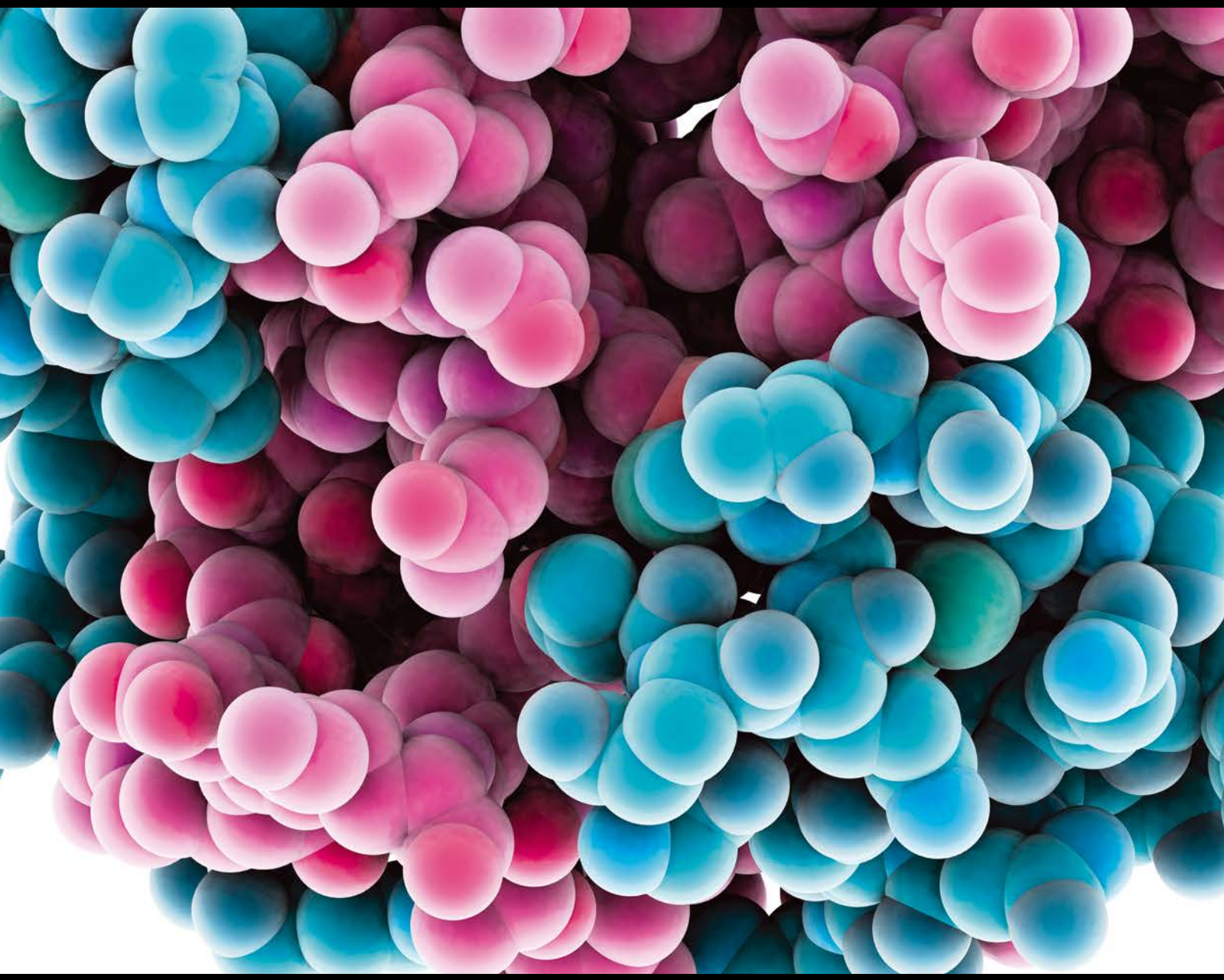
Journal of Diabetes Research

Nutritional Biomarkers, Gene-Diet Interaction, and Risk Factors for Type 2 Diabetes

Guest Editors: Ju-Sheng Zheng, Kaijun Niu, Simone Jacobs, Hassan Dashti, and Tao Huang



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Editorial

Nutritional Biomarkers, Gene-Diet Interaction, and Risk Factors for Type 2 Diabetes

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Prevalence of type 2 diabetes (T2D) has been increasing globally, gaining high public health priority [1]. T2D is a complex disease, resulting from an interplay between lifestyle and genetic factors. Evidence from several randomized controlled trials suggests that physical activity and dietary intervention could prevent or delay progression of T2D [2–4]. In addition, genome-wide association studies have identified hundreds of genetic variants associated with T2D [5]. In the present special issue, each paper investigated different aspects of T2D prevention strategy, ranging from physical activity and diet/nutritional biomarker to gene-epigenetic mechanisms, in animal models and human studies.

J. Qi et al. revealed that, in rats with insulin resistance induced by a high-fat diet, swimming exercise could improve insulin sensitivity, potentially via a reduction of TRIM72 expression, and upregulation of PI3-K/AKT signaling pathway, including IRS-1, p-AKT^{Ser473}, and AKT expression. The PI3-K/AKT signaling pathway is critically important for the regulation of insulin sensitivity and other biological processes related to aging [6, 7]. This study further showcased the intricate mechanism linking physical activity (swimming in this study) to insulin resistance. With similar design in a high-fat induced nonalcoholic fatty liver disease rodent model, Y. Xie et al. found that total alkaloid from Nelumbinis Plumula (NPA) could reduce insulin resistance via mechanism of restoring IRS1 and suppressing the JNK phosphorylation. Indeed, natural products derived from food resources have been proved to be promising natural remedies for T2D prevention and treatment [8].

Objectively measured nutritional biomarker may overcome recall bias and measurement error resulting from traditional dietary assessment tools, such as food frequency questionnaires. Emerging metabolomics studies are revealing more and more promising nutritional biomarkers and linking them to health outcomes. One of the examples is Branched-Chain Amino Acid (BCAA). In this issue, X. Zhao et al. systematically reviewed the current evidence on the relationship between BCAA and insulin resistance and concluded that circulating BCAAs (including valine, leucine, and isoleucine) are positively associated with insulin resistance and are useful biomarkers to predict future T2D cases. In a recent Mendelian randomization analysis, researchers identified a causal association of high circulating BCAAs with T2D risk and proved the causal role of BCAA metabolism in the T2D etiology [9]. Thus, use of nutritional biomarkers has obtained increasing popularity given their nature of objective measurement and potential usage to test causality in observational studies.

Lastly, L. Yang et al. updated the association of genetic variations in *LEPR* gene with T2D risk with a meta-analysis, providing robust evidence for the association between rs1137101 at *LEPR* and T2D risk. In addition, T. Matsha et al. demonstrated the role of DNA methylation-genetic variations in screen-detected diabetes or known diabetes on treatment in an African population. All the above efforts add

to the current knowledge on the role of genetic and epigenetic modulation on T2D etiology [10].

Taken together, articles in this special issue highlight several important fields in the prevention and treatment of T2D, focusing on the important role of diet and physical activity, incorporating natural remedies from food resources and new omics (metabolomics and genomics) strategies. The issue also suggests the importance of integrative strategies for the prevention and treatment of T2D in future research.

Ju-Sheng Zheng
Kaijun Niu
Simone Jacobs
Hassan Dashti
Tao Huang

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Research Article

Glucose Tolerance, *MTHFR* C677T and *NOS3* G894T Polymorphisms, and Global DNA Methylation in Mixed Ancestry African Individuals

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The aim of this study is to quantify global DNA methylation and investigate the relationship with diabetes status and polymorphisms in MTHFR C677T and NOS3 G894T genes in mixed ancestry subjects from South Africa. Global DNA methylation was measured, and *MTHFR* rs1801133 and *NOS3* rs1799983 polymorphisms were genotyped using high throughput real-time polymerase chain reaction and direct DNA sequencing. Of the 564 participants, 158 (28%) individuals had T2DM of which 97 (17.2%) were screen-detected cases. Another 119 (21.1%) had prediabetes, that is, impaired fasting glucose, impaired glucose tolerance, or the combination of both, and the remainder 287 (50.9%) had normal glucose tolerance. Global DNA methylation was significantly higher in prediabetes and screen-detected diabetes than in normal glucose tolerance (both $p \leq 0.033$) and in screen-detected diabetes compared to known diabetes on treatment ($p = 0.019$). There was no difference in global DNA methylation between known diabetes on treatment and normal glucose tolerance ($p > 0.999$). In multivariable linear regression analysis, only *NOS3* was associated with increasing global DNA methylation ($\beta = 0.943$; 95% CI: 0.286 to 1.560). The association of global DNA methylation with screen-detected diabetes but not treated diabetes suggests that glucose control agents to some extent may be reversing DNA methylation. The association between *NOS3* rs1799983 polymorphisms and DNA methylation suggests gene-epigenetic mechanisms through which vascular diabetes complications develop despite adequate metabolic control.

1. Introduction

Type 2 diabetes mellitus (T2DM) results from an interaction of environmental and genetic factors. Despite the initial enthusiasm from the identification of risk loci for T2DM [1, 2], the clinical utility of these genetic markers for T2DM risk prediction and reduction has remained limited. It is becoming increasingly evident that the gene-environment interaction in T2DM and other diseases can be explained in part by epigenetics. Indeed, a number of recent studies using different types of biological tissues from pancreas to peripheral blood mononuclear cells (PBMCs) are showing that DNA methylation patterns are altered in subjects with diabetes [3–6]. T2DM rates are increasing rapidly in African populations, with traditional diabetes risk factors failing to explain a great deal of these increases. Emerging alternate pathways involve the interplay between epigenetics, microbiome, and the immune system. Epigenetic mechanisms include DNA

methylation, lysine methylation, histone methylation, histone phosphorylation, RNA interference (RNAi), and genomic imprinting [7]. DNA methylation is the most extensively investigated epigenetic mechanism; however, DNA methylation profiling of populations from Africa remains to be investigated.

DNA methylation is an epigenetic process characterized by a covalent modification with the addition of a methyl group to the carbon at position 5 of cytosine nucleotides (H5), process catalyzed DNA methyltransferases (DNMTs) [8]. S-Adenosyl-methionine (SAM-CH3) donates the methyl group in a process catalyzed by methylenetetrahydrofolate reductase (MTHFR), an important enzyme in the folate metabolic pathway. Collectively, this folate and methionine cycles form the transmethylation pathway, which is modulated by a number of genetic and environmental factors that in turn affect DNA methylation [9]. In this regard, DNA hypomethylation has been reported in liver biopsies of subjects with type 2 diabetes (T2DM) with low folate levels [10], while *MTHFR* polymorphisms have been associated with decreased enzyme activity. Two *MTHFR* polymorphisms, +677C/T (rs1801133) and +1298A/C (rs1801131), have been widely studied and in vitro studies have shown these polymorphisms to decrease the enzyme activity by 35% and 60%, respectively [11, 12], consequently causing DNA hypomethylation. Another gene that has been shown to be affected by folate supplementation is nitric oxide synthase (NOS) [13, 14]. Nitric oxide synthase (eNOS, also referred to as NOS3) is an endothelial enzyme whose function is to synthesize nitric oxide (NO) from L-arginine [15, 16]. Nitric oxide is a unique molecule with diverse physiologic regulatory functions such as smooth muscle relaxation, inhibition of platelet aggregation, immune regulation, neurotransmission, and blood pressure regulation [17–19].

Therefore, this study was designed to examine global DNA methylation in individuals with and without diabetes and to evaluate whether polymorphisms in genes involved in DNA methylation and/or folate metabolism, namely, *MTHFR and NOS3*, have an effect on global DNA methylation in mixed ancestry subjects from South Africa.

2. Materials and Methods

2.1. Ethical Approval of the Study. The study was approved by the Faculty of Health and Wellness Sciences Ethics Committee of the Cape Peninsula University of Technology (CPUT) (NHREC: REC-230408-014) and was conducted according to the code of ethics of the World Medical Association (Declaration of Helsinki, 1975). All participants who were recruited for the study voluntarily signed written consent after the procedures had been fully explained in the language of their choice. Permission to conduct the study was granted by relevant authorities such as city and community authorities as previously described [20].

2.2. Study Design and Population. The present study was cross-sectional by design, involving participants from a mixed ancestry ethnic population group residing in Bellville South Township in Cape Town, South Africa. A detailed description of the survey and procedures conducted in the study are available elsewhere [21, 22]. Eligible participants were those who are older than 20 years, are residing in Bellville South, are of mixed ancestry origin, are not pregnant, and are not acutely ill.

2.3. Clinical Data. Clinical data which has been previously described [20] was collected in the form of a standardized questionnaire. During this time physical examination was conducted with data collection on blood pressure according to World Health Organization (WHO) guidelines [23] using a semiautomatic digital blood pressure monitor (Rossmax PA, USA) on the right arm in sitting position and anthropometric measurements. Body weight was measured to the nearest 0.1 kg with a Sunbeam EB710 digital bathroom scale, which was calibrated and standardized using a weight of known mass. Measurements were recorded with each subject wearing light clothing, without shoes and socks. Waist circumference was determined using a nonelastic tape at the level of the narrowest part of the torso, as seen from the anterior view. All anthropometric measurements were performed three times and their average was used for analysis. Participants with no history of doctor diagnosed diabetes mellitus underwent a 75 g oral glucose tolerance test (OGTT) as recommended by WHO [24].

2.4. Biochemical Analysis. Blood samples were collected from participants after overnight fast and processed as described in [20]. Plasma glucose levels and glycated haemoglobin (HbA1c) in whole blood collected in an EDTA tube were measured, respectively, by enzymatic hexokinase method and the turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics, Germany). Serum insulin was determined by a microparticle enzyme immunoassay (AxSYM, Abbott). High-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were estimated by enzymatic colorimetric methods in serum (Cobas 6000, Roche Diagnostics). Low-density lipoprotein cholesterol (LDL-C) was calculated using Friedwald's formula [25]. Body mass index (BMI) was calculated as weight/height $*$ height (kg/m^2) and waist-hip-ratio (WHR) as waist/hip circumference. Diabetes was based on a history of doctor diagnosis, fasting blood glucose concentration $\geq$ 7.0 mmol/L (or 126 mg/dL), and/or 2-hour post-OGTT plasma glucose $\geq$ 11.1 mmol/L (or 200 mg/dL).

2.5. Genotyping and DNA Methylation. Genomic DNA was extracted from whole blood samples collected in EDTA tubes using the salt extraction method and quantified with the NanoDrop ND-1000 instrument (Nanodrop Technologies, Wilmington, USA). Global DNA methylation was measured with the 5mC DNA ELISA kit according to the manufacturer's instructions (Zymo Research Corp., Irvine, CA, USA). Briefly, 100 ng of genomic DNA was allowed to bind to the enzyme linked immunosorbent assay (ELISA) plate, and the methylated fraction of DNA was detected using a 5-methylcytosine monoclonal antibody and quantified by an ELISA-like reaction and measuring the absorbance at

450 nm. The *MTHFR* rs1801133 and *NOS3* rs1799983 polymorphisms were genotyped using high throughput real-time polymerase chain reaction (RT-PCR) on the BioRad Optica (BioRad, USA) platform using Taqman genotyping assay (Applied Biosystems, USA). Conventional polymerase chain reaction followed by direct DNA sequencing was performed for analytical validation of high throughput genotyping.

2.6. Statistical Analysis. General characteristics of the study participants are summarized as count and percentage for dichotomous traits and median and 25th–75th percentiles for quantitative traits. Group comparisons used chi square and Kruskal-Wallis tests. Monotonous trends in the distribution of characteristics across quarters of global DNA methylation were assessed using the Cochran-Armitage trend test for proportions and Jonckheere-Terpstra trend test for medians. Robust regressions were then used to assess the effect of various traits on global DNA methylation in models accounting for age, gender, smoking, and glucose tolerance status. SNPs were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectation via a chi square goodness of fit test, and their effect on DNA methylation was investigated in linear regressions models, while always assuming a log-additive genetic model. Results corresponding to p values below 5% are described as significant. All analyses used the statistical software R (version 3.2.2 (2015-08-14), R Foundation for statistical computing, Vienna, Austria). SNPs analyses used the packages "*genetic*" and "*SNPassoc*."

3. Results

3.1. Global DNA Methylation and Cardiometabolic Profile. The study cohort consisted of 564 participants, including 438 (78%) females. One hundred and fifty-eight (28%) individuals had T2DM of which 97 (17.2%) were screen-detected cases. Another one hundred and nineteen (21.1%) had prediabetes, that is, impaired fasting glucose, impaired glucose tolerance, or the combination of both, and the remainder 287 (50.9%) had normal glucose tolerance. Global DNA methylation (%) was significantly higher in subjects with prediabetes or diabetes when compared to individuals with normoglycemia ($p < 0.05$). However, no significant differences were observed between subjects with prediabetes and diabetes. On further analysis, higher global DNA methylation in diabetics was driven by those with screen-detected diabetes. As shown in Figure 1, global DNA methylation was significantly higher in subjects with screen-detected diabetes than in those with normoglycemia ($p = 0.0003$) and remained significantly higher when compared to individuals with known diabetes ($p = 0.019$). However, there was no significant difference between known diabetic and normoglycemia subjects ($p > 0.999$). The characteristics of participants by glucose tolerance status are summarized in Table 1, mostly showing the expected differences.

The baseline characteristics of participants across quarters of global DNA methylation are summarized in Table 2. The proportion of participants with any diabetes increased across increasing quarters of global DNA methylation

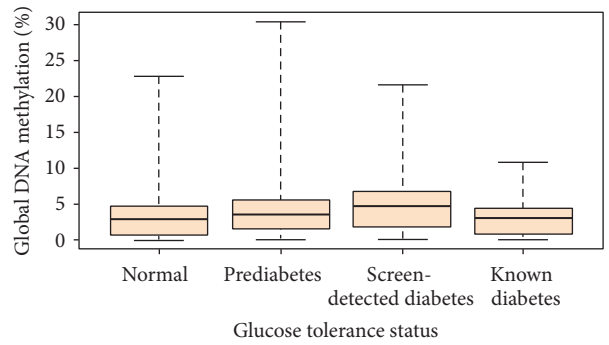

Figure 1: Boxplot showing the distribution of global DNA methylation across glucose tolerance status. Global DNA methylation was significantly higher in diabetic participants than in normotolerant participants ($p = 0.0003$), significantly higher in screen-detected diabetes than in known diabetes ($p = 0.0188$), and significantly higher in prediabetes than in normal glucose tolerance ($p = 0.0328$). But there was no difference in global DNA methylation between known diabetes and normal glucose tolerance ($p > 0.999$) and between known diabetes and prediabetes ($p = 0.408$); all p values are adjusted for multiple comparisons. Median global DNA methylation (25th–75th percentiles): normal: 2.81 (0.68–4.62; $N = 284$); prediabetes: 3.40 (1.53–5.45; $N = 120$); screen-detected diabetes: 4.62 (1.71–6.77; $N = 97$); known diabetes: 3.07 (0.80–4.45; $N = 61$).

($p = 0.028$) and in a linear fashion ($p = 0.008$ for linear trend). Across quarters of global DNA methylation levels, significant differences were also observed in the distribution of fasting ($p = 0.006$) and 2-hour glucose levels ($p = 0.002$), with increasing trends across quarters of global DNA methylation, always in linear fashions (both $p \leq 0.003$). Significant correlations were also observed between global DNA methylation and diabetes ($r = 0.101$; $p = 0.016$), body mass index and waist circumference (both, $r = 0.09$; $p = 0.033$), fasting blood glucose ($r = 0.126$; $p = 0.029$), and after 2-hour blood glucose ($r = 0.167$; $p = 0.0002$). In robust linear regression analysis adjusted for age, gender, status for hyperglycemia, and smoking, any diabetes ($\beta = 0.621$; $p = 0.036$) was associated with global DNA methylation. When participants with diabetes were distinguished into those with screen-detected diabetes or known diabetes, the association remained significant only for screen-detected diabetes ($\beta = 1.069$; $p = 0.004$) but not for known diabetes. In similar regression analyses, no other characteristics emerged as a significant predictor of DNA methylation, although borderline associations were observed with female gender, gamma-GT, and systolic blood pressure (Table 2). The association of any diabetes with DNA methylation also remained significant after expansion of the basic models to include the following correlates of diabetes status in the sample: BMI, waist circumference, systolic blood pressure, drinking status, HDL-cholesterol, total cholesterol, triglycerides, log-transformed CRP, and log-transformed gamma-GT ($\beta = 0.653$; $p = 0.038$).

3.2. Genetic Polymorphisms and DNA Methylation. Table 3 shows the genotype distribution of polymorphisms across the quarters of global DNA methylation. None of the single

Table 1: Characteristics according to glucose tolerance status.

Characteristic	Normotolerant	Prediabetes	Screen-detected diabetes	Known diabetes	p value
n (%)	283 (50.5)	119 (21.2)	97 (17.3)	61 (10.9)	
Median actual% 5mc	2.83 (0.71–4.62)	3.41 (1.55–5.45)	4.62 (1.71–6.77)	3.07 (0.80–4.45)	0.0001
Women, n (%)	215 (76.0)	98 (82.3)	81 (83.5)	40 (65.6)	0.030
Current smoking, n (%)	121 (42.8)	44 (37.0)	35 (36.1)	20 (32.8)	0.363
Current drinking, n (%)	80 (28.3)	33 (27.7)	25 (25.8)	12 (19.7)	0.573
Age (years)	49.7 (13.5)	54.0 (12.0)	58.0 (13.3)	58.3 (11.4)	<0.0001
Body mass index (kg/m^2)	28.7 (7.2)	31.2 (7.1)	32.5 (7.3)	30.5 (6.6)	<0.0001
Waist circumference (cm)	93 (14)	99 (15)	102 (16)	101 (14)	<0.0001
Hip circumference (cm)	108 (15)	112 (14)	114 (15)	109 (15)	0.007
Waist-to-hip ratio	0.86 (0.07)	0.89 (0.09)	0.89 (0.08)	0.92 (0.08)	<0.0001
Systolic blood pressure (mmHg)	119 (18)	122 (15)	129 (21)	128 (18)	<0.0001
Diastolic blood pressure (mmHg)	74 (11)	74 (10)	76 (12)	75 (12)	0.323
Fasting blood glucose (mmol/L)	5.0 (0.7)	5.7 (0.5)	8.5 (3.4)	11.1 (4.2)	<0.0001
2-hour glucose (mmol/L)	5.9 (1.0)	8.6 (1.1)	13.9 (5.7)	NA	<0.0001
HbA1c (%)	5.7 (0.4)	5.8 (0.4)	7.1 (1.8)	8.3 (1.8)	<0.0001
Fasting insulin (μU/mL)	6.2 (3.0–10.6)	7.2 (1.9–12.7)	9.3 (3.7–16.1)	8.0 (2.8–12.8)	0.013
2-hour insulin (μU/mL)	33.9 (17.1–54.4)	58.2 (26.1–97.9)	56.6 (21.2–108.6)	NA	<0.0001
C-reactive protein (mg/L)	2.8 (0.7–7.1)	6.6 (1.8–12.6)	6.4 (2.1–11.8)	4.0 (1.3–7.6)	<0.0001
Cotinine (ng/mL)	10.0 (9.0–307.5)	10.0 (9.0–270.2)	10.0 (9.0–213.0)	10.0 (9.0–125.2)	0.514
Gamma-glutamyltransferase (IU/L)	25.0 (17.0–36.5)	29.0 (22.0–41.0)	33.0 (24.0–54.0)	29.5 (20.0–45.7)	<0.0001
Triglycerides (mmol/L)	1.1 (0.8–1.4)	1.4 (1.1–1.8)	1.6 (1.2–2.1)	1.6 (1.2–2.2)	<0.0001
HDL-cholesterol (mmol/L)	1.3 (0.3)	1.3 (0.3)	1.3 (0.3)	1.1 (0.3)	0.007
LDL-cholesterol (mmol/L)	3.6 (1.0)	3.6 (1.0)	4.0 (1.1)	3.3 (1.1)	0.0001
Total cholesterol (mmol/L)	5.5 (1.2)	5.6 (1.1)	6.1 (1.3)	5.3 (1.1)	<0.0001

Values are count (percentages), mean (standard deviation), or median (25th–75th percentiles).

nucleotide polymorphisms (SNPs) deviated from Hardy-Weinberg Equilibrium (HWE) (all $p \geq 0.582$), and their distribution showed neither significant difference (all $p \geq 0.173$) nor linear trends (all $p \geq 0.205$ for linear trend) across the quarters of global DNA methylation. In linear regression analysis adjusted for age, gender, status for hyperglycemia, and smoking, only T allele of *NOS3* was associated with increasing global DNA methylation with a beta coefficient of 0.943 (95% confidence interval: 0.286 to 1.560) assuming a log-additive genetic model. This association remained significant in sensitivity analysis after exclusion of participants with known diabetes. In this subgroup, the beta coefficient was 1.102 (95% CI: 0.401 to 1.802) for the effect of T allele of *NOS3* on global DNA methylation.

4. Discussion

In this study, we measured global DNA methylation in PBMCs of mixed ancestry individuals with different levels of glucose tolerance, in parallel with genetic screening of polymorphisms in the *MTHFR* and *NOS3* genes. Our results show that global DNA methylation is increased in both prediabetic and diabetic states, but this increase was more pronounced in those with screen-detected diabetes, even after adjustment for extraneous factors such as age, gender, smoking, and glucose tolerance status. The distribution of the investigated SNPs did not differ across quarters of DNA methylation, but regression analysis under log-additive genetic model assumption revealed *NOS3* to be an independent determinant of global DNA methylation.

The strengths of our study include a well-characterized cohort and using the reference standard OGTT and a detailed drug history to carefully assign individuals to glucose tolerance status. Both T2DM and DNA methylation are affected by age, obesity, and genetic factors. In this regard, we also evaluated the effect of genes involved in DNA methylation and/or folate metabolism. We quantified DNA methylation across the whole genome using enzyme linked immunosorbent assay (ELISA), which is cost-effective and easy technique that is amenable for routine analysis in developing countries. The use of robust analytic methods also increases the reliability of our findings. For instance, by implementing robust correlations to eliminate the effect of outliers, we provide better estimates of the measures of association without loss of power [26].

In this study, we show that DNA methylation is significantly increased in subjects with screen-detected diabetes compared to those with diabetes and those on treatment. We believe that the lack of association between known diabetes and global DNA methylation in our sample is likely due to the reversal of DNA methylation changes by glucose controls agents in these individuals. These findings are in contrast with a study that quantified global DNA methylation by

Table 2: Baseline characteristics across quarters of global DNA methylation.

Characteristic	Global DNA methylation Q1	Q2	Q3	Q4	p difference*	p-trend	r (p value)**	β (p value)***
n	141	141	140	142				
Actual% 5mc								
Median	0.14	2.14	3.97	7.27				
Women, n (%)	100 (70.9)	109 (77.3)	115 (82.1)	114 (80.3)	0.117	0.036	0.076 (0.070)	0.543 (0.063)
Any diabetes	31 (22.0)	38 (26.9)	36 (25.7)	53 (37.3)	0.028	0.008	0.101 (0.016)	0.621 (0.036)
Diabetes status, n (%)					0.0007	0.145	0.082 (0.052)	
None	110 (78.0)	103 (73.0)	104 (74.3)	89 (62.7)				*Reference*
Screen-detected	15 (10.6)	23 (16.3)	17 (12.1)	42 (29.6)				1.069 (0.004)
Known diabetes	16 (11.3)	15 (10.6)	19 (13.6)	11 (7.7)				−0.026 (0.947)
Current smoking, n (%)	55 (39.0)	60 (42.5)	59 (42.1)	50 (35.2)	0.562	0.519	−0.038 (0.367)	−0.162 (0.505)
Current drinking, n (%)	40 (28.4)	37 (26.2)	37 (26.4)	36 (25.3)	0.950	0.594	−0.019 (0.648)	0.211 (0.494)
Age (years)	51 (41–64)	52 (44–60)	53 (44–62)	53 (44–64)	0.883	0.523	0.036 (0.391)	0.007 (0.443)
Body mass index (kg/m^2)	29.1 (24.4–33.4)	28.8 (23.6–34.7)	28.6 (24.3–34.3)	31.0 (26.3–34.9)	0.209	0.051	0.090 (0.033)	0.015 (0.464)
Waist circumference (cm)	96.0 (85.5–104.0)	96.5 (85.1–108.0)	97.0 (87.5–106.4)	99.5 (88.4–108.2)	0.256	0.053	0.090 (0.033)	0.007 (0.444)
Hip circumference (cm)	107.8 (98.0–116.5)	108.0 (99.2–118.0)	109.2 (100.2–117.5)	110.0 (101.5–121.0)	0.385	0.083	0.083 (0.050)	0.004 (0.685)
Waist-to-hip ratio	0.87 (0.82–0.93)	0.87 (0.83–0.93)	0.87 (0.84–0.92)	0.88 (0.83–0.93)	0.871	0.477	0.031 (0.465)	1.265 (0.470)
Systolic blood pressure (mmHg)	121 (109–137)	120 (109–134)	119 (108–129)	120 (109–133)	0.570	0.514	−0.025 (0.557)	−0.012 (0.090)
Diastolic blood pressure (mmHg)	75 (66–82)	74 (67–83)	73 (66–79)	73 (67–82)	0.529	0.379	−0.039 (0.357)	−0.013 (0.207)
Fasting blood glucose (mmol/L)	5.4 (4.9–6.0)	5.7 (5.0–6.3)	5.4 (5.0–6.3)	6.0 (5.0–7.1)	0.006	0.003	0.126 (0.029)	0.066 (0.225)
2-hour glucose (mmol/L)	6.6 (5.4–9.0)	6.9 (6.0–8.8)	7.0 (5.6–8.6)	7.8 (6.0–10.0)	0.002	0.0003	0.167 (0.0002)	0.011 (0.824)
HbA1c (%)	5.7 (5.5–6.1)	5.8 (5.5–6.3)	5.9 (5.6–6.3)	5.9 (5.5–6.3)	0.519	0.195	0.057 (0.181)	−0.039 (0.782)
Fasting insulin (μU/mL)	7.3 (3.7–11.7)	6.1 (2.1–12.9)	6.7 (2.9–12.4)	8.3 (3.0–12.8)	0.435	0.961	−0.005 (0.900)	−0.016 (0.133)
2-hour insulin (μU/mL)	36.3 (19.9–64.3)	38.7 (18.3–68.4)	39.7 (10.2–68.1)	44.1 (19.5–89.4)	0.485	0.131	0.070 (0.120)	0.0004 (0.860)
C-reactive protein (mg/L)	3.6 (0.9–9.2)	4.4 (1.1–9.6)	2.8 (0.9–9.4)	5.3 (1.7–11.7)	0.091	0.121	0.080 (0.059)	0.005 (0.641)
Cotinine (ng/mL)	10.0 (9.0–282.1)	10 (9–310)	10 (9–270)	10.0 (9.0–197.2)	0.417	0.323	−0.059 (0.160)	−0.002 (0.109)
Gamma-glutamyltransferase (IU/L)	27.0 (18.0–40.0)	28.0 (20.0–45.7)	27.5 (19.0–39.2)	28.0 (20.2–44.5)	0.820	0.625	0.0005 (0.991)	−0.002 (0.081)
Triglycerides (mmol/L)	1.23 (0.93–1.74)	1.24 (0.90–1.69)	1.27 (0.87–1.81)	1.34 (1.06–1.92)	0.096	0.048	0.079 (0.061)	−0.065 (0.642)
HDL-cholesterol (mmol/L)	1.28 (1.11–1.46)	1.22 (0.99–1.45)	1.24 (1.03–1.45)	1.16 (0.99–1.44)	0.138	0.058	−0.081 (0.055)	−0.509 (0.130)
LDL-cholesterol (mmol/L)	3.49 (2.84–4.28)	3.43 (2.80–4.05)	3.62 (3.00–4.26)	3.68 (2.90–4.39)	0.279	0.136	0.079 (0.062)	0.078 (0.538)
Total cholesterol (mmol/L)	5.52 (4.70–6.40)	5.33 (4.59–6.05)	5.59 (4.75–6.37)	5.54 (4.93–6.46)	0.183	0.275	0.061 (0.147)	−0.009 (0.933)

* p values from Kruskal-Wallis and chi square tests for the differences across quarters of global DNA methylation.
** Spearman correlation coefficients and p values for the continuous associations of global DNA methylation with covariates.
*** Beta coefficients and p values from age, gender, and status for hyperglycemia and smoking adjusted robust linear regressions for the prediction of global DNA methylation by various traits.

Table 3: Genotype distribution across the quarters of global DNA methylation.

Characteristic	Global DNA methylation				p difference*	p-trend	p HWE	β (95% CI)***
	Q1	Q2	Q3	Q4				
n	111	110	112	111				
Actual% 5mc								
Median	0.11	2.12	3.94	7.24				
MTHFR rs1801133					0.665	0.255	0.697	−0.387 (−1.054 to 0.281)
C/C	75 (68.8)	78 (71.6)	85 (76.6)	86 (77.5)				
C/T	32 (29.4)	27 (24.8)	24 (21.6)	23 (20.7)				
T/T	2 (1.8)	4 (3.7)	2 (1.8)	2 (1.8)				
NOS3 rs1799983					0.173	0.205	0.582	0.943 (0.286 to 1.560)
G/G	82 (76.6)	73 (68.9)	81 (77.1)	65 (62.5)				
G/T	23 (21.5)	28 (26.4)	22 (20.9)	36 (34.6)				
T/T	2 (1.9)	5 (4.7)	2 (1.9)	3 (2.9)				

* p values from Kruskal-Wallis and chi square tests for the differences across quarters of global DNA methylation.
*** Beta coefficients and 95% confidence interval from age, gender, and status for hyperglycemia and smoking adjusted linear regressions for the prediction of global DNA methylation by various SNPs assuming a log-additive genetic model.
HWE: Hardy-Weinberg equilibrium.

bisulfite pyrosequencing and showed a null effect of diabetes medication on the association between DNA methylation and insulin resistance [27]. The difference between Zhao et al. [27] and our study may be attributed to the sample size of these two studies. Their study analyzed global DNA methylation of 17 diabetics on glucose control agents, whereas 61 diabetics on medication were included in our study. Genetic polymorphisms have been shown to be responsible for individual responses to many oral antidiabetic drugs [28, 29], and these polymorphisms may also be subject to epigenetic regulation. It is thought that antidiabetic drugs could influence methylation trends through gene expression mechanisms and may represent a possible confounding factor for the identification of T2DM related epigenetic profiles [3]. Furthermore, our results are in contrast with those reported in liver biopsies of subjects with T2DM where genome-wide analysis of DNA methylation identified 94% hypomethylated CpG sites in these individuals [10]. Although diabetic subjects in this study were similarly obese as in Nilsson et al. [10], the differences in the cell types and liver versus blood leucocytes as well as the different methods for quantifying DNA methylation may explain differences in DNA methylation.

Although global DNA methylation correlated positively with adiposity indices, there was no correlation with age. The lack of association between DNA methylation and age is at variance with a number of studies that have repeatedly shown decreasing DNA methylation with increasing age [30–32]. The narrow age range in our study has likely masked any association between DNA methylation and age. We also did not find an association between the MTHFR C677T polymorphism and global DNA methylation, despite previous reports that have shown an association between MTHFR C677T, methyl group generation, and subsequent DNA methylation [33, 34]. Contrary to our prior hypothesis that *MTHFR* gene polymorphisms will affect global DNA methylation, we observed that global DNA methylation was associated with *NOS3* G894T polymorphism. Reduced production of NO is one of the most important contributors to endothelial dysfunction, an initiator of vascular complications [15–19]. NOS3 is encoded by the *NOS3* gene located on chromosome 7q35-36 and three polymorphisms (G894T, 4b/a, and T786C) in the *NOS3* gene are associated with diabetes and diabetes related traits and reduced enzyme activity and consequently bioavailability of NO and endothelial function [19, 35, 36]. It is not clear how NOS3 may influence global DNA methylation, but a study involving obese children and type 1 diabetes with *NOS3* polymorphisms demonstrated an improved endothelial function after supplementation with folate [37]. Similarly, the authors obtained differing results with respect to MTHFR as no improvement in endothelial function was evident in those with *MTHFR* polymorphisms suggesting two independent mechanisms for vascular response to folate for both NOS3 and MTHFR [37]. Folate plays an important role in DNA methylation and several studies have shown aberrant DNA methylation profiles in relation to folate levels [10, 38]. Individuals with T2DM are at an increased risk of both micro- and macrovascular complications and it has previously been shown that glycemic control is not adequate to halt the progression of vascular complications [39–41]. Thus, the observed association between *NOS3* G894T gene polymorphisms and global DNA methylation suggests gene-epigenetic mechanisms through which vascular complications develop despite adequate glycemic control in individuals with T2DM.

The current study represents a significant contribution in the African setting, where research about the contribution of epigenetics to the growing burden of noncommunicable disease in the region is lacking [42]. In summary, our data indicates that global DNA methylation is significantly associated with screen-detected diabetes compared to known diabetes on treatment, leading us to speculate that glucose control agents to some extent may be reversing DNA methylation. Furthermore, we showed that DNA methylation is

independent of *MTHFR* polymorphism but is influenced by *NOS3* gene polymorphism G894T. It is known that epigenetic variations are regulated in a tissue-specific manner; however, the use of peripheral blood to assess DNA methylation is recommended for epidemiologic samples [43]. Furthermore, DNA methylation in blood presents a feasible marker for the diagnosis, prevention, and management of T2DM. To fully elucidate the role of DNA methylation in this population, future studies should explore gene-specific methylation together with global DNA methylation analysis.

Disclosure

Any opinion, finding, and conclusion or recommendation expressed in this material is that of the authors and the MRC does not accept any liability in this regard.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Tandi E. Matsha made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, and final approval of the version to be published. Carmen Pheiffer made substantial contributions to conception and design, acquisition of data, revising it critically for important intellectual content, and final approval of the version to be published. Tinashe Mutize made substantial contributions to design, acquisition of data, revising it critically for important intellectual content, and final approval of the version to be published. Rajiv T. Erasmus revised the paper critically for important intellectual content and contributed to final approval of the version to be published. Andre P. Kengne contributed to analysis and interpretation, revising the paper critically for important intellectual content, and final approval of the version to be published.

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Research Article

Swimming Exercise Alleviated Insulin Resistance by Regulating Tripartite Motif Family Protein 72 Expression and AKT Signal Pathway in Sprague-Dawley Rats Fed with High-Fat Diet

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We aimed to investigate whether swimming exercise could improve insulin resistance (IR) by regulating tripartite motif family protein 72 (TRIM72) expression and AKT signal pathway in rats fed with high-fat diet. Five-week-old rats were classified into 3 groups: standard diet as control (CON), high-fat diet (HFD), and HFD plus swimming exercise (Ex-HFD). After 8 weeks, glucose infusion rate (GIR), markers of oxidative stress, mRNA and protein expression of TRIM72, protein of IRS, p-AKT^{Ser473}, and AKT were determined in quadriceps muscles. Compared with HFD, the GIR, muscle SOD, and GSH-Px were significantly increased ($p < 0.05$, resp.), whereas muscle MDA and 8-OHdG levels were significantly decreased ($p < 0.05$ and $p < 0.01$) in Ex-HFD. Expression levels of TRIM72 mRNA and protein in muscles were significantly reduced ($p < 0.05$ and $p < 0.01$), whereas protein expression levels of IRS-1, p-AKT^{Ser473}, and AKT were significantly increased in Ex-HFD compared with HFD ($p < 0.01$, $p < 0.01$, and $p < 0.05$). These results suggest that an 8-week swimming exercise improves HFD-induced insulin resistance maybe through a reduction of TRIM72 in skeletal muscle and enhancement of AKT signal transduction.

1. Introduction

Insulin resistance (IR) is the important pathophysiological basis of diseases such as type 2 diabetes mellitus (T2DM), hypertension, hyperlipidemia, and obesity [1, 2]. Insulin primarily acts on liver and fatty tissues, as well as skeletal muscles. Skeletal muscle, which is responsible for more than 30% of energy consumption, is the major peripheral tissue of glucolipid metabolism under insulin stimulation [3]. Several cytokines and peptides, also called myokines, can be synthesized by skeletal muscle cells in type 2 diabetes and are possibly involved in IR [4]. Previously, tripartite motif family protein 72 (TRIM72), also named MG53, is the one with three conserved domains known as "molecular bandage" for muscle cell damage [5–7], while recent studies [8, 9] found that TRIM72 expression is obviously upregulated in pathogenesis of IR and acts as an E3 ligase targeting the insulin receptor and insulin receptor substrate-1 (IRS-1) for ubiquitin-dependent degradation, subsequently inhibiting PI3-K/AKT signal transduction. During IR pathogenesis, abnormally high TRIM72 expression in skeletal muscles was higher than that in other organs, which was the important originating mechanism leading to systemic metabolic disorders [10–12]; it suggests that TRIM72 may be a specific muscle factor in early stage of IR pathological genesis. Moreover, TRIM72 expression is increased under the condition of oxidative stress induced with high-fat diet [5]. These findings suggest that regulation of TRIM72 to AKT signal pathway is involved in the IR pathogenesis.

The insulin receptor substrate (IRS) family of proteins plays a central role in insulin signal transduction. Following activation of the insulin receptor kinase, IRS-1, IRS-2, IRS-3, and IRS-4 undergo phosphorylation of multiple tyrosine residues in unique sequence motifs throughout the molecule.

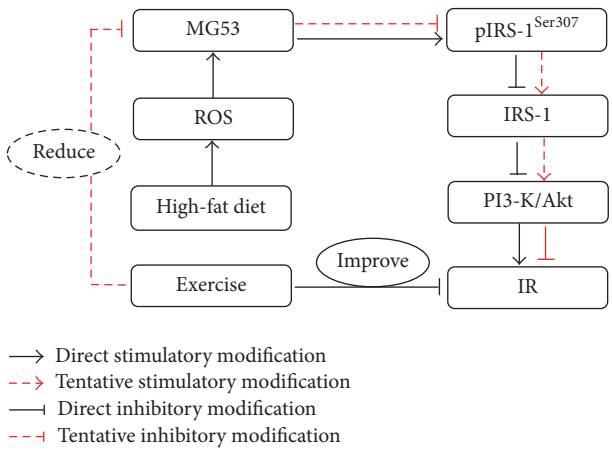

Figure 1: The signaling pathway of high-fat diet induced IR through TRIM72 and PI3-K/AKT in skeletal muscle. Whether the swimming exercise can improve insulin resistance in Sprague-Dawley rats fed with high-fat diet through inhibiting TRIM72 expression and activating PI3-K/AKT signal pathway should be further testified.

As the studies shown mice with targeted disruption of the IRS genes lend some support to both situations. Despite the absence of IRS-1 and IRS-2, which are the major IRS expressed in all tissues could induce IR, IRS-1 and IRS-2 produce different phenotypes [13–18]; this suggests that IRS-1 and IRS-2 play significant and nonredundant roles in growth and regulation of glucose homeostasis. Studies also demonstrated that IRS-3 and IRS-4 show a restricted tissue distribution and are not critically involved in regulating growth and glucose homeostasis [19–21]. Although studies showed that TRIM72 was targeting IRS-1, whether expression of IRS-2, IRS-3, and IRS-4 is also involved in the TRIM72 in IR rats fed with high-fat diet should need further studies.

Convincing evidences demonstrate that exercise can improve IR [22, 23] partially through PI3-K/AKT signal pathway [24, 25]. However, the detailed mechanisms involved remain to be verified. We therefore established the IR model in Sprague-Dawley rats fed with high-fat diet to observe the effect of swimming exercise on parameters such as lipid deposition, oxidative stress, and TRIM72 expression in the skeletal muscles of the IR rats (see Figure 1). Moreover, we aimed to reveal the possible rule of TRIM72 in IR prevention. We hypothesized that IR can be effectively improved by exercise through regulating TRIM72/IRS-1/AKT pathway and that the decreased TRIM72 may be mediated by the lower levels of ROS which are induced by exercise.

2. Material and Methods

2.1. Animals. Male Sprague-Dawley rats, aged 5 weeks old with body weight of 130 ± 20 g, provided by the experimental animal center of Jiangsu University (certification number SCXK (Su) 2009-0002), were housed under standard conditions (22 ± 2°C, humidity 50 ± 10%, cycles of 12 h light/12 h dark). Experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China in 1998, and were approved by the Animal Ethics Committee of Shanghai Normal University. Thirty Sprague-Dawley rats were randomly divided into 3 groups: standard diet as control (CON), high-fat diet (HFD), and HFD plus swimming exercise (Ex-HFD). The control diet contained 65% carbohydrates, 22% protein, and 13% fat and the high-fat diet consisted of 40% carbohydrates, 22% protein, and 38% fat.

2.2. Exercise Program. The modifications of exercise program were conducted in accordance with the Bendford program [26]. In brief, rats in Ex-HFD group were trained to swim 10–30 min per session for 5 days to reduce water-induced stress. Rats were placed in a plastic pool of $150 \times 60 \times 70\ cm^3$ with a water temperature at 30–32°C; the rats underwent swimming exercise for 45 min twice/day, 6 days/week, for 8 weeks.

2.3. Blood Samples Collection and Blood Biochemistry. Twenty hours after the final session of swimming exercise, all rats were anesthetized by single-dose intraperitoneal injection of 10% chloral hydrate (50 mg/kg). Blood samples were collected from abdominal aorta and serum was separated by centrifugation at 1200x rpm for 10 min. Serum glucose level was measured using an autoanalyzer (RT-1904C; Rayto, China). Insulin concentration in serum was determined using a radioimmunoassay described by Laemmli [27].

2.4. Calculation of Homeostasis Model Assessment of Insulin Resistance (HOMA-IR). The HOMA-IR was calculated using the following formula: FBG (mmol/L) × FIN (mIU/L)/22.5 [28], where FBG is fasting blood glucose and FINS is fasting insulin concentration.

2.5. Euglycemic Clamp Test. The improved Kraegen method [29] was used to measure the glucose infusion rate (GIR). Briefly, the rats were injected with 50 mg/kg anesthesia containing 10% chloral hydrate and then fixed in supine position. A thin silicon tube was inserted into the jugular vein to connect with peristaltic pump for infusion of insulin and peristaltic pump for infusion of 10% glucose through a three-way tube. Insulin at $4\ mU \cdot kg^{-1} \cdot min^{-1}$ and 10% glucose were infused through the bilateral veins (constant speed). Blood sample was collected every 10 min through the venule to measure the blood glucose level, and GIR was constantly adjusted until glucose homeostasis was achieved. Six GIR values were recorded within 60 min under glucose homeostasis, and the average value was used as the GIR under glucose homeostasis.

2.6. Skeletal Muscle Sampling. After injecting peritoneal anesthesia with 10% chloral hydrate for 50 mg/kg, the red quadriceps femoris of the bilateral thighs was isolated, placed into liquid nitrogen immediately, and stored at −80°C. Subsequently, 0.9% normal saline was added to the skeletal muscle tissue with a ratio of 50 mg : 50 μL (quality fraction: 10%) to prepare a tissue homogenate by using an ultrasonic crasher (40 amp, 5 sec each time, 10 sec for each interval; operation was repeated five times). The homogenate was centrifuged at 3000 rpm for 15 min under 4°C. Supernatant was collected

and stored at −20°C for further testing the level of skeletal muscle TG and oxidative stress parameters.

2.7. Measurement of Free Fatty Acid (FFA), Triglyceride (TG), and Oxidative Stress Parameters in Skeletal Muscle. The levels of FFA, TG, SOD, GSH-Px, and MDA in skeletal muscle were determined with free fatty acid determination kit (Sigma), triglyceride determination kit (Sigma), superoxide dismutase determination kit (Sigma), and glutathione peroxidase determination kit (Sigma), respectively, following the manufacturer's instructions. The 8-hydroxyl-deoxyguanosine (8-OHdG) content was measured using enzyme-linked immunosorbent assay (ELISA) (R&D, Minneapolis, USA).

2.8. Quantitative Real-Time PCR Analysis. Total RNA was extracted and purified from the cells using the RNA isolator Total RNA Extraction Reagent (TaKaRa, Kusatsu, Japan) and subjected to reverse transcription using the PrimeScript™ RT Master Mix Kit (TaKaRa, Kusatsu, Japan). The real-time PCR and data collection were subsequently performed as described previously [30] using the AceQ® qPCR SYBR® Green Master Mix kit (TaKaRa, Kusatsu, Japan) on ABI 7500 system (ABI, New York, USA). Primers used for the amplification were as follows: TRIM72-F: 5′-CGAGCAGGACCGCACACTT-3′, TRIM72-R: 5′-CCAGGAACATCCGCATCTT-3′; insulin receptor substrate-1 (IRS-1) F: 5′-GAAGAAGTGGCGGCACAAGT3′, IRS-1 R: 5′-GTCAGGCAGAGGCGGTAGAT-3′; IRS-2 F: 5′-ATACCGCCTATGCCTGTCTG-3′, IRS-2 R: 5′-AGAAGAAGCTGTCCGAGTGG-3′; IRS-3 F: 5′-GCAGAGCAGCAAACATGGTA-3′, IRS-3 R: 5′-GCGAAGATCCAAGACTCAGG-3′; IRS-4 F: 5′-TTGCTGACAGTGCCATTTGC-3′, IRS-4 R: 5′-TGCACTTCTTCCTGCCTAGC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) F: 5′-GC ACCGTCAAGGCTGAGAAC-3′, GAPDH R: 5′-TGGTGAAGACGCCAGTGG A-3′. The relative expression levels of the indicated mRNA normalized against GAPDH mRNA were calculated using the $2^{-\Delta\Delta CT}$ methods.

2.9. Protein Extraction and Western Blot. Western blot was performed following standard methods. Briefly, the samples were placed in protein extraction solution (lysis in RIPA) and ultrasonicated at maximum speed at 4°C for 30 s (Sonics, Newtown, USA). The homogenate was centrifuged at 12000 rpm at 4°C for 30 min. After denaturation, the samples were subjected to 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, USA). The membranes were then blocked with 5% nonfat dried milk and incubated with primary antibodies and followed by incubation with horseradish peroxidase-(HRP-) linked secondary antibodies. The antibodies used in the current study were rabbit polyclonal anti-IRS-1 (1 : 2000, Cell Signaling Technology, MA, USA), rabbit polyclonal anti-pIRS-1 (Ser307) (1 : 2000), rabbit polyclonal anti-IRS-2 (1 : 1000, CST), and HRP-linked anti-rabbit IgG (1 : 2000); rabbit polyclonal anti-IRS-3 (1 : 200, Santa Cruz Biotechnology, CA, USA), goat polyclonal anti-IRS-4 (1 : 200), goat polyclonal anti-TRIM72 (1 : 200), and HRP-linked anti-goat IgG (1 : 2000); and mouse monoclonal anti-GAPDH (1 : 1000) and HRP-linked donkey anti-goat IgG (1 : 3000) (KangChen Biotech, Shanghai, China). Immunoreactive protein bands were visualized with the Pierce ECL Plus Western Blot Substrate (Thermo Fisher Scientific, Rockford, IL, USA). GAPDH was performed as an internal loading control, and quantification of the band intensity was performed using ImageJ software (NIH) and defined as the fold-change of the CON after being normalized against GAPDH.

2.10. Statistical Analysis. Firstly, we test the normality of data distribution; the data with normal distribution were expressed as mean (M) ± standard deviation (SD) and the data with skewed distribution were log-transformed. Pearson correlation analysis was performed for the parameters, and one-way ANOVA for significance was employed for intergroup data. Multiple comparisons were performed using LSD. The level of significant difference was set at $p < 0.05$. All analyses were completed with SPSS (version 17.0, SPSS Inc., 233 S Wacker Drive, 11th Floor, Chicago, IL 60606).

3. Results

3.1. Blood Glucose, Blood Insulin, and Insulin Resistance Index. To evaluate insulin sensitivity, euglycemic clamp was firstly performed; the results showed that the GIR of group HFD considerably decreased ($p < 0.01$) compared with that in group CON, while the GIR in rats of group Ex-HFD significantly increased ($p < 0.01$) compared with that in group HFD (Figure 2(a)). Furthermore, to assess IR, we next detected fasting blood glucose (FBG) and fasting blood insulin (FIN) to calculate HOMA-IR index. As shown in Figures 2(b), 2(c), and 2(d), there were no significant differences of FBG among group CON, group HFD, and group Ex-HFD ($p > 0.05$, Figure 2(b)); the level of the FIN in group HFD was significantly higher than that in group CON ($p < 0.01$); the FIN in the Ex-HFD group was significantly lower than that in HFD group ($p < 0.01$, Figure 2(c)); and the HOMA-IR was markedly increased ($p < 0.01$) compared with that in group CON. However, the HOMA-IR was greatly decreased ($p < 0.01$) compared with that in group HFD (Figure 2(d)). These results proved that the animal model prepared using high-fat diet is suitable for the current study, and an 8-week swimming exercise can improve IR in rats induced by high-fat diet.

3.2. FFA, TG, and Oxidative Stress Parameters in Skeletal Muscles. To observe the lipid accumulation in skeletal muscles after a long-time high-fat diet in rats, we measured the levels of FFA and TG. As shown in Table 1, the FFA and TG of the skeletal muscle in group HFD were significantly higher than those in group CON ($p < 0.01$, resp.). The FFA and TG of the skeletal muscle in group Ex-HFD were significantly lower than those in group HFD ($p < 0.05$). Moreover, to confirm the oxidative stress in skeletal muscles after a long-time high-fat diet in rats, we also detected the levels of SOD, GSH-Px, MDA, and 8-OHdG in rat skeletal muscles. For the test, the levels of SOD and GSH-Px in group HFD were significantly decreased ($p < 0.05$, resp.) than those in group CON, while MDA and 8-OHdG contents of the skeletal muscles in group

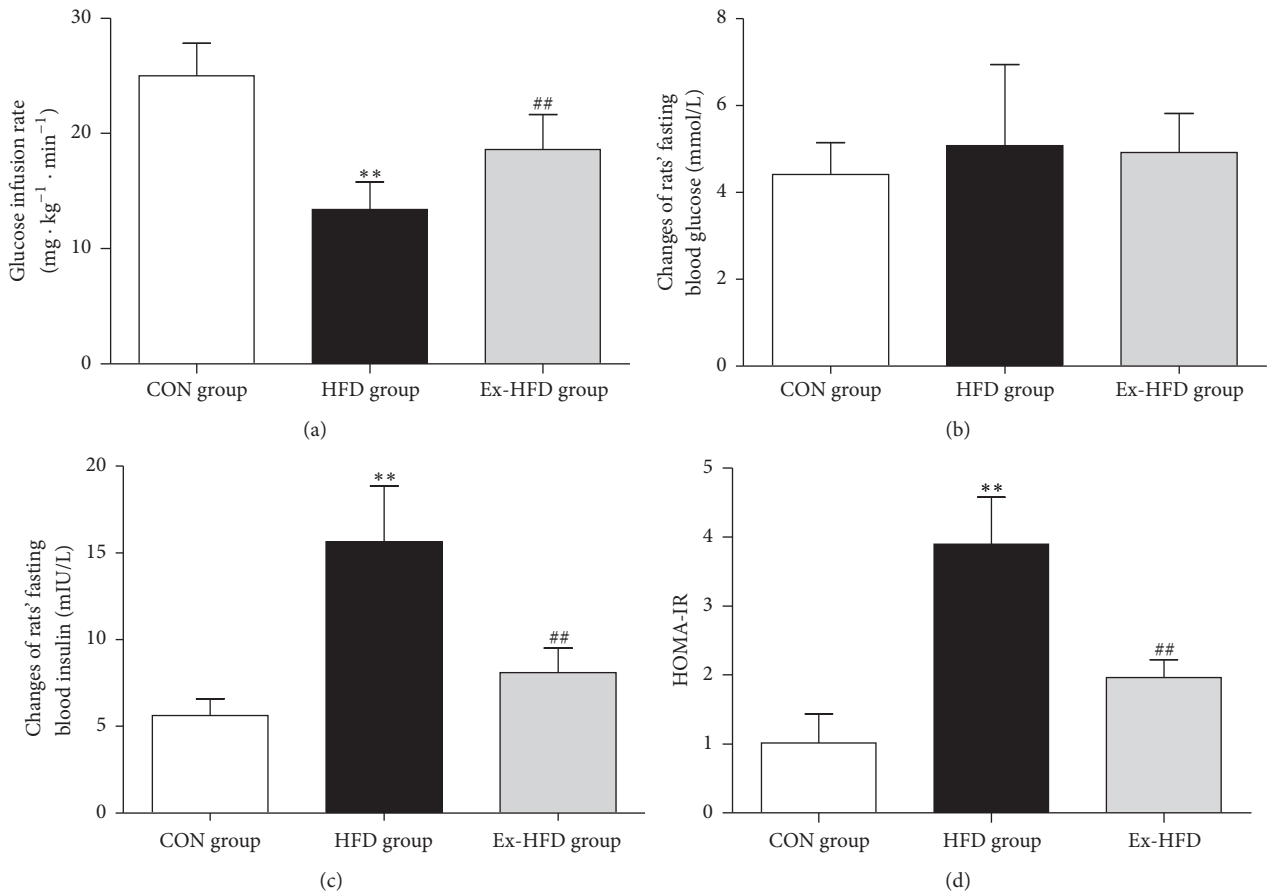

Figure 2: Blood glucose, insulin, and insulin resistance index in the three groups. (a) The fasting blood glucose in three groups at the 8th week. There were no significant differences in the fasting blood glucose measured after 8-week swimming exercise intervention compared with the untreated high-fat-diet group. (b) The fasting blood insulin in three groups at the 8th week. There was significant decrease in the fasting blood insulin that was measured after 8-week swimming exercise intervention compared with the untreated high-fat-diet group. (c) The GIR of three groups. The GIR is commonly used in evaluating insulin resistance. After training with swimming exercise, GIR in group Ex-HFD was significantly increased. (d) HOMA-IR value of three groups at the 8th week, which was also selected to evaluate insulin resistance. After training with swimming exercise, HOMA-IR was improved. $^{**}p < 0.01$, versus CON group; $^{\#\#}p < 0.01$, versus group HFD group.

HFD obviously increased ($p < 0.05$ and $p < 0.01$, resp.). After 8 weeks of exercise, the SOD and GSH-Px activities in group Ex-HFD significantly increased compared with those in group HFD ($p < 0.05$ and $p < 0.01$, resp.). However, the MDA and 8-OHdG contents in group Ex-HFD significantly decreased ($p < 0.05$ and $p < 0.01$, resp.). These findings indicate that 8-week swimming exercise can reduce the lipid accumulation and the level of oxidative stress in skeletal muscles of rats after being fed with high-fat diet.

3.3. Quantitative Real-Time PCR Analysis. Since high-fat diet could induce oxidative stress, we further verified the effects of exercise on TRIM72 and IRS gene expression in skeletal muscle induced by oxidative stress after high-fat diet, and qPCR was performed. As shown in Figure 3, the mRNA expression of TRIM72 and IRS-3 in group HFD was significantly higher than those in group CON ($p < 0.01$ and $p < 0.01$, resp.); mRNA expression of IRS-1 and IRS-2 in group HFD was significantly lower than that in group CON ($p < 0.01$, resp.). Compared with group HFD, the mRNA expression of TRIM72 and IRS-3 in group Ex-HFD significantly decreased ($p < 0.01$ and $p < 0.05$, resp.); the mRNA expression of IRS-1 and IRS-2 in group Ex-HFD was significantly increased than those in group HFD ($p < 0.01$). No significant differences of mRNA expression of IRS-4 were shown among the group CON, group HFD, and group Ex-HFD. These findings demonstrate that exercise can decrease the mRNA expression of TRIM72 and IRS-3 and increase the mRNA expression of IRS-1 and IRS-2 of skeletal muscles in rats fed with high-fat diet.

3.4. Western Blot Analysis. TRIM72, pIRS-1^{Ser307}, IRS-1, IRS-2, IRS-3, IRS-4, pAKT^{Ser473}, and AKT protein expression levels were detected by western blot method in skeletal muscle. To prove the effects of exercise on protein expression of TRIM72, IRS, and AKT in skeletal muscle induced by long-term high-fat diet, we performed western blot. As shown in Figure 4, the TRIM72 protein expression in group HFD was significantly higher than that in group CON ($p < 0.01$); the TRIM72 protein expression in group Ex-HFD

Table 1: Effect of swimming exercise on FFA, TG, and oxidative stress parameters in skeletal muscle.

	CON	HFD	Ex-HFD
FFA (nmol/g pro)	2.32 ± 0.77	5.59 ± 1.39**	3.28 ± 0.83*
TG (mmol/g pro)	0.35 ± 0.12	0.28 ± 0.09**	0.38 ± 0.18#
SOD (U/g pro)	19.57 ± 6.55	12.09 ± 5.72*	18.15 ± 5.15#
GSH-Px (U/g pro)	0.84 ± 0.10	0.41 ± 0.09*	0.66 ± 0.12##
MDA (nmol/g pro)	15.31 ± 2.07	19.34 ± 3.32*	15.51 ± 3.72#
8-OHdG (ng/g pro)	289.35 ± 19.84	538.72 ± 61.71**	418.77 ± 42.87##

FFA, triglycerides, SOD, GSH-Px, MDA, and 8-OHdG of all rats were measured at the 8th week of the experiment. Results were expressed as mean (M) ± standard deviation (SD) ($n = 10$). Differences between groups were compared by one-way analysis of variance (ANOVA). We could observe an increase in FFA, TG, MDA, and 8-OHdG and a decrease in SOD and GSH-Px in IR rats, but exercise could decrease the levels of FFA, TG, MDA, and 8-OHdG and increase SOD and GSH-Px in skeletal muscle of rats. $^{**}p < 0.01$: versus CON group; $^{*}p < 0.05$: versus CON group; $^{\#\#}p < 0.01$: versus group HFD group; and $^{\#}p < 0.05$: versus group HFD group.

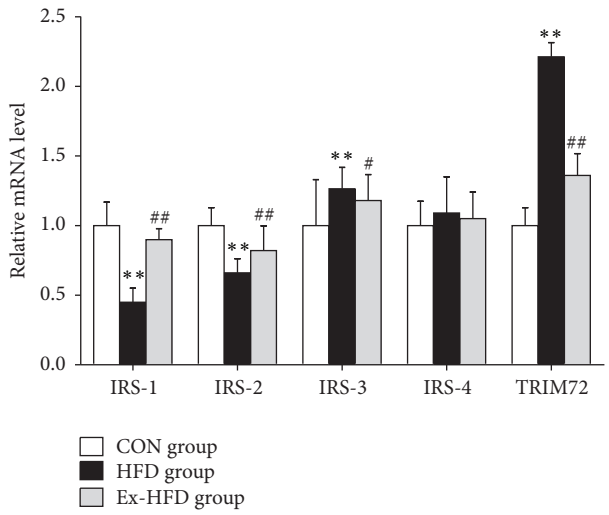

Figure 3: Effect of swimming exercise on mRNA expression of IRS-1 and TRIM72 in skeletal muscle. Data are presented as mean ± SD. For each group $n = 10$. IRS-1: insulin receptor substrate-1, IRS-2: insulin receptor substrate-2, IRS-3: insulin receptor substrate-3, IRS-4: insulin receptor substrate-4, and TRIM72: tripartite motif family protein 72. $^{**}p < 0.01$: versus CON group; $^{\#\#}p < 0.01$: versus group HFD group; $^{\#}p < 0.05$: versus group HFD group.

significantly decreased compared with that in group HFD ($p < 0.01$). Furthermore, compared with group CON, the phosphorylation level of IRS-1^{Ser307} was significantly increased ($p < 0.01$); the expression level of IRS-1 and IRS-2 protein was significantly decreased in group HFD ($p < 0.01$). After swimming exercise for 8 weeks, compared with group HFD, the phosphorylation level of IRS-1^{Ser307} significantly decreased ($p < 0.01$); the expression level of IRS-1 and IRS-2 protein significantly increased ($p < 0.01$) in group Ex-HFD while the expression of IRS-3 and IRS-4 showed no significance among group CON, group HFD, and group Ex-HFD. In addition, the proteins of pAKT^{Ser473} and AKT in group HFD were significantly lower than those in group CON ($p < 0.01$ and $p < 0.05$, resp.), but significantly increased in group Ex-HFD than those in group HFD ($p < 0.01$ and $p < 0.05$, resp.). These findings suggest that swimming exercise could decrease the protein expression of TRIM72, upregulate the protein expression of IRS-1 and IRS-2, and phosphorylation level of AKT (Ser473) of skeletal muscles in rats fed with high-fat diet.

4. Discussion

In present study, we tested the levels of oxidative stress parameters and performed qPCR and western blot to detect the mRNA and protein of TRIM72, IRS (IRS-1, IRS-2, IRS-3, and IRS-4), and AKT and protein of phosphorylation of IRS-1 (Ser307) and AKT (Ser473). The results showed that the elevation of oxidative stress, TRIM72, and pIRS-1^{Ser307} expression and the reduction of protein expression of IRS-1 and pAKT^{Ser473} of skeletal muscle in rats fed with high-fat diet, while the swimming exercise was effective in reversing IR induced by high-fat diet. Our findings indicate that an 8-week of swimming exercise not only effectively reduced the oxidative stress of skeletal muscle, but also alleviated the expression of TRIM72, upregulated the AKT signaling pathway, and reversed IR.

It has been widely accepted that lipid accumulation and oxidative stress are strongly correlated with the IR in peripheral tissues (muscle and liver) [31–37]. Long-term excessive intake of high calories could lead to excessive fat accumulation in fat cells, consequently resulting in fats flowing to other tissues except fat tissues. Ectopic lipid deposition subsequently occurs, thereby causing cell damage. Studies [4, 38] have shown that excessive TG accumulation in nonfat tissue has been known to have a toxic effect on cells and to reduce sensitivity to insulin, eventually leading to diabetes and metabolic syndrome [31, 39]. We fed the rats with high-fat diet, and we noticed that high-fat diet induced the increase in visceral fat content along with fat decomposition, which could lead to massive FFA and TG in the skeletal muscle. The present study also demonstrated that GIR greatly decrease in rats fed with high-fat diet, and the ISI significantly increased simultaneously, while HOMA-IR was obviously decreased, suggesting that the 8-week high-fat diet led to a presence of IR in rats. However, the GIR and ISI were significantly increased in rats from the exercise group that were also fed with high-fat diet, whereas HOMA-IR considerably decreased, implying that IR genesis and progress in rats fed with high-fat diet can be effectively relieved by swimming exercise.

Changes in in vivo metabolism (such as massive lipid accumulation and increased ROS) [10, 40] eventually lead to cell damage. TRIM72, as a specific cell repair factor of skeletal muscle, is rapidly and massively transposed toward

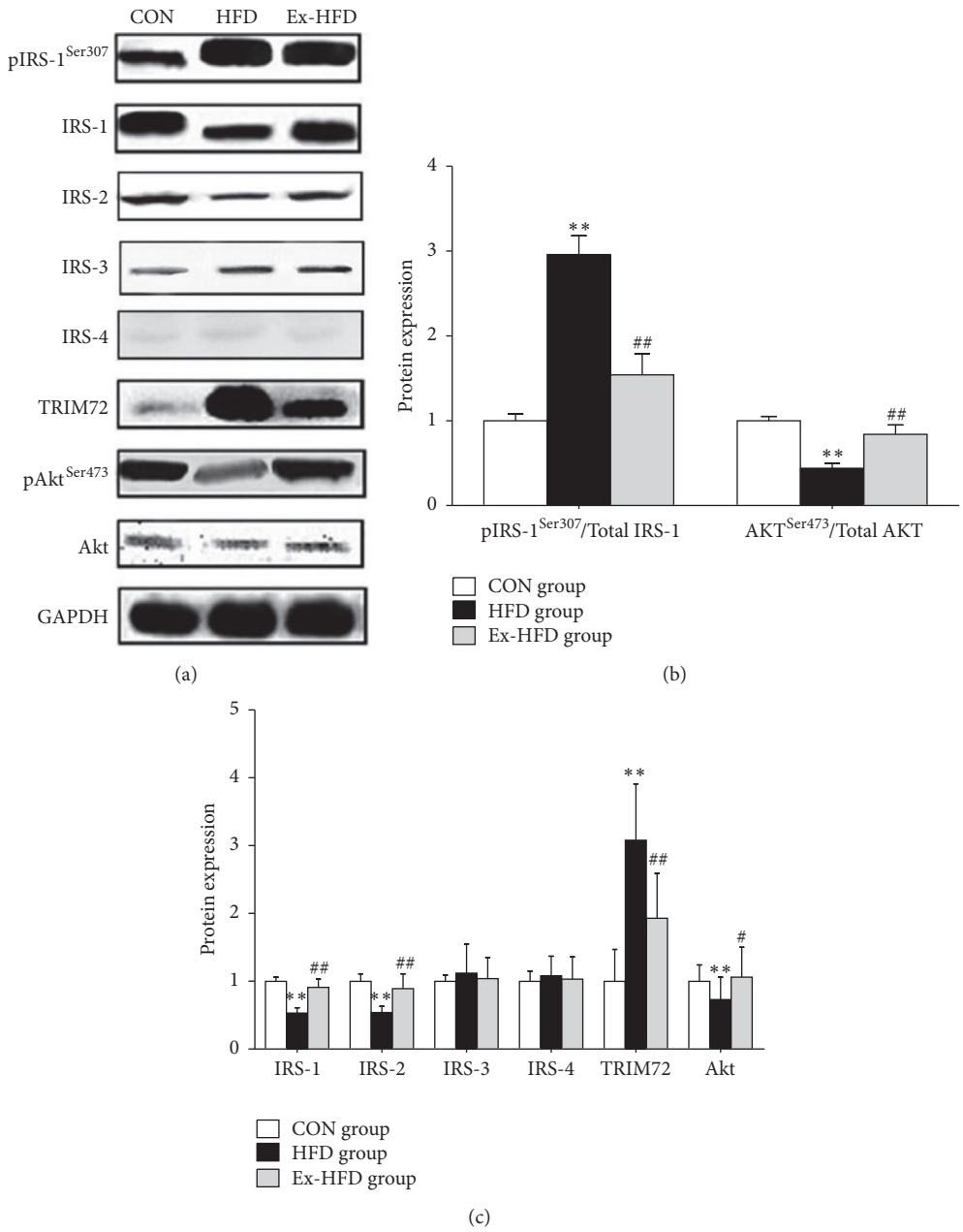

Figure 4: Protein expression of TRIM72, IRS-1, IRS-2, IRS-3, and IRS-4 and phosphorylation of IRS-1 (Ser307) and AKT (Ser473) levels in skeletal muscle were tested by western blot. $^{**}p < 0.01$: versus CON group; $^{*}p < 0.05$: versus CON group; $^{\#\#}p < 0.01$: versus group HFD group; and $^{\#}p < 0.05$: versus group HFD group.

the damaged sites [5]. When experiencing a long-term high-fat diet, the oxidative stress could occur, which consequently results in oxidative damage in skeletal muscle cells. Subsequently, TRIM72 expression is initiated. Previous studies have demonstrated that SOD, MDA, GSH-Px, and 8-OHdG can evaluate the oxidative stress [41–45]. Several studies have shown that systemic IR can be effectively improved by exercise through reduction of oxidative stress [46, 47]. In the present study, we tested SOD, MDA, GSH-Px, 8-OHdG, and TRIM72 levels in the skeletal muscles of rats in all the groups; the oxidative stress was found increased after long-term feeding with high-fat diet, and, in HFD group, the TRIM72 showed a significant-level promotion on both mRNA and protein expression; this is in accordance with Song et al. [8] (published in "*Nature*" 2013), although some publications from different groups showed that HFD treatment does not alter expression of TRIM72 [48–53]. We analyzed and inferred the possible reasons: firstly, the differences of the high-fat diet composition: the multiple researchers use the high-fat diet with 45% or more calories from fat or extra cholesterol added. In our study, the high-fat diet only contains 38% calories from fat. Meanwhile, multiple researchers

performed feeding mice with high-fat diet for a long time (8 weeks, 16 weeks, or more weeks); secondly, Song et al. showed that TRIM72 expression increased at the early stage of IR, in other words, TRIM72 expression increased when mild IR existed. While the multiple studies fed mice with high-fat diet (45%, 60.9%, or more calories from fat) for long, the data showed the seriously impaired insulin action; it indicated severe IR progression in mice. However, we fed rats with high-fat diet (38% calories from fat) for 8 weeks, the IR was milder than those mice fed with high-fat diet (45%, 60.9%, or more calories from fat); it might be the early stage of IR. Therefore, we observed that TRIM72 expression markedly increased in group HFD, in keeping with Song et al. In addition, we found that, in HFD group, the mRNA and protein expression of IRS-1 and IRS-2 were both notably elevated; the mRNA expression of IRS-3 was greatly increased, while its protein expression showed no significance; the mRNA and protein expression of IRS-4 have not obviously changed. However, these levels can be effectively reduced by an 8-week swimming exercise. In the present study, we also detected the expression of IRS (IRS-1, IRS-2, IRS-3, and IRS-4). Meanwhile, the AKT signal was determined. In the group HFD, pIRS-1^{Ser307}, IRS-1, IRS-2, and pAKT^{Ser473} expressions were at a lower level. While 8 weeks of swimming exercise later, these levels of protein were all increased. Therefore, we infer that 8-week high-fat diet can result in high expression of TRIM72 mRNA and protein, and TRIM72 acts as an E3 ligase targeting the insulin receptor and IRS-1 for ubiquitin-dependent degradation, then inhibiting PI3-K/AKT signal transduction and subsequently producing IR. In the study, we found that the level of IRS-2 increasing is not relevant to the pathway that we studied, which may be through other ways.

Previous studies have shown that systemic IR can be effectively improved by exercise through reduction of oxidative stress [46]. In the present experiment, relevant parameters such as expression of TRIM72, IRS, and AKT in skeletal muscles of rats in Ex-HFD groups showed that oxidative stress reduced after long-term exercise with high-fat diet. Subsequently, the expression of TRIM72 mRNA and protein levels was decreased, and insulin signal transduction was enhanced through swimming exercise. These results suggest that an 8-week swimming exercise improves HFD-induced insulin resistance, maybe through a reduction of TRIM72 in skeletal muscle and enhancement of AKT signal transduction.

5. Summary

IR induced by high-fat diet can be effectively improved during an 8-week swimming exercise. The possible mechanism in rats fed with high-fat diet during exercise implied that the degree of damage in the skeletal muscle cells can be relieved by the decrease in lipid deposition and inhibition of oxidative stress in skeletal muscles. Consequently, this finding indicates reduced TRIM72 expression level and increased IRS-1 protein expression level can enhance skeletal muscular PI3-K/AKT signal transduction in rat skeletal muscles, then finally improving IR.

Competing Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Jie Qi contributed to data analysis and developed and wrote the paper. Bo Yang developed the experimental design, contributed to data generation, and reviewed the paper. Cailing Ren conducted the ceramide analysis, conducted the mRNA analysis from skeletal muscle tissue, and contributed to paper writing. Jian Fu analyzed the protein expression in skeletal muscle and contributed to paper writing. Jun Zhang contributed to data analysis and commented on the paper. Jie Qi and Bo Yang contributed to the work equally.

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Research Article

Effect of Alkaloids from Nelumbinis Plumula against Insulin Resistance of High-Fat Diet-Induced Nonalcoholic Fatty Liver Disease in Mice

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This study aimed to investigate the effects of total alkaloids from Nelumbinis Plumula (NPA) on insulin resistance (IR) of high-fat diet- (HFD-) induced nonalcoholic fatty liver disease (NAFLD). Rats were fed with HFD for 8 weeks to induce NAFLD. Then, the effect of NPA on ameliorating IR in HFD-induced NAFLD was evaluated. Fasting serum insulin was determined using an enzyme-linked immunosorbent assay (ELISA) kit for insulin following the manufacturer's protocol. Some inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were determined using ELISA kits to assess the inflammatory burden in rats. The results showed that HFD could induce a significant increase in blood glucose and IR in rats. However, rats treated with NPA (400 or 600 mg/kg) showed improved IR and reduction in serum inflammatory cytokines TNF-α and IL-6. Further investigation indicated that NPA could inhibit IR by restoring the insulin receptor substrate-1 (IRS-1) and suppressing the expression of c-Jun N-terminal kinase (JNK) phosphorylation. The present results supported the view that the pathogenesis of NAFLD was complex with inflammation, together with increasing serum glucose and IR. Also, JNK and IRS phosphorylation were suggested for their involvement in the modulating of IR during NAFLD progression. Therefore, NPA may serve as a potential natural remedy against IR in NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to the accumulation of hepatic steatosis not due to excess alcohol consumption. NAFLD has become a serious medical problem and an increasing threat to public health. It is estimated that approximately 20%–40% of the people in various countries have excess fat accumulation in the liver [1]. NAFLD encompasses a spectrum of disorders ranging from hepatic steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, or hepatocellular carcinoma [2]. Despite its severity and high prevalence, currently no pharmacological agent is available for treating NAFLD. Many factors including lipid metabolic disturbance, insulin resistance (IR), oxidative stress, and mitochondrial dysfunction are known to cause the occurrence and aggregation of NAFLD [3].

According to the prevailing theory of NAFLD pathogenesis, IR and lipid dysregulation are the "first hit" toward NAFLD, which may trigger following "hits" spontaneously such as oxidative stress, inflammation, necrosis, apoptosis, and fibrosis [4]. Therefore, targeting steatosis and IR in the early stage of NAFLD is extremely important for the retardation of NAFLD in both basic research and clinical trials. Although the initial accumulation of fat in the liver can occur through several mechanisms, most previously done research implicates IR as the key mechanism in primary NAFLD, leading to hepatic steatosis [5]. IR is associated with an increase in peripheral lipolysis, uptake of fatty acids by the liver, and synthesis of triglycerides, which promote the accumulation of hepatic fat [6].

Nowadays, the methods for treating NAFLD involve rational diet, exercise, and medicines including metformin,

statins, and fibrates. However, these drugs have some adverse effects or contraindications, and no consensus exists on the most effective drug therapies [7]. Recently, emerging evidence suggests the possible use of herbal derivatives in treating NAFLD, such as green tea [8], garlic [9], resveratrol [10], and coffee [11] in both preclinical studies and clinical trials.

Lotus seeds have been widely consumed by people in Asia. The therapeutic hepatoprotective activity of ethanolic extracts of edible lotus leaves was studied, indicating that the hepatoprotective activity of lotus leaf extract was comparable with that of a standard treatment comprising silymarin, a known hepatoprotective drug [12]. NP is the germ of the lotus seeds, which tastes bitter and generally can be removed. A previous research demonstrated the protective effect of total alkaloid and alkaloid compounds including liensinine, isoliensinine, or neferine from NP against oxidative stress induced by tertiary-butyl hydroperoxide, suggesting their involvement in the cytoprotective effect on oxidative stress in liver [13]. Furthermore, total alkaloid and alkaloid compounds from NP displayed a significant cytoprotective effect against hepatic steatosis (data not reported). However, no papers have reported on the total alkaloid from NP against NAFLD.

Therefore, the aim of the present study was to investigate the effects of NPA on ameliorating IR in high-fat diet- (HFD-) induced nonalcoholic fatty liver disease in mice. And the possible mechanisms were also studied.

2. Materials and Methods

2.1. Reagents. HFD, composed of 5% lactalbumin, 10% cholesterol, 20% lard oil, and 1% yolk powder, was obtained from Research Diets, Inc. Serum blood glucose, free fatty acid, alanine amino transferase (ALT) and aspartate aminotransferase (AST) detection kits, and bicinchoninic acid protein assay kit were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Phosphorylated insulin receptor substrate 1 (IRS-1) and c-Jun N-terminal kinase (JNK) antibody were purchased from Sigma Chemicals Co. The insulin enzyme immunoassay kit was purchased from Puer Biotechnology Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were supplied by Glory Science Co. Ltd. (TX, USA).

2.2. Preparation of Total Alkaloid from NP. NP was purchased from Fujian Xin Zi Jin Curative Co. Ltd. (Fuzhou, Fujian, China). NPA was prepared as described previously [13]. Briefly, the peel powder was extracted with 80% ethanol. Then, the extraction was centrifuged at 1500 g for 15 min, and the supernatant was collected. Next, 5% HCl solution was used to immerse the extract, followed by the addition of NaOH to regulate pH to 9-10. The crude sample was then extracted with chloroform using liquid-liquid separation. The collected soluble fraction from chloroform was evaporated to dryness and called NPA. The alkaloid composition of NPA was also reported in previous studies [13, 14], which indicated that 3 alkaloid components of liensinine, isoliensinine, and neferine make up the majority in NPA.

2.3. Animals. Male Wistar rats (180–200 g) were provided by the Experimental Animal Center of Fujian University of Traditional Chinese Medicine (Fuzhou, China). The animals were housed in a room with a controlled temperature (21 ± 3°C), relative humidity (65%–70%), and 12 h light/dark cycles. They were given ad libitum access to water. After adaptation for 1 week, the rats were used for the experiment approved by the ethical committee and the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine.

The rats were randomly divided into six groups (n = 10). Group I (normal control) was given distilled water (10 mL/kg body weight) and normal feeding. Groups II–VI were administered with HFD feeding for 8 weeks to induce NAFLD in the animal. Among them, Group II was designed as the model control group. Groups III–V were administered with NPA (200, 400, and 600 mg/kg, resp., dissolved in water) for another 4 weeks. Group VI was given compound methionine and choline bitartrate tablets as positive control medicine. At the end of the experiment, blood samples of all rats were obtained via the abdominal vein under anesthesia after an overnight fasting. Liver sample from each rat was collected for molecular measurement and stored at −80°C for the assays.

2.4. Serum Biochemistry. Serum was obtained from blood after centrifugation (2500 ×g, 10 min). The serum blood glucose, free fatty acid, ALT, and AST levels of rats were detected using corresponding detection kits according to the manufacturer's protocols.

2.5. Assessment of the Level of Serum Insulin and IR. Fasting serum insulin was determined using ELISA kit for insulin following the manufacturer's protocol. Using an ELISA reader, the absorbance of the reaction product was read at 450 nm.

The homeostasis model assessment of IR (HOMA-IR) was calculated as [fasting blood glucose (mmol/L) × fasting serum insulin (mU/L)/22.5] [15].

2.6. Determination of Serum Levels of TNF-α and IL-6 Using ELISA Kits. Some inflammatory cytokines were determined to assess the inflammatory burden in rats. An ELISA kit for TNF-α or interleukin-6 (IL-6) was employed for estimating the serum levels of these parameters. The assays were performed in accordance with the manufacturer's protocol.

2.7. Western Blotting Assay. Western blotting assay for detecting phosphorylation of JNK or IRS-1 in liver tissue was determined according to the method described by Li et al. [16] with some modification. Briefly, total protein was obtained from the livers. Then, it was denatured, resolved with sodium sulfate poly acrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (PVDF). After blocking, the membranes were individually incubated overnight with mouse anti-JNK (1 : 700 dilution), mouse anti-p-JNK (1 : 700 dilution), mouse anti-IRS-1 (1 : 700

Table 1: Body weight changes of rats during NAFLD development.

Group	Body weight at week 0 (P value) (g)	Body weight at week 12 (P value) (g)
Control	185.3 ± 6.2^{a}	310.2 ± 19.1^{e}
NAFLD	188.9 ± 5.1^{a}	372.3 ± 22.6^{a}
NAFLD + NPA (L)	190.3 ± 9.2^{a}	362.0 ± 17.6^{ab}
NAFLD + NPA (M)	181.5 ± 5.6^{a}	347.3 ± 22.5^{bc}
NAFLD + NPA (H)	180.2 ± 4.7^{a}	338.8 ± 31.6^{cd}
Positive control	184.1 ± 5.4^{a}	322.5 ± 19.2^{de}

Values are means ± SD, $n = 10$.
Control group means the normal group of rats; NAFLD group means the model group of rats; NAFLD + NPA (L) group means the NAFLD model of rats which were administrated with 200 mg/kg NPA; NAFLD + NPA (M) group means the NAFLD model of rats which were administrated with 400 mg/kg NPA; NAFLD + NPA (H) group means the NAFLD model of rats which were administrated with 600 mg/kg NPA. Different letters (a, b, c, d, or e) in the same column represent significant differences between the treated groups ($P < 0.05$).

Table 2: Effect of NPA on serum levels of glucose, insulin, and insulin sensitivity index in all groups of rats.

Group	FBG (P value) (mmol/L)	Insulin (P value) (mU/L)	HOMA-IR (P value)
Control	3.41 ± 0.37^{c}	21.15 ± 3.26^{d}	3.23 ± 0.75^{d}
NAFLD	4.61 ± 0.52^{a}	37.57 ± 3.21^{a}	7.65 ± 1.34^{a}
NPA (200 mg/kg)	4.34 ± 0.49^{ab}	35.89 ± 5.18^{a}	7.07 ± 1.45^{a}
NPA (400 mg/kg)	4.12 ± 0.28^{b}	30.69 ± 3.86^{b}	5.60 ± 0.61^{b}
NPA (600 mg/kg)	3.73 ± 0.27^{c}	26.71 ± 3.11^{c}	4.53 ± 0.36^{c}
Positive group	3.65 ± 0.13^{c}	25.58 ± 3.26^{c}	4.16 ± 0.57^{c}

Different letters (a, b, c, or d) in the same column represent significant differences between the treated groups ($P < 0.05$).

dilution), or mouse anti-P-IRS-1 (1 : 700 dilution), respectively, followed by washing and incubation with secondary antibodies to show the resulting bands. Parallel blotting of β-actin was used as the internal control.

2.8. Statistical Analysis. All values for each group were given as mean and standard deviation. The data were analyzed using one-way analysis of variance coupled with Duncan post hoc multiple range test at 95% confidence level using SPSS (version 16.0, Chicago, USA).

3. Results

3.1. Effects of NPA on Body Weight of Rats. Before the start of the experiment, the average body weight of each group was similar. As showed in Table 1, after feeding with HFD for 8 weeks, the body weights of rats in the NAFLD group significantly increased compared with the control group, indicating that HFD had a significant influence on body weight. However, after oral administration of NPA (400 and 600 mg/kg) for 4 weeks, the body weight of rats significantly reduced compared with the NAFLD group. Therefore, the consumption of NPA could significantly reduce the body weight of NAFLD rats.

3.2. Serum AST and ALT Activities. Serum levels of amino transferases were indexed for general hepatic injury. To correlate hepatic injury with NAFLD, serum ALT and AST measurements were conducted in each group of rats. As shown in Figure 1, the progression of NAFLD significantly induced the higher levels of both serum ALT and AST compared with the control rats. However, the levels of ALT and AST significantly decreased in a dose-dependent manner after NPA treatment, and the hepatoprotective effects produced by NPA at the dose of 600 mg/kg reach the highest as well as the positive control group. Therefore, NPA treatment potently alleviated hepatic injury by reducing the levels of aminotransferases.

3.3. Effects of NPA on Serum Blood Glucose and Insulin Levels. As noted, IR and glucose metabolism dysfunction are typical clinical symptoms of NAFLD and metabolic syndrome [17]. The levels of blood glucose are given in Table 2. Eight weeks of daily ingestion of HCD contributed to the insulin resistance of the model group, which exhibited a significant increase in fasting blood glucose (FBG) compared with the control group. However, after NPA exposure, the concentrations of FBG in blood significantly decreased in a dose-dependent manner compared with the model group. These findings indicated that NPA had obvious glucose-lowering effects against NAFLD. The fasting serum insulin level increased significantly in association with the elevated FBG in the model group; however, NPA intake at higher dose significantly decreased the fasting insulin level in Groups IV-V compared with the NAFLD group.

3.4. HOMA-IR. A high-fat, cholesterol-rich diet induced a significant increase of IR in the NAFLD model group

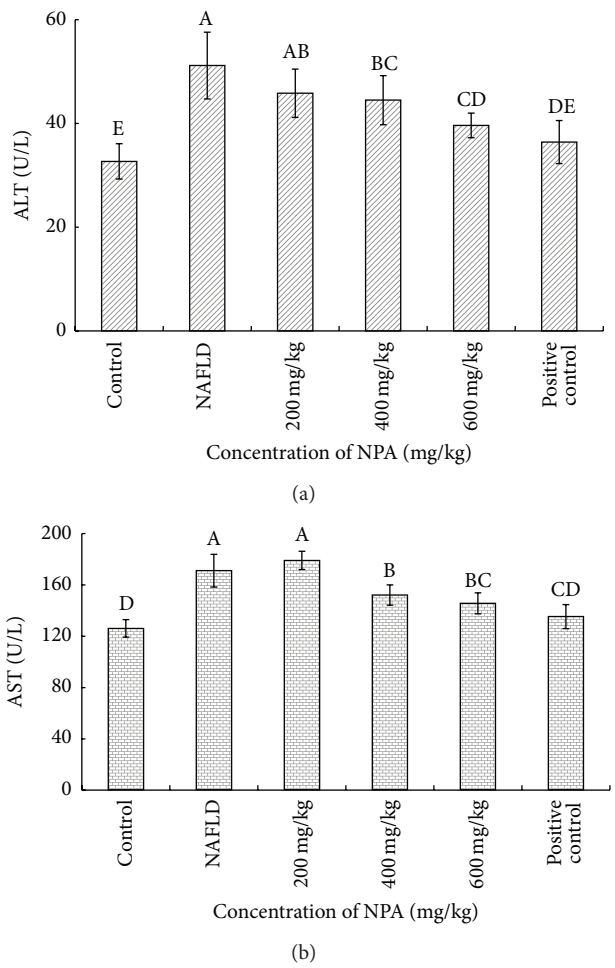

Figure 1: Effect of different concentrations of NPA on serum levels of ALT (a) and AST (b) in each group of rats. Different letters (A, B, C, or D) upon each column represent significant differences between the treated groups ($P < 0.05$).

animals compared with the control group, as indicated by the high HOMA-IR. NPA administration in Groups IV-V could decrease their HOMA-IR level compared with the model group, reaching the control range (Table 2).

3.5. Effects of NPA on the Level of Serum Free Fatty Acid. Our results showed that the NAFLD model rats could exhibit a much higher level of free fatty acid (FFT) compared with the control group (Figure 2). However, treating the NAFLD model with NPA at a concentration of 400 or 600 mg/kg could significantly inhibit the FFT level in serum, suggesting its significant effect on alleviating steatosis in rats.

3.6. Inhibitory Effects of NPA on Key Inflammatory Mediators of TNF-α and IL-6. Rats fed with HFD for 8 weeks showed a significant increase in serum TNF-α and IL-6 levels compared with the rats in the control group. Treatment with NPA (400 or 600 mg/kg) significantly decreased these high levels compared with the control group. Notably, NPA at a higher dose could decrease the serum IL-6 level to a much greater extent, which was comparable with the positive control group (Figures 3(a) and 3(b)).

3.7. JNK Phosphorylation Was Associated with IR. The hypothesis that phosphorylated JNK is a crucial mediator for IR was tested. The results showed that JNK phosphorylation significantly increased in the NAFLD rats compared with the normal group. However, JNK phosphorylation in liver tissue could be significantly reversed by NPA treatment. Thus, increased JNK phosphorylation might explain the increased IR (Figure 4(a)).

3.8. Improvement in NAFLD by NPA Was Partially Attributed to the Modulation of IRS-1 Phosphorylation. IRS phosphorylation was strongly impaired in the liver of rats with NAFLD. It is well established that the insulin signaling pathway is impaired in obese objects as a result of decreased IRS phosphorylation [18]. In the present NAFLD model, western blot results showed that hepatic tissue levels of phosphorylated IRS-1 were inhibited by the induction of NAFLD. However, cotreatment with NPA completely restored the levels of these proteins (Figure 4(c)), indicating the basis of improving IR in NAFLD.

4. Discussion

The aim of the present study was to evaluate the hepatoprotective effect of NPA by ameliorating IR in a model of HFD-induced NAFLD. HFD is well known to increase body weight and induce IR, in addition to an inflammatory and oxidative burden in rodent models [19]. In the present study, high-fat, high-glucose diet was administered to rats to induce the occurrence of NAFLD. The aforementioned findings confirmed that 8 weeks of HFD feeding in rats resulted in increments in body weight, adiposity index, systemic IR, and inflammatory markers. HFD feeding could also increase serum FFA, causing the overloads of FFA in hepatocytes and promoting lipogenesis.

NAFLD is defined histologically as the presence of lipid droplets in more than 5% of hepatocytes, not caused by excessive alcohol consumption, drugs, or viruses. Numerous studies have demonstrated a correlation between NAFLD and IR [20]. IR represents the main link among obesity, metabolic syndrome, and liver disease with fat accumulation, that is, NAFLD [21]. Studies have also shown that hepatic steatosis may induce hepatic IR [22]. The present research investigated the effect of NPA on the metabolic alterations in the glucose level and IR in HFD-induced NAFLD. Feeding the rats with HFD resulted in dramatic increases in the FBG, FFA, and IR. The clustering of these metabolic risk factors was identified as "IR syndrome" [23].

The causes for IR during NAFLD development are numerous. It is well known that infusions of triglyceride emulsions to rapidly increase FFA levels would cause a decrease in insulin sensitivity [24]. Therefore, controlling IR, as well as associated glucose metabolism and lipid accumulation, is the first but most important step toward the therapy of NAFLD.

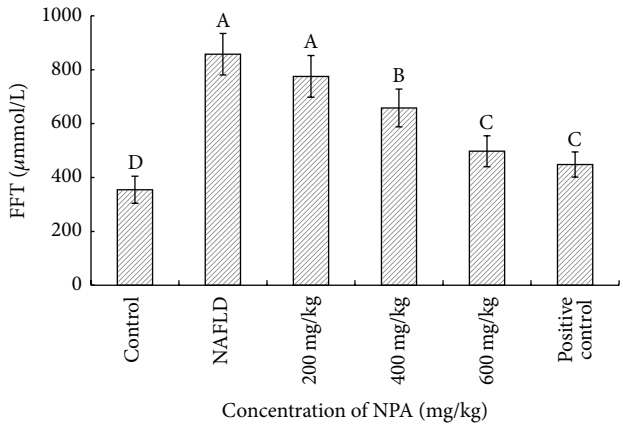

Figure 2: Effect of different concentrations of NPA on serum levels of FFT in each group of rats. Different letters (A, B, C, or D) upon each column represent significant differences between the treated groups ($P < 0.05$).

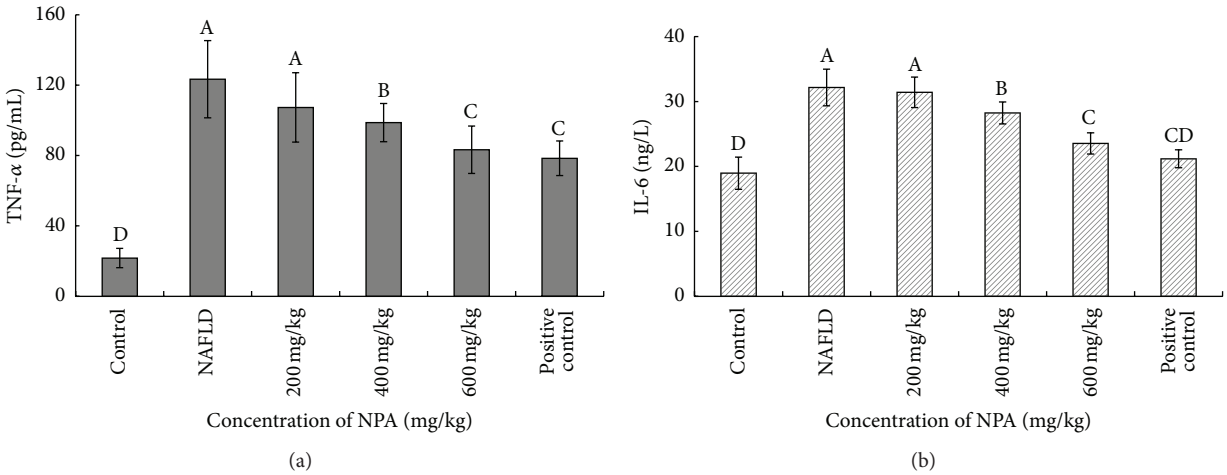

Figure 3: Effect of different concentrations of NPA on serum key inflammatory mediators of TNF-α (a) and IL-6 (b) in each group of rats. Different letters (A, B, C, or D) upon each column represent significant differences between the treated groups ($P < 0.05$).

Fat accumulation and hepatocellular injury can lead to nonalcoholic steatohepatitis and advanced fibrosis [25]. The adipocytes are no longer considered passive cells that store excessive triglycerides but are instead considered active cells that regulate the energy balance and secrete the proinflammatory cytokines IL-6 and TNF-α [26]. TNF-α is an important mediator of IR due to its ability to influence the tyrosine kinase activity of the insulin receptor [27]. In the present study, the serum level of TNF-α and IL-6 increased after HFD feeding. This suggested that multiple immunomodulatory factors could contribute to the chronic inflammatory condition and hepatocytes injury observed in NAFLD. It is assumed that inflammatory factors may be mediators of IR because the plasma levels of both markers are correlated negatively with insulin sensitivity [28]. Excessive fat accumulation in hepatocytes, regardless of its cause, tends to induce the activation of nuclear factor-κB, which is the key regulator of inflammation [29]. Subsequently, the expression of inflammatory cytokines TNF-α and IL-6 may lead to infiltration of neutrophils and inflammation in liver injury.

IR is the major abnormality in NAFLD with metabolic syndrome. Impaired IRS-1 phosphorylation is responsible for reduced insulin signaling and impaired downstream PI3K/Akt signal transduction [30]. It is well established that the insulin signaling pathway is impaired in obese patients as a result of decreased IRS phosphorylation [18]. The pathways by which increased visceral adiposity leads to IR are not fully understood. Some authors suggest that lipolysis induced by TNF-α and IL-6, resulting in the inhibition of IRS, may represent a major mechanism [31]. Thus, it could be considered that the restoration of IRS-1 might be beneficial for the amelioration of NAFLD. The present results showed that insulin hypersecretion was prevalent in the group of NAFLD model rats, which significantly decreased on administering NPA. Considering the survival-promoting role of IRS-1 in several hepatic diseases, particularly NAFLD,

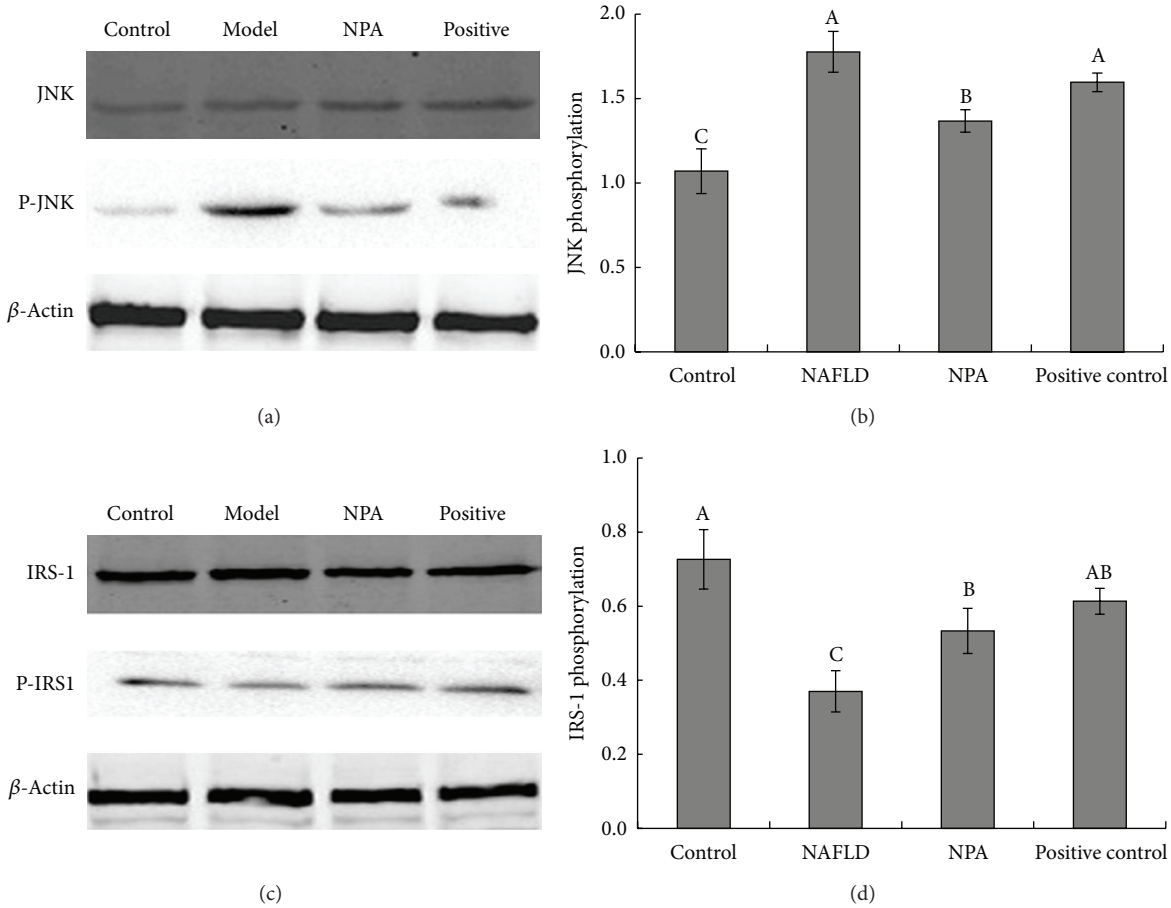

Figure 4: Effect of NPA on phosphorylation of JNK (a) and IRS-1 (c) in the hepatic tissue of NAPLD rats analyzed by western blot. Phosphorylation levels were evaluated by the band strength of P-JNK/JNK or P-IRS1/IRS (b and d). Liver and adipose tissue protein blots were normalized to endogenous β-actin. Different letters (A, B, or C) upon each column represent significant differences between the treated groups ($P < 0.05$).

the restoration of this pathway would definitely contribute to the improvement in IR.

Lipid peroxidation products can activate transcription factor NF-kB in patients with NAFLD [32], a key transcription factor that regulates the expression of several proinflammatory cytokines and hepatic inflammation. In fact, it is now generally established that JNK1 is central to obesity-induced IR, although JNK2 might also play a contributing role [33]. The present results suggested that JNK phosphorylation might be responsible for aggravating IR, as NAFLD group advanced to more severe form. However, this progress can be reversed by administration with NPA. Hence, inhibition of JNK phosphorylation by NPA adds to its modulation effect on IR during NAPLD progression.

5. Conclusions

In conclusion, the present results supported the view that the pathogenesis of NAFLD was complex with inducing inflammation and IR, as well as increasing serum glucose and free fatty acid level. Administration with NPA in NAFLD rats had beneficial effects on reversing these metabolic indexes. Moreover, cotreatment with NPA could regulate IR through restoring phosphorylation of IRS-1 and alleviating phosphorylation of JNK in liver tissue, suggesting its involvement in modulating IR by protein phosphorylation during NAFLD progression. Further investigations are needed to ensure the safety, benefits, and utility of NPA in patients with NAFLD.

Competing Interests

The authors wish to confirm that there are no known competing interests associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Review Article

The Relationship between Branched-Chain Amino Acid Related Metabolomic Signature and Insulin Resistance: A Systematic Review

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Recent studies have shown the positive association between increased circulating BCAAs (valine, leucine, and isoleucine) and insulin resistance (IR) in obese or diabetic patients. However, results seem to be controversial in different races, diets, and distinct tissues. Our aims were to evaluate the relationship between BCAA and IR as well as later diabetes risk and explore the phenotypic and genetic factors influencing BCAA level based on available studies. We performed systematic review, searching MEDLINE, EMASE, ClinicalTrials.gov, the Cochrane Library, and Web of Science from inception to March 2016. After selection, 23 studies including 20,091 participants were included. Based on current evidence, we found that BCAA is a useful biomarker for early detection of IR and later diabetic risk. Factors influencing BCAA level can be divided into four parts: race, gender, dietary patterns, and gene variants. These factors might not only contribute to the elevated BCAA level but also show obvious associations with insulin resistance. Genes related to BCAA catabolism might serve as potential targets for the treatment of IR associated metabolic disorders. Moreover, these factors should be controlled properly during study design and data analysis. In the future, more large-scale studies with elaborate design addressing BCAA and IR are required.

1. Introduction

Obesity, one of the escalating global health problems, is a major risk factor for the onset and development of diabetes mellitus, metabolic syndrome, cancer, and other chronic disorders [1, 2]. Insulin resistance (IR) is known to play a significant role in obesity-related metabolic disturbances [3, 4]. Several mechanisms are known to be responsible for IR, including carbohydrate metabolism, fat metabolism, and protein metabolism [5]. Despite many years of research, the exact mechanisms between IR and obesity-related metabolic complications are still not fully established. Thus, accurate biomarkers or parameters reflecting insulin resistant state and metabolic risks are essential to understand its mechanism and to prevent obesity-related complications.

Metabolomics is an analytical approach that aims to detect and quantify endogenous small molecule metabolites (<1,500 Da) [6]. Recent studies have suggested that altered metabolites or metabolomics profiles could predict specific metabolic diseases with high accuracy and help to understand related fundamental mechanisms as well as affected metabolic pathways [7, 8]. In addition, metabolomics approach is beneficial to classify personalized "metabolic signature" and make it possible to suggest ideal and individualized therapies effectively [9]. Variable kinds of metabolomics techniques have been applied to generate metabolic profiles from blood, urea, or tissues in human, such as ultraperformance liquid chromatography (UPLC), nuclear magnetic resonance spectroscopy (NMR), and tandem mass spectrometry (MS/MS).

BCAAs are essential amino acids, changing along with the consumption of a protein-containing meal, which include valine, leucine, and isoleucine [10]. Previous studies using metabolomics approaches reported that insulin resistant rats and human had an increased level of circulating BCAA-related metabolites, including valine, leucine, isoleucine, and

the sum of the above three amino acids [11–13]. Moreover, some prospective studies found that the plasma concentrations of BCAAs are prognostic for the onset and progress of type 2 diabetes in long-term follow-up [14, 15]. These indicated that BCAA, known as important nutrient signals, might play an important role in the pathogenesis of IR and T2DM [16]. In recent years, several studies have performed many investigations on BCAA and IR as well as diabetes risks across different races, sex, and so on. The results have shown big differences in the BCAA level due to variances in ethnicity, sex, gene expressions, and dietary pattern. However, studies focusing on phenotypic and genetic factors influencing BCAA level are largely lacking. Thus, this systematic review aims to evaluate the relationship between BCAA and insulin resistance as well as later diabetes risks and explore the phenotypic and genetic factors influencing BCAA level and its relationship with IR based on the available studies.

2. Method

2.1. Literature Search. We searched papers available on MEDLINE, EMASE, ClinicalTrials.gov, the Cochrane Library, and Web of Science for relevant studies from inception to March 2016. A search strategy was applied for MEDLINE according to medical subject headings (MeSH®) terms and some key words. Different possible variations and combinations of the following search terms were used: "metabolomic profiles", "metabolomic approach", "serum metabolites", "metabolic signature", "branched-chain amino acids", "BCAA", "leucine", "valine", "isoleucine", "amino acids metabolism", "insulin resistance", "nuclear magnetic spectroscopy," "NMR", and "mass spectrometry". Two persons (Xue Zhao and Qing Han) independently did the search process in order to minimize selection bias. If there were disagreements, a third person will join in and resolve disagreements by consensus (Guixia Wang).

2.2. Inclusion and Exclusion Criteria. Inclusion and exclusion criteria were set up before starting paper selection. The following inclusion criteria were applied: (1) all subjects should be adults, which means that age is >18 years and <65 years; (2) participants should be free of any thyroid or metabolic disorders requiring treatment such as diabetes, hypertension, severe dyslipidemia, and coronary heart disease; (3) studies need to include adiposity measures (such as BMI or waist circumference) and HOMA-IR according to the formula glucose (mmol/L) × insulin (pg/mL)/22.5 [17], and serum BCAAs and related metabolites measurement results should be the outcome; (4) metabolomic techniques, such as MS, NMR spectroscopy, and UPLC, were applied to detect metabolite profiles in human blood; (5) principal components analysis (PCA) was used as a means to reduce the complexity of the variables; and (6) only papers published in English were included in our paper.

Studies were excluded if subjects were <18 or >65 years old, or were pregnant, or had diabetes, or had other chronic diseases, or had medication history. The metabolomics profiles extracted from urea are ruled out. Also, reviews, conference abstracts, case reports, and meta-analyses were excluded.

2.3. Data Extraction and Analysis. Data on population characteristics and metabolomic profiles were extracted. This procedure was done by two different persons (Xue Zhao and Qing Han). Because of differences in study design, method, and population characteristics, it is not appropriate to perform the quantitative meta-analysis.

BCAAs are known as essential amino acids, including valine, leucine, and isoleucine. Some included studies using BCAA to represent the sum of the above three amino acids. However, other studies point out the specific changes of valine, leucine, or isoleucine. Thus, in this review, we also use BCAA to represent the sum of three amino acids and each amino acid to represent itself in order to sustain the original state of included papers.

2.4. Methodological Quality Assessment. To assess the methodological quality of included studies, QUADOMICS tool was applied. QUADOMICS was developed to assess quality issues specific to "-omics" research, including the quality assessment of studies included in systematic reviews [18]. To differentiate high or low quality, studies will be scored from 1 to 16. And studies which scored more than 11/16 are regarded as "high quality," and studies which scored less than 11 are regarded as "low quality."

3. Results

3.1. Literature Search and Study Characteristics. After careful selection, 23 studies (including 20,091 participants) were included in our systematic review, which met the inclusion/exclusion criteria. Figure 1 presented the procedure of the literature selection based on PRISMA statement [19]. At first, 510 records were identified, and 94 full papers were retrieved. After reading all the papers, twenty-three unique studies providing adequate data were included in this systematic review.

Table 1 showed the details about general population characteristics of the twenty-three studies with 20,091 participants. The included studies were published between 2009 and 2016. Seven studies were conducted in the United States [12, 14, 20–24], four studies were from Finland [25–28], three studies were from China [29–31], two studies were from Canada [32, 33] and Japan [34, 35], and one study was from each of the following countries: UK [36], Korea [37], Singapore [38], Germany [15], and Mexico [39]. The studies varied in sample size from 30 to 7098 with a median of 873. There were seventeen studies enrolling both males and females, and two studies focused on women only [26, 32], while the rest of the four studies address novel findings from males [31, 36–38]. The age of participants in our studies ranged from 18 to 59 years. All studies focused on the serum metabolites and insulin resistance (HOMA-IR). The analytical platforms used for metabolite detection included amino acid analyser, HPLC/fluorescence spectroscopy, ultraperformance LC-MS, GC-MS, FIA-MS/MS, UPLC-Q-TOF MS,

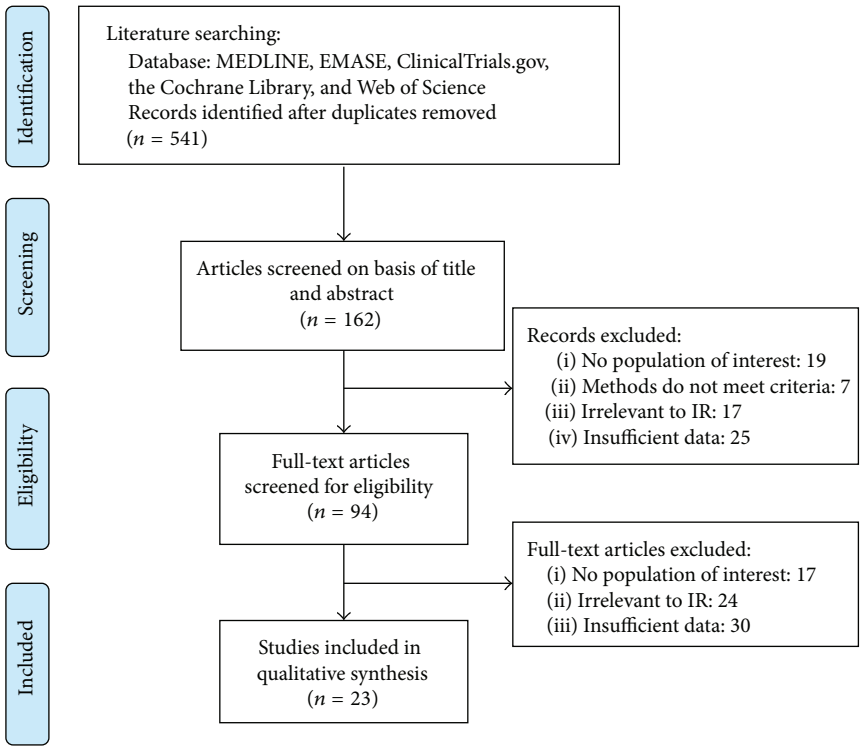

Figure 1: The flow chart of paper selection procedure in this systematic review.

NMR spectroscopy, CE-MS, ESI-LC-MS/MS, and UPLC-TQ-MS.

3.2. BCAA and Insulin Resistance. Included studies revealed significant alterations of BCAA in obese adults and the intimate association between BCAA and IR. Newgard et al. [12] revealed that obese subjects presented 2.3-fold higher HOMA-IR than lean controls ($P < 0.0001$). Notably, some metabolites showed significant differences between obese and lean controls, which mainly refer to leucine/isoleucine, valine, the aromatic amino acids, and C3 and C5 acylcarnitines ($P < 0.0001$). Furthermore, a significant linear relationship was shown between these BCAA-related metabolites and HOMA-IR ($r = 0.58$, $P < 0.0001$). Consistent results were shown in the study of Würtz et al. [28] on 7,098 young adults, comprising two Finnish cohorts, the Northern Finland Birth Cohort 1966 (NFBC) and the Cardiovascular Risk in Young Finns Study (YFS). They also showed that the BCAA together with other related metabolites was positively associated with HOMA-IR ($P < 0.0005$). Boulet et al. [32] suggested the positive relationship between AA related factors and HOMA-IR ($r = 0.35$, $P < 0.01$). And compared with valine and isoleucine, leucine showed highest correlation with HOMA-IR ($r = 0.26$). Another recent twin study from Bogl et al. [25] consisting of 286 subjects (MZ: 136, DZ: 150) showed HOMA-IR correlated significantly with higher valine, leucine, and aromatic amino acids (AAA) as well as lipid profiles ($r = 0.30$–0.40). In the study by Tai et al. [38] on 263 nonobese Asian Indian and Chinese men, they divided enrolled subjects into low HOMA and high HOMA groups; the results showed individuals with high HOMA presented higher level of valine ($P = 0.0033$) and leucine/isoleucine ($P = 0.0321$) in both Chinese and Asian Indians. Yamada et al. [35] showed the consistent results with previous studies in 94 nonobese Japanese people. Another study [34] on Japanese with normal glucose tolerance also showed positive correlation between HOMA-IR and valine, glutamate, and tyrosine levels, but negative correlation was found in citrulline, glutamine, and glycine levels.

Despite the altered BCAA level in peripheral circulation, BCAA metabolism in target tissue such as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) also showed significant changes in obese and insulin resistant patients [27, 32, 33]. Naukkarinen et al. [27] presented a close correlation between IR and downregulated mitochondrial BCAA catabolism in the adipose tissue of obese cotwins, who showed significantly lower insulin sensitivity and higher plasma insulin concentrations than their lean cotwins.

3.3. Elevated BCAAs and Later Risk for Diabetes and Metabolic Disorders. After evaluating all included studies, six prospective studies were found reporting the relationship between elevated BCAAs and later risk of type 2 diabetes [14, 15, 20, 28, 29, 36]. Wang et al.'s study on Framingham cohort [14] showed that 1 SD increment in five target amino acids (isoleucine, leucine, valine, tyrosine, and phenylalanine) was associated with a 57–102% increase in the risk of future diabetes ($P = 0.0002$–0.002). In addition, moderate association was found between baseline amino acids level and HOMA-IR as well as HOMA-beta ($r = 0.24$–0.37, $P < 0.001$).

Table 1: General population characteristics of included studies in this systematic review.

Study item	Year	Number	Type	Mean age	BMI (kg/m^2)	HOMA-IR	Detection platform
Chen et al.[a] [29]	2016	213	Cohort study	FDM: 53.78 ± 1.75 Control: 38.51 ± 0.95	FDM: 25.53 ± 0.50 Control: 24.63 ± 0.27	FDM: 2.13 ± 0.61 Control: 1.67 ± 0.30	UPLC-TQ-MS
Chen et al.[b] [29]	2016	216	Observational study	52.81 ± 0.71	Obese: 27.26 ± 0.25 Lean: 20.48 ± 0.07	Obese: 2.08 ± 0.54 Lean: 1.11 ± 0.17	UPLC-TQ-MS
Lee et al. [20]	2016	685	Cohort study	54 (47–62)	LT: 26.9 ± 5.0; MT: 28.5 ± 5.6; UT: 29.7 ± 5.4	LT[c]: 2.19 ± 0.39 MT[c]: 1.64 ± 0.6 UT[c]: 1.27 ± 0.25	MS/MS
Takashina et al. [34]	2016	83	Observational study	34.0 ± 5.7	Obese: 26.7 ± 2.3 Nonobese: 21.9 ± 1.8	Obese: 2.1 ± 1.6 Nonobese: 1.1 ± 0.5	HPLC
Bogl et al. [25]	2016	286	Observational study	28.7 ± 0.2	25.4 ± 0.3	1.4 ± 0.1	NMR
Boulet et al. [32]	2016	59	Observational study	47 ± 5.0	Lean: 23.2 ± 1.3 Overweight: 27.0 ± 1.3 Obese: 34.0 ± 3.3	Lean: 1.9 ± 1.4 Overweight: 1.7 ± 0.9 Obese: 2.6 ± 1.0	ESI-LC-MS/MS, ESI-MS/MS
Tillin et al. [36]	2015	2286	Cohort study	European: 52.9 ± 7.3 South Asian 50.6 ± 7	European: 25.65 ± 0.73 South Asian 25.46 ± 0.67	European: 1.71 ± 0.26 South Asian 2.43 ± 0.35	NMR
Yamada et al. [35]	2015	94	Observational study	40.1 ± 9.6	22.7 ± 3.9	1.12 ± 0.72	LC/MC
Liu et al. [31]	2015	30	Observational study	20.5 ± 1.8	Obese: 32.62 ± 2.70 Control: 20.77 ± 1.25	Obese: 1.55 ± 0.12 Control: 1.25 ± 0.11	GC/MS, UPLC-TQ-MS
Badoud et al. [33]	2014	30	Observational study	LH: 51 ± 3 MHO: 50 ± 4 MUO: 48 ± 2	LH: 22.1 ± 0.6 MHO: 30.6 ± 1.1 MUO: 33.0 ± 1.9	LH: 0.65 ± 0.28 MHO: 1.18 ± 0.16 MUO: 2.19 ± 0.42	GC-MS, CE-MS
Xie et al.[a] [30]	2014	211	Cohort study	Obese: 46.8 ± 6.9 Lean: 45.9 ± 7.2	Obese: 26.7 ± 1.25 Lean: 20.6 ± 0.52	Obese: 1.97 ± 0.83 Lean: 1.11 ± 0.52	UPLC-QTOFMS; GC-TOFMS

Table 1: Continued.

Study item	Year	Number	Type	Mean age	BMI (kg/m^2)	HOMA-IR	Detection platform
Xie et al.[b] [30]	2014	105	Cohort study	Obese: 47.0 ± 8.0 Lean: 49.9 ± 8.3	Obese: 27.1 ± 1.2 Lean: 18.8 ± 0.7	Obese: 2.20 ± 0.73 Lean: 1.31 ± 0.5	UPLC-QTOFMS; GC-TOFMS
Serralde-Zúñiga et al. [39]	2014	115	Observational study	Non-IR 41 ± 10 IR: 39 ± 11	Non-IR: 29.8 ± 10.9 IR: 44.2 ± 11.8	Non-IR: 1.6 ± 0.6 IR: 5.4 ± 3.2	N/A
Wiklund et al. [26]	2014	78	Observational study	MHO: 39.7 ± 7 MetS: 44 ± 6	MHO: 28.9 ± 3.2 MetS: 30.6 ± 3.4	MHO: 1.6 ± 1.0 MetS: 2.3 ± 0.9	NMR
Batch et al. [21]	2013	1872	Observational study	59.49 ± 11.8	30.05 ± 6.44	2.24 ± 1.87	MS/MS
Floegel et al. [15]	2013	2282	Cohort study	49.5 ± 8.9	26.1 ± 0.09	1.6 ± 0.4	FIA-MS/MS
Xu et al. [22]	2013	734	Cohort study	51.2 ± 9	32.65 ± 3.9	2.36 ± 0.36	N/A
Naukkarinen et al. [27]	2014	32	Observational study	27.4 ± 1.0	Obese: 30.4 ± 1.3 Nonobese: 24.75 ± 1.3	Obese: 2.95 ± 0.5 Nonobese: 1.35 ± 0.3	GC-TOFMS
Cheng et al. [23]	2012	1015	Observational study	56 ± 9	28.3 ± 5.0	2.5 ± 2.2	LC/MS
Würtz et al. [28]	2012	7098	Observational study	31 ± 3	Men: 31.2 ± 2.6 Women: 31.2 ± 2.8	Men: 0.98 ± 0.09 Women: 0.92 ± 0.08	NMR
Shah et al. [24]	2012	500	Cohort study	55.9 ± 8.7	33.9 ± 4.7	2.45 ± 0.5	MS/MS
Wang et al. [14]	2011	2422	Cohort study	Case: 56 ± 9 Con: 57 ± 8	Case: 30.5 ± 5 Control: 30.0 ± 5.5	Case: 3.5 ± 2.6 Control: 3.1 ± 2.3	LC-MS
Kim et al. [37]	2010	60	Observational study	39.55 ± 1.24	Obese: 28.9 ± 0.2 Nonobese: 20.9 ± 0.14	Case: 3.34 ± 0.3 Control: 1.72 ± 0.11	UPLC-Q-TOF MS
Newgard et al. [12]	2009	141	Observational study	51 ± 9	Obese: 36.6 ± 1.33 Lean: 23.2 ± 0.33	Obese: 5.73 ± 0.74 Lean: 2.61 ± 0.22	MS/MS, GC/MS
Tai et al. [38]	2010	263	Observational study	49.25 ± 10.6	Chinese: 24.2 ± 2 India Asian: 24.7 ± 2.1	Chinese: 1.8 ± 0.8 India Asian: 2.63 ± 2	MS/MS, GC/MS

Note: BMI: body mass index; HOMA-IR: homeostasis model of assessment for insulin resistance index; BCAA: branched-chain amino acids; LH: lean healthy; MHO: metabolically healthy obese; MUO: metabolically unhealthy obese; FDM: future diabetes development; LT: lowest third of BCAA; MT: middle third of BCAA; UT: upper third of BCAA; MetS: metabolic syndrome; IR: insulin resistance; non-IR: noninsulin resistance; N/A: not applicable; UPLC-TQ-MS: ultraperformance liquid chromatography triple quadruple mass spectrometry; MS/MS: tandem mass spectrometry; HPLC: ultraperformance liquid chromatography; NMR: nuclear magnetic resonance; ESI-LC-MS/MS: liquid chromatography-electrospray ionization mass spectrometry; ESI-MS/MS: electrospray ionization mass spectrometry; LC/MC: liquid chromatography/mass spectrometry; GC/MS: gas chromatography-mass spectrometry; CE-MS: capillary electrophoresis-mass spectrometry; GC-TOFMS: gas chromatography/time-of-flight mass spectrometry; FIA-MS/MS: flow injection analysis tandem mass spectrometry; [a]study I; [b]study II for validation; [c]data for insulin sensitivity.

Table 2: The influence of race in BCAA level and its relationship with insulin resistance.

Study	Country	Race	BCAA-related metabolites identified	Relation between BCAA and IR
Lee et al. [20]	Canada	Caucasians, African Americans, Hispanics	Valine, leucine, isoleucine, total BCAA	Positive, Caucasians plus Hispanics > African Americans
Tillin et al. [36]	Finland	Europeans, South Americans	Isoleucine, leucine, valine, phenylalanine, tyrosine, alanine, glutamine, glycine, histidine	Positive, South Americans > Europeans
Tai et al. [38]	Singapore	Chinese, Asian Indians	Valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamine, ornithine, alanine, proline	Positive, Chinese > Asian Indians

Note: IR: insulin resistance; BCAA: branched-chain amino acids.

Besides, similar results have been validated in another four large cohorts with long-term follow-up [15, 20, 29, 36]. In Tillin et al.'s study [36] on 801 European and 643 South Asian participants with 19-year follow-up, 227 South Asian men (35%) and 113 European men (14%) developed diabetes [36]. And amino acid metabolites (isoleucine, leucine, valine, phenylalanine, and tyrosine) were associated with incidence of diabetes in both ethnic groups after logistic regression analyses ($OR_{isoleucine} = 3.11$; $OR_{leucine} = 3.36$; $OR_{valine} = 3.34$). In recent studies on Chinese population [29], 51 individuals presented similar level of metabolic markers but higher amino acids level (valine, leucine, isoleucine, tyrosine, and phenylalanine) and 162 controls were enrolled. After 10-year follow-up, the positive correlation was found between the baseline five AAs and future diabetes incidents in these 51 individuals (ORs per SD > 1.5 and $P < 0.001$). However, the baseline metabolic markers failed to predict the risk of diabetes ($P > 0.05$). In the EPIC-Potsdam study with 7-year follow-up, Floegel et al. [15] showed positive correlation between isoleucine, valine, and future risk of T2DM (RR per SD 1.30 [95% CI 1.17–1.43] and 1.27 [1.16–1.40], resp.). Also, the ethnical difference in using BCAA to predict diabetes is presented by Lee et al. [20]; the positive relationship with later diabetes risk was more obvious in Caucasians or in the combined Caucasian and Hispanic group but not in African Americans during 5-year follow-up. In a word, compared with already established metabolic factors, elevated BCAA level can largely improve the accuracy of prediction on future metabolic risk [15].

3.4. Factors Influencing BCAA Level and IR. Multiple factors have shown to influence the concentration of BCAA and its correlation with IR. Based on current evidence, these factors can be divided into two main aspects: phenotypic modification and genetic modification. Phenotypic part refers to race, gender, and dietary pattern; and genetic part refers to relative gene variants in BCAA metabolism.

3.4.1. Race Difference in BCAA and Its Relationship with IR. Three studies compared BCAAs outcomes across different races, including Europeans versus South Asians [36], Chinese versus Indian Asians [38], and Caucasians versus African Americans versus Hispanics [20]. The detailed study characteristics about the influence of different race in BCAA level and its relationship with IR were shown in Table 2. Tillin et al. [36] reported that South Asian participants (1,279), compared with European (1,007), had higher serum concentrations of isoleucine ($P < 0.0001$) but weaker correlation with obesity measurements. During 19-year follow-up in this study, higher risk of incident diabetes was also found in South Asian (34%) compared to European subjects (14%). Tai et al. [38] revealed that Chinese individuals with high HOMA had higher levels of amino acids (valine and leucine/isoleucine) than Chinese individuals with low HOMA ($P_{valine} = 0.005$ and $P_{leucine/isoleucine} = 0.011$, resp.). Similar association was also observed in Asian Indians in other amino acids but did not reach statistical significance. The Insulin Resistance Atherosclerosis Study (IRAS) from Lee et al. [20] demonstrated the variances in ethnicity among Caucasians ($n = 290$), Hispanics ($n = 230$), and African Americans ($n = 165$). In stratified analysis by ethnicity, they found negative association between BCAA levels and insulin sensitivity (S_I) in Caucasians and Hispanics [β-coefficients: −0.0027 (−0.0035, −0.0020)]. After 5-year follow-up, positive correlation between BCAA and the risk of developing diabetes was only found in Caucasians and Hispanic group but not in African Americans. As for other races (such as Japanese, Chinese, and Finns), similar positive association between BCAA and IR was also presented [29, 30].

3.4.2. Gender Difference in BCAA and Its Relationship with IR. Seven studies addressed gender difference in BCAAs levels [20, 25, 28, 30, 32, 34, 35]. The detailed study characteristics about the comparison of altered BCAA level between male and female were shown in Table 3. The study on 7,098 young adults from Würtz et al. [28] displayed positive association between BCAA (leucine, isoleucine, and valine) and IR, and stronger correlation was found in men compared with women ($P_{leucine} < 0.001$; $P_{isoleucine} = 0.006$; $P_{valine} = 0.001$). In addition, a study from 94 Japanese people [35] revealed that BCAAs (leucine, isoleucine, valine) were positively associated with IR. However, this positive association was only found in women ($r = 0.354$, $P = 0.016$), which did not reach statistical significance in men ($r = 0.245$, $P = 0.094$), while, in a study on 685 participants, Lee et al. [20] found that men

Table 3: Comparison of altered BCAA level between male and female.

Study	Number	Men	Female	BCAA and related metabolites identified	Relation between BCAA and IR
Lee et al. [20]	685	45%	55%	Valine, leucine, isoleucine, total BCAA	Positive, male > female
Takashina et al. [34]	83	80%	20%	Valine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, alanine, arginine, asparagine, α-aminobutyric acid, citrulline, cystine, glutamate, glutamine, glycine, ornithine, proline, serine, taurine, tyrosine, histidine	Positive, male > female
Boulet et al. [32]	59	0%	100%	Leucine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, alanine, arginine, citrulline, cystine, glycine, ornithine, proline, serine, taurine, tyrosine, C3	Positive (leucine)
Yamada et al. [35]	94	51%	49%	Valine, leucine, isoleucine, total BCAA, alanine, tryptophan, phenylalanine, tyrosine, ornithine, glycine, glutamate/glutamine, methionine, lysine, cysteine, aspartic acid, total AA	Positive, male > female
Xie et al.[a] [30]	211	36%	64%	Valine, isoleucine, leucine, glutamic acid, tryptophan, tyrosine, carnitine, phenylalanine, alanine, beta-tyrosine, creatine	Positive, male > female
Xie et al.[b] [30]	105	38%	62%	Valine, isoleucine, leucine, glutamic acid, tryptophan, tyrosine, carnitine, phenylalanine, alanine, beta-tyrosine, creatine	Positive, male > female
Würtz et al. [28]	7098	48%	52%	Leucine, isoleucine, valine, glutamine, pyruvate, creatinine, alanine, phenylalanine, phospholipids, fatty acids	Positive, male > female

Note: IR: insulin resistance; BCAA: branched-chain amino acids; AA: amino acids; [a]study I; [b]study II for validation.

Table 4: The influence of dietary pattern and weight loss in BCAA level.

Study	Diet	Weight loss	BCAA and related metabolites identified	Relationship with BCAA and IR
Batch et al. [21]	BCAA supplement	No	Leucine/isoleucine, valine, tyrosine, methionine, alanine, histidine, phenylalanine; C3; C5	Not related to BCAA and IR
Xu et al. [22]	High & low fat	Yes	Valine, isoleucine, leucine, tyrosine, phenylalanine; tryptophan, BCAA/AAA	Corresponded to gene variants
Würtz et al. [28]	Protein intake	No	Leucine, isoleucine, valine, glutamine, pyruvate, alanine, creatinine, phenylalanine, phospholipids, fatty acids	Positive for valine level Not related to IR
Shah et al. [24]	BCAA intake	Yes	Alanine, leucine/isoleucine, valine, methionine, phenylalanine, tyrosine, glutamate/glutamine, ornithine	Slightly positive for BCAA level Not related to IR
Newgard et al. [12]	BCAA supplement	No	Alanine, valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamine, aspartate/asparagine	Positive for BCAA and IR (in rats)

Note: IR: insulin resistance; BCAA: branched-chain amino acids; AAA: aromatic amino acids.

had higher level of BCAA as well as BMI, waist-hip ratio, IR, and dietary energy intake compared with women. The gender-dependence was also reported by Xie et al. [30]. They presented that BCAA was correlated with IR only in obese Chinese men; similar results were validated by another two cohorts on Chinese and Americans. Consistent results were also presented by Takashina et al. [34]. Other studies did not show difference in BCAA or IR between male and female.

3.4.3. Dietary Pattern and Weight Loss in BCAA and Their Relationship with IR. Five studies showed the relationship between diet and BCAA [12, 21, 22, 24, 28]. The detailed study characteristics about the influence of dietary pattern and weight loss in BCAA level were shown in Table 4. In the study of Shah et al. [24], BCAAs (valine and leucine/isoleucine) not only correlated positively with baseline HOMA-IR ($r = 0.50$, $P < 0.0001$) but also correlated with 6-month HOMA-IR ($r = 0.28$, $P < 0.0001$) as well as ΔHOMA-IR ($r = -0.38$, $P < 0.0001$) after weight loss. Moreover, during the weight-loss period, valine and leucine/isoleucine presented remarkable reduction ($P = 0.005$, $P < 0.0001$). To test the influence of BCAA intake on plasma BCAA level, they

Table 5: BCAA-related gene variants in specific tissues based on included studies.

Study	BCAA and related metabolites identified	Identified gene variants	Specific tissue
Boulet et al. [32]	Leucine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, alanine, arginine, citrulline, cystine, glycine, ornithine, proline, serine, taurine, tyrosine, C3	*BCKDHA, BCKDHB, BCAT1, BCAT2, BCKDK*	Visceral adipose tissue, subcutaneous adipose tissue
Badoud et al. [33]	Valine, isoleucine, ornithine, alanine, aspartic acid, glutamine, phenylalanine, methionine, tyrosine, glycine, cysteine, aspartic acid, glutamine/glutamic acid	*BCAT2, BCKDHA, BCKDHB, DBT,*	Subcutaneous adipose tissue
Serralde-Zúñiga et al. [39]	Total BCAA, isoleucine, leucine, valine	*BCAT2, BCKDH E1a, SREBP-1, FTO*	Omental adipose tissue
Xu et al. [22]	Valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, BCAA/AAA	*PPM1K (rs 1440581), C allele*	Blood
Naukkarinen et al. [27]	Leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, glutamine, glutamate/glutamine	*Genes of BCAA catabolism (monozygotic twin study)*	Subcutaneous adipose tissue
Würtz et al. [28]	Leucine, isoleucine, valine, glutamine, pyruvate, creatinine, alanine, phenylalanine, phospholipids, fatty acids	*GCKR (rs 1260326)*	Blood

Note: IR: insulin resistance; BCAA: branched-chain amino acids; AAA: aromatic amino acids; *BCKDK*: branched-chain keto acid dehydrogenase kinase; *BCKDHE1a (BCKDHA)*: branched-chain keto acid dehydrogenase E1, alpha polypeptide; *BCKDHB*: branched-chain keto acid dehydrogenase E1 subunit beta; *BCAT1*: branched-chain amino acid transaminase 1; *BCAT2*: branched-chain amino acid transaminase 2; *DBT*: dihydrolipoamide branched-chain transacylase E2; *SREBP-1*: sterol regulatory element binding protein 1; *FTO*: fat mass and obesity associated; *PPM1K*: protein phosphatase, Mg2+/Mn2+ dependent 1K; *GCKR*: glucokinase (hexokinase 4) regulator.

performed baseline and 6-month intake of BCAA in enrolled participants. In Shah et al.'s study, BCAA intake refers to a sum of dietary intake on valine, leucine, and isoleucine intake. The results showed that BCAA intake was weakly related to peripheral BCAA level ($r = 0.14$, $P = 0.003$), but change in BCAA intake was not correlated with plasma BCAA level ($P = 0.39$) and ΔHOMA-IR ($P = 0.82$). The study from Würtz et al. [28] demonstrated that dietary intake, especially for protein intake, was related to fasting metabolites levels (valine, phenylalanine, and tyrosine). However, no relationship was found in protein intake and IR. Batch et al. also presented no difference in glucose metabolism and HOMA-IR between controls and BCAA-supplement group [21]. However, study from Newgard et al. [12] on rats showed positive relationship in BCAA intake and IR, indicating the existence of species difference.

3.4.4. Gene Variants in BCAA and Its Relationship with IR. There were six studies reporting relevant gene variants in BCAA metabolism [22, 27, 28, 32, 33, 39]. The detailed study characteristics about BCAA-related gene variants in specific tissues were shown in Table 5. In study of Badoud et al. [33], plasma valine and isoleucine were positively correlated with HOMA-IR [lean healthy (LH) < metabolic healthy obese (MHO) < metabolic unhealthy obese (MUO)]. To explore the alterations in BCAA-related gene expression, they found downregulations of BCAA catabolism and TCA cycle in subcutaneous adipose tissue (SAT) from MHO and MUO patients compared with LH patients. The key mitochondrial genes involved in BCAA catabolism were significantly influenced, including the increase in *BCAT1* and decrease in *BCKDHA* as well as *BCAT2* in obese group compared with lean group. In two obese groups, MUO group had more significant reduction in *BCAT2* and *BCKDHA* than MHO group. In other studies, Serralde-Zúñiga et al. [39] revealed significant reduction in the gene expressions of *BCAT2* and *BCKDH E1a* in omental adipose tissue of insulin resistant subjects. Similar changes of gene expression were reported by Boulet et al. [32] in visceral adipose tissue (VAT) in obese women with BMI > 30 kg/m^2 ($P < 0.05$), while, in SAT, only expression of *BCAT2* was decreased. Naukkarinen et al. [27] studied gene variants in weight-discordant monozygotic twins and found that genes related to BCAA catabolism, oxidative phosphorylation, and fatty acid β oxidation were downregulated in SAT of obese cotwins compared with their lean counterpart. Due to the impairment in BCAA clearance, higher BCAA level was found in obese cotwins ($P = 0.016$). Another study from Würtz et al. [28] presented the positive correlation between metabolites (isoleucine, alanine, total fatty acids, etc.) and SNP rs1260326 in *GCKR* which was associated with IR ($P = 0.001$). Xu et al. [22] found that individuals carrying C allele of the branched-chain amino acid/aromatic amino acid (BCAA/AAA) ratio-associated variant rs1440581 near *PPM1K* gene may benefit less during weight loss than those without this allele when undertaking an energy-restricted high-fat diet.

3.5. Quality Assessment. The study team conducted quality assessment process through QUADOMICS tool [18, 40]. In all the included studies, sixteen studies were defined as high quality, fulfilling more than 11 scores. Two different persons double-checked the general characteristics of included studies (Xue Zhao and Qing Han).

4. Discussion

4.1. BCAA Metabolism and Its Correlation with IR. Leucine, isoleucine, and valine are known as BCAA. The metabolism of BCAA has been known as a significant factor in the pathophysiology of many multifactorial diseases, such as metabolic syndrome, cancer, and hepatic disease [41]. The catabolism of BCAA contains two important steps. The first step is a reversible transamination reaction converting BCAA to branched-chain a-keto acids (BCKAs). In this procedure, the enzyme branched-chain amino acid aminotransferase (BCAT) played an important role. At the same time, the other partner pair a-ketoglutarate/glutamate takes place with this transamination reaction. The next step in the BCAAs catabolism is irreversible, mediated by the mitochondrial branched-chain keto-acid dehydrogenase complex (BCKDC). This enzymatic step confers oxidation on the BCKAs, leading to the production of NADH, CO_2, and different end-products [42].

Changes in *BCAT* and *BCKDC* can have significant effects on BCAA catabolism [43, 44]. Because of BCAA dysmetabolism, some potentially toxic intermediates in BCAA catabolic pathway might be accumulated, leading to the impaired cellular or organ function. In some studies, humans or animal models with impaired BCAA metabolism presented higher susceptibility to IR and T2DM compared with controls [41]. Olson et al. [43] showed that high level of toxic BCAA metabolites, rather than BCAAs themselves, could result in mitochondrial dysfunction and apoptosis in β-cell.

However, the mechanism between BCAA and IR remains unclear [45]. Studies have shown that persistent high nutrient signaling might lead to the onset of IR mediated by mTORC1 signaling pathway [16, 46]. In recent years, mTOR has been considered as the important link between amino acids and insulin action. Once mTOR has been stimulated, it will activate p70 ribosomal S6 kinase (p70S6K). p70S6K regulates protein synthesis cascade and subsequently leads to the phosphorylation of its downstream target, ribosomal protein S6 kinase (S6K) [44, 47]. Moreover, activation of S6K1 leads to serine/threonine phosphorylation and therefore the inhibition of insulin receptor substrate (IRS)-1. This procedure not only impacts normal insulin signaling but also causes the degradation of IRS-1 [48]. However, much more studies are required in the future to figure out the role of mTOR pathway in elevated BCAA and IR.

4.2. Factors Influencing BCAA and Its Relationship with Insulin Resistance. The factors influencing BCAA level and its relationship with IR in this study mainly refer to race, gender, diet, and gene variants. First of all, after generating available studies on different ethnicities, we found that Asian people (Chinese or Japanese) seemed to be more susceptible to higher HOMA-IR and higher BCAA level compared with western people such as Caucasians or Europeans. Although Asian people usually hold lower BMI, studies have shown their higher susceptibility to impaired islet function at the early stages of metabolic disorders [29], while, in studies focusing on western people, Hispanics and Caucasians seemed to have stronger positive association between BCAA and IR than African Americans. Thus, the elevated BCAA level and its relationship with IR seem to be race-dependent. Future studies should extend their sample size in different races in order to validate the above findings. For gender difference, majority of included studies showed obese men had higher BCAA and stronger positive relationship with IR compared with female. This indicated future studies should pay much attention to the gender difference during data analysis and study design. In a word, diverse race difference and gender difference can have significant effects on BCAAs level and insulin resistant state, which should also be validated in studies with larger population.

The influence of dietary pattern on BCAA level and IR was not established yet. For a long time, beneficial effects were presented on the regulation of body weight, muscle protein synthesis, and glucose homeostasis in patients or animals applying BCAA supplementation or BCAA-rich diets [49, 50]. In this study, the results showed that a BCAA-rich diet might have a weakly positive impact in peripheral BCAA level, which was most frequently explained by the insulinotropic properties of amino acids [51] or the decreased glycemic load of high-protein diets. Also, Xu et al.'s study [22] presented a new gene-diet interaction, illustrating that patients with C allele of BCAA/AAA might lose less weight during a weight-loss diet. As for the relationship between BCAA supplement and IR, most studies showed negative results, indicating that the ectogenic BCAA intake constituted an extremely small portion in the onset of IR. However, how BCAAs influence insulin signaling remains unclear. Thus, the relationship between diet, circulating BCAA, and IR deserves further exploration.

Although studying blood metabolites can uncover novel markers of potential clinical relevance, it is difficult to determine the relevant gene variants and specific tissues underlying changes in metabolic pathways. As mentioned, *BCAT* and the *BCKD* complex in BCAA catabolism are broadly expressed in human tissues, indicating that extensive tissues hold the ability of BCAA's transamination and oxidation [13]. Among these tissues, skeletal muscle, adipose tissue, and liver seem to play the central role [45]. In our study, we mainly talked about the gene expressions of BCAA metabolism in adipose tissue based on included studies and revealed the significant role of gene expression changes in BCAA catabolism of adipose tissue regarding pathogenesis of IR. Consistent results were also reported by Pietiläinen et al. [52] and Solini et al. [53]. Another study validating the function of adipose tissue in peripheral BCAA level was performed by Herman et al. [54]. They showed that the transplanted adipose tissue had the capability of catabolizing circulating BCAAs in vivo and indicated the possibility that adipose tissue modulates circulating BCAA levels through regulating BCAA enzymes. All these showed that genetic variants of BCAA catabolism in adipose tissue might have important influences on altered BCAA level and the presence of IR to some extent.

In addition to adipose tissue, several studies have demonstrated the existence of BCAA dysmetabolism in other tissues, while few studies detected relative gene expressions. Shin et al. [55] showed that decreased hepatic *BCKDH*

might contribute to the increased plasma BCAAs. Moreover, hypothalamus presented an important role in impaired BCAA metabolism in obesity and diabetes. Their study on rats revealed that short-term overfeeding impaired brain insulin ability to lower BCAAs, which was also associated with reduced *BCKDH* protein in liver. In addition, in a study of 35 subjects, Chevalier et al. [56] revealed the increased postabsorptive protein catabolism with elevated BCAA in obesity was associated with increased gluconeogenesis and led to dysglycemia. In skeletal muscle, Krebs et al. [57] reported that elevated plasma amino acid can induce IR in skeletal muscle through inhibiting the glucose transport/phosphorylation and later glycogen synthesis. As for gut microbiota, the amino acid fermenting bacteria in human gastrointestinal tract present the important role in the digestion and absorption of protein and amino acids [58, 59]. Beyond that, gut bacteria themselves also hold a higher level of BCAA-related amino acids [60]. However, it remains to be known if BCAA from gut bacteria can impact the homeostasis of BCAA in the host and how they influence each other. Future studies should focus on the BCAA catabolic alteration in different tissues and explore the mechanism of altered BCAA and its relationship with IR.

4.3. Prospects in BCAA and IR Treatment. BCAAs (valine, leucine, and isoleucine) can be an important and useful biomarker of IR. Besides the reflection of insulin resistant state, BCAA can also give feedback of drug effect. In a randomized, double-blind, controlled study on 25 overweight/obese adults [61], patients using insulin sensitizer therapy showed improved insulin sensitivity and reduced functional metabolites (BCAA) compared to placebo treatment after three months of intervention. Walford et al. [62] found administration of glipizide and metformin can influence the ratio–BCAA/AAAs acutely, and the magnitude of change was dependent on the IR status of participants. The above results demonstrate that BCAA or BCAA/AAAs may be useful biomarkers for monitoring the early response to therapeutic interventions in T2DM patients. Since protein intake or BCAA supplement will not influence plasma BCAA level too much, we highly recommend measurement of BCAA levels in order to capture the whole course of disease progress, including obesity, insulin resistant state, T2DM, and later drug effect. Improvement in this field will definitely deepen our understanding that BCAA (as a biomarker) can reflect not only IR but also pharmacological effects of drug intervention.

4.4. Strength and Limitation. Based on current studies, we found that BCAAs could be useful biomarker for IR and predictor for later diabetes risks. To our knowledge, this is the first review focusing on the relationship between BCAA and IR as well as the factors influencing BCAA level. All the eligible studies were published between 2009 and 2016, manifesting the latest development in this field. Although much more studies are largely needed, our study sheds light on the differences in ethnicity, sex, gene expression, and dietary pattern, which are important factors influencing BCAA level that should be controlled and considered when conducting clinical or basic studies. However, several limitations should be addressed here. There were huge heterogeneities in subject and study design; the control group and experimental group were not consistent across studies. This limitation restricts further comparison and data analyzed for meta-analysis.

5. Conclusion

In conclusion, this systematic review highlighted BCAAs (valine, leucine, and isoleucine) as useful biomarkers for early detection and diagnosis of IR in nondiabetic patients with obesity and as a valuable predictor for later risk of T2DM. Based on included studies, we found that differences in ethnicity, sex, gene expression, and dietary pattern can influence BCAA level and its relationship with IR. Future studies should focus on the mechanism of BCAA and IR in different tissues and explore the potential treatment targets for obesity and T2DM. Despite the long-time exploration of BCAA and its applications so far, much more studies are still required to bring it to light.

Competing Interests

No competing interests were declared.

Authors' Contributions

Xue Zhao and Guixia Wang designed this review; Xue Zhao and Qing Han conducted the paper selection; Chenglin Sun, Xiaokun Gang, and Yujia Liu performed the statistical analysis and data extraction; Xue Zhao and Guixia Wang wrote the manuscript and were responsible for the final content of the manuscript. All authors read and approved the final manuscript.

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Review Article

Variations in the Obesity Gene "*LEPR*" Contribute to Risk of Type 2 Diabetes Mellitus: Evidence from a Meta-Analysis

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Leptin is a hormone protein regulating food intake and energy expenditure. A number of studies have evaluated the genetic effect of leptin (*LEP*) and leptin receptor (*LEPR*) genes on T2DM. This study aimed to investigate the association between these gene polymorphisms and T2DM by a systematic review and meta-analysis. Published studies were identified through extensive search in PubMed and EMBASE. A total of 5143 T2DM cases and 5021 controls from 14 articles were included in this study. Five functional variants in *LEPR* were well evaluated. Meta-analysis showed that rs1137101 (p.R223Q) was significantly associated with T2DM in all genetic models: allele model (OR = 1.27, 95% confidence interval (CI) = 1.13–1.42), dominant model (OR = 1.19, 95% CI = 1.05–1.35), homozygote model (OR = 1.82, 95% CI = 1.38–2.39), and recessive model (OR = 1.75, 95% CI = 1.35–2.28), with minimal heterogeneity and no indication of publication bias. Similar associations with T2DM were also found for rs62589000 (p.P1019P) and 3′UTR ins/del, although the data was obtained from a small number of studies. For the other two polymorphisms rs1137100 (p.R109K) and rs8179183 (p.K656N), they were not significantly associated with T2DM. Our results provide robust evidences for the genetic association of rs1137101 (p.R223Q) in *LEPR* with T2DM susceptibility.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a group of metabolic disorders with insulin secretion deficiency or insulin resistance (IR) and characterized by hyperglycemia. The prevalence of diabetes increases significantly in recent decades, affecting about 6% of adult population globally. Therefore, it is one of the major health care challenges in the world [1]. T2DM is a heterogeneous and polygenic disease associated with increased risk of several complications, such as cardiovascular disease, ischemic heart disease, and diabetic retinopathy [2]. Over the past decades, great achievements have been made in clinical diagnosis and interventions as well as elucidating the underlying pathogenesis of diabetes. Obesity, food intake, and energy expenditure have long been recognized as important key factors in the etiology of diabetes [3]. Besides, genetic factors also play a pivotal role in the development of diabetes and identified multiple T2DM-associated genes, providing additional insights into the disease mechanisms [4].

Leptin is a hormone protein important in regulating food intake and energy expenditure for energy balance, fertility, and metabolism, which are mediated by the cell surface leptin receptor (LepR) [5, 6]. It is suggested that there is a connection between energy metabolism and obesity [7]. Under normal condition, leptin can reduce appetite and increase sympathetic activity. Notably, leptin is also known to facilitate glucose utilization and improves insulin sensitivity [8, 9]. Apart

from its role in obesity, recent studies have drawn attention to the role of leptin in the pathogenesis of T2DM and insulin resistance.

LepR is encoded by the leptin receptor gene (*LERP*) on chromosome 1p31. Several functional variants with possible biological effects on metabolism regulation have been extensively investigated for the genetic predispositions on diabetes and its complications. These gene polymorphisms include rs1137101 (p.Arg223Gln or p.R223Q), rs1137100 (p.Arg109Lys or p.R109K), rs8179183 (p.Lys656Asn or p.K656N), rs62589000 (p.Pro1019Pro or p.P1019P), 3′-untranslated region (UTR) ins/del, rs1805134 (p.Ser343Ser or p.S343S), and rs2228301 (p.Asn567Asn or p.N567N) [10–23].

Although many studies have elucidated the association between T2DM and *LEPR* gene, the conclusion is still controversial because of small sample size in each study and lack of robust replication. We therefore conducted a systematic review and meta-analysis to evaluate the genetic impacts of *LEPR* gene polymorphisms on the risk of T2DM.

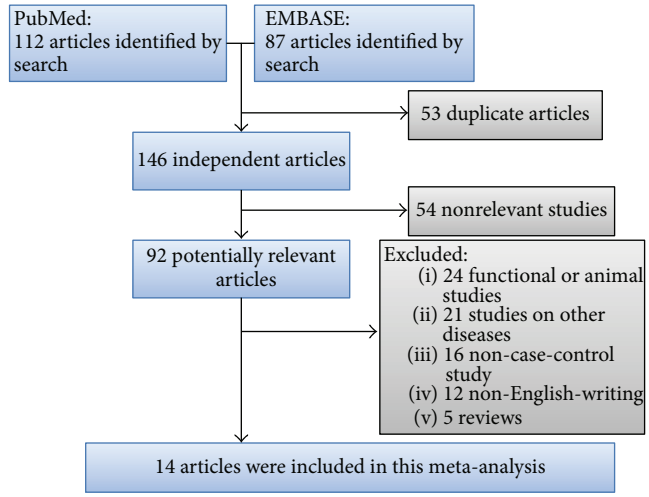


Figure 1: A schematic representation of the search strategy and selection process.

2. Methods

2.1. Searching Strategy and Inclusion Criteria. A comprehensive literature search was conducted in online databases, MEDLINE (Medical Literature Analysis and Retrieval System Online) and EMBASE (via Ovid) engines up to July 2015. The following medical subject headings and keywords were used for search strategy: "leptin", "leptin receptor", "LEP", "LEPR", "gene(s)", "polymorphism(s)", "mutation(s)", "variant(s)", "diabetes", "diabetic", "DM", and "diabetes mellitus". References lists of the retrieved articles and reviews were also screened for additional articles not captured by electronic search. Eligible studies were defined as the following criteria: (1) case-control study; (2) investigating the association between T2DM and *LEPR* polymorphisms; (3) sufficient genotype distribution data in case and control groups; (4) study samples being unrelated individuals drawn from clearly defined populations; (5) written in English. The exclusion criteria were defined as studies on animals, case reports, reviews, abstracts, editorial comments, and reports with incomplete data.

2.2. Data Extraction and Quality Appraisal. The following information was extracted from each study: first author, year of publication, country of studies, ethnicity, sample size, age, gender, allele/genotypic frequencies, and body mass index (BMI). If the test for Hardy-Weinberg equilibrium (HWE) was not reported, it was tested by the genotype data. The quality of studies was evaluated independently by two investigators (M. M. Yang and J. Wang) according to the Newcastle-Ottawa Scale (NOS). Uncertainties were resolved by discussions or by consensus with a third reviewer (J. J. Fan). NOS evaluated studies with a star-rating system ranging from 0 (lowest) to 9 (highest) stars, which was based on three study components, including selection, comparability, and outcome assessment. Studies with more than 5 points were evaluated as qualified.

2.3. Statistical Analysis. Pooled odds ratios (ORs) and corresponding 95% confidence intervals (CI) were used to estimate the association strength between *LEPR* polymorphisms and T2DM risk. The regression coefficients and the associated standard errors (SE, 95% CI) were combined using meta-analytic software. The combined ORs were, respectively, calculated by four genetic models (allele, dominant, recessive, and homozygous). HWE among controls was evaluated by χ^2 test and $P < 0.05$ was considered as significant disequilibrium. Both Cochran's Q statistic testing degree of heterogeneity across studies and the index I^2 statistic quantifying the proportion of heterogeneity between studies were calculated; P value less than 0.10 for the Q-test and I^2 above 50% were considered as statistically significant. If there was significant heterogeneity, the random effects model would be used to analyze the pooled ORs; otherwise, the fixed effects model would be applied. We used univariate random effects metaregression to investigate the potential sources of heterogeneity, such as, ethnicity, sample size, source of control, and publication years. Sensitivity analysis was used to assess the stability of results by systematically removing each study and reassessing the significance, whereas funnel plot and Egger's test were used to assess the potential publication bias. Statistical analyses were conducted by Review Manager (version 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

3. Results

3.1. General Characteristics of the Included Studies. The search and selection process of included studies was presented in Figure 1. According to our searching strategy, 199 potentially relevant studies were retrieved initially; after screening, 14 studies and 30 extracted SNP outcomes met the inclusion criteria and were used for the meta-analysis. The general characteristics of the included studies were summarized in Table 1. Genotypic distribution was in agreement with HWE in all

Table 1: General characteristics of the studies included in this meta-analysis.

SNP	First author	Ethnicity	Case	Control	Age (mean ± SD)		Genotype frequency		BMI (kg/m^2)		MAF
					Case	Control	Case	Control	Case	Control	
R223Q rs1137101	Jiang [13]	Chinese	369	176	68.0 ± 6.4*	67.1 ± 7.1	4/65/273	3/33/117	25.0 ± 4.0*	23.1 ± 3.3*	0.42
	Mohammadzadeh [16]	Iranian	144	147	54.3 ± 8.9	52.5 ± 7.3	5/59/80	5/62/80	27.6 ± 4.5	28.0 ± 4.0	
	Gan [11]	Chinese	301	172	52.7 ± 10.7*	52.8 ± 8.0	18/83/200	4/47/121	24.8 ± 2.3*	24.8 ± 1.7*	
	Murugesan [19]	Indian	150	150	NA	NA	53/67/30	22/55/73	26.2 ± 3.6*	24.1 ± 3.4*	
	Etemad [10]	Malaysian	145	133	NA	NA	42/17/86	22/20/91	NA	NA	
	Etemad [10]	Chinese	49	71	NA	NA	13/0/36	6/5/60	NA	NA	
	Etemad [10]	Indian	90	77	NA	NA	37/7/46	23/15/39	NA	NA	
	Han [12]	Korean	407	345	59.7 ± 9.62	64.5 ± 3.4	4/92/311	8/69/266	24.8 ± 3.1	24.0 ± 3.2	
	Takahashi-Yasuno [20]	Japanese	220	377	NA	NA	66/154†	97/279†	NA	NA	
	Liao [14]	Taiwanese	999	45	NA	NA	8/194/796	1/8/36	NA	NA	
	Kyong [17]	Korean	775	688	58.9 ± 10.5	64.2 ± 4.2	11/177/578	13/148/523	24.4 ± 2.9	23.6 ± 3.1	
R109K rs1137100	Jiang [13]	Chinese	369	176	68.0 ± 6.4*	67.1 ± 7.1	3/81/184	3/35/72	25.0 ± 4.0	23.1 ± 3.3	0.32
	Murugesan [19]	Indian	150	150	NA	NA	10/40/100	10/48/91	25.7 ± 4.8*	24.7 ± 3.6*	
	Cruz [21]	Mexican	519	547	53.4 ± 7.4	43.6 ± 6.6	NA	NA	29.3 ± 4.8	27.5 ± 3.6	
	Kyong [17]	Korean	775	688	58.9 ± 10.5	64.2 ± 4.2	31/238/496	22/200/461	24.4 ± 2.9	23.6 ± 3.1	
	Qu [18]	Chinese	317	282	49.3 ± 13.7	45.2 ± 5.7	11/93/213	8/71/203	25.5 ± 4.1	23.8 ± 2.9	
	Liao [14]	Taiwanese	999	80	NA	NA	23/265/705	1/29/50	NA	NA	
	Han [12]	Korean	407	345	59.7 ± 9.62	64.5 ± 3.4	16/122/269	11/97/235	24.8 ± 3.1	24.0 ± 3.2	
K656N rs8179183	Jiang [13]	Chinese	369	176	68.0 ± 6.4*	67.1 ± 7.1	0/34/226	0/9/96	25.0 ± 4.0	23.1 ± 3.3	0.14
	Murugesan [19]	Indian	150	150	NA	NA	9/52/89	13/51/86	25.8 ± 4.3*	24.7 ± 4.0*	
	Kyong [17]	Korean	775	688	58.9 ± 10.5	64.2 ± 4.2	4/111/650	3/84/596	24.4 ± 2.9	23.6 ± 3.1	
	Qu [18]	Chinese	317	282	49.3 ± 13.7	45.2 ± 5.7	1/27/289	1/32/249	25.5 ± 4.1	23.8 ± 2.9	
	Han [12]	Korean	407	345	59.7 ± 9.62	64.5 ± 3.4	0/57/350	0/38/305	24.8 ± 3.1	24.0 ± 3.2	
P1019P rs62589000	Lu [15]	Chinese	216	108	NA	NA	132/33/51	49/17/42	27.0 ± 4.1*	22.3 ± 2.2	0.31
	Takahashi-Yasuno [20]	Japanese	220	377	NA	NA	57/163†	102/274†	NA	NA	
	Qu [18]	Chinese	317	282	49.3 ± 13.7	45.2 ± 5.7	4/69/244	4/53/225	25.5 ± 4.1	23.8 ± 2.9	
3′UTR Ins/Del	Lakka [22]	Finland	41	81	52.9 ± 6.0	52.9 ± 6.4	1/3/37	0/19/62	NA	NA	NA
	Nannipieri [23]	Mexican	503	609	NA	NA	7/124/372	29/167/413	NA	NA	
S343S rs1805134	Kyong [17]	Korean	775	688	58.9 ± 10.5	64.2 ± 4.2	4/103/659	3/63/616	24.4 ± 2.9	23.6 ± 3.1	0.25
N567N rs2228301	Kyong [17]	Korean	775	688	58.9 ± 10.5	64.2 ± 4.2	0/3/769	0/7/676	24.4 ± 2.9	23.6 ± 3.1	NA

BMI: body mass index; NA: not available; MAF: minor allele frequency.
For allele and genotype frequency, the rare allele was defined as reference.
*Data was calculated manually by combining subgroup data.
†Only genotype counts in dominant model can be extracted from the original study.

Table 2: Pooled analyses on the association of *LEPR* gene polymorphisms with T2DM.

Polymorphism	Cases/controls	Genetic model	OR (95% CI)	*P* value	I^2 (%)	P_Q
p.Arg223Gln (R223Q)	3649/2381	Allele	1.27 [1.13, 1.42]	<0.0001	38	0.13
		Dominant	1.19 [1.05, 1.35]	0.007	28	0.13
		Homozygote	1.82 [1.38, 2.39]	<0.0001	17	0.22
		Recessive	1.75 [1.35, 2.28]	<0.0001	37	0.29
p.Arg109Lys (R109K)	3536/2268	Allele	1.02 [0.92, 1.12]	0.73	0	0.45
		Dominant	1.02 [0.86, 1.20]	0.86	27	0.23
		Homozygote	1.15 [0.81, 1.65]	0.44	0	0.8
		Recessive	1.17 [0.82, 1.66]	0.39	0	0.84
p.Lys656Asn (K656N)	2018/1641	Allele	1.08 [0.91, 1.30]	0.38	20	0.26
		Dominant	1.09 [0.90, 1.33]	0.36	10	0.35
		Homozygote	0.79 [0.38, 1.65]	0.53	0	0.79
		Recessive	0.78 [0.38, 1.61]	0.5	0	0.83
p.Pro1019Pro (P1019P)	753/767	Allele	1.49 [0.89, 2.50]	0.13	78	0.03
		Dominant	1.28 [0.84, 1.96]	0.25	67	0.05
		Homozygote	1.86 [1.14, 3.02]	0.01	10	0.29
		Recessive	1.75 [1.12, 2.72]	0.01	1	0.31
3′UTR ins/del	544/690	Allele	0.69 [0.55, 0.86]	0.001	0	0.5
		Dominant	0.71 [0.55, 0.92]	0.008	34	0.22
		Homozygote	0.75 [0.05, 11.7]	0.84	66	0.08
		Recessive	0.35 [0.16, 0.76]	0.92	69	0.07

studies. The detailed information of the corresponding pooled odds ratios and *P* values of each SNP were presented in Table 2. The NOS results showed that the methodological quality was generally good (data not shown).

3.2. The Effect of p.Arg223Gln on T2DM. Eleven studies, containing 3649 T2DM cases and 2381 controls, were eligible for pooling of genetic effects of p.R223Q on T2DM. The allele model (R versus Q) yielded a pooled OR of 1.27 (95% CI: 1.13–1.42) with minimal heterogeneity ($P_Q = 0.13$, $I^2 = 38\%$). Our result demonstrated a positive correlation between R allele of p.R223Q and T2DM risk. Significant association was also observed under other genetic models (dominant: OR = 1.19, 95% CI 1.05–1.35; homozygote: OR = 1.82, 95% CI 1.38–2.39; and recessive: OR = 1.75, 95% CI 1.35–2.28, resp.) (Figure 2).

3.3. The Effect of p.R109K on T2DM. For p.R109K variant, seven studies containing 3536 cases and 2268 controls were included. The pooled analysis showed that R109K had no significant association with T2DM susceptibility for all genetic models: allele (OR = 1.02, 95% CI 0.92–1.12), dominant model (OR = 1.04, 95% CI 0.91–1.19), homozygote model (OR = 1.15, 95% CI 0.81–1.65), and recessive model (OR = 1.17, 95% CI 0.82–1.66) (Figure 3). The ORs for all genetic effects were homogeneous across all studies ($I^2 = 0\%$) except mild heterogeneity in the dominant model ($I^2 = 27\%$).

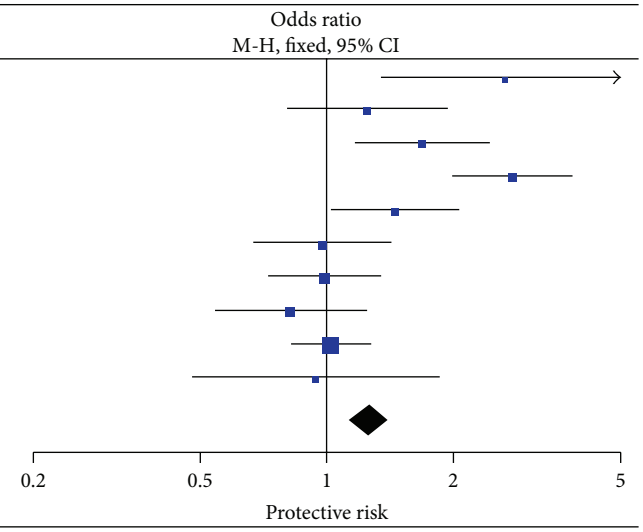
3.4. The Effect of p.K656N on T2DM. The association between p.K656N and T2DM has been examined in five studies, including 2018 cases and 1641 controls. Overall, no significant association was observed in any genetic models. The genetic effects were homogeneous across studies ($I^2 \leq 25\%$, Figure 4).

3.5. Other Loci. Three studies were carried out to assess the association of p.P1019P with susceptibility to T2DM, involving 753 cases and 767 controls. Only dominant genotype data can be extracted from the original study by Takahashi-Yasuno et al. [20]. Overall, the recessive and homozygote model showed homogeneity and the fixed effects pooled ORs were found to be significant (OR = 1.75, 95% CI = 1.12–2.72, and OR = 1.86, 95% CI = 1.14–3.02, resp.). The allele and dominant model yielded a strong heterogeneity between studies ($I^2 \geq 67\%$; $P = 0.001$), and the random effect model was therefore used but no significant association was observed (OR = 1.49, 95% CI = 0.89–2.50, and OR = 1.28, 95% CI = 0.84–1.96, resp.) (Figure 5). Regarding the association of 3′UTR ins/del, variable results were observed across different models, whose significant association was detected between 3′UTR ins/del and T2DM under allele and dominant models with no evidence of heterogeneity, but lack of significant association in other genetic models. Moreover, the ORs yielded strong heterogeneity among the studies ($I^2 = 66\%$ and 69% in homozygote and recessive model, resp.) (Figure 6). Two variants of rs1805134 (p.S343S) and rs22283014 (p.N567N) were reported by only one article by Kyong et al. with 775 cases and 688 healthy controls [17]. This study found no significant association between these two polymorphisms and risk of T2DM.

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Etemad et al. 2013 (China)	1.9%	2.66 [1.35, 5.22]
Etemad et al. 2013 (India)	6.6%	1.25 [0.81, 1.93]
Etemad et al. 2013 (Malay)	8.0%	1.69 [1.16, 2.44]
Murugesan et al. 2010	7.7%	2.77 [1.98, 3.86]
Gan and Yang 2012	9.8%	1.45 [1.02, 2.07]
Mohammadzadeh et al. 2013	9.9%	0.98 [0.67, 1.43]
Han et al. 2008	14.8%	0.99 [0.73, 1.35]
Jiang et al. 2014	8.8%	0.82 [0.54, 1.24]
Kyong et al. 2006	29.3%	1.02 [0.82, 1.27]
Liao et al. 2012	3.1%	0.94 [0.48, 1.84]
Total (95% CI)	**100.0%**	**1.27 [1.13, 1.42]**

Heterogeneity: $\chi^2 = 11.23$, df = 9 ($P = 0.13$); $I^2 = 38\%$

Test for overall effect: $Z = 4.13$ ($P < 0.0001$)

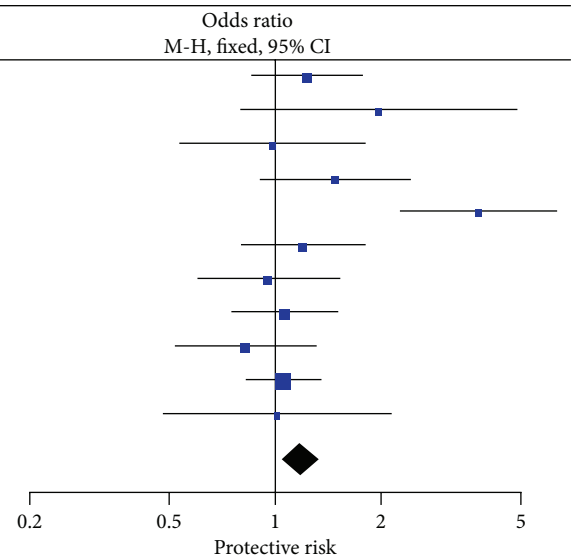


(a)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Takahashi-Yasuno et al. 2004	11.3%	1.24 [0.86, 1.79]
Etemad et al. 2013 (China)	1.5%	1.97 [0.80, 4.86]
Etemad et al. 2013 (India)	4.7%	0.98 [0.53, 1.80]
Etemad et al. 2013 (Malay)	5.8%	1.49 [0.91, 2.43]
Murugesan et al. 2010	3.5%	3.79 [2.27, 6.33]
Gan and Yang 2012	9.7%	1.20 [0.80, 1.80]
Mohammadzadeh et al. 2013	8.3%	0.96 [0.60, 1.52]
Han et al. 2008	14.4%	1.07 [0.76, 1.50]
Jiang et al. 2014	8.9%	0.82 [0.52, 1.30]
Kyong et al. 2006	28.9%	1.06 [0.83, 1.35]
Liao et al. 2012	3.1%	1.01 [0.48, 2.14]
Total (95% CI)	**100.0%**	**1.19 [1.05, 1.35]**

Heterogeneity: $\chi^2 = 13.96$, df = 10 ($P = 0.13$); $I^2 = 28\%$

Test for overall effect: $Z = 2.72$ ($P = 0.007$)



(b)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Etemad et al. 2013 (China)	4.8%	3.61 [1.26, 10.34]
Etemad et al. 2013 (India)	18.6%	1.36 [0.70, 2.67]
Etemad et al. 2013 (Malay)	20.0%	2.02 [1.12, 3.66]
Murugesan et al. 2010	9.4%	5.86 [3.05, 11.27]
Gan and Yang 2012	5.9%	2.72 [0.90, 8.23]
Mohammadzadeh et al. 2013	6.0%	1.00 [0.28, 3.59]
Han et al. 2008	10.7%	0.43 [0.13, 1.44]
Jiang et al. 2014	5.2%	0.57 [0.13, 2.59]
Kyong et al. 2006	17.0%	0.77 [0.34, 1.72]
Liao et al. 2012	2.4%	0.36 [0.04, 2.97]
Total (95% CI)	**100.0%**	**1.82 [1.38, 2.39]**

Heterogeneity: $\chi^2 = 17.47$, df = 9 ($P = 0.22$); $I^2 = 17\%$

Test for overall effect: $Z = 4.30$ ($P < 0.0001$)

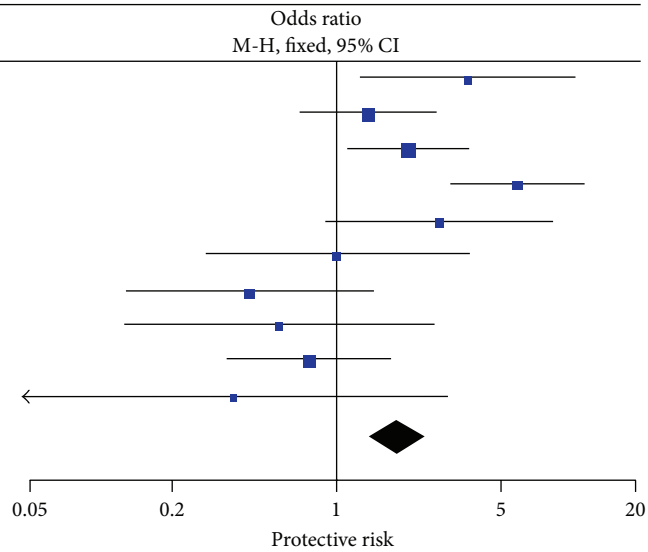


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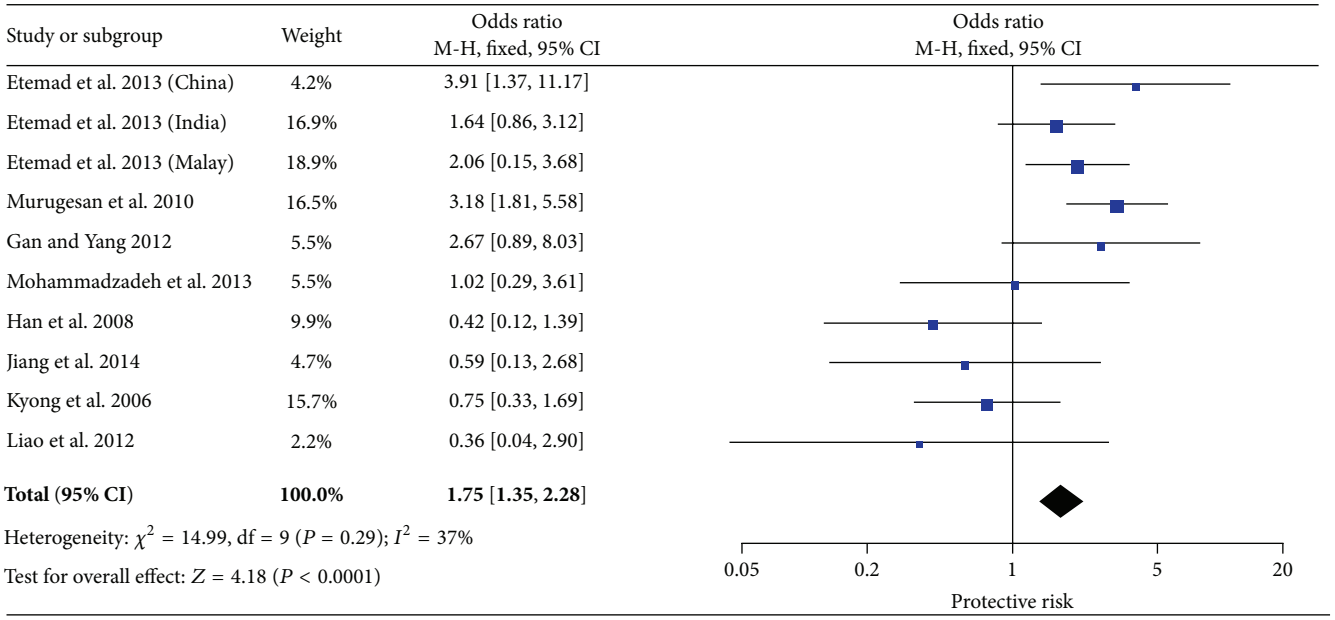
Figure 2: Continued.



(d)

Figure 2: Forest plots of the association of *LEPR* p.R223Q with T2DM. The size of the box is proportional to the weight of the study, horizontal lines indicate 95% CI, and a diamond indicates the summary OR with its corresponding 95% CI. (a) Allele model, (b) dominant model, (c) homozygote model, and (d) recessive model; LEPR: leptin receptor gene; T2DM: type 2 diabetes mellitus; OR: odds ratio; CI: confidence interval.

3.6. Heterogeneity and Publication Bias. In order to examine the influence of each study set to the pooled ORs, sensitivity analysis was performed by omitting one study in each time. For the variants, rs1137101 (p.R223Q), rs1137100 (p.R109K), and rs8179183 (p.K656N), the ORs were not significantly influenced by individual data, indicating that our results were statistically stable and robust. Metaregression was performed to detect the source of heterogeneity for the significant finding of rs1137101; the results indicated that sample size (P = 0.015) and source of control (P = 0.029) contributed heterogeneity, while ethnicity (P = 0.648) and publication year (P = 0.429) did not. Regarding rs62589000 (p.P1019P), the sensitivity analysis showed that the data from Lu et al. apparently influenced the overall results; therefore, the pooled ORs need be taken into account [15]. For 3′UTR ins/del, only two studies were performed in Finland and Mexican populations; among the two included articles, the results in one study performed by Nannipieri et al. were overweight and significantly influenced the overall results [23]. Publication bias was investigated by funnel plot and Egger's test; for all SNPs, funnel plot shapes did not reveal any evidence of obvious asymmetry (Figure 7). Egger's test also suggested no publication bias (P = 0.524 for rs1137101).

4. Discussion

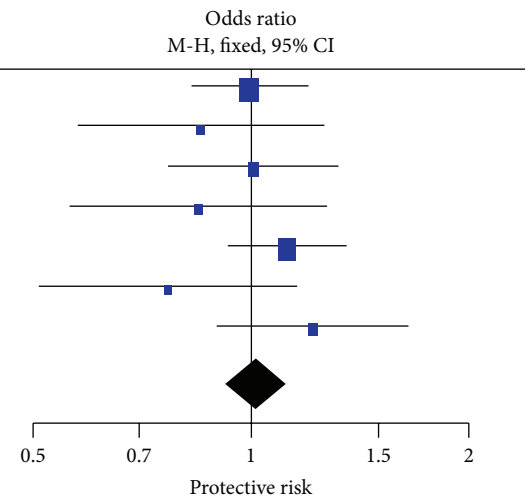
In recent years, numerous studies have been conducted to evaluate the genetic influence of leptin to T2DM susceptibility. While the reports in different ethnic groups often yielded contradictory results, the inconsistencies could be due to the lack of power in each individual study with limited sample size as well as the heterogeneous data and methods. To confirm the association of *LEPR* and T2DM, we, for the first time, conducted a systematic review and meta-analysis to examine the associations of *LEPR* polymorphisms with T2DM risk. For this meta-analysis, five of the most commonly investigated *LEPR* SNPs were analyzed. Among them, p.R223Q was found to be significantly associated with T2DM, which remained significant even after sensitivity analysis. Genetic effect with allele R showed a positive association with T2DM from 1.19- to 1.82-fold under different genetic models. This result provides robust evidence for the genetic impact of leptin on diabetes, which will lead to further biological function investigation.

It is well known that obesity is a major link to T2DM, especially characterized by insulin resistance [24]. As leptin has long been linked with obesity, recent studies have depicted the role of leptin in T2DM and insulin resistance. Leptin can facilitate glucose utilization and improve insulin sensitivity, which has been implicated in the development of diabetes. Meanwhile, lower leptin expression in the adipose tissue and serum leptin levels were observed in T2DM patients [25]. The *LEPR* p.R223Q (G>A polymorphism, rs1137101) leads to an amino acid alteration from Arg to Gln located in the regulatory domain of the LEPR protein. Study by Murugesan et al. found that the levels of leptin, insulin, and body mass index (BMI) were significantly increased with homozygous and heterozygous variants of p.R223Q and showed significant difference between cases and controls [19]. These results were consistent with findings of Yiannakouris

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Cruz et al. 2010	30.5%	0.99 [0.83, 1.19]
Murugesan et al. 2010	7.0%	0.85 [0.58, 1.26]
Han et al. 2008	13.7%	1.01 [0.77, 1.31]
Jiang et al. 2014	6.3%	0.85 [0.56, 1.27]
Kyong et al. 2006	26.7%	1.12 [0.93, 1.35]
Liao et al. 2012	6.2%	0.77 [0.51, 1.16]
Qu et al. 2008	9.7%	1.21 [0.90, 1.65]
Total (95% CI)	**100.0%**	**1.02 [0.92, 1.12]**

Heterogeneity: $\chi^2 = 5.79$, df = 6 ($P = 0.45$); $I^2 = 0\%$

Test for overall effect: $Z = 0.35$ ($P = 0.73$)

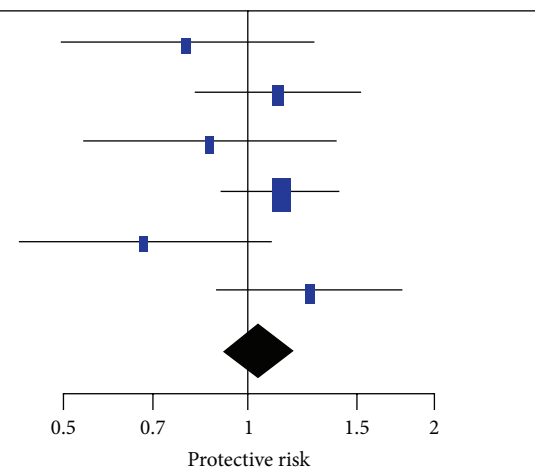


(a)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Murugesan et al. 2010	9.6%	0.79 [0.49, 1.27]
Han et al. 2008	19.3%	1.12 [0.82, 1.52]
Jiang et al. 2014	9.2%	0.86 [0.54, 1.38]
Kyong et al. 2006	37.9%	1.13 [0.91, 1.40]
Liao et al. 2012	9.9%	0.68 [0.42, 1.08]
Qu et al. 2008	14.0%	1.25 [0.88, 1.78]
Total (95% CI)	**100.0%**	**1.04 [0.91, 1.19]**

Heterogeneity: $\chi^2 = 6.88$, df = 5 ($P = 0.23$); $I^2 = 27\%$

Test for overall effect: $Z = 0.58$ ($P = 0.56$)



(b)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Murugesan et al. 2010	16.8%	0.91 [0.36, 2.29]
Han et al. 2008	20.4%	1.15 [0.52, 2.55]
Jiang et al. 2014	7.5%	0.39 [0.08, 1.98]
Kyong et al. 2006	38.3%	1.31 [0.75, 2.29]
Liao et al. 2012	3.2%	1.63 [0.22, 12.33]
Qu et al. 2008	13.9%	1.31 [0.52, 3.32]
Total (95% CI)	**100.0%**	**1.15 [0.81, 1.65]**

Heterogeneity: $\chi^2 = 2.34$, df = 5 ($P = 0.80$); $I^2 = 0\%$

Test for overall effect: $Z = 0.78$ ($P = 0.44$)

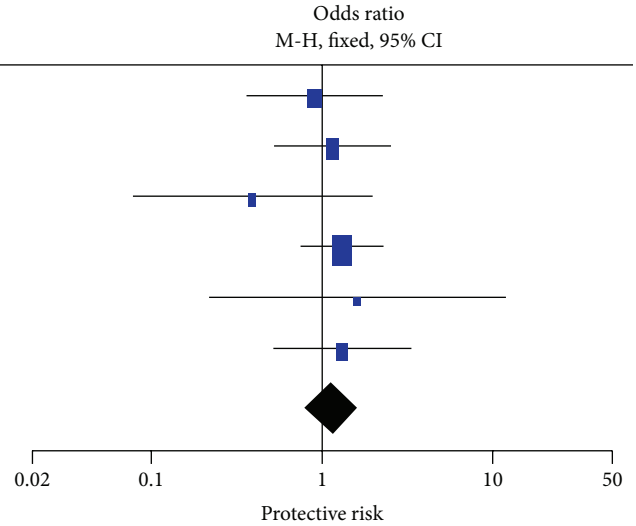


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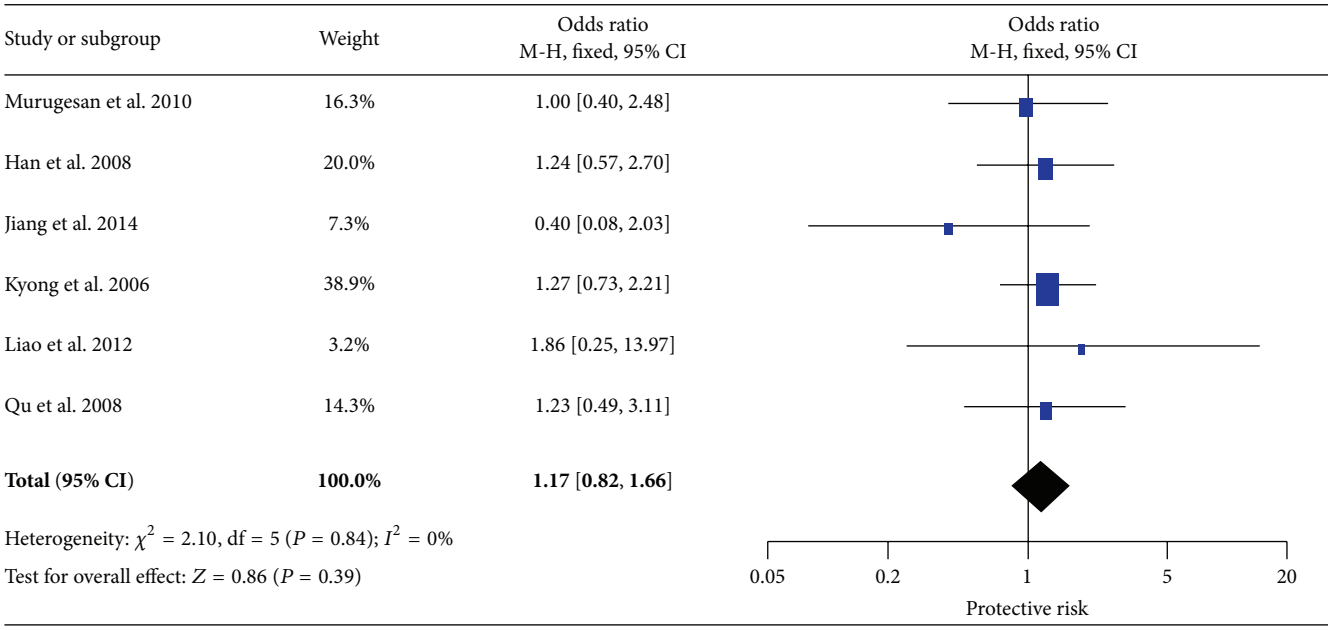
Figure 3: Continued.



(d)

Figure 3: Forest plots of the association of *LEPR* p.R109K with T2DM. (a) Allele model, (b) dominant model, (c) homozygote model, and (d) recessive model.

et al. suggesting that the p.R223Q polymorphism is associated with obesity and predicts a small percentage of body weight and body composition variability in a genetically homogeneous population [26]. Regarding this SNP, majority of studies were conducted in Asian populations; this is because some studies in other ancestries were excluded during the process of narrowing the studies; additionally, the MAF of this polymorphism is similar across different populations. Therefore, this significant association reported in the present study is more likely to be predictive of diabetes in overall population.

For other two functional variants, p.R109Q and p.K656N, no significant associations were found with T2DM under any genetic models, implying that these two variants might not contribute to the risk of T2DM. With regard to p.P1019P and 3′UTR ins/del, significant association was identified with T2DM, but the results were largely influenced by particular article and a limited number of studies were available. Therefore, these associations should be carefully interpreted, and further examinations in larger cohorts are warranted.

There are a number of limitations in our current meta-analysis. Firstly, only 14 available studies were enrolled. The number is not enough for every variant in the meta-analysis, such as p.P1019P and 3′UTR ins/del. Secondly, the small sample size conferred limited statistical power for exploring real association, especially for the subgroup analysis. Thirdly, as a multifactorial disease, a more precise analysis on T2DM-related factors should be conducted, such as BMI, age, and environmental exposure; however, some studies did not provide the detailed information.

To the best of our knowledge, this is the first meta-analysis to investigate the association of *LEPR* gene with T2DM. Our results suggested that the polymorphism rs1137101 (p.R223Q) has strong association with T2DM susceptibility. More studies in larger cohort and functional analyses of *LEPR* are required to reinforce the results and elucidate its biological roles in diabetes.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Ming Ming Yang, Jun Wang, and Yan-Bo Li designed the experiments. Ming Ming Yang, Jiao Jie Fan, and Jun Wang performed the experiments. Ming Ming Yang, Tsz Kin Ng, and Jun Wang performed the analysis and wrote the paper. Dian Jun Sun and Yan Teng revised the paper. All authors contributed to the editing of the paper and to scientific discussions. Ming Ming Yang and Jun Wang contributed equally to this work.

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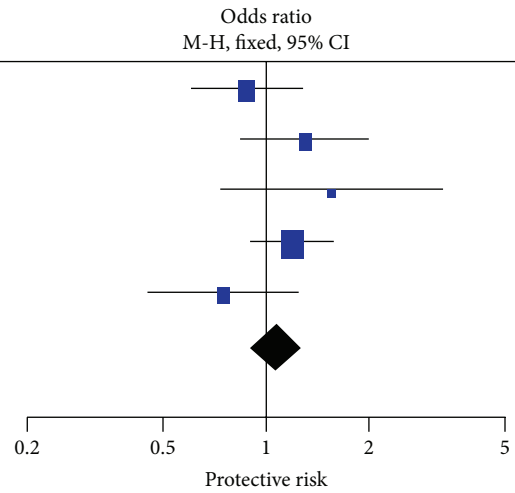
Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Murugesan et al. 2010	25.8%	0.88 [0.61, 1.28]
Han et al. 2008	15.5%	1.31 [0.84, 2.03]
Jiang et al. 2014	5.2%	1.56 [0.74, 3.32]
Kyong et al. 2006	38.4%	1.20 [0.90, 1.59]
Qu et al. 2008	15.0%	0.75 [0.45, 1.24]
Total (95% CI)	**100.0%**	**1.08 [0.91, 1.30]**

Heterogeneity: $\chi^2 = 5.30$, df = 4 ($P = 0.26$); $I^2 = 25\%$

Test for overall effect: $Z = 0.87$ ($P = 0.38$)

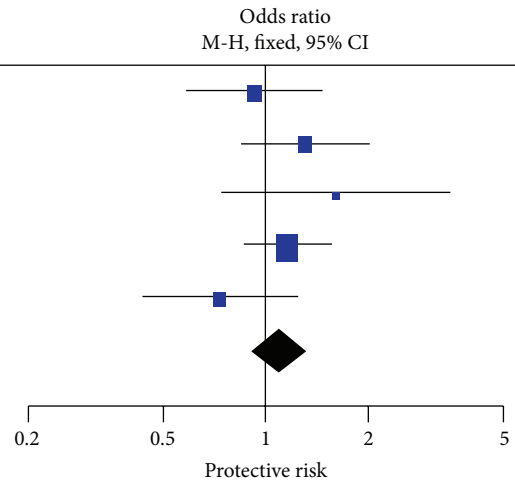


(a)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Murugesan et al. 2010	19.4%	0.92 [0.58, 1.46]
Han et al. 2008	18.1%	1.31 [0.84, 2.03]
Jiang et al. 2014	5.7%	1.60 [0.74, 3.47]
Kyong et al. 2006	40.6%	1.16 [0.86, 1.56]
Qu et al. 2008	16.2%	0.73 [0.43, 1.24]
Total (95% CI)	**100.0%**	**1.09 [0.90, 1.33]**

Heterogeneity: $\chi^2 = 4.46$, df = 4 ($P = 0.35$); $I^2 = 10\%$

Test for overall effect: $Z = 0.92$ ($P = 0.36$)

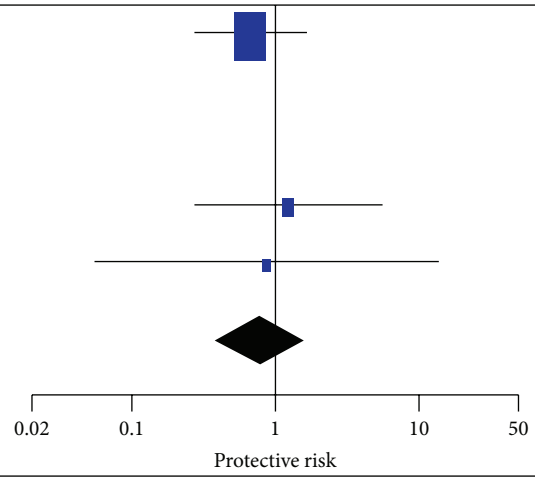


(b)

Study or subgroup	Weight	Odds ratio M-H, fixed, 95% CI
Murugesan et al. 2010	73.7%	0.67 [0.27, 1.65]
Han et al. 2008		Not estimable
Jiang et al. 2014		Not estimable
Kyong et al. 2006	19.5%	1.22 [0.27, 5.49]
Qu et al. 2008	6.7%	0.86 [0.05, 13.85]
Total (95% CI)	**100.0%**	**0.79 [0.38, 1.65]**

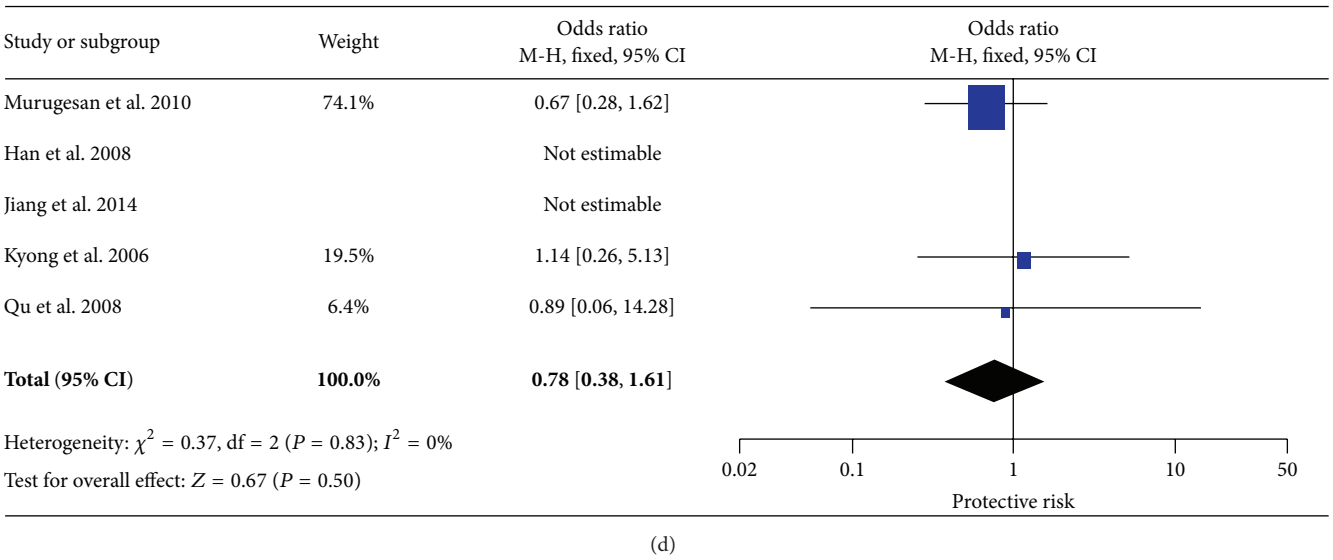
Heterogeneity: $\chi^2 = 0.46$, df = 2 ($P = 0.79$); $I^2 = 0\%$

Test for overall effect: $Z = 0.63$ ($P = 0.53$)



(c)

Figure 4: Continued.



(d)

Figure 4: Forest plots of the association of *LEPR* p.K656N with T2DM. (a) Allele model, (b) dominant model, (c) homozygote model, and (d) recessive model.

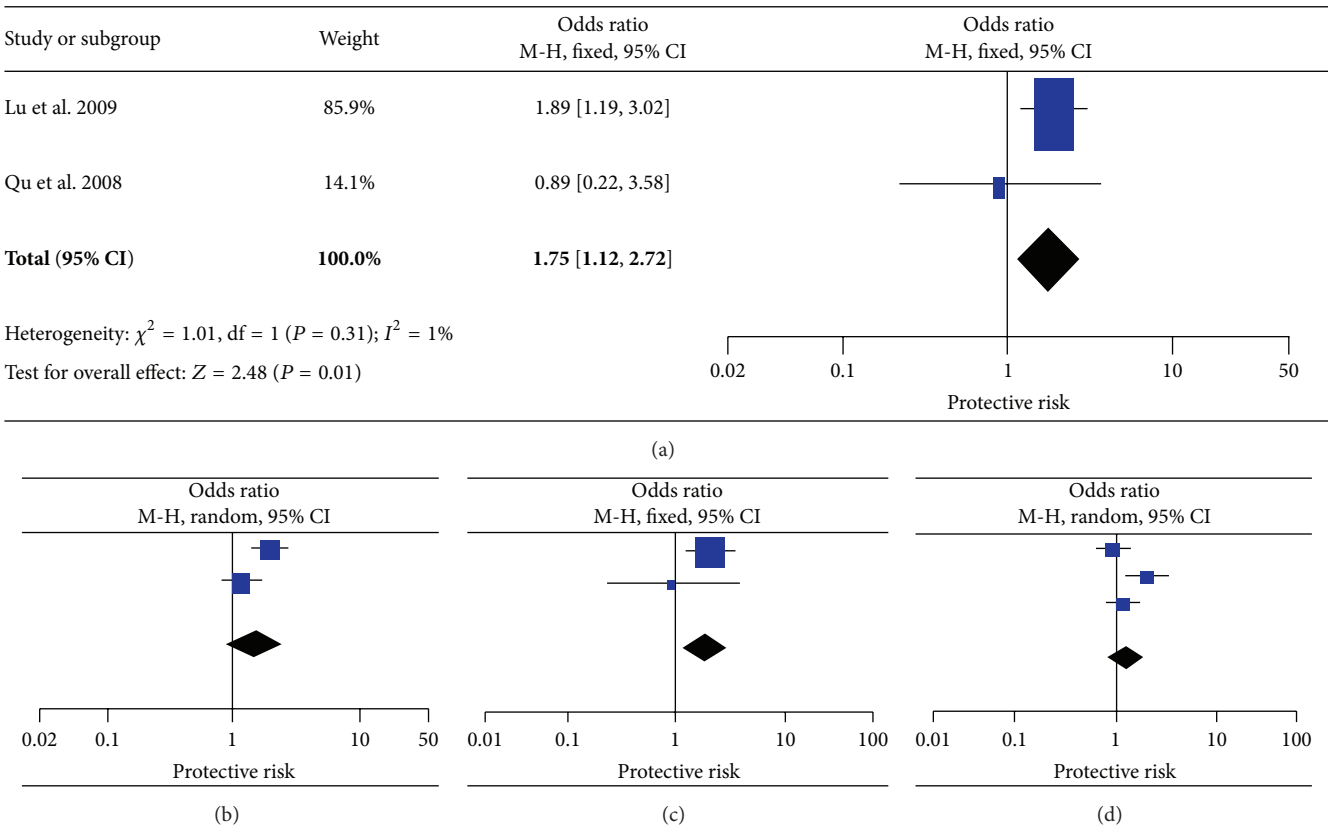


Figure 5: Forest plots of the association of *LEPR* p.P1019P with T2DM. (a) Recessive model, (b) allele model, (c) homozygote model, and (d) dominant model (study by Takahashi-Yasuno et al. only provides dominant data for this SNP).

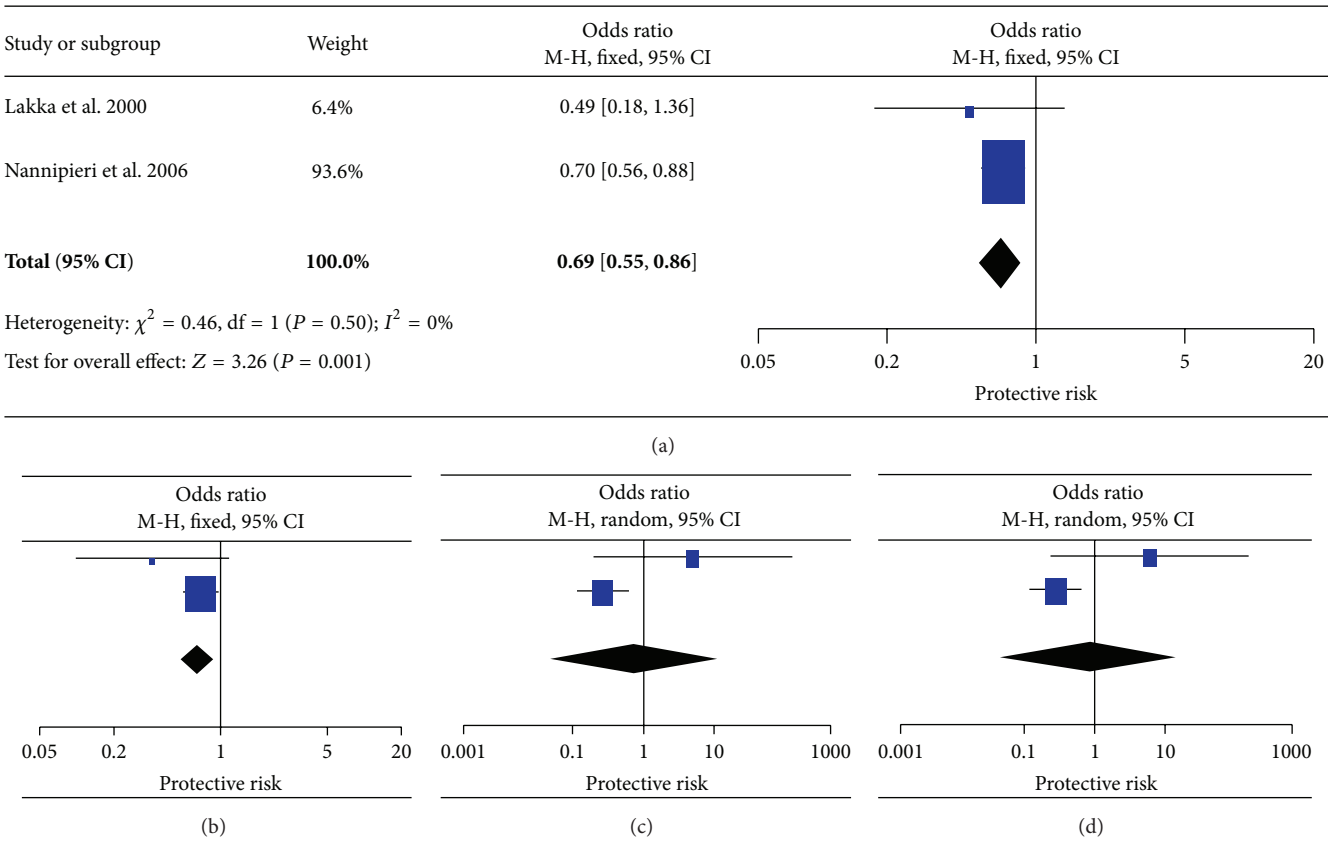


Figure 6: Forest plots of the association of *LEPR* 3′UTR with T2DM. (a) Allele model, (b) dominant model, (c) homozygote model, and (d) recessive model.

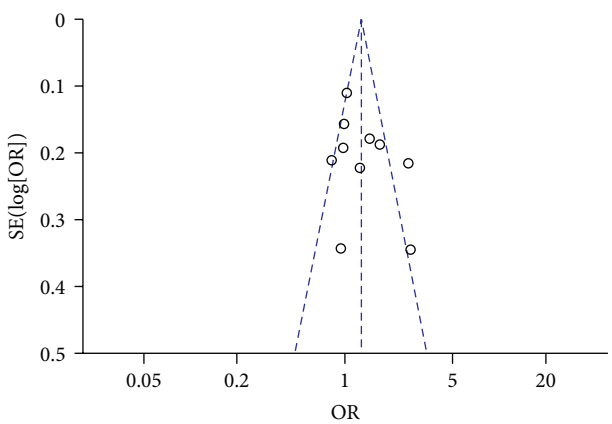


Figure 7: Funnel plot of studies conducted on the association between rs1137101 (R223Q) and T2DM risk (G>A).

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