer and Nutrition (EPIC) study: the InterAct project," *Diabetes Care*, vol. 34, no. 9, pp. 1913–1918, 2011.

[35] J. Cobb, A. Eckhart, A. Motsinger-Reif, B. Carr, L. Groop, and E. Ferrannini, "α-Hydroxybutyric acid is a selective metabolite biomarker of impaired glucose tolerance," *Diabetes Care*, vol. 39, no. 6, pp. 988–995, 2016.

[36] H. Poudyal, S. K. Panchal, J. Waanders, L. Ward, and L. Brown, "Lipid redistribution by alpha-linoleic acid-rich chia seed inhibits stearoyl-CoA desaturase-1 and induces cardiac and hepatic protection in diet-induced obese rats," *The Journal of Nutritional Biochemistry*, vol. 23, no. 2, pp. 153–162, 2012.

[37] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest," *Nature*, vol. 444, no. 7122, pp. 1027–1031, 2006.

[38] P. Kovatcheva-Datchary and T. Arora, "Nutrition, the gut microbiome and the metabolic syndrome," *Best Practice and Research. Clinical Gastroenterology*, vol. 27, no. 1, pp. 59–72, 2013.

[39] S. M. Harakeh, I. Khan, T. Kumosani et al., "Gut microbiota: a contributing factor to obesity," *Frontiers in Cellular and Infection Microbiology*, vol. 6, article 95, pp. 1–11, 2016.

[40] T. M. Marques, R. Wall, O. O'Sullivan et al., "Dietary trans-10, cis-12-conjugated linoleic acid alters fatty acid metabolism and microbiota composition in mice," *British Journal of Nutrition*, vol. 113, no. 5, pp. 728–738, 2015.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 1039161, 7 pages
https://doi.org/10.1155/2017/1039161

Research Article

Irisin: A Potential Link between Physical Exercise and Metabolism—An Observational Study in Differently Trained Subjects, from Elite Athletes to Sedentary People

Stefano Benedini,[1,2] Elena Dozio,[1] Pietro Luigi Invernizzi,[1] Elena Vianello,[1] Giuseppe Banfi,[3,4] Ileana Terruzzi,[5] Livio Luzi,[1,2,6] and Massimiliano Marco Corsi Romanelli[1,7]

[1]*Department of Biomedical Sciences for Health, Università degli Studi di Milano, Via L. Mangiagalli 31, 20133 Milan, Italy*
[2]*Endocrinology Unit, IRCCS Policlinico San Donato, Via R. Morandi 30, San Donato Milanese, 20097 Milan, Italy*
[3]*Laboratory of Experimental Biochemistry and Molecular Biology, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy*
[4]*Vita-Salute San Raffaele University, Milan, Italy*
[5]*Diabetes Research Institute, Metabolism, Nutrigenomics and Cellular Differentiation Unit, San Raffaele Scientific Institute, Milan, Italy*
[6]*Metabolism Research Center, IRCCS Policlinico San Donato, Via R. Morandi 30, San Donato Milanese, 20097 Milan, Italy*
[7]*SMEL-1 Laboratory Medicine Unit, IRCCS Policlinico San Donato, Via R. Morandi 30, San Donato Milanese, 20097 Milan, Italy*

Correspondence should be addressed to Stefano Benedini; stefano.benedini@unimi.it

Received 16 November 2016; Revised 10 January 2017; Accepted 1 March 2017; Published 13 March 2017

Academic Editor: Joseph F. Ndisang

We compared irisin levels among groups of differently trained healthy individuals to explore the role of irisin as a physiological linker between exercise and metabolic health. Irisin and biochemical parameters of glucose and lipid metabolism were assessed in 70 healthy volunteers stratified for sport performance level into four groups: (1) 20 elite athletes of national level, (2) 20 subelite athletes of local level, (3) 20 recreational athletes, and (4) 10 sedentary subjects. All biochemical parameters were within the ranges of normality. Fasting glucose, HOMA-IR, and total cholesterol levels were inversely related to the degree of physical activity. HbA1c was higher in elite athletes compared to all the other groups ($p < 0.01$). A U-shaped relation between free fatty acids and the degree of physical activity was observed. All groups showed similar plasma irisin levels. After correction for the degree of insulin resistance (irisin/HOMA-IR), elite athletes showed higher levels compared to sedentary and recreational subjects ($p < 0.01$ and $p < 0.05$, resp.). In addition, the number of metabolic parameters correlated with irisin increased at increasing the training status. Our study suggests a correlation between sport performance, insulin sensitivity, and irisin levels. Irisin may be one potential mediator of the beneficial effects of exercise on metabolic profile.

1. Introduction

The role of physical activity in maintaining good health and preventing insulin resistance, type 2 diabetes mellitus (DMT2), obesity, metabolic syndrome, atherosclerosis, and other cardiovascular complications is well recognized [1–3]. The protective effect of physical activity may be ascribed, to some extent, to the release of myokines from contracting skeletal muscles. These molecules, in fact, may mediate the beneficial effects of exercise on glucose and lipid metabolism and on inflammation, which characterizes both metabolic and cardiovascular diseases [4]. An interesting editorial by Polyzos and colleagues highlighted the possibility that irisin in humans could be a link between physical activity and metabolic homeostasis and could be implicated in processes involved in weight balance [5].

Irisin is a myokine firstly identified for its ability to induce browning of white adipose tissue, to increase energy expenditure and to protect against insulin resistance and obesity [6]. The beneficial effects of exercise on bone metabolism seem to be mediated by different myokines as irisin [7]. The real effect of physical activity in promoting expression and secretion of irisin in human is still unclear. In fact, besides some studies which described a great effect of exercise in promoting irisin increase [8–10], other studies reported that neither acute nor chronic exercise promotes changes in irisin level [11–14]. Probably, most of these controversies may depend on the type of exercise (resistance/endurance), its duration (acute/prolonged), the training status of individuals before enrollment in the studies, and the type of assay for irisin quantification.

A review published in 2014 explains that different studies in humans have shown inconsistent results related to the proposed induction of irisin by exercise [15]. Human studies till now performed were almost interventional studies aimed to evaluate acute changes in irisin level after a training program. The relationship among the sporting behavior and chronic irisin level is poorly explored, instead.

To this aim, we compared irisin level among groups of differently trained healthy individuals (elite athletes of international and national level, athletes of local level, recreational athletes, and sedentary individuals) and we explored the role of irisin as a physiological linker between exercise and metabolic health.

2. Methods

2.1. Aim. The purpose of this work is to compare irisin level among groups of differently trained healthy individuals (elite athletes of international and national level, athletes of local level, recreational athletes, and sedentary individuals) and to explore the role of irisin as a physiological linker between exercise and metabolic health.

2.2. Design and Setting of the Study. All subjects involved in this protocol were admitted to the UO Endocrinology after an overnight fast and before any exercise activity. At 08.00, a polyethylene catheter was inserted into the antecubital vein of one forearm. At 08.30, a basal blood sample for the measurement of hormones and metabolites was drawn. Weight and height were then recorded with standard scales and stadiometers and BMI was calculated. Then the subjects were discharged.

2.3. Biochemical Assays. Blood samples were collected after overnight fasting into pyrogen-free tubes with EDTA as anticoagulant or in tubes for serum separation. Plasma and serum samples were prepared by centrifugation at 4°C within 1.5 h of sampling and aliquots were frozen at −20°C until later analyses.

Fasting glucose, glycated hemoglobin (HbA1c), insulin, total cholesterol, and triglycerides were quantified with commercial kits using Cobas 6000 analyzer (Roche Diagnostics, Milan, Italy), as previously reported [16, 17]. Briefly, total cholesterol has been determined by an enzymatic-colorimetric method. The lower detection limit was 3.86 mg/dL. Intra- and interassay coefficient of variations (CV%) were 1.1% and 1.6%, respectively. The hexokinase method was used to quantify glucose. The lower detection limit was 2 mg/dL. Intra- and interassay CV% were 1.0% and 1.3%, respectively. Turbidimetric inhibition immunoassay method was used for the determination of HbA1c. Lower detection limit was 0.18 mmol/L. Intra- and interassay CV% were 1.6% and 2.0%, respectively. Triglycerides were quantified using an enzymatic-colorimetric method. Lower detection limit was 8.85 mg/dL. Intra- and interassay CV% were 1.1% and 2.0%, respectively. Insulin was measured by electrochemiluminescent immunoassay. The lower detection limit was 0.2 μU/mL. Intra- and interassay CV% were 1.5% and 4.9%, respectively. Free fatty acids (NEFA) were quantified on a Uvikon analyzer using the colorimetric method provided by Randox Laboratories (Crumlin, County Antrim, UK). The method is linear up to 2.24 mmol/L. The minimum detectable level with acceptable precision has been determined at 0.072 mmol/L. Within run assay CV% and total assay CV% were 4.81% and 4.51%, respectively.

2.4. Irisin Quantification. Circulating irisin levels have been quantified on serum samples by a specific competitive enzyme immunoassay kit which has been previously validated by MS spectrometry analysis (Cat. EK-067-29, Phoenix Europe GmbH, Karlsruhe, Germany). The minimum detectable concentration was 1.43 ng/mL. The intra- and interassay variations were less than 10% and 15%, respectively. The assays used to detection of irisin were previously validated [18].

2.5. HOMA-IR. Insulin action was assessed using the equations provided by HomeOstasis Model Assessment for estimating insulin resistance (HOMA-IR): HOMA-IR = $G0 \cdot I0/22.5$, where $I0$ (μU/mL) is the fasting insulin concentration, $G0$ (mmol/L) is the fasting glucose concentration, and 22.5 represents a constant applied to correct the value to unity as previously described [19].

2.6. Characteristics of Participant. In a case-control study, we studied 4 groups of healthy subjects who routinely perform different levels of aerobic physical activity (elite athletes of international and national level, athletes of local level, recreational athletes, and sedentary subjects). Elite athlete is defined as a highly specialized athlete whose performances correspond to the best national results in his or her respective sports or discipline. Athlete of local level is a subject who trains at least 4 times a week for a total of at least 10 hours per week. Subjects who performed recreational physical activity are defined as subjects who train at least 2 times a week for a total of at least 3 hours per week. The sedentary is a subject who does not perform any train session in the week and does not perform physical exercise during habitual work.

All the subjects recruited for the study gave their informed written consent after being given an explanation of purposes and nature of the experimental protocol, conducted in accordance with the declaration of Helsinki, as revised in 2013.

2.7. Ethics Approval and Consent to Participate. The study was approved by the ethics committee of Ospedale San Raffaele, reference number: 88/int/2016. All the subjects recruited for the study gave their informed written consent after being given an explanation of purposes and nature of the experimental protocol. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.8. Statistical Analysis. All statistical analyses and graphical data representations were done using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, San Diego, CA). Data are expressed as mean ± SD. The Kolmogorov-Smirnoff test was used to assess the normality of data distribution. Comparisons between groups were performed using ANOVA or Kruskal-Wallis tests followed by Bonferroni or Dunn's posttests, as appropriate. The univariate associations between irisin, demographic, anthropometric, and biochemical parameters were examined by Pearson or Spearman correlation tests. A p value < 0.05 was considered significant.

3. Results

3.1. Anthropometric Parameters of the Participants, Group Size, and Gender. Of the 70 subjects enrolled for the study 20 were elite athletes [17 males and 3 females, age 22.4 ± 2.8 mean ± standard deviation (SD), and body mass index (BMI) 22.4 ± 1.7 Kg/m2], 20 were athletes of local level [15 males and 5 females, age 21.2 ± 2.1 mean ± SD, BMI 22.4 ± 2.4 Kg/m2], 20 were recreational [13 males and 7 females, age 24.6 ± 8.1 mean ± SD, BMI, 22.1 ± 2.7 Kg/m2], and 10 were sedentary individuals [6 males and 4 females, age 25.2 ± 8.1 mean ± SD, BMI, 22.8 ± 2.8 Kg/m2]. Total physical activity score of participants was classified as inactive (less than 600 MET minutes per week), recreational (between 600 and 3000 MET minutes per week), subelite (between 3000 and 4000 MET minutes per week), and elite (more than 4000 MET minutes per week) according to recommendations in the International Physical Activity Questionnaire (IPAQ) [20].

3.2. Glucose Metabolism. Fasting glucose, HbA1c, and insulin levels were within the normal ranges in all groups. Fasting glucose was higher in the sedentary group compared to both elite and subelite groups ($p < 0.01$ and $p < 0.05$, resp.) (Figure 1). No statistically significant differences were detected in fasting insulin levels among the groups, although a trend of increase may be observed at decreasing the training status (Figure 1). To be noted, in sedentary, the HOMA-IR was higher compared to both elite and subelite groups ($p < 0.05$ for both), thus suggesting a mild insulin resistance status (Figure 1). HbA1c was increased only in elite athletes compared to all the other groups ($p < 0.01$ for all).

3.3. Lipid Profile. Plasma lipid profile was normal in all groups. Total cholesterol was higher in sedentary group than the others ($p < 0.05$, for all). Triglycerides were similar in all the four groups. In contrast, nonesterified fatty acids (NEFA) were higher in sedentary and elite groups compared to recreational ($p < 0.01$ for all) (Figure 1).

3.4. Irisin. All groups showed similar plasma irisin level. After irisin correction for the degree of insulin resistance (irisin/HOMA-IR) elite and subelite athletes showed significantly increased ratios compared both to sedentary ($p < 0.01$ for elite and $p < 0.05$ for subelite) and to recreational subjects ($p < 0.05$, for both) (Figure 2). The associations among irisin, demographic, anthropometric, and biochemical parameters were explored using Pearson or Spearman correlation coefficients. For this analysis elite and subelite athletes were considered jointly because these parameters are very similar in these two groups. An increased number of correlations among irisin and biochemical parameters were observed at increasing the training status. In sedentary group, it was only one direct correlation between irisin and irisin/HOMA-IR ratio (Table 1). In recreational group, irisin was also inversely associated with fasting glucose (Table 1). In elite/subelite group, 3 further inverse correlations were observed: with total cholesterol, triglycerides, and free fatty acid (Table 1).

4. Discussion

It has been suggested that the myokine irisin could play an endocrine control of metabolism in the humans. Irisin level seems to be negatively correlated with age, insulin concentrations, triglyceride, and adiponectin levels, suggesting that this hormone may be involved in mechanisms for metabolic regulation. In particular in the study of Huh and colleagues an acute bout of vibration exercise increases circulating irisin, whereas chronic training does not change the values of irisin in humans [21]. Another study of this group shows exercise-induced irisin secretion independently of age or fitness level [22]. In our study, we evaluated the concentrations of irisin in a population of athletes who regularly perform different loads of physical activity and we did not find significant differences in the basal concentrations of this hormone in the different groups studied. These data seem to be similar to results of another study performed on adolescent athletes in a paper of Singhal and colleagues [23]. In this study irisin was compared in two groups (eumenorrheic athletes and nonathletes, resp.) in amenorrheic athletes. The data about irisin in the same condition in eumenorrheic athletes and nonathletes were similar in these groups [23]. Another study showed that 12 weeks of heavy strength training is not able to increase serum irisin levels in women [24]. In contrast a study showed, during an endurance exercise routine in sedentary men, a twofold rise in plasma irisin levels at ten weeks [6], while another study in adults reported an increase in irisin following acute exercise, but a decrease in irisin after a 12-week of endurance and strength training [25].

Previous studies focused on insulin resistance [26, 27] assumed that this condition may represent an independent risk factor affecting the circulating levels of different molecules, that is, adipokines, and suggested the use of the adjusted ratio as a more metabolically relevant measure. Since also in our study HOMA-IR is the main parameter that

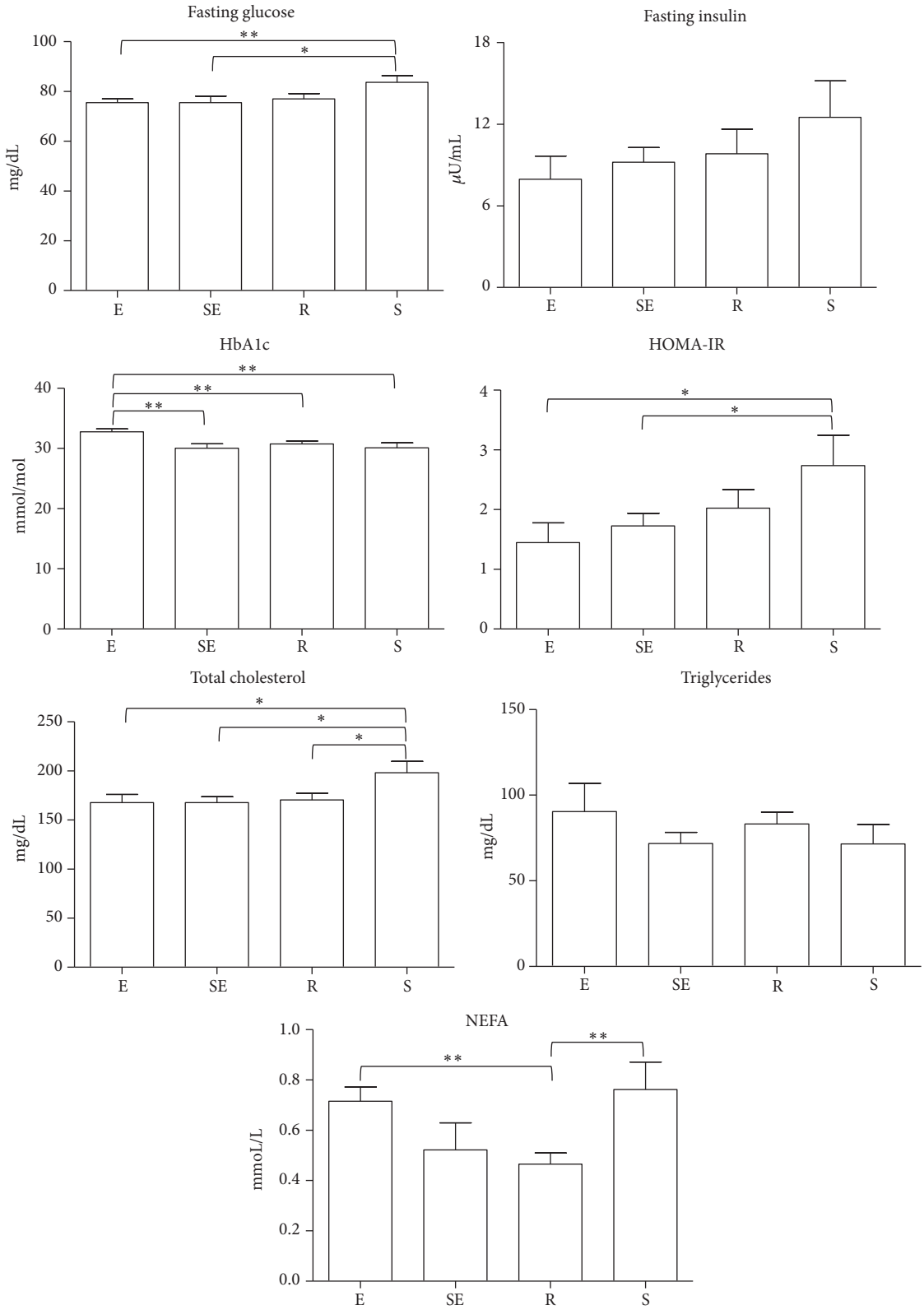

Figure 1: Fasting glucose, insulin, glycated hemoglobin (HbA1c), total cholesterol, triglycerides, free fatty acids (NEFA) levels, and HomeOstasis Model Assessment for estimating insulin resistance (HOMA-IR) values in differently trained subjects. Fasting glucose, insulin, HbA1c, total cholesterol, triglycerides, NEFA levels, and HOMA-IR values were evaluated in elite (E), subelite (SE), recreational (R), and sedentary (S) subjects. $^{*}p < 0.05$; $^{**}p < 0.01$.

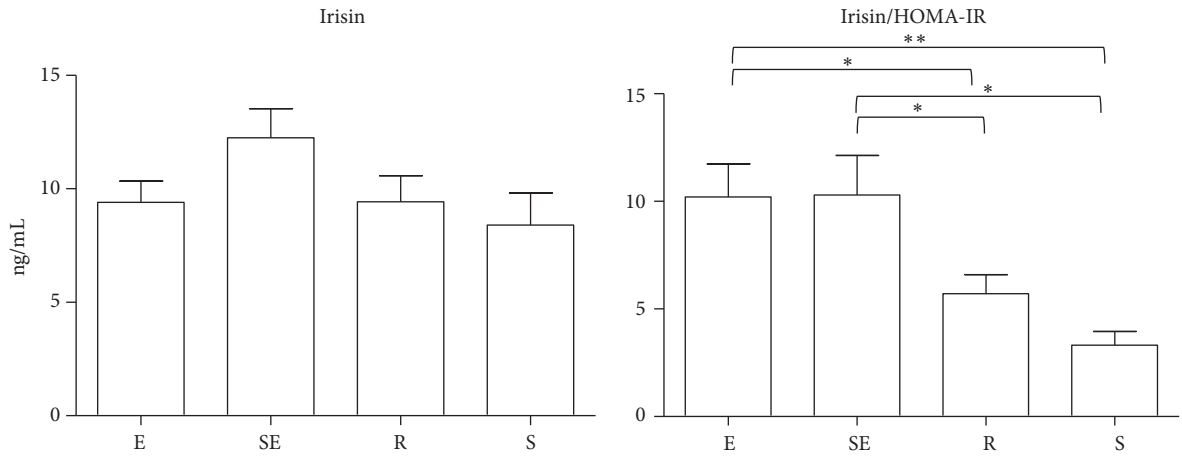

Figure 2: Irisin and irisin/HomeOstasis Model Assessment for estimating insulin resistance (HOMA-IR) ratio levels in differently trained subjects. Irisin and irisin/HOMA-IR ratios were evaluated in elite (E), subelite (SE), recreational (R), and sedentary (S) subjects. $^{*}p < 0.05$; $^{**}p < 0.01$.

changes more between the different groups, we hypothesized that insulin resistance could affect irisin values and we decided to include also the adjusted ratio (irisin/HOMA-IR) as a new independent parameter. Notably, differently from irisin, whose levels were not different among groups, values of irisin/HOMA-IR were significantly increased when compared to sedentary and recreational subjects, thus confirming a potential influence of insulin resistance on irisin levels.

In our study HOMA-IR correlate inversely with the degree of physical activity (in particular there is a strict correlation between insulin sensitivity and METS expressed as MET minutes per week, $p < 0.001$); logically in subjects that do more physical activities HOMA-IR values are lower and also the values of absolute irisin seem to be high; although not reaching significance this hormone shows a trend of higher values in subjects that perform more physical activity as elite and subelite athletes versus sedentary (10.6±5.1 versus 8.4±4.5 ng/mL). Despite not showing a statistically significant difference these data are interesting and suggest the presence of a "memory" of physical activity performed.

Here are not current data on irisin in elite athletes in literature. The possibility of analyzing irisin on subjects with a high load of exercise (>11 METS: Metabolic Equivalent of Task) is critical to understand the real correlation between this myokine and physical activity.

Another study highlights a significant decrease of irisin in type 2 diabetic subjects compared to nonobese controls [28]; in particular the irisin values found in the lean control subjects are comparable to those found in our group of sedentary subjects.

A recent study showed that irisin concentrations of the endurance trained athletes were higher than that of the sedentary control subjects [29].

At baseline values irisin seems to increase or not different in insulin resistance models such as polycystic ovary syndrome (PCOS) as reported by recent papers [30, 31].

The numerous works in the literature cited in the discussion often have not adequate control group and therefore the findings of these paper must be interpreted and partially downsized in their conclusions.

The values of irisin could be more closely related to insulin sensitivity of peripheral tissues and in particular with striated muscle and this difference seems to be detectable also in basal condition, independently of physical activity performed in acute exercise. In fact, irisin is a myokine produced by striated muscle and the muscle's ability to modulate the input of energy substrates in the muscle is essential to maintain glucose homeostasis.

In fact, inverse correlation between irisin and blood glucose in subjects who performed physical exercises seems to indicate a direct action of irisin on glucose metabolism. In addition high values of HbA1c in elite athletes have already been described in another paper [32] and seem to correlate with increased endogenous glucose production during exercise and secondary to exogenous implementation during and after the performance. The euglycemia that is present in these athletes at rest is probably also mediated by irisin.

Moreover, the presence of lower values of circulating lipids (total cholesterol, triglycerides, and NEFA) in the group of subjects who performed more hours of physical training also indicates a modulatory role of irisin on lipid metabolism.

In particular the possibility of developing a form of "irisin-resistance" linked to the increase in insulin resistance may in part explain the high levels of this hormone in obese subjects and in women with PCOS, while, as already mentioned above, in elite athletes the value of irisin may remain slightly elevated as a "memory" of physical exercise performed in the previous day.

In fact, the absence of changes in irisin values in women with PCOS after taking therapy with metformin suggests, at least in this condition of insulin resistance, a different mechanism of myokine increases [33].

In conclusion our data indicate a possible key role of irisin on glucose and lipid metabolism in health young people, which is reflected on high value of ratio irisin/HOMA-IR in the group of athletes who perform more exercise.

Table 1: Univariate association between plasma irisin level and demographic, anthropometric, and biochemical parameters in differently trained individuals: (a) elite + subelite group, (b) recreational group, and (c) sedentary group.

(a) Elite and subelite

	r	p
Age[(2)]	−0.16	0.336
BMI[(1)]	−0.11	0.512
Fasting glucose[(2)]	−0.477	**0.003**
Fasting insulin[(2)]	−0.058	0.730
Total cholesterol[(1)]	−0.329	**0.047**
Triglycerides[(2)]	−0.441	**0.006**
NEFA[(2)]	−0.545	**0.001**
HbA1c[(1)]	−0.206	0.221
HOMA-IR[(2)]	−0.065	0.698
Irisin/HOMA-IR[(2)]	0.633	<**0.0001**

(b) Recreational

	r	p
Age[(2)]	−0.667	0.967
BMI[(1)]	0.268	0.090
Fasting glucose[(1)]	−0.306	**0.006**
Fasting insulin[(2)]	0.142	0.399
Total cholesterol[(1)]	0.004	0.792
Triglycerides[(2)]	−0.137	0.411
NEFA[(1)]	0.075	0.66
HbA1c[(1)]	−0.139	0.413
HOMA-IR[(2)]	0.203	0.203
Irisin/HOMA-IR[(2)]	0.603	<**0.0001**

(c) Sedentary

	r	p
Age[(2)]	−0.278	0.436
BMI[(1)]	−0.217	0.548
Fasting glucose[(1)]	0.103	0.777
Fasting insulin[(2)]	0.395	0.333
Total cholesterol[(1)]	0.09	0.803
Triglycerides[(2)]	0.4001	0.251
NEFA[(1)]	−0.134	0.752
HbA1c[(1)]	−0.601	0.087
HOMA-IR[(2)]	0.288	0.419
Irisin/HOMA-IR[(2)]	0.833	**0.003**

Associations between variables were explored using Pearson or Spearman correlation coefficients, as appropriate. [(1)]Pearson correlation; [(2)]Spearman correlation.
BMI, body mass index; NEFA, free fatty acids; HbA1c, glycated hemoglobin; HOMA-IR, HomeOstasis Model Assessment for estimating insulin resistance.

It will need additional studies with a larger number of subjects to better understand the mechanisms that regulate the production of irisin during different levels of physical activity.

5. Study Limitations

There are some possible limitations to consider in the present study. First, the number of subjects in the sample is somewhat small. Second, this is a case-control study and the power analysis of sample size cannot draw a cause-effect conclusion about irisin and insulin sensitivity in elite athletes. Further large studies are needed to elucidate the real role of irisin in modulation of insulin sensitivity in humans.

Moreover in some papers cited as references it is not entirely clear whether the blood sample for irisin assay was performed immediately after exercise or after a few hours; this could be a confounding factor that may partially justify the contradictory data.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission. Stefano Benedini and Elena Dozio equally contributed to the paper.

Acknowledgments

The authors thank Dr. Elena Costa and Dr. Roberta Rigolini, IRCCS Policlinico San Donato, Milan, Italy, for clinical chemistry data and Ms. Judith Baggott for English language editing. The study was supported by funds from Italian Ministry for Health "Ricerca Corrente" IRCCS Policlinico San Donato *(no.* 9.16.2*)*.

References

[1] E. J. Mayer-Davis, R. D'Agostino Jr., A. J. Karter et al., "Intensity and amount of physical activity in relation to insulin sensitivity the Insulin Resistance Atherosclerosis Study," *Journal of the American Medical Association*, vol. 279, no. 9, pp. 669–674, 1998.

[2] J. E. Manson, P. Greenland, A. Z. LaCroix et al., "Walking compared with vigorous exercise for the prevention of cardiovascular events in women," *The New England Journal of Medicine*, vol. 347, no. 10, pp. 716–725, 2002.

[3] T. Christiansen, J. M. Bruun, E. L. Madsen, and B. Richelsen, "Weight loss maintenance in severely obese adults after an intensive lifestyle intervention: 2- to 4-year follow-up," *Obesity*, vol. 15, no. 2, pp. 413–420, 2007.

[4] K. M. Choi, "The impact of organokines on insulin resistance, inflammation, and atherosclerosis," *Endocrinology and Metabolism*, vol. 31, no. 1, pp. 1–6, 2016.

[5] S. A. Polyzos, J. Kountouras, K. Shields, and C. S. Mantzoros, "Irisin: a renaissance in metabolism?" *Metabolism: Clinical and Experimental*, vol. 62, no. 8, pp. 1037–1044, 2013.

[6] P. Boström, J. Wu, M. P. Jedrychowski et al., "A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis," *Nature*, vol. 481, no. 7382, pp. 463–468, 2012.

[7] G. Lombardi, F. Sanchis-Gomar, S. Perego, V. Sansoni, and G. Banfi, "Implications of exercise-induced adipo-myokines in bone metabolism," *Endocrine*, vol. 54, no. 2, pp. 284–305, 2016.

[8] S. S. Daskalopoulou, A. B. Cooke, Y.-H. Gomez et al., "Plasma irisin levels progressively increase in response to increasing exercise workloads in young, healthy, active subjects," *European Journal of Endocrinology*, vol. 171, no. 3, pp. 343–352, 2014.

[9] J. Y. Huh, A. Siopi, V. Mougios, K. H. Park, and C. S. Mantzoros, "Irisin in response to exercise in humans with and without metabolic syndrome," *Journal of Clinical Endocrinology and Metabolism*, vol. 100, no. 3, pp. E453–E457, 2015.

[10] Y. Tsuchiya, T. Ijichi, and K. Goto, "Effect of sprint training on resting serum irisin concentration—sprint training once daily vs. twice every other day," *Metabolism: Clinical and Experimental*, vol. 65, no. 4, pp. 492–495, 2016.

[11] J. Y. Huh, G. Panagiotou, V. Mougios et al., "FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise," *Metabolism: Clinical and Experimental*, vol. 61, no. 12, pp. 1725–1738, 2012.

[12] S. Pekkala, P. K. Wiklund, J. J. Hulmi et al., "Are skeletal muscle FNDC5 gene expression and irisin release regulated by exercise and related to health?" *Journal of Physiology*, vol. 591, no. 21, pp. 5393–5400, 2013.

[13] S. Raschke, M. Elsen, H. Gassenhuber et al., "Evidence against a beneficial effect of irisin in humans," *PlOS One*, vol. 8, no. 9, Article ID e73680, 2013.

[14] L. Gamas, P. Matafome, and R. Seicą, "Irisin and myonectin regulation in the insulin resistant muscle: implications to adipose tissue: muscle crosstalk," *Journal of Diabetes Research*, vol. 2015, Article ID 359159, 8 pages, 2015.

[15] T. Hofmann, U. Elbelt, and A. Stengel, "Irisin as a muscle-derived hormone stimulating thermogenesis—a critical update," *Peptides*, vol. 54, pp. 89–100, 2014.

[16] E. Dozio, A. E. Malavazos, E. Vianello et al., "Interleukin-15 and soluble interleukin-15 receptor α in coronary artery disease patients: association with epicardial fat and indices of adipose tissue distribution," *PLoS ONE*, vol. 9, no. 3, Article ID e90960, 2014.

[17] E. Dozio, S. Briganti, E. Vianello et al., "Epicardial adipose tissue inflammation is related to vitamin D deficiency in patients affected by coronary artery disease," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 25, no. 3, pp. 267–273, 2015.

[18] S. A. Polyzos and C. S. Mantzoros, "An update on the validity of irisin assays and the link between irisin and hepatic metabolism," *Metabolism: Clinical and Experimental*, vol. 64, no. 9, pp. 937–942, 2015.

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

[20] M. Hagströmer, P. Oja, and M. Sjöström, "The International Physical Activity Questionnaire (IPAQ): a study of concurrent and construct validity," *Public Health Nutrition*, vol. 9, no. 6, pp. 755–762, 2006.

[21] J. Y. Huh, V. Mougios, A. Skraparlis, A. Kabasakalis, and C. S. Mantzoros, "Irisin in response to acute and chronic whole-body vibration exercise in humans," *Metabolism*, vol. 63, no. 7, pp. 918–921, 2014.

[22] J. Y. Huh, V. Mougios, A. Kabasakalis et al., "Exercise-induced irisin secretion is independent of age or fitness level and increased irisin may directly modulate muscle metabolism through AMPK activation," *Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 11, pp. E2154–E2161, 2014.

[23] V. Singhal, E. A. Lawson, K. E. Ackerman et al., "Irisin levels are lower in young amenorrheic athletes compared with eumenorrheic athletes and non-athletes and are associated with bone density and strength estimates," *PLoS ONE*, vol. 9, no. 6, Article ID e100218, 2014.

[24] S. Ellefsen, O. Vikmoen, G. Slettaløkken et al., "Irisin and FNDC5: effects of 12-week strength training, and relations to muscle phenotype and body mass composition in untrained women," *European Journal of Applied Physiology*, vol. 114, no. 9, pp. 1875–1888, 2014.

[25] F. Norheim, T. M. Langleite, M. Hjorth et al., "The effects of acute and chronic exercise on PGC-1α, irisin and browning of subcutaneous adipose tissue in humans," *FEBS Journal*, vol. 281, no. 3, pp. 739–749, 2014.

[26] K. R. King-Morris, S. M. Deger, A. M. Hung et al., "Measurement and correlation of indices of insulin resistance in patients on peritoneal dialysis," *Peritoneal Dialysis International*, vol. 36, no. 4, pp. 433–441, 2016.

[27] M. Sperling, T. Grzelak, M. Pelczyńska et al., "Concentrations of omentin and vaspin versus insulin resistance in obese individuals," *Biomedicine and Pharmacotherapy*, vol. 83, pp. 542–547, 2016.

[28] T. Kurdiova, M. Balaz, M. Vician et al., "Effects of obesity, diabetes and exercise on Fndc5 gene expression and irisin release in human skeletal muscle and adipose tissue: in vivo and in vitro studies," *Journal of Physiology*, vol. 592, no. 5, pp. 1091–1107, 2014.

[29] M. Belviranli, N. Okudan, B. Kabak, M. Erdoğan, and M. Karanfilci, "The relationship between brain-derived neurotrophic factor, irisin and cognitive skills of endurance athletes," *Physician and Sportsmedicine*, vol. 44, no. 3, pp. 290–296, 2016.

[30] R. Abali, I. Temel Yuksel, M. A. Yuksel et al., "Implications of circulating irisin and Fabp4 levels in patients with polycystic ovary syndrome," *Journal of Obstetrics and Gynaecology*, vol. 36, pp. 897–901, 2016.

[31] A. Adamska, M. Karczewska-Kupczewska, A. Lebkowska et al., "Serum irisin and its regulation by hyperinsulinemia in women with polycystic ovary syndrome," *Endocrine Journal*, vol. 63, no. 12, pp. 1107–1112, 2016.

[32] G. Lippi, M. Montagnana, G. L. Salvagno, M. Franchini, and G. C. Guidi, "Glycaemic control in athletes," *International Journal of Sports Medicine*, vol. 29, no. 1, pp. 7–10, 2008.

[33] M. Li, M. Yang, X. Zhou et al., "Elevated circulating levels of irisin and the effect of metformin treatment in women with polycystic ovary syndrome," *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, pp. 1485–1493, 2015.