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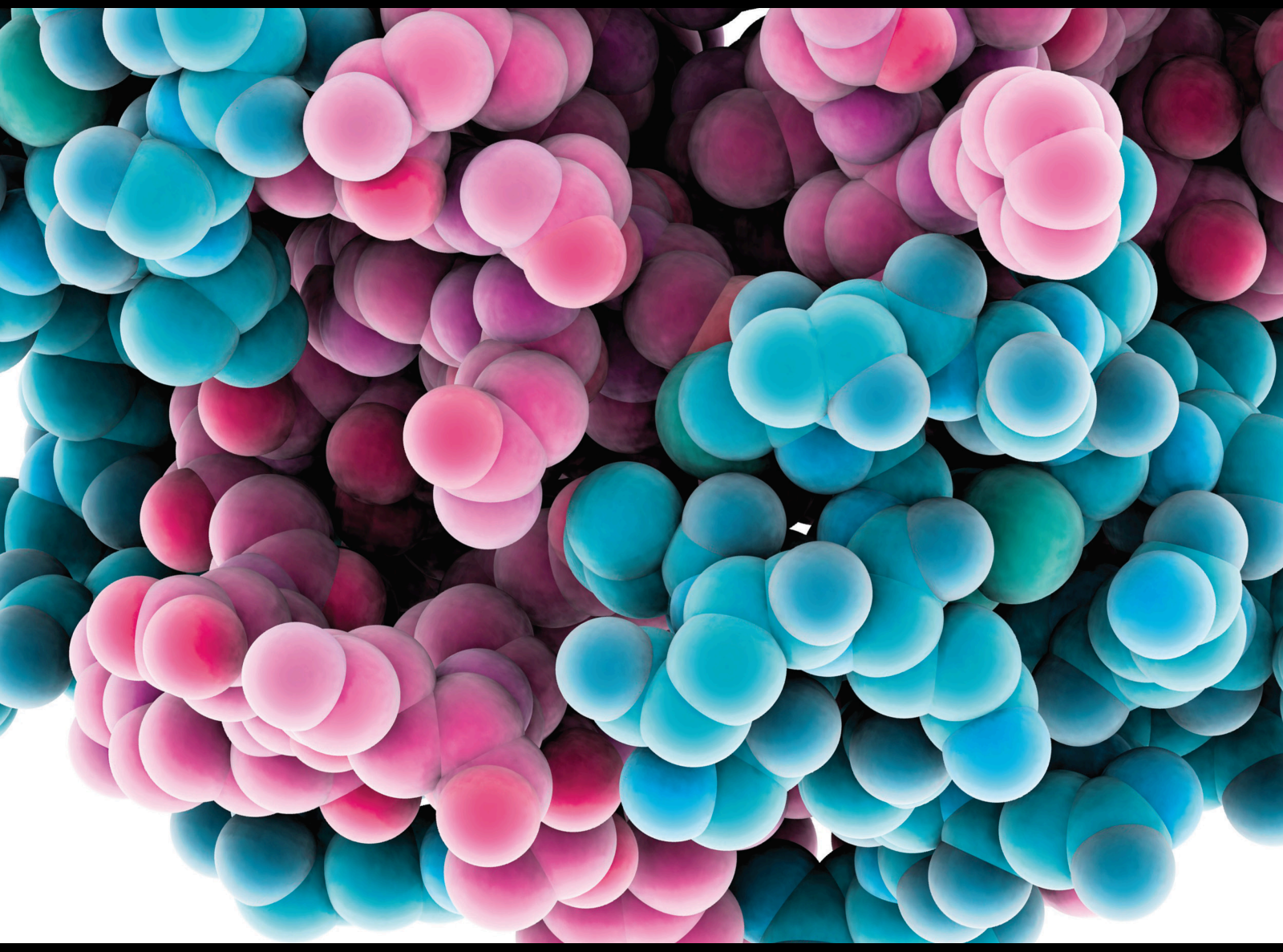

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# Contents

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*Editorial*

# New Insights into the Role of Oxidative Stress in the Development of Diabetes Mellitus and Its Complications

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The special issue addresses new insights into the role of oxidative stress in the development of diabetes mellitus and its complications. Diabetes is a pandemic that has reached alarming proportions; it is estimated that the global prevalence in adults (between 20- and 79-year-olds) will reach 12.2%, which is over 783 million people, by 2045 [1]. Poor glycemic control, the common threat for individuals with all types of diabetes, impacts micro- and macrovasculature homeostasis [2]. As a result, several complications, such as cardiovascular events, retinopathy, neuropathy, and nephropathy, are induced in the population with diabetes and are the leading cause of mortality and morbidity [3–5]. Together, diabetes and its complications generate a significant economic burden, and its global health-related expenditures were estimated to reach almost a trillion USD in 2021 [1].

A large number of cutting-edge studies have established that oxidative stress is one of the central pieces of the development of diabetes and its complications, as highlighted in the previous special issue published in this journal [6]. The big challenge in the field is the fact that molecules involved in oxidative stress response, such as reactive oxygen species (ROS), cannot be eliminated completely from the human body, since they control vital processes in the human body such as the immunological defense, mitochondrial biogenesis, and antioxidant system. The establishment of the "ideal" balance between generation and scavenging of ROS is the target of controlling diabetes development and the complications related to this disease. Therefore, scientists worldwide have focused on characterizing ROS-related sources and behaviors, their associated pathways, and scavenging components in diabetes.

Novel insights linking oxidative stress in the field of diabetes and its complications have been well addressed in the present thematic issue. This special issue includes seven research articles (two reviews of literature and five original articles) focusing on the role of ROS in the development of diabetes and its associated diseases. The guest editors are thrilled to present a collection of innovative, original research and review scientific reports as follows.

Several researchers have shown that regular exercise inhibits and delays the development of type 2 diabetes [7]; however, most of the studies in the diabetes field were carried out analyzing the effect of aerobic (mainly running) or resistance exercise. In the review article "The Effects of Tai Chi Exercise for Patients with Type 2 Diabetes Mellitus: An Overview of Systematic Reviews and Meta-Analyses," the impact of regular practice of tai chi, which is a series of calm physical exercises and stretches' movement in

individuals with type 2 diabetes, was evaluated [8]. The authors concluded that Tai Chi is beneficial and safe for individuals with type 2 diabetes; however, due to few experimental research on the topic, professionals in the area should approach this conclusion with caution.

In the original research, "Serum Uric Acid Levels Are Related to Diabetic Peripheral Neuropathy, Especially for Motor Conduction Velocity of Tibial Nerve in Type 2 Diabetes Mellitus Patients" and determine the role of serum uric acid in the neuropathy of patients with type 2 diabetes mellitus [9]. Analyzing 106 type 2 diabetes patients with and without diabetic neuropathy, the authors suggest that serum uric acid levels could impact the function of the tibial nerve motor fiber independent from the control of glycated hemoglobin. Altogether, the authors concluded that subnormal serum uric acid is a risk factor for neuropathy of patients with type 2 diabetes mellitus.

Another original research is about a traditional Chinese medicine prescription (GuaLouQuMaiWan) that has been described as an alternative treatment for type 2 diabetes mellitus (T2DM) with positive results [10]. With a mix of compounds, with unclear small molecular components, the study by Feng et al. used network pharmacology and transcriptomics to reveal its mechanism in treating diabetes. After gene expression analysis, up- and downregulated genes were investigated, related to insulin secretion and proinflammatory profile. In an interestingly health-to-disease process, from no disease to glucose intolerance to type 2 diabetes profile, seventeen genes were highlighted by the authors. The progression until established diabetes involves several abnormalities in $\beta$ cells, including a decrease in the number of pancreatic $\beta$ cells and their function during the secretion and synthesis of insulin. Thus, they started to test whether someone within the fifty compounds of traditional medicine met the criteria by interaction of target proteins. In this way, this study found associations between genes related to inflammatory targets such as cytokines, nuclear transcription factors, growth factors, and energy balance. Thus, the results of this study [10] help to explain the molecular pathways involved in diabetes pathogenesis and provide new strategies for treatment, such as specifically reducing the degree of inflammation in pancreatic islets. Since pharmacological and nonpharmacological interventions for diabetes have been discussed based on inflammatory, oxidative, and proteostasis pathways [11], it is possible to propose that the compound could restore the number of $\beta$ cells and islet function by connecting *in silico*, *in vitro*, animal models, and clinical data pieces of evidence, to possibly becoming effective in patients with failed insulin control, based on anti-inflammatory and insulin resistance properties.

Traditional Chinese medicine is not the only alternative intervention beneficial to patients with diabetes, multiple vitamins' supplementation including vitamins B, C, and E decreases the development of diabetic retinopathy. The original research "The Amelioration of Detrimental Biochemical Anomalies by Supplementing B, C, and E Vitamins in Subjects with Type 2 Diabetes Mellitus May Reduce the Rate of Development of Diabetic Retinopathy" was a prospective placebo control trial. In this study, Pramanik et al. followed 185 patients with T2DM who received vitamin B, vitamin C, and vitamin E together with antidiabetic medication and 175 patients with T2DM who were treated with only antihyperglycemic drugs for five years. The group with vitamin supplementation had a slower rate of the development of diabetic retinopathy and reduced ROS markers [12]. It is concluded that vitamin supplementation decreases the development of diabetic microvascular complications via their antioxidant properties.

Myrrh resin, a natural substance obtained from the bark of the *Commiphora myrrha*, has been tested as a therapeutical approach against several diseases. In the original research "Pharmacological Studies on the Antidiabetic, Antioxidant, and Antimicrobial Efficacies of *Commiphora myrrha* Resin in Streptozotocin-Induced Diabetes in Rats: A Preclinical Study" [13], the authors tested the hypotheses that aqueous extract of *Commiphora myrrha* resin has an antioxidant, antimicrobial, and antidiabetic effect for type one diabetes (T1D). Oleo-gum resins of *Commiphora myrrha* were collected from a wild tree growing in Wadi Noeman at Makkah, Saudi Arabia, and aqueous resin oleum were extracted as described by the authors [13]. Streptozotocin-induced diabetes in rats were treated *Commiphora myrrha* powder (0.5 mL of 0.5 g/kg body weight) dissolved in water for 30 days. The results of the study successfully supported the hypothesis, which opens a window of opportunity for testing myrrh resin administration to control blood glucose of T1D in clinical trials.

Chronic inflammation impairs wound healing in individuals with diabetes. Controlling oxidative stress is one of the therapeutics approaches to improve diabetic-induced cutaneous wounds that precede diabetic foot ulcers. Using cell culture of alternatively activated macrophage (M2 phenotypes) that is responsible to inflammation and tissue regeneration, the authors of the original article "ceAF Ameliorates Diabetic Wound Healing by Alleviating Inflammation and Oxidative Stress via TLR4/NF-$\kappa$B and Nrf2 Pathways" tested the hypotheses that chick early amniotic fluids (ceAF) ameliorate inflammation and wound healing [14]. The results of the study indicate that ceAF downregulates inflammatory response by the regulation of TLR4/NF-$\kappa$B and Nrf2 signaling pathways in M2 macrophage and that could improve diabetic wound healing.

The global incidence and the prevalence of type 1 diabetes mellitus (T1DM) are increasing rapidly. Vitamin D, which underlies calcium and phosphorus metabolism, also has an immunomodulatory role. Therefore, researchers have been testing the hypothesis that decreased levels of vitamin D underlie chronic infections, specific types of cancer, and autoimmune rheumatic diseases [14]. Scientific reports on the relationship between vitamin D deficiency and the development of T1DM are discussed in the review article "Progress in the Relationship between Vitamin D Deficiency and the Incidence of Type 1 Diabetes Mellitus in Children" [15]. Together, studies indicated that vitamin D could protect pancreatic $\beta$ cells from immune attack by regulating T cell response via reducing oxidative stress. However, according to the reports analyzed in the present literature review, there is no consensus regarding the protective effect of

supplementation of vitamin D on $\beta$ cell function in T1DM. Therefore, large-scale epidemiological studies are needed to evaluate the role of vitamin D in the development of T1DM.

The guest editors expect this special issue to be of huge interest to scientists and clinicians working in the field of diabetes and/or oxidative stress, especially those focusing their work on alternative therapeutical approaches against the development of diabetes and its complication. We hope that investigators worldwide continuously make additional advances in expanding the knowledge in the field of diabetes and its complication.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

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*Qing Zhong*
*Sandra A. Benite-Ribeiro*
*Thiago Gomes Heck*

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*Research Article*

# Serum Uric Acid Levels Are Related to Diabetic Peripheral Neuropathy, Especially for Motor Conduction Velocity of Tibial Nerve in Type 2 Diabetes Mellitus Patients

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*Background*. Oxidative stress is one of the most critical factors that contribute to the pathogenesis of neuronal damage, including diabetic peripheral neuropathy (DPN). Uric acid is a kind of natural antioxidant that plays a major role in the antioxidant capacity against oxidative stress. Here, we aim to determine the role of serum uric acid (SUA) in the DPN of patients with type 2 diabetes mellitus (T2DM). *Patients and Methods*. 106 patients with T2DM were recruited and divided into the DPN group and the control group. Clinical parameters, especially for motor nerve fiber conduction velocity and sensory nerve fiber conduction velocity, were collected. Differences between T2DM patients with and without DPN were compared. Correlation and regression analyses were performed to explore the association between SUA and DPN. *Results*. Compare with 57 patients with DPN, 49 patients without DPN showed lower HbA1c and elevated SUA levels. Additionally, SUA levels are negatively associated with the motor conduction velocity of tibial nerve with or without adjusting for HbA1c. Besides, it is suggested that decreased SUA levels may influence the motor conduction speed of the tibial nerve by multiple linear regression analysis. Moreover, we demonstrated that decreased SUA level is a risk factor for DPN in patients with T2DM by binary logistic regression analysis. *Conclusion*. Lower SUA is a risk factor for DPN in patients with T2DM. Additionally, decreased SUA may influence the damage of peripheral neuropathy, especially for motor conduction velocity of the tibial nerve.

## 1. Introduction

According to estimates, there are 451 million adults worldwide who have been diagnosed with diabetes. It is projected that by 2045, this number will increase to 693 million [1]. There were approximately 1.09 billion adults in mainland China in 2013. Additionally, the overall prevalence of diabetes of adults was 10.9% [2]. Type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycemia, insulin resistance and usually along with lipid disorder [3], and hyperuricemia [4]. Increased serum uric acid (SUA) is associated with diabetes [5] and several diabetic complications [6–12] including diabetic peripheral neuropathy (DPN). Indeed, hyperuricemia is common in patients with T2DM and associated with the C-peptide incremental effect of islet beta cell function in T2DM, especially in female patients [13]. Except for diabetes

itself, SUA levels are higher in T2DM patients with DPN than those without DPN [14, 15]. It is also suggested that elevated SUA levels increased the chance of developing peripheral polyneuropathy in patients with T2DM [16]. Another Chinese clinical study indicated that there is a significant association between elevated SUA levels and DPN. Additionally, SUA may be a valuable indicator to predict the occurrence of DPN in T2DM patients [17]. However, a large sample study with 2809 individuals found that SUA is not an independent risk factor of DPN [18]. However, Zhuang et al. demonstrated that the low SUA level is closely associated with DPN [19].

DPN is a major risk factor for diabetes-related lower-extremity complications and presents clinically as distal symmetrical sensorimotor polyneuropathy [20]. It affects about 50% of T2DM patients with a diabetic duration of more than 10 years [21]. Additionally, it also appears in newly diagnosed patients with T2DM [22]. As the mechanism is not clear, there is no effective therapy for patients with DPN. Oxidative stress is one of the critical factors that contribute to the pathogenesis of neuronal damage, including DPN [23–25]. Further laboratory experiments demonstrated that oxidative stress is one of the potential mechanisms of DPN [26]. A recent study indicated that oxidative stress is involved in the apoptosis of Schwann cells and takes part in DPN [27]. Thus, it is suggested that reducing of oxidative stress may improve DPN. Interestingly, uric acid is a kind of natural antioxidant and plays a major role in the antioxidant capacity against oxidative stress [28].

In general, SUA may be a double-edged sword in different studies. Although a great number of researches instant that elevated SUA is a risk factor for DPN, low SUA levels may also contribute to DPN in patients with T2DM. These conflicting results have indicated that the relationship between SUA and DPN in patients with T2DM is still unclear and needs further exploration. Here, we performed this study to investigate the relationship between SUA and DPN, in T2DM patients without uric acid treatments.

## 2. Material and Methods

*2.1. Experiment Design and Ethics.* This cross-section study was conducted in The First Affiliated Hospital of University of Science and Technology of China (USTC). 106 patients certificated the standard of T2DM were recruited. In these individuals, 57 patients with T2DM were diagnosed as DPN and 49 T2DM patients without DPN. All participants were informed about the process of this experiment and given a handwritten signature on the informed consent before the experiment. The study was approved by the Ethics Committee of The First Affiliated Hospital of USTC and complied with the Declaration of Helsinki. This study was registered on the Chinese Clinical Trial Registry (ChiCTR2100046905).

*2.2. Inclusion and Exclusion Criteria.* The World Health Organization 1999 [29] criteria for T2DM diagnosis were used for patient recruitment in this present work. Exclusion criteria were the following: any other clinically evident causes of neuropathy apart from diabetes and taking drugs affecting the serum level of SUA such as diuretics, cyclosporine, allopurinol, estrogen, and cytotoxic drugs. DPN patients were diagnosed based on the Toronto consensus of diabetic neuropathy [30]. Patients without DPN were defined as control.

*2.3. Clinical Data Collection.* Age, gender, and education as well as the duration of diabetes mellitus (DM) and the duration of high blood pressure (HBP) were collected. Triglyceride (TG) (Roche Group, Basel, Switzerland; 0.1-10.0 mmol/L), total cholesterol (TC) (Roche Group, Basel, Switzerland; 0.1-20.7 mmol/L), low-density lipoprotein cholesterol (LDL-C) (Ningbo Ruiyuan Biotechnology Co., Ltd., Ningbo, China; 0.2-11.6 mmol/L), high-density lipoprotein cholesterol (HDL-C) (Roche Group, Basel, Switzerland, 0.08-3.88 mmol/L), and SUA (Beckman Coulter, Brea, USA; 89-1785 umol/L) were determined by their kits described above. Additionally, microcolumn ion-exchange chromatography was employed to detect glycosylated hemoglobin (HbA1c) levels. These measurements were conducted in The First Affiliated Hospital of USTC, Center Laboratory for medical usage. These data were collected for further analysis. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$.

*2.4. Neurophysiological Examinations.* All patients underwent neurophysiological examinations by electromyographic evoked potential meter according to the protocol of the manufacturer (Natus Neurology, USA). These neurophysiological examination tests were performed in our hospital by staff in the electrophysiology room for medical use. The information of motor conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, tibial nerve, and common peroneal nerve, as well as the sensory conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, and sural nerve, was collected from the medical histories of patients.

*2.5. Sample Size Calculation.* The minimum sample size was calculated by PASS V11.0.7 (NCSS, USA). Before we finished the work of volunteer recruitment, we had estimated the minimum sample size by mean and standard deviation of SUA (data from recruited patients at that time). When we finished the volunteer recruitment work, we confirmed that the sample size is sufficient. Minimum sample sizes of patients without DPN and those with DPN are 48 and 56, respectively, according to the data from all recruited patients.

*2.6. Statistical Methods.* Data was analyzed by SPSS 22.0 (IBM, USA). *T*-tests were carried out to compare the difference of normally distributed variables in patients with and without DPN. Mann–Whitney *U* Tests were performed to compare the difference of asymmetrically distributed variables in the control group and the DPN group. Chi-squared tests were applied to compare the difference of the binary variable in the two groups. Pearson's correlation, partial correlation analysis, binary logistic regression analysis, and multiple linear regression analysis were performed to explore the relationships between DPN and SUA. $p < 0.05$ was defined as statistical significance. The methods (including statistical methods and some other descriptions) are similar with our previous published manuscript [31].

## 3. Results

*3.1. Clinical Parameter Result of T2DM Patients with and without DPN.* To explore the potential risk factors of DPN in patients with T2DM, baseline data of diabetic patients with DPN and those without DPN were compared. As shown in Table 1, there was no significant difference in age, gender, BMI, duration of diabetes, duration of hypertension, TG, TC, LDL-C, and HDL-C in T2DM patients with DPN and those without DPN (all $p > 0.05$). While increased HbA1c was found in 57 patients with DPN, decreased SUA was detected in patients with DPN, compared to 49 diabetic patients without DPN (All $p < 0.05$) (Table 1).

*3.2. Neurophysiological Examination Result of T2DM Patients with and without DPN.* In this present work, we compared the difference in motor conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, tibial nerve, and common peroneal nerve, as well as the sensory conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, and sural nerve patients. Compared with 49 patients in the control group, patients in the DNP group showed not only decreased motor conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, tibial nerve, and common peroneal nerve but also impaired sensory conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, and sural nerve (all $p < 0.05$) (Table 2).

*3.3. Pearson's Correlation between SUA and Nerve Conduction Velocity.* To confirm the relationship between SUA and DPN in patients with T2DM, Pearson's correlation was conducted. Here, it is demonstrated that SUA is not only associated with tibial nerve motor conduction velocity ($R = 0.247$, $P = 0.011$) but also related to median nerve ($R = 0.211$, $P = 0.030$) and sural nerve ($R = 0.223$, $P = 0.022$) sensory conduction velocity in T2DM patients (Table 3).

*3.4. Partial Correlation between SUA and Nerve Conduction Velocity Adjusted for HbA1c.* As HbA1c levels are higher in patients with DPN than those without DPN, partial correlation analysis was carried out and adjusted for HbA1c to further explore the relationship between SUA and DPN in diabetic patients without uric acid lowering drugs. It is different from the results of Pearson's correlation. We found an association between SUA and tibial nerve motor conduction velocity ($R = 0.197$, $p = 0.044$), rather than median nerve and sural nerve sensory conduction velocity (all $p > 0.05$) (Table 4).

*3.5. Comparison of SUA Levels in Different Patients with T2DM.* As shown in the baseline data, decreased SUA was found in diabetic patients with DPN, compared to those without DPN. Here, we showed this result in a scatter diagram (Figure 1(a)). The above result indicated that SUA is associated with tibial nerve motor conduction velocity. We also compared the difference in SUA levels between T2DM patients with or without tibial nerve motor fiber damage. Interestingly, we also found decreased SUA levels in patients with slow tibial nerve motor conduction velocity (Figure 1(b)).

*3.6. Low SUA Level Is One of the Risk Factors of DPN in T2DM Patients.* To further investigate the role of SUA in diabetic patients with DPN, binary logistic regression analysis was conducted and adjusted for HbA1c. It is showed that lower SUA is one of the risk factors of DPN in T2DM patients independent from HbA1c (OR = 0.994, $p = 0.043$) (Table 5).

*3.7. Low SUA Levels May Influence Tibial Nerve Motor Conduction Velocity.* To further investigate the effect of SUA levels on the details of DPN in patients with T2DM patients, multiple linear regression analysis was performed. After adjusting for HbA1c, low SUA may influence the tibial nerve motor conduction velocity of diabetic patients with DPN ($\beta = 0.012$, $p = 0.044$) (Table 6).

## 4. Discussion

DPN is one of the most important complications of diabetes associated with hyperglycemia [32, 33]. Indeed, increased HbA1c levels were detected in patients with DPN, compared with those without DPN. It is agreed with a previous study [34]. Except for chronic hyperglycemia, oxidative stress may also be involved in the development of DPN. Indeed, oxidative stress plays an important role in painful diabetic peripheral neuropathy [23]. Additionally, the occurrence of oxidative stress may also result from hyperglycemia [35]. Moreover, it demonstrated the relationship between oxidative stress and DPN in humans [24], animals [26, 36], and in vitro [37] studies. As described in the introduction, SUA may be involved in DPN, which is associated with oxidative stress [38–40]. We also compared with levels of SUA, which is a kind of natural antioxidant [28], in T2DM patients with or without DPN. Interestingly, decreased SUA was observed in diabetic patients with DPN. Similarly, a previous study showed lower SUA in T2DM patients with mild cognitive impairment than those without cognition decline [41], which is a kind of dysfunction in the central nervous system [42, 43]. To confirm the occurrence of DPN, the conduction velocity of nerve was compared. Undoubtedly, motor conduction fiber velocity of the ulnar nerve, radial nerve, median nerve, tibial nerve, and common peroneal nerve as well as sensory conduction fiber velocity of ulnar nerve, radial nerve, median nerve, and sural nerve was faster in patients without DPN than those with DPN.

Due to the decreased SUA levels in patients with DPN, Pearson's correlation was conducted to detect the association between SUA and the above conduction velocity of nerves. We found that levels of SUA were positively associated with the conduction speed of tibial nerve motor fibers, median nerve sensory fibers, and sural nerve sensory fibers. Additionally, we described the increased HbA1c levels in DPN patients. Here, we performed a partial correlation adjusted for HbA1c, to further investigate the association between SUA and DPN. SUA was associated with tibial nerve motor conduction velocity adjusted for HbA1c in this present study.

After adjusting by HbA1c, SUA was associated with tibial nerve motor conduction velocity in patients with T2DM.

Table 1: Comparation of clinical parameters between control and DPN group.

| | Control group ($n = 49$) | DPN group ($n = 57$) | $p$ |
|---|---|---|---|
| Age (years) | 55.43 ± 10.59 | 56.02 ± 13.66 | 0.807[a] |
| Female ($n$, %) | 17, 34.7 | 23, 40.4 | 0.177[c] |
| BMI ($m^2$/kg) | 24.61 (22.57-26.38) | 24.30 (21.62-26.53) | 0.606[b] |
| Duration of DM (years) | 7 (3-10) | 7 (1-15) | 0.610[b] |
| Duration of HBP (years) | 0 (0-7) | 0 (0-4.5) | 0.840[b] |
| HbA1c (%) | 8.80 (6.85-9.85) | 10.6 (9.20-12.15)* | 0.001[b] |
| TG (mmol/l) | 1.75 (1.26-2.49) | 1.42 (1.07-2.20) | 0.194[b] |
| TC (mmol/l) | 4.54 (3.84-5.12) | 4.47 (3.95-5.10) | 0.917[b] |
| HDL-C (mmol/l) | 0.85 (0.67-1.07) | 0.84 (0.69-1.11) | 0.646[b] |
| LDL-C (mmol/l) | 2.38 (1.69-2.79) | 2.32 (1.92-3.19) | 0.219[b] |
| Scr (umol/l) | 63.00 (49.50-72.50) | 58.00 (49.50-72.00) | 0.375[b] |
| BUN (mmol/l) | 5.84 (5.15-6.84) | 5.74 (4.97-7.53) | 0.807[b] |
| SUA (mmol/l) | 326.27 ± 69.79 | 284.24 ± 83.17* | 0.006[a] |

The data are presented as $n$ (%) or the median (interquartile range) unless otherwise specified. [a]Student's $t$-test was employed for normally distributed variables. [b]The Mann–Whitney $U$ test was employed for asymmetrically distributed variables. [c]The Chi-square test was employed for categorical variables. *$p < 0.05$, DPN group vs. control group. Abbreviations: DPN: diabetic peripheral neuropathy; BMI: body mass index; DM: diabetes mellitus; HBP: high blood pressure; TG: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; Scr: serum creatinine; BUN: blood urea nitrogen; SUA: serum uric acid.

Table 2: Comparation of neurophysiological test results between control and DPN group.

| | Control group ($n = 49$) | DPN group ($n = 57$) | $p$ |
|---|---|---|---|
| *Motor conduction* | | | |
| Ulnar nerve (m/s) | 61.95 (60.05-63.15) | 54.60 (51.85-60.68)* | <0.001[b] |
| Radial nerve (m/s) | 64.85 (62.60-66.53) | 63.55 (61.25-65.68)* | 0.014[b] |
| Median nerve (m/s) | 60.20 (56.93-62.20) | 54.25 (50.50-56.50)* | <0.001[b] |
| Tibial nerve (m/s) | 47.37 ± 3.10 | 40.95 ± 4.17* | <0.001[a] |
| Common peroneal nerve (m/s) | 46.75 (45.13-48.68) | 43.45 (40.03-44.98)* | <0.001[b] |
| *Sensory conduction* | | | |
| Ulnar nerve (m/s) | 56.70 (52.33-60.50) | 49.00 (43.23-53.43)* | <0.001[b] |
| Radial nerve (m/s) | 57.79 ± 8.41 | 49.82 ± 5.96* | <0.001[a] |
| Median nerve (m/s) | 58.15 (54.00-62.50) | 45.55 (40.48-51.55)* | <0.001[b] |
| Sural nerve (m/s) | 52.35 ± 5.03 | 44.63 ± 5.48* | <0.001[a] |

[a]Student's $t$-test was employed for normally distributed variables. [b]The Mann–Whitney $U$ test was employed for asymmetrically distributed variables. *$p < 0.05$, DPN group vs. control group. Abbreviations: DPN: diabetic peripheral neuropathy.

So, not only the levels of SUA in diabetic patients with and without DPN but also those in patients with normal and decreased tibial nerve motor conduction velocity were observed. Both decreased SUA levels in DPN patients and patients with decreased tibial nerve motor conduction velocity were found.

To confirm the risk factor of DPN in patients with T2DM, binary logistic regression analysis was conducted and adjusted for HbA1c. It is showed that lower SUA is one of the risk factors of DPN in T2DM patients independent from HbA1c. To further investigate the relationship between SUA and nerve injury details, multiple linear regression analysis was performed by adjusting for HbA1c. Decreasing SUA may influence the tibial nerve motor conduction velocity of diabetic patients with DPN. It is demonstrated that low SUA levels are associated with DPN. However, increased SUA levels are also associated with the occurrence of DPN in T2DM patients [17]. These contradictions may result from different populations included. In this present study, we included hospitalized patients without SUA lowering treatments. Patients with high levels of SUA were excluded due to the use of SUA lowering drugs. The neuroprotective effect of antioxidant SUA may be offset in patients with abnormally increased SUA levels and other metabolic disorders associated with hyperuricemia. As a kind of metabolic disorder, extremely high levels of SUA may lead to an increased risk of DPN along with other factors of metabolic disorder [19].

Table 3: Pearson's correlation between SUA and nerve conduction velocity.

| | Pearson correlation ($R$) | $p$ |
|---|---|---|
| *Motor conduction* | | |
| Ulnar nerve | 0.144 | 0.142 |
| Radial nerve | -0.026 | 0.094 |
| Median nerve | 0.178 | 0.067 |
| Tibial nerve | 0.247 | 0.011* |
| Common peroneal nerve | 0.097 | 0.321 |
| *Sensory conduction* | | |
| Ulnar nerve | 0.131 | 0.182 |
| Radial nerve | 0.151 | 0.123 |
| Median nerve | 0.211 | 0.030* |
| Sural nerve | 0.223 | 0.022* |

*$p < 0.05$. Abbreviations: SUA: serum uric acid.

Table 4: Partial correlation between SUA and nerve conduction velocity adjusted for HbA1c.

| | Partial correlation ($R$) | $p$ |
|---|---|---|
| *Motor conduction* | | |
| Ulnar nerve | 0.090 | 0.360 |
| Radial nerve | -0.040 | 0.684 |
| Median nerve | 0.144 | 0.144 |
| Tibial nerve | 0.197 | 0.044* |
| Common peroneal nerve | 0.023 | 0.820 |
| *Sensory conduction* | | |
| Ulnar nerve | 0.090 | 0.361 |
| Radial nerve | 0.098 | 0.318 |
| Median nerve | 0.160 | 0.103 |
| Sural nerve | 0.159 | 0.104 |

*$p < 0.05$. Abbreviations: SUA: serum uric acid.

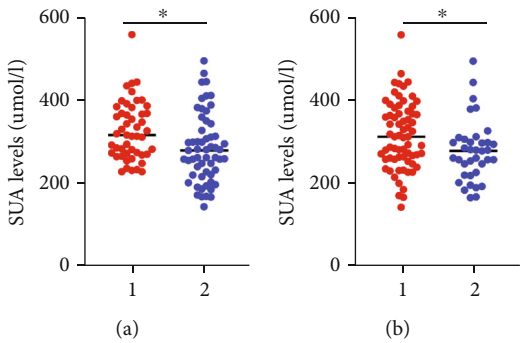


Figure 1: SUA levels in different patients with T2DM. *$p < 0.05$. "1" in (a) showed T2DM patients without DPN; "2" in (a) showed T2DM patients with DPN; "1" in (b) showed T2DM patients without decreased tibial nerve motor conduction velocity; "2" in (b) showed T2DM patients with decreased tibial nerve motor conduction velocity. Abbreviations: T2DM: type 2 diabetes mellitus; SUA: serum uric acid.

Table 5: Binary logistic regression analysis for risk factors of DPN in T2DM patients.

| | Binary logistic regression (OR) | 95% CL of OR | | $p$ |
|---|---|---|---|---|
| HbA1c | 0.348 | 1.112 | 1.633 | 0.002* |
| SUA | 0.994 | 0.989 | 1.000 | 0.043* |

*$p < 0.05$. Abbreviations: DPN: diabetic peripheral neuropathy; T2DM: type 2 diabetes mellitus; SUA: serum uric acid.

Table 6: Multiple linear regression analysis for factors that influence the tibial nerve motor conduction velocity of T2DM patients.

| | Multiple linear regression ($\beta$) | 95% CL of $\beta$ | | $p$ |
|---|---|---|---|---|
| HbA1c | -0.496 | -0.839 | -0.100 | 0.013* |
| SUA | 0.012 | ≤0.001 | 0.023 | 0.044* |

*$p < 0.05$. Abbreviations: T2DM: type 2 diabetes mellitus; SUA: serum uric acid.

Generally, previous research has mainly investigated the risk factors associated with DPN in individuals with diabetes. The study investigated the characteristics of T2DM patients with and without peripheral neuropathy, focusing particularly on nerve conduction velocities and SUA levels. The present study, however, not only explored the risk factors for DPN but also examined the association between SUA levels and specific neuronal damage in patients with T2DM. Nonetheless, there are several limitations that require addressing. Firstly, the study design was cross-sectional, thus allowing for the establishment of only an association rather than a causal relationship between SUA and DPN. Secondly, uric acid-lowering drugs were used as an essential factor in uric acid disorder treatment. Here, patients with uric acid-lowering drugs were excluded in this research. So, the analysis of the kind and dosage of uric acid-lowering drug treatment was not sufficient in this study. Lastly, the study only considered the effect of decreased SUA levels on DPN, neglecting the potential impact of elevated SUA levels.

## 5. Conclusion

To the best of our knowledge, this is the first study focusing on the relationship between the DPN, especially for certain nerve conduction velocities, and SUA in T2DM patients without uric acid lowering treatments. We demonstrated that low SUA is a risk factor for DPN in patients with T2DM. Additionally, decreased SUA may influence the function of the tibial nerve motor fiber independent from the control of HbA1c. This may result from the antioxidant effect of SUA. Increasing SUA to a certain level may be a novel method to reduce the burden of DPN in T2DM patients. Further well-designed prospective cohort studies and basic researches are needed to clarify the causal association between SUA and DPN as well as the mechanisms associated with oxidative stress of SUA in DPN of T2DM patients.

## Data Availability

All data in this manuscript have been submitted to our institute for records. Additionally, all IDs of recruited patients were also submitted for further use. The datasets analyzed are available from authors on reasonable request.

## Conflicts of Interest

The authors report no conflicts of interest in this work.

## Authors' Contributions

Haoqiang Zhang and Wei Wang contributed to the idea. Hui Zhang, Carvalho Vladmi, and Haoqiang Zhang wrote the manuscript draft and (or) revised the manuscript. Zhen Zhang, Wan Zhou, Jiang Xu, Wanwan Zhao, Yang Chen, Mengting He, and Ya Zhang performed the tests, collected the data, checked the statistical analysis, and helped to revise the final version of the paper. All authors read and approved the final manuscript.

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*Research Article*

# ceAF Ameliorates Diabetic Wound Healing by Alleviating Inflammation and Oxidative Stress via TLR4/NF-κB and Nrf2 Pathways

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*Background*. With the rise in diabetes incidence, diabetic foot ulcers have become the most common clinically chronic refractory wounds. Persistent chronic inflammation is a typical feature of diabetic cutaneous wounds, and diabetic wound healing can be improved by alleviating inflammation and oxidative stress. Chick early amniotic fluids (ceAF) consist of native conglutinant substances with balanced amounts of growth factors, cytokines, and chemokines. However, whether ceAF modulates inflammation and oxidative stress and thus promotes diabetic wound healing remains unknown. *Materials and Methods*. RAW264.7 cells were categorized into four groups: negative control, LPS, LPS + ceAF, and ceAF. 10% of ceAF was selected to treat different groups of mice with a full-thickness skin defect wound. Then, RT-qPCR, western blot, immunofluorescence, and other assays were carried out to explore the effect of ceAF on wound healing and its molecular mechanism. *Results*. Topical administration of ceAF improved M2 macrophage polarization and inflammatory response in the wound tissues, thereby ameliorating delayed wound healing. Histological improvement could be observed in the grade of inflammation, collagen deposition, and neovascularization in wound edge tissues. ceAF also increased M2 macrophage-specific markers expression and exogenous ceAF suppressed LPS-induced cellular inflammatory response *in vitro* high glucose environment. Additionally, ceAF could activate TLR4/NF-κB and Nrf2 signal transductions to promote M2 macrophage polarization *in vitro*. *Conclusions*. In summary, ceAF downregulates inflammatory response, regulates M2 macrophage transition via TLR4/NF-κB and Nrf2 signaling pathways, and thus improves diabetic wound healing.

## 1. Introduction

As a kind of chronic wound, the diabetic wound has been vexing diabetic patients and clinicians [1]. Due to the complex microenvironment of diabetic wounds such as hypoxia, infection, ischemic condition, inflammation, and oxidative stress, diabetic ulcer is often prolonged and recurrent [2, 3]. Traditional debridement and dressing change therapy has limited effect on diabetic wounds [4]. The application of the flap is also dependent on the patient's own condition because occlusion of distal blood vessels in the limb is common in diabetics [5]. Therefore, how to treat diabetic ulcer efficiently and economically has become an urgent problem.

Oxidative stress and inflammation are closely related to wound healing in diabetes mellitus [6]. According to the activators, macrophages can be classified into classically-

activated M1 or alternatively-activated M2 phenotypes [7]. During wound healing, M1 macrophages are responsible for the phagocytosis of necrotic tissue and cell debris, while M2 macrophages are involved in inhibiting inflammation and promoting tissue regeneration [8]. Diabetic wounds are often in a state of excessive inflammation. There is a large amount of M1 macrophages in wound tissue and the transformation of macrophages to M2 type is interrupted [9]. Notably, previous research has demonstrated that increasing the M2 phenotype could be a vital factor in diabetic wound repair [10, 11]. The combination of suppressing oxidative stress damage and promoting M2 macrophage polarization can be desirable for diabetic ulcer.

Transcription factors Nrf2 and NF-$\kappa$B are classical prototypical proinflammatory effectors that regulate cell proliferation, apoptosis, and differentiation [12]. TLR4 is an important upstream regulator of NF-$\kappa$B signal transduction [13]. Previous reports have shown that TLR4/NF-$\kappa$B signal path is associated with macrophage polarization [14, 15]. The induction of iNOS in macrophages was dependent on NF-$\kappa$B signal transduction [16]. miR-146a can promote M2 macrophage polarization through inhibiting TLR4/NF-$\kappa$B axis during diabetic wound healing [17]. Heme oxygenase 1(HO-1) is regulated by Nrf2 and plays an important immune-modulatory role in macrophages; the induction of HO-1 could switch these cells from the proinflammatory (M1) to anti-inflammatory (M2) phenotype. [18].

In many species, amniotic fluid is a nourishing, protective fluid that surrounds the embryo throughout the pregnancy [19]. It contains stem cells derived from embryos, and thus, is a favorable substance for wound healing [20]. ceAF is a compound that we extract from chick embryos that have been incubated for 6-8 days. In view of the previous studies mentioned that embryonic amniotic fluid contains stem cells and growth factors which were conducive to wound healing. We designed experiments on wound healing *in vivo* and *in vitro* to investigate the therapeutic effects and possible mechanisms of ceAF.

## 2. Materials and Methods

*2.1. Antibodies and Reagents.* APC CD206 antibody and FITC F4/80 antibody were procured from eBioscience. Arginase1 (Arg-1), $\alpha$-SMA, and CD206 were purchased from CST. GAPDH, iNOS, TNF-$\alpha$, IL-6, IL-1$\beta$, Nrf2, HO-1, NQO1, and CD31 were purchased from Abcam. TLR4, NF-$\kappa$B-p65, and p-IKB were purchased from ABclonal. The Trizol Reagent and SYBR green were purchased from Vazyme Biotech. STZ and glucose were purchased from Sigma-Aldrich.

*2.2. Preparation of ceAF.* Fertile chicken eggs were hatched at 38°C and 50% humidity. Between days 6 and 8 of hatching, ceAF was isolated from the eggs. After centrifugation (2500 × g, 20 min), a 0.22 $\mu$m sterile filter (Millipore, USA) was used to filter the supernatant. The filtered specimens were aliquoted and kept at -80°C.

*2.3. Cell Culture.* RAW264.7 cells were supplied by the Chinese Academy of Sciences Cell Bank and then cultured in high-glucose DMEM medium containing 10% of FBS and 1% of double antibody at 37°C with 5% of $CO_2$. The DMEM with 40 mM glucose was employed as high-glucose conditions. Various concentrations of ceAF were added to the medium for subsequent experiments.

*2.4. Animals and Wound Procedure.* The experimental protocols were approved by the Animal Care and Ethics Committee of Nanjing University. C57BL/6 mice (male, 8 weeks old) were obtained from the Model Animal Research Center of Nanjing University, and maintained in a specific pathogen-free environment with unlimited access to water and food. Eighteen mice in each group were injected intraperitoneally every day with 50 mg/kg STZ (in sodium citrate buffer) for five days in order to construct an STZ-induced diabetes model. The mice were given blood glucose measurements after three weeks, and those with blood glucose levels > 16.7 mM were classified as diabetes. To establish an excisional wound model, an 8 mm circular biopsy punch was performed on the back skin of mice following hair removal. After modeling, 10% ceAF was topically applied to the wound surface daily and the control mice were given equal volume PBS. The wound images were captured on days 0,3,5,7, and 11, and wound areas were measured with ImageJ software (National Institutes of Health). Wound tissue samples were collected on days 5 and 10 postinjury for subsequent experiments.

*2.5. Histological and Immunofluorescent Staining.* The wound margin tissues were fixed, dehydrated, embedded in paraffin, and sectioned at 5 $\mu$m thickness. Masson's trichrome (MT), hematoxylin-eosin (H&E), and Sirius red staining were conducted according to standardized histological procedures. To assess macrophage polarization and angiogenesis, CD206, iNOS, CD31, and $\alpha$-SMA monoclonal antibody (1 $\mu$g/ml) staining was carried out at 4°C overnight. Then, a specific fluorescent secondary antibody was incubated, followed by DAPI staining.

RAW264.7 cells were sequentially rinsed with PBS, fixed in paraformaldehyde (4%), perforated with 0.1% Triton X-100, and blocked with BSA (3%). Then, the cells were incubated with corresponding primary and secondary antibodies according to the instructions. All photographs were taken using an Olympus FluoView FV3000 confocal microscope (Tokyo, Japan).

*2.6. RNA Isolation and RT-qPCR.* Cells and wound margin tissue were treated with Trizol Reagent to isolate total RNA by following the manufacturer's instructions. RT-qPCR was conducted with SYBR green dye using the StepOne RT-qPCR system (Applied Biosystems, USA). After normalization with GAPDH, the relative gene levels were determined using the $2^{-\Delta\Delta CT}$ method. The primer pairs are listed in Table 1.

*2.7. Western Blot (WB) Analysis.* Protein samples were isolated from lysed skin tissues and cells using RIPA lysis buffer (KeyGEN, China). BCA assay was performed to determine

Table 1: Primer sequences used for RT-qPCR.

| Gene | Forward | Reverse |
|---|---|---|
| *CD206* | GAGGGAAGCGAGAGATTATGGA | GCCTGATGCCAGGTTAAAGCA |
| *Arg-1* | TTGGGTGGATGCTCACACTG | GTACACGATGTCTTTGGCAGA |
| *iNOS* | ACATCGACCCGTCCACAGTAT | CAGAGGGGTAGGCTTGTCTC |
| *TNF-α* | CTGAACTTCGGGGTGATCGG | GGCTTGTCACTCGAATTTTGAGA |
| *IL-6* | CCAAGAGGTGAGTGCTTCCC | CTGTTGTTCAGACTCTCTCCCT |
| *IL-1β* | GAAATGCCACCTTTTGACAGTG | TGGATGCTCTCATCAGGACAG |
| *GAPDH* | CCAGTATGACTCCACTCACG | GACTCCACGACATACTCAGC |

the total protein concentration after centrifugation. The protein specimens were separated through 10% SDS-PAGE gel and transferred onto the PVDF membrane (Millipore, USA). After blocking with 5% BSA, the membrane was incubated with the corresponding primary for overnight and secondary antibody for 1 h. The visualization of protein bands was conducted using an ECL substrate kit (Vazyme, China).

*2.8. Flow Cytometry.* To determine the polarization trend of RAW264.7 macrophages, the cells were preincubated with FITC-conjugated anti-mouse F4/80 antibody and APC-conjugated anti-mouse CD206 antibody at 4°C in the dark for 30 min. The cell phenotype was determined using a flow cytometer (FACSCanto II, BD, USA), and data analysis was conducted with FlowJo software.

*2.9. Cell Viability Test.* CCK-8 assays (Beyotime, China) were used to assess RAW264.7 cell viability. After 12 h of starvation, the cells were exposed to 0%, 1%, 5%, 10%, or 20% of ceAF and then incubated for 24 h. The cells were rinsed with PBS 3 times, and then covered with 200 $\mu$L in a complete medium containing CCK-8 mixture (10 $\mu$L) and incubated at 37°C. Absorbance was measured using a microplate reader at 450 nm.

*2.10. Measurement of SOD, MPO, MDA, GSH-Px, and ROS Levels.* RAW264.7 cells were stimulated with 100 ng/mL LPS for 48 h to induce cellular inflammation. Protein concentration was determined quantitatively with a BCA protein assay kit (Pierce Biotechnology, Rockford, Illinois, USA). SOD, MPO, MDA, GSH-Px, and ROS levels in cells were measured according to a previously described method using a relevant assay kit (Nanjing KeTeng Biotech Co. Ltd., Nanjing, China).

*2.11. ELISA.* RAW264.7 cells were exposed to 10% of ceAF for 48 h, and cellular supernatants were collected for testing. The secreted IL-6, IL-10, TGF-$\beta$1, and TNF-$\alpha$ were measured via ELISA kit according to the kit instruction (Elabscience, China).

*2.12. Statistical Analysis.* Experimental data were analyzed with GraphPad Prism v8.0 software and presented as mean ± SEM. Parametric tests were used for data that conform to the normal distribution (Shapiro–Wilk test). If the data were normally distributed, the statistical differences among multiple groups were compared with one-way ANOVA and Newman-Keuls post hoc test. The two-tailed Student's t test was utilized for the comparison of two groups if the data passed the normality test. The combined effects of two factors were analyzed with two-way ANOVA followed by Tukey's posttest. At least three independent assays were conducted, and $P$ values of $<0.05$ were defined as statistically significant.

## 3. Results

*3.1. ceAF Attenuates the Inflammation and Oxidative Stress of LPS-Stimulated RAW 264.7 Cells via TLR4/NF-κB and Nrf2 Axis.* To explore the regulatory role of ceAF on LPS-induced cellular inflammation, we stimulated RAW264.7 with 100 ng/mL LPS for 48 h to induce cellular inflammation. Firstly, the proliferative capability of RAW264.7 cells was tested with different concentrations of ceAF. CCK8 results demonstrated that the proliferative capability of RAW264.7 increased with the progressive of ceAF concentration and reached the peak at 10% concentration (Figure 1(a)). Next, we validated macrophages and inflammatory markers at the transcriptional level. RT-qPCR analysis indicated that the mRNA levels of M2 macrophage markers (Arg-1 and CD206) were remarkably decreased in the LPS group and increased in the LPS+ceAF group (Figure 1(b)). While the mRNA levels of the M1 marker (iNOS) and inflammatory factors (IL-1$\beta$, IL-6, and TNF-$\alpha$) were markedly decreased in the LPS+ceAF group (Figure 1(b)). LPS utilization significantly increased ROS, MDA, and MPO levels, while the levels of SOD and GSH-Px were significantly decreased, and these changes could be reversed by ceAF treatment (Figure 1(c)). Further, TLR4/NF-κB pathway-associated proteins were significantly activated and Nrf2 pathway-associated proteins were markedly decreased in the LPS group. However, the trend was reversed in the LPS+ceAF group (Figures 1(d) and 1(e)).

*3.2. ceAF Induces RAW264.7 to Polarize M2 Macrophages In Vitro.* Next, flow cytometry was conducted to assess the polarization state of macrophages after exposure to LPS and ceAF. It was found that the proportion of M2 macrophages was greatly enhanced in the LPS+ceAF group than in the LPS group at 48 and 72 h (Figures 2(a) and 2(b)). Similar results of changes in macrophage polarization were also observed by immunofluorescent staining with reaching statistical significance (Figures 2(c) and 2(d)). We further detected the inflammatory-related cytokines in the cellular supernatant using ELISA. The secretion of TNF-$\alpha$ and IL-6

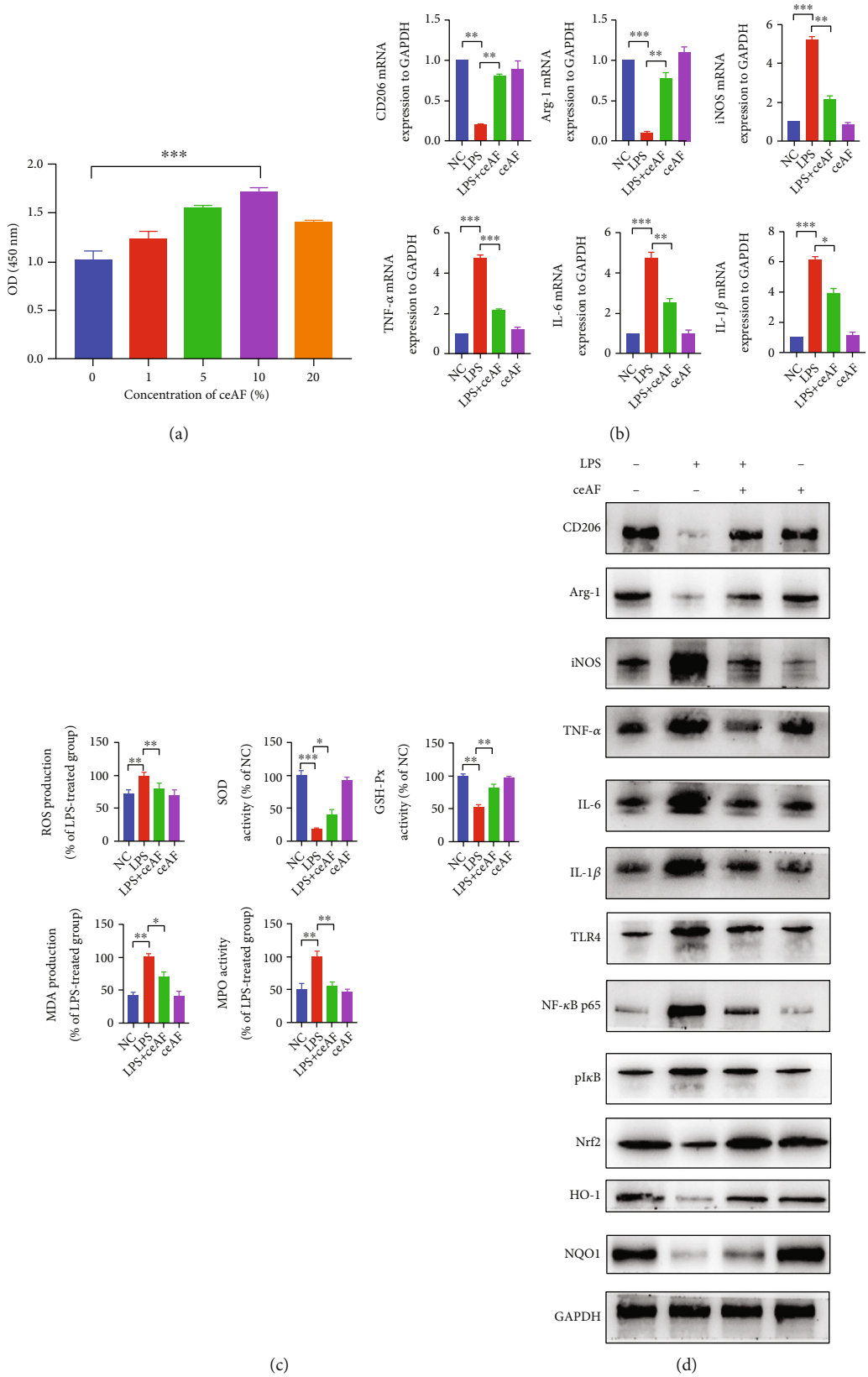
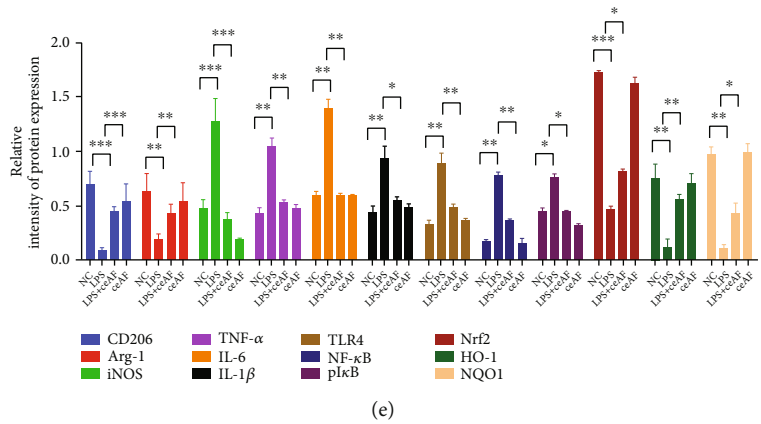



Figure 1: Continued.




(e)

Figure 1: ceAF was able to suppress inflammation and oxidative stress via TLR4/NF-κB and Nrf2 pathways in LPS-stimulated RAW 264.7 cells via TLR4/NF-κB and Nrf2 pathways. (a) The viability of RAW 264.7 cells exposed to ceAF at various concentrations. (b) The expression levels of CD206, Arg-1, iNOS, IL-1β, IL-6, and TNF-α were assessed by RT-qPCR. (c) The levels of ROS SOD, GSH-Px, MDA, and MPO were measured. (d) WB results of the influence of ceAF on macrophage markers and TLR4/NF-κB axis. CD206, Arg-1, iNOS, IL-1β, IL-6, TLR4, TNF-α, NF-κB p65, pIκB Nrf2, HO-1, and NQO1 were tested, and GAPDH served as a standard reference. (e) Bands from the WB in (d) were analyzed by densitometry. $n = 3$, Mean ± SEM. NC: negative control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

in the LPS+ceAF group was markedly attenuated compared to that in the LPS group (Figure 2(e)). On the contrary, TGF-β1 and IL-10 were dramatically increased as anti-inflammatory factors in the LPS+ceAF group (Figure 2(e)).

*3.3. ceAF Promotes Wound Healing in Diabetic Mice.* To evaluate the rates of wound healing in diabetes mellitus (DM) and treatment (DM+ceAF) groups, 8 mm full-thickness wounds were created on the back of the diabetic mice. As shown in Figures 3(a) and 3(b), the wound healing slowed down obviously in the DM group. The adverse trend was reversed in the DM+ceAF group from the fifth day (Figures 3(a) and 3(b)). To exclude confounding factors, we measured the body weight and blood glucose of the mice on day 11 and found no statistical difference between the two groups (Figures 3(c) and 3(d)).

*3.4. ceAF Ameliorates the Wound Histological Indicators of Diabetic Mice.* The protective effects of ceAF on diabetic wounds in mice were further evaluated by histopathological analysis. H&E staining analysis revealed an obvious difference in skin margin tissue between DM and DM+ceAF groups (Figure 4(a)). After ceAF administration, the inflammatory cells of diabetic mice wounds were markedly decreased compared to the control group (Figure 4(b)). Wound samples were collected on day 10 for the MT test. The results demonstrated that the proportion of collagen deposition was much higher in the DM+ceAF group than in the DM group (Figures 4(c) and 4(d)). The content of type I and III collagen in the wound after healing can reflect the healing quality to a certain degree. Therefore, we used Sirius red staining to determine the type of collagen in the newborn skin after wound healing. The staining results showed that the green coloration of type III collagen is more obvious in the DM+ceAF group. In contrast, the DM group contained more yellow-red type I collagen (Figure 4(e)).

*3.5. ceAF Promotes M2 Macrophage Polarization and Neovascularization in the Wound of Diabetic Mice.* Subsequently, we assessed the trend of macrophage polarization and angiogenesis *in vivo*. The immunofluorescence staining data indicated that the number of $CD206^{+}$ macrophages was much higher in the DM+ceAF group than in the DM group on day 5 after wounding (Figure 5(a)). Meanwhile, the M1 macrophage population notably reduced in diabetic mice treated with ceAF (Figure 5(b)). The rate of wound vascularization is a crucial indicator for determining the effect of wound healing. Therefore, CD31 and α-SMA expression were further confirmed by immunofluorescence. It was observed that the DM+ceAF group had lesser $CD31^{+}$ and $\alpha\text{-SMA}^{+}$ cells compared to the DM group on day 10 (Figures 5(c) and 5(d)).

*3.6. ceAF Inhibits Inflammation through Inducing M2 Macrophage Polarization in the Wound of Diabetic Mice.* To verify the changes induced by ceAF therapy between macrophage polarization and inflammation *in vivo*, we collected mouse wound tissue on day 5 to verify the indicators of macrophages and inflammation using WB and RT-qPCR. The protein levels of CD206 and Arg-1 in the DM+ceAF group were remarkably elevated, and the expression of iNOS was dramatically weakened in comparison with that of the DM group (Figures 6(a) and 6(b)). The changes in proinflammatory factors were consistent with the M1 macrophage indicator. ceAF treatment markedly reduced the protein levels of TNF-α, IL-1β, and IL-6 compared to the DM group (Figures 6(a) and 6(b)). The transcriptional levels of the above indicators were measured by RT-qPCR. The experiments were repeated 3 times and similar trends were observed (Figures 6(c)–6(h)).

## 4. Discussion

A diabetic ulcer is a common complication in diabetic patients, and one of the reasons for a nonhealing wound is an excessive inflammatory reaction [21]. Herein, the anti-

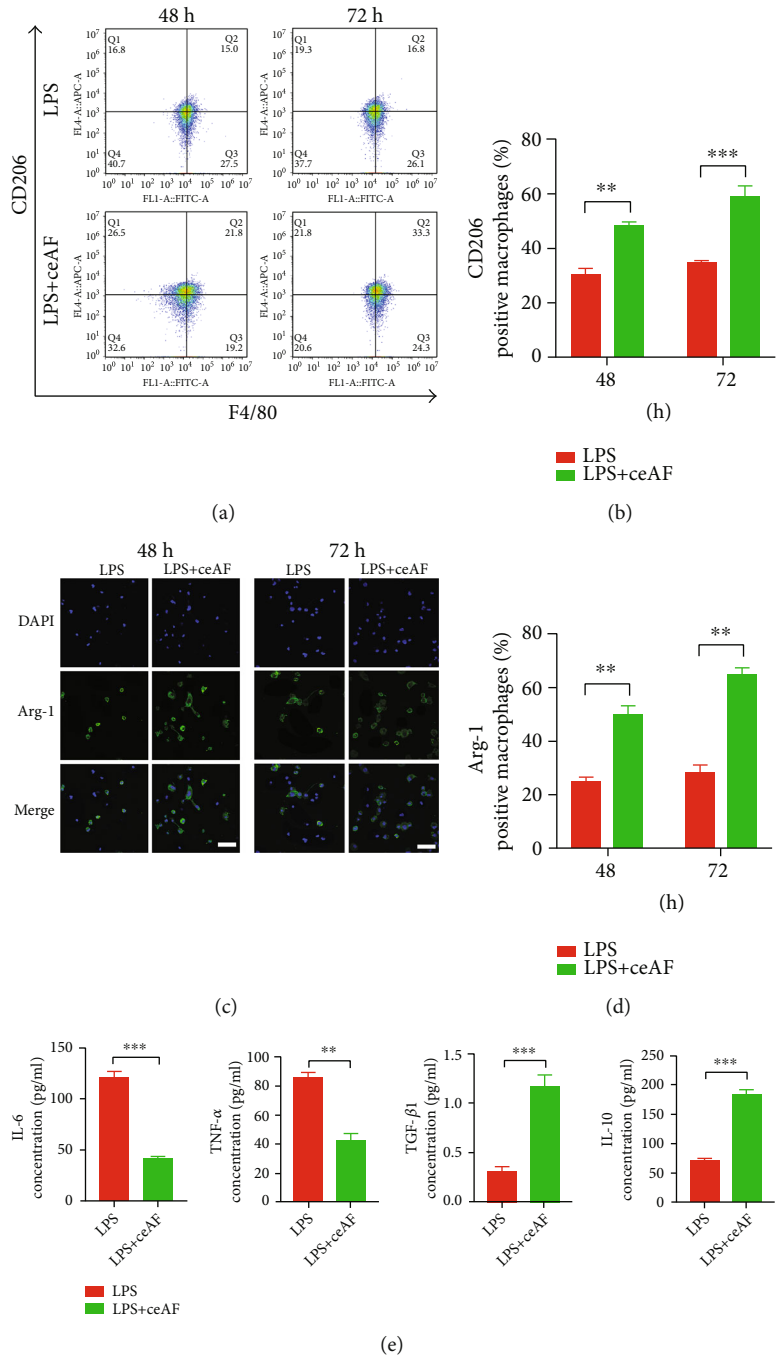



Figure 2: ceAF augments M2 macrophage polarization *in vitro*. (a) Flow cytometric analysis of M2 macrophage proportions after ceAF treatment in a time-dependent manner. (b) The quantified results of (a) are presented in a bar chart. (c) Immunofluorescence staining indicated that Arg-1$^+$ cells were treated with LPS and LPS+ceAF at 48 and 72 h. Scale bar: 40 $\mu$m. (d) The quantified results of (c) are presented in a bar chart. (e) ELISA detection results of IL-6, TNF-$\alpha$, TGF-$\beta$1, and IL-10. $n = 3$, Mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

inflammatory effects of ceAF on diabetic wound healing and its underlying mechanism were elucidated. The main results are as follows: (i) ceAF could inhibit cellular inflammation by deactivating the TLR4/NF-$\kappa$B axis and activating the Nrf2 axis; (ii) ceAF can promote wound healing and improve histological indicators in diabetic mice; (iii) ceAF regulates the macrophage polarization both *in vivo* and *in vitro*; and (iv) ceAF ameliorates inflammation and oxidative stress by promoting M2 macrophage polarization. It is speculated ceAF exerts an anti-inflammatory function by promoting a rapid transition from the inflammatory stage to the remodeling stage. Our findings provide an important theoretical basis for treating refractory skin wounds in diabetes patients.

Amniotic fluid is essential for fetal development and survival, and its role varies at different stages of embryonic development [22]. The reason we chose early amniotic fluid is that it contains more growth-promoting substances such as stem cells, growth factors, and chemokines [23, 24]. Late

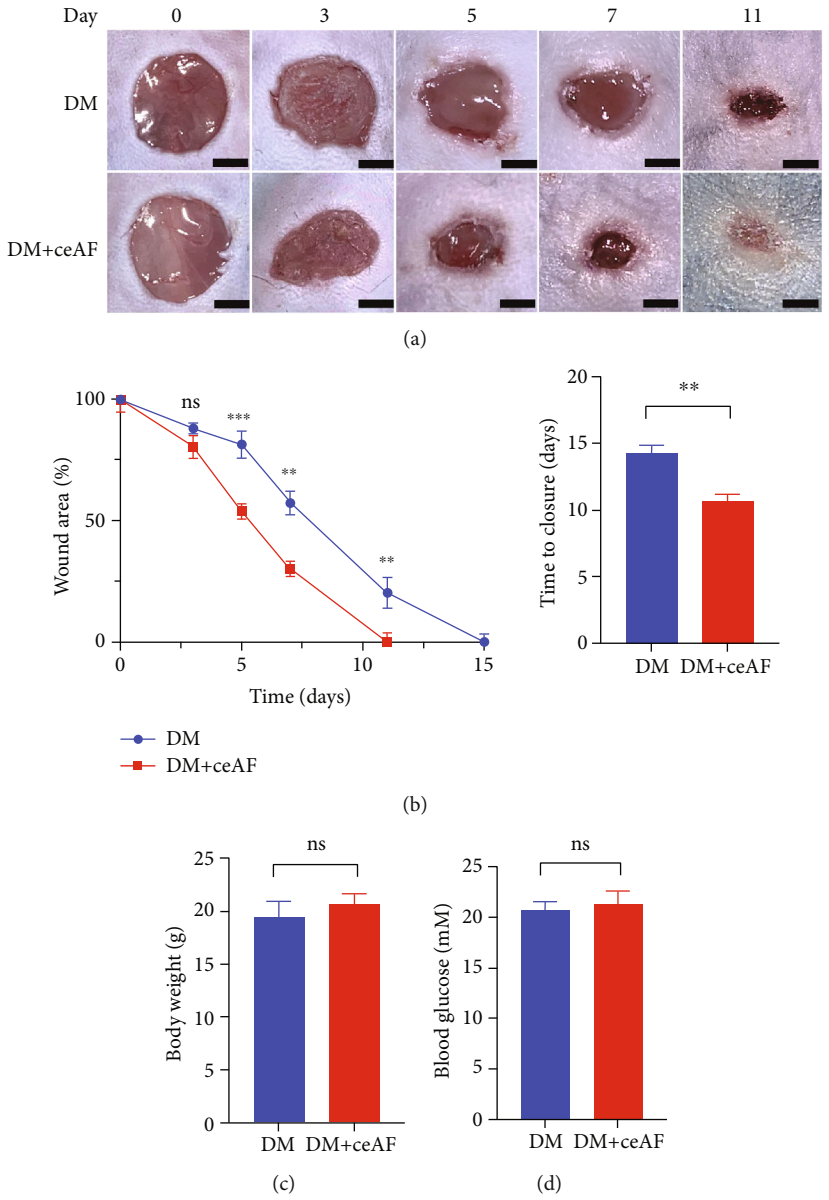



Figure 3: Treatment with ceAF ameliorates wound healing in STZ-induced diabetic mice. (a) Data for wound changes were recorded daily from day 1 to day 11. (b) Statistical graph representation of wound area and time to closure. (c) The measured body weight of the experimental mice on day 11. (d) The measured blood glucose of the experimental mice on day 11. Scale bar: 3 $\mu$m. $n = 6$, ns: no statistical significance, $^{**}P < 0.01$, $^{***}P < 0.001$.

amniotic fluid contains some urine and feces and may not be a good choice. It has been recently reported that amniotic fluids from different species have essential factors that can be used in a broad range of therapeutic areas such as corneal wound regeneration, diabetic wound care, and fetal wound healing [25, 26]. Nevertheless, the precise mechanisms remain unknown. Although we are still in the early stages of exploring the mechanism, the unique advantage of the current study is that we use eggs to separate amniotic fluid, which can be prepared on an industrial scale. The approach is economical, safe, and ethical, which further can be used for in-depth mechanism studies and preclinical trials.

Wound healing is mainly classified into four phases: hemostasis, inflammatory, proliferative, and remodeling [27]. All processes are intertwined, and persistent inflammation can have a range of negative effects on wound healing [28]. The evolution of a diabetic wound does not follow the normal healing time process and is affected by several factors such as hyperglycemia, chronic inflammation, microcirculation disorder, hypoxia, and autonomic neuropathy [29]. In diabetic wound, there are more inflammatory cell infiltration around the dermis and blood vessels, releasing a large number of reactive oxygen species and proteolytic enzymes, which continue to damage normal tissues [30]. In addition, the ratio of M1 (proinflammatory) to M2 (anti-inflammatory) macrophages has been in a heightened state [7]. The expression of inflammatory factors (e.g., TNF-$\alpha$, IL-6, and IL-1) also continued to be high in diabetic wounds [6].

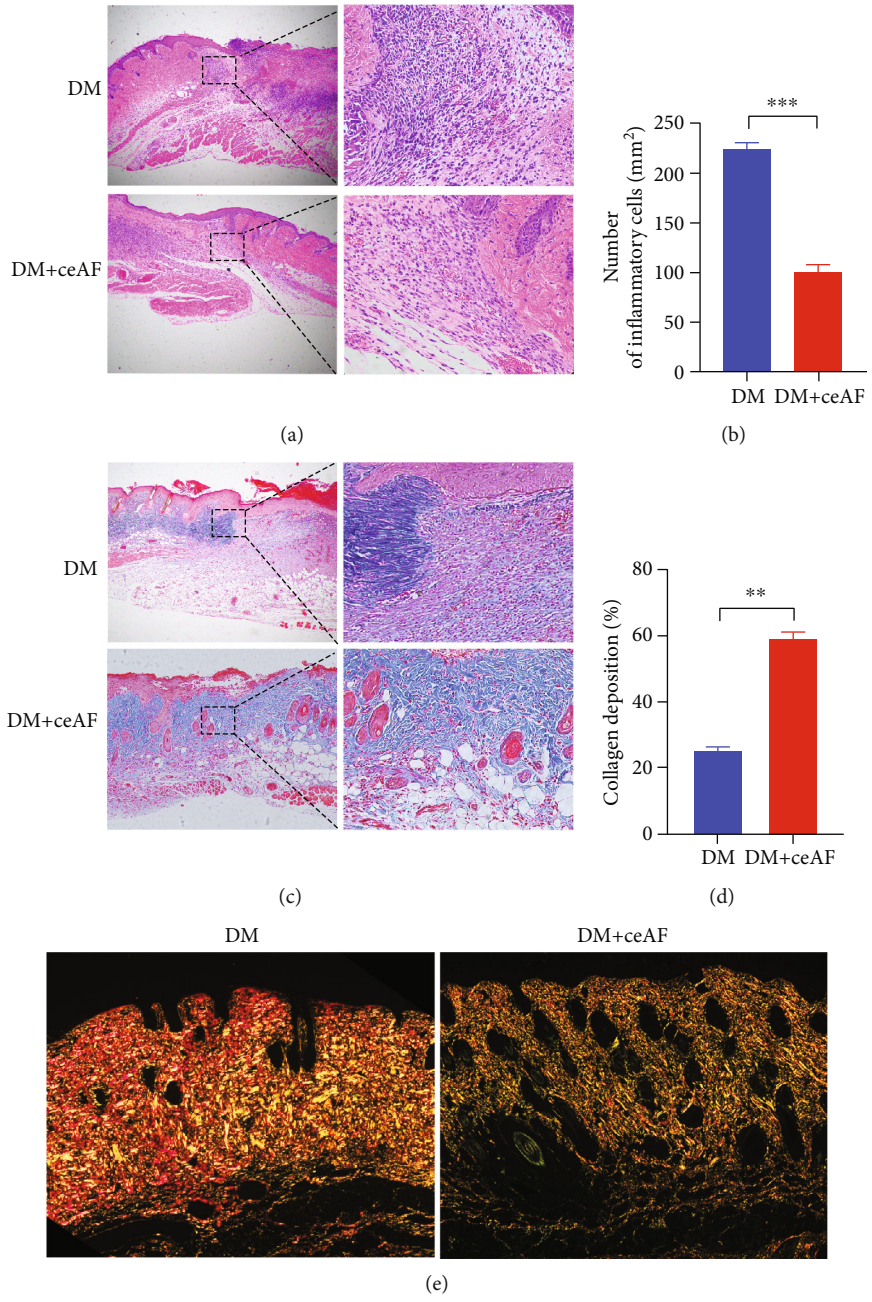



Figure 4: Effect of ceAF treatment on the histological changes in wound tissues. (a) H&E staining of skin wounds on day 5 indicated an enhanced inflammatory cell infiltration in diabetic mice treated with ceAF (Left 40×, Right 400×). (b) Histogram representation of inflammatory cell number. (c) MT staining of skin wounds on day 10 indicated an enhanced collagen deposition in the ceAF treatment group (Left 40×, Right 400×). (d) Histogram representation of collagen deposition. (e) Sirius red staining of DM group and ceAF+DM group after healing (40×). $n = 6$, $^{**}P < 0.01$, $^{***}P < 0.001$.

According to our results, ceAF treatment increased the ratio of M2 to M1 macrophages, thus suppressing inflammatory responses and promoting tissue repair.

It is believed that oxidative stress plays a critical role in diabetic wound healing. The imbalance of free radicals and antioxidants in patients leads to the overproduction of reactive oxygen species, which leads to cellular and tissue damage and delayed wound healing. ROS are key regulators of several stages of wound healing. In fact, low levels of ROS are necessary to combat external damage. However, excessive oxidative stress and decreased antioxidant capacity of tissues lead to redox imbalance, which is the main cause of diabetic wound nonhealing. Histological investigations have shown that nonhealing diabetic wounds are infiltrated by a highly oxidized environment, which is associated with hyperglycemia and tissue hypoxia, resulting in delayed wound repair [31]. Thus, reducing overproduction ROS levels and suppressing oxidative stress through the antioxidant system may reduce tissue damage and thus improve diabetic wound healing.

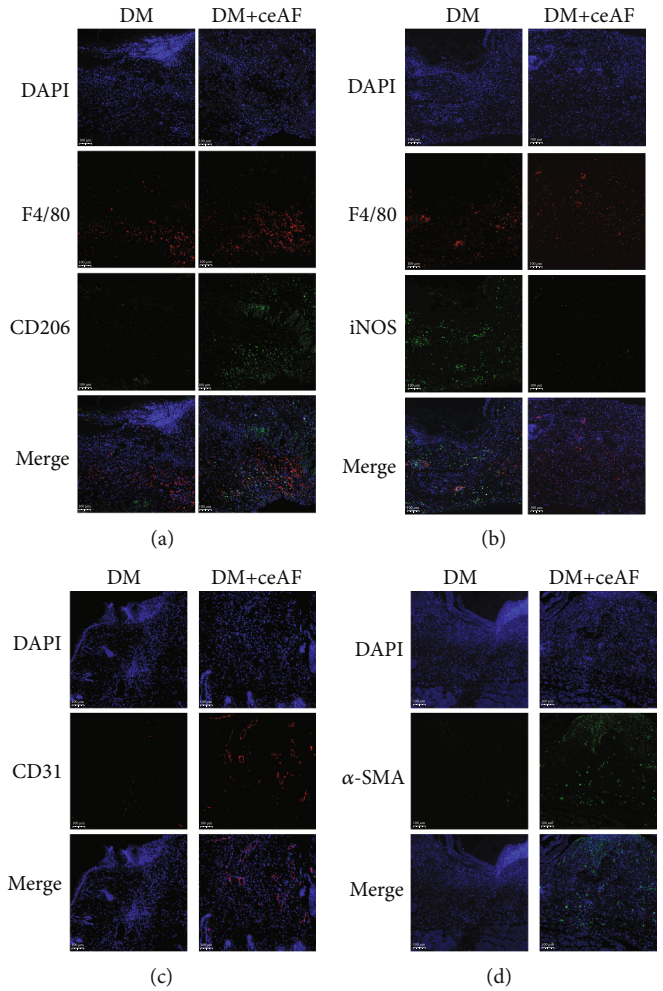



Figure 5: (a, b) Immunofluorescence assessment of $CD206^+$ (green), $iNOS^+$ (green), and $F4/80^+$ (red) cells in DM and DM+ceAF on day 5 postinjury. (c, d) Immunofluorescence analysis of $CD31^+$ (red) and $\alpha$-$SMA^+$ (green) cells in DM and DM+ceAF on day 10 postinjury. $n = 6$, Scale bar: 100 $\mu$m.

The polarization of macrophages is closely associated with wound healing. In normal wound healing, M1 macrophages dominate on days 1-3 and then transform into M2 macrophages [8]. The phenotype of macrophages in diabetic wounds is mostly M1 type, which will not decrease over time and rarely transforms into M2 macrophages [32]. There is an urgent need to suppress M1 polarization since the increased levels of M1 macrophages can exacerbate the progression of chronic ulcers [33]. There were several inflammation-associated factors present during diabetic wound healing, contributing to delayed wound healing during the inflammation phase. In chronic wounds, TNF-$\alpha$ and IL-6 increased, which led to elevated levels of metalloproteinases, degrading the extracellular matrix in the area and impairing cell migration [34, 35]. Flow cytometry and cell immunofluorescence showed that ceAF could induce RAW264.7 polarization to M2 type after 48 h. This preliminary evidence suggests that ceAF can inhibit inflammation by inducing polarization direction. *In vivo* experiments further confirmed this phenomenon, both the transcriptional and translational levels of M2-related genes in the wound tissue of ceAF-treated mice significantly elevated. Increasing M2 marker expression was accompanied by decreasing M1 marker and inflammatory cytokine expression, both of which occurred simultaneously. It proves partially that ceAF inhibits inflammation by improving the polarization of M2 macrophages.

In the canonical pathways, TLR4/NF-$\kappa$B and Nrf2 pathway activation is a fundamental step of inflammation launch [36]. TLR4 plays a pivotal role in the innate immune system and modulates it might offer therapeutic benefits for inflammatory diseases [37]. NF-$\kappa$B activation is responsible for the secretion of proinflammatory cytokines when TLR4 acts as a receptor for LPS [38]. As a result, NF-$\kappa$B has been reported as a potential target for treating inflammation. Furthermore, activation of the Nrf2 pathway could provide an endogenous defense system to resist cellular oxidative stress and mitigate oxidative damage, making it a dependable therapeutic method for suppressing wound inflammation. To explore the exact mechanisms underlying LPS-induced inflammation in RAW264.7 cells, we investigated whether ceAF affects TLR4 and NF-$\kappa$B activation. It was found that ceAF significantly decreased the LPS-stimulated upregulated

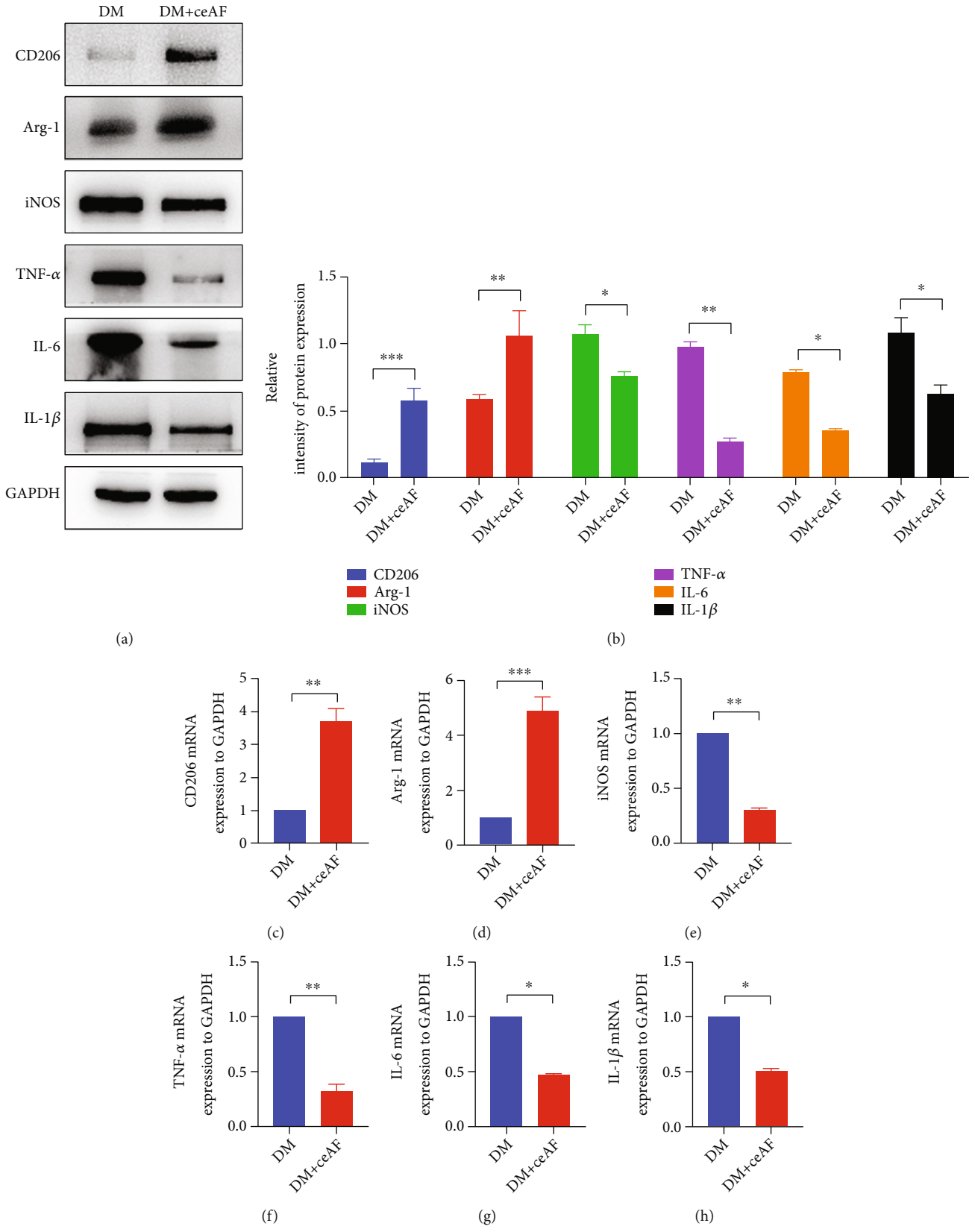



Figure 6: ceAF was able to suppress inflammation via promoting M2 macrophage polarization *in vivo*. (a) The expression of CD206, Arg-1, iNOS, IL-1$\beta$, IL-6, and TNF-$\alpha$ in the diabetic wound of mice was evaluated by WB. GAPDH served as a standard reference. (b) WB blots in (a) were quantified by densitometric analysis. (c–h) The expression of CD206, Arg-1, iNOS, IL-1$\beta$, IL-6, and TNF-$\alpha$ in the diabetic wound of mice was tested by RT-qPCR. $n = 6$, Mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression of TLR4/NF-$\kappa$B pathway proteins, and the expression of related inflammatory factors was also inhibited. Combined with flow cytometry results, we speculate that ceAF switching RAW264.7 to M2 phenotype may contribute to TLR4/NF-$\kappa$B and Nrf2 axes.

In addition, macrophages polarized to M2 in diabetic wounds are beneficial to collagen production and angiogenesis [39]. M2 macrophages are responsible for the production of many proangiogenesis factors during wound healing, such as VEGF and EGF [40]. It was shown that macrophages can also induce fibroblast activation, as the paracrine factors from M1/M2 macrophage polarization provoked distinct fibroblast phenotypes [41]. Activation of fibroblasts forces extracellular matrix (ECM) formation which is closely related to collagen deposition [42]. Although excessive collagen deposition can lead to scar formation, the priority for diabetic wounds is to heal as soon as possible rather than scar treatment. The results of our study showed that ceAF-treated mice had increased angiogenesis on wound tissue and the main component of new collagen is type III. It suggests that ceAF can both improve the speed and quality of healing. This may be related to the fact that ceAF promotes macrophages to secrete more TGF-$\beta$1 and IL-10. More studies are needed to confirm the mechanism due to we only conducted relevant cell experiments.

## 5. Conclusion

In summary, our data suggest that ceAF can improve the inflammation and oxidative stress of LPS-stimulated RAW264.7 cells *in vitro* and promote STZ-induced diabetic wound healing *in vivo*. This function is achieved by regulating TLR4/NF-$\kappa$B and Nrf2 axis. Our findings provide a promising therapeutic strategy for treating diabetic wounds through the anti-inflammatory activity of ceAF.

## Data Availability

The datasets used and analyzed are available from the corresponding author upon reasonable request.

## Conflicts of Interest

There is no conflict of interest between the author/editor and reviewer.

## Authors' Contributions

Shiyan Li and Xiaofeng Ding contributed equally to this work.

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*Research Article*

# Pharmacological Studies on the Antidiabetic, Antioxidant, and Antimicrobial Efficacies of *Commiphora myrrha* Resin in Streptozotocin-Induced Diabetes in Rats: A Preclinical Study

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*Background and Objectives*. *Commiphora myrrha* resin is the traditionally used herbal remedy in the Middle East against a variety of diseases, including diabetes, due to its multiple pharmacological activities. The present study investigates the antioxidant, antimicrobial, and antidiabetic efficacies of aqueous extract of *Commiphora myrrha* resin (MAE) in streptozotocin-induced (STZ) diabetes in the female Sprague Dawley rats. *Material and Methods*. The thirty (30) female adult Sprague Dawley rats were randomly and equally segregated into three sets of experimental groups: group I (normal control): the rats were given an intraperitoneal injection of sodium citrate buffer solution and marked as a normal control group (NCG); group II (diabetic control): the rats were injected with STZ (60 mg/kg body weight (b.w.)) and marked as the diabetic control group (DCG); and group III (MAE treated): the rats were injected with STZ (60 mg/kg b.w.) for induction of diabetes and treated with MAE powder (0.5 mL of 0.5 g/kg b.w.) dissolved in distilled water. The treatment was given for 30 days. All rats were sacrificed after 30 days of treatment. The blood samples from each rat were collected for biochemical analysis, and the pancreas was taken for histopathological examination. *Results*. The aqueous extracts of MAE were phytochemically analyzed, and the results revealed the presence of high concentrations of tannins, sterols, and isoprenoids (terpenoids), while steroids and flavonoids were found in moderate concentrations. The plant extract showed promising inhibition of the growth of gram-positive and gram-negative pathogens. It also showed that MAE has potential antihyperglycemic and antioxidant activities. Microscopic examination of the pancreas showed degenerative changes and atrophy associated with dilatation of the exocrine ducts in the STZ-induced diabetic rats, while the treatment revealed that the Langerhans islets were close to normal without any histopathological alteration. *Conclusion*. The present results suggested that an aqueous extract of MAE could be considered an efficient antidiabetic, antioxidant, and antimicrobial treatment in the future.

## 1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder, which is characterized by a deficiency of insulin secretion by the pancreas and or insulin resistance in peripheral tissues. Both of these conditions lead to the accumulation of glucose in the blood commonly referred to as hyperglycemia. Long-term hyperglycemia is an identifying parameter for diabetes, and this increased level for a long time creates a severe injury to multiple organ systems of the body. In long-term hyperglycemic conditions, the excessive glucose in the blood reacts with hemoglobin and forms glycosylated hemoglobin (HbA1C). Accordingly, HbA1C measurement is directly related to blood glucose concentration and is considered a very sensitive index for glycemic control [1]. Furthermore, DM can cause glycation of body proteins, leading to secondary complications that might primarily affect various vital organs, mainly the kidneys, eyes, nerves, and blood vessels. Uncontrolled diabetes may lead to blindness, heart attacks, renal failure, stroke, and sometimes amputation of limbs [2]. In addition, DM is shown to be accompanied by an increment in oxidative stress [3, 4]. Malondialdehyde (MDA) is known as one of the common oxidative stress primary biomarkers, and high levels of MDA were in different tissues, including serum and plasma of patients suffering from diabetes [5]. Hence, it is of utmost importance to control the hyperglycemia to prevent the secondary complications of diabetes. The existing antidiabetic medications have numerous undesirable side effects; therefore, there is an increasing demand for natural antidiabetic products by diabetic patients to avoid the side effects of available marketed drugs [6]. The prevalence of DM is continuously rising, especially in developing countries and undeveloped and low-income countries. The International Diabetes Federation (IDF) reported that the global prevalence of diabetes is continuously rising. It is a big global challenge for the well-being of peoples and to make a healthy society. This is reported in the IDF that currently, about 537 million adults are suffering from diabetes mellitus. These figures are further predicted to increase to 643 million by the year 2030 and again to 783 million by the year 2045 [7]. Lifestyle change is a critical feature of diabetes care. To control type 2 DM, the consumption of healthy food, increase physical work and exercise, control of body weight, psychosocial care, and stopping tobacco use are important steps [8]. Medical plants have been reported to possess antidiabetic efficacy by different mechanisms of action like increasing insulin secretion by stimulating $\beta$ cells, decreasing insulin resistance, preventing reabsorption of renal glucose, and rejuvenation of beta cells of pancreas in terms of size and numbers [9]. Several studies have been reported the antihyperglycemic efficacy of the medicinal plants. The hypoglycemic effects of these medicinal plants are helpful to rectify the metabolic abnormalities and also delay the progress of diabetes-associated complications [9]. As per the World Health Organization (WHO) guidelines, it is essential to prevent the diabetes and its associated complications to attain the better life. There have been strong emphases in the search of medicinal plants with antidiabetic potential. Natural products of plant origin might be promising lead candidates in the discovery of drug development with antidiabetic potential. Myrrh resin is one of the naturally derived substances obtained from the bark of the *Commiphora myrrha*. *Commiphora myrrha* is a tree of the Commiphora genus, which belongs to the Burseraceae family. Many species of the genus Commiphora are primary source of the production of oleo-gum resin known as myrrh. The word "myrrh" was derived from the Arabic word *murr*, meaning bitter [10]. Myrrh has unique medicinal properties: it can act as a carminative, anti-inflammatory, astringent, analgesic, antiseptic, diuretic, emmenagogue, and expectorant. Unani physicians traditionally used myrrh for the treatment of different illnesses, including inflammation, asthma, cough and cold, cancer, ulcers, indigestion, spasms, respiratory disorders, congestion of lungs, arthritic pain, wounds, leprosy, and syphilis. It is also used as a stimulant. It is traditionally used to increase menstrual flow and for the management of various gynecological problems including leucorrhea, menorrhagia, and amenorrhea. It is also found to be beneficial in the cervical stenosis and pelvic inflammatory diseases. This is also used as abortifacient and galactagogue [11]. Although myrrh has extensive uses in traditional medicine, few studies have examined its effects on the pancreas and its potential antidiabetic properties. Some medicinal plants may show antioxidant activities by reducing the reactive oxygen species (ROS) that may appear due to free radicals in the pancreas [12]. The phenolic compounds have a major role to play in protecting living tissues from the severe effects of ROS that are considered to be an important risk factor for causing acute cell damage [13]. Various studies have mentioned that reinforcing the antioxidant system could decrease the harmful effects of diabetes [14, 15]. Streptozotocin (STZ) is a broad-spectrum antibiotic. This is toxic to beta cells of pancreas, which causes destruction of these $\beta$-cells of pancreas in the mammals. STZ is widely used in the medical research to induce experimental diabetes in animals. The induction mechanism includes the generation of oxygen free radicals by STZ, which consequently damages the pancreas and destruction of $\beta$-cells. The pathological conditions of STZ-induced diabetes are identical to the type 2 DM in humans [16]. Therefore, the current study is aimed at examining the protective efficacy of aqueous extract of *C. myrrha* resin (MAE) on STZ-induced diabetes in the pancreas of female Sprague Dawley rats, in addition to its antioxidant and antimicrobial activities.

## 2. Material and Methods

*2.1. Plant Resin Collection and Identification.* Oleo-gum resins of *C. myrrha* (used as an aromatic plant and in traditional medicine in Saudi Arabia) were collected (August 2019) from a wild tree growing in Wadi Noeman at Makkah, Saudi Arabia ($21^{\circ}21'55.98''$N and $40^{\circ}11'27.03''$E). Prof. M. Fadl (professor of plant taxonomy at Taif University, Saudi Arabia) identified the tree. The collected samples were deposited in the herbarium of the Biology Department at Taif University, and the ID number for the voucher

specimen is Wadi Noeman, 2019, 10512 (TUH)-Roushdy M.M. The criteria for choosing the best and ideal form of oleo-gum resin of *C. myrrha* included important characters such as its transparency, color, odor, and time of storage. The gum should not be stored for more than three months and should be transparent with a golden to brownish yellow color.

*2.2. C. myrrha Resin Aqueous Extraction (MAE).* The dried powdered resin (100 g) of *C. myrrha* was washed with distilled water and left to dry at 60°C overnight. It was then subjected to extraction with 500 mL tap water at room temperature for 48 hours. The extraction step was followed by a filtration process using a Whatman No. 1 paper. The filtrates were concentrated using a rotary evaporator under reduced pressure and controlled temperature, followed by room temperature drying. A stock solution was prepared by dissolving the dried extract powder (400 mg) in distilled water (1000 mL) and stored in the refrigerator at 2-4°C for further investigations.

*2.3. Preliminary Phytochemical Study.* To identify the chemical constituents of the plant extract, the MAE powder was dissolved in distilled water and subjected to preliminary phytochemical screening. The aqueous extracts of *C. myrrha* were subjected to preliminary phytochemical investigations to determine the different phytoconstituents like terpenoids, sterols, and tannins, while steroids and flavonoids using standard official procedures [17, 18].

*2.4. Experimental Animals.* A total of 30 adult female albino Sprague Dawley rats of similar age and weight (120-140 g), obtained from the Egyptian Organization for Biological Products and Vaccines (Helwan, Cairo, Egypt), were used in the present study. For a 48-hour adaptation period, all rats were housed in individual stainless-steel cages (three rats/cage) and maintained at appropriate temperature ($23 \pm 2 \circ C$) and humidity ($55\% \pm 10$) with a standard 12 h light/dark cycle and ad libitum access to water and standard food. Body weights of all experimental rats were recorded weekly throughout the feeding period, and the body weight gain was calculated at the end of the experiment. The experimental animals were handled in compliance with the principles of good laboratory practices and ethical guidelines on animal use in research, and the study protocol was approved by the research ethics committee (REC/NHTMRI/A5-2021).

*2.5. Induction of Diabetes.* DM was induced in the rats that had fasted overnight by one intraperitoneal injection (60 mg/kg body weight) of STZ (Sigma Chemicals Co., St. Louis, USA) that was prepared in a fresh and cold sodium citrate buffer (0.1 M citric acid and 0.1 M trisodium citrate dihydrate) at pH 4.5 [19]. Blood samples were collected by tail snip method, and the sugar level of each animal was measured before the treatment (day 0) and 72 h post-STZ treatment. Rats were considered to be hyperglycemic based on blood glucose levels > 200 mg/dL [20].

*2.6. Study Design.* The thirty rats were randomly allocated into three main groups ($n = 10$ per group) as required by the present study. Animals were randomly allocated into three groups as follows:

(1) Group I (normal control group): the rats were given intraperitoneal injection of sodium citrate buffer solution and marked as NCG. The rats were fed on a standard diet and left without treatment under the same laboratory condition

(2) Group II (diabetic control group): the rats were given with a single intraperitoneal injection of 60 mg/kg, of body weight of STZ, and marked as DCG. They were also fed a standard diet under the same laboratory condition

(3) Group III (MAE-treated group): the rats were first injected with STZ (60 mg/kg body weight) for the induction of diabetes and marked as aqueous extract of *Commiphora myrrha* resin-treated group (MAETG). The diabetic rats were treated with MAE powder at 0.5 mL of 0.5 g/kg body weight dissolved in distilled water. The treatment was given for 30 days. The treatment with MAE was performed orally by gastric intubation between 9:00 am and 11:00 a.m. for 30 days. The body weight was measured at the beginning and the end of the experiment

*2.7. Sample Collection.* At the end of the experimental period (30 days), the rats were fasted overnight and anesthetized with urethane (99%, Aldrich) at a dose of 1 g/kg body weight intraperitoneally. Blood samples were taken from the retro-orbital venous plexus. The samples were allowed to coagulate at room temperature and centrifuged at 4000 revolutions per minute (RPM) for 15 minutes until the serum was separated and stored at −20°C for further biochemical investigations.

*2.8. The Antimicrobial Activities of MAE*

*2.8.1. Microorganisms.* The antimicrobial activities of MAE were evaluated against various pathogenic bacterial strains including both gram-positive and gram-negative bacteria. The strains used for the antimicrobial assays were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Gram-negative strains were *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 27853), *Pseudomonas fluorescens* (ATCC 13525), and *Klebsiella pneumoniae* (ATCC 10031), while the gram-positive bacteria comprised *Bacillus subtilis* (ATCC 11774), *Streptococcus pyogenes* (ATCC 12344), and *Staphylococcus epidermidis* (ATCC 12228). Bacterial cells were cultivated on Mueller-Hinton agar medium at pH 7.4. The agar plates were incubated at 37°C for 24 h.

*2.9. Antibacterial Assay Using Agar Disc Diffusion Method.* The antibacterial activity of MAE was carried out using the agar disc diffusion method [21]. Each bacterial strain was first cultivated in nutrient broth at 37°C for 24 h. Each

bacterial suspension was diluted with nutrient broth to obtain inocula of $\sim 1 \times 10^6$ CFU/mL [22]. One milliliter of the standardized inoculum of each test bacterium was spread with the help of a sterile spreader onto a sterile nutrient agar plate. The plates were allowed to dry. A Whatman No. 1 sterile filter paper disc (6 mm diameter) was impregnated with 100 $\mu$L of 10 mg/mL of the aqueous solution of myrrh resin. The preparation of negative controls was carried out using sterilized distilled water. Subsequently, the plates were refrigerated for at least 1 h for diffusion to take place and then incubated at 37°C for 24 h. Evaluation of antibacterial activity was determined by measuring the resulting inhibition zones' diameter against the tested bacteria. Three replicates of the experiment were carried out, and the zone of inhibition was measured in millimeters [23]. One hundred microliters of ciprofloxacin was loaded onto filter papers and used as a positive control.

*2.10. Biochemical Analysis.* Biochemical tests, including fasting plasma glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL-C) cholesterol, HbA1C, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and total and direct bilirubin (T and DB) were measured in a Roche Cobas 6000/c501 chemistry automated analyzer device (Roche Diagnostics, Mannheim, Germany) using the Roche laboratory kit reagent according to the reference range of the Cobas c501 biochemistry analyzer. Fasting insulin was measured in the Roche Cobas 6000/e601 hormone automated analyzer device (Roche Diagnostics, Mannheim, Germany) using the Roche laboratory kit reagent according to the reference range of the Cobas e601 hormone analyzer. The homeostasis model assessment of insulin resistance (HOMA IR) was calculated as the product of the fasting serum glucose (mg/dL) and fasting insulin levels (mU/L) divided by a constant (405) according to the formula of Pickavance et al. [24]. Serum levels of total antioxidant capacity (TAC), as well as MDA, were assessed by the enzyme-linked immunosorbent assay (ELISA) method (Spectrum, Egypt) according to the method of Miller et al. [25] and Jiang et al. [26], respectively. Low-density lipoprotein (LDL-C) cholesterol levels were calculated according to the formula of Mousavi et al. [27]:

$$\text{LDL-C} = \text{Cholesterol} - \left(\text{HDL-C} + \frac{\text{TG}}{5}\right). \quad (1)$$

*2.11. Pancreatic Harvesting and Tissue Homogenate Preparation.* At the end of 30 days, all the rats were anesthetized with urethane (99%, Aldrich) at a dose of 1 g/kg body weight intraperitoneally, and the pancreas of each rat was quickly removed and washed in ice-cold saline immediately. The harvested pancreas was divided into two halves: one-half was saved for the histopathological examination, and the other half was used to prepare the pancreatic homogenate for biochemical analysis. The pancreatic homogenate was prepared in 10 mL of ice-cold phosphate-buffered saline (PBS) using a mechanical homogenizer. Samples were then centrifuged at $1000 \times g$ for 10 min at 4°C to remove large insoluble particles. Finally, the supernatant was separated and stored at -80°C in aliquots for further biochemical analysis and measurement of MDA levels [26].

*2.12. The Histopathological Analysis of Pancreatic Tissue.* The harvested pancreas from the tested animals was fixed in 10% formalin and embedded in paraffin. Paraffin-embedded tissue blocks were prepared, and 5 $\mu$m thick sections were taken using a microtome for further staining. Briefly, the sections for histopathological examination were placed on glass slides, deparaffinized, rehydrated, and stained with routine hematoxylin and eosin (H&E) stain. The stained slides were covered with coverslips after mounting and examined under a light microscope [28].

*2.13. Statistical Analysis.* All data were expressed as mean ± SD (standard deviation). Analysis of variance (ANOVA) was done, followed by the post hoc least significant difference test (LSD) to test the research hypothesis. Data analyses were performed using the statistical package for social sciences (SPSS version 26) (IBM Corp., Armonk, N.Y., USA). The differences between the groups were considered statistically significant if $p$ value was $<0.05$.

## 3. Results

*3.1. The Phytochemical Screening of MAE.* The results of the phytochemical screening of MAE revealed the presence of high concentrations of terpenoids, sterols, and tannins, while steroids and flavonoids were found in moderate concentration (Table 1).

*3.2. Effect of MAE on Bacterial Activity.* MAE showed promising inhibition of the growth of the tested pathogens, as shown in Table 2. The maximum inhibition zones were found against *E. coli* (ATCC 10536) followed by *S. epidermidis* (ATCC 12228), *B. subtilis* (ATCC 11774) (27 mm), *S. pyogenes* (ATCC 12344), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 27853), *P. fluorescens* (ATCC 13525), and *S. typhimurium* (ATCC 13311), respectively. The extract showed high activity against all the tested strains, when compared to the referenced antibiotic (ciprofloxacin).

*3.3. Effect of MAE on Body Weight, Food Intake, and Water Intake Changes.* The effect of MAE on body weight, food intake, and water intake in the diabetic control group (DCG) and MAETG is observed in Table 3. The results showed that there were no significant differences between groups in body weight, food intake, and water intake at the beginning of the experiment.

However, by the end of the study, the STZ-induced DG exhibited a greater loss of body weight in comparison to NCG. In contrast, the body weight of MAETG was shown to be significantly increased compared with DG and significantly decreased compared to NCG ($p < 0.001$). The opposite changes were seen with food and water intake. Therefore, MAE could induce weight loss but increase food and water intake ($p < 0.003$).

Table 1: Phytochemical screening of *C. myrrha* extract.

| S. no. | Constituents | Level* |
|---|---|---|
| 1 | Terpenoids | +++ |
| 2 | Sterols | +++ |
| 3 | Steroids | ++ |
| 4 | Tannins | +++ |
| 5 | Flavonoids | ++ |

*: ++: moderate concentration; +++: high concentration.

Table 2: *In vitro* antibacterial activity of *C. myrrha* oleo-gum extract against tested bacteria.

| Test species | Zone of inhibition (mm)* | |
|---|---|---|
| | Resin extract | Ciprofloxacin |
| *S. typhimurium* (ATCC 13311) | $18.33 \pm 0.056^{f}$ | $15.67 \pm 0.029^{f}$ |
| *E. coli* (ATCC 10536) | $28.33 \pm 0.044^{b}$ | $21.00 \pm 0.076^{b}$ |
| *P. aeruginosa* (ATCC 27853) | $22.33 \pm 0.012^{e}$ | $17.00 \pm 0.088^{e}$ |
| *P. fluorescens* (ATCC 13525) | $21.00 \pm 0.015^{e}$ | $15.00 \pm 0.009^{f}$ |
| *K. pneumoniae* (ATCC 10031) | $25.33 \pm 0.035^{d}$ | $18.33 \pm 0.053^{d}$ |
| *B. subtilis* (ATCC 11774) | $28.33 \pm 0.048^{b}$ | $20.67 \pm 0.039^{c}$ |
| *S. pyogenes* (ATCC 12344) | $26.67 \pm 0.103^{c}$ | $21.33 \pm 0.011^{b}$ |
| *S. epidermidis* (ATCC 12228) | $28.67 \pm 0.099^{a}$ | $23.67 \pm 0.047^{a}$ |

*Concentration of extracts 10 mg/mL (100 $\mu$g/disc). Inhibition zones were the mean of three replicates. The mean results were expressed as mean ± SD. Different superscript letters (a, b, and c) denote significance, while similar letters denote no significance between groups. The mean difference is significant at $p < 0.05$.

*3.4. Effect of MAE on Liver Function.* Serum ALP ($p < 0.0012$), ALT ($p < 0.001$), AST ($p < 0.0316$), GGT ($p < 0.001$), TB ($p < 0.001$), and DB ($p < 0.001$) levels in STZ-induced diabetic rats (DG) were significantly elevated when compared to the NCG and MAETG (Figure 1).

*3.5. Effect of MAE on Serum Lipid Profile.* Although there were no significant changes in HDL-C values between the groups ($p < 0.864$), DG exhibited significant elevation in serum levels of TC ($p < 0.001$), TG ($p < 0.001$), and LDL-C ($p < 0.001$) when compared to NCG and MAETG. In contrast, MAETG showed an obvious reduction in the serum levels of these parameters when compared to DG, even though they were still higher than the normal group (Figure 2).

*3.6. Effect of MAE on Fasting Blood Glucose and Insulin Levels, HOMA-IR, and HbA1C.* As shown in Table 4, STZ injection was shown to significantly increase the levels of the fasting serum glucose ($p < 0.001$), HbA1C ($p < 0.001$), and HOMA-IR ($p < 0.001$) in DG when compared to NCG and MAETG, and it caused a significant decrease in the fasting serum insulin ($p < 0.005$) within DG.

*3.7. Effect of MAE on Antioxidant Activity.* Correspondingly, the intraperitoneal injection of STZ in albino rats showed an imbalance in the oxidative status which was confirmed by a significant reduction in serum TAC ($p < 0.008$) and a significant elevation in serum MDA ($p < 0.005$) compared to NCG and MAETG (Figure 3). The results showed that MAETG improved the levels of serum TAC and MDA when compared to the DG rats.

Similarly, MDA in the pancreatic tissue of diabetic rats (DG) was significantly increased ($p < 0.001$) in comparison with NCG and MAETG.

*3.8. Effect of MAE on the Histological Structure of the Pancreas.* The light microscopic observation of pancreatic islet cells from normal rats (NCG) showed no histopathological alteration, with a normal histological structure of the islet of Langerhans cells as endocrine portion as well as the acini and duct system of the exocrine portion (Figure 4(a)). However, the microscopic examination of DG showed degenerative changes and atrophy associated with dilatation of the exocrine ducts. This was a result of STZ action as a diabetic induction agent (Figure 4(b)). MAETG, on the other hand, showed marked improvement in the histological appearance of Langerhans islets with normal pattern (Figure 4(c)).

## 4. Discussion

Although there are different types of drugs available to lower blood glucose levels in humans, these medications are known to cause numerous adverse effects. Therefore, researchers are focusing on assessing other treatment options, including the search for active natural products.

Natural products have exhibited a range of biological properties, including anticancer, antioxidant, antimicrobial, and anti-inflammatory. *C. myrrha* has been investigated and reported to exhibit a wide range of therapeutic efficacies since the discovery of this medicinal plant [10]. Therefore, the present study was conducted to assess the antimicrobial and hypoglycemic potential activities of *C. myrrha* resin aqueous extract.

In DM patients, uncontrolled hyperglycemia conditions are among the most serious factors that may disrupt the immune system [29]. Another factor that may lead to type 2 DM is malnutrition (especially deficiency in vitamin D). Vitamin D deficiency has been reported to be associated with insulin resistance, type 2 diabetes, cancer, obesity, and cardiovascular diseases [30–32].

Our results revealed that MAE has strong potency as an antimicrobial agent compared to the antibiotic, ciprofloxacin. The obtained result is in agreement with Alqahtani et al. [10], who stated that *C. myrrha* extract showed strong antimicrobial activity against gram-positive bacteria like *Enterococcus faecalis and S. aureus*. The research study ascribed the reason for this antimicrobial action of the plant extract due to its high concentrations of active compounds like 2-acetoxy-furano-diene, furano-eudesma-1,3-diene, and 2-methoxyfuranodiene including some other phytochemical constituents. The present results are in agreement with several studies that have investigated the potential effects of exercise and a low-calorie vegetarian diet on oxidative stress and insulin resistance in type 2 diabetic patients [33]. On the other hand, another study by Jeevandran et al.

Table 3: Effects of treatment with MAE on body weight, food intake, and water intake in STZ-induced diabetic rats.

| Groups* | Body weight (g) | | | Food intake (g/day) | | | Water intake (mL/day) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 15 | Day 30 | Day 1 | Day 15 | Day 30 | Day 1 | Day 15 | Day 30 |
| NCG | 116.3 ± 8.52 | 136.5 ± 10.64 | 160.6 ± 8.41[a] | 17 ± 0.34 | 19.8 ± 0.93 | 24.1 ± 0.97[b] | 46.1 ± 2.13 | 51.6 ± 3.55 | 56.3 ± 1.43[b] |
| DCG | 120.2 ± 7.27 | 107.1 ± 10.59 | 95.4 ± 9.25[b] | 18.4 ± 0.91 | 22.6 ± 2.26 | 29.2 ± 2.98[a] | 45.5 ± 2.99 | 54 ± 3.94 | 62.7 ± 4.16[a] |
| MAE | 118.3 ± 8.06 | 123.5 ± 11.06 | 134.3 ± 8.94[a] | 18.1 ± 0.49 | 20.6 ± 1.56 | 25.5 ± 1.50[b] | 45.7 ± 2.26 | 52.3 ± 4.74 | 57.9 ± 2.89[b] |

*Each group contained 10 rats. The mean results were expressed as mean ± SD. Different superscript letters (a, b, and c) denote significance, while similar letters denote no significance between groups. The mean difference is significant at $p < 0.05$.

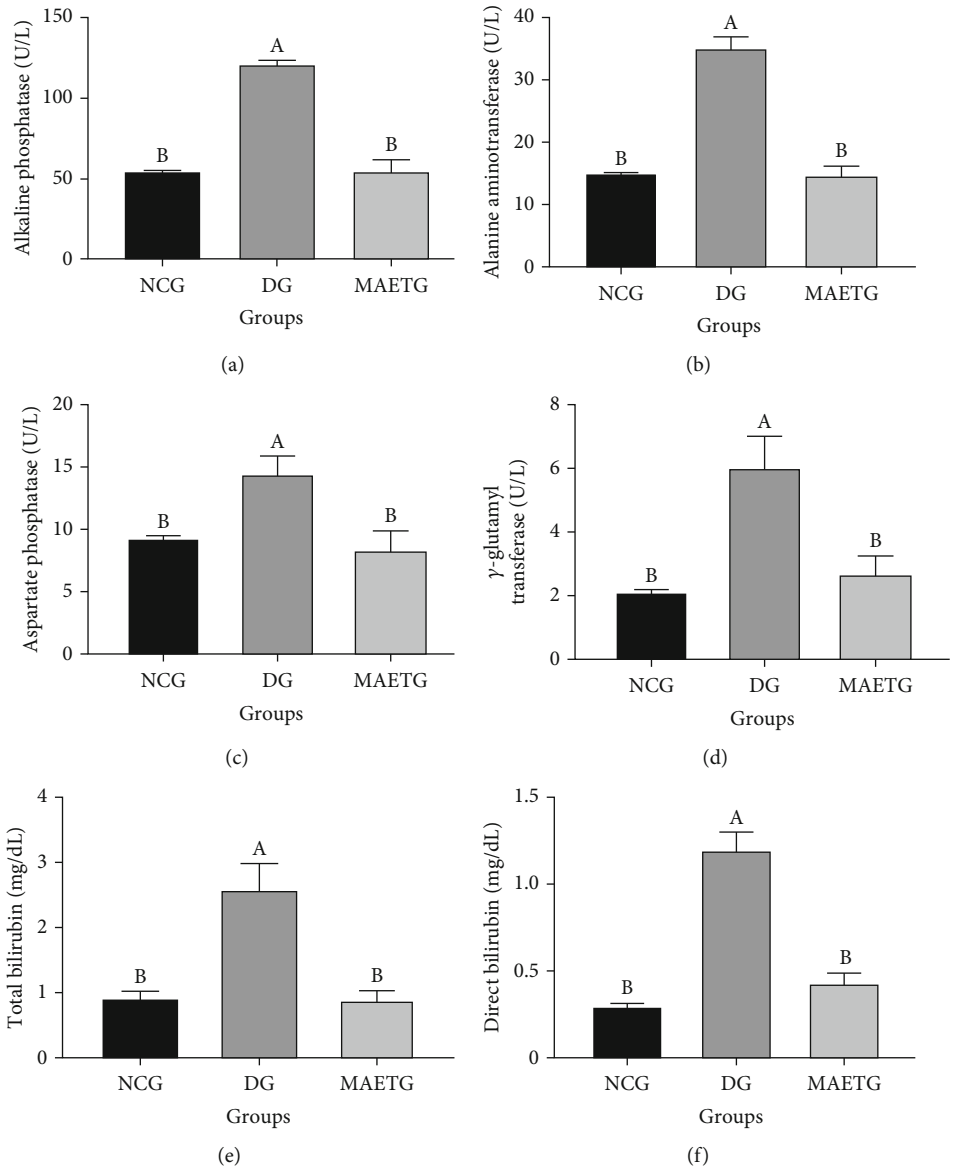



Figure 1: The effects of MAE of *C. myrrha* on liver function parameters ((a) alkaline phosphatase, (b) alanine aminotransferase, (c) aspartate phosphatase, (d) gamma-glutamyl transferase, (e) total bilirubin, and (f) direct bilirubin) of STZ-induced diabetic rats. Data were presented as mean ± SD. Data were analyzed using ANOVA followed by LSD. The mean difference is significant at $p < 0.05$. Each group contained 10 rats. Different superscript letters (A and B) denote significance, while similar letters denote no significance between groups.

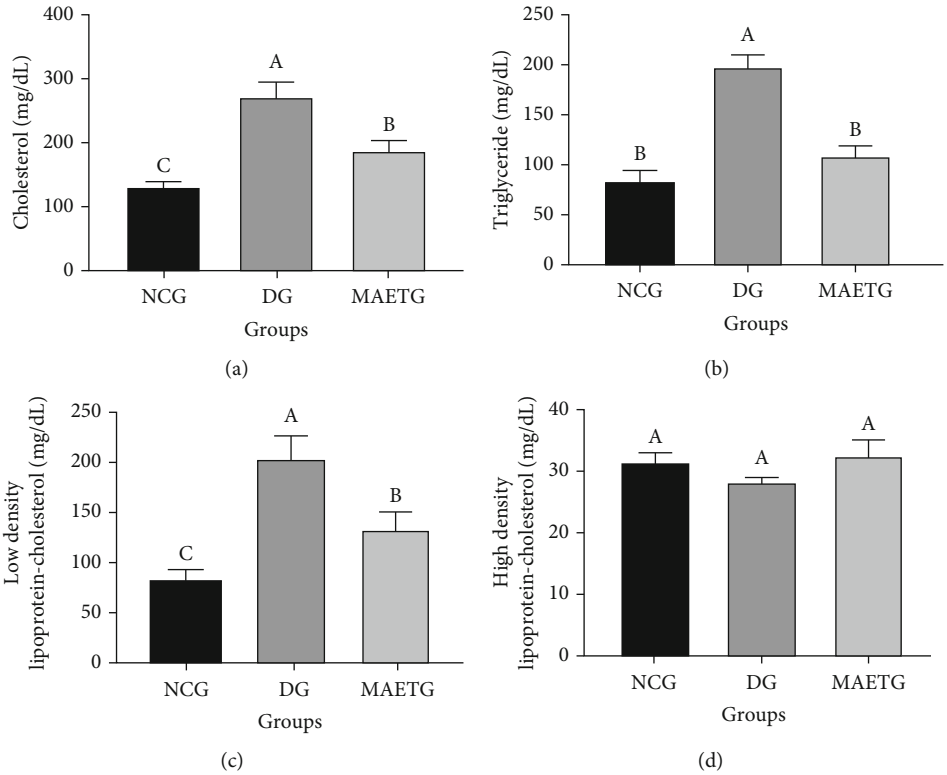



Figure 2: Effect of MAE on (a) total cholesterol, (b) triglyceride, (c) low-density lipoprotein cholesterol, and (d) high-density lipoprotein cholesterol in STZ-induced diabetic rats after 30 days of treatment. Data were presented as mean ± SD. Data were analyzed using ANOVA followed by LSD. The mean difference is significant at $p < 0.05$. Different superscript letters (A, B, and C) denote significance while similar letters denote no significance between groups. Each group contained 10 rats.

Table 4: Effect of MAE of *C. myrrha* on serum glucose, fasting insulin, HbA1C, and HOMA-IR index in STZ-induced diabetic rats after 30 days of treatment.

| Groups* | Fasting glucose (mg/dL) | HbA1C (%) | Fasting insulin (mU/L) | HOMA-IR |
|---|---|---|---|---|
| NCG | $92.3 \pm 7.7^{b}$ | $4.998 \pm 0.311^{b}$ | $1.917 \pm 0.13^{a}$ | $0.44 \pm 0.04^{b}$ |
| DG | $437.9 \pm 45.36^{a}$ | $8.754 \pm 0.59^{a}$ | $0.885 \pm 0.13^{c}$ | $0.96 \pm 0.29^{a}$ |
| MAETG | $84.7 \pm 10.55^{b}$ | $4.751 \pm 0.59^{c}$ | $1.579 \pm 0.12^{b}$ | $0.33 \pm 0.04^{b}$ |

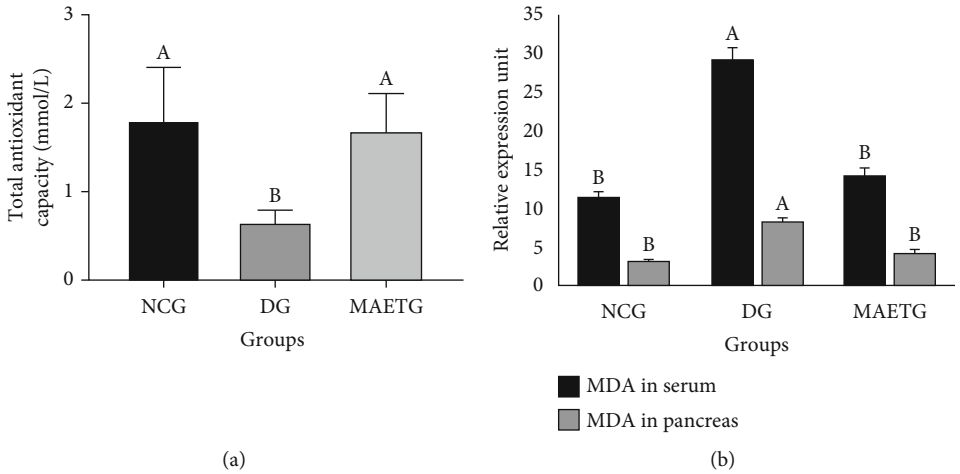
*Each group contained 10 rats. The mean results were expressed as mean ± SD. Different superscript letters (a, b, and c) denote significance while similar letters denote no significance between groups. The mean difference is significant at $p < 0.05$. HbA1C: glycated hemoglobin; HOMA-IR: homeostasis model assessment of insulin resistance.

[34] investigated the effect of ethanolic extracts of seeds of *Archidendron pauciflorum* (ESAP), where the ESAP did not exhibit an antihyperglycemic effect in diabetic rodents. As a result, it concluded that not all plants or even plant parts have effects on diabetes.

To induce DM, STZ was intraperitoneally injected in the experimental animals [35]. From the present observations, STZ-induced hyperglycemia is characterized by a high level of low serum insulin level, high glucose level, and high HbA1C level with an elevation of calculated HOMA-IR. These results were in accordance with different studies. Iftikhar et al. [36] showed that the administration of MAE (0.5 g/kg b.w.) for 30 days considerably reduced the hyperglycemic action of STZ.

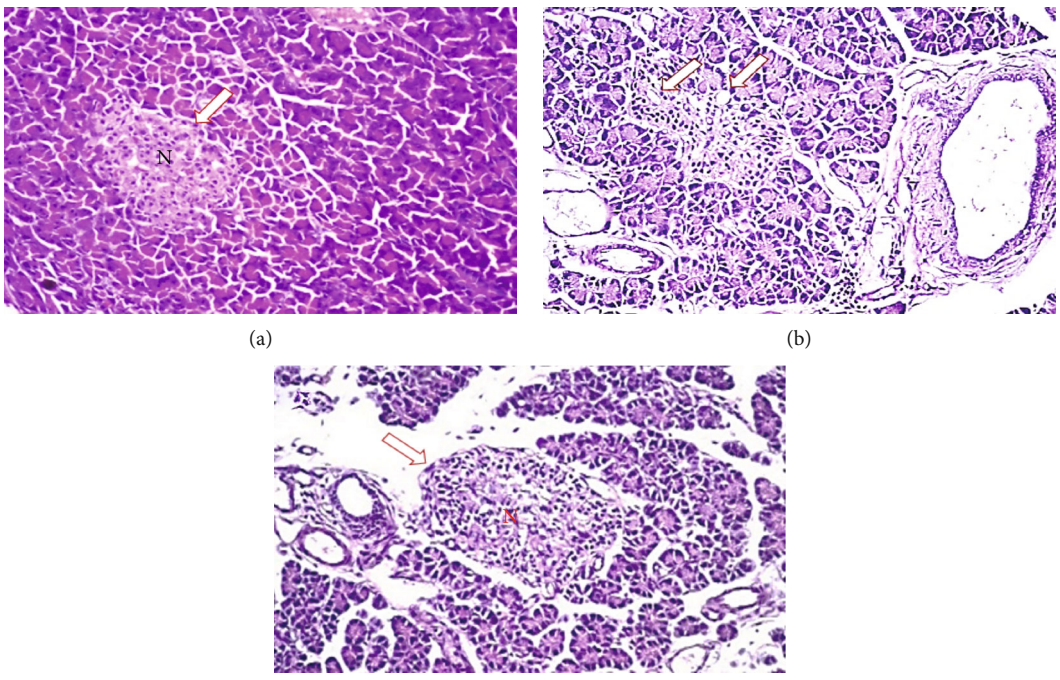
Furthermore, Al-Romaiyan et al. [37] stated that the uses of aqueous *C. myrrha* extract (2 mg/mL) rapidly and reversibly increased the secretion of insulin at both stimulatory and substimulatory glucose levels in islets of the pancreas of humans and mice.

In addition, DM is a metabolic disorder with hyperglycemia. The uncontrolled elevated level of blood glucose causes serious complications in many vital organs like the kidney, pancreas, liver, and heart [4]. Lipid abnormalities have been found in diabetic patients. Diabetes is associated with dyslipidemia like elevated serum triglycerides, increased cholesterol, elevated LDL, and reduced HDL. These lipid abnormalities have been observed in nearly 40% of patients suffering from diabetes [38, 39]. In the current study, the results of the lipid profiles are at par with the previously reported studies. The administration of STZ significantly increased the serum concentration of cholesterol, TG, and LDL, while it decreased the HDL-C levels. Another study




(a) (b)

Figure 3: Effect of MAE on (a) TAC (total antioxidant capacity) and (b) MDA (malondialdehyde) in STZ-induced diabetic rats after 30 days of treatment. Data were presented as mean ± SD. Data were analyzed using ANOVA followed by LSD. The mean difference is significant at $p < 0.05$. Different superscript letters (A and B) denote significance while similar letters denote no significance between groups. Each group contained 10 rats.




(a) (b)

(c)

Figure 4: Effect of MAE of *C. myrrha* on pancreatic Langerhans islet cells after 30 days in albino rats: (a) histological appearance of Langerhans islets of normal control rats (NCG) showing no histopathological changes, (b) histological appearance of Langerhans islets of STZ-induced diabetic rats (DG) showing degenerative changes and atrophy associated with dilatation of the exocrine ducts (see arrow), and (c) histological appearance of Langerhans islets of the treated group (MAETG) showing the normal histopathological pattern.

reported similar results which investigated the association between diabetes and lipid profile in the blood. This study illustrated an increase in the levels of serum triglyceride, cholesterol, and LDL-C that indicated the incidence of hyperlipidemia in the rats. The underlying mechanism of hyperlipidemia could be due to the hyperactivity of hormone-sensitive lipase, which causes the flow of fatty acids from triglycerides deposited in the adipocytes [40]. In contrast, the results from our study demonstrated that the administration of MAE to diabetic rats significantly improved the lipid profile. Therefore, one can conclude that MAE proved to have antidiabetic and antihyperlipidemic activities against STZ-induced DM. Our results are at par with Ojiako et al. [41] and Ota and Ulrih [42], who stated that the *Commiphora myrrha* contains numerous active phytoconstituents like alkaloids, flavonoids, glycosides, and

terpenoids, which exhibited substantial antioxidant antidiabetic properties. The flavonoid component of the plant extract could be responsible for the antidiabetic activities. The flavonoids might be responsible for the regeneration and survival of pancreatic beta cells since flavonoids are potential alpha-amylase inhibitors [7]. The sodium glucose cotransporter-1(SGLT1) is an intestinal glucose transporter that facilitates the transport of glucose into the bloodstream. The tannins and polyphenolic components of the MAE might act by inhibiting the SGLT1, hence inhibiting glucose uptake from the rat's intestine [43].

Therefore, the antidiabetic efficacy of the MAE might be explained due to the presence of a high amount of polyphenolic compounds and flavonoids including alkaloids in the aqueous extract.

Plants with clinical applications play a major role in the control of plasma glucose through various mechanisms, one of which is the increase in the number of beta cells in the pancreas and the activation of their regeneration ability [10]. Induction of diabetes by STZ could severely damage the pancreatic beta cells and subsequently decrease serum insulin levels [44]. This fact was also confirmed by several studies that revealed that STZ directly causes substantial destruction of the pancreatic beta cells [45, 46]. So, treatment with MAE could increase the serum insulin level, and this could be explained by the presence of flavonoids, tannins, and steroids in the investigated extract. Therefore, the plant extract may have a high antioxidant ability that may support the protection of beta cells from harmful oxidative stress and other damaging factors [47]. Coskun et al. [48] reported that flavonoids could significantly decrease blood sugar levels and also exhibited protective effects on the beta cells from oxidative stress and preserve the integrity of beta cells of the pancreas.

MDA is a byproduct of lipid peroxidation and is commonly known as a marker of oxidative stress. The present study revealed a significant increase in both serum and pancreatic MDA and a significant decrease in total antioxidant capacity (TAC) in the STZ-induced diabetic rats (DG).

The present study also revealed that there was a significantly increased level of MDA both in serum and pancreatic tissues. There was a significant reduction in the total antioxidant capacity (TAC) in the STZ-induced diabetic rats (DG). Interestingly, the administration of MAE to the diabetic animals significantly decreased the MDA in serum and pancreatic tissues but exhibited significant antioxidant activity, where TAC was significantly increased.

Several previous studies on STZ-induced diabetes also reported similar results [49–51]. These studies showed that the level of MDA in the tissues of the pancreas was significantly elevated as compared to the normal control group [52–54]. Furthermore, Jagtap and Patil [55] and Abou Khalil et al. [56] reported that MDA value was increased in the plasma as well as the pancreatic tissue of diabetes-induced rats, while it was significantly decreased by the treatment of diabetic animals with plant extracts (family Apiaceae). These results could be explained as mentioned by Alqahtani et al. [10], who reported that *C. myrrha* has a great variation in furano-sesquiterpenoids, 2-methoxyfuranodiene and 2-acetoxyfuranodiene contents, possessing maximum antioxidant activity.

Pancreatic histopathological studies showed degenerative changes and atrophy associated with dilatation of the exocrine ducts in the STZ-diabetic rats (DG) [56]. On the other hand, normal control animals as well as MAE-treated animals showed normal pancreatic Langerhans islets. Similar results were obtained by Parasuraman et al. [9], who found vacuolar degeneration in islet cells in sections from the pancreas of diabetic rats with the atrophic islet.

The present study gives hope for using this plant extract in many applications of clinical importance. The plant extract could be used as a mouthwash due to its antiseptic and antimicrobial properties. It also could be used for wound dressing for the prevention of serious infections. Its antimicrobial ingredients could be extracted in the future and tested to be used as an antibiotic after studying its side effects.

## 5. Conclusion

Our experiments focused on the extraction, identification, and purification of the active constituents of MAE, having promising biological activities. According to the results from the current study, one can conclude that the administration of *C. myrrha aqueous* extract has the ability to reduce the plasma glucose level in STZ-induced diabetes in rodents. The MAE also had antimicrobial and antioxidant potential activities. The antihyperglycemic, antioxidant, and antimicrobial effects of MAE may be due to the presence of high amounts of various active constituents like polyphenolic components and flavonoids including alkaloids in the aqueous extract. In addition, the histological microscopic examination of MAETG pancreases revealed that the Langerhans islets turned out to be normal without any histopathological alteration despite the injection of animals with STZ, which has a destructive effect on the pancreatic cells. The outcome of these findings advocates that MAE might be pondered as an effective oral antidiabetic therapy, including additional antioxidant and antimicrobial agents in the future.

*C. myrrha* showed high antioxidant as well as hypoglycemic activities due to the presence of various important constituents and compounds inside the plant. Therefore, the future prospect of the present study is to use simple, viable, effective, and rapid methodologies which will be essential for the extraction of these active phytochemicals and to study their effects on humans to acquire more information about this promising plant.

## Data Availability

All the data are included in the manuscript.

## Ethical Approval

This study protocol was approved by the research ethics committee at the National Hepatology and Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt (approval no. REC/NHTMRI/A5-2021).

## Conflicts of Interest

The authors declared that there is no conflict of interest.

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*Research Article*

# Network Pharmacology and Transcriptomics Reveal the Mechanism of GuaLouQuMaiWan in Treatment of Type 2 Diabetes and Its Active Small Molecular Compound

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The main pathophysiological abnormalities in type 2 diabetes (T2D) include pancreatic $\beta$-cell dysfunction and insulin resistance. Due to hyperglycemia, patients receive long-term treatment. However, side effects and drug tolerance usually lead to treatment failure. GuaLouQuMaiWan (GLQMW), a common traditional Chinese medicine (TCM) prescription, has positive effects on controlling blood sugar and improving quality of life, but the mechanism is still unclear. To decipher their molecular mechanisms, we used a novel computational systems pharmacology-based approach consisting of bioinformatics analysis, network pharmacology, and drug similarity comparison. We divided the participants into nondisease (ND), impaired glucose tolerance (IGT), and type 2 diabetes groups according to the WHO's recommendations for diabetes. By analyzing the gene expression profile of the ND-IGT-T2D (ND to IGT to T2D) process, we found that the function of downregulated genes in the whole process was mainly related to insulin secretion, while the upregulated genes were related to inflammation. Furthermore, other genes in the ND-IGT (ND to IGT) process are mainly related to inflammation and lipid metabolic disorders. We speculate that 17 genes with a consistent trend may play a key role in the process of ND-IGT-T2D. We further performed target prediction for 50 compounds in GLQMW that met the screening criteria and intersected the differentially expressed genes of the T2D process with the compounds of GLQMW; a total of 18 proteins proved potential targets for GLQMW. Among these, RBP4 is considerably related to insulin resistance. GO/KEGG enrichment analyses of the target genes of GLQMW showed enrichment in inflammation- and T2D therapy-related pathways. Based on the RDKit tool and the DrugBank database, we speculate that (-)-taxifolin, dialoside A_qt, spinasterol, isofucosterol, and 11,14-eicosadienoic acid can be used as potential drugs for T2D via molecular docking and drug similarity comparison.

## 1. Background

Type II diabetes (T2D) is a chronic disease characterized primarily by abnormally high blood sugar levels. The number of patients, a high proportion of whom are young adults, suffering from T2D is increasing annually [1]. The World Health Organization (WHO) recommends that T2D be divided into two pathological states, impaired glucose tolerance (IGT) and T2D [2, 3], upon which doctors can adjust treatment strategies. The pathophysiological characteristics of T2D include insulin resistance and insulin deficiency [4]. Obesity due to disordered glucose and lipid metabolism is a common cause of T2D. Insulin deficiency is mainly related to glucose toxicity [5] and lipotoxicity [6], which

result in pancreatic $\beta$-cell dysfunction. After an irregular diet, an imperceptible and cumulative increase in fasting and 2 h postprandial blood glucose levels occurs [5], which is known as glucotoxicity. Glucose toxicity can cause pancreatic $\beta$-cell dysfunction and senescence. Moreover, compared with the loss of $\beta$-cells, supplementation through the differentiation of islet stem/progenitor cells [7] and self-replication of pancreatic $\beta$-cells [8] is insignificant, which leads to insufficient insulin secretion and abnormal blood sugar levels. In a cohort study of T2D, researchers measured the quality of various parts of the pancreatic tissue and found that the $\beta$-cell content of T2D and IGT patients showed a downward trend [9]. Insulin resistance refers to a phenomenon in which the biological effects of insulin in muscle tissue and the liver, such as promoting the production of muscle glycogen and liver glycogen, are reduced [10]. Insulin can regulate blood glucose homeostasis by stimulating the phosphatidylinositol 3-kinase (PI3K)-independent pathway through binding and activating membrane-localized receptors using tyrosine kinase activity [11]. Similarly, insulin has a regulatory effect on lipids and glycogen, including lipid conversion, glycogen decomposition and synthesis, and glucose transporters activity increase on the cell surface, affecting mRNA synthesis, cell proliferation, and survival [12]. In insulin resistance, tyrosine kinase receptor (the main insulin receptor) cannot adequately activate downstream reactions because the expression of protein-tyrosine phosphatase 1B (PTP1B) tends to be upregulated, exerting a dephosphorylation effect that counteracts the biological effects of insulin [13]. Moreover, insulin resistance can cause chronic inflammation and a decrease in cell surface receptors. First, members of the cytokine signaling inhibitor protein family activate degradation of the cell membrane surface insulin receptor substrate through the ubiquitin-proteasome pathway [14]. Second, the massive release of free fatty acids and cellular inflammatory factors, such as tumor necrosis factor (TNF) and interleukin-6 (IL6), also has a negative impact on the biological effects of insulin [15, 16]. These factors accelerate T2D progression.

In China, GuaLouQuMaiWan (GLQMW), a traditional prescription, synergizes with antidiabetic drugs in T2D treatment by reducing drug resistance [17] and stabilizing blood sugar [18]. GLQMW, which is composed of *Trichosanthis* Radix (THF), *Aconiti Lateralis Radix Praeparata* (FZ), *Poria Cocos* (Schw.) *Wolf.* (FL), *Dianthi Herba* (QM), and *Rhizoma Dioscoreae* (SY), can invigorate the spleen and kidney and eliminate dampness and diuresis, according to the principles of TCM for T2D. SY and FL proved to be effective in intervening blood glucose and blood lipid levels in animal models [19, 20], and QM has an anti-inflammatory effect [21]. Although recent research has shown that some components of GLQMW are beneficial to T2D, there has been no research to interpret the specific molecular mechanism of GLQMW intervention in the progression of T2D.

T2D is a nonmutated disease, hence, its progression is laboriously illustrated through the change of a single gene. However, the changes in gene expression profiles during disease progression can be identified and visualized by transcriptome sequencing analysis [22], providing insight on potential therapeutic targets. TCM is a multitarget and multipath process for the treatment of diseases, so we need to separate and analyze natural compounds in herbs that meet potential drug standards. We can summarize the effective compounds of GLQMW in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) as it collects the herbs contained in the Chinese Pharmacopoeia and the basic physical and chemical properties of their related compounds [23]. However, we must choose the PharmMapper server, a tool based on the pharmacophore mapping algorithm [24], to predict potential targets of the compound since TCMSP only includes the confirmed information for the herbal compound. The PharmMapper server is designed to identify potential target candidates for the given probe small molecules (drugs, natural products, or other newly discovered compounds with binding targets unidentified) using a pharmacophore mapping approach in the pharmacophore database (PharmTargetDB) and lists the annotation and classification of the predicted target [25]. The principle of drug similarity means that the structure of a compound determines its basic properties, therefore, the more similar the structure of a compound, the more similar the basic properties, such as physical and chemical properties and physiological metabolism [26]. This principle helps us to further decipher the potential pharmacological activities of compounds that are not included in the PharmMapper server. Furthermore, RDKit can help us compare the structure of small molecule compounds, especially in the field of pharmacological identification of TCM [27]. Based on the Python environment, RDKit, an open-source toolkit suitable for chemical informatics [27], can convert 2D/3D to 3D/2D compound structures, generate compound fingerprints, and calculate the structure of compound similarity via machine learning methods [28]. Following RDKit processing, we deduced that GLQMW contains small-molecule compounds that interfere with the process of T2D progression. Finally, molecular docking and LIGPLOT can predict binding poses and affinities through the interactions between receptors and drug molecular ligands, which involve spatial matching and energy matching between molecules [29]. These methods are powerful tools for pharmacological mechanistic and drug research and development. Therefore, transcriptomic analysis, network pharmacology, and molecular docking were used to explore the pharmacological targets and mechanisms of GLQMW against T2D.

## 2. Materials and Methods

The overall process of the research is shown in Figure 1.

*2.1. Raw Data.* RNA-seq data of T2D (including 411 islet tissue samples) and the corresponding clinical data were downloaded from GEO dataset. By filtering out the sample missing diagnosis, the expression data for 326 islet tissue were kept for

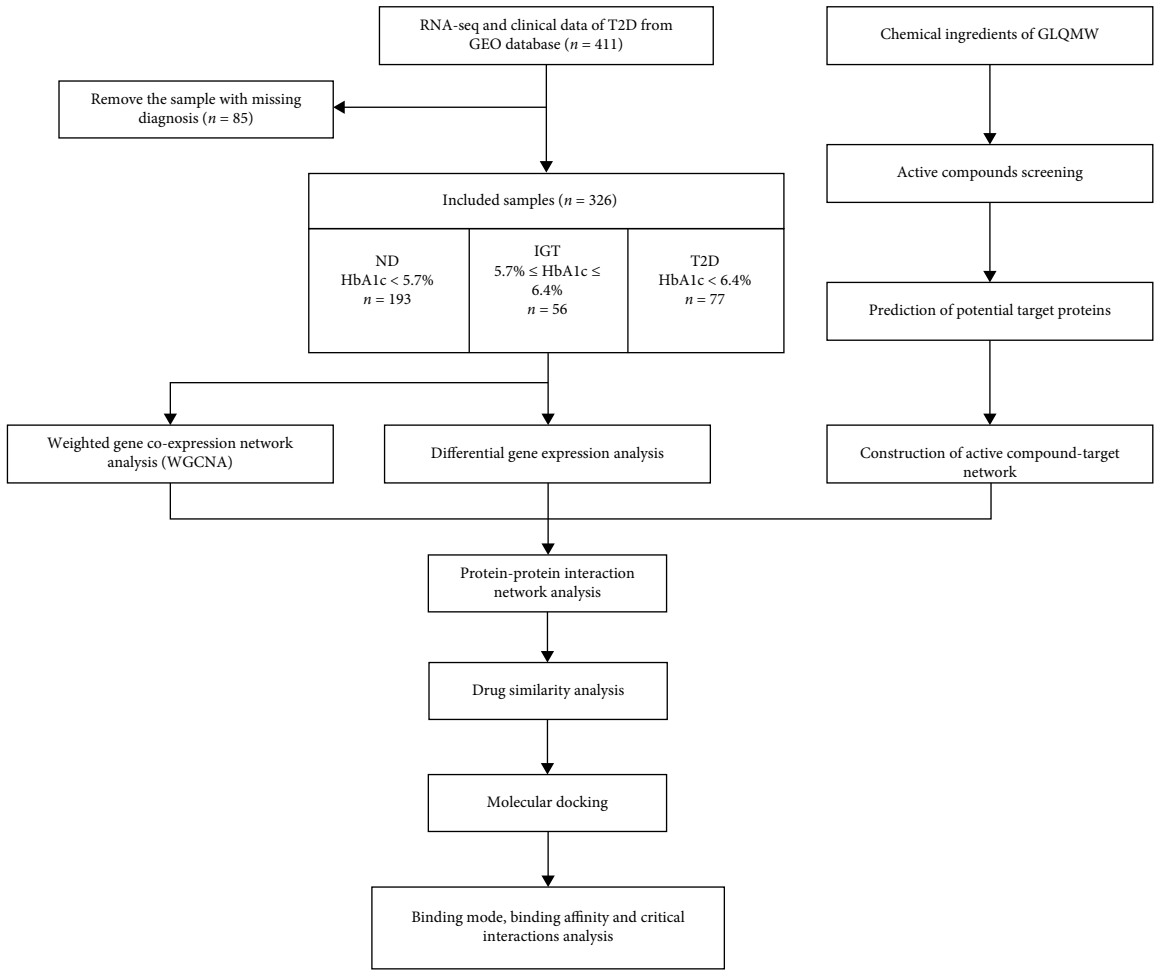



Figure 1: Experimental design for elucidating the mechanisms of action of GLQMW in the treatment of T2D.

downstream analysis. Four cohort used in this study as validation datasets (GSE38642, GSE50397, GSE76894, and GSE76895) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo). Compounds of GLQMW and its' target proteins origin from TCMSP (https://tcmspw.com/) and PharmMapper server, respectively.

*2.2. Type 2 Diabetes Process Classification.* Using the diagnostic criteria (ND < 5.7%, IGT5.7%-6.4%, T2D : >6.4%) which is recommended by WHO, we distinguished the T2D process of diabetic patients via glycosylated hemoglobin (HbA1c), and divided the samples into 3 groups (including 193 ND, 56 IGT, and 77 T2D islet tissue samples).

*2.3. Differential Gene Expression Analysis.* Differential expression analysis was conducted using the R package "limma". The screening conditions for the differential genes were $|\log 2\text{FoldChange}| > 0.3$, $p.\text{adj} < 0.05$. Heatmaps of differential genes were drawn using the R package "pheatmap". For process-specific genes, only genes with significant differences and consistent trend in expression ($|\log 2\text{FC}| > 0.3$, $p.\text{adj} < 0.05$) in all three possible comparisons were considered T2D-specific genes.

*2.4. Weighted Gene Coexpression Network Analysis (WNGCA) and Identification of Clinically Significant Modules.* We use the R package "WGCNA" to construct a gene-weighted coexpression network, when R package "limma" has processed the gene expression data of removing the batches. First, compared to the Pearson method, we chose the biweight midcorrelation method to construct the adjacency matrix to describe the correlation strength between nodes. Subsequently, choosing the soft threshold $\beta = 4$ (scale free $R^2 = 0.85$), we convert the adjacency matrix to a topological overlap matrix (TOM), and set the type of TOM to a signed network. Next, we perform hierarchical clustering to identify modules which contains at least 30 genes (minModuleSize = 30), calculated feature genes, hierarchically clustered the modules, and merged similar modules.

The module feature gene (ME) is the first principal component of the module and represents the expression pattern of the module in each sample. The degree of module membership (MM) refers to the correlation coefficient between genes and the characteristic genes of the module, which is used to describe the reliability of the gene belonging to the module. Based on ME and MM, we calculated the correlation between modules and clinical data to determine important clinical modules. Finally, we selected the modules whose

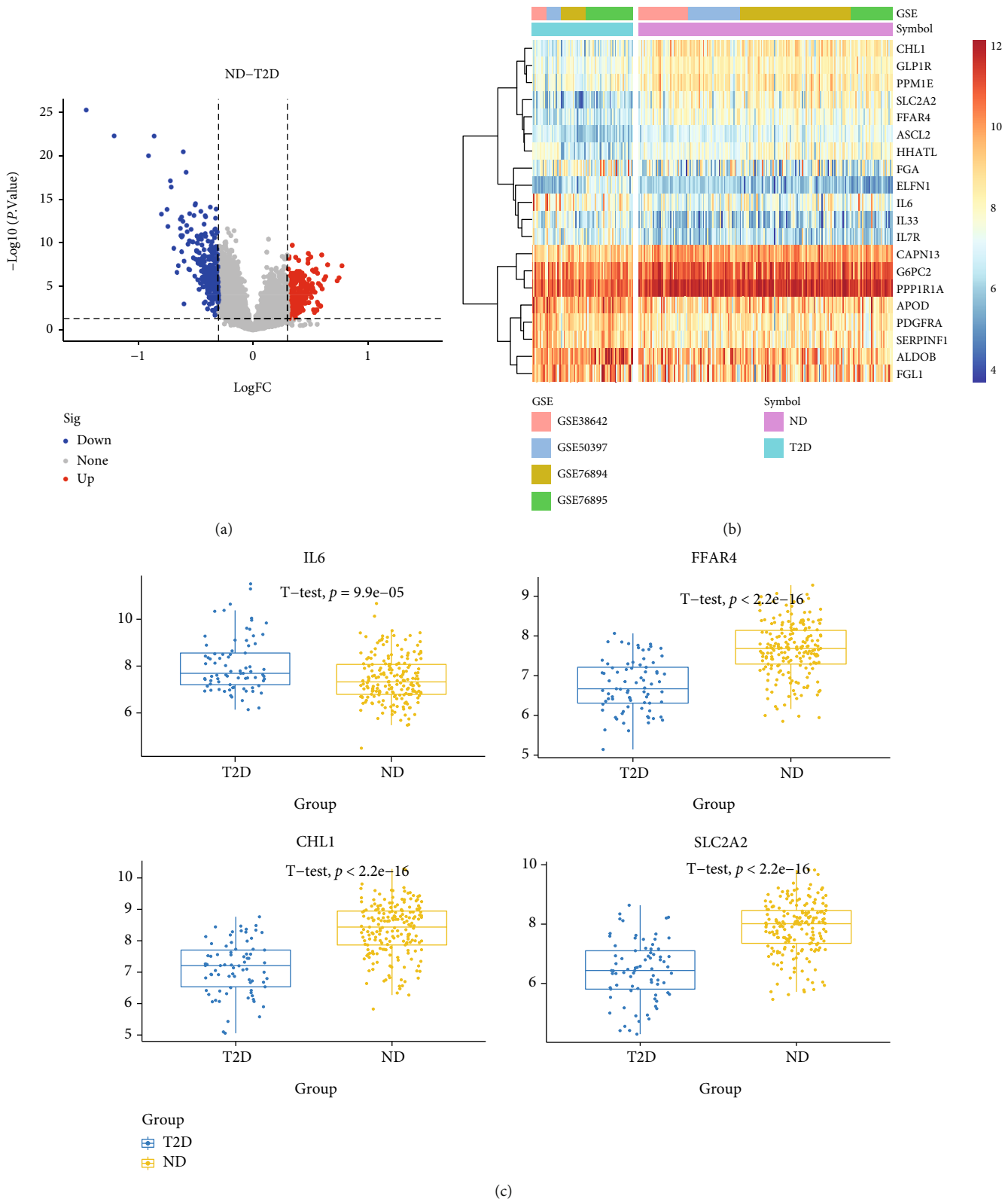



FIGURE 2: Volcano plots, heatmaps, and gene expression profiles of DEGs in ND-T2D. (a) Number and distribution of up and downregulated genes. (b) Heatmap for DEGs generated by comparison in ND and T2D. Row is the gene, and column name is the samples, which is not shown in plot. DEGs were determined by Wilcoxon rank sum test with $q < 0.05$ and $\log 2FC > 0.3$ as the significance threshold. (c) Expression levels of some DEGs in ND and T2D.

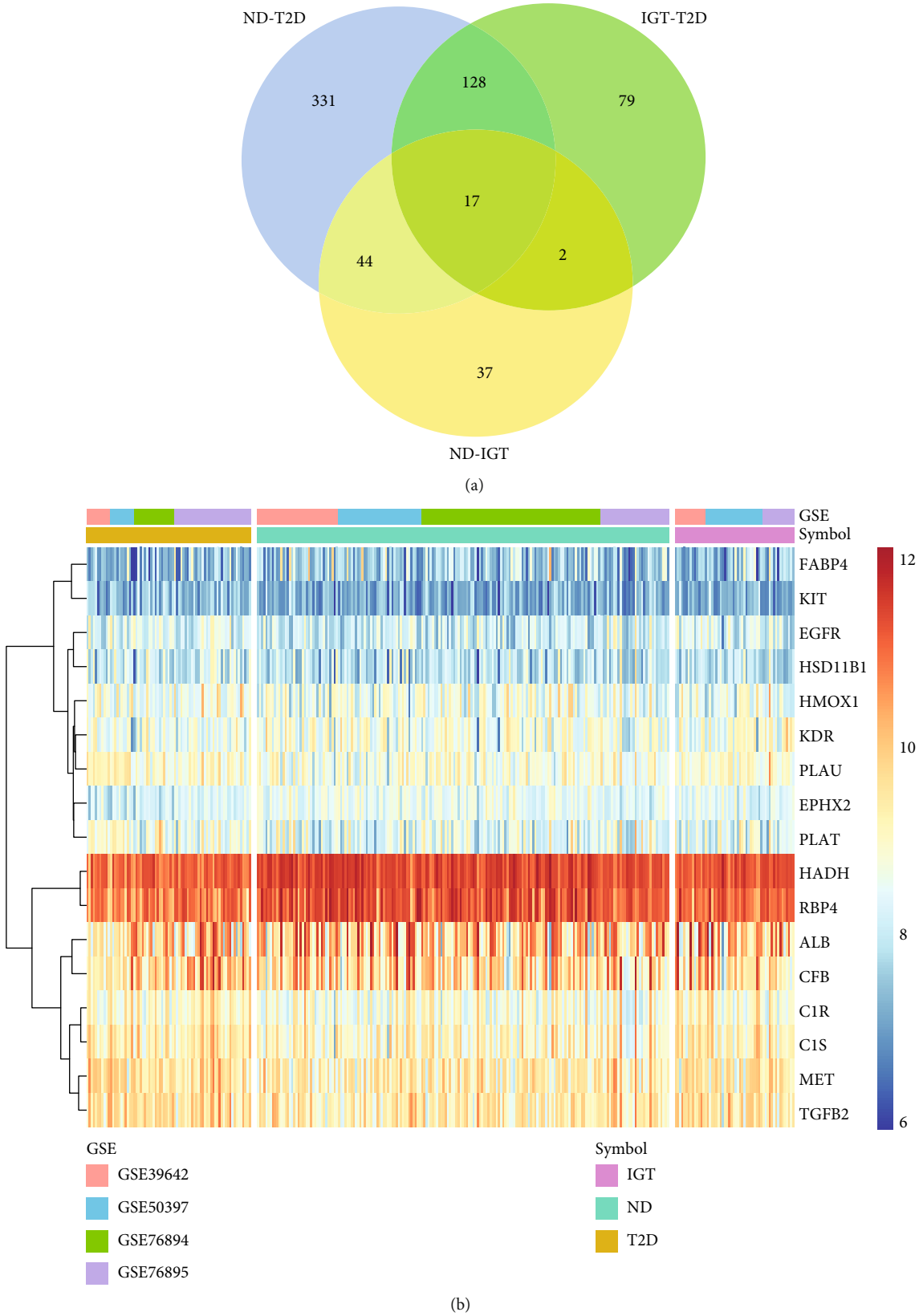



Figure 3: Venn diagrams and heatmaps of intersecting genes in ND-IGT-T2D. (a) Venn plots showing common upregulated and downregulated DEGs shared by ND-IGT, IGT-T2D, and ND-T2D. (b) The expression level of common up and downregulated DEGs in ND, IGT, and T2D.

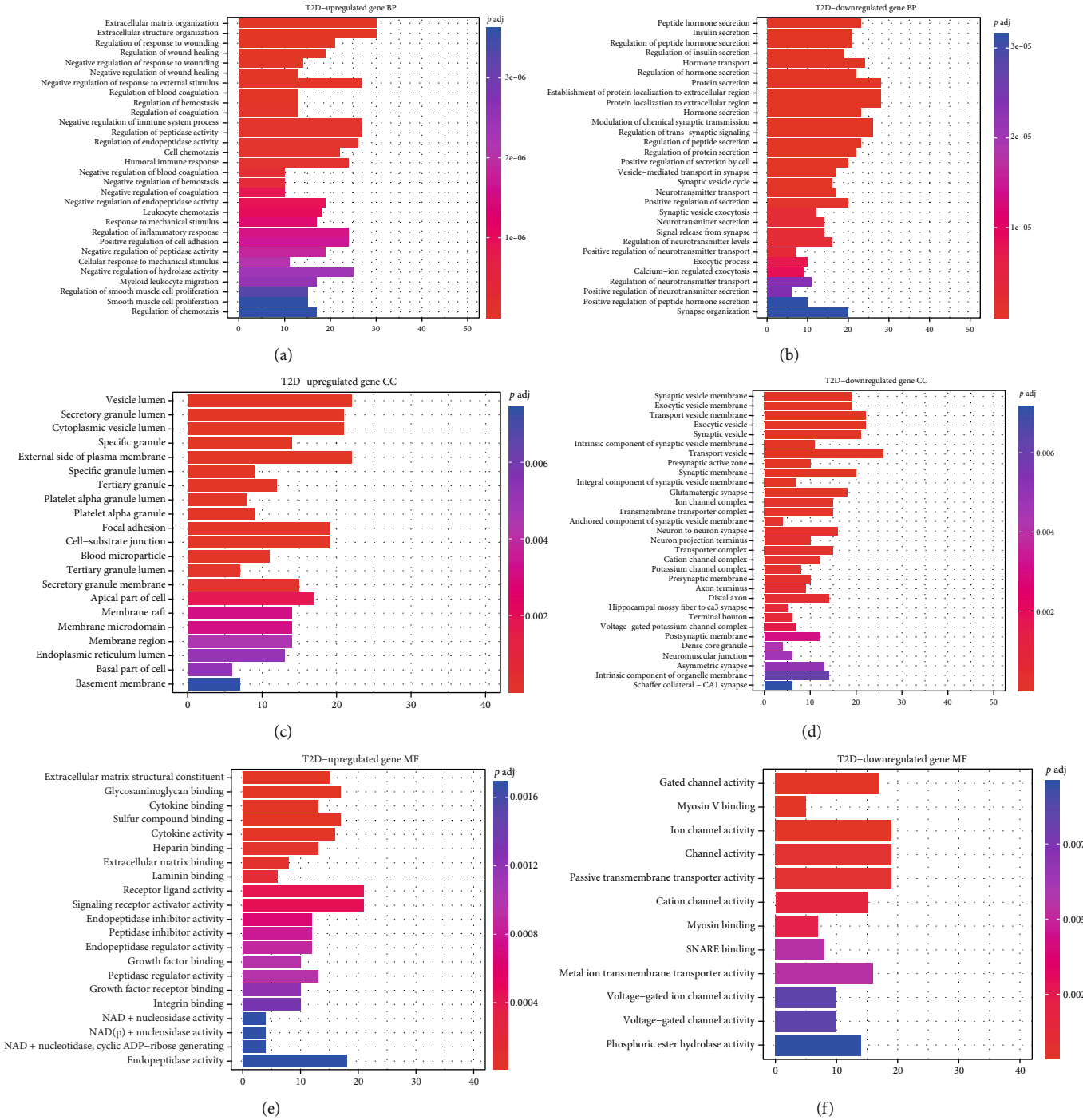



Figure 4: GO analysis of the up/downregulated DEGs involved in ND-T2D regarding biological process, cellular component, and molecular function.

expression changes were in line with the process of ND-IGT-T2D, and extracted the hub gene and core genes which were further analyzed.

*2.5. GO and KEGG Enrichment Analysis.* GO and KEGG enrichment analyses were performed with the aid of R packages "clusterProfiler," "enrichplot," and "ggplot2." Only terms with both $p$ and $q$ value of $< 0.05$ were considered significantly enriched. For T2D, GO and KEGG enrichment analyses were based on upregulated and downregulated expressed genes. For GLQMW, we used "http://org.hs.eg.db/" to convert the target protein into a gene, and then performed GO and KEGG enrichment analysis.

*2.6. Active Compounds Screening.* All of the chemical ingredients of GLQMW were obtained from TCMSP. The active compounds of GLQMW were mainly filtered with oral bioavailability (OB), drug-likeness (DL), and Caco-2

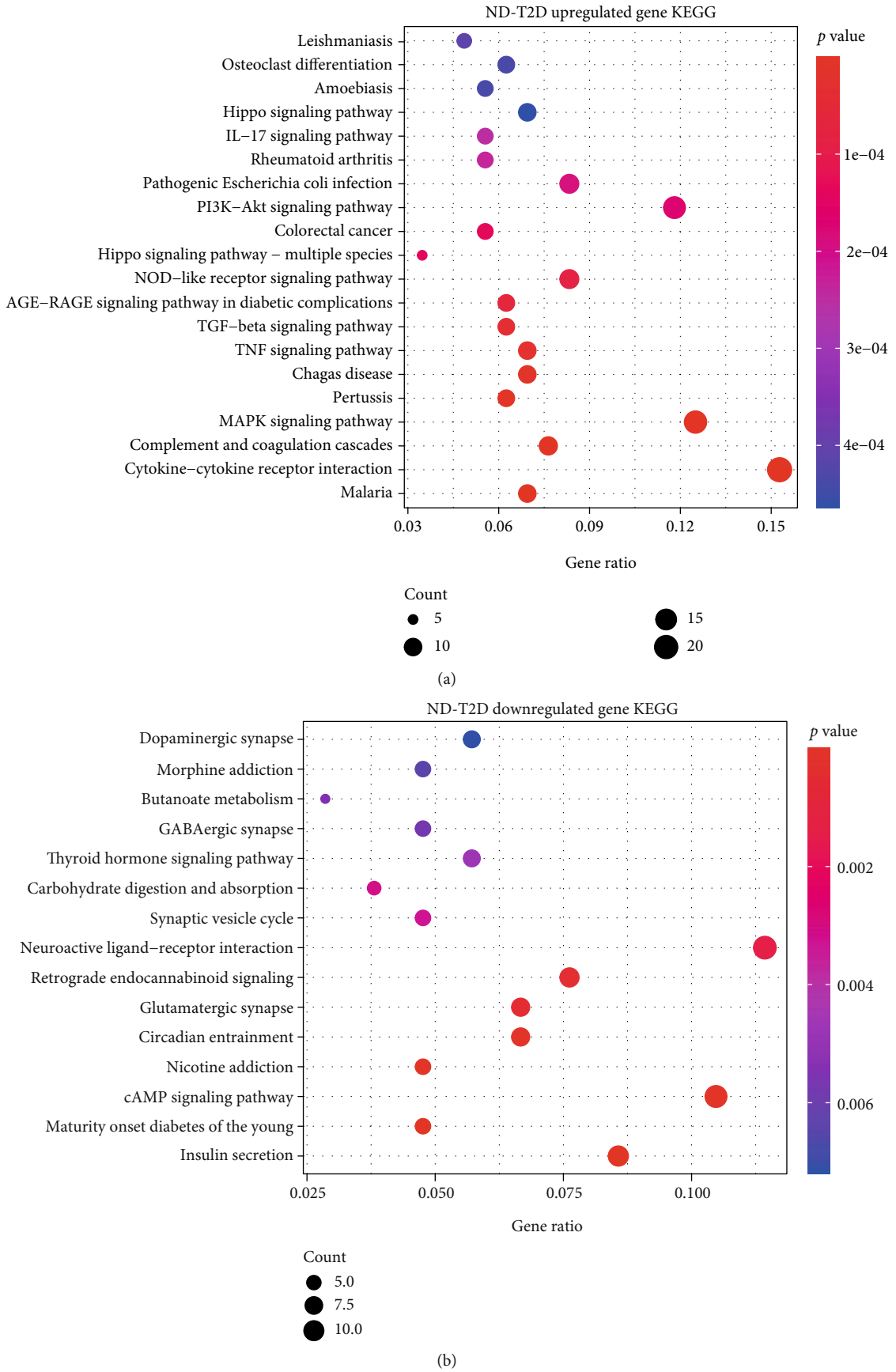
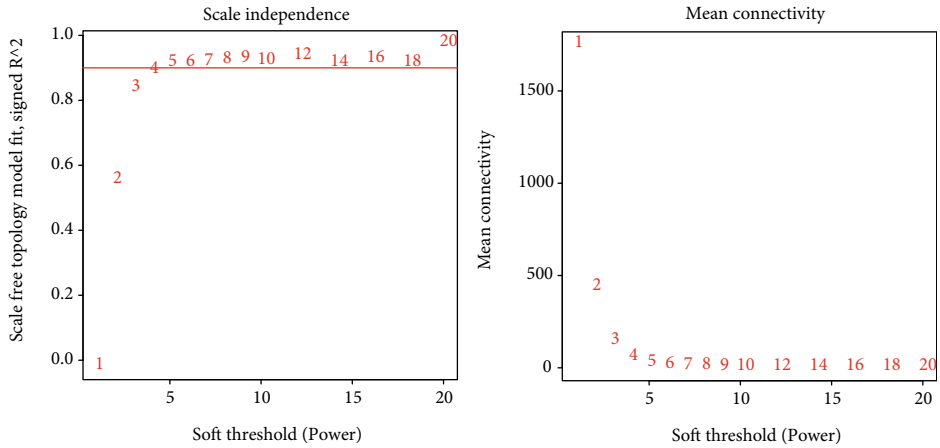



Figure 5: KEGG analysis of the up/downregulated DEGs involved in ND-T2D.




(a)

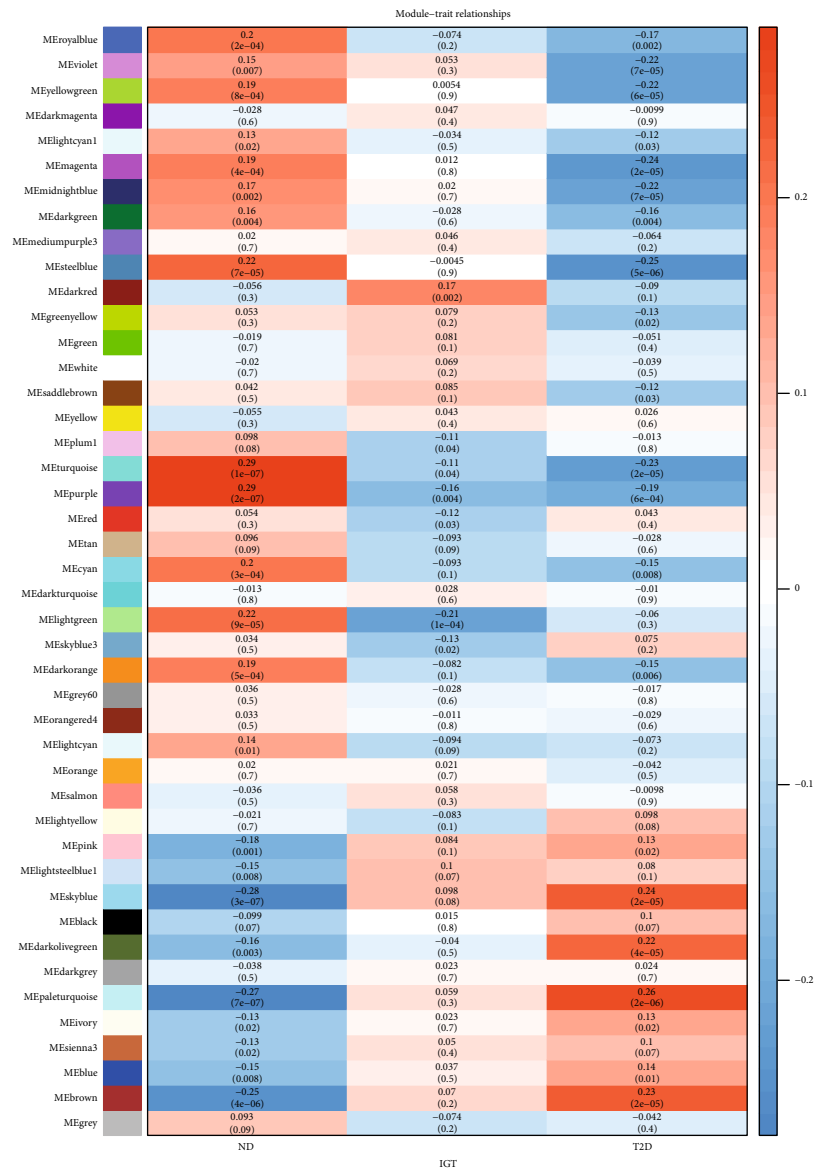



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Figure 6: Continued.

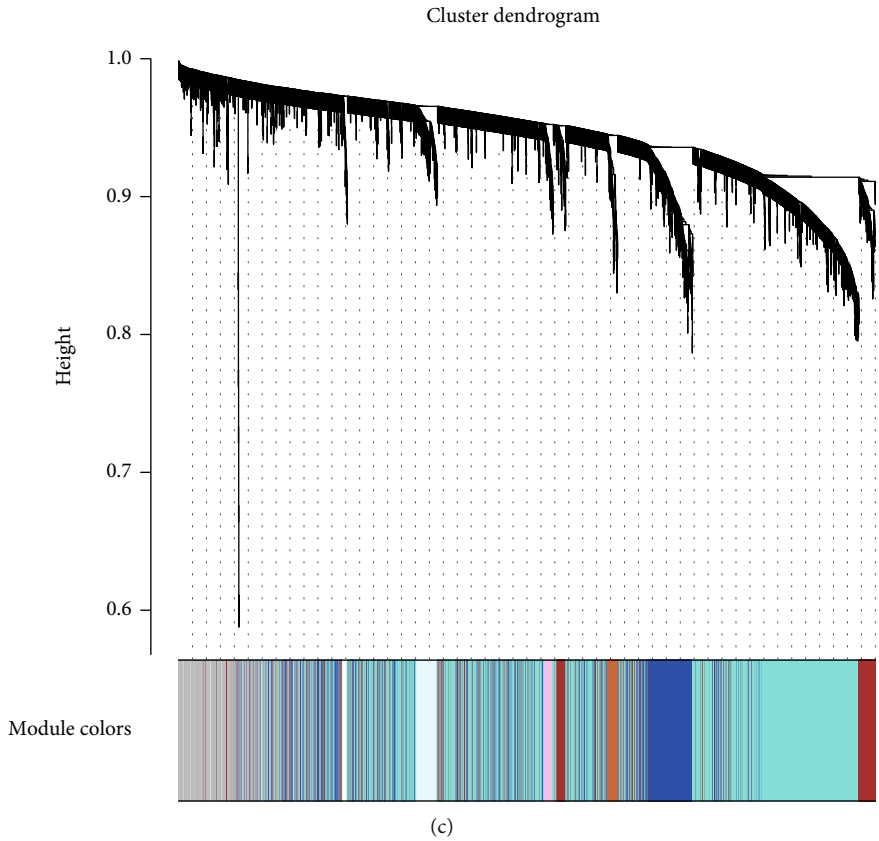



Figure 6: WGCNA in ND-IGT-T2D. (a) Analysis of network topology for various soft-thresholding powers. (b) Module–trait associations. Each row corresponds to a module, and each column corresponds to a trait. Each cell contains the corresponding correlation and $p$ value. The table is color-coded by correlation according to the color legend. (c) Clustering dendrogram of genes, with dissimilarity based on topological overlap, together with assigned module colors.

permeability (Caco-2), while those compounds in GLQMW with OB ≥ 30%, DL ≥ 0.18, and Caco − 2 ≥ −0.4 were preserved [30]. But, we selected 5 compounds with reports of pharmacological activity to join the results, and those compounds' OB and DL meet the threshold but Caco-2 does not.

*2.7. Network Pharmacology Analysis.* Download the 3D structure of the GLQMW compound from TCMSP. The PharmMapper server recognizes the pharmacophore of the compound, and uses the pharmacophore as the recognition group of given probe molecule to predict potential target proteins. PharmMapper server output results, including target name and protein number, among which $z$-score is an important basis for target fit. We choose 1.0 as the threshold, and extract all protein targets greater than the threshold for subsequent analysis. Based on cytoscape, we construct an active compound-target network, and select compounds with a larger number of nodes for key analysis.

*2.8. Drug Similarity Analysis.* Download the "full database" document from the DrugBank database (https://go.drugbank.com/) [31], which contains all the drug details. In the process of processing the document, select the drug information whose type is "small molecule," and remove the drug whose category is protein, due to focusing on small molecule compounds in GLQMW.

RDkit generates compound descriptors and compound fingerprints, and calculates the similarity of compound structures, based on the 2D and 3D structure of compounds.

Our study compares active compounds in GLQMW and small molecule drugs in the DrugBank database, and outputs information on the top 10 drugs with drug similarity.

*2.9. Molecular Docking and LIGPLOT.* The 3D structure of protein: RBP4 (PDB ID:2WR6, Uniprot:P02753), PLAT (PDB ID:1A5H, Uniprot:P00750), MET (PDB ID:2ZGH, Uniprot:P51124), KIT (PDB ID:4U0i, Uniprot:P10721), C1S (PDB ID:5UBM, Uniprot:P09871), and HSD11B (PDB ID:2RBE, Uniprot:P28845) were downloaded from the protein data bank (PDB) database (https://www.rcsb.org/). The ligand and water macromolecule in these targets were removed, and the hydrogen atoms were added with pymol2.3. The targets were set to rigid and saved as pdbqt file format by AutoDock Tools 1.5.6. Finally, molecular docking was performed using Vina. Based on the top one minimal binding energy of each target, compounds in GLQMW were selected for further analysis of their binding mode, binding affinity, and critical interactions using PyMOL2.3 and LIGPLOT2.2.

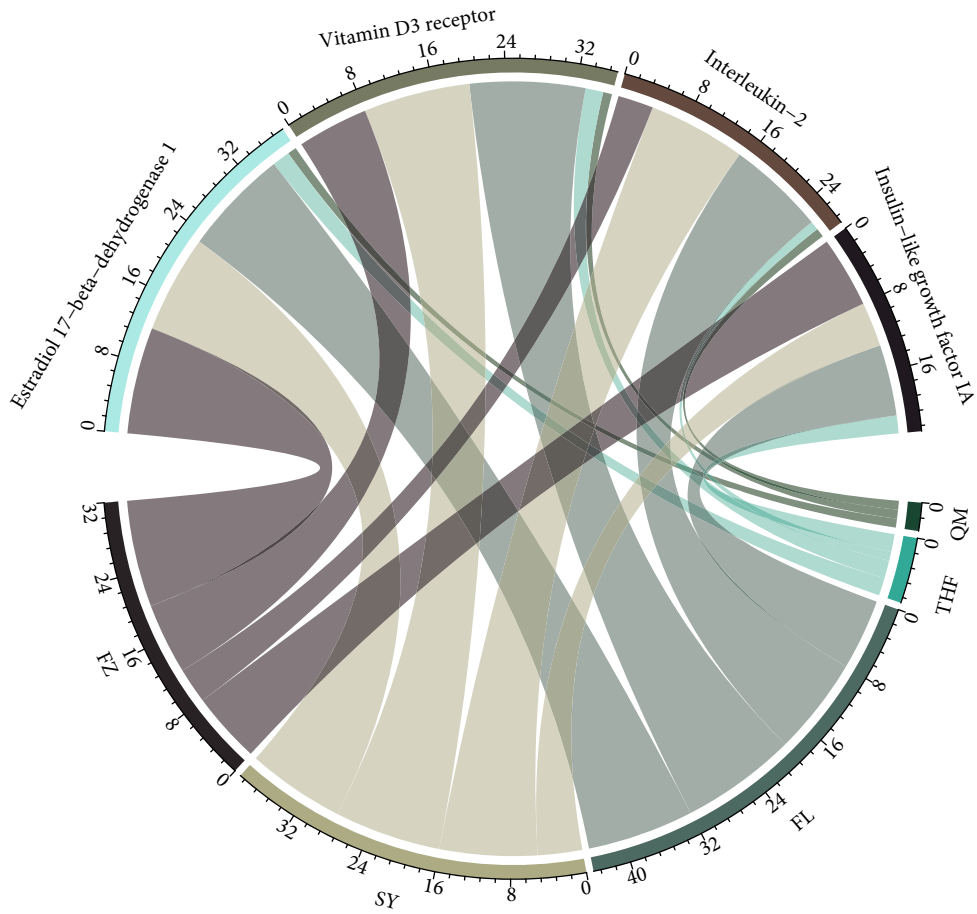



Figure 7: Correspondence between GLQMW and main target proteins.

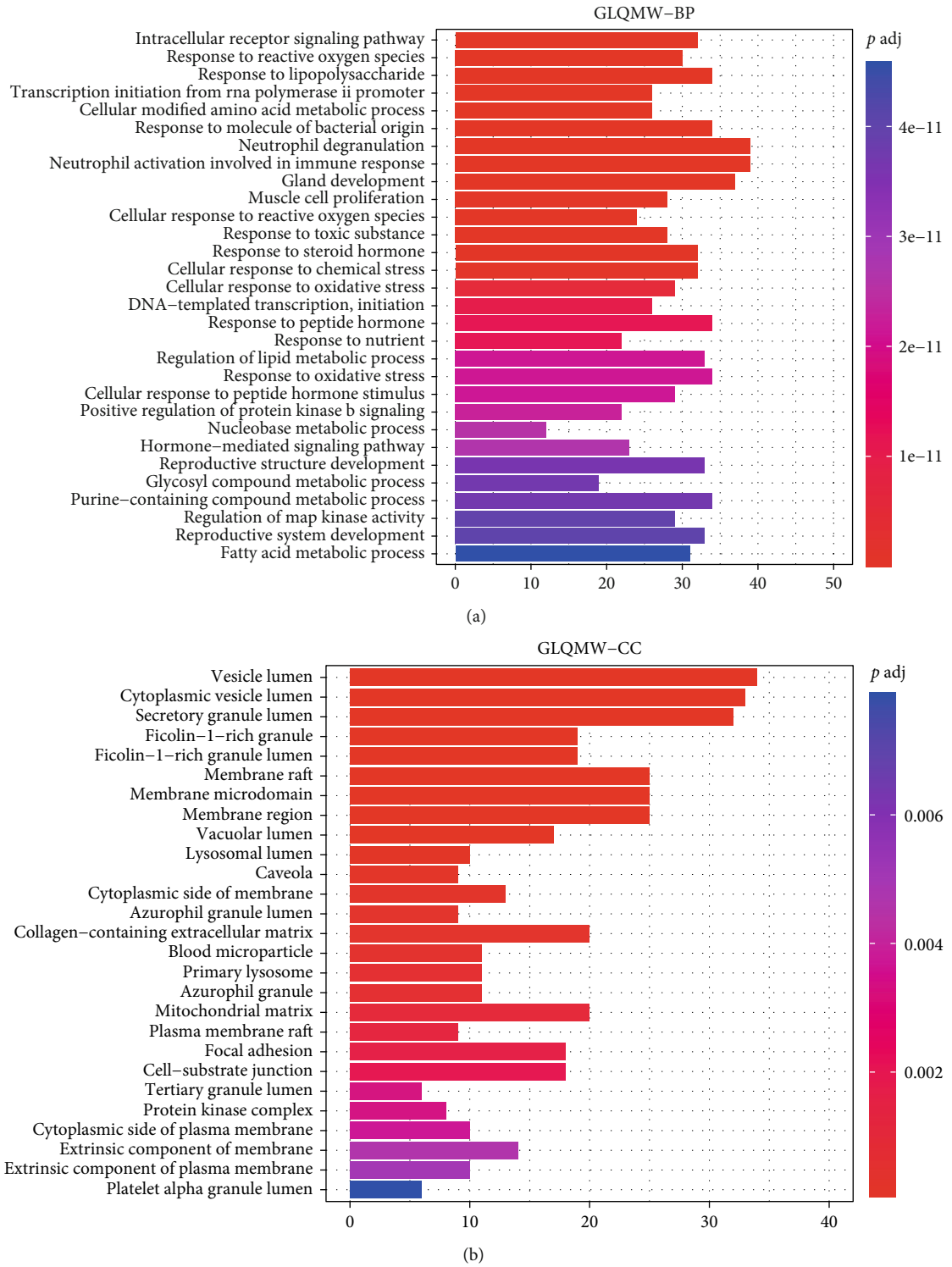
## 3. Results

*3.1. Remove Different Batch Effect.* This study included high-throughput gene expression profiling data of 326 pancreatic islet tissue samples from 4 datasets. Since the data comes from different sequencing platforms, the data has a relatively obvious batch effect, which will adversely affect the results of subsequent analysis. Therefore, we eliminate the batch effect on the combined data, and the results are shown in Supplementary Figure 1.

*3.2. Identification of Type 2 Diabetes Progress Important DEGs by Transcriptome Analysis.* Using ND as the control, 520 differential genes were identified in the ND-T2D group, of which 259 were upregulated and 261 were downregulated. Using ND as the control, 58 upregulated genes and 42 downregulated genes were identified in the IGT group. In the comparison between the IGT-T2D groups and IGT as the control, there were 156 upregulated genes and 70 downregulated genes in T2D. Figure 2 shows DEGs expression in ND-T2D, Supplementary Figures 2 and 3 show DEGs expression in ND-IGT and IGT-T2D, respectively.

In ND-T2D, the most significantly upregulated and downregulated genes were APOD (logFC = 0.77, $p$.adj < 0.05) and SLC2A2 (logFC = −1.45, $p$.adj < 0.05), respectively. In the ND-IGT, the most significantly upregulated and downregulated genes were PTGS2 (logFC = 0.58, $p$.adj < 0.05) and TMED6 (logFC = −0.68, $p$.adj < 0.05), respectively. In IGT-T2D, the most significantly upregulated and downregulated genes were CEACAM7 (logFC = 0.91, $p$.adj < 0.05) and SLC2A2 (logFC = −0.99, $p$.adj < 0.05), respectively.

We found that the number of DEGs decreased among the three groups of ND-T2D (520), IGT-T2D (226), and ND-IGT (100), Figure 3. This phenomenon coincides with the clinical observation that the states of ND and IGT are somewhat similar. However, T2D is often difficult to reverse, so the transcriptome difference is higher than that in other states. There were 17 DEGs that met the DEGs screening criteria in all disease stages and had the same disease expression trend (Supplementary Table 1). Thus, these genes may play a central role in T2D progression. Moreover, 61% of the DEGs in the ND-IGT process were also identified in ND-T2D, indicating that the DEGs of the predisease progression, such as the inflammation-related genes IL1RL1 and IL6, may continue to affect disease progression, providing evidence that inflammation is a risk factor for disease progression. Similarly, in the process of IGT-T2D, in addition to inflammation-related genes, glucose transport-related genes such as SLC2A2 and SLC26A4 also had different expression levels, which show that the development of T2D into an irreversible state is related to the imbalance of the glucose transport system.

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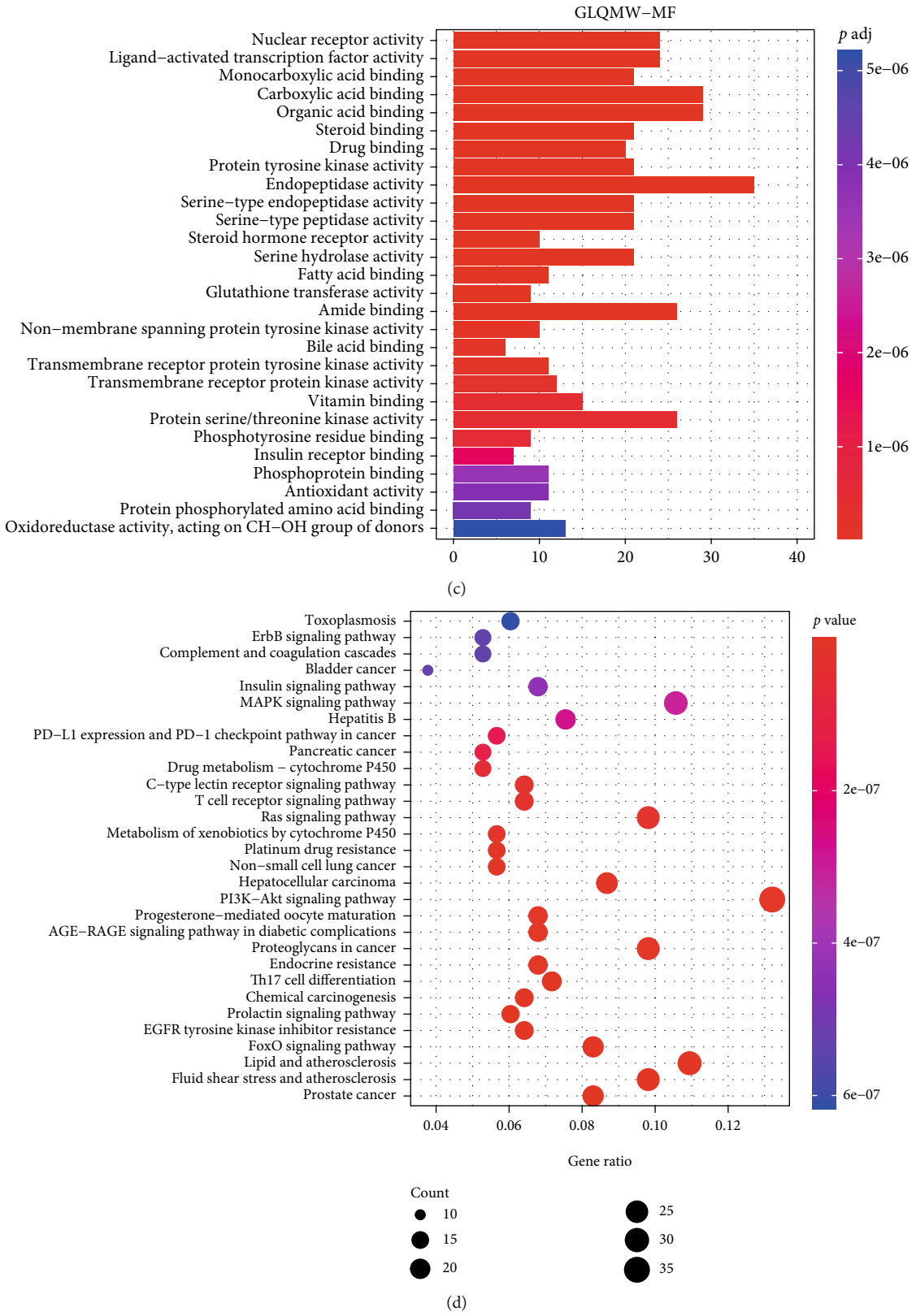



Figure 8: GO and KEGG analysis of the GLQMW target proteins.

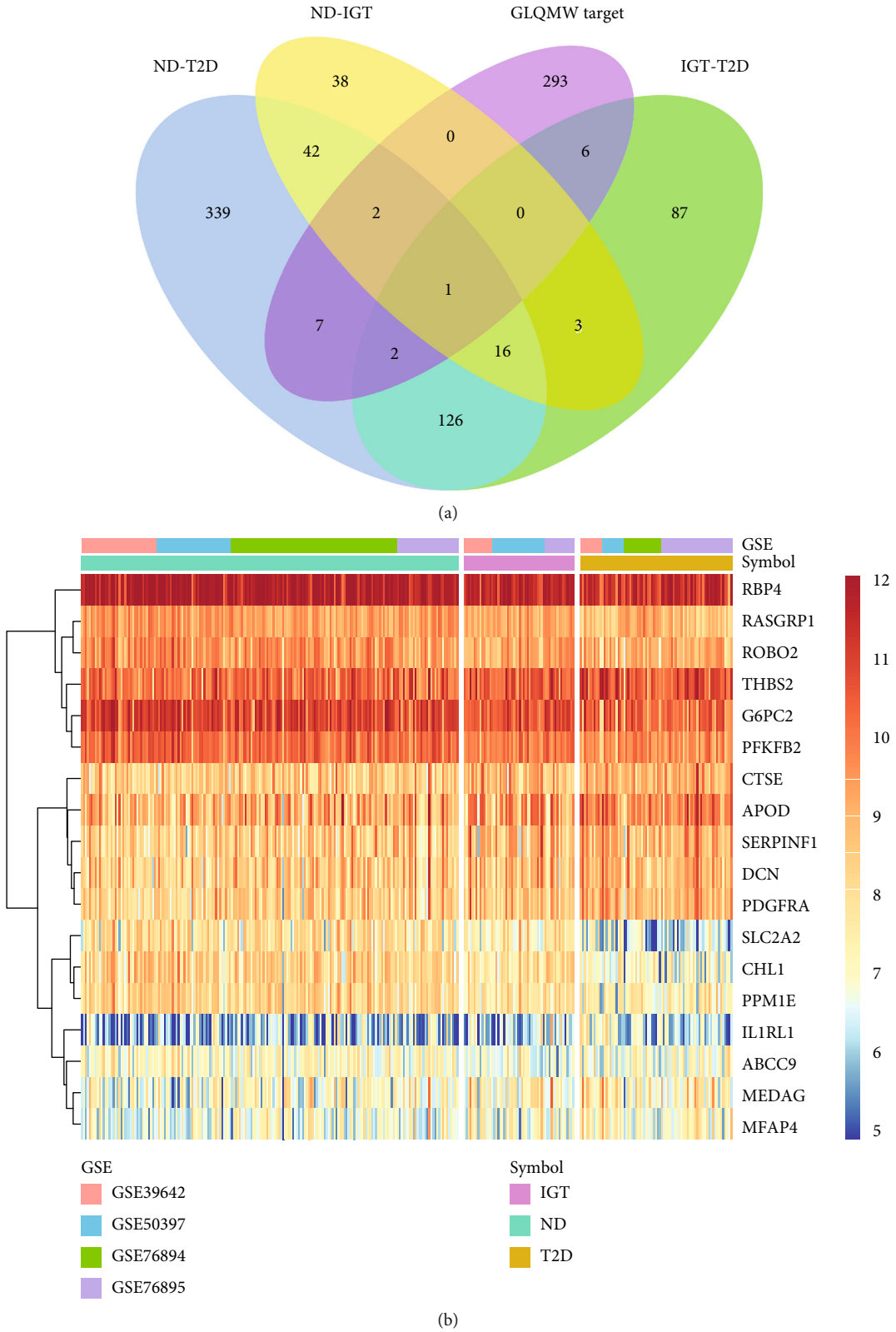



Figure 9: Venn diagram and heatmap of intersecting DEGs between process of ND-IGT-T2D and GLQMW target proteins. (a) Venn plots showing the intersecting targets between common DEGs in ND-IGT-T2D and GLQMW target proteins. (b) The expression level of intersecting DEGs in ND, IGT, and T2D.

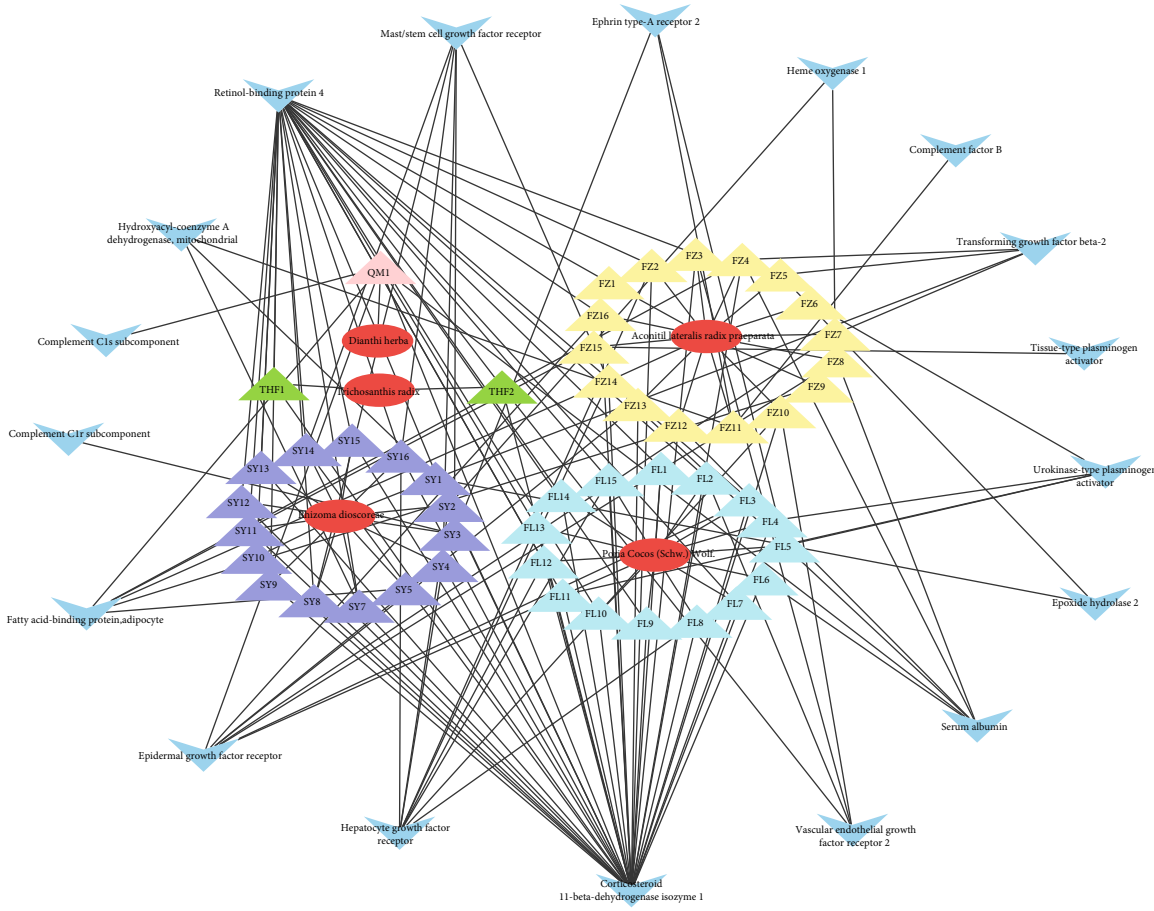
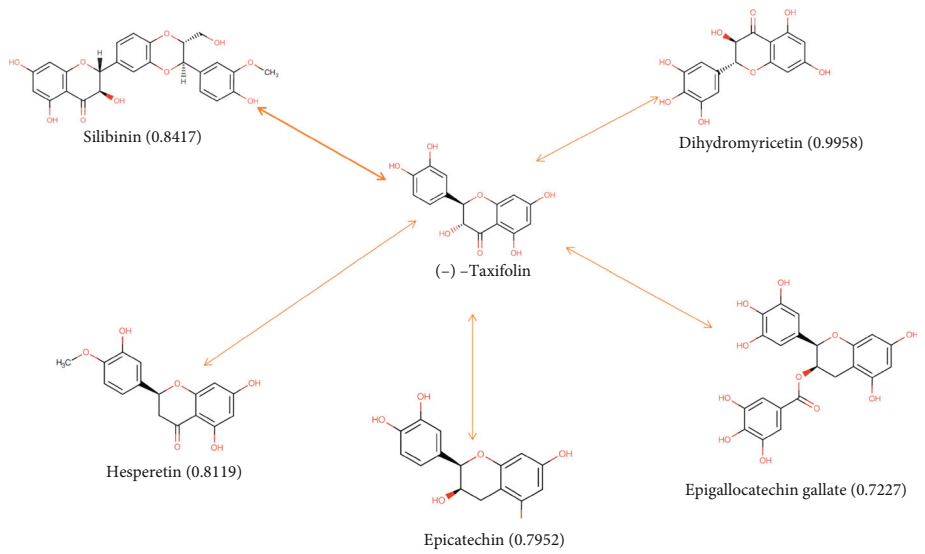



Figure 10: Interactions between herbs and target proteins for compounds. (a) The ovals represent herbs; the triangles represent the compounds of herbs, and different colors represent the source of the compound; the blue darts represent the intersecting target proteins between GLQMW and process of ND-IGT-T2D. (b) Hexagons represent T2D; the triangles represent the compounds of herbs, and different colors represent the source of the compound; blue darts represent clinical drug targets.

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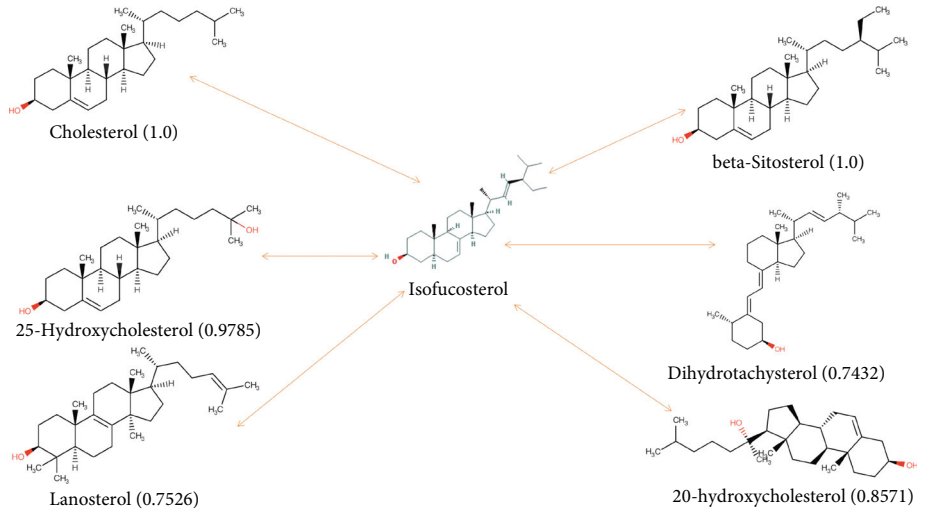


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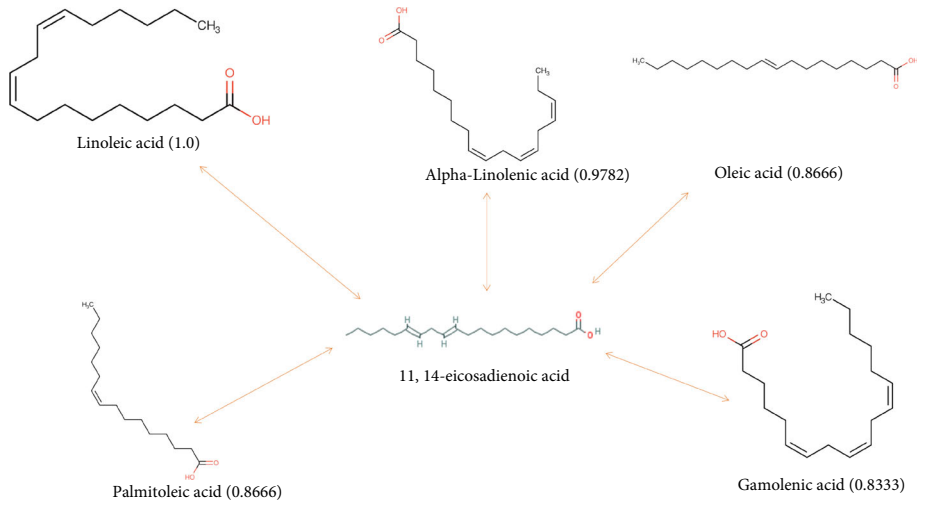
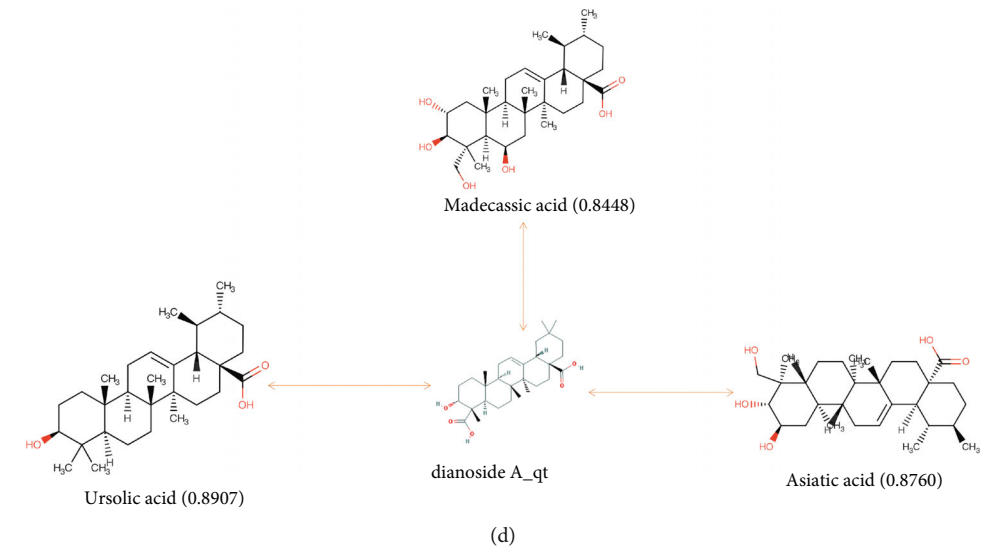
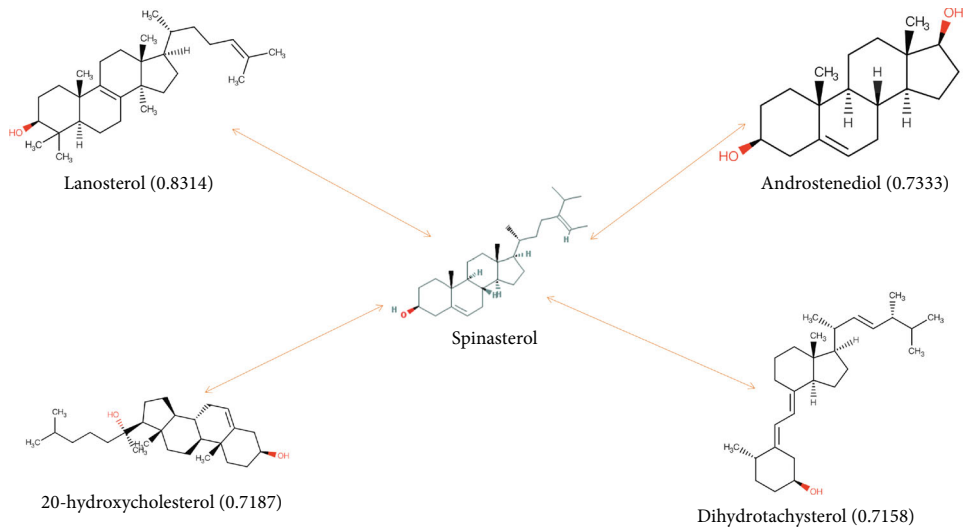



(c)

Figure 11: Continued.




(d)




(e)

Figure 11: Drug similarity between compounds and drugs of DrugBank. The two-way line represents the comparative relationship between the compound and the drug, and the numbers represent the drug similarity. (a) drug similarity of (-)-taxifolin; (b) drug similarity of isofucosterol; (c) drug similarity of 11,14-eicosadienoic acid; (d) drug similarity of dianoside A_qt; (e) drug similarity of spinasterol.

## 4. GO/KEGG Enrichment Analysis for ND-IGT-T2D DEGs and WGCNA

*4.1. GO Enrichment Analysis.* The degrees of ND-T2D progression were grouped according to upregulated and downregulated expression, after which GO enrichment analysis was performed ($p < 0.01$). For GO enrichment results, a total of 409 GO entries of upregulated genes were obtained, including 354 BP, 33 MF, and 22 CC, and 115 GO entries of downregulated genes were obtained, including 72 BP, 12 MF, and 31 CC.

The BP entry ($p$.adj < 0.01) of upregulated genes in ND-T2D mainly included regulatory response to injury, regulation of inflammatory response, regulation of cell adhesion, and acute inflammatory response regulation (Figure 4(a)), which may prove that the inflammatory response or inflammatory environment in the pancreatic islets is one of the reasons for the accelerated consumption of pancreatic islet $\beta$-cells. The BP entries enriched by downregulated genes ($p$.adj < 0.01) were mainly related to peptide hormone secretion, insulin secretion, protein secretion, etc. (Figure 4(b)). This result is consistent with the pathological phenotype of T2D; insulin secretion is reduced, and the ability of tissues to use insulin is downregulated.

GO-MF enrichment results showed that upregulated genes ($p$.adj<0.01) were mainly mapped to glycosaminoglycan binding, cytokine binding, cytokine activity, integrin binding, and other pathways related to immune stress, which matched the BP results (Figure 4(c)). Downregulated genes mainly enriched gated channel activity, ion channel activity, passive transmembrane transport protein activity, etc. (Figure 4(d)), all of which is related to the utilization of glucose by tissues due to downregulation, causing an imbalance in glucose transport. The enrichment analysis results for GO-CC ($p$.adj < 0.01) are shown in Figures 4(e) and 4(f).

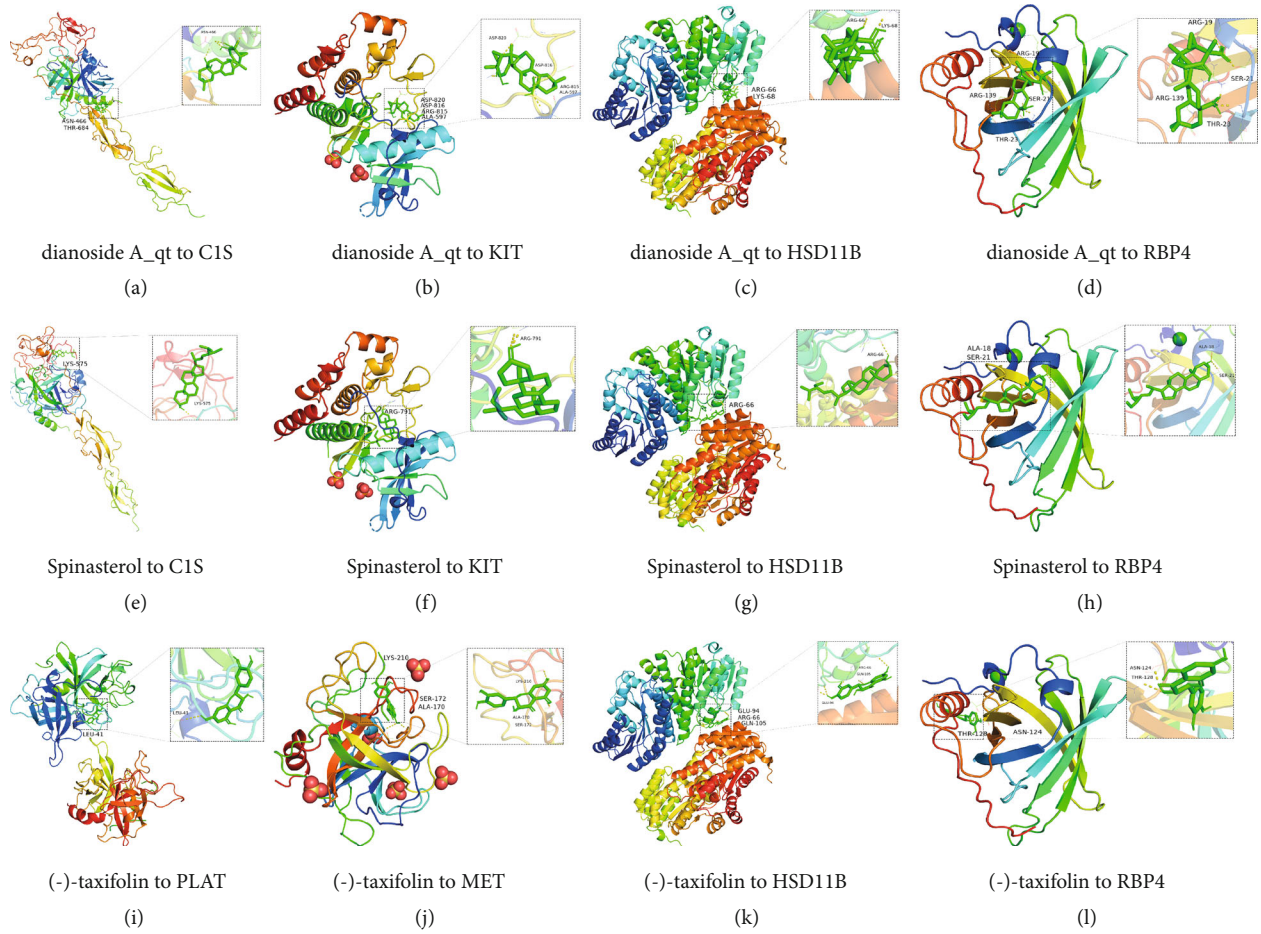



Figure 12: The docking mode and interactions between compounds and target proteins.

In the ND-IGT process, the two physiological statuses are similar, and the number of DEGs is small. The GO enrichment results were included in ND-T2D and IGT-T2D, indicating that inflammation and decreased insulin secretion are two factors that accelerate the process in the early stage of T2D. For BP terms in the IGT-T2D process, upregulated genes were enriched in the humoral immune response, neutrophil activation-mediated immune response, immune response effector, lymphocyte-mediated immune response, and leukocyte-mediated immune response. This shows that during the progression of IGT to T2D, the inflammatory response that accelerates the consumption of pancreatic islet $\beta$-cells is an important factor leading to the progression of T2D. Downregulated genes are enriched in pathways such as insulin secretion, positive feedback of hormone secretion, positive regulation of insulin secretion, hormone transport, and lipid digestion and utilization. Contrary to the ND-T2D process, IGT-T2D downregulated genes are also enriched in lipid-related pathways, which shows that lipotoxicity may also affect the course of T2D. The results of the CC and MF items were similar to those of the ND-T2D enrichment analysis.

*4.2. KEGG Enrichment Analysis.* In the ND-T2D group, the upregulated expression gene enrichment ($p < 0.05$) was found in cytokine-cytokine receptor interaction, MAPK signaling pathway, Hippo signaling pathway, TGF-beta signaling pathway, TNF signaling pathway, diabetes AGE-RAGE signaling pathway, IL-17 signaling pathway, ECM-receptor interaction, NF-kappa B signaling pathway, Toll-like receptor signaling pathway, and NOD-like receptor signaling pathway (Figure 5(a)). The Hippo signaling pathway regulates cell proliferation and apoptosis to ensure that organ size is normal [32]. Studies have shown that the imbalance of this pathway may be one of the reasons why T2D patients are more likely to develop tumors [33], and the MAPK and TGF-beta signaling pathways have also been shown to be considerably related to cell proliferation. Cytokine-cytokine receptor interaction, Hippo signaling pathway, TNF signaling pathway, AGE-RAGE signaling pathway in diabetes, IL-17 signaling pathway, and ECM-receptor interaction, NF-kappa B signaling pathway, Toll-like receptor signaling pathways, and NOD-like receptor signaling pathways are all related to the onset of inflammation. Excessive levels of inflammatory factors in the cellular environment accelerate cell senescence and death, resulting in excessive consumption of abnormal pancreatic islet $\beta$-cells [34]. Downregulated genes were enriched in pathways such as insulin secretion, young diabetes, and cAMP signaling (Figure 5(b)). The results of KEGG and GO enrichment analyses are consistent, which enhances the credibility of the GO enrichment results and further shows that the cause

of T2D is inflammation and insufficient insulin secretion. However, KEGG was enriched in the MAPK signaling pathway, TGF-beta signaling pathway, and other pathways related to cell proliferation, indicating that the cell cycle is also affected in the process of T2D. KEGG results of ND-IGT showed that the main signaling pathways affected were immune-related NF-kappa B signaling pathway, cytokine-cytokine receptor interaction, TNF signaling pathway, and lipolysis of fat cells. The regulatory pathways of serotonin were also enriched, which means that when the patients progressed to the IGT stage, the lipid metabolism disorder was a relatively apparent phenomenon. During the progression from the intermediate IGT state to the irreversible T2D state, the cAMP signaling pathway, insulin secretion, fat digestion and absorption, and other pathways indicated that the level of insulin secretion, cell growth, and the ability of the body to utilize lipids have declined.

*4.3. WGCNA Analysis.* The height cut-off value was set at 80, and 2 outlier samples were excluded in our analysis (Supplementary Figure 4).

Since scale independence reached 0.9 and average connectivity was high (Figure 6(a)), the soft threshold power $\beta$ was set to 4 in subsequent analyses. Furthermore, we constructed a gene network and identified the modules. For cluster splitting, the soft thresholding power was set to 4, the minimum module size was set to 30, and DeepSplit was set to 2 (which implies medium sensitivity). Finally, 45 gene coexpression modules were constructed (Figure 6(b)).

We correlated the modules with clinical characteristics (ND/IGT/T2D) and searched for the most significant associations. The results of this analysis showed that the turquoise and purple modules were markedly correlated with T2D (Figure 6(c)).

We conducted GO and KEGG analyses of genes in the turquoise and purple modules (Supplementary Figures 5 and 6). The results of these analyses showed that regarding BP, the genes of the turquoise module were mainly enriched in synapse organization, and the genes of the purple module were mainly enriched in the regulation of protein secretion. As for CC, the genes of the turquoise module were mainly enriched in microtubules, and the genes of the purple module were mainly enriched in the extracellular exosome. Finally, regarding MF, the genes of the turquoise module were mainly enriched in tubulin binding, and the genes of the purple module were mainly enriched in calcium ion binding. We then performed KEGG analysis of the genes in the two modules and identified the module-regulated pathways. The results of this analysis showed that the purple module-regulated pathways included herpes simplex virus 1 infection and amino acid degradation, but the turquoise module was enriched for insulin resistance, glucagon signaling pathway, and insulin secretion.

The turquoise module contained 2597 genes. Using a gene significance (GS) over 0.2 and an MM over 0.8 as the cut-off criteria, 158 genes were identified as hub genes (Supplementary Table 2). Using GS > 0.2 and MM > 0.6 as the cut-off criteria, 156 genes were identified as hub genes from 225 genes in the purple module (Supplementary Table 2). There are genes that have been reported to be associated with T2D: TSPAN7, CNTN1, NOL4, NMNAT2, and TMEM196 in the turquoise module, and THNSL1, ZBED8, ZNF420, ZNF512, and KBTBD7 in the purple module.

The WGCNA results reinforce previous findings and suggest that inflammation and islet function are responsible for T2D because the hub genes are associated with the main T2D phenotype.

## 5. Active Compounds in GLQMW

This study is based on the fact that GLQMW can stabilize the blood glucose levels of patients with T2D in the long-term clinical practice of TCM, and even when the patient reduces the use of hypoglycemic drugs and exogenous insulin, it can ensure that the blood glucose level of the patients is maintained at a normal level. Therefore, in our study, we investigated the components of GLQMW, whether its compounds can have potential hypoglycemic effects. GLQMW is composed of five herbs: THF, FZ, FL, QM, and SY. The compounds in GLQMW that met the screening criteria were QM 1, THF 2, and FL 15 (two compounds, poricoic acid A, and poricoic acid B were selected to join the study based on literature reports [35]), FZ 16 (choose 5 have been reported, OB and DL meet the conditions but Caco-2 does not meet the screening criteria, add the drug screening results [36, 37]), and yams 16 (add a compound that does not meet the Caco-2 screening criteria [38]). A total of 50 compounds were included in our study (Supplementary Table 3).

## 6. Target of Compounds Prediction

The PharmMapper platform was used to input the 3D structure of the drug-like compound and predict the human target protein by matching the pharmacophore. In this study, the $Z$-score was used as the basis for judging the strength of the correlation between the compound and the target protein, and the predicted targets that met the standard ($Z - \text{score} > 1.0$) were included in further studies. Figure 7 shows the correspondence between drug-like compounds and the predicted targets. We then performed a statistical analysis on these predicted targets. Several proteins, such as corticosteroid 11-b-dehydrogenase isoenzyme 1 (HSD11B1), estradiol 17-b-dehydrogenase 1, vitamin D3 receptor (VDR), insulin-like growth factor, glutathione S-transferase A1, SEC14-like protein 2, mineralocorticoid receptor, 72 kDa Type IV collagenase, oxysterol receptor LXR-alpha, and bile acid receptor, that are frequently targeted by the compounds may be the specific targets for GLQMW in patients with T2D.

## 7. GO/KEGG Enrichment Analysis for GLQMW Targets

GO enrichment analysis was performed on the target genes of GLQMW ($p.\text{adj} < 0.05$). The top 30 significantly enriched GO-BP terms are listed in Figure 8(a). The results clearly demonstrate that numerous targets are involved in various biological processes associated with fatty acid metabolism, regulation

of MAP kinase activity, hormone-mediated signaling pathways, positive regulation of protein kinase B signal transduction, and response to oxidative stress. The GO-CC enrichment analysis results shown in Figure 8(b) demonstrated that GLQMW acts mainly on the cell membrane, plasma membrane, extracellular space, and ficolin-1 rich granular cavity, which are closely related to insulin secretion. The results of GO-MF enrichment analysis shown in Figure 8(c) demonstrate that GLQMW targets are involved in antioxidant activity, phosphotyrosine residue binding, protein serine/threonine kinase activity, bile acid binding, and insulin receptor binding.

KEGG analysis of the GLQMW target ($p < 0.05$), Figure 8(d), showed significant enrichment in inflammation-related pathways such as focal adhesion, chemokine signaling pathway, NF-$\kappa$B signaling pathway, NOD-like receptor signaling pathways, natural killer cell-mediated cytotoxicity, IL-17 signaling pathway, relaxin signaling pathway, and T-cell receptor signaling pathway. These pathways, as per our clinical observations, regulate the levels of related inflammatory factors in pancreatic islet tissues. Pathways related to glucose and lipid metabolism, such as those of type II diabetes, diabetic cardiomyopathy, endocrine and insulin resistance, insulin signaling, atherosclerosis, and adipocytokine signaling, play an important role in insulin resistance.

## 8. Network Analysis of GLQMW Active Compounds

The predicted target of GLQMW and the degrees of all stages of T2D were intersected, and 18 target proteins were identified (Figures 9(a) and 9(b)). The targets of GLQMW in ND-IGT are C1S, RBP4, and C1R. Targeted genes in IGT-T2D include KDR, HADH, HSD11B1, ALB, RBP4, KIT, HMOX1, PLAT, and CFB. In the T2D stage, the targeted genes were MET, HADH, FABP4, EGFR, C1S, RBP4, PLAU, C1R, EPHA2, EPHX2, TGFB2, and PLAT.

Hepatocyte growth factor (HGF) binds to MET, activating signal transduction and the RAS and PI3K/Akt signaling pathways [39] to regulate cell growth and the cell cycle. Fatty acid-binding protein 4 (FABP4), abundantly expressed in adipocytes, plays an important role in adipocyte differentiation and lipid metabolism [40]. FABP4 in the serum is responsible for the transport of free fatty acids and can affect the regulation of systemic insulin sensitivity [41, 42]. Retinol-binding protein 4 (RBP4) is present in the serum and can assist the liver in releasing retinol into the systemic circulation to meet tissue needs, but high concentrations of RBP4 in the serum are associated with an increased risk of T2D [43, 44]. Doctors use vitamin A analogs such as fenretinide to increase urine excretion, reduce the high expression of RBP4 caused by a high-fat diet, and restore insulin sensitivity [45]. Therefore, these may be the potential targets by which GLQMW can treat T2D.

In our research, small-molecule compounds (Figure 10(a)) such as FZ16 and SY3, having multiple targets and a potential therapeutic effect on them, were included in the follow-up analysis to further interpret their mechanisms of action. Based on previous results, we analyzed the compounds with the target proteins of clinical drugs (Figure 10(b)) and found that THF2, FZ10, QM1, FZ16, and SY3 also act on the targets of drug action.

## 9. Similarity Analysis of Active Compounds

Based on the RDKit tool, the small-molecule compounds obtained were compared with all small-molecule drugs released from the DrugBank database, and the top 10 drug compounds with similarity were output. The drug similarities of (-)-taxifolin, dihydromyricetin, silibinin, hesperetin, epicatechin, and epigallocatechin gallate were 0.9958, 0.8417, 0.8119, 0.7952, and 0.7227, respectively (Figure 11(a)). Dihydromyricetin, a research drug, can inhibit oxidative stress, enhance neuroprotection, and reverse cognitive impairment caused by T2D and abnormal glucose and lipid metabolism in mouse models [46]. Silibinin has PPAR$\gamma$ agonist properties, and PPAR$\gamma$ is the specific target of thiazolidinedione in the treatment of T2D [47]. Hesperetin can regulate glucose metabolism by changing the activity of glucose-regulating enzymes and reducing lipid levels in the serum and liver [48]. Epicatechin works by increasing the expression of antioxidant enzymes, reversing the production of reactive oxygen species (ROS) in skeletal muscle and regulating autophagy involving mitochondria. Epicatechin can also increase the oxidation of muscle lipids and stimulate insulin-resistant skeletal muscle absorption of glucose [49]. Epigallocatechin gallate has a significant insulin-like effect on erythrocyte membrane-bound AChE to achieve a therapeutic effect [50]. The similarities between isofucosterol and cholesterol, beta-sitosterol, 25-hydroxycholesterol, 20-hydroxycholesterol, lanosterol, and dihydrotachysterol were 1.0, 1.0, 0.9785, 0.8571, 0.7526, and 0.7432, respectively (Figure 11(b)). Beta-sitosterol has been shown to lower blood sugar in clinical trials and T2D model mice [51], inhibit the serine phosphorylation of IRS induced by hyperlipidemia, and restore the expression of GLUT4 in adipose tissue [52]. Kim et al. showed that 20-hydroxycholesterol can inhibit fat formation in mice through a hedgehog-dependent mechanism [53], which is related to alleviating insulin resistance. 11,14-eicosadienoic acid is highly similar to linoleic, alpha-linoleic, oleic, palmitoleic, and gamolenic acids (Figure 11(c)). Linoleic acid and alpha-linoleic acid, essential fatty acids, have been shown to improve liver fat deposition and insulin resistance [54] and inhibit the expression of IL-1$\beta$ and Toll-like receptors, exerting anti-inflammatory effects [55]. Gamolenic acid inhibits the expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) to reduce the degree of inflammatory response, and it also affects the aggregation of the extracellular matrix (ECM) in patients with diabetic nephropathy [56]. Drugs with high similarity to dianoside A_qt were madecassic acid, ursolic acid, and asiatic acid (AA) (Figure 11(d)). The anti-inflammatory effect of madecassic acid regulates the dynamic balance of Th17/Treg cells because the low proportion of Treg cells leads to chronic inflammation [57]. Madecassic acid restores the Th17/Treg balance by regulating the PPAR$\gamma$/AMPK/ACC1 pathway, and therefore can reduce local inflammation [58]. Ursolic acid inhibits $\alpha$-amylase and $\alpha$-glucosidase activity by binding to

their inactivation sites and has been shown to rapidly reduce the blood glucose concentrations 2 h after a meal in animal models [59]. AA reduces the decomposition of glycogen to glucose and is released into the blood by inhibiting the translation of GSK-3$\beta$ and glucose 6-phosphatase [60]. Particularly, AA prevents islet dysfunction by downregulating islet fibrosis caused by fibronectin [61]. In the results of spinasterol (Figure 11(e)), dihydrotachysterol, a vitamin D analog has proven that vitamin D and its analogs can be used to assist in the treatment of T2D [62]. Androstenediol can increase PPAR-$\gamma$ DNA-binding activity, activate the PPAR-$\gamma$ pathway to inhibit IL-6 and iNOS gene expression, and achieve the purpose of slowing down local inflammation [63]. 20-hydroxycholesterol has a regulatory function of circulating lipid levels. These compounds may serve as the basis for GLQMW in T2D treatment.

## 10. Molecular Docking and LIGPLOT

The docking results of the active compounds with C1S, HSD11B, KIT, MET, PLAT, and RBP4 are presented in Supplementary Table 4. Compound (-)-taxifolin showed the lowest binding energy with HSD11B (−7.5 kcal/mol), MET (−7.1 kcal/mol), PLAT (−7.6 kcal/mol), and RBP4 (−6.2 kcal/mol). Dianoside A_qt was found to have the lowest binding energy with C1S (−8.4 kcal/mol), HSD11B (-8.8 kcal/mol), KIT (−8.6 kcal/mol), and RBP4 (−7.5 kcal/mol). Spinasterol had the lowest binding energy with C1S (−9.2 kcal/mol), HSD11B (−8.5 kcal/mol), and KIT (−9.2 kcal/mol). Binding energy analyses showed that the active compounds in GLQMW formed stable conformations with the target proteins. The docking analysis between the selected compounds and target proteins is shown in Figure 12. Compound dianoside A_qt bound to C1S, forming hydrogen bond interactions with residues Asn466 and Thr684; HSD11B, forming hydrogen bond interactions with residues Arg66 and Lys68; KIT, forming hydrogen bond interactions with residues Asp820, Asp816, Arg815, and Ala597; and RBP4, forming hydrogen bond interactions with residues Arg139, Ser21, Arg19, and Thr23. When binding to HSD11B, (-)-taxifolin formed hydrogen bonds with residues Glu94, Arg66, and Gln105. Furthermore, when binding to RBP4, (-)-taxifolin formed hydrogen bond interactions with residues Asn124, Thr128, MET, Lys210, Ser172, and Ala170 (Figure 12). In addition, when binding to RBP4, C1S, HSD11B, and KIT, spinasterol formed hydrogen bond interactions with residues Ala18, Ser21, Lys575, Arg66, and Arg791, respectively. The ligand plot results, Supplementary Figure 7, show the hydrophobic interaction sites of the target protein.

## 11. Discussion

With societal development and improvements in living standards, T2D has become a major chronic disease that threatens human health because of its gradually increasing incidence. Currently, physicians tend to use drugs for T2D control, supplemented by diet and rhythmic exercise. However, due to the continuous increase in insulin resistance in the body, weakening of drug sensitization, and increase in drug resistance in the body, it is often difficult for T2D patients in the later stage to maintain normal blood glucose ranges. The patient's quality of life significantly deteriorates due to constant medication and insulin injections, resulting in decreased treatment efficacy. Therefore, it is necessary to develop a new clinically effective treatment strategy. TCM is a medical discipline that has been summarized and developed by the Chinese nation in long-term practice. GLQMW, which are briefly recorded in the "JinGuiYaoLue," have the function of treating T2D, but no scholars have summarized and discussed the effectiveness and treatment mechanism of TCM prescription. It is generally believed that the developmental trend of type 2 diabetes is ND-IGT-T2D, in which IGT is an intermediate state and not a stage of irreversible diabetes damage [3]. Therefore, if the T2D stage can be reverted to the IGT stage through adjustment of self-living habits, the T2D patient can then more appropriately achieve the purpose of curing T2D. In our study, during IGT, we found that inflammation and lipid metabolism disorders played a significant role in the process of ND-IGT. For the treatment of patients with IGT, focus on fighting inflammation and adjusting our diet to avoid high fat and sugar intake is mandatory. The progression of IGT to T2D involves abnormalities in $\beta$ cells, which mainly include a significant decrease in the number of pancreatic $\beta$-cells and a decrease in the function of $\beta$-cells to secrete and synthesize insulin. The decrease in the number of pancreatic $\beta$-cells is related to the disorder of the $\beta$-cell cycle [64], but the decrease or loss of islet $\beta$-cell function is associated with islet $\beta$-cell dedifferentiation, glucotoxicity caused by high concentrations of glucose [65], lipotoxicity caused by free fatty acids and their related products [6], and chronic inflammation in islet tissue [66] to a certain extent. Accordingly, doctors can design rational drug regimens according to the etiology of patients with T2D.

Based on the results of gene expression profiling and WGCNA, we derived the disease patterns and key targets of T2D. IL1RL1, a membrane receptor whose expression is continuously upregulated during the progression of T2D, can bind to IL-33 to activate the TH2 inflammatory response and eosinophilia [67]. IL-33 is also markedly upregulated in the T2D stage, which can competitively inhibit angiotensin II and phenylephrine, and over-activate the NF-$\kappa$B and MAPK signaling pathways [68]; thus, the IL-33 and IL1RL1 complex can sustainably induce local inflammation in islet tissue during ND-T2D progression. Similarly, IL-17, produced by Th17 cells, can effectively mediate neutrophil mobilization and acts as a proinflammatory factor that causes an inflammatory storm and enhances the intensity of the inflammatory response [69]. However, high expression levels of IL-17 cause chronic inflammation in pancreatic islets [70]. According to the results of WGCNA, RRAGD, PPM1E, PFKFB2, and CHL1 were hub genes in the major modules of T2D, and the expression of these genes continued to be downregulated with the prolongation of the disease course. RRAGD, a monomeric guanine nucleotide-binding protein, plays a crucial role in the regulation of the mTORC1 signaling cascade, promoting growth in response

to growth factors, energy levels, and amino acids [71]. When the expression of RRAGD is reduced, mTORC1 signaling pathway function is dysregulated, which affects insulin sensitivity [72]. PPM1E is in cAMP-activated protein kinase (AMPK) phosphatases and is a potential antidiabetic drug target [73]. PFKFB2 is one of the key enzymes of glycolysis, and its low expression will inevitably reduce glucose utilization, while CHL1 affects the migration and cell cycle of islet $\beta$-cells. Therefore, the results in this study further explain the pathogenesis of T2D from the molecular mechanism and provide new strategies for treatment, such as specifically reducing the degree of inflammation in the pancreatic islets. According to the results of the Connectivity map (CMap) database (Supplementary Figure 8), the HDAC inhibitor [74] may be a promising drug that can antagonize the ND-T2D process (Supplementary Table 5), and the spleen tyrosine kinase (SYK) inhibitor, fostamatinib [75], can reverse the progression of IGT-T2D (Supplementary Table 6).

In the results of network pharmacology, the main targets of the active ingredients of GLQMW were HSD11B1, VDR, TGR5, FXR, and RBP4, all of which mainly related to T2D. Such finding may be the basis for the therapeutic effectiveness of GLQMW. According to the GO/KEGG results of GLQMW targets, in addition to inflammation-related and diabetes phenotype-related pathways, GLQMW was shown to be involved in pathways related to cell proliferation, such as the MAPK, JAK-STAT, Ras, and PI3K-Akt signaling pathways. GLQMW may restore the number of $\beta$-cells and restore islet function based on its effect on cell proliferation. This is in line with the clinical observation that GLQMW is effective in T2D patients with failed insulin control; based on anti-inflammatory and insulin resistance properties, GLQMW stimulates the proliferation of $\beta$-cells to achieve therapeutic purposes. In the results of drug similarity analysis, we found that compounds in GLQMW, including spinasterol, isofucosterol, dianoside A_qt, 11,14-eicosadienoic acid, and (-)-taxifolin, have potential therapeutic functions in T2D. We can isolate compounds for further experimental validation to determine whether they can be used as parents for new T2D drugs. Therefore, in the future, purified natural compounds can be prepared with a composition ratio similar to that of TCM prescriptions to replace existing decoction application methods [76]. Although some of the compounds involved in this study have demonstrated pharmacological activity, the efficacy of GLQMW in T2D has not been fully explained. In order to further improve the reliability of the study, we still need to comprehensively verify the effectiveness of GLQMW from cells, animals, and clinics, and enhance its efficacy.

## 12. Conclusion

GLQMW treat T2D by anti-inflammatory and restoring islet cell function, and its potential therapeutically active compounds are (-)-taxifolin, dianoside A_qt, isofucosterol, 11,14-eicosadienoic acid, and spinasterol.

## Data Availability

The data in this study were obtained from public databases.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

## Authors' Contributions

Jiahao Feng drafted the manuscript. Jiahao Feng and Yuheng Zhou collected and analyzed the data. Liping Yu, Ping Yuan, and Li Liao analyzed immumohistochemical staining and drug search results. Jun Zhang reviewed this manuscript. All authors approved the final version of this paper.

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## Supplementary Materials

Supplementary figures and tables show transcriptome profiles of different stages of diabetes, protein molecular forces, and CMap predictions of diabetes-reversing drugs. *(Supplementary Materials)*

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*Review Article*

# Progress in the Relationship between Vitamin D Deficiency and the Incidence of Type 1 Diabetes Mellitus in Children

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Type 1 diabetes mellitus (T1DM) is an autoimmune disease, due to a large number of islet $\beta$ cells damaged, resulting in an absolute lack of insulin, ultimately relying on insulin therapy. Vitamin D is a fat-soluble sterol derivative that not only participates in calcium and phosphorus metabolism but also acts as an immunomodulatory role by binding to nuclear vitamin D receptors to regulate the expression of transcription factors. Increasing evidence has shown that vitamin D has immunoregulation and anti-inflammatory effects, and it may play a role in T cell regulatory responses due to downregulation in the expression of cathepsin G and inhibition of CD4+ T cell activation and protection of $\beta$ cells from immune attack and is beneficial in decreasing oxidative stress in T1DM patients. Epidemiologic evidence demonstrates involvement of vitamin D deficiency in T1DM pathogenesis, with the immune system improperly targeting and destroying its own islet $\beta$ cells. In addition, polymorphisms in genes critical for vitamin D metabolism may increase the risk of islet autoimmunity and T1DM. In this paper, the relationship between vitamin D deficiency and the molecular mechanism of T1DM was discussed.

## 1. Introduction

Diabetes is one of the major chronic diseases and seriously threatens human health. The global diabetes prevalence in 20–79-year-olds in 2021 was estimated to be 536.6 million people (10.5%), rising to 783.2 million (12.2%) in 2045 [1]. Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease that tends to occur in children and adolescents, resulting from autoimmune degradation of pancreatic $\beta$ cells leading to lifelong dependence on exogenous insulin. The number of children and adolescents (0–19 years old) with T1DM exceeded 1.1 million worldwide, and the number of new children and adolescents with T1DM was 128,900 per year, growing nearly 3% in the incidence rate [2]. The incidence of T1DM is increasing worldwide. Recent studies have shown that the overall population incidence of T1DM in China is 1.01 per 100,000 people per year [3], and the annual incidence of childhood with T1DM is about 2.02~5.3 per 100,000 [4]. The pathogenesis of T1DM has not been clearly defined so far, but it is generally thought to be due to multiple factors such as gene susceptibility and environment [5].

Serum vitamin D is bound to its binding protein, by which it is transported to the liver where it is converted to 25-hydroxy vitamin D [25(OH)D] by 25-hydroxylase; then, 25(OH)D is transformed to $1,25(OH)_2D$, the biological active form of vitamin D in the kidneys, through the action of the enzyme 25-hydroxyvitamin D-1alpha-hydroxylase (CYP27B1) [6]. The presence of CYP27B1 along with the vitamin D receptor (VDR) is found in several tissues [7, 8]. 1,25(OH)2D not only is involved in calcium and phosphorus metabolism but also acts in an immunomodulatory role by binding to nuclear VDRs to regulate the expression of transcription factors [9]. Vitamin D is closely related to the occurrence of autoimmune diseases [10, 11], which can play an important role in the pathogenesis of diabetes and glycemic control by inhibiting inflammatory and autoimmune responses, promoting insulin synthesis and secretion, and enhancing insulin sensitivity and vitamin D-related gene polymorphisms [12]. Vitamin D may play a role in T cell regulatory responses and defend $\beta$ cells from immune attack [13]. Research progress on the relationship between vitamin D deficiency and the pathogenesis of T1DM is reviewed.

## 2. Pathogenic Relationship between Vitamin D Levels and T1DM

Vitamin D deficiency is closely related to the occurrence, development, and complications of T1DM [14–16]. T1DM patients were reported to have lower 25(OH)D levels compared to age-matched controls in children and adolescents [17–20]. A case-control study in northern India with Borkar et al. [21] suggested that 58% of T1DM had a 25(OH)D deficiency (25-OHD level < 20 ng/mL or <50 mmol/L), while the control group was only 32%. A similar previous study in our team found that serum 25(OH)D levels in patients with T1DM were 48.69 ± 15.26 nmol/L, compared with 57.93 ± 19.03 nmol/L in the control group [22]. According to the criteria of serum, 25(OH)D <50 nmol/L was insufficient and <30 nmol/L was deficient, and 49.66% of the patients had vitamin D insufficiency or deficiency, compared with only 30.51% in the control group. In the newly diagnosed T1DM children, the deficient and insufficient patients were 64.2%, while it was 41.60% in established T1DM. A systematic review analysis concluded that insufficiency or deficiency of 25(OH)D was associated with the development of T1DM in children [15]. Meanwhile, animal experiments have also shown that vitamin D deficiency in early life could accelerate T1DM in nonobese diabetic mice [23]. These studies suggest that deficiency of vitamin D will increase the risk of developing type 1 diabetes.

## 3. Pathogenesis of Autoimmune Response in T1DM

T1DM is a multifactorial chronic autoimmune disease in which blood glucose levels increase due to impaired secretion of islet $\beta$ cells. It generally occurs in genetically susceptible individuals. During the development of T1DM, the immune system improperly targets and destroys its own islet $\beta$ cells, resulting in progressive damage to insulin production and secretion function, and when T1DM is diagnosed, approximately 70–80% of $\beta$ cell mass is destructed [24].

According to the genetic susceptibility to the disease and the targeting classification system [25–30], T1DM can be followed by four distinct stages:

(i) Stage 1 (islet autoimmunity). Subjects exhibit islet autoimmunity, as evidenced by the persistent presence of at least two islet autoantibodies [islet cell antibodies (ICAs), insulin autoantibodies (IAAs), glutamic acid decarboxylase 65 (GAD65), insulinoma-associated antigen 2 (IA-2), or zinc transporter 8 (ZnT8)]. During this stage, subjects remain normoglycemic and asymptomatic

(ii) Stage 2 (abnormal glucose tolerance). Subjects maintain multiple islet autoantibody positivity and remain asymptomatic, but display dysglycemia, as evidenced by impaired fasting glucose, an abnormal oral glucose tolerance test (OGTT), or HbA1c ≥5.7%

(iii) Stage 3 (symptomatic disease). Subjects experience the onset of clinical T1DM, which is often accompanied by symptoms such as polyuria, polydipsia, fatigue, weight loss, and diabetic ketoacidosis

(iv) Stage 4. Established/long-term disease

## 4. Molecular Mechanism of Vitamin D Inhibiting T Cell Activation in T1DM

The process of pancreatic islet infiltration by immune cells represents the histological hallmark of the autoimmune destruction of $\beta$ cells within the pancreatic islets. T1DM is mostly characterized by an inflammatory lesion [31], which is infiltrated by helper T cells (CD4+ or Th cells), cytotoxic T cells (CD8+), B lymphocytes, and macrophages, eventually leading to the destruction of islet $\beta$ cells [32, 33], and CD8 + cytotoxic T lymphocytes are the most frequent among the islet infiltrating immune cells. Helper T cells also play an important role in T1DM pathophysiology [34]. Autoreactive CD8+ T cells recognize major histocompatibility complex (MHC) class I-restricted islet autoantigens on the $\beta$ cell surface and exert cytotoxic effects through several effector mediators, particularly cytokines released by T helper type 1 (Th1) cells such as interferon (IFN)-$\gamma$ [35]. T1DM patients exhibit defects in the ability of regulatory T cells (Treg cells) to suppress the activity and proliferation of autoreactive CD4+ and CD8+ T cells [36, 37].

Vitamin D has multiple roles in the body and can mediate immunomodulatory functions related to innate immune responses, antigen presentation, and adaptation to the immune system [38]. VDRs are expressed not only in islet $\beta$ cells but also in immune cells, including activated T cells. Vitamin D has the effect of regulating islet $\beta$ cells, increasing insulin sensitivity, and reducing the expression of inflammatory factor-induced apoptosis gene-related proteins to protect islet cells [39]. Vitamin D deficiency can cause abnormal glucose tolerance, impair the transcription of islet cell function genes [40], and increase the risk of developing type 1 diabetes and type 2 diabetes [41]. Vitamin D regulates the immune system by binding to receptors [42, 43].

Vitamin D can regulate T cells, promote CD4+ T cells to Th2 and Treg cell differentiation, reduce the production of Th1 and Th17 cells, and decrease the proportion of Th1/Th2. Vitamin D will also affect the production of cytokines, stimulating immune cells to release anti-inflammatory cytokines including IL-4, IL-10, and TGF-$\beta$ while weakening the production of proinflammatory cytokines such as IFN-$\gamma$, IL-1$\beta$, IL-2, IL-6, IL-12, IL-17, IL-22, and TNF-$\alpha$ [44].

Autoantigens are presented by MHC at the beta-cell surface, and both CD4+ and CD8+ T cells isolated from peripheral blood could recognize epitopes in major islet autoantigens [32, 33]. Vitamin D promotes the maturation of monocytes into macrophages but at the same time decreases the ability of monocytes to present antigens to T cells by reducing the expression of superficial major histocompatibility complex MHC-II [45]. It also impairs dendritic cell maturation, leading to the formation of tolerant dendritic cells without surface MHC molecules and consequently being unable to present antigens [46]. Impaired antigen presentation by antigen-presenting cells (APCs)

leads to unresponsive T cells, thereby inhibiting and/or impinging B cell proliferation, plasma cell differentiation, formation of memory $\beta$ cells, and production of autoantibodies [47].

When vitamin D is deficient, the inhibitory effect on T cells is weakened and the chemokines secreted by T cells increase, which can recruit immune cells to participate in autoimmune attacks on $\beta$ cells [48]. At the same time, cytokines released by immune cells can upregulate the expression of MHC-I and MHC-II and adhesion molecules on islet $\beta$ cells, promoting the interaction of $\beta$ cells with cytotoxic T cells and inducing apoptosis. The continued progression of vitamin D deficiency is also accompanied by impaired proliferative activity of T cells and imbalances in the proportion of regulatory T cells (CD4+) and CD8+ T cells while vitamin D supplementation normalizes the proliferative activity of T cells and the proportion of T cell subsets. Figure 1 displays the immunomodulatory and anti-inflammatory actions of $1,25(OH)_2D$.

The immunomodulatory effects of vitamin D, promoting immune tolerance and induction of T cell anergy, impinging on B cell activity and antibody production, and reducing the inflammatory response, suggest the therapeutic potential of calcitriol in autoimmune diseases including T1DM.

## 5. Mechanism of Vitamin D Downregulating CatG Expression in T1DM

Numerous studies in vivo and in vitro have revealed potent anti-inflammatory actions of vitamin D that affect the major cellular players associated with autoimmune disease [49–51]. T1DM is an autoimmune disease attributed to progressive injury of islet $\beta$ cells mediated by T cells. Cathepsin G (CatG) is involved in the antigen presentation of proinsulin. It was demonstrated by Zou et al. that CatG could impact the activation of CD4+ T cells in nonobese diabetic (NOD) mice. During the pathogenesis of diabetes, the expression level of CatG in NOD mice gradually increased and the CD4+ T cells were gradually activated, resulting in more Th1 cells and fewer Th2 and Treg cells. Treatment with a CatG-specific inhibitor could reduce the blood glucose level, improve the function of islet $\beta$ cells, and reduce the activation of CD4+ T cells [52]. A new study further discovered that vitamin D supplementation could improve pancreatic $\beta$ cell function and suppress immunological and inflammatory reactions in the T1DM mice. The overexpression of CatG in diabetes tissue samples was documented and then showed that vitamin D supplementation normalizes the islet immune microenvironment through downregulating CatG expression in T1DM mice. Experiments in vitro subsequently elucidated that vitamin D supplementation could impede CD4+ T activation by downregulating CatG expression and thereby enhancing pancreatic $\beta$ cell function [53]. Therefore, vitamin D could downregulate the expression of CatG to inhibit CD4+ T cell activation and prevent the destruction of islet $\beta$ cells by immune cells and is expected to play a therapeutic role as an immunomodulator in type 1 diabetes.

## 6. Vitamin D Inhibiting Oxidative Stress in T1DM

Environmental factors can also trigger the onset of T1DM and influence its progression. The factors include viral infections, toxins, reactive oxygen species (ROS), and chronic inflammation, which are triggers of endoplasmic reticulum (ER) stress. ER stress initiates the unfolded protein response (UPR), which acts through inositol-requiring protein-1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6), all of which are localized to the ER membrane and respond to stress by transmitting ER signals to the cytoplasm and nucleus. Although UPR initially attempts to mitigate ER stress, if the stress is prolonged or severe, it switches from a proapoptotic to a problematic response [54, 55]. It has been reported that modulating the unfolded protein response (UPR) in $\beta$ cells of nonobese diabetic (NOD) mice by deleting the UPR sensor IRE1$\alpha$ prior to insulitis induced transient dedifferentiation of $\beta$ cells, resulting in substantially reduced islet immune cell infiltration and $\beta$ cell apoptosis, and IRE1$\alpha$-deficient mice exhibited significantly fewer cytotoxic CD8+ T cells in their pancreata, and adoptive transfer of their total T cells did not induce diabetes [54]. Inflammation in pancreatic islets occurs early during the pathogenesis of T1DM. Proinflammatory signaling in $\beta$ cells might be conducive to the immunogenicity of $\beta$ cells. The recent study supports the contention that inflammatory signaling in $\beta$ cells promotes autoimmunity during T1DM progression [55]. It has been shown that $\beta$ cells of prediabetic NOD mice display dysfunction and overt ER stress that may be driven by NF-$\kappa$B signaling, and strategies that attenuate pathways leading to ER stress may preserve $\beta$ cell function in T1DM [56].

Vitamin D may reduce oxidative markers such as superoxide dismutase (SOD) in T1DM, which have high insulin and C-peptide levels in the treatment group compared to other groups [57]. Interestingly, in a study, calcitriol plays a prominent role in suppressing ROS, regenerating glutathione (GSH), and reversing the pro-atherogenic phenotype in human umbilical vein endothelial cells caused by hyperketonemia [58]. One main source of oxidative stress in pancreatic $\beta$ cells appears to be the reactive oxygen species producer NADPH oxidase (NOX) enzyme, which has a role in $\beta$ cell death [59].

Presumably, vitamin D supplementation may be beneficial in reducing oxidative stress in patients with T1DM. Further studies need to be carried out to demonstrate the effect of vitamin D in the prevention of T1DM where inducing $\beta$ cell dedifferentiation, prior to insulitis, allows these cells to escape immune-mediated destruction.

## 7. Polymorphisms of Vitamin D Metabolism Genes and T1DM

Different polymorphisms of genes, such as encoding vitamin D hydroxylases and VDR, may influence the risk of islet autoimmunity and T1DM [27]. Serum vitamin D levels are influenced by variants in genes involved in the synthesis,

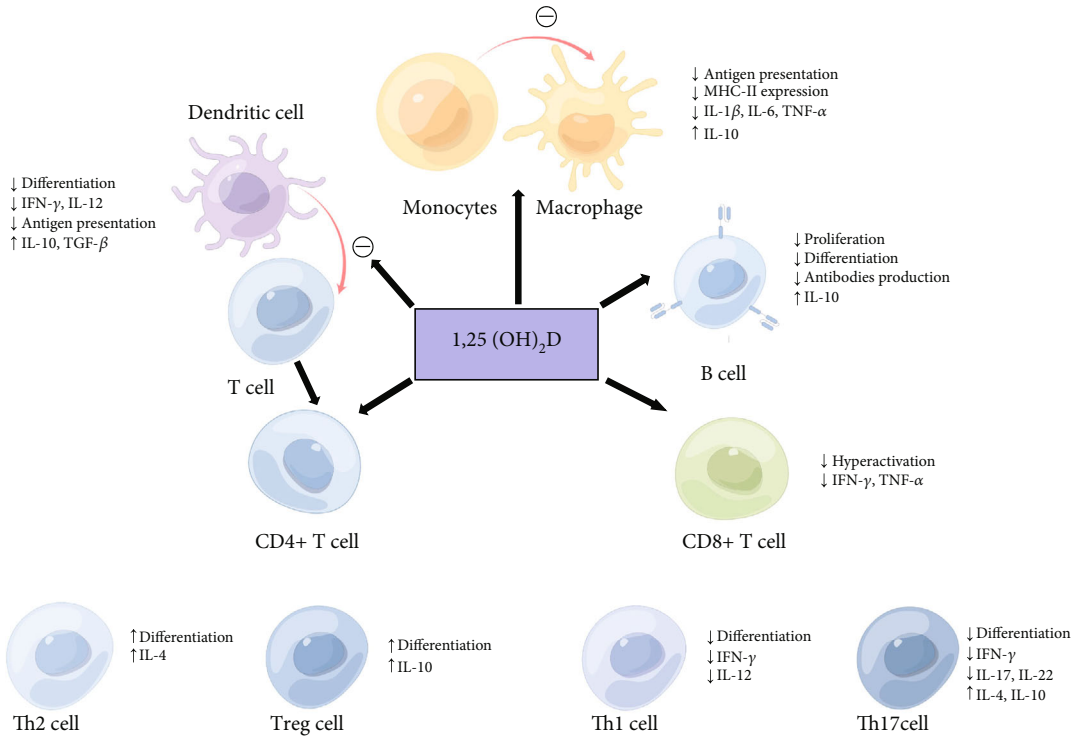


Figure 1: A review of anti-inflammatory and immunoregulation of 1,25(OH)2D on immune systems [27, 44]. ↓ or represents downregulation, and ↑ represents upregulation. IFN-$\gamma$, interferon gamma; IL-1$\beta$, interleukin 1$\beta$; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-12, interleukin 12; IL-17, interleukin 17; IL-22, interleukin 22; MHC-II, major histocompatibility complex-II; TGF-$\beta$, transforming growth factor-beta; TNF-$\alpha$, tumor necrosis factor-alpha.

transport, hydroxylation, and degradation of vitamin D. A relationship between single-nucleotide polymorphisms (SNPs) of the gene encoding the vitamin D 25-hydroxylase in CYP2R1 and T1DM was studied. A case-control study of 252 children less than 20 years old found that vitamin D deficiency was more prevalent in children with T1DM than in healthy controls. CYP2R1 rs12794714 and rs10766196 polymorphisms were associated with a high risk of T1DM, and polymorphisms in vitamin D metabolism could lead to susceptibility to T1DM in Korean children [60]. Another similar study showed a cumulative effect of SNPs at the CYP2R1 (rs2060793), DHCR7 (rs12785878), GC (rs2282679), and CYP24A1 (rs6013897) loci on the susceptibility to type 1 diabetes [61]. Tangjittipokin et al. [62] found SNPs and T1DM in CYP2R1 (rs10741657) (GA, OR: 1.83, 95% CI: 1.01–3.31; $p = 0.04$). CYP27B1 (rs4646536) was negatively associated with 25(OH)D) levels, and CYP27B1 (rs4646536) and GC (rs2282679) were positively associated with TNF-$\alpha$ levels. Hussein et al. [63] reported that the GG genotype of CYP2R1 (SNP rs10741657) or CC genotype of CYP27B1 (SNP rs10877012) increased the risk of developing T1D in Egyptian children.

A potential role of single-nucleotide polymorphisms (SNPs) of the VDR gene in T1D, including FokI (rs10735810), ApaI (rs7975232), TaqI (rs731236), and BsmI (rs1544410), has been reported. Results by Rasoul et al. [64] demonstrated a significant effect of two VDR gene polymorphisms (FokI and TaqI) (FokI, C>T, rs10735810, and TaqI, C>T, rs731236) on the genetic susceptibility of T1DM in Kuwaiti Arabs; meanwhile, the VDR gene ApaI (G>T, rs7975232) and BsmI (A>G, rs1544410) polymorphisms were not associated with T1DM.

Tangjittipokin et al. [62] found that VDR gene-related variations of ApaI (rs7975232), TaqI (rs731236), and BsmI (rs1544410) were negatively associated with vitamin D and IL-10 levels in children with T1DM. Norris et al. [65] showed that higher serum 25(OH)D levels are associated with a lower risk of islet autoimmunity in children at increased genetic risk of T1DM, and the association between childhood 25(OH)D status and islet autoimmunity was modified by the ApaI (rs7975232) SNP in VDR (interaction $p = 0.0072$), where for each additional minor allele, higher 25(OH)D concentrations were associated with a greater reduction in islet autoimmunity risk. Therefore, vitamin D and VDR may play a combined role in the development of islet autoimmunity among children with increased genetic risk for T1DM.

The relationship between inherited variation in vitamin D genes and diabetes has been addressed in recent reviews and meta-analyses [66–68]. However, there were many studies that did not confirm these results [69]. There was a lack of association of vitamin D receptor gene polymorphisms of VDR genes including FokI, (rs10735810), ApaI (rs7975232), TaqI (rs731236), and BsmI (rs1544410) with susceptibility to T1DM in the Portuguese population [70]. Thorsen et al. [71] did not find an association between SNPs in CYP2R1, CYP27B1, VDR, and GC and the risk of T1DM in a juvenile Danish population, though 25(OH)D levels

were associated with variants in the GC gene. A systematic review and meta-analysis of genetic evidence showed no large effect of a genetically determined reduction in serum 25(OH)D concentrations by selected polymorphisms on T1DM risk, despite the strong association seen in some observational studies [72].

These studies suggest that SNPs in genes critical for the synthesis, transport, and action of vitamin D may affect the risk of T1D development. The polymorphisms may be associated with decreased VDR, 25-hydroxylase, and 1α-hydroxylase activity and expression. In order to investigate the relationship between T1DM pathogenesis and SNPs in genes involved in vitamin D metabolism, more prospective studies are needed.

## 8. Effects of Vitamin D Interventions on T1DM

Vitamin D deficiency can affect T1DM, and low vitamin D levels are strongly associated with ketoacidosis in children with new-onset type 1 diabetes [73]. Even in patients with T1DM who have been diagnosed for several months, vitamin D deficiencies are still a cause for concern [22, 74]. Studies have shown that by simply regulating the intake of vitamin D in children with T1DM without changing the amount of insulin, glycated hemoglobin (HbA1c) can also be better controlled [75]. From another perspective, vitamin D deficiency can affect blood glucose control in children with T1DM. Studies have revealed that T1DM children with vitamin D deficiency are more likely to have hypoglycemia and metabolic diseases [76]. The possible role of vitamin D supplementation, as an additional therapy, to increase glycemic control and insulin sensitivity opens new perspectives to increase the control of the disease and improve the health of these patients [77]. In a double-blind randomized controlled study by Treiber et al. [78], vitamin D supplementation in T1DM children was shown to enhance the inhibitory capacity of Treg cells and increase the proportion of Treg cells, while reducing the need for fasting blood glucose, HbA1c, and exogenous insulin. This suggests that vitamin D supplementation can affect the onset and development of T1DM and that patients with T1DM with inadequate vitamin D levels should be treated with vitamin D.

Regular vitamin D supplementation in early infancy may reduce the risk of T1DM [79–81]. A birth cohort study of more than 10,000 children found similar protective effects [82]. Therefore, in the first years of life, vitamin D deficiency should be promptly diagnosed and appropriately treated, especially in children at high genetic risk for T1DM, which is defined by a family history of T1DM and islet autoantibodies and/or human leukocyte antigen (HLA) positivity. Vitamin D should be considered an additional therapy.

## 9. Conclusion

T1DM is an autoimmune disease characterized by the T cell-mediated destruction of insulin-producing $\beta$ cells in pancreatic islets. Vitamin D has immunomodulatory and anti-inflammatory actions, and its deficiency may play a role in the pathogenesis of T1DM, indicating hypovitaminosis D as an important environmental factor for the development of the disease. Vitamin D has a role in T cell regulatory response by protecting $\beta$ cells from immune attack and is beneficial in reducing oxidative stress in patients with T1D. However, studies on vitamin D supplementation and preservation of $\beta$ cell function in T1DM have no conclusion. In the future, large-scale prospective trials are needed to fully evaluate the role of vitamin D as a disease modifier for T1DM.

## Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

He LP and Zhu T wrote the first draft; Gu W, Song YX, and Liu CW contributed to the writing and editing of the manuscript; He LP conceptualized the topic and proofread the manuscript. All authors provided supervision and approved the submission of this minireview.

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*Research Article*

# The Amelioration of Detrimental Biochemical Anomalies by Supplementing B, C, and E Vitamins in Subjects with Type 2 Diabetes Mellitus May Reduce the Rate of Development of Diabetic Retinopathy

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Excessive intracellular glucose in insulin-independent tissues including nerve, nephron, lens, and retina invites mishandling of metabolism of glucose resulting in a background of increased oxidative stress, advanced glycation end products (AGE) formation, lipid peroxidation, and failure of antioxidant defense systems in type 2 diabetes mellitus (T2DM). All these detrimental biochemical anomalies ultimately attack biological membranes and especially capillary beds of the retina, resulting in the breakdown of the inner blood-retinal barrier and the initiation of diabetic retinopathy (DR). If these disarrays are corrected to a large extent, the development of DR can be avoided or delayed. In this prospective clinical trial, 185 patients with T2DM who received B vitamins, vitamin C, and vitamin E along with antidiabetic medication for five years demonstrated a slower rate of the development of DR and reduced abnormal biochemical mediators like reactive oxygen species (ROS), malondialdehyde (MDA), AGE, and vascular endothelial growth factor (VEGF) compared to 175 T2DM individuals who were treated with only antihyperglycemic drugs.

## 1. Introduction

Though duration and uncontrolled hyperglycemia are considered to be the contributors to the pathogenesis of DR, some individuals with T2DM keep away from the development of complications from this disease for a prolonged period [1, 2]. We have illustrated in our previous studies that DR is a two-stage disease where the initial unseen part of apoptosis of pericytes and endothelial cells is guided by toxic mediators of hyperglycemia-induced biochemical derangements, and the manifested stage from microaneurysms to neovascularization is dictated by an increased secretion of VEGF [3, 4].

Initial enormous intracellular glucose activates enhanced glycolysis and tricyclic acid (TCA) pathway as far as possible. All of the useful energy liberated during oxidation of nearly all of the carbohydrates is made accessible within the mitochondria as reducing equivalents (-H or electrons). Hypermetabolic retinal cells generate energy by reducing molecular oxygen to water. During this process, some amounts of partially reduced reactive oxygen forms are produced as an unavoidable byproduct of mitochondrial respiration. Some of these forms are liberated as free radicals that can damage lipids, proteins, and nucleic acids [5]. They are defined as ROS. Consequently, cells create a defense system to prevent injury caused by these free radical products.

An imbalance between free radical generating and radical scavenging systems results in oxidative stress, a condition that is associated with endothelial injury and dysfunction [6, 7]. Enhanced glycolysis and TCA cycle loaded with excessive fuel cannot continue for an indefinite period. There must be gradual exhaustion of oxidized cofactors which are integral parts of these two essential metabolic pathways. Therefore, the sequential paths will follow increased anaerobic glycolysis resulting in lactate-induced lowering of cellular pH and a decrease in essential enzymatic activities. The other side of this pathway invites glutamate cascade leading to lipid peroxidation and lipid-derived free radicals. Above all, significant portions of unutilized glucose are forced to enter into nonenzymatic glycation end product formation which takes an important role in the pathogenesis of DR [8].

Among the different detrimental pathways regarding enhanced glycation, oxidative stress, and lipid peroxidation derived from chronic hyperglycemia, cells attempt to create a barrier or defense system to maintain normal physiology. Superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), and reduced glutathione (GSH), which are the normal cellular protectors against the development of damaging toxicity of free radicals and lipid peroxides, are severely affected by advanced AGEs, MDA, and ROS [3, 4, 6].

Considering the above-mentioned detrimental biochemical pathways occurring in T2DM, we attempted to reduce the toxicity of these routes to prevent the development of DR by treating with antihyperglycemic drugs supplemented with B vitamins, which act as the source of nicotinamide adenine dinucleotide (NAD+); flavin adenine dinucleotide (FAD+), an inhibitor of glycation; and vitamin E ($\alpha$-tocopherol) and vitamin C (ascorbic acid), which function as the inhibitor of lipid peroxidation and scavengers of free radicals. This prospective cohort study tests the effectiveness of alternative management of T2DM in preventing or delaying DR development.

## 2. Methodology

The study was conducted on consecutively selected individuals who were diagnosed with T2DM between March 2012 and December 2012 at the "Diabetic Clinic" of the Medical College, Kolkata, and referred to the "Retina Clinic" of the "Regional Institute of Ophthalmology" (RIO), Medical College, Kolkata, for an eye check-up. A trained ophthalmologist examined each subject, and those who did not have any clinical evidence of DR (DWR) were included in to the study. As the study attempted to determine if treating DWR patients with antioxidants (vitamins C and E), B vitamins, and antihyperglycemic drugs could reduce development rates of DR compared to treating them only with antihyperglycemic drugs, study subjects were divided into two groups, A and B, and followed up each one-year interval throughout the following five years. The number of subjects in each study group was estimated by using the following formula from Chow et al. [9].

$$n = \frac{\left[Z_{\alpha/2}\sqrt{p_0(1-p_0)} + Z_{\beta}\sqrt{p_1(1-p_1)}\right]^2}{(p_1 - p_0)^2}. \quad (1)$$

The sample size is based on the testing problem $H_0 : p = p_0$ against $H_a : p = p_1$, where $p_1 \neq p_0$. In our context, $p_0$ is the cumulative five-year incidence of DR following standard treatment regime, and $p_1$ is the expected cumulative five-year incidence of DR under the proposed treatment regime. Following Cikamatana et al. [10], we assume $p_0 =$ 22.2% while we expect $p_1$ to be 12.2%, indicating that the proposed treatment regime may reduce the progression of DR by $(p_0 - p_1) = 10\%$, the effect size for our study. Considering 5% level of significance ($\alpha$) and 90% desired power $(1 - \beta)$, the minimum size for each group was set as $n =$ 183 at the baseline, considering 20% rate of attrition. Finally, 185 subjects in Group A and 175 subjects in Group B were enrolled at the baseline into the study.

Subjects with T2DM were diagnosed based on the guidelines of the American Diabetic Association [11]. The study was approved by the "Institutional Ethics Committee" of the RIO, Medical College, Kolkata (Ref. No. RIO/MC/KOL/NON-SPON/119/11-2011), and informed consent was collected from all the patients according to the declaration of Helsinki. Further, the trial was registered with the clinical trial registry of India at http://ctri.nic.in (CTRI/2011/091/000192). The inclusion criteria were (1) subjects with T2DM who can only be treated with oral antihyperglycemic medicines and expected to remain physically stable during the course of the study with such medication; (2) best-corrected visual acuity $(VA) \geq 6/9$ in each eye with no DR; and (3) literate patients who could give informed written consent and could read the Snellen VA chart. The exclusion criteria were (1) presence of any types of DR with or without diabetic macular edema (DME) as determined by dilated fundoscopic examination, spectral-domain optical coherence tomography (SD-OCT), and digital fundus photography; (2) hyperglycemic individuals who needed insulin to manage their condition; (3) the presence of concomitant conditions such as branch retinal vein occlusion, Eales' disease, or glaucoma in the eyes could alter the normal occurrence of microvascular complications; and (4) patients suffering from hypertension, coronary artery diseases, or strong family history of coronary artery diseases, peripheral vascular disease, chronic infection, thromboembolic events, and urinary microalbumin > 300 mg/dl were some of the conditions considered as exclusion criteria for this study. The presence of any type of DR among study subjects was diagnosed according to the modified guideline of "Early Treatment of Diabetic Retinopathy Study (ETDRS)" [12].

*2.1. Treatment Protocol.* Among study groups, Group A was given standard doses of the following: (i) vitamin B containing thiamine mononitrate 10 mg, (ii) riboflavin 10 mg, (iii) nicotinamide 45 mg, (iv) pyridoxamine HCl 3 mg, (v) calcium pantothenate 50 mg, (vi) cyanocobalamin 15 mcg (Neurobion Forte, MERCK PHARMA), (vii) ascorbic acid 500 mg (Celin, GlaxoSmithKline Pharmaceuticals), and (viii)

lastly, $\alpha$-tocopherol 400 (Evion, MERCK LTD India) along with required oral antihyperglycemic drugs. Group B was treated with only oral antihyperglycemic drugs. General physical health and ocular condition were assessed at baseline and at 12-month interval using standard clinical procedures for evaluating vision and ocular pathology, including, (i) best-corrected visual acuity by Snellen's VA Chart; (ii) intraocular pressure by applanation tonometry; (iii) contrast sensitivity by "Rabin Contrast Sensitivity Chart"; (iv) dilated fundus examination by direct, indirect ophthalmoscopy and slit-lamp biomicroscopy with +90D lens; and (v) central retinal thickness measurement by spectral-domain optical coherence tomography (Spectralis, Heidelberg Engineering, Germany).

*2.2. Biochemical Investigations.* About 10 ml of venous blood sample was collected from each study subject after 12 hours of the overnight fast and was aliquoted in the following manner: 6 ml in the ethylenediamine tetraacetic acid (EDTA) and another 4 ml in the clot activating vials. From this 6 ml blood, 4 ml was taken in to a 15 ml sterile centrifuge tube for the isolation of peripheral blood mononuclear cells (PBMC), and another 2 ml sample containing EDTA vial was then centrifuged at 3000 revolutions per minute for 10 minutes at 4°C to separate plasma and cellular components. Buffy coat was removed by careful aspiration, and packed erythrocytes were then washed with cold phosphate-buffered saline (pH—7.2) by maintaining 4°C temperature for estimation of HbA1C (%), SOD, GR, and GSH activities. Plasma samples were collected in cryovials for the assessment of glucose. The serum sample was collected from the remaining 4 ml sample and preserved at -80°C for further estimation of AGE, MDA, and VEGF. The level of the ROS generation was investigated in the PBMC.

*2.3. Measurement of ROS Generation in the PBMC.* Intracellular ROS generation in PBMC cells ($5 \times 10^5$ pelleted cells) was measured by flow cytometric assay method [13] using a ROS-sensitive cell-permeable dye 2′ 7′ dihydrodichlorofluorescein diacetate (2′ 7′ H2DCFDA). In this method, 2′ 7′ $H_2$DCFDA oxidized to highly fluorescent 2′ 7′-dichlorofluorescein (2′ 7′ DCF) presence of ROS in the PBMC. The PBMC exhibited an increased fluorescence of oxidized DCF, as measured by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) fitted with an argon-ion laser (15 mW) set to a wavelength of 488 nm. The fluorescence of DCF was collected in FL1 channel, equipped with a 530/30 nm band-pass filter. Fluorescence was measured in the long mode using "Cell Quest Pro" software (BD Bioscience, San Jose, CA) and expressed as geometrical mean fluorescence channel (GMFC). Cells were gated on the basis of their characteristic morphology, i.e., forward scatter and side scatter of monocytes and lymphocytes. Acquisitions were performed on 10000 gated events; while data analysis was carried out with "Cell Quest Pro" software (BD Bioscience).

*2.4. Measurement of MDA.* The MDA content of the serum was measured by the thiobarbituric acid (TBA) assay method as described by Satoh [14]. In the assay, MDA reacts with TBA to produce a chromogenic adduct. The color product was measured using a spectrophotometer (Halo DB-20, Dynamica, Salzburg-Mayrwise, Austria) at 532 nm wavelength.

*2.5. Measurement of SOD Activity.* Erythrocyte SOD activity was measured using the kit of BioVision (catalog no. K335-100; Mountain View, CA 94043, USA). The SOD assay kit utilizes WST-1 solution and enzyme solution. WST-1 solution produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of reduction with a superoxide anion is linearly related to the xanthine oxidase activity, which was used as an enzyme solution in this assay and was inhibited by SOD present in the sample. The inhibition activity of SOD was determined spectrophotometrically at a 450 nm filter using a microplate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). The activity of SOD was calculated according to the formula given below:

$$\text{SOD activity (inhibition rate\%)} = \frac{(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})}{(A_{\text{blank1}} - A_{\text{blank3}})} \times 100. \quad (2)$$

*2.6. Measurement of GR Activity.* Erythrocyte GR activity was measured using the kit of BioVision (catalog no. K 761-200, 155 S. Milpitas Boulevard, Milpitas, CA 95035 USA). In the assay, GR reduces oxidized glutathione (GSSG) to reduced glutathione (GSH), which reacts with 5,5′ -dithiobis (2-nitrobenzoic acid) (DTNB) to generate $TNB2^-$. GR present in the sample generates yellow-colored TNB2 from DTNB. Absorbance of the color product was measured at the initial stage of reaction (first reading) and after 10 min (second reading) at 25°C of incubation at a 405 nm filter by using a microplate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). The GR activity detection range of the assay is 0.1–40 mU/ml. The amount of TNB2 ($\Delta B$) produced by GR was determined by the TNB standard curve ranging from 0 to 50 nmol/well, and finally, the activity of GR was calculated using the following equation:

$$\text{GR activity} = \frac{\Delta B \times \text{sample dilution factor}}{(T_2 - T_1) \times 0.9 \times V}, \quad (3)$$

where $\Delta B$ is the TNB amount from the TNB standard curve (in nmol), $(T_2 - T_1)$ is the time difference of the first and second reading (in minute), $V$ is the volume of pretreated sample added into the reaction well (in ml), and 0.9 is the sample volume change factor during sample pretreatment procedure.

*2.7. Measurement of GSH.* A hundred microliters of pelleted erythrocytes and an equal volume of 5% sulfosalicylic acid (SSA) solution were taken in a microcentrifuge tube and vortexed vigorously and kept on the ice for 10 minutes. The content of the tube was then centrifuged at 12000 × g and 4°C for 10 minutes. After, the supernatant was collected, it was diluted 10-fold and used for the measurement of GSH

by using commercial kits of Sigma-Aldrich (catalog no. MAK364, St Louis, MO, USA). Assay kit was based on an enzymatic cycling method in the presence of GSH and a chromophore. The reduction of the chromophore produced a stable product, which was measured kinetically using a 450 nm (A450) filter in a microplate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). The absorbance is directly proportional to the amount of GSH in the sample. The assay was reproducible and able to detect a GSH concentration of 50 pmol/well in a 100-microliter reaction.

*2.8. Measurement of the Serum AGE.* The serum level of total AGE was measured by the competitive enzyme-linked immunosorbent assay (ELISA) method using the Cell Biolabs kits (Cat no. STA-817 Cell Biolabs, San Diego, CA, USA). The kit included an ELISA plate coated with AGE conjugate. The unknown samples or AGE-bovine serum albumin (BSA) standards were then added to the AGE conjugate preabsorbed ELISA plate. After a brief incubation, an anti-AGE polyclonal antibody was added, followed by an HRP-conjugated secondary antibody. Then, a color developed, and the absorbance of that color product was read at 450 nm as the primary wavelength using a microplate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat) against the reduced BSA standard as the absorbance blank.

*2.9. Measurement of Human Serum VEGF.* The VEGF content in serum of each study subject was estimated by the ELISA method and using a commercially available kit (REY Biotech, Cat. No. ELH-VEGF-001, Norcross USA). The kit included an antibody specific to a human VEGF-coated well plate. The standards and samples were added into the wells. VEGF protein present in the sample was bound to the wells by the immobilized antibody. The wells were then washed several times, and a biotinylated anti-human VEGF antibody was added. Following a buffer wash, HRP-conjugated streptavidin was pipetted into the wells. Further, the wells were subjected to washing again. The TMB substrate solution was then added to wells and allowed to incubate for 30 minutes at room temperature. Then, a final color developed, whose intensity was proportional to the concentration of VEGF protein in the sample. The absorbance of the color product was measured colorimetrically by using a 450 nm filter in an ELISA plate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). The concentration of the VEGF in the sample was calculated using the standard curve and expressed in picograms per milliliter. The minimum detectable dose of VEGF in this method was <10 pg/ml. The intra- and interassay coefficient of variations (CV) for this method was <10% and <12%, respectively.

## 3. Statistical Analysis

We assume that 185 and 175 subjects in Group A and Group B are random samples from their respective populations. Baseline demographic, clinical, and biochemical parameters of two study groups were represented as mean ± SD (standard deviation) and compared by unpaired two-tailed Student's $t$-test. The baseline level of biochemical parameters in each group was compared with their levels after 5 years of treatment by paired two-tailed Student's $t$-test. Categorical data of two groups were presented as percentages and further evaluated by using the $Z$ test. Pearson correlation coefficients were used to evaluate the association between two continuous variables. Survival analysis was performed to compare the progression rate of DR between two groups. Further, a Cox regression analysis was also conducted to assess factors that influence DR progression. A $p$ value < 0.05 was considered statistically significant.

Table 1: Baseline demographic and clinical characteristics of study subjects of two groups.

| Parameters | Group A0 | Group B0 | $p$ value |
|---|---|---|---|
| Age (years) | 54.6 ± 8.96 | 54.6 ± 9.12 | 0.962 |
| Sex (M/F) | 117/68 | 105/70 | 0.527 |
| BMI (kg/m$^2$) | 24.63 ± 4.46 | 24.57 ± 4.49 | 0.904 |
| FPG (mg/dl) | 126.55 ± 39.08 | 128.58 ± 42.34 | 0.637 |
| PPG (mg/dl) | 264.62 ± 74.72 | 267.06 ± 78.82 | 0.764 |
| HbA1c (%) | 7.85 ± 1.75 | 7.95 ± 1.71 | 0.647 |

Group A0: baseline levels of different parameters of Group A; Group B0: baseline levels of different parameters of Group B; FPG: fasting plasma glucose; PPG: postprandial plasma glucose; HbA1c: glycated haemoglobin. Continuous observations were presented as mean ± SD (standard deviation) and compared by using the unpaired $t$-test. Categorical data of the two groups were presented as ratios and compared using the $Z$ test; $p$ value < 0.05 was considered statistically significant.

## 4. Results

(i) Study results showed that 177 individuals (95.67%) in Group A and 168 individuals (96%) in Group B completed the 5-year follow-up period. Eight subjects (4.32%) from Group A and 7 (4%) subjects from Group B failed to attend the usual follow-up at each 1-year interval

(ii) The comparison of the baseline demographic and clinical parameters like age, sex distribution, BMI, FPG, PPG, and HbA1c between the groups showed no significant differences (Table 1)

(iii) Different biochemical parameters like PBMC ROS, MDA, GSH, SOD, GR, AGE, and VEGF demonstrated no significant differences when comparing A0 (baseline levels of different biochemical parameters of Group A) with A5 (levels of different biochemical parameters of Group A after five years) and B0 (baseline levels of different biochemical parameters of Group B), respectively. However, B5 (levels of different biochemical parameters of Group B after five years) showed significantly higher levels of PBMC ROS, MDA, AGE, and VEGF and lower levels of GSH, SOD, and GR compared to B0 (Table 2)

(iv) The correlation analysis revealed a significant positive correlation of VEGF with PBMC ROS, MDA, and AGE and a negative correlation with GSH, SOD,

Table 2: Comparison of different biochemical parameters between the groups at baseline and within the groups after 5 years.

| Parameters | Group A0 | Group B0 | $p$ value for A0 vs. B0 | A5 | $p$ value for A0 vs. A5 | B5 | $p$ value for B0 vs. B5 |
|---|---|---|---|---|---|---|---|
| PBMC ROS (geomean of DCF/$10^5$ cells) | 97.35 ± 11.31 | 97.61 ± 14.38 | 0.846 | 96.3 ± 15.98 | 0.539 | 101.0 ± 14.95 | 0.026 |
| MDA (nmol/ml) | 2.59 ± 0.57 | 2.63 ± 0.61 | 0.569 | 2.56 ± 0.89 | 0.804 | 2.89 ± 0.84 | 0.0006 |
| SOD (U/ml) | 42.10 ± 3.39 | 42.06 ± 3.13 | 0.891 | 42.61 ± 3.96 | 0.337 | 40.84 ± 3.56 | 0.0003 |
| GR (mU/ml) | 23.12 ± 3.64 | 23.09 ± 3.31 | 0.946 | 23.26 ± 3.97 | 0.701 | 22.0 ± 3.25 | 0.0009 |
| GSH (nmol/ml) | 26.14 ± 5.18 | 26.34 ± 4.94 | 0.699 | 26.27 ± 4.76 | 0.546 | 21.41 ± 4.15 | $<2.2 \times 10^{-16}$ |
| AGE ($\mu$g/ml) | 3.09 ± 0.62 | 3.12 ± 0.71 | 0.699 | 3.09 ± 0.79 | 0.938 | 3.53 ± 1.04 | $3.15 \times 10^{-5}$ |
| VEGF (pg/ml) | 96.5 ± 9.78 | 96.7 ± 9.31 | 0.841 | 96.4 ± 11.8 | 0.921 | 99.5 ± 9.15 | 0.0002 |

A0: baseline levels of different parameters of Group A; A5: levels of different parameters of Group A after 5 years; B0: baseline levels of different parameters of Group B; B5: levels of different parameters of Group B after 5 years; PBMC ROS: peripheral blood mononuclear cell reactive oxygen species; MDA: malondialdehyde; SOD: superoxide dismutase; GR: glutathione reductase; GSH: reduced glutathione; AGE: advanced glycation end products; VEGF: vascular endothelial growth factor. Two sets of paired observations were compared by paired $t$-test; $p$ value < 0.05 was considered statistically significant.

Table 3: Correlation of VEGF with different biochemical parameters (represented with correlation coefficient "$r$" with "$p$ value") at baseline and after 5 years of two study groups.

| Group | MDA | PBMC ROS | SOD | GR | AGE | GSH |
|---|---|---|---|---|---|---|
| A0 | 0.40 ($1.61 \times 10^{-8}$) | 0.321 ($8.13 \times 10^{-6}$) | -0.262 (0.0003) | -0.223 (0.002) | 0.262 (0.0003) | -0.222 (0.002) |
| A5 | 0.14 (0.059) | 0.098 (0.195) | -0.065 (0.388) | -0.155 (0.039) | 0.12 (0.112) | -0.13 (0.087) |
| B0 | 0.239 (0.001) | 0.318 ($1.81 \times 10^{-5}$) | -0.275 (0.0002) | -0.187 (0.013) | 0.257 (0.0006) | -0.213 (0.005) |
| B5 | 0.405 ($5.23 \times 10^{-8}$) | 0.398 ($8.96 \times 10^{-8}$) | -0.388 ($2.05 \times 10^{-7}$) | -0.354 ($2.54 \times 10^{-6}$) | 0.342 ($5.67 \times 10^{-6}$) | -0.306 ($5.44 \times 10^{-5}$) |

Pearson's correlation was used, and $p$ value < 0.05 was considered statistically significant.

and GR levels for A0, B0, and B5, respectively. Hence, for A5, the study demonstrated only a significant negative correlation between VEGF and GR (Table 3)

(v) The Cox regression model demonstrated that treating patients with only oral antidiabetic medication increases the hazard of DR by a factor of 3.795, (95% LCL = 2.08, UCL = 6.93): keeping other predictors constant, without vitamin B, C, and E supplements, the risk of DR is increased by 279%. Similarly, for one-unit increase in VEGF, the risk of DR is increased by 3.6% (95% LCL = 1.01, UCL = 1.07) and a unit increase in MDA increases the risk of DR by a factor of 7.5% (95% LCL = 0.71, UCL = 1.64) (Table 4). Further, survival probability plot exhibited a significant difference ($p$ value of the log-rank test: $2.21 \times 10^{-5}$) between the two groups (Figure 1)

(vi) There were no adverse effects of B vitamins, vitamin C, or vitamin E supplementation reported during the study

Table 4: Cox regression showing variables that influence the development of DWR to DR.

| Variables | Coef | Exp (coef) | SE (coef) | $Z$ | $p$ value |
|---|---|---|---|---|---|
| Group B | 1.334 | 3.795 | 0.308 | 4.336 | $1.45 \times 10^{-5}$ |
| VEGF | 0.036 | 1.036 | 0.015 | 2.329 | 0.0199 |
| MDA | 0.072 | 1.075 | 0.214 | 0.337 | 0.7359 |

MDA: malondialdehyde; VEGF: vascular endothelial growth factor. The best three-predictor model was selected by carrying out forward and backward stepwise regression procedure. The final model demonstrated that treating patients with only oral antidiabetic medication (Group B) increases the hazard of diabetic retinopathy (DR) by a factor of 3.795, (95% LCL = 2.08, UCL = 6.93): keeping other predictors constant, without vitamin B, C, and E supplements, the risk of DR is increased by 279%. Similarly, for one-unit increase in VEGF, the risk of DR is increased by 3.6% (95% LCL = 1.01, UCL = 1.07) and a unit increase in MDA increases the risk of DR by a factor of 7.5% (95% LCL = 0.71, UCL = 1.64). All the asymptotically equivalent tests (likelihood ratio, Wald, and score tests) unanimously rejected the omnibus null hypothesis that all the regression coefficients are zero with $p$ value $\leq 2 \times 10^{-5}$.

## 5. Discussion

It has been demonstrated by landmark studies that improving glycemic control reduces the risk of development and progression of DR, though there is no lower limit of glycemic control that is protective against the onset of retinopathy [15, 16]. Our sequential studies have suggested that hyperglycemic and dyslipidemia-induced biochemical derangements result in tissue hypoxia and upregulation of VEGF [17, 18]. The important anomalies related to mishandling of glucose and lipids leading to increased generation of advanced glycation and lipid peroxidation end products, oxidative stress, and failure of the body's antioxidant defense systems ultimately invite inflammation, hypoxia, and increased VEGF secretion [17, 18].

This study included 185 diabetic patients who had been treated with antihyperglycemic medication along with

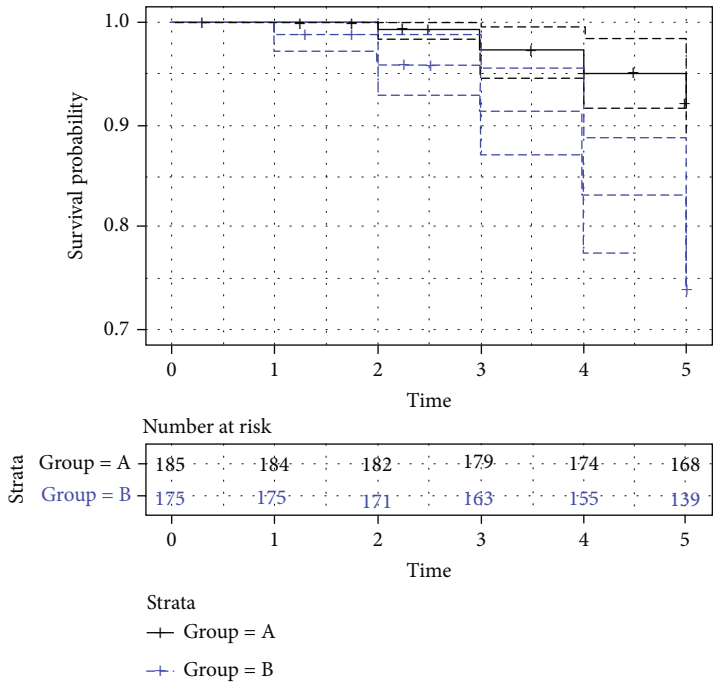


Figure 1: Kaplan–Meier survival analysis of progression to DR in Group A and Group B. The survival probability plot showed a significant difference ($p$ value $2.21 \times 10^{-5}$) in the two groups.

vitamins B, C, and E since their diagnosis in the year 2012 and 175 patients who were treated only with antihyperglycemic medications. Conventional control of hyperglycemia with average HbA1c between 7 and 7.5% did not illustrate any significant visual disturbance and microangiopathy on dilated fundus examination and 3-D spectral OCT. It is hypothesized that niacin and riboflavin administered exogenously provide the steady supply of NAD+, FAD+, and flavin mononucleotide (FMN), the most important oxidized cofactors for continuity of tricarboxylic acid cycle (TCA) and glycolysis. Energy liberated from oxidation of all food stuffs generates reducing equivalents that are directly collected and carried to mitochondrial respiratory systems to reduce oxygen, yielding adenosine triphosphate (ATP), water, and some unavoidable byproducts such as ROS, which are not only detrimental to essential cellular components but also responsible for diminished activities of oxidant scavenging enzymes like SOD and GR.

Administration of ascorbic acid, $\alpha$-tocopherol, and B vitamins containing thiamine, niacin, riboflavin, pyridoxamine, and pantothenic acid eliminates inhibition of glycolysis and TCA cycle and reduces lipid peroxidation and free radical accumulation. Two experimental animal studies have demonstrated beneficial effects of vitamin thiamine and niacin to ameliorate different detrimental pathways related to hyperglycemia [19, 20]. Another groundbreaking animal model diabetic study by Kowluru et al. revealed that a diet supplemented with multinutrients like ascorbic acid, cholecalciferol, d-alpha tocopherol, Fish Oil EE 70%, eicosapentaenoic acid, docosahexaenoic acid, benfotiamine, $\alpha$ lipoic acid, tocomin, zeaxanthin, lutein, and proprietary blend containing Polygonum cuspidatum SE (resveratrol), green tea, turmeric root (curcuminoids), N-acetyl-cysteine, Pycnogenol® pine bark, grape seed extract, coenzyme Q10 and zinc, and soybean oil prevents DR and also maintains normal retinal function, mitochondrial homeostasis, and inflammatory mediators [21]. Inhibition of AGE formation and accumulation by pyridoxamine has been tested in an experimental diabetic model [22]. In our previous study, we also demonstrated the effectiveness of vitamin supplements like B, C, and E in reducing oxidative stress-induced structural and functional abnormalities of red blood cells (RBCs) in preventing DR development [23].

The present study clearly demonstrates that regular intake of niacin, riboflavin, thiamine, pyridoxal phosphate, ascorbic acid, and $\alpha$-tocopherol reduces the circulatory levels of surrogate markers of lipid peroxidation (MDA), ROS, and VEGF and, on the contrary, increases serum level of intracellular antioxidants, SOD, GR, and GSH. GR is an ever-present enzyme that reduces oxidized glutathione (GSSH) to reduced forms of GSH which is an omnipotent intracellular antioxidant [24]. Another study has suggested that decreased activity of superoxide dismutase is associated with microvascular complications in T2DM individuals [6].

Nonenzymatic glycation end products and excessive free radicals may alter the conformation and activities of cellular antioxidant enzymes SOD and GR, whereas vitamins C and E and pyridoxamine might attenuate these injurious pathways and rid the cellular system of malfunctioning. Riboflavin and niacin, the precursors of FAD+ and NAD+, the important electron carriers in mitochondria, for oxidative phosphorylation, help the continuity of glycolysis and TCA at a steady state.

It is hypothesized from this prospective study that glucose metabolism machinery requires a sufficient supply of

NAD+ and flavoproteins, FAD+, and FMN to run the glycolysis and TCA cycle. Prevention of nonenzymatic glycation which disturbs the normal function of proteins by cross-linking, disulfide bond formation, and chemical rearrangement is very essential to stop or delay diabetic microvascular complications. Stoppa et al. demonstrated that reduced antioxidant enzyme activity owing to hyperglycemia is lessened by aminoguanidine [25].

A similar detrimental pathway of glutamate-induced increased intracellular $Ca^{++}$-mediated lipid peroxidation is believed to take an important part in the pathogenesis of the development of diabetic complications [8, 26]. Regular intake of vitamin E inhibits lipid peroxidation and production of MDA which are toxic to capillary endothelium [27]. Consequently, oxidative stress, i.e., increased formation of free radicals due to increased TCA cycle and lipid peroxidation, is considered a crucial player in the diminished ability of the intracellular antioxidant defense system and invitation of proinflammatory and inflammatory cytokines [28]. As the involvement of retinal vascular endothelial cell dysfunction in the pathogenesis of DR is suggested by AGE activation, ROS generation, and lipid peroxidation, systemic administration of antioxidants like vitamin C, vitamin E, and pyridoxal phosphate (vitamin B6) may be adequate to suppress these detrimental pathways. Isolated experimental studies have highlighted the efficacy of vitamins B1, B3, and B6 to prevent diabetes-induced retinal vascular lesions, whereas complex multiple pathways derived from hyperglycemia-induced biochemical derangements need multiple blockers.

The novelty of this study is the addition of attention beyond AGE formation and ROS production in diabetes mellitus, i.e., assistance in the uninterrupted running of glycolysis and Krebs cycle and pentose phosphate pathway to generate adequate ATP and intracellular antioxidant defense.

As the "Age-Related Eye Disease Study" suggested that a nutritional supplement could prevent the progression of age-related macular degeneration [29], we took this clinical trial to evaluate initially the efficacy of vitamins B, C, and E to ameliorate the detrimental pathways involved in the development of DR in T2DM. A large multicenter, double-blind randomized controlled clinical trial is required to validate these findings.

## 6. Conclusion

The present study demonstrated that vitamin B, C, and E supplements in combination with conventional management of hyperglycemia decrease the risk of development of DR by inhibiting oxidative stress, AGE formation, lipid peroxidation, and VEGF secretion. This finding may suggest a new approach to DR management.

## Data Availability

The data presented in this study are available on a reasonable request from the corresponding author.

## Ethical Approval

We followed all ethical guidelines of institutional and/or national research committees when conducting human subject studies, as well as the Helsinki Declaration and its revision in 2013 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki). The study was approved by the 'Institutional Ethics Committee of the RIO, Medical College, Kolkata (Ref. No. RIO/MC/KOL/NON-SPON/119/11-2011).

## Consent

Written informed consents were collected from all patients according to the Declaration of Helsinki.

## Conflicts of Interest

The authors declare no conflict of interest.

## Authors' Contributions

S.P. collected samples, performed experiments, and wrote the paper; K.B analyzed and interpreted the data and wrote the paper; L.K.M. conceived and designed experiments, contributed reagents and materials, and wrote the paper. The manuscript has been reviewed and approved by all the authors.

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*Review Article*

# The Effects of Tai Chi Exercise for Patients with Type 2 Diabetes Mellitus: An Overview of Systematic Reviews and Meta-Analyses

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*Objectives*. Tai chi (TC) is a potential complementary treatment for type 2 diabetes mellitus (T2DM). This overview systematically summarizes and evaluates the existing evidence of TC in the treatment of T2DM. *Methods*. Systematic reviews (SRs)/meta-analyses (MAs) on TC interventions for T2DM were comprehensively searched in seven databases. Methodological quality, risk of bias, reporting quality, and quality of evidence were assessed using the Assessment of Multiple Systematic Reviews 2 (AMSTAR-2), the Risk of Bias in Systematic (ROBIS) scale, the list of Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA), and the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) system. *Results*. Eight published SRs/MAs were included in our study. Based on the methodology and quality of evidence assessment, all SRs/MAs are considered to be of very low quality, and only 1 SR/MA has been assessed as low risk of bias, and none of the SR/MA has been fully reported on the checklist. A total of 65 outcome indicators extracted from the included SRs/MAs were evaluated, and only 1 item was assessed as high quality. *Conclusions*. TC may be an effective and safe complementary treatment for T2DM. However, this conclusion must be treated with caution because the quality of the evidence provided by the included SRs/MAs is generally low.

## 1. Introduction

As a chronic metabolic disease, type 2 diabetes mellitus (T2DM) is characterized by elevated blood glucose levels, which induces disturbances in glucose, fat, and protein metabolism in the body [1]. The pathophysiological hallmark of T2DM is insulin resistance, accompanied by decreased insulin secretion due to pancreatic $\beta$ cell dysfunction, and oxidative stress (OS) is also considered to be a major hallmark of the pathogenesis and progression of T2DM [2]. T2DM accounts for 90% of all diabetes cases, and the International Diabetes Federation (IDF) estimates that the number of people with type 2 diabetes worldwide is expected to reach 578 million by 2030 and 700 million by 2045 [3]. T2DM is generally associated with a higher risk of vascular complications, including macrovascular complications (e.g., cardiovascular complications) and microvascular complications (including neuropathy, nephropathy, and retinopathy) [4]. The current mainstream treatments for T2DM include oral hypoglycemic drugs and subcutaneous insulin injections. In addition, body movement constitutes a significant part of a diabetes management program and plays a specific role in preventing diabetes complications and managing blood sugar in patients with T2DM [5].

OS occurs when the accumulation of the by-products of oxygen metabolism eventually exceeds the antioxidant capacity [6]. Experimental and clinical studies have shown that OS is involved in the pathogenesis of cardiovascular disease, carcinogenesis, and other diseases. It is known to play a key role in the etiology and pathophysiology of diabetes [7]. Mitochondrial dysfunction and dysregulation of prooxidase appear to be major factors involved in chronic reactive

oxygen species (ROS) generation, resulting in a chronic OS state [2]. Chronic hyperglycemia is considered to be a major contributor to the development of microvascular and macrovascular complications in type 2 diabetes and is known to cause DNA, lipid, and protein damage, the extent of which is related to the hyperglycemia-induced reactive oxygen species production and the degree of OS [8]. Physical exercise intervention induces an adaptive response characterized by a reduction in markers of OS damage and an increase in the body's antioxidant response [9].

Originated in ancient China, TC has a history of thousands of years. It was formed under the guidance of traditional Chinese medicine theories and Chinese folk martial arts, and it is a gem of the Chinese nation [10]. The pathogenesis of T2DM is closely related to the physical and mental state of the individual. TC is a movement that emphasizes the unity of energy, breath, and spirit and combines physical activity with breathing [11]. A meta-analysis showed that regular TC exercise increased superoxide dismutase and catalase levels while decreasing lipid peroxide levels [12]. Therefore, TC plays an active role in glycemic control and OS regulation in T2DM patients, and the ADA recommends TC as a mind-body therapy for T2DM patients to increase balance, muscle strength, and flexibility [13].

Systematic review (SR)/meta-analysis (MA) is an important tool for evidence-based clinical work, but its methods must strictly follow a series of guidelines to minimize the possibility of deviation in answering specific questions [14]. A growing number of SRs/MAs based on TC interventions for T2DM have shown that TC can reduce fasting glucose (FGB) and glycated hemoglobin (HbA1c) and improve the quality of life in patients with T2DM. However, the methodological and evidentiary quality of these SRs/MAs has not been assessed, and it remains controversial whether these findings could provide credible evidentiary support for clinical staff [15]. The SR/MA overview is a newly emerged approach that combines multiple SRs/MAs to assess their quality and various findings in an attempt to resolve inconsistencies between them [16]. The purpose of this overview is to objectively and comprehensively evaluate the scientific validity and applicability of SRs/MAs regarding the effects of TC exercise on T2DM.

## 2. Methods

*2.1. Research Methods.* The SR/MA overview is based on the guidelines specified in the *Cochrane Handbook* [17], and we followed the methods of Shi et al. [18], Liu et al. [19], and Shen et al. [20].

*2.2. Eligibility Criteria*

*2.2.1. Literature Inclusion Criteria*

*(1) Type of Research.* This overview includes SRs/MAs of randomized controlled trials (RCTs) of the effects of TC exercise on T2DM.

*(2) Type of Participants.* Subjects were patients diagnosed with T2DM by any international or national standard.

*(3) Type of Intervention.* The intervention for the control group was usual care or standard treatment (ST) or any type of other exercises, and the intervention for the experimental group was TC exercise or TC combined with the treatments received by the control group.

*(4) Types of Outcomes.* Outcomes assessed in this overview include fasting blood glucose (FBG), glycated hemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), fasting serum insulin (FINs), postprandial blood glucose (PBG), low-density lipoprotein (LDL), triglycerides (TG), high-density lipoprotein (HDL), total cholesterol (TCL), diastolic blood pressure (DBP), systolic blood pressure (SBP), body mass index (BMI), and quality of life.

*2.2.2. Exclusion Criteria.* The exclusion criteria were as follows: (1) animal studies and (2) network MAs, research protocols, narrative reviews, overviews, dissertation, and conference abstracts.

*2.3. Data Sources and Search Strategy.* The literatures were retrieved from PubMed, Cochrane Library, Embase, Chongqing VIP, Wanfang Database, CNKI, and SINOMED on January 1, 2022. We adopted a strategy that combines keyword search with free word search, and the keywords include "Type 2 Diabetes Mellitus", "Tai Chi", "Systematic Review", and "Meta-Analysis". The literature search strategy of the PubMed database is shown in Table 1, which was reasonably tuned for each database. We also reviewed the references of all retrieved literatures to avoid missing topic-related SRs/MAs.

*2.4. Literature Screening and Data Extraction.* The literature screening (HS-S and YF-Z) and information extraction (PL-L and CD-C) were performed independently by two researchers. We imported the retrieved documents into Endnote X9 document management software and removed the duplicates. The literatures that potentially met the inclusion and exclusion criteria were then obtained by reading the titles and abstracts of these literatures. Ultimately, we finalized the included SRs/MAs by reading the full text. All SRs/MAs were read by two independent researchers, and the following data were extracted from the SRs/MAs: first author, publication year, country, number of RCTs included, interventions for experimental and control groups, included RCT quality assessment tools, and main conclusion. The disagreement between the two researchers was resolved through discussion.

*2.5. SR/MA Quality Assessment.* Two researchers (HS-S and CD-C) independently assessed the methodological and evidentiary quality of the included SRs/MAs.

*2.6. Assessment of Methodological Quality*

*2.6.1. Estimate of Methodological Quality.* The methodological quality of the included SRs/MAs was assessed by the Assessment System for Evaluating Methodological Quality 2 (AMSTAR-2) [21]. Seven (2, 4, 7, 9, 11, 13, and 15) of the 16 items in the tool were critical areas.

Table 1: Search strategy for the PubMed database.

| Query | Search term |
|---|---|
| #1 | "Tai Ji"[Mesh] |
| #2 | "Tai-ji" OR "Tai Chi" OR "Chi, Tai" OR "Tai Ji Quan" OR "Ji Quan, Tai" OR "Quan, Tai Ji" OR "Taiji" OR "Taijiquan" OR "T'ai Chi" OR "Tai Chi Chuan" OR "Tai ji" |
| #3 | #1 OR #2 |
| #4 | "Diabetes Mellitus, Type 2"[Mesh] |
| #5 | "Diabetes Mellitus, Noninsulin-Dependent" OR "Diabetes Mellitus, Ketosis-Resistant" OR "Diabetes Mellitus, Ketosis Resistant" OR "Ketosis-Resistant Diabetes Mellitus" OR "Diabetes Mellitus, Non Insulin Dependent" OR "Diabetes Mellitus, Non-Insulin-Dependent" OR "Non-Insulin-Dependent Diabetes Mellitus" OR "Diabetes Mellitus, Stable" OR "Stable Diabetes Mellitus" OR "Diabetes Mellitus, Type II" OR "NIDDM" OR "Diabetes Mellitus, Noninsulin Dependent" OR "Diabetes Mellitus, Maturity-Onset" OR "Diabetes Mellitus, Maturity Onset" OR "Maturity-Onset Diabetes Mellitus" OR "Maturity Onset Diabetes Mellitus" OR "MODY" OR "Diabetes Mellitus, Slow-Onset" OR "Diabetes Mellitus, Slow Onset" OR "Slow-Onset Diabetes Mellitus" OR "Type 2 Diabetes Mellitus" OR "Noninsulin-Dependent Diabetes Mellitus" OR "Noninsulin Dependent Diabetes Mellitus" OR "Maturity-Onset Diabetes" OR "Diabetes, Maturity-Onset" OR "Maturity Onset Diabetes" OR "Type 2 Diabetes" OR "Diabetes, Type 2" OR "Diabetes Mellitus, Adult-Onset" OR "Adult-Onset Diabetes Mellitus" OR "Diabetes Mellitus, Adult Onset" |
| #6 | #4 OR #5 |
| #7 | Meta-Analysis as Topic [Mesh] |
| #8 | "Systematic review" OR "meta-analysis" OR "meta analysis" OR "meta-analyses" OR "Review, Systematic" |
| #9 | #7 OR #8 |
| #10 | #3 AND #6 AND #9 |

*2.6.2. Assessment of Risk of Bias.* The Risk of Bias in Systematic Review (ROBIS) [22] scale was used in this overview to evaluate the risk of bias of the inclusion of SRs/MAs. The scale was used to assess the overall risk of bias in the inclusion of SRs/MAs in three stages.

*2.6.3. Assessment of Reporting Quality.* The quality of each SR/MA report of the included SRs/MAs was evaluated by the list of PRISMA [23] which consisted of 27 items focusing on the reporting methods and results that were incorporated into the SRs/MAs.

*2.6.4. Assessment of Quality of Evidence.* The quality of evidence for each SR/MA outcome was evaluated by the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) [24], according to which, five aspects will lead to the degradation of evidence quality, including limitations, inconsistencies, indirectness, imprecision, and publication bias.

## 3. Results

*3.1. Results on Literature Search and Selection.* Using our search strategy, a total of 83 articles were identified. After removing 33 duplicate articles, the researchers screened the remaining 50 articles by reading titles and abstracts. Subsequently, 16 articles were obtained. After reading the full text, 6 articles [25–30] were found irrelevant to SRs/MAs in RCTs, and 2 SRs/MAs [31, 32] were not about people with T2DM. Thus, 8 SRs/MAs [33–40] were finally included in this overview. The process of study selection is shown in Figure 1.

*3.2. Description of Included SRs/MAs.* The characteristics included in the overview are shown in Table 2. These SRs/MAs were all published between 2017 and 2021, 5 [33–37] of which were in English, and the remaining 3 [38–40] were in Chinese. One [34] of the SRs/MAs was published by Korean researchers, and the remaining 7 SRs/MAs [33, 35–40] were published by Chinese researchers. The number of RCTs was between 10 and 23, and the sample size was between 740 and 1,800. In terms of quality evaluation scales, 6 SRs/MAs [34–36, 38–40] used the Cochrane risk of bias standard, 1 SR/MA [37] used the Physiotherapy Evidence Database scale, and 1 SR/MA [33] used the Jadad scale.

*3.3. Results on SR/MA Quality Assessment*

*3.3.1. Methodological Quality Assessment.* Regarding the methodological quality of the included SRs/MAs, all were considered to be of very low quality because more than one key item was missing from the SRs/MAs included in the quality assessment. Methodological quality limitations come from the following items: item 2 (none of the SR/MA registers the study protocol), item 7 (no SR/MA provided by SR/MA provided for exclusion), and item 13 (when interpreting the evaluation results, only 2 SRs/MAs [34, 36] considered the risk of bias in the main study). The evaluation details of the included SRs/MAs on the AMSTAR-2 are shown in Table 3.

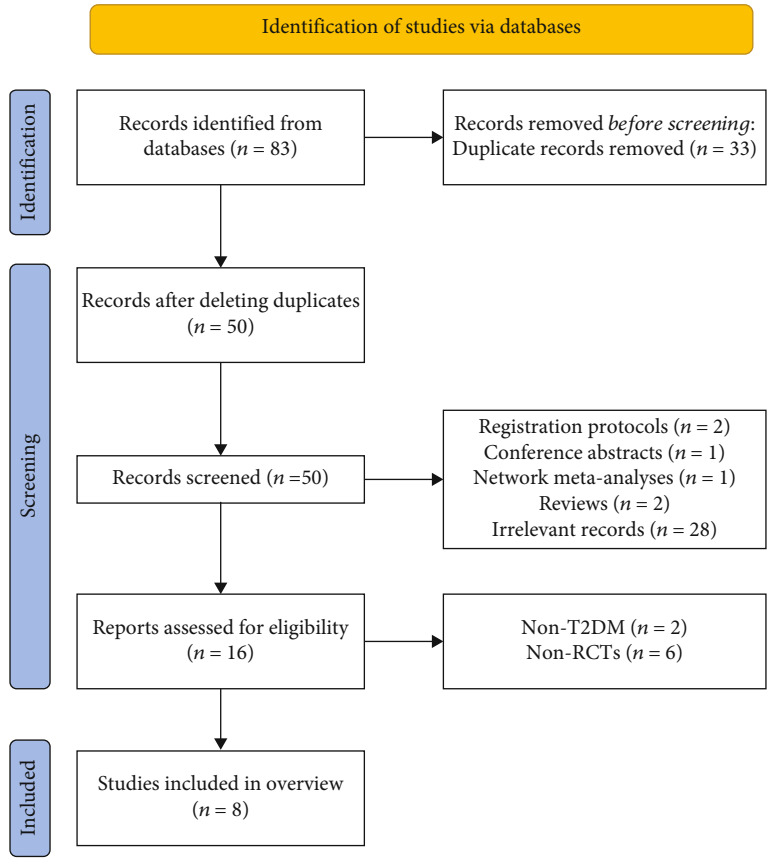


Figure 1: The flowchart of the screening process.

*3.3.2. Risk of Bias of the Included SRs/MAs.* Regarding the results of the ROBIS assessment, phase 1 assessed the relevance of the study topic and domain 1, and all SRs/MAs were rated as low risk of bias in both items. Five [33–37] of the SRs/MAs were assessed as low risk in domain 2, 5 SRs/MAs [33, 35–37, 39] were assessed as low risk of bias in domain 3, and only 3 SRs/MAs [33, 36, 39] were assessed as low risk of bias in domain 4. In phase 3, only 2 SRs/MAs [34, 36] had a low risk of bias. The evaluation details of the included SRs/MAs on the ROBIS scale are shown in Table 4.

*3.3.3. Report Quality of the Included SRs/MAs.* The results of the PRISMA inventory evaluation are shown in Table 5. 22 out of 27 items have a "yes" response rate of more than 70%, and this shows that the report was relatively complete. However, there were some reporting deficiencies in other items. The reports of item 5 (protocol and registration), item 8 (search), and item 15 (risk of bias across studies, methods) were incomplete (the "yes" response rate was less than 50%).

*3.3.4. Evidence Quality of the Included SRs/MAs.* The 8 SRs/MAs included 65 outcomes related to the effectiveness of TC for T2DM. By means of the GRADE evaluation, 1 was rated as high quality, 7 moderate quality, 10 low quality, and 47 very low quality in terms of the quality of evidence for all outcome indicators. Risk of bias ($n = 58$) was the most common downgrading factor, followed by publication bias ($n = 47$), inconsistency ($n = 37$), imprecision ($n = 37$), and indirectness ($n = 0$). GRADE-specific assessment details are shown in Table 6.

*3.4. Summary Results of the Included Studies.* The result indicators extracted from the included studies are listed in Table 6.

*3.4.1. Outcomes Related to Glucose Metabolism.* Eight SRs/MAs [33–40] reported the effect of TC on FGB in patients with T2DM, and all SRs/MAs indicated that TC could significantly reduce FGB. However, 3 subreports [33–35] also showed that compared with other aerobic exercises, TC had no significant effect on FGB. Eight SRs/MAs [33–40] reported the effect of TC on HbA1c in T2DM patients, of which 7 SRs/MAs [33, 35–40] indicated that TC could significantly reduce HbA1c levels. Two SRs/MAs [33, 35] reported the effect of TC on PBG in T2DM patients. Two reports [33, 35] showed that TC could significantly reduce PBG level, and 1 report [33] showed that TC had no significant effect on PBG compared with other aerobic exercises. Two SRs/MAs [33, 37] reported the effect of TC on FINs, and one SR/MA [35] showed that TC was beneficial for lowering FINs. In addition, 2 SRs/MAs [33, 37] reported that TC reduced HOMA-IR in T2DM patients.

*3.4.2. Outcome-Related Lipid Metabolism.* Six SRs/MAs [35–40] reported the effect of TC on TCL in T2DM patients,

Table 2: Characteristics of the included SRs/MAs. Note: N: nonexercise; AE: aerobic exercise; ST: standard treatment.

| Author, year (country) | Trials (subjects) | Intervention group | Control group | Quality assessment | Main results |
|---|---|---|---|---|---|
| Mengyao Chao, 2018 (China) [33] | 14 (798) | TC | N, AE | Jadad | TC can effectively influence the management of blood glucose and HbA1c in patients with T2DM. Long-term adherence to TC has a better effect on reducing blood sugar and HbA1c levels in patients with T2DM |
| Myeong Soo Lee, 2015 (South Korea) [34] | 15 (754) | TC, TC+control group | N, ST, AE | Cochrane criteria | In conclusion, the evidence that TC may benefit people with T2DM compared with exercise therapy is not convincing. In addition, evidence from RCTs comparing TC with conventional antidiabetic drugs appears to be mixed |
| Shuai Guo, 2021 (China) [35] | 23 (1,800) | TC, TC+control group | ST, AE | Cochrane criteria | Compared with routine clinical treatment, TC has better effects on blood sugar control, lipid metabolism, and body composition and is superior to aerobic exercise in improving partial metabolic control. The optimal intervention time window for TC may vary for different metabolic markers |
| Ting-Wei Xia, 2019 (China) [36] | 17 (774) | TC, TC+control group | N, ST, AE | Cochrane criteria | TC appears to be effective in treating T2DM compared to control interventions. Different training times and methods will lead to differences in effects |
| Zonglei Zhou, 2019 (China) [37] | 23 (1,235) | TC | AE, ST | PEDro scale | TC was effective in controlling biomedical outcomes and improving the quality of life-related outcomes in patients with T2DM, but no effects on balance and fasting insulin were observed |
| Yao Ge, 2020 (China) [38] | 13 (856) | TC, TC+control group | N, ST | Cochrane criteria | TC exercise can control blood sugar level and regulate lipoprotein concentration in patients with T2DM, which can provide the basis for exercise therapy for later stage diabetes |
| Yongjin Liu, 2017 (China) [39] | 10 (740) | TC | N, AE | Cochrane criteria | TC exercise can regulate the level of glucose and lipid metabolism and improve the quality of life in patients with T2DM and can be used as an important part of exercise therapy for diabetes |
| Qing Tang, 2017 (China) [40] | 11 (764) | TC | CT | Cochrane criteria | TC helps improve blood sugar control, weight loss, blood lipid regulation, and quality of life in patients with T2DM |

Table 3: Result of the AMSTAR-2 assessments. Note: Y: yes; PY: partial yes; N: no; VL: very low; L: low. Note: key items are marked in italic.

| Author, year (country) | Q1 | *Q2* | Q3 | *Q4* | Q5 | Q6 | *Q7* | Q8 | *Q9* | Q10 | *Q11* | Q12 | *Q13* | Q14 | *Q15* | Q16 | Quality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mengyao Chao, 2018 (China) [33] | Y | PY | Y | Y | Y | Y | N | Y | Y | N | Y | Y | N | Y | Y | Y | VL |
| Myeong Soo Lee, 2015 (South Korea) [34] | Y | PY | Y | Y | Y | Y | N | Y | Y | N | Y | Y | Y | Y | N | Y | VL |
| Shuai Guo, 2021 (China) [35] | Y | PY | Y | Y | Y | Y | N | Y | Y | N | Y | Y | N | Y | N | Y | VL |
| Ting-Wei Xia, 2019 (China) [36] | Y | PY | Y | Y | Y | Y | N | Y | Y | N | Y | Y | Y | Y | Y | Y | VL |
| Zonglei Zhou, 2019 (China) [37] | Y | PY | Y | Y | Y | Y | N | Y | Y | N | Y | Y | N | Y | Y | Y | VL |
| Yao Ge, 2020 (China) [38] | Y | PY | Y | PY | Y | Y | N | N | Y | N | Y | Y | N | Y | N | Y | VL |
| Yongjin Liu, 2017 (China) [39] | Y | PY | Y | PY | Y | Y | N | Y | Y | N | Y | Y | N | Y | Y | Y | VL |
| Qing Tang, 2017 (China) [40] | Y | PY | Y | PY | Y | Y | N | Y | Y | N | Y | Y | N | Y | N | Y | VL |

Table 4: Results of the ROBIS assessments. Note: √: low risk; ×: high risk.

| Author, year (country) | Phase 1 | Phase 2 | | | Phase 3 |
|---|---|---|---|---|---|
| | Assessing relevance | Domain 1: study eligibility criteria | Domain 2: identification and selection of studies | Domain 3: collection and study appraisal | Domain 4: synthesis and findings | Risk of bias in the review |
| Mengyao Chao, 2018 (China) [33] | √ | √ | √ | √ | √ | × |
| Myeong Soo Lee, 2015 (South Korea) [34] | √ | √ | √ | × | × | √ |
| Shuai Guo, 2021 (China) [35] | √ | √ | √ | √ | × | × |
| Ting-Wei Xia, 2019 (China) [36] | √ | √ | √ | √ | √ | √ |
| Zonglei Zhou, 2019 (China) [37] | √ | √ | √ | √ | × | × |
| Yao Ge, 2020 (China) [38] | √ | √ | × | × | × | × |
| Yongjin Liu, 2017 (China) [39] | √ | √ | × | √ | √ | × |
| Qing Tang, 2017 (China) [40] | √ | √ | × | × | × | × |

of which 4 reports [35–37, 40] showed that TC could significantly reduce TCL level, while 3 reports [35, 38, 39] showed that the effect of TC on TCL was not statistically significant. Five SRs/MAs [35, 36, 38–40] reported the effect of TC on TG in T2DM patients. Five reports [35, 36, 38–40] suggested that TC could significantly reduce TG level, while 1 report [35] showed that TC had no significant effect on TG compared with other aerobic exercises. Four SRs/MAs [35, 36, 38, 39] reported the effect of TC on LDL in patients with T2DM, of which 2 SRs/MAs [36, 38] showed that TC could significantly lower LDL levels. Four SRs/MAs [35, 36, 38, 39] reported the effect of TC on HDL in patients with T2DM, and 3 of the SRs/MAs [35, 38, 39] indicated that TC could significantly improve HDL levels.

*3.4.3. Other Outcomes.* Four SRs/MAs [35–37, 40] reported that TC reduced BMI in T2DM patients. One SR/MA [37] reported that TC reduced SBP and DBP in T2DM patients. Two SRs/MAs [37, 40] reported that TC could improve the quality of life of patients with T2DM. One SR/MA [37] result showed that TC had no statistically significant effect on balance.

*3.4.4. Adverse Event.* There are 3 SR/MA narrative reviews suggesting that TC is safe.

## 4. Discussion

Exercise therapy is recommended for T2DM management because regular physical activity improves glycemic control as well as lipid index, blood pressure, cardiovascular disease, and quality of life [41]. TC is a low-to-moderate-intensity mind-body exercise that originated in China and is very popular around the world [42]. This overview is the first comprehensive and systematic assessment of the SRs/MAs associated with TC intervention for T2DM and will help to establish a clear link between the need to resolve uncertainty and prior clinical knowledge [43].

*4.1. Summary of the Main Findings.* This overview includes 8 SRs/MAs on the impact of TC on T2DM. All SRs/MAs are

Table 5: Results of the PRISMA checklist. Note: Y: yes; N: no.

| Section/ topic | Items | Mengyao Chao, 2018 (China) [33] | Myeong Soo Lee, 2015 (South Korea) [34] | Shuai Guo, 2021 (China) [35] | Ting-Wei Xia, 2019 (China) [36] | Zonglei Zhou, 2019 (China) [37] | Yao Ge, 2020 (China) [38] | Yongjin Liu, 2017 (China) [39] | Qing Tang, 2017 (China) [40] | Number of yes (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Title | Q1. Title | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| Abstract | Q2. Structured summary | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| Introduction | Q3. Rationale | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q4. Objectives | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| Methods | Q5. Protocol and registration | N | N | N | N | N | N | N | N | 0% |
| | Q6. Eligibility criteria | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q7. Information sources | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q8. Search | N | N | Y | N | Y | N | N | N | 25% |
| | Q9. Study selection | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q10. Data collection process | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q11. Data items | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q12. Risk of bias in individual studies | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q13. Summary measures | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q14. Synthesis of results | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q15. Risk of bias across studies | N | N | N | Y | Y | N | N | N | 25.00% |
| | Q16. Additional analyses | Y | N | Y | Y | Y | N | N | Y | 62.50% |
| Results | Q17. Study selection | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q18. Study characteristics | Y | Y | Y | Y | Y | N | Y | Y | 88% |
| | Q19. Risk of bias within studies | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q20. Results of individual studies | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q21. Synthesis of results | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q22. Risk of bias across studies | Y | N | N | Y | Y | N | Y | N | 50.00% |
| | Q23. Additional analysis | Y | N | Y | Y | Y | N | Y | Y | 75.00% |
| Discussion | Q24. Summary of evidence | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q25. Limitations | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| | Q26. Conclusions | Y | Y | Y | Y | Y | Y | Y | Y | 100% |
| Funding | Q27. Funding | Y | Y | Y | Y | Y | Y | Y | Y | 100% |

Table 6: Results of evidence quality. ①The included studies have a large bias in methodology such as randomization, allocation concealment, and blinding. ②The confidence interval overlaps less, or the $I^2$ value of the combined results was larger. ③The sample size from the included studies does not meet the optimal sample size, or the 95% confidence interval crosses the invalid line. ④The funnel chart is asymmetry. *The 95% confidence interval does not cross the invalid line.

| Author, year (Country) | Outcomes | Studies (participants) | Limitations | Inconsistency | Indirectness | Imprecision | Publication bias | Relative effect (95% CI) | Quality |
|---|---|---|---|---|---|---|---|---|---|
| Mengyao Chao, 2018 (China) [28] | FBG (tai chi versus nonexercise) | 10 (489) | -1① | -1② | 0 | 0 | -1④ | MD = −1.39 (-1.95, -0.84)* | Very low |
| | FBG (tai chi versus other aerobic exercises) | 7 (342) | -1① | -1② | 0 | -1③ | -1④ | MD = −0.21 (-0.61, 0.19) | Very low |
| | HbA1c (tai chi versus nonexercise) | 7 (293) | -1① | 0 | 0 | -1③ | -1④ | MD = −0.73 (-0.95, -0.52)* | Very low |
| | HbA1c (tai chi versus other aerobic exercises) | 7 (372) | -1① | -1② | 0 | -1③ | -1④ | MD = −0.19 (-0.37, 0.00)* | Very low |
| | PBG (tai chi versus nonexercise) | 5 (162) | -1① | 0 | 0 | -1③ | -1④ | MD = −2.07 (-2.89, -1.26)* | Very low |
| | PBG (tai chi versus other aerobic exercises) | 3 (84) | -1① | 0 | 0 | -1③ | -1④ | MD = −0.44 (-1.42, 0.54) | Very low |
| Myeong Soo Lee, 2015 (South Korea) [29] | HbA1c (tai chi versus ST) | 3 (127) | -1① | 0 | 0 | -1③ | -1④ | MD = −0.54 (−1.23, 0.15) | Very low |
| | HbA1c (tai chi versus other aerobic exercises) | 2 (148) | -1① | 0 | 0 | -1③ | -1④ | MD = 0.00 (−0.31, 0.31) | Very low |
| | HbA1c (no) | 2 (84) | -1① | -1② | 0 | -1③ | -1④ | MD = −1.58 (−3.83, 0.67) | Very low |
| | FBG (tai chi versus ST) | 4 (188) | -1① | 0 | 0 | -1③ | -1④ | MD = −1.57 (−2.34, −0.80)* | Very low |
| | FBG (tai chi versus other aerobic exercises) | 4 (212) | -1① | 0 | 0 | -1③ | -1④ | MD = −0.03 (−0.49, 0.42) | Very low |
| Shuai Guo, 2021 (China) [30] | FBG (tai chi versus ST) | 15 (1,023) | -1① | -1② | 0 | 0 | -1④ | MD = −1.04 (-1.42, -0.66)* | Very low |
| | FBG (tai chi versus other aerobic exercises) | 8 (619) | -1① | -1② | 0 | -1③ | -1④ | MD = −0.03 (-0.30, 0.23) | Very low |
| | HbA1c (tai chi versus ST) | 9 (749) | -1① | -1② | 0 | 0 | -1④ | MD = −0.73 (-1.03, -0.43)* | Very low |
| | HbA1c (tai chi versus other aerobic exercises) | 5 (504) | -1① | 0 | 0 | 0 | -1④ | MD = −0.33 (-0.61, 0.04)* | Low |
| | PBG (tai chi versus ST) | 2 (260) | -1① | 0 | 0 | -1③ | -1④ | MD = −1.58 (-1.94,-1.22)* | Very low |
| | TCL (tai chi versus ST) | 11 (868) | -1① | -1② | 0 | 0 | -1④ | MD = −0.51 (-0.88, -0.14)* | Very low |

Table 6: Continued.

| Author, year (Country) | Outcomes | Studies (participants) | Limitations | Inconsistency | Indirectness | Imprecision | Publication bias | Relative effect (95% CI) | Quality |
|---|---|---|---|---|---|---|---|---|---|
| | TCL (tai chi versus other aerobic exercises) | 5 (423) | -1① | 0 | 0 | -1③ | -1④ | MD = −0.08 (-0.24, 0.09) | Very low |
| | TG (tai chi versus ST) | 9 (745) | -1① | -1② | 0 | 0 | -1④ | MD = −0.40 (-0.72, -0.07)* | Very low |
| | TG (tai chi versus other aerobic exercises) | 4 (332) | -1① | -1② | 0 | -1③ | -1④ | MD = 0.04 (−0.22, 0.31) | Very low |
| | HDL (tai chi versus ST) | 9 (798) | -1① | -1② | 0 | 0 | -1④ | MD = 0.39 (0.14, 0.63)* | Very low |
| | HDL (tai chi versus other aerobic exercises) | 5 (538) | -1① | 0 | 0 | 0 | -1④ | MD = 0.24 (0.07, 0.41)* | Low |
| | LDL (tai chi versus ST/other aerobic exercises) | 9 (730) | -1① | -1② | 0 | 0 | -1④ | MD = −0.79 (-1.27, -0.30)* | Very low |
| | BMI (tai chi versus ST) | 5 (358) | -1① | 0 | 0 | 0 | -1④ | MD = −1.15 (-1.79, -0.51)* | Low |
| | SBP (tai chi versus ST) | 5 (390) | -1① | 0 | 0 | -1③ | -1④ | MD = −11.86 (-14.47, -9.25)* | Very low |
| | DBP (tai chi versus ST) | 5 (390) | -1① | -1② | 0 | -1③ | -1④ | MD = −7.93 (-12.39, -3.46)* | Very low |
| | FINs (tai chi versus ST) | 3 (255) | -1① | 0 | 0 | -1③ | -1④ | MD = −1.02 (-1.39, -0.64)* | Very low |
| | HOMA-IR (tai chi versus ST) | 3 (255) | -1① | -1② | 0 | -1③ | -1④ | MD = −0.65 (-1.01, -0.30)* | Very low |
| Ting-Wei Xia, 2019 (China) [31] | FBG | 13 (616) | -1① | -1② | 0 | 0 | -1④ | SMD = −0.54 (-0.91, -0.16)* | Very low |
| | HbA1c | 9 (517) | -1① | -1② | 0 | 0 | -1④ | SMD = −0.68 (-1.17, -0.19)* | Very low |
| | TCL | 8 (343) | -1① | 0 | 0 | -1③ | -1④ | SMD = −0.35 (-0.54, -0.16)* | Very low |
| | TG | 8 (359) | -1① | 0 | 0 | -1③ | -1④ | SMD = −0.19 (-0.31, -0.07)* | Very low |
| | HDL | 6 (290) | -1① | 0 | 0 | -1③ | -1④ | SMD = 0.04 (− 0.01, 0.09) | Very low |
| | LDL | 6 (290) | -1① | -1② | 0 | -1③ | -1④ | SMD = −0.49 (− 1.06, 0.08) | Very low |
| | BMI | 6 (296) | -1① | 0 | 0 | -1③ | -1④ | SMD = −0.61 (− 0.85, − 0.38)* | Very low |

Table 6: Continued.

| Author, year (Country) | Outcomes | Studies (participants) | Limitations | Inconsistency | Indirectness | Imprecision | Publication bias | Relative effect (95% CI) | Quality |
|---|---|---|---|---|---|---|---|---|---|
| Zonglei Zhou, 2019 (China) [32] | FBG | 21 (1,115) | -1① | -1② | 0 | 0 | 0 | SMD = −0.67 (-0.87, -0.47)* | Low |
| | HbA1c | 12 (714) | 0 | 0 | 0 | 0 | 0 | MD = −0.53 (-0.62, -0.44)* | High |
| | FINs | 8 (500) | -1① | -1② | 0 | -1③ | 0 | SMD = −0.32 (-0.71, 0.07) | Very low |
| | HOMA-IR | 5 (332) | 0 | 0 | 0 | -1③ | 0 | MD = −0.41 (-0.78, -0.04)* | Moderate |
| | TCL | 10 (658) | -1① | -1② | 0 | 0 | 0 | SMD = −0.59 (-0.90, -0.27)* | Low |
| | BMI | 7 (388) | 0 | 0 | 0 | -1③ | 0 | MD = −0.82 (-1.28, -0.37)* | Moderate |
| | Balance | 2 (107) | 0 | -1② | 0 | -1③ | 0 | MD = 2.71 (-3.29, 8.71) | Low |
| | SBP | 5 (290) | 0 | -1② | 0 | -1③ | 0 | MD = −10.03 (-15.78, -4.29)* | Low |
| | DBP | 5 (290) | 0 | 0 | 0 | -1③ | 0 | MD = −4.85 (-8.23, -1.47)* | Moderate |
| | Physical function | 5 (389) | -1① | -1② | 0 | -1③ | 0 | MD = 7.07 (0.79, 13.35)* | Very low |
| | Bodily pain | 5 (389) | -1① | 0 | 0 | -1③ | 0 | MD = 4.30 (0.83, 7.77)* | Low |
| | Social function | 6 (426) | 0 | -1② | 0 | 0 | 0 | MD = 13.84 (6.22, 21.47)* | Moderate |
| Yao Ge, 2020 (China) [33] | FBG | 9 (560) | -1① | -1② | 0 | 0 | -1④ | SMD = −0.607 (-0.930, -0.284)* | Very low |
| | HbA1c | 7 (434) | -1① | -1② | 0 | 0 | -1④ | SMD = −0.585 (-0.784, -0.386)* | Very low |
| | TCL | 7 (533) | -1① | -1② | 0 | -1③ | -1④ | SMD = −0.418 (-0.897, 0. 061) | Very low |
| | TG | 6 (480) | -1① | -1② | 0 | 0 | -1④ | SMD = −0.833 (-1.383, -0.283)* | Very low |
| | HDL | 4 (420) | -1① | -1② | 0 | 0 | -1④ | SMD = 0.458 (0. 063, 0.852)* | Very low |
| | LDL | 3 (76) | -1① | -1② | 0 | -1③ | -1④ | SMD = −1.252 (-2.305, -0.199) | Very low |

Table 6: Continued.

| Author, year (Country) | Outcomes | Studies (participants) | Limitations | Inconsistency | Indirectness | Imprecision | Publication bias | Relative effect (95% CI) | Quality |
|---|---|---|---|---|---|---|---|---|---|
| Yongjin Liu, 2017 (China) [34] | FBG | 9 (727) | -1① | 0 | 0 | 0 | 0 | SMD = −0.39 (-0.54, -0.24)* | Moderate |
| | HbA1c | 7 (645) | -1① | 0 | 0 | 0 | 0 | MD = −0.59 (-0.73, -0.44)* | Moderate |
| | TCL | 7 (612) | -1① | -1② | 0 | -1③ | 0 | SMD = −0.24 (-0.58, 0.10) | Very low |
| | TG | 7 (612) | -1① | -1② | 0 | 0 | 0 | SMD = −0.52 (-0.85, -0.19)* | Low |
| | HDL | 6 (566) | -1① | 0 | 0 | 0 | 0 | SMD = 0.31 (0.14, 0.47)* | Moderate |
| | LDL | 6 (462) | -1① | -1② | 0 | 0 | 0 | MD = −0.32 (-0.59, -0.05)* | Low |
| Qing Tang, 2017 (China) [35] | FBG | 7 (354) | -1① | -1② | 0 | -1③ | -1④ | MD = −0.74 (-1.32, -0.16)* | Very low |
| | HbA1c | 7 (572) | -1① | -1② | 0 | 0 | -1④ | MD = −0.77 (-1.16, -0.39)* | Very low |
| | BMI | 4 (316) | -1① | 0 | 0 | -1③ | -1④ | MD = −1.64, (-2.35, -0.92)* | Very low |
| | TG | 6 (518) | -1① | -1② | 0 | 0 | -1④ | MD = −0.33 (-0.49, -0.17)* | Very low |
| | TCL | 6 (518) | -1① | -1② | 0 | 0 | -1④ | MD = −0.08 (-0.33, -0.48)* | Very low |
| | Quality of Life | 2 (264) | -1① | 0 | 0 | -1③ | -1④ | MD = 45.47 (18.24, 72.71)* | Very low |

based on RCTs and published from 2015 to 2021. Among them, 7 (7/8, 87.5%) SRs/MAs were published in the past five years, indicating that the improvement effect of TC on T2DM has attracted increasing attention over the years.

Based on the results of the AMSTAR-2 evaluation in this overview, the methodological quality of all SRs/MAs was assessed as very low, especially in item 2 (protocol registration, 0/8, 0%), item 7 (exclusion list, 0/8, 0%), and item 13 (RoB account, 2/8, 25%). None of the SR/MA registered study protocols. No SR/MA contained initial research protocol registrations, which could lead to greater than expected adjustments to the research process, increasing the risk of bias and impacting the rigor and credibility of the final SR/MA results [44]. None of the SR/MA provides a complete exclusion of the lists for each study, which may affect the reliability of the results and assessment of publication bias. Providing a list of exclusion researches is a more strong demonstration of the rigor of the literature screening process. The authors of the 6 SRs/MAs did not consider the risk of bias of including RCTs when interpreting or discussing the study results, which may reduce the reliability of the final results. The risk of bias assessment of the ROBIS scale indicated that only one SR/MA was at low risk. Further analysis revealed that inadequate interpretation of the risk of bias and inadequate evaluation of publication bias were the main factors contributing to the high risk of bias. Similar to the results of the AMSTAR-2, the PRISMA assessment results indicate a lack of registration of programs. In addition, the included literatures only provided search keywords without elaborating specific search strategies, which reduces the reproducibility and credibility of the research.

According to the evidence quality assessment for the 65 outcomes by means of GRADE, 1 was rated as high quality, 7 moderate quality, 10 low quality, and 47 very low quality. Further generalization revealed the following common pitfalls in the inclusion of RCTs: only randomization was mentioned, without elaborating specific randomization method; allocation was not concealed; and only single blinding was performed. Therefore, the low methodological quality of the included trials was the underlying factor contributing to the decline of evidence quality. Besides, the lack of publication bias assessment was also an important reason for the downgrading of the evidence quality. The high heterogeneity of relevant outcome measures may be related to the unreasonable design of the original study. In addition, the insufficient scale included in the RCTs was also an important downgrading factor. Descriptive analysis showed that TC is an effective and safe method for the treatment of T2DM. Due to the low quality of methodology and evidence from the included studies, the conclusions of SRs/MAs may differ from real results, so we cannot draw firm conclusions about TC for T2DM.

*4.2. Implications for Practice and Research.* As a regular small-to-medium-intensity aerobic exercise, the main benefit of TC is not to consume calories but to promote the metabolism of cells and tissues, enhance cardiopulmonary function, activate antioxidant and anti-inflammatory activities, promote blood return to the heart, and improve the body's ability to respond to glucose. It can improve the utilization rate of target cells, improve the body's glucose tolerance, prevent the composition of HbA1c, and accelerate the combination of hemoglobin and oxygen [45]. In addition to this, the antioxidant effect of TC can be explained by the excitatory process, in which TC acts as a moderate exercise and the continuous stimulation of ROS generated by the adaptive process promotes the antioxidant response [46]. In this case, the main associated proteins are MAPK and FNkB [47] and the antioxidant gene Nrf2. Regular exercise leads to the upregulation of endogenous antioxidant defenses and counteracts the harmful effects of ROS [48]. Given the nature of TC exercise, the antioxidant effects may be attributed not only to the described excitatory processes but also to the interplay among several mechanisms related to meditation and diaphragmatic respiration. Studies have shown that psychological stress is positively correlated with increased production of free radicals and OS [49]. As for diaphragmatic breathing exercise, one study showed that the relaxation induced by diaphragmatic breathing increases the antioxidant defense status of athletes after exhaustive exercise [50].

In conclusion, when using the AMSTAR-2, PRISMA, ROBIS, and GRADE assessments to assess and standardize various aspects of the included SR/MA, researchers are expected to register or publish research protocols in advance when conducting SRs/MAs to minimize the risk of bias and ensure the accuracy of SRs/MAs results, and they should also provide a list of excluded literatures to ensure transparency and avoid publication bias. For literatures at high risk of bias, researchers should conduct separate analyses and provide reasonable explanations to ensure the quality of the evidence. In addition, a complete assessment of publication bias would also improve the accuracy of the meta-analysis results. Although the peculiarities of TC therapy can make blinding difficult to implement, a carefully designed and rigorously implemented RCT can minimize or avoid bias, which is the gold standard for evaluating interventions [51]. Clinical researchers should improve the top-level design of clinical trials through comprehensive evaluation and sophisticated analysis. Notably, Consolidated Standards of Reporting Trials (CONSORT) should be used to improve the quality of evidence from RCTs [52]. In view of the low evidence quality regarding the effect of TC exercise on T2DM, researchers should strictly describe all stages of their research, specify the research scheme, and register study protocols in their future researches, so as to facilitate publication and subsequent inclusion in SRs/MAs. At the same time, during SRs/MAs, researchers should also strictly follow relevant methodologies to improve the evidence system.

Although TC originated from traditional Chinese medicine theory, the duration, frequency, and mode of TC movement vary greatly in different studies. Therefore, we propose to use a standardized TC training program, where duration, frequency, and pattern are formalized, so as to better study the impact of TC on T2DM. Researchers should also pay attention to the effect of TC on related biochemical indicators in T2DM patients, including indicators related to OS. In addition to this, most studies focused on the therapeutic

effects of TC on T2DM, but it still remains unclear whether TC can reduce the risk of associated complications in individuals with T2DM. Further research on the relationship between TC and the risk of developing various complications of T2DM is recommended.

*4.3. Strength and Limitations.* Our overview is the first to use AMSTAR2, ROBIS, PRISMA, and GRADE to evaluate SRs/MAs regarding the impact of TC on T2DM. Based on the current results, TC may be an effective adjunctive replacement therapy for T2DM. Furthermore, the evaluation process revealed clear limitations of the current relevant SRs/MAs and RCTs, which may help guide future high-quality clinical studies. However, this overview has certain limitations because the assessment is subjective. While our assessments were assessed and reviewed by two independent assessors, different assessors may have their personal judgment on each factor, so the results may vary.

## 5. Conclusion

In conclusion, TC is beneficial and safe for T2DM. However, due to the generally low methodological and evidentiary quality of the included SRs/MAs, clinicians should approach these findings with caution in their practice.

## Data Availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## Conflicts of Interest

The authors declare no conflict of interest.

## Authors' Contributions

LYJ, WWB, and SGM participated in the research design. SHS, DCD, WD, and, LPL conducted a literature search and screened data extraction. LPL, ZYF, and WD analyzed the data, did a statistical analysis, and wrote a manuscript. SHS, WWB, and LPL participated in the correction of the manuscript. The manuscript was revised by WST. All authors reviewed the manuscript. All authors read and approved the final version of the manuscript. Hongshuo Shi and Shaoting Wang are the co-first authors.

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