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ABOUT COVER

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EDITORIAL

Effects of Tai Chi in diabetes patients: Insights from recent research

Hidetaka Hamasaki

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Abstract

Tai Chi, a practice that combines elements of both exercise and mindfulness, offers a wide range of health benefits. The body of evidence concerning the impact of Tai Chi on diabetes has recently been growing. This editorial aims to provide a concise summary of the current state of evidence for Tai Chi's effects on individuals with type 2 diabetes (T2D). The review includes 3 randomized controlled trials (RCTs) and 5 systematic reviews and meta-analyses, all of which investigate the effectiveness of Tai Chi on various health outcomes in individuals with T2D. Tai Chi demonstrates a significant effect to enhance glycemic control, lower blood pressure, improve serum lipid profiles, reduce insulin resistance, positively influence obesity-related indices, and improve overall quality of life in individuals with T2D. However, it is noteworthy that recent RCTs have reported inconsistent findings regarding the effects of Tai Chi on glycemic control and insulin resistance. The author also delves into potential mechanisms by which Tai Chi may exert its influence on the human body. Finally, the editorial highlights the critical issues that warrant further exploration in the future.

Key Words: Type 2 diabetes; Tai Chi; Exercise; Mind-body exercise; Chinese traditional exercise; Randomized controlled trial; Systematic review and meta-analysis

Core Tip: Exercise therapy plays a crucial role in the management of diabetes. Tai Chi offers a unique approach, serving not only as a moderate-intensity exercise but also as a mindfulness intervention that incorporates deep breathing and meditation. While prior systematic reviews have highlighted the favorable effects of Tai Chi on glycemic control and metabolic parameters in individuals with type 2 diabetes, recent randomized controlled trials have yielded inconsistent findings. This suggests that Tai Chi holds potential as an addition to diabetes management, yet there remains an insufficiency of scientific evidence, particularly in terms of elucidating its biological mechanisms of action.

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INTRODUCTION

Tai Chi, a traditional Chinese martial art, provides various health benefits. In 2016, Huston and McFarlane[1] summarized the evidence regarding the effects of Tai Chi on health as follows:

Strong evidence supports the positive impact of Tai Chi exercise in managing depression, aiding in cardiac and stroke rehabilitation, and addressing dementia. There is also moderate evidence suggesting that Tai Chi exercise can improve the quality of life (QOL) for patients with cancer, fibromyalgia, hypertension, and osteoporosis. However, there is currently no clear evidence directly linking Tai Chi exercise to benefits for diabetes, rheumatoid arthritis, or chronic heart failure. Systematic reviews on overall health and fitness consistently demonstrate strong evidence for improving balance and aerobic capacity, particularly in individuals with low fitness levels. Furthermore, there is good evidence indicating that Tai Chi exercise can enhance lower limb strength, with moderate evidence suggesting improvements in overall well-being and sleep quality.

Tai Chi serves as a dual intervention, encompassing both exercise and mindfulness. Mindfulness-based interventions have demonstrated a positive impact on glycemic control, leading to an approximate 0.3% reduction in hemoglobin A1c (HbA1c) levels in individuals with type 2 diabetes (T2D)[2]. In addition, traditional martial arts, such as Tai Chi, incorporate breathing techniques aimed at harmonizing the human body. For instance, diaphragmatic breathing can effectively alleviate both physiological and psychological stress, potentially benefiting the brain and cardiovascular (CV) system by enhancing autonomic nervous system (ANS) function[3]. Notably, a recent systematic review has revealed that Tai Chi enhances heart rate variability, which is closely associated with ANS function, when compared to control groups [4].

Patients with diabetes frequently experience ANS dysfunction, and CV autonomic neuropathy plays a pivotal prognostic role in CV morbidity and mortality. Exercise holds the potential to rectify sympatho-vagal balance by elevating parasympathetic nervous system activity while reducing sympathetic nervous activity in individuals with T2D. This adjustment could be particularly valuable in the management of diabetes patients at risk of severe hypoglycemia unawareness[5]. Consequently, Tai Chi, which incorporates both exercise and mindfulness, may offer additional benefits to diabetes patients compared to conventional structured exercise.

The body of evidence regarding the effects of Tai Chi on diabetes has been steadily growing. A PubMed search utilizing the terms "Tai Chi" and "diabetes" yielded 168 articles as of September 30, 2023. Among these, 52 articles were randomized controlled trials (RCTs) and systematic reviews. In this paper, the author reviewed representative RCTs and systematic reviews published within the last five years, summarizing the current state of evidence on this topic.

RANDOMIZED CONTROLLED TRIALS

The author identified three eligible RCTs published between 2018 and 2023.

In the study by Chan *et al*[6], the effects of Tai Chi on reducing CV risk factors were compared to brisk walking in adults with hypertension. Although the study participants were not necessarily patients with diabetes, 58.5% of them had diabetes, prompting the author's review. A total of 246 adults with hypertension were randomly assigned to three groups: Tai Chi (n = 82), brisk walking (n = 82), or a control group (n = 82). However, 52 participants dropped out at the 9-month follow-up point, resulting in a dropout rate of 21.1%. An intention-to-treat analysis was conducted accordingly. The adherence rate to the exercise intervention was 90% in the Tai Chi group and 88% in the brisk walking group during the 3-mo study period. In the Tai Chi group, participants performed a 24-form Yang style Tai Chi for 60 min, twice a week, for a duration of three months. All training sessions were led by the same qualified and experienced Tai Chi Master. Additionally, participants were encouraged to engage in daily Tai Chi practice at home for 30 min, on at least five days each week, both during and after the 3-mo study period. Adherence to the intervention was defined as successfully completing at least 80% of the recommended sessions.

In contrast, individuals in the brisk walking group were instructed to walk at a pace of 5 to 6 km/h for 30 min a day, on at least five days per week. Each participant was provided with a pulse oximeter to monitor their heart rate during brisk walking and was advised to aim for a personalized heart rate corresponding to a moderate-intensity exercise, determined based on their age. The primary outcome measured was the change in blood pressure. Secondary outcomes

included fasting blood glucose and HbA1c levels.

At the 9-mo follow-up, Tai Chi demonstrated significant improvements in various CV risk factors compared to the control group. These improvements encompassed a substantial decrease in systolic blood pressure (−13.33 mmHg) and diastolic blood pressure (−6.45 mmHg), lower fasting blood glucose levels (−0.72 mmol/L), reduced HbA1c levels (−0.39%), decreased perceived stress, and improved mental health and self-efficacy in exercising. Notably, the Tai Chi group exhibited even more pronounced improvements compared to the brisk walking group. Specifically, the Tai Chi group experienced more substantial decreases in systolic blood pressure (−12.46 mmHg), diastolic blood pressure (−3.20 mmHg), and fasting glucose levels (−1.27 mmol/L), more significant drops in HbA1c levels (−0.56%), lower levels of perceived stress, and more substantial enhancements in perceived mental health and exercise self-efficacy in contrast to the brisk walking group. However, no significant differences emerged in body mass index (BMI), waist circumference, serum cholesterol levels, aerobic endurance, and perceived physical health among the groups at the 9-mo follow-up.

The authors concluded that Tai Chi displayed more favorable effects on CV risks compared to brisk walking. However, it is worth noting that they did not describe whether the participants in the brisk walking group were encouraged to continue brisk walking after the 3-mo intervention, whereas the participants in the Tai Chi group were encouraged to engage in daily Tai Chi practice at home for 30 min. This disparity in post-intervention activities may have introduced some bias. Indeed, at the 3-mo mark, no significant differences in glycemic control appeared to exist between the Tai Chi group and brisk walking group.

Li *et al*[7] conducted a study to examine the therapeutic effects of Tai Chi and Qigong exercises in middle-aged and older patients with T2D. Initially, 103 eligible patients were randomly allocated to the Tai Chi group, the Qigong group, and the control group. However, 16 participants dropped out, leaving 24 participants in the Tai Chi group, 34 participants in the Qigong group, and 29 participants in the control group who completed the study. Each group engaged in 60 min of exercise, five times a week, over a span of 12 wk.

For the Tai Chi intervention, the authors employed the classical Chen style (18 forms), and these Tai Chi classes were led by an experienced instructor. Each session adhered to a structured format, commencing with a 10-min warm-up and self-massage, followed by a 10-min review of principles and essential movements, 30 min of practice and skill-building, and concluding with a 10-min relaxation period.

In contrast, the Qigong exercise was specifically tailored based on the theory of meridians in traditional Chinese medicine, with a focus on the unique characteristics of diabetic patients. Each Qigong movement was designed to promote meridian circulation, emphasizing the benefits of clearing various meridians to prevent and treat diseases related to internal organs. Professional instructors from the Fitness Qigong Association of Jiaozuo led the fitness Qigong classes, and similar to the Tai Chi sessions, these classes followed a structured pattern. They started with a 10-min warm-up and self-massage, followed by a 10-min review of movement principles and breathing techniques, a 30-min practice session, and ended with a 10-min relaxation period. Throughout the intervention period, participants were encouraged to practice Qigong for approximately 60 min at home.

As for the control group, participants engaged in low-intensity stretching exercises. Each class session consisted of 40 min of activity, which included a 10-min warm-up and self-massage, followed by 30 min of supervised stretching exercises. These stretching exercises primarily focused on the upper body, trunk, and lower body, incorporating controlled breathing and relaxation techniques.

Fasting blood glucose levels increased in the control group, slightly decreased in the Qigong group, and remained unchanged in the Tai Chi group. However, there was no significant difference in the effects of the interventions between the groups. Although no significant difference was observed between the groups, HbA1c levels decreased in the control group and showed no change in the Qigong group. On the other hand, HbA1c levels increased in the Tai Chi group compared to those in the control group. This result is unexpected, as regular exercise typically leads to improvements in glycemic control. The author speculates that other factors, such as dietary intake and changes in medications, which were not mentioned in the paper, may have contributed to this outcome.

Furthermore, blood C-peptide levels significantly decreased in the Tai Chi group compared to both the Qigong group and the control group. The authors mentioned the possibility that Tai Chi exercise may lead to a decrease in insulin secretion in patients with T2D. However, given that Tai Chi involves moderate-intensity exercise and offers various health benefits, including insulin sensitivity[8], caution is needed in interpreting these results.

Most recently, Chen *et al*[9] assessed the effectiveness of Tai Chi exercise in improving cognitive function compared to fitness walking in patients with T2D who had mild cognitive impairment. A total of 328 patients were randomly assigned to three groups: The Tai Chi group (n = 107), the fitness walking group (n = 110), and the control group (n = 111). All participants were included in the intention-to-treat analysis, and 289 participants (88.1%) completed the study, with 282 participants (86.0%) evaluated at the 36-wk follow-up. The adherence rate for the intervention was 88.8% in the Tai Chi group and 90.0% in the fitness walking group.

The primary outcome measured was cognitive function, assessed using the Montreal Cognitive Assessment (MoCA) at 36 wk. Secondary outcomes included fasting blood glucose, insulin, homeostasis model assessment-insulin resistance (HOMA-IR), HbA1c, advanced glycation end products (AGEs), and soluble receptor of AGE (sRAGE) levels. All groups attended educational seminars focused on T2D management, with each seminar lasting 30 min. These seminars were held once every four weeks during the 24-wk study period.

Participants in the Tai Chi group underwent supervised 24-wk training in the 24-form Tai Chi, with 1-h training sessions three times a week. Additionally, participants were encouraged to continue exercising beyond the 24-wk supervised period, up until the 36-wk follow-up evaluation. Certified instructors guided the fitness walking and Tai Chi exercises. Participants in the control group did not receive any exercise intervention and maintained their usual lifestyle.

In comparison to fitness walking, Tai Chi exercise at 36 wk demonstrated a greater improvement in mean MoCA scores (24.67 ± 2.72 *vs* 23.84 ± 3.17; between-group mean difference (MD) of 0.84; 95%CI: 0.02 to 1.66). Furthermore, Tai Chi was more effective in enhancing other cognitive subdomain test results, fasting blood glucose levels (129.4 ± 25.9 mg/dL *vs* 139.5 ± 36.2 mg/dL; between-group MD of −10.3; 95%CI: −18.6 to −2.3), and the AGE/sRAGE ratio (0.05 ± 0.03 *vs* 0.07 ± 0.05; between-group MD of −0.02; 95%CI: −0.03 to −0.01). However, no significant differences were observed in HOMA-IR and HbA1c levels.

At 24 wk, Tai Chi did not exhibit a significantly greater improvement in MoCA scores compared to fitness walking. Nonetheless, Tai Chi was more effective in enhancing certain cognitive subdomain test results compared to fitness walking. Additionally, there were no differences between groups in fasting blood glucose and HbA1c levels, HOMA-IR, and the AGE/sRAGE ratio.

Compared with the control group, Tai Chi significantly improved mean MoCA scores at 24 wk (23.99 ± 3.10 *vs* 22.54 ± 3.29; between-group MD of 1.45; 95%CI: 0.59 to 2.32). However, no significant improvements were observed in fasting blood glucose and HbA1c levels, HOMA-IR, and the AGE/sRAGE ratio.

At 36 wk, the Tai Chi group exhibited a significant improvement in cognitive subdomain test results, fasting blood glucose levels (129.4 ± 25.9 mg/dL *vs* 140.0 ± 29.7 mg/dL; between-group MD of -10.6; 95%CI: −18.9 to −2.3), and the AGE/sRAGE ratio (0.05 ± 0.03 *vs* 0.07 ± 0.04; between-group MD of −0.02; 95%CI: −0.03 to −0.01). However, HbA1c levels and HOMA-IR did not differ significantly between groups.

Although one patient in the control group reported hospital admission and one patient in the Tai Chi group visited the emergency department, there was no significant difference in the number of adverse events among the three groups. These findings suggest that Tai Chi exercise has a beneficial effect on cognitive function and may offer protection to the vasculature. However, it may not significantly improve glycemic control in patients with T2D.

Table 1 summarizes RCTs investigating the effects of Tai Chi in patients with T2D.

SYSTEMATIC REVIEWS

The author identified a total of five systematic reviews and meta-analyses investigating the effects of Tai Chi on health outcomes in patients with T2D.

In a review by Zhou *et al*[10], 25 studies were assessed, focusing on the effects of Tai Chi on physiological parameters, balance function, and QOL in T2D patients. It's worth noting that all the included studies were RCTs; however, 17 out of 25 (68%) articles were of Chinese origin and were not listed in major English databases such as PubMed.

The review found that Tai Chi was effective in reducing fasting blood glucose levels [21 studies; standardized MD (SMD) = −0.67; 95%CI: −0.87 to −0.47], HbA1c levels (14 studies; MD = −0.88%; 95%CI: −1.45% to −0.31%), HOMA-IR (5 studies; MD = −0.41; 95%CI: −0.78 to −0.04), total cholesterol levels (10 studies; SMD = −0.59; 95%CI: −0.90 to −0.27), systolic blood pressure (5 studies; MD = −10.03 mmHg, 95%CI: −15.78 to −4.29 mmHg), diastolic blood pressure (5 studies; MD = −4.85 mmHg, 95%CI: −8.23 to −1.47 mmHg), and BMI (7 studies; MD = −0.82 kg/m^2, 95%CI: −1.28 to −0.37 kg/m^2). Moreover, Tai Chi improved QOL in terms of physical function, pain, and social function. However, Tai Chi exhibited no significant effect on fasting insulin levels and balance function. The quality of the included studies was assessed using the PEDro scale. Most of the studies received scores of 4 or 5, with only 3 studies scoring 6 to 10 (indicating high quality).

Xia *et al*[11] investigated the differences in the effectiveness of Tai Chi for glycemic control in T2D patients based on different durations and styles of interventions. The analysis included a total of 17 RCTs. Among these, 12 studies (70.6%) were conducted in China, while others were carried out in Taiwan, Australia, and Thailand. Various Tai Chi styles were utilized, including the simplified style (n = 4), Yang-style (n = 3), Lin-style (n = 1), Da Yuan Jiang Tang-style (n = 1), Chen-style (n = 2), Sun-style and Yang-style (n = 2), Tai Chi Ball (n = 2), and an unknown style (n = 2). The number of sessions per week and the duration of Tai Chi exercise also varied.

The findings revealed that Tai Chi significantly reduced fasting blood glucose levels (13 studies; SMD = −0.54; 95%CI: −0.91 to −0.16) and HbA1c levels (9 studies; SMD = −0.68; 95%CI: −1.17 to −0.19) in comparison to control groups. However, Yang-style Tai Chi and 24 movements (simplified style) did not show improvements in glycemic control, and other styles with a duration of ≤ 12 wk also had no significant effects on glycemic control. On the other hand, Tai Chi, excluding the simplified style and Yang-style Tai Chi with a duration of > 12 wk, led to reductions in fasting blood glucose (2 studies; SMD = −0.90; 95%CI: −1.28 to −0.52) and HbA1c levels (2 studies; SMD = −0.90; 95%CI: −1.28 to −0.52).

Furthermore, Tai Chi was effective in reducing total cholesterol (7 studies; SMD = −0.35; 95%CI: −0.54 to −0.16), triglycerides (8 studies; SMD = −0.19; 95%CI: −0.31 to −0.07), and BMI (6 studies; SMD = −0.61; 95%CI: −0.85 to −0.38). However, it is important to note that the methodological quality of the included studies was generally low due to potential biases, including the inability to blind participants to the intervention.

Palermi *et al*[12] examined the impact of Tai Chi programs on balance function in patients with T2D. This systematic review included only 3 RCTs and one before-after quasi-experimental study written in English. The findings revealed that Tai Chi effectively improved balance function, as measured by the single-leg stance test, tandem walk score, or Berg Balance Scale (SMD = 0.52; 95%CI: 0.20 to 0.84). The overall quality of the included RCTs, assessed using the Cochrane risk-of-bias tool, was categorized as "some concerns."

Qin *et al*[13] assessed the impact of Tai Chi on BMI, waist-to-hip ratio, and QOL in patients with T2D. The analysis included 15 RCTs and 3 quasi-experimental studies. Among these, 14 studies (77.8%) were conducted in China, while the others were carried out in Australia, South Korea, and Thailand. The results showed that compared with control groups, Tai Chi led to a reduction in BMI (11 studies; MD = −1.53 kg/m^2; 95%CI −2.71 kg/m^2 to −0.36 kg/m^2). However, Tai Chi

Table 1 Randomized controlled trials investigating the effects of Tai Chi on glycemic control, insulin resistance, and cognitive function in patients with type 2 diabetes

Ref.	Country	Study design	Study period (follow-up period)	Subjects (baseline characteristics)	Study outcomes	Intervention/Control	Results
Chan *et al* [6], 2018	China	Three-arm, randomized, controlled, parallel-group trial	12 wk (9 month)	246 patients with T2D. Tai Chi group (32 men and 50 women): Age: 64.70 ± 7.59 years, BMI: 26, 38 ± 4.26 kg/m^2, HbA1c: 6.66% ± 1.17% Walking group (42 men and 40 women): Age: 63.22 ± 11.11 years, BMI: 25.90 ± 4.39 kg/m^2, HbA1c: 7.10% ± 1.61% Control group (38 men and 44 women): Age: 65.13 ± 10.22 years, BMI: 25.72 ± 4.04 kg/m^2, HbA1c: 6.87 %± 1.25%	Primary outcome: Blood pressure Secondary outcomes: BMI, waist circumference, aerobic endurance, fasting blood glucose, HbA1c, TC, TG, HDL-C, LDL-C, perceived stress, quality of life, exercise self-efficacy	Tai Chi/brisk walking/usual physical activity	Blood pressure↓[1]. Fasting blood glucose↓, HbA1c↓, perceived stress↓. Exercise self-efficacy↑[2]
Li *et al*[7], 2020	China	Three-arm, randomized, controlled, parallel-group trial	12 wk	87 patients with T2D. Tai Chi group (12 men and 12 women): Age: 61.71 ± 6.91 years, BMI: 24.04 ± 2.98 kg/m^2, HbA1c: 8.20% ± 2.46% Qigong group (21 men and 13 women): Age: 59.71 ± 6.67 years, BMI: 25.21 ± 2.71 kg/m^2, HbA1c: 7.99% ± 1.66% Control group (14 men and 15 women): Age: 58.66 ± 10.89 years, BMI: 25.69 ± 2.57 kg/m^2, HbA1c: 7.63% ± 1.74%	Primary outcome: Fasting blood glucose, HbA1c, C-peptide	Tai Chi/Qigong/stretching exercise	Fasting blood glucose→[3], HbA1c↑, C-peptide↓
Chen *et al* [9], 2023	China	Three-arm, randomized, controlled, parallel-group trial	24 wk (36 wk)	328 patients with T2D. Tai Chi group (49 men and 58 women): Age: 67.56 ± 4.99 years, BMI: 24.32 ± 3.03 kg/m^2, HbA1c: 7.04% ± 1.20% Fitness walking group (61 men and 49 women): Age: 67.46 ± 4.73 years, BMI: 23.86 ± 2.90 kg/m^2, HbA1c: 6.84% ± 1.41% Control group (51 men and 60 women): Age: 67.62 ± 5.35 years, BMI: 23.98 ± 3.40 kg/m^2, HbA1c: 7.14% ± 1.48%	Primary outcome: MoCA assessment at 36 wk. Secondary outcomes: MoCA assessment at 24 wk, cognitive subdomain tests, fasting blood glucose, HbA1c, AGE, sRAGE, HOMA-IR	AE + RT/usual care	MoCA score at 36 wk↑ *vs* fitness walking group: (at 24 wk). Fasting blood glucose→, HbA1c→, HOMA-IR→, AGE: sRAGE ratio→. (at 36 wk) Fasting blood glucose↓, HbA1c→, HOMA-IR→, AGE: sRAGE ratio↓ *vs* control group: (at 24 wk) Fasting blood glucose→, HbA1c→, HOMA-IR→, AGE: sRAGE ratio→. (at 36 wk) Fasting blood glucose↓, HbA1c→, HOMA-IR→, AGE: SRAGE ratio↓

[1]Decreased.
[2]Increased/improved.
[3]Unchanged.
BMI: Body mass index; HbA1c: Hemoglobin A1c; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; MoCA: The Montreal Cognitive Assessment; AGE: Advanced glycation end products; sRAGE: Soluble receptor of advanced glycation end products; T2D: Type 2 diabetes; HOMA-IR: Homeostasis model assessment-insulin resistance.

did not have a significant effect on BMI when compared to other types of exercise, such as aerobic exercise, walking, and dancing. In contrast, Tai Chi did not demonstrate a beneficial effect on the waist-to-hip ratio. Moreover, Tai Chi improved QOL, as measured by Medical Outcomes Study Short Form-36 (*e.g.*, physical function: MD = 7.73; 95%CI: 1.76 to 13.71; body pain: MD = 8.49; 95%CI: 1.18 to 15.8; overall health: MD = 9.80; 95%CI: 5.77 to 13.82; social functioning: MD = 9.1; 95%CI: 4.75 to 13.45; mental health: MD = 5.62; 95%CI: 1.57), compared to control groups. The mean methodological quality score, as measured by the PEDro scale, was 5.3, indicating that the overall quality of the included studies was "fair".

Recently, Guo *et al*[14] reported that Tai Chi had a favorable impact on metabolic parameters in patients with T2D; however, its superior effects were observed only in relation to HbA1c and high-density lipoprotein cholesterol when compared to aerobic exercise. The analysis encompassed a total of 23 RCTs, with 19 studies (82.6%) conducted in China, 3 studies in Australia, and one study in South Korea.

Tai Chi was found to significantly reduce fasting blood glucose levels (15 studies; SMD = −1.04; 95%CI: −1.42 to −0.66) compared to conventional therapies; however, there was no significant difference in fasting blood glucose levels observed between the Tai Chi group and the aerobic exercise group. Furthermore, Tai Chi exhibited a positive effect on postprandial glucose levels (2 studies; MD = −1.58, units not specified but assumed to be mmol/L; 95%CI: −1.94 to −1.22) when compared to conventional therapies. The reduction in HbA1c levels was also more significant in the Tai Chi group (9 studies; MD = −1.28%; 95%CI: −2.06 to −0.51) compared to conventional therapies, and Tai Chi showed a trend towards statistical significance in decreasing HbA1c levels (5 studies; MD = −0.24%; 95%CI: −0.49 to 0.00, P = 0.05) compared to the aerobic exercise group.

Moreover, Tai Chi significantly reduced total cholesterol levels (11 studies; SMD = −0.50; 95%CI: −0.86 to −0.13), triglycerides (9 studies; SMD = −0.38; 95%CI: −0.65 to −0.10), and low-density lipoprotein cholesterol levels (9 studies; SMD = −0.38; 95%CI: −0.65 to −0.10) compared to conventional therapies. However, when compared to aerobic exercise, Tai Chi did not offer additional benefits in reducing total cholesterol, triglycerides, and low-density cholesterol levels.

On the other hand, Tai Chi significantly increased high-density cholesterol levels compared to both conventional therapies (9 studies, SMD = 0.13; 95%CI: 0.06 to 0.20) and aerobic exercise (5 studies, SMD = 0.07; 95%CI: 0.01 to 0.12). Moreover, Tai Chi was effective in decreasing BMI (5 studies; MD = −1.15 kg/m^2; 95%CI: −1.79 kg/m^2 to −0.51 kg/m^2), fasting insulin levels (7 studies; MD = −2.63, units not specified but assumed to be μIU/mL; 95%CI: −4.51 to −0.76), HOMA-IR (3 studies; MD = −1.02; 95%CI: −1.39 to −0.64), systolic blood pressure (5 studies; MD = −11.86 mmHg; 95%CI: −14.47 to −9.25 mmHg), and diastolic blood pressure (5 studies; MD = −9.58 mmHg; 95%CI: −11.52 to −7.63 mmHg) compared to conventional therapies. These reductions in BMI and blood pressure were observed regardless of whether the study duration was < 12 wk or 12-24 wk. However, the quality of the included studies remained an issue to be addressed, as there were no studies with a low risk of bias.

Table 2 summarizes systematic reviews and meta-analyses reporting the effectiveness of Tai Chi on health outcomes in T2D patients.

INSIGHTS FROM RECENT RESEARCH

Based on the findings of recent systematic reviews and meta-analyses, Tai Chi significantly reduces fasting blood glucose and HbA1c levels. Furthermore, Tai Chi improves blood pressure, serum lipid profiles, insulin resistance, obesity-related indices, and the QOL in patients with T2D. However, recent RCTs have shown inconsistent effects of Tai Chi on glycemic control and insulin resistance. This inconsistency may be attributed to variations in Tai Chi styles[11] and differences in control group interventions (*e.g.*, usual care, sham exercise, aerobic exercise)[13,14].

Most systematic reviews, except Palermi *et al*[12], have reported significant heterogeneity among the included RCTs. Therefore, the effectiveness of Tai Chi on glycemic control may vary based on demographic characteristics of study subjects, physical fitness, medications, the severity of diabetes, and comorbidities, including diabetic complications and CV diseases. Furthermore, the majority of the studies have been conducted in China, which could introduce regional bias and limit the generalizability of the evidence. In addition, there is a lack of well-designed studies investigating the effects of Tai Chi in patients with type 1 diabetes, a condition characterized by depleted insulin secretion. Since the pathophysiology of type 1 diabetes differs significantly from that of T2D, further studies in patients with type 1 diabetes are warranted to understand the potential benefits of Tai Chi.

Tai Chi, considered a moderate-intensity exercise, reduces oxidative stress in obese patients with T2D[15]. Exercise is known to have an anti-inflammatory effect mediated by various myokines, such as interleukin (IL)-6, tumor necrosis factor-α, and IL-10[16,17], suggesting that Tai Chi may also reduce inflammation[18]. Yeh *et al*[19] revealed that Tai Chi exercise decreased HbA1c levels along with an increase in IL-12 Levels and the T-cell helper type 1 reaction in patients with T2D, indicating that Tai Chi benefits immune function. Moreover, a 12-wk Tai Chi exercise improves physical function, vascular function, reduces anxiety and mental fatigue when compared to baseline[20], indicating Tai Chi's potential to help prevent frailty in older adults.

The molecular mechanisms underlying the effects of Tai Chi on noncommunicable diseases (NCDs) remain unclear. However, recent research provides valuable insights that helped elucidate the benefits of Tai Chi in patients with diabetes. Tai Chi exercise has reduced epicardial adipose tissue volume and heart rate by inactivating the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, along with an increase in serum miR-126 Levels in patients with coronary heart disease[21]. Additionally, Tai Chi influences specific gene expression involved in neutrophil activation, T-cell activation, and the Nod-like receptor signaling pathway in patients with Parkinson's disease. The key candidate genes play a role in modulating peripheral immunity and inflammation[22]. A recent study revealed

Table 2 Systematic reviews and meta-analyses reporting the effects of Tai Chi on glycemic control, metabolic parameters, physical function, and the quality of life in patients with type 2 diabetes

Ref.	Subjects	Interventions (Tai Chi style)	Comparators	Outcomes	Results
Zhou *et al*[10], 2019	1235 patients with T2D. Age: 35.6–69.5 years, sex: No description, BMI: No description, HbA1c: 6.9%–11.9%	Time: 15–120 min/session, Number of sessions: 2–14 sessions/wk, Duration of the intervention: 4–24 wk, (Simplified style, Chen style, Yang style, Sun and Yang style, Lin style, Da-yuan-jiang-tang style)	Usual care, usual exercise, or sham exercise	BMI, fasting blood glucose, HbA1c, insulin, HOMA-IR, TC, blood pressure, QoL (SF-36), balance (single-leg stance test)	BMI↓[1], fasting blood glucose ↓, HbA1c↓, HOMA-IR↓, insulin→[3], TC↓, Systolic blood pressure↓, diastolic blood pressure↓, QOL↑[2]
Xia *et al* [11], 2019	774 patients with T2D. Age: No description, sex: No description, BMI: No description, HbA1c: No description	Time: 30–60 min/session, Number of sessions: 2–14 sessions/wk, Duration of the intervention: 8–24 wk, (Simplified style, Chen style, Yang style, Sun and Yang style, Lin style, Da-yuan-jiang-tang style, Tai Chi Ball, Unknown style)	Usual care, wait list, dancing, walking or running, conventional exercise, sham exercise, no intervention	BMI, fasting blood glucose, HbA1c, TC, TG, HDL-C, LDL-C	BMI↓, fasting blood glucose ↓, HbA1c↓, TC↓, TG↓, HDL-C→, LDL-C→
Palermi *et al*[12], 2020	144 patients with T2D. Age: 62.73–66.05 years (mean), sex: No description, BMI: No description, HbA1c: No description	Time: 55–120 min/session Number of sessions: 2–3 sessions/wk, Duration of the intervention: 12–16 wk, (Yang and Sun style)	Usual care, sham exercise, no intervention	Balance function measured by single-leg stance test, tandem walk test, Balance Index, and Berg Balance Scale	Balance function↑
Qin *et al* [13], 2020	1418 patients with T2D. Age: 47–70 years, sex: No description, BMI: No description, HbA1c: No description	Time: 30–120 min/session, Number of sessions: 1–7 sessions/wk, Duration of the intervention: 12–24 wk	Usual care, wait-list, walking, brisk walking, aerobic exercise, dancing, Baduanjin, sham exercise, no intervention	BMI, waist-to-hip ratio, QOL measured by SF-36 or DSQOL	BMI↓, QOL↑
Guo *et al* [14], 2021	1549 patients with T2D, Age: 46.1–70.4 years (mean), sex: No description, BMI: No description, HbA1c: No description	Time: No description, Number of sessions: No description, Duration of the intervention: No description (Simplified style, Yang and Sun style, Yang style, Chen style)	Usual care, walking, brisk walking, dancing, aerobic exercise, sham exercise	BMI, fasting blood glucose, HbA1c, insulin, HOMA-IR, TC, TG, HDL-C, LDL-C, blood pressure	BMI↓, fasting blood glucose ↓ (→ *vs* aerobic exercise), HbA1c↓, insulin↓, HOMA-IR↓, TC↓ (→ *vs* aerobic exercise), TG↓ (→ *vs* aerobic exercise), HDL-C↓, LDL-C↓ (→ *vs* aerobic exercise), blood pressure↓

[1]Decreased.
[2]Increased/improved.
[3]Unchanged.
BMI: Body mass index; HbA1c: Hemoglobin A1c; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; QOL: Quality of life; SF-36: Medical Outcomes Study Short Form-36; DSQOL: Diabetes-specific quality of life; T2D: Type 2 diabetes; HOMA-IR: Homeostasis model assessment-insulin resistance.

that Tai Chi intervention reduced the expressions of inflammatory factors, including Never in Mitosis A-related Kinase 7, nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3, reactive oxygen species, nuclear factor-kappa B, and IL-1β in individuals with prediabetes[23]. Further, Tai Chi exercises may induce beneficial epigenetic changes[24]. Furthermore, Tai Chi may modulate the ANS and the hypothalamus–pituitary–adrenal axis, providing potential therapeutic effects on depression, mood disorders, stress, and gut dysbiosis[25-29] (Figure 1).

However, the effect of gene–environment interactions is likely substantial, considering the etiology of complex diseases, such as diabetes, making the combined effects of environmental and lifestyle factor assessment essential along with Tai Chi on metabolic mechanisms[30]. The combination of a healthy diet, nonsmoking habits, and appropriate alcohol intake with Tai Chi practice demonstrated potential for improving health outcomes in patients with diabetes in terms of the exercise aspect of Tai Chi. No studies directly compare the effectiveness of Tai Chi with these lifestyle factors on a one-to-one basis, while holistic lifestyle interventions are crucial for addressing metabolic disturbances and improving the prognosis of patients with diabetes[31-33]. Moreover, abnormal bowel health, such as chronic diarrhea or constipation, is associated with an increased risk of cancer, CV diseases, and diabetes. Chronic constipation contributes to a higher risk of CV mortality (hazard ratio = 1.698; 95%CI: 1.144 to 2.520)[34]. Indeed, high-fiber diets have improved glycemic control and insulin sensitivity, thereby decreasing all-cause mortality (risk ratio = 0.55; 95%CI: 0.35 to 0.86) when comparing the highest with the lowest fiber intakes[35]. In summary, Tai Chi exercise should be integrated with various lifestyle modifications to improve the management of patients with diabetes.

Molecular pathological epidemiology (MPE) research, which investigates the combined effects of Tai Chi in association with molecular pathologies and clinical outcomes, demonstrates the potential for elucidating the biological mechanisms of Tai Chi in the human body. Traditional epidemiological studies may underestimate true associations between diet, physical activity, smoking, drinking habits, and other lifestyle-related factors concerning molecular markers of genetic pathways and NCD risk. In contrast, MPE studies can help determine the association of such factors with the risk of colorectal cancer[36] and the relationship of host and microbial tryptophan metabolites with T2D risk[37]. The MPE

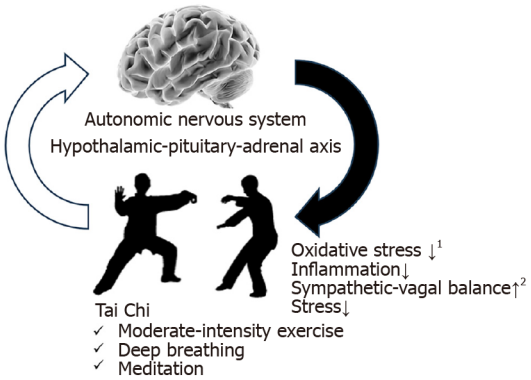

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Figure 1 Tai Chi exercise reduces oxidative stress, inflammation, and stress, while improving sympathetic-vagal balance. [1]Reduced; [2] Increased/improved.

research paradigm not only provides future perspectives on the dynamics among the environment, NCDs, and hosts but also introduces new areas for investigation. Emerging advancements, including computational digital pathology, systems biology, big data analytics, and artificial intelligence, will continue to revolutionize the fields of pathology and MPE[38]. This approach offers a promising direction for fully investigating the effects of Tai Chi on patients with diabetes.

However, high-quality evidence related to the physiological, endocrinological, and biochemical mechanisms of Tai Chi intervention is still insufficient due to methodological limitations, such as the blinding of study participants and the number of well-designed studies, including large-scale RCTs with extended study periods, compared to structured exercise. A PubMed search using the term 'Tai Chi' yielded a limited number of articles (approximately 4000 articles), while a search using the term 'aerobic exercise' resulted in significantly more articles (540000 articles). This suggests that research on Tai Chi is still relatively sparse. Additionally, Tai Chi, unlike other structured exercise programs, is a martial art that typically requires several years to master the various skills, including breathing techniques, specific physical manipulations, and form. If study participants are beginners who engage in mere physical exercise, they may not fully realize Tai Chi's health benefits during the short study duration.

CONCLUSION

In conclusion, Tai Chi may be considered as an option for exercise therapy in the management of diabetes by clinicians who possess the requisite skills and knowledge of Tai Chi. However, future studies should further explore the differences between conventional exercise and Tai Chi, as well as the underlying biological mechanisms explaining Tai Chi's positive clinical effects in diabetes patients. More research into these areas is essential for building a stronger evidence base.

FOOTNOTES

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EDITORIAL

Individualized intensive insulin therapy of diabetes: Not only the goal, but also the time

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Abstract

Intensive insulin therapy has been extensively used to control blood glucose levels because of its ability to reduce the risk of chronic complications of diabetes. According to current guidelines, intensive glycemic control requires individualized glucose goals rather than as low as possible. During intensive therapy, rapid blood glucose reduction can aggravate microvascular and macrovascular complications, and prolonged overuse of insulin can lead to treatment-induced neuropathy and retinopathy, hypoglycemia, obesity, lipodystrophy, and insulin antibody syndrome. Therefore, we need to develop individualized hypoglycemic plans for patients with diabetes, including the time required for blood glucose normalization and the duration of intensive insulin therapy, which deserves further study.

Key Words: Diabetes; Intensive therapy; Insulin; Treatment-induced neuropathy

Core Tip: Intensive insulin therapy is popular in the treatment of patients with diabetes. This article highlighted the effects and side effects of intensive insulin therapy. It is a warning against the use of insulin therapy without any limitations, such as the speed of blood glucose lowering and the duration of insulin therapy.

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INTRODUCTION

Intensive insulin therapy refers to the control of blood glucose levels within the normal range using insulin therapy in patients with poor glycemic control. Intensive insulin therapy has been demonstrated to effectively decrease the risk of chronic complications in both patients with type 1 and type 2 diabetes[1,2]. The Diabetes Control and Complications Trial [3] in 1993 and the United Kingdom Prospective Diabetes Study[4] in 1998 are landmark studies that demonstrated the benefits of intensive insulin therapy in type 1 and type 2 diabetes, respectively. Moreover, intensive insulin therapy has favorable outcomes in the recovery and maintenance of β-cell function and protracted glycemic remission compared to treatment with oral hypoglycemic agents in patients newly diagnosed with type 2 diabetes[5]. Therefore, intensive insulin therapy is administered widely among patients with diabetes due to its benefits. Furthermore, the standards of insulin intensive therapy are constantly updated, and the side effects identifiedare summarized briefly in the present commentary.

INDIVIDUAL GOALS FOR INTENSIVE THERAPY

Most current diabetes guidelines recommend individualized goals for intensive glycemic control. The Action to Control Cardiovascular Risk in Diabetes study found that low glycemic control with a goal of HbA1c < 6.0% led to increased mortality in patients with type 2 diabetes[6]. As such, the goal of intensive therapy is not as low as possible, and the increased risk of hypoglycemia should be considered. According to the guidelines of the American Diabetes Association and the Chinese Diabetes Society, the reasonable HbA1c goal for most nonpregnant adults is < 7%, which is beneficial for reducing microvascular and macrovascular complications in type 1 and type 2 diabetes[7,8]. The East African Diabetes Study Group recommended a target HbA1c of 7.5% for all children with type 1 diabetes mellitus[9]. More stringent HbA1c targets (such as < 6.5%, or even close to the normal reference value) and less stringent HbA1c goals (such as < 8.0%) are indicated depending on the duration of disease, life expectancy, complications, risk of hypoglycemia, and other adverse effects of treatment[10,11].

SIDE EFFECTS OF RAPID BLOOD GLUCOSE REDUCTION

Clinicians and even patients usually recommend blood glucose recovery to the glycemic target as soon as possible during intensive therapy, usually within a week[5,12], and this is the same when patients initially use an automatic insulin delivery system[13,14]. During intensive therapy, HbA1c can be dramatically reduced by more than 1.5%-2% in 3-4 mo [15,16], and 3%-4% in a year[16,17]. Several studies have reported that rapid blood glucose reduction can aggravate various complications, including cardiovascular events[16], retinopathy[17], nephropathy[18] and neuropathy[15,19]. Neuropathy induced by an abrupt improvement in glycemic control is called treatment-induced neuropathy in diabetes (also referred to as insulin neuritis). All these complications commonly occur in patients with chronic hyperglycemia, the incidence rate and severity are positively correlated with the magnitude and speed of the decrease in HbA1c[15,16]. Therefore, the planning of a individualized intensive therapy program to prevent these complications requires further research. Hence, the duration of hyperglycemia, HbA1c levels, and preexisting complications at baseline should be considered.

OVERUSE OF INSULIN IN PATIENTS WITH TYPE 2 DIABETES

With the popularity of short-term intensive therapy, many patients with type 2 diabetes are prescribed insulin therapy at the time of the new diagnosis; However, some of these patients do not evaluate the possibility of insulin withdrawal in time[20]. Some patients had been using insulin for several years. Although these patients can maintain good glycemic control, the excessive and prolonged use of insulin can result in certain side effects. More treatment-induced neuropathy and retinopathy have been reported in patients receiving insulin therapy than in patients treated with oral hypoglycemic agents[21]. Not only because insulin reduces HbA1c the most[22] but because the abnormal activation of the insulin-IGF-1-AKT signaling pathway may exacerbate these complications[22,23]. In our previous study using flash glucose monitoring, about 40% of patients with type 2 diabetes using premixed insulin had time below range ≥ 4%, illustrating a high proportion of hypoglycemia; Meanwhile, the proportion of oral hypoglycemic agents treatments combination was less than 50%[24]. Moreover, the long-term use of insulin and hyperinsulinemia in patients with type 2 diabetes may lead to obesity[25] and insulin resistance, lipodystrophy[26,27], and exogenous insulin antibody syndrome[28]. These problems

lead to the deterioration of glycemic control. Therefore, when and under what circumstances intensive insulin therapy can be stopped and switched to oral hypoglycemic agents must be emphasized in patients newly diagnosed with type 2 diabetes.

CONCLUSION

To control the side effects of intensive insulin therapy, individualized glycemic goals and hypoglycemic plans need to be developed for patients, including the time required for blood glucose levels to reach the target and the duration of intensive insulin therapy. Oral glucose-lowering drugs and the GLP-1 receptor agonist adjunct to insulin can help reduce the insulin dose and improve glycemic variations[29,30], and should be initiated simultaneously with intensive insulin therapy in patients with type 2 diabetes and even in some patients with type 1 diabetes who have insulin resistance[31]. Furthermore, some nerve and microvascular protectors, such as epalrestat[32], mecobalamin[33], and pancreatic kininogenase[34], may helpprevent these complications of intensive therapy, which needs further clinical studies.

FOOTNOTES

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MINIREVIEWS

Management of monogenic diabetes in pregnancy: A narrative review

Mohammad Sadiq Jeeyavudeen, Sarah R Murray, Mark W J Strachan

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Abstract

Pregnancy in women with monogenic diabetes is potentially complex, with significant implications for both maternal and fetal health. Among these, maturity-onset diabetes of the young (MODY) stands out as a prevalent monogenic diabetes subtype frequently encountered in clinical practice. Each subtype of MODY requires a distinct approach tailored to the pregnancy, diverging from management strategies in non-pregnant individuals. Glucokinase MODY (GCK-MODY) typically does not require treatment outside of pregnancy, but special considerations arise when a woman with GCK-MODY becomes pregnant. The glycemic targets in GCK-MODY pregnancies are not exclusively dictated by the maternal/paternal MODY genotype but are also influenced by the genotype of the developing fetus. During pregnancy, the choice between sulfonylurea or insulin for treating hepatocyte nuclear factor 1-alpha (HNF1A)-MODY and HNF4A-MODY depends on the mother's specific circumstances and the available expertise. Management of other rarer MODY subtypes is individualized, with decisions made on a case-by-case basis. Therefore, a collaborative approach involving expert diabetes and obstetric teams is crucial for the comprehensive management of MODY pregnancies.

Key Words: Diabetes; Pregnancy; Maturity-onset diabetes of the young; Insulin; Sulphonylurea; Glucokinase; Hepatocyte nuclear factor 1-alpha, hepatocyte nuclear factor 1-beta, and hepatocyte nuclear factor 4-alpha

Core Tip: Management of monogenic diabetes in pregnancy, particularly maturity-onset diabetes of the young (MODY), requires tailored approaches due to the unique challenges encountered in pregnancy. While glucokinase MODY often doesn't require treatment outside pregnancy, managing it during pregnancy is complex due to its impact on fetal growth. Monitoring fetal genotype and growth patterns is essential for adjusting treatment. Non-invasive methods for fetal genotype determination, such as cell-free DNA analysis, hold promise but require further research.

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INTRODUCTION

Monogenic diabetes is an umbrella term encompassing all diabetes forms caused by pathogenic mutations in a single gene[1]. Maturity-onset diabetes of the young (MODY) is the most common monogenic diabetes, and generally presents in later childhood or early adulthood. Other specific gene abnormalities can give rise to neonatal diabetes, which as its name suggests causes diabetes in early life. This review will focus on the management of the most common forms of MODY in pregnancy.

Autosomal dominant mutations in genes affecting pancreatic β cell function are responsible for the development of MODY[2,3]. A total of 1%-5% of all cases of diabetes and, in particular, 1%-2% of all cases of diabetes in the white European population have MODY[3]. Since MODY is a rare form of diabetes, and because of variable access to diagnostic genetic testing, it is often mis-classified as one of the more common forms of diabetes - type 1 or type 2 diabetes mellitus. The distinguishing features between MODY and type 1 or type 2 diabetes are early onset fasting hyperglycemia, lean body habitus, absence of pancreatic islet autoantibodies and a family history of diabetes with autosomal dominant inheritance[4].

Classification of MODY

MODY is classified into 14 subtypes based on the gene involved (Table 1)[5,6]. Mutations in the hepatocyte nuclear factor 1-alpha (HNF1A), HNF4A, and glucokinase (GCK) genes account for > 95% of all MODY cases[7].

Prevalence

The prevalence of MODY has been extensively studied in European, North American, and Australian populations, while data on its prevalence in other regions are limited[3,8]. The prevalence of MODY is 1:10000 in adults and 1:23000 in children in the European population[9]. HNF1A-MODY is the most common MODY subtype, followed by GCK-MODY, HNF4A-MODY and HNF1B-MODY in European cohorts[10]. In a United States based study focused on GCK-, HNF1A- and HNF4A-MODY genes, the prevalence was estimated to be 1.2% in children with diabetes[8]. In contrast, prevalence data from other ethnic groups and regions, including Asia, Africa, and South America, remain scarce[8]. The lack of data in these populations may be attributed to limited access to genetic testing, the diverse genetic background of different ethnic groups and different testing strategies.

GCK-MODY is estimated to account for approximately 1% of all cases of gestational diabetes mellitus (GDM), with a majority of affected individuals remaining asymptomatic and undiagnosed outside of pregnancy[3,11]. In the population-based Atlantic Diabetes in Pregnancy study involving 5500 participants, the population prevalence of GCK-MODY was found to be 1.1 in 1000 [95% confidence interval (CI): 0.3-2.9 in 1000][3]. Within women with GDM in this study, the prevalence of GCK-MODY was 0.9% (95%CI: 0.3-2.3). The combined criteria of having a body mass index < 25 kg/m² and fasting glucose ≥ 5.5 mmol/L demonstrated a sensitivity of 68%, specificity of 96% for the detection of GCK-MODY. The number of women with GDM to test to identify one case of GCK-MODY was 2.7[3].

Testing for MODY in pregnancy

MODY screening is not routinely performed in pregnant women due to its low prevalence in the general population. Any woman who is diagnosed with GDM at < 35 years of age and with a lean body mass index should be screened for MODY if auto-antibodies for type 1 diabetes mellitus are negative[10,12-14]. In the context of MODY testing during pregnancy, two key sequencing methods have been utilised: Sanger sequencing and next-generation sequencing (NGS)[15]. Sanger sequencing, also known as chain termination sequencing, has been a fundamental technique in genetic testing for several decades. It allows for the identification of specific DNA sequences by synthesising new DNA strands complementary to the target region. While Sanger sequencing is accurate and reliable, it is best suited for examining individual genes or specific genomic regions[16]. Due to its relatively lower throughput and higher cost per sample, Sanger sequencing is often employed when targeting a particular known mutation or a limited set of candidate genes associated with MODY. On the other hand, NGS has revolutionised the field of genetic testing by enabling high-throughput sequencing of millions of DNA fragments simultaneously[17]. NGS platforms can process large-scale genomic data rapidly, making it a more efficient approach for detecting mutations in multiple genes concurrently. This technology is particularly valuable in the context of MODY testing during pregnancy since it allows for comprehensive screening of a wide range of MODY-

Table 1 Classification of maturity-onset diabetes of the young

MODY type	MODY sub-type	Genes affected	Characteristics features
HNF4A-MODY	MODY 1	HNF4A	Young-onset hyperglycemia, pancreatic beta cell dysfunction, sensitive to sulfonylurea treatment, macrosomia, transient neonatal hyperinsulinism
GCK-MODY	MODY 2	GCK	Mild fasting hyperglycemia, stable glucose levels, lack of complications
HNF1A-MODY	MODY 3	TCF1	Young-onset hyperglycemia, sensitive to sulfonylurea treatment, renal cysts, genital tract anamolies
PDX1-MODY	MODY 4	PDX1	Young-onset hyperglycemia, pancreatic agenesis, hypopituitarism, growth retardation
HNF1B-MODY	MODY 5	HNF1B	Young-onset hyperglycemia, renal abnormalities, genital tract malformations, gout
NEUROD1-MODY	MODY 6	NEUROD1	Young-onset hyperglycemia, retinal dystrophy, cerebellar ataxia, epilepsy, intellectual disability, sensorineural hearing loss
KLF11-MODY	MODY 7	KLF11	Young-onset hyperglycemia, hepatic steatosis
CEL-MODY	MODY 8	CEL	Neonatal diabetes, pancreatic atrophy, exocrine pancreatic insufficiency, transient neonatal hyperinsulinism
PAX4-MODY	MODY 9	PAX4	Adult onset diabetes, multisystem disorder, mutation inhibits beta-cell proliferation, ketosis prone
INS-MODY	MODY 10	INS	Neonatal diabetes, insulin gene mutation, requires lifelong insulin treatment
BLK-MODY	MODY 11	BLK	Young-onset hyperglycemia, reduced beta-cell mass
ABCC8-MODY	MODY 12	ABCC8	Neonatal diabetes, potassium channel gene mutation, responsive to high-dose sulfonylurea
KCNJ11-MODY	MODY 13	KCNJ11	Neonatal diabetes, potassium channel gene mutation, responsive to high-dose sulfonylurea
APPL1-MODY	MODY 14	APPL1	Young-onset diabetes and decreased glucose mediated insulin release, dysmorphic features and developmental delay in animal models

HNF4A: Hepatocyte nuclear factor 4 alpha; GCK: Glucokinase; TCF1: Transcription factor 1 (also known as HNF1A); PDX1: Pancreatic and Duodenal Homeobox 1; HNF1B: Hepatocyte nuclear factor 1 beta (also known as TCF2); NEUROD1: Neurogenic differentiation 1; KLF11: Krüppel-like factor 11; CEL: Carboxyl ester lipase; PAX4: Paired box 4; BLC2L1: B-cell CLL/lymphoma 2 like 1; INS: Insulin; BLK: B-lymphocyte kinase; APPL1: Adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper containing 1; ABCC8: ATP-binding cassette subfamily c member 8 (also known as SUR1); KCNJ11: potassium voltage-gated channel subfamily J member 11 (also known as Kir6.2).

associated genes, facilitating a more accurate and comprehensive diagnosis[18]. Hence awareness of the testing type available locally will be useful to guide the diagnosis and treatment strategies.

Direct mutation assessment of the foetus to identify whether the foetus has inherited the given mutation is challenging. Chorionic villi sampling or amniocentesis testing are available for predicting foetus genotype and consequently, the likelihood of developing MODY[19]. However, these invasive methods are not recommended solely for detecting MODY genotyping purposes, as they carry a risk of miscarriage[14]. The emergence of cell-free circulating DNA has shown promise as a non-invasive method for determining fetal genotype[20]. Nonetheless, extensive studies in this area are currently lacking, and it is not ready yet for routine clinical use. Therefore, the importance of evaluating fetal growth through ultrasound remains a crucial tool in assessing the impact of maternal hyperglycemia on the fetus[21]. Additionally, ultrasound can aid in identifying structural anomalies associated with specific genotypes. Nevertheless, it is important to note that these indicators are not definitive and cannot conclusively determine the fetal genotype. Until more robust diagnostic tools become available, monitoring fetal growth by ultrasound after 26 wk of gestation using ethnicity-specific growth charts may offer insights into the fetal genotype and guide the initiation of appropriate therapeutic interventions for optimal fetal growth and maternal glycemic control.

GCK-MODY

GCK-MODY results from a loss of function mutation in the GCK gene[22]. GCK codes for the enzyme GCK that converts glucose to glucose-6-phosphate during the first stage of glycolysis. In the pancreatic beta-cells, GCK acts as the glucose sensor facilitating insulin release in response to rising blood glucose levels (Figure 1)[23]. The inactivating heterozygous mutation in GCK reduces the insulin secreting function of β-cells and causes mild stable hyperglycemia in the prediabetic range starting from birth. Affected individuals respond to glucose-stimulated insulin release, but at a higher set-point

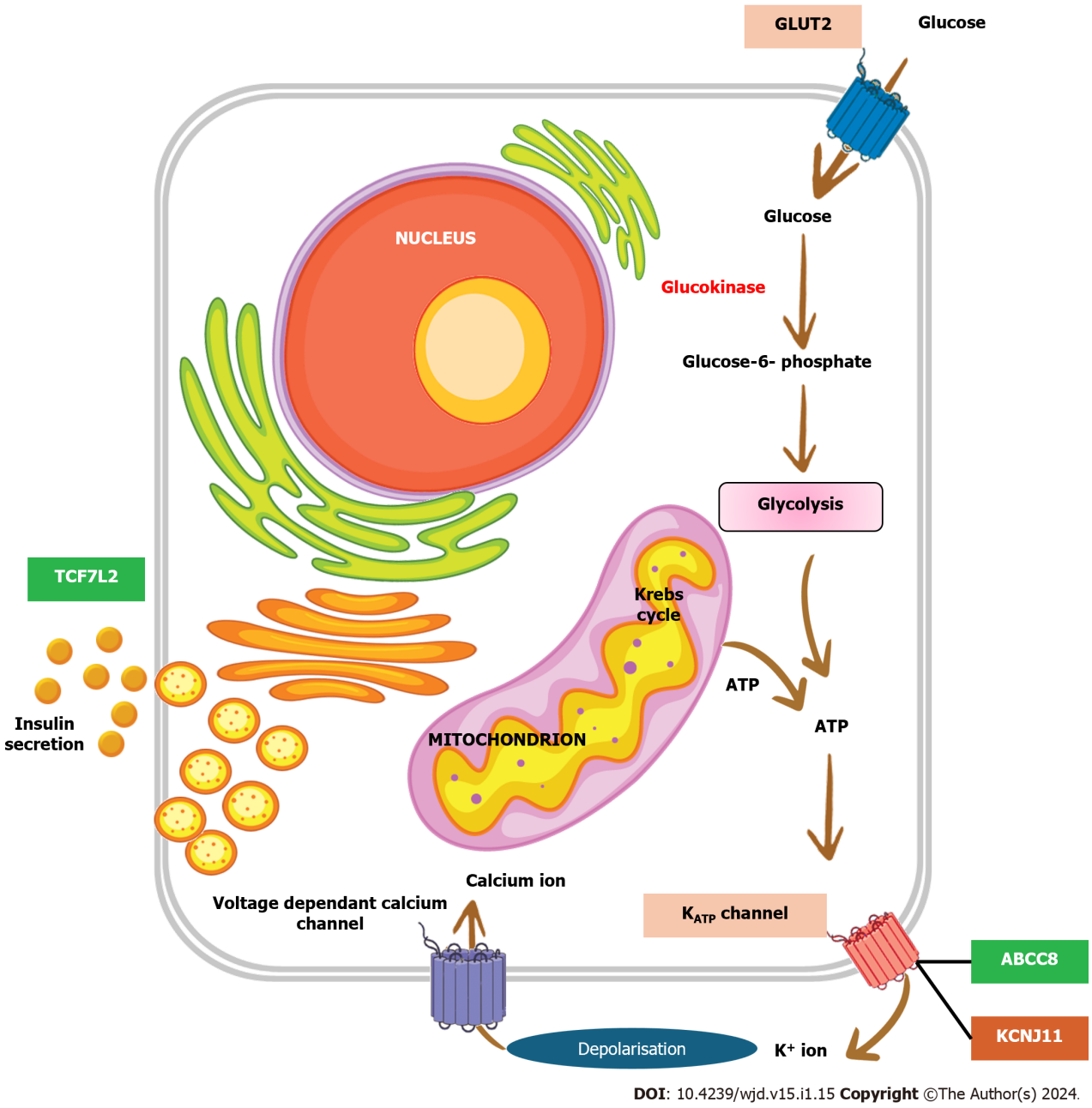

Figure 1 The key process of glucose mediated insulin secretion in the pancreas. Glucose enters the cell through GLUT2 transported and gets phosphorylated to glucose-6-phosphate by glucokinase, a rate limiting step in the process.

than those with normal GCK function[24]. As the hyperglycemia is mild in GCK-MODY, affected individuals do not develop major microvascular or macrovascular complication of diabetes.

Complications associated with GCK-MODY pregnancy

An increase in miscarriage rate of up to 33% has been reported with GCK-MODY pregnancies in some cohorts, but not all [13]. Caudal regression syndrome in offspring of mothers carrying the mutant allele has recently been reported[25]. Furthermore, despite the increase in birth weight, there is no long-term effect of maternal hyperglycemia on the offspring's glucose tolerance (Table 2)[26]. There are some reported cases of homozygous deletion of the GCK gene leading to intrauterine or neonatal death due to fetal growth retardation[29]. Maternal hyperglycemia in GCK-MODY pregnant mothers causes an increase in fetal insulin production and in fetuses with no GCK-MODY mutation this leads to accelerated fetal growth (and potentially macrosomia) due to the anabolic effect of insulin. Macrosomia is well known to be associated with an increased risk of shoulder dystocia and an increased likelihood of cesarean birth. Furthermore, neonates carrying the wild-type GCK gene born to GCK-MODY mother are faced with an increased vulnerability to postnatal hypoglycemia. Having been exposed to a glucose-rich environment in utero accompanied by hyperinsulinemia, the abrupt cessation of maternal glucose supply postnatally can result in some neonates experiencing hypoglycemia, persisting until the neonate's glucose-insulin equilibrium is reestablished.

Table 2 Complications of glucokinase maturity-onset diabetes of the young in pregnancy

Outcome feature	Percentage	Ref.
Miscarriage	15%-33%	[9,27]
Preterm birth	12%	[27]
Low birth weight	6%	[28]
Macrosomia	3%	[23]
Neonatal hypoglycemia	10%	[23]
Congenital malformations	2%-3%	[23]

Management of GCK-MODY during pregnancy

The impact of maternal GCK-MODY on the fetus is closely linked to the fetal genotype, i.e. whether or not the fetus has inherited the abnormal maternal GCK gene. This is summarised in Figure 2. A fetus with wild-type GCK is at risk of accelerated growth, diabetic fetopathy and increased birth weight. This is due to enhanced fetal insulin secretion in response to maternal hyperglycemia[23], as is seen in conventional GDM. If the fetus has inherited the abnormal GCK gene from the mother, the fetus does not develop hyperinsulinaemia in response to maternal hyperglycaemia and birthweight is not increased[30]. For completeness, Figure 2 also shows that if the fetus has an abnormal GCK, which has been inherited from the father, then the fetus will produce lower insulin levels than normal in the face of maternal normoglycaemia, resulting in reduced birthweight[3].

Chorionic villus sampling or amniocentesis are available for determining fetal genotype[19]. However, these invasive methods are not recommended solely for MODY genotyping purposes, as they carry a risk of miscarriage of around 1%-2%[14]. The emergence of circulating cell-free fetal DNA has shown promise as a non-invasive method for determining fetal genotype[20]. Nonetheless, extensive studies in this area are currently lacking, and it is not yet available for routine clinical use.

Most women with GCK-MODY do not require anti-diabetic therapy out with pregnancy. It is generally recommended that anti-diabetic therapy should not be commenced as a matter of routine during the first and second trimesters of pregnancy, even though maternal blood glucose levels are likely to be above typical pregnancy targets. Thereafter, the requirement for treatment should be determined by looking at the trajectory of fetal growth. Pregnant woman with GCK-MODY are recommended to undergo ultrasound scanning every two weeks from 26 wk of gestation[22]. Accelerated fetal growth in pregnancy (implying that the fetus has not inherited the abnormal GCK gene) should lead to the commencement of maternal insulin therapy[7,9,19,24,27]. Pregnant women with GCK-MODY generally require higher insulin doses (0.6 to 1 U/kg) to lower maternal glucose levels and it is often difficult to achieve standard pregnancy glucose targets. If the fetus shows a normal growth pattern from 26 wk (implying that the fetus has inherited the abnormal GCK gene), maternal insulin may not be required and if treated may cause growth restriction.

HNF1A MODY

The gene responsible for developing HNF1A MODY is HNF1A. HNF-1A MODY, also known as MODY 3, manifests with hyperglycemia in adolescence or during early adulthood[31].

Management during HNF1A-MODY pregnancy

The first line of treatment for HNF1A-MODY in non-pregnant individuals is a low dose of a sulphonylurea. DPP-IV inhibitors and GLP1 receptor agonist are the second line available options[14]. However, data related to management during pregnancy in HNF1A-MODY is limiting. Some reports suggest that offspring inheriting the mutant allele do not show an increase in birthweight or neonatal hypoglycemia. However, the usage of sulphonylurea and insulin therapy need to be carefully decided in pregnant women as sulphonylurea's have been shown to cross the placenta[32]. Glyburide, the only sulphonylurea approved in pregnancy, might cause an increased risk of neonatal hypoglycemia and macrosomia when administered during pregnancy as compared to insulin therapy[33,34]. In a meta-analysis conducted in 2014 of women with gestational diabetes, macrosomia (risk ratio = 3.07) and neonatal hypoglycemia (risk ratio = 2.30) were more common in those treated with glyburide compared with insulin therapy[33]. These data are leading to a shift towards insulin therapy as opposed to sulphonylurea therapy during HNF1A-MODY pregnancy[33]. Fetal monitoring at an early gestational stage, with fetal echocardiography to identify birth defects followed by growth ultrasound scanning at periodic intervals after 26 wk of pregnancy are recommended[4]. Post-partum mothers can restart glybruide and it is safe to continue during breastfeeding as a low dose of glyburide is neither excreted in breast milk nor leads to neonatal hypoglycemia[14].

HNF4A MODY

HNF4A-MODY arises from genetic mutations within the HNF4A gene. The clinical attributes of HNF4A-MODY parallel those observed in HNF1A-MODY, as both are marked by a progressive decline in insulin secretion[22]. Furthermore, this gene is recognized as an upstream regulator of the HNF1A transcription factor.

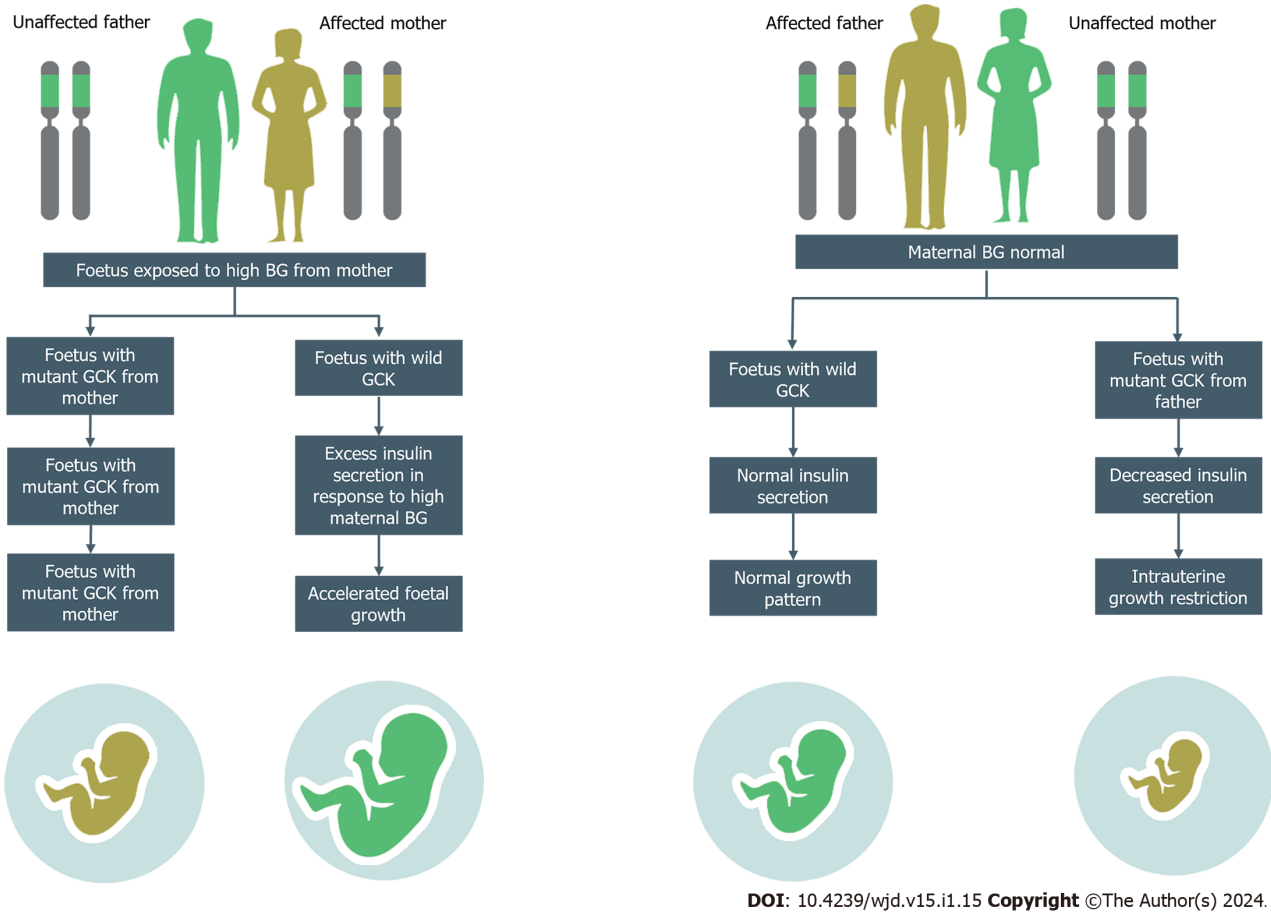

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Figure 2 Management of glucokinase maturity-onset diabetes of the young in pregnancy. GCK: Glucokinase; BG: Blood glucose.

Management during HNF4A-MODY pregnancy

Babies carrying the mutation are macrosomic in approximately half of the cases, along with an elevated risk of transient neonatal hypoglycemia[31]. Both of these outcomes are linked to fetal hyperinsulinism[35]. In a non-pregnant state, low-dose sulfonylureas prove effective in achieving glycemic control. Given the significant risks associated with macrosomia and hypoglycemia, maintaining a tight maternal glycemic control during pregnancy is of paramount importance. Currently, no validated interventions exist to enhance fetal outcomes or manage macrosomia in HNF4A-MODY pregnancies. The therapeutic approach mirrors that employed for HNF1A-MODY pregnancies, necessitating the discontinuation of sulfonylureas before pregnancy and a transition to insulin therapy, accompanied by periodic fetal growth surveillance from 26 wk[31]. The occurrence of macrosomia is significantly higher in offsprings carrying HNF4A mutations (56% *vs* 13%) when compared to the offsprings carrying no mutuation[35]. Additionally, neonatal hyperinsulinemic hypoglycemia has been observed in 15% of infants with HNF4A mutations, compared to those without the mutation[26,35].

HNF1B-MODY

HNF1B-MODY, also known as MODY5, occurs due to a mutation in HNF1B, which is involved in embryonic development of pancreas, kidney, liver and genitourinary tract[36,37]. The clinical manifestations are not limited to insulin secretion resulting in hyperglycemia, but individuals may also develop genital tract malformation, cystic renal disease, hypomagnesaemia, abnormal liver function, gout and hyperuricemia. The mean age of diabetes onset is 24 years [38]. However, the age of onset can range from the neonatal period to middle age[39,40]. Diabetes is managed with insulin and during pregnancy management is along the same lines as for type 1 diabetes[22]. Taken together, the major complications associated with HNF1B-MODY are the variable age of onset and the variable clinical phenotypes that progress with age. Moreover, the dependency on insulin therapy is the only way to manage the disease. These clinical phenotypes and available treatment strategies raise concern for the identification of the causative disease mutation in the family at an early age and also identification of better and novel treatment drugs for this MODY.

Management during HNF1B-MODY pregnancy

There is a paucity of evidence with regards to management of MODY5 in pregnancy compared to the other MODY subtypes. Fetus' carrying the mutant allele from a normal mother show reduced birthweight and increased risk of being small for gestational age. On the other hand, if both the fetus and the mother carry the mutant allele then the baby tends to grow larger for their gestational age. The fetal growth should be regularly monitored during pregnancy and the

offspring should be subjected to genetic screening with renal abnormalities monitored in carriers as renal abnormalities are more common than diabetes in MODY 5[22].

CONCLUSION

Managing MODY pregnancy remains an area of ongoing investigation, and current protocols are based on expert judgement, small studies rather than large clinical trial data. Each form of MODY needs an individualised approach to management. Improving the early detection of MODY during the initial stages of pregnancy requires several key changes: Better implementation of MODY guidelines in general diabetes clinics for pre-pregnancy diagnosis, raising awareness among the treating medical team, ensuring accessibility to genetic testing facilities, and fostering familiarity with appropriate treatment strategies. Once pregnancy is confirmed, establishing a well-structured and comprehensive care plan is vital to avoid unnecessary interventions for the pregnant mother and ensures the optimal well-being of the fetus. Advancements in non-invasive pre-natal testing methods, such as cell-free fetal DNA analysis, hold promise for the identification of fetal genotypes.

FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prediabetes: An overlooked risk factor for major adverse cardiac and cerebrovascular events in atrial fibrillation patients

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Abstract

BACKGROUND

Prediabetes is a well-established risk factor for major adverse cardiac and cerebrovascular events (MACCE). However, the relationship between prediabetes and MACCE in atrial fibrillation (AF) patients has not been extensively studied. Therefore, this study aimed to establish a link between prediabetes and MACCE in AF patients.

AIM

To investigate a link between prediabetes and MACCE in AF patients.

METHODS

We used the National Inpatient Sample (2019) and relevant ICD-10 CM codes to identify hospitalizations with AF and categorized them into groups with and without prediabetes, excluding diabetics. The primary outcome was MACCE (all-cause inpatient mortality, cardiac arrest including ventricular fibrillation, and stroke) in AF-related hospitalizations.

RESULTS

Of the 2965875 AF-related hospitalizations for MACCE, 47505 (1.6%) were among patients with prediabetes. The prediabetes cohort was relatively younger (median 75 *vs* 78 years), and often consisted of males (56.3% *vs* 51.4%), blacks (9.8% *vs* 7.9%), Hispanics (7.3% *vs* 4.3%), and Asians (4.7% *vs* 1.6%) than the non-prediabetic cohort (P < 0.001). The prediabetes group had significantly higher rates of hypertension, hyperlipidemia, smoking, obesity, drug abuse, prior myocardial infarction, peripheral vascular disease, and hyperthyroidism (all P < 0.05). The prediabetes cohort was often discharged routinely (51.1% *vs* 41.1%), but more frequently required home health care (23.6% *vs* 21.0%) and had higher costs. After adjusting for baseline characteristics or comorbidities, the prediabetes cohort with AF admissions showed a higher rate and significantly higher odds of MACCE compared to the non-prediabetic cohort [18.6% *vs* 14.7%, odds ratio (OR) 1.34, 95% confidence interval 1.26-1.42, P < 0.001]. On subgroup analyses, males had a stronger association (aOR 1.43) compared to females (aOR 1.22), whereas on the race-wise comparison, Hispanics (aOR 1.43) and Asians (aOR 1.36) had a stronger association with MACCE with prediabetes *vs* whites (aOR 1.33) and blacks (aOR 1.21).

CONCLUSION

This population-based study found a significant association between prediabetes and MACCE in AF patients. Therefore, there is a need for further research to actively screen and manage prediabetes in AF to prevent MACCE.

Key Words: Prediabetes; Atrial fibrillation; Cardiovascular disease risk; Major adverse cardiovascular and cerebrovascular events; Stroke; Mortality

Core Tip: In our study, we've shed light on a critical yet often overlooked connection between prediabetes and major adverse cardiac and cerebrovascular events (MACCE) in atrial fibrillation (AF) patients. Our research revealed that AF patients with prediabetes are at significantly higher risk of experiencing MACCE, highlighting the importance of identifying and managing prediabetes in this population. This finding emphasizes the need for proactive screening and targeted interventions to reduce the burden of MACCE in AF patients with prediabetes. Further research and dedicated efforts are essential to enhance care and outcomes for these individuals.

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INTRODUCTION

In 2019, more than one-third of the adult population, that is, 96 million adults aged 18 and above, received a diagnosis of prediabetes[1]. Prediabetes is a condition where people are associated with an increased risk of developing type 2 diabetes mellitus (DM), cardiovascular disease, and stroke due to elevated blood glucose levels that haven't reached the threshold for diabetes. Impaired glucose tolerance (IGT) test was prevalent in 7.5% men and women in 2019 as per the International Diabetes Federation[2]. Among the arrhythmia's, atrial fibrillation (AF) was the most common arrhythmia

and poses a significant healthcare burden due to its sequalae like stroke, heart failure, and increased mortality[3,4]. AF is often associated with other comorbid conditions like diabetes, hypertension, and obesity, which increases the major adverse cardiac and cerebrovascular events (MACCE)[4-6]. There is increasing evidence suggesting that prediabetes acts as a risk factor for MACCE in patients with AF; we have limited understanding regarding the underlying mechanisms[7, 8].

The 2019 American Heart Association/American College of Cardiology/Heart Rhythm Society focused update for the management of patients with AF reported that increased risk of thromboembolic events may be due to the underlying vascular changes and prothrombotic state commonly seen with diabetes, however, the contemporary data and guidelines on the long-term impact of prediabetes on AF risk and outcomes remain largely unknown. Despite increasing evidence suggesting that prediabetes is one of the risk factors for MACCE in patients with AF, the understanding of the pathophysiology causing these adverse outcomes is still unclear. Previously proposed mechanisms of prediabetes leading to AF and associated cardiovascular complications include such as insulin resistance, oxidative stress, inflammation, autonomic dysfunction, fibrosis, and a prothrombotic state. Although these mechanisms are involved there is still a lack of understanding regarding the pathophysiology of how prediabetes contributes to AF. It entails an interaction, between factors that encompass the heart, metabolism and chronic inflammation. As it is crucial to understand the relationship between prediabetes and MACCE in patients with AF, which can help develop strategies for screening and managing this high-risk population, we analyzed the modern-day data from a nationally representative sample in the United States.

MATERIALS AND METHODS

Source of data

The study utilized the National Inpatient Sample (NIS) database for the year 2020, which is a part of the Healthcare Cost and Utilization Project (HCUP) sponsored by the Agency for Healthcare Research and Quality. NIS is the largest all-payer inpatient healthcare dataset in the United States, representing about 20% of United States hospitals from 48 states, comprising average 7 million un-weighted discharges per year that approximate more than 35 million weighted nationwide discharges. The database provides one primary diagnosis and up to 24 secondary discharge diagnoses for each inpatient admission. As the NIS database contains deidentified data, Institutional review board approval was not necessary. Additional information about the database can be accessed from the HCUP website.

Study population and characteristics

Patients with prediabetes (ICD code: R73. 03) and AF (ICD codes: I48.0, I48.1, I48.2, I48.3, I48.4, I48.9) were identified using relevant ICD-10 CM codes[9,10]. Patients AF are divided into two groups consisting of with and without prediabetes respectively excluding diabetic population. Patients with both prediabetes and AF are considered as exposure group and the other group is considered as control then the co-morbidities and outcomes are identified using Revised Clinical Classification Software codes. Cardiovascular and extracardiac comorbidities were determined by utilizing the Elixhauser comorbidity indices. Predefined criteria found in the NIS database, which are based on ICD-10 CM codes (Figures 1 and 2).

Study outcomes

The primary objective of the study was to determine the relationship between prediabetes and in-hospital MACCE, including all-cause inpatient mortality, cardiac arrest including ventricular fibrillation and stroke. The secondary objective were length of hospital stays, hospital costs, and comorbidities related to AF hospitalizations.

Statistical analyses

Descriptive statistics were used to describe study population and initial characteristics were obtained. Categorical variables and continuous variables were reported as frequency and percentage, interquartile ranges respectively. Pearson's chi-square test and Mann Whitney *U* tests were utilized for categorical and continuous variables (non-normal distribution), respectively, to compare baseline demographics and hospital characteristics and other comorbidities between the two groups. Discharge weight provided in the database was used to generate national estimates. To evaluate the relationship between prediabetes (pDM) and MACCE in AF hospitalizations, multivariable logistic regression model was used to assess the risk of in-hospital outcomes. In conducting the regression analysis, we took into account factors including age, at admission, gender, race, income level, payment status, type of admission, hospital size teaching status of the facility geographical location and relevant medical conditions relevant cardiac and extra cardiac comorbidities and prior history of myocardial infarction or revascularization with percutaneous coronary intervention or coronary artery bypass grafting, stroke, venous thromboembolic events and cardiac arrest. Adjusted odds ratio (OR), 95% confidence interval (CI), and *P*-values were used to present logistic regression results. IBM SPSS Statistics 25.0 (IBM Corp, Armonk, New York) software was used for all statistical analyses using complex sample modules. A two-tailed *P*-value of less than 0.05 was used to determine statistical significance.

RESULTS

The study investigated the relationship between prediabetes and MACCE in individuals with AF in individuals hospit-

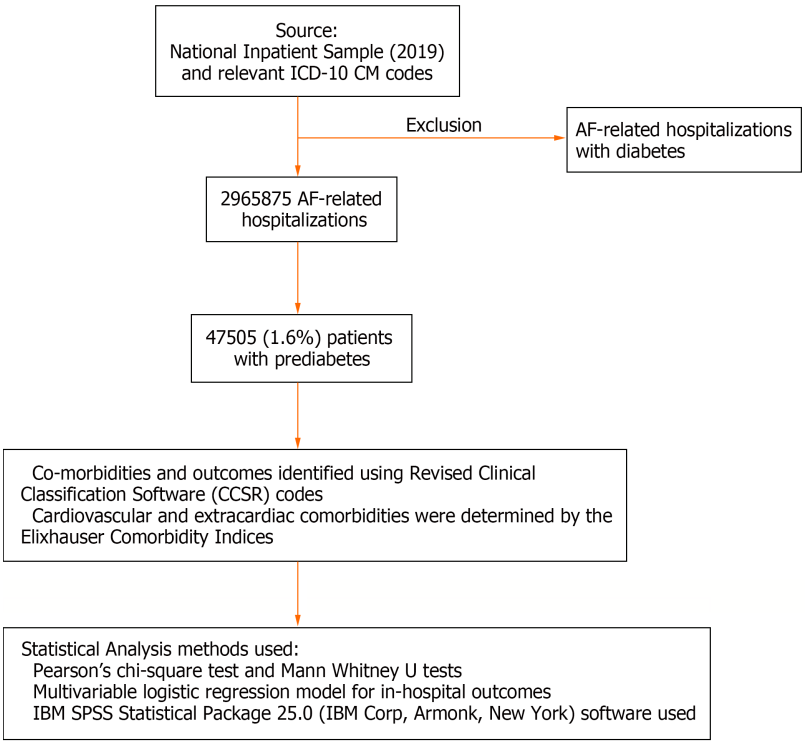

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Figure 1 Flow diagram showing exclusion criteria and how the final study cohorts were reached. AF: Atrial fibrillation.

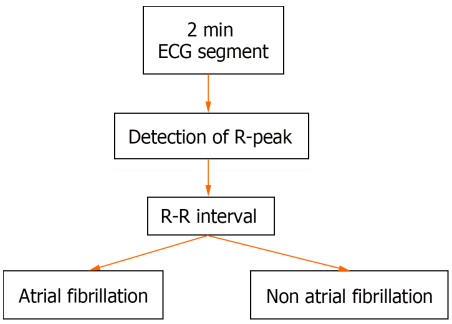

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Figure 2 Algorithm for atrial fibrillation. ECG: Electrocardiogram.

alized with AF using 2019 NIS, cohorts were divided into hospitalizations with AF into groups with and without prediabetes (pDM), excluding diabetics. MACCE defined as all-cause mortality, cardiac arrest including ventricular fibrillation, and stroke in AF- related hospitalizations, was the primary outcome. Of 2965875 total AF-related hospitalizations 47505 (1.6%) patients were identified with prediabetes.

The pDM cohort comprised a higher percentage of male population (56.3% *vs* 51.4%), were mostly younger (Median 75 *vs* 78 years) and had a greater proportion of black (9.8% *vs* 7.9%), Hispanic (7.3% *vs* 4.0%), and Asian or Pacific Islander patients (4.7% *vs* 1.6%) than the non-prediabetic cohort ($P < 0.001$) (Table 1).

Comorbidities

Of the comorbid conditions hypertension, hyperlipidemia, smoking, obesity, drug misuse, prior myocardial infarction (MI), peripheral vascular disease (PVD), and hyperthyroidism were more prevalent in the pDM group ($P < 0.05$ for all the comorbidities).

Outcomes

Interestingly pDM were more often routinely discharged (23.6% *vs* 21.0%), however more often required home healthcare and incurred greater charges compared to patients without prediabetes.

After multivariable regression analysis and adjustment for baseline characteristics, the pDM cohort with AF hospitalizations had a substantially higher rate and chances of MACCE than the non-pDM cohort (18.6% *vs*. 14.7%, OR 1.34, 95%CI 1.25-1.45, $P < 0.001$). Figure 3 sub group analysis showed that males had a larger correlation (1.43 adjusted OR) than

Table 1 Baseline characteristics of atrial fibrillation-related hospitalizations with and without prediabetes (%)

Variable	Prediabetes	No prediabetes	*P* value
N	47505	2918370	
Age (yr)	75 (67-82)	78 (68-85)	< 0.001
Male sex	56.3	51.4	< 0.001
Race/ethnicity			< 0.001
White	75.5	84	
Black	9.8	7.9	
Hispanic	7.3	4	
API	4.7	1.6	
Comorbidities			
Hypertension (uncomplicated)	34.6	31.3	< 0.001
Hyperlipidemia	64.7	48.7	< 0.001
Smoking	10.5	10.2	0.033
Obesity	29.9	14.6	< 0.001
Drug abuse	2.5	2.1	< 0.001
Prior myocardial infarction	10.8	9.6	< 0.001
PVD	20.2	12.7	< 0.001
Hypothyroidism	18.1	20.4	< 0.001
Outcomes			
Discharge disposition			
Routine	51.1	43.1	< 0.001
Home health care	23.6	21	< 0.001
Total charges (USD), Median	41155	41276	< 0.001

API: Asian or Pacific Islanders; PVD: Peripheral vascular disease.

females (adjusted OR 1.22). Hispanics (1.43 adjusted OR) and Asians (1.36 adjusted OR) had a greater connection between MACCE and pDM than whites (1.33 adjusted OR) and blacks (1.21 adjusted OR) (Table 2).

DISCUSSION

This study, based on the 2019 NIS, was conducted to understand and examine the effects of prediabetes in AF patients. According to the findings, 47505 (1.6%) of the 2965875 AF-related hospitalizations for MACCE were among patients with prediabetes. After correcting baseline characteristics or comorbidities, the prediabetes cohort had a greater frequency of MACCE, including all-cause inpatient mortality, cardiac arrest, including ventricular fibrillation, and stroke, in AF-related hospitalizations than the non-prediabetic cohort. This study points out that further research is needed to effectively manage prediabetes in AF patients to reduce MACCE (Figure 1).

Patients with prediabetes are thought to be responsible for endothelial dysfunction, chronic inflammation, and oxidative stress, which could lead to atherosclerosis, MI, stroke, and other cardiovascular disorders. The exact mechanisms underlying these changes are not well known[11,12]. Most of the patient population were younger males (56.3%); Hispanics or Asians had a stronger association with MACCE with prediabetes, followed by whites and blacks. As per the literature, certain ethnic groups and men were more predisposed to prediabetes and diabetes than others[13].

The prediabetic cohort was discharged from the hospital early, was associated with more home health care, and had greater overall cost. In addition, the prediabetes cohort with AF admissions showed a higher rate and significantly higher odds of MACCE compared to the non-prediabetic cohort. Despite having shorter hospital stays, they had higher healthcare charges, possible due to the requirement for frequent monitoring, more medications for comorbid conditions and management, of AF and subsequent in-hospital MACCE. Taking steps to tackle prediabetes and its potential progression towards diabetes can play a crucial role, in reducing these expenses and enhancing overall health outcomes in patients with multiple comorbidities and higher atherosclerotic cardiovascular disease risk.

Table 2 Association between prediabetes and major adverse cardiac and cerebrovascular events in patients with atrial fibrillation and by subgroups (sex and race)

Outcome	Adjusted odds ratio (95%CI)	*P* value
MACCE	1.34 (1.26-1.42)	< 0.001
All-cause mortality	0.62 (0.55-0.71)	< 0.001
Cardiac arrest including ventricular fibrillation	0.85 (0.072-0.99)	0.043
Stroke	1.69 (1.55-1.84)	< 0.001
Subgroup	Adjusted odds ratios of MACCE (95%CI)	*P* value
Sex		
Male	1.43 (1.33-1.54)	< 0.001
Female	1.22 (1.11-1.33)	< 0.001
Race/ethnicity		
White	1.33 (1.24-1.42)	< 0.001
Black	1.21 (1.03-1.44)	< 0.001
Hispanic	1.43 (1.18-1.74)	< 0.001
API	1.36 (1.00-1.85)	0.05

Major adverse cardiac and cerebrovascular events included all-cause mortality, cardiac arrest and stroke. In the multivariable regression analysis, the following covariates were adjusted for: Age at admission, sex, race, median household income quartile, payer status, type of admission, hospital bed size, location/teaching status, region, and relevant cardiac and extra cardiac comorbidities and prior history of myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting venous thromboembolism. API: Asian or Pacific Islanders; MACCE: Major adverse cardiac and cerebrovascular events.

Autonomic dysfunction associated with prediabetes can cause arrhythmias, which can increase the chances of developing AF[14]. In this study, involving 17943 patients with newly diagnosed AF, it was discovered that 20.7% of them had prediabetes while 56.4% had diabetes. Over a span of 4.7 years, heart failure occurred in 14, 15.7% and 17.7% in those with normal glucose levels, prediabetics and diabetics. This study concluded that prediabetes is associated with an elevated risk of HF in patients with AF. Moreover, individuals with a prediabetic state who develop diabetes within a span of two years are at a heightened risk of heart failure. On the hand those who revert to normal blood sugar levels experience a decreased risk of heart failure. It is essential for healthcare practitioners to diligently observe and control pre-diabetes in patients with AF in order to hinder the progression, towards diabetes and minimize the chances of developing heart failure. Additionally, interventions targeting glycemic control should be implemented early on to improve cardiovascular outcomes in this high-risk population.

Comorbid conditions such as hypertension, hyperlipidemia, smoking, obesity, substance abuse, prior MI, PVD, and hyperthyroidism are all prevalent among the exposure group, which is the prediabetic group with AF. These comorbid conditions are all identified as independent risk factors for MACCE[15], which brings to the point that the results could have been an effect of comorbidities rather than prediabetes itself. The presence of underlying health conditions in individuals with prediabetes as compared to those without prediabetes has significant implications for their overall outcomes. Among the comorbid conditions, hypertension is a well-recognized risk factor for MACCE, like stroke, MI, and cardiovascular mortality, irrespective of prediabetes status. An interesting correlation was found in recent literature in a retrospective analysis[16]. Hypertension and MACCE events are found in individuals with prediabetes. Other than hypertension, hyperlipidemia is another well-known risk factor for MACCE[17]. Hyperlipidemia is a well-established risk factor for coronary artery disease and can lead to premature cardiovascular mortality if not managed.

Smoking, alcohol, and obesity, major contributors to atherosclerosis, have been associated with MACCE in people with prediabetes[18-20]. PVD was also significantly higher in the AF cohort with prediabetes, PVD being a marker of systemic atherosclerosis and inflammation, could contribute to worsening AF prognosis and subsequent MACCE in patients with prediabetes.

The findings of this study shed light on the relationship between prediabetes and AF. Previous research has already established that prediabetes increases the risk of conditions like stroke and MI. The changes in insulin sensitivity and resistance observed in prediabetes may contribute to MACCE[21], which is concerning. Individuals with prediabetes have a 34% risk of MACCE compared to those without this condition in this study. However, it is important to note that correlation does not imply causation, and further research is needed to establish a direct link between prediabetes and AF. There is a link between prediabetes and complications such as retinopathy, nephropathy, and chronic kidney disease suggested in the literature. Furthermore, it has been observed that patients who have recently experienced stroke or transient ischemic attack are more likely to have prediabetes compared to the general population[21]. This indicates that prediabetes could potentially be a contributing factor to these events and warrant monitoring and interventions in order to prevent the progression to DM and these complications.

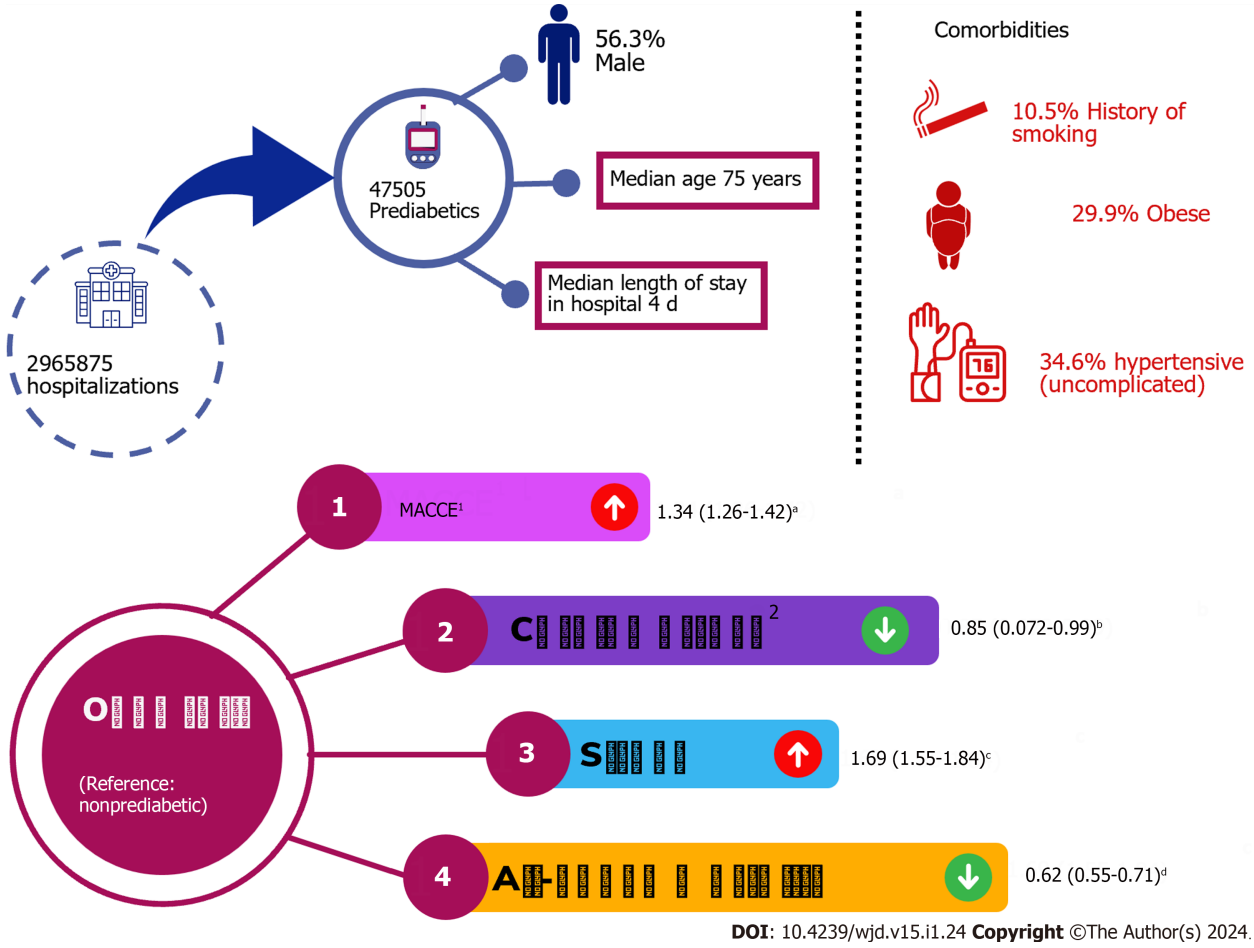

Figure 3 Outcomes related to occurrence of prediabtes in the study cohort. [1]Major adverse cardiac and cerebrovascular events; [2]Including ventricular fibrillation. [a]*P* < 0.001 *vs* non-prediabetic; [b]*P* = 0.043 *vs* non-prediabetic; [c]*P* < 0.001 *vs* non-prediabetic; [d]*P* < 0.001 *vs* non-prediabetic.

To effectively manage prediabetes, it's essential to understand the factors involved, such as IGT and impaired fasting glucose (IFG). Patients with IFG and IGT experience impaired beta cell function and insulin resistance, which is similar to type 2 diabetes. However, their pathophysiology differs slightly. IFG is mainly characterized by insulin resistance in the liver, while IGT involves muscle insulin resistance and mild hepatic resistance. Recognizing these differences is vital for tailoring treatment approaches that can prevent the progression of DM.

Future direction

Patients who have AF face the risk of experiencing MACCE. These events can be attributed to both the pathological changes caused by AF itself well as the presence of other comorbid conditions associated with AF that directly or indirectly contribute to these adverse events. While research suggests that factors, like endothelial dysfunction, chronic inflammation, oxidative stress and autonomic dysfunction may be involved in the pathophysiology of MACCE, further investigation is necessary to confirm and expand upon these theories[22-24]. Moreover, future studies should prioritize developing strategies for reducing MACCE in patients, with prediabetes who also have AF. This is particularly important since diabetes is a known factor that leads to microvascular and macrovascular changes associated with disease.

Additionally, demographic differences revealed a greater risk of MACCE in men, Latinos, and Asians, which shows that understanding patient group diversity in terms of cardiovascular risk factors is critical for successful risk assessment and treatment. Additional studies quantifying the effects of multiple comorbidities in AF patients with prediabetes causing MACCE. Finally, it can be useful to find out potential advantages in lowering cardiovascular risk factors in prediabetes and AF patients with therapies and guidelines directed towards decreasing the inciting events. The findings of our study shed light on a topic that has not received much attention and could have important clinical implications. Our findings, in our opinion, should make physicians more aware of the prediabetes disease and any potential long term side effects. Considering the findings of this study, we would advocate for aggressive prediabetes treatment. This would entail behavioral modification, nutritional assessment and intervention, drug therapy, and optimization of risk factors.

Limitation of study

To our knowledge, this is the largest study providing valuable insights into the relationship between prediabetes and AF-related MACCE in the United States adult population, however, it has a few drawbacks to consider while inferring results. Firstly, this study was mainly retrospective and was conducted on data provided by the administration, which

could have coding errors and variable misclassification of different diseases and diagnoses. Furthermore, this study did not account for other potential confounding variables such as nutrition, physical exercise, the effects of other comorbid conditions. As a retrospective observational analysis, this study evaluated association and not causation. This study could not establish their causal relationship. Other study designs such as randomized controlled trials are needed to validate the association between prediabetes and MACCE in AF patients. Long-term follow up data were not available. Lastly, we could not evaluate whether any medications could have impacted AF outcomes in patients with prediabetes. Coding inaccuracies. Lack of lab value, residual confounding, absence of outpatient data.

CONCLUSION

This study suggests that AF patients with prediabetes are at increased risk of MACCE even after adjusting baseline characteristics and comorbidities compared to AF patients without prediabetes. These enlighten the importance of screening for prediabetes in AF patients and strict hyperglycemia control to prevent MACCE. Further study is required, as this study suggests that pre-diabetes may affect cardiovascular risk differently depending on the demographic group. Therefore, pre-diabetes screening should be done as a part of routine care for AF patients, especially if they are male, Hispanic, or Asian, to reduce the risk of MACCE.

ARTICLE HIGHLIGHTS

Research background

Prediabetes is a well-established risk factor for major adverse cardiac and cerebrovascular events (MACCE). This observational retrospective cohort study examines relationship between prediabetes and MACCE in patients with atrial fibrillation (AF).

Research motivation

The study aims to fill a knowledge gap by studying the connection between prediabetes and major cardiac and cerebrovascular events in AF patients. The goal is to better understand the risks and implications for clinical practice in managing prediabetes in this population.

Research objectives

Our objective is to investigate and establish a link between prediabetes and MACCE in patients with AF.

Research methods

Using National Inpatient Sample (2019) and relevant ICD-10 CM codes, hospitalizations with AF were categorized into groups with and without prediabetes, excluding diabetics. The primary outcome was MACCE (all-cause inpatient mortality, cardiac arrest including ventricular fibrillation, and stroke) in AF-related hospitalizations.

Research results

Key findings include: Prediabetes was present in 1.6% of AF-related hospitalizations. The prediabetes cohort was younger (median age 75 years) with a higher proportion of males, blacks, Hispanics, and Asians. Males had a stronger association between prediabetes and MACCE than females, and among different racial groups, Hispanics and Asians had a stronger association compared to whites and blacks. The prediabetes cohort with AF admissions had a higher rate of MACCE compared to the non-prediabetic cohort with an odds ratio of 1.34 and a 95% confidence interval of 1.26-1.42.

Research conclusions

This study highlights the relationship between prediabetes and MACCE in AF patients, therefore emphasizing the importance of further research, awareness as well as the importance of screening and managing prediabetes in AF patients to prevent MACCE.

Research perspectives

The research perspective is primarily epidemiological and clinical. It aims to understand the prevalence, risk factors, and clinical implications of prediabetes in the context of cardiovascular health.

FOOTNOTES

Author contributions: Desai R investigation writing-original draft, visualization, writing-review and editing, investigation, formal analysis, administration, supervision; Katukuri N, Goguri SR, Kothawala A, Alle NR, Bellamkonda MK, Dey D, Ganesan S, Biswas M, Sarkar K, Prattipati P, writing-original draft; writing-review and editing, investigation, visualization; Chauhan S conceptualization, methodology, resources, writing original, draft; writing-review and editing, administration, supervision; all authors have read and approved the final manuscript.

Institutional review board statement: Not applicable, data is obtained from a publicly available data set, patient identifiers are not used.

Informed consent statement: Informed consent statement was not needed because the study was carried out on a publicly available data set without patient identifiers.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at drshaylikachauhan@gmail.com. Participants consent was not obtained but the presented data are anonymized and risk of identification is low.

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Retrospective Study

Predictive value of bilirubin and serum γ-glutamyltranspeptidase levels in type-2 diabetes mellitus patients with acute coronary syndrome

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Abstract

BACKGROUND

Cardiovascular disease is a major complication of diabetes mellitus (DM). Type-2 DM (T2DM) is associated with an increased risk of cardiovascular events and mortality, while serum biomarkers may facilitate the prediction of these outcomes. Early differential diagnosis of T2DM complicated with acute coronary syndrome (ACS) plays an important role in controlling disease progression and improving safety.

AIM

To investigate the correlation of serum bilirubin and γ-glutamyltranspeptidase (γ-GGT) with major adverse cardiovascular events (MACEs) in T2DM patients with ACS.

METHODS

The clinical data of inpatients from January 2022 to December 2022 were analyzed retrospectively. According to different conditions, they were divided into the T2DM complicated with ACS group (T2DM + ACS, n = 96), simple T2DM group (T2DM, n = 85), and simple ACS group (ACS, n = 90). The clinical data and laboratory indices were compared among the three groups, and the correlations of serum total bilirubin (TBIL) levels and serum γ-GGT levels with other indices were discussed. T2DM + ACS patients received a 90-day follow-up after discharge and were divided into event (n = 15) and nonevent (n = 81) groups according to the occurrence of MACEs; Univariate and multivariate analyses were further used to screen the independent influencing factors of MACEs in patients.

RESULTS

The T2DM + ACS group showed higher γ-GGT, total cholesterol, low-density lipoprotein cholesterol (LDL-C) and glycosylated hemoglobin (HbA1c) and lower TBIL and high-density lipoprotein cholesterol levels than the T2DM and ACS groups ($P < 0.05$). Based on univariate analysis, the event and nonevent groups were significantly different in age ($t = 3.3612$, $P = 0.0011$), TBIL level ($t = 3.0742$, $P = 0.0028$), γ-GGT level ($t = 2.6887$, $P = 0.0085$), LDL-C level ($t = 2.0816$, $P = 0.0401$), HbA1c level ($t = 2.7862$, $P = 0.0065$) and left ventricular ejection fraction (LEVF) levels (t=3.2047, $P = 0.0018$). Multivariate logistic regression analysis further identified that TBIL level and LEVF level were protective factor for MACEs, and age and γ-GGT level were risk factors ($P < 0.05$).

CONCLUSION

Serum TBIL levels are decreased and γ-GGT levels are increased in T2DM + ACS patients, and the two indices are significantly negatively correlated. TBIL and γ-GGT are independent influencing factors for MACEs in such patients.

Key Words: Acute coronary syndrome; Type-2 diabetes mellitus; Total bilirubin; Major adverse cardiovascular events

Core Tip: Serum total bilirubin (TBIL) and serum γ-glutamyltranspeptidase (γ-GGT) levels can be used as predictors of acute coronary syndrome (ACS) in patients with type-2 diabetes mellitus (T2DM). This study aims to investigate the levels and their correlations with major adverse cardiovascular events in T2DM patients with ACS. TBIL levels are found to be decreased and γ-GGT increased in T2DM + ACS patients, both of which can be used as indicators to assess patients' condition and predict long-term adverse cardiovascular events in such patients.

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INTRODUCTION

Acute coronary syndrome (ACS) is a common cardiovascular disease (CVD) manifested by chest tightness and chest pain, which can cause heart failure, arrhythmia and even sudden death[1]. In recent years, ACS, as the most serious ischemic heart disease, has become one of the primary causes of death worldwide[2]. Despite the treatment recommended by current guidelines, some ACS patients are still at high risk of recurrent cardiovascular events. This risk is especially high among patients with type-2 diabetes mellitus (T2DM), who account for approximately one-third of ACS cases[3-5]. T2DM is a frequently occurring disease, and diabetes, as is well known, has become one of the primary causes of morbidity and mortality in most countries[6]. The incidence of diabetes is rising, affecting an estimated 346 million people worldwide [7], and the World Health Organization (WHO) predicts that it will be the seventh leading cause of death worldwide by 2030[8]. CVD is one of the major complications of diabetes, causing 50% to 80% of early deaths. According to related research, ACS often occurs in patients with diabetes rather than nondiabetic patients. Overall, mortality from ACS is four times higher in men with diabetes and seven times higher in women with diabetes than in those without diabetes[9]. In addition, a linear positive association has been reported between hyperglycemia on admission and post-ACS mortality [10]. Therefore, early differential diagnosis of T2DM complicated with ACS plays an important role in controlling disease progression and improving safety.

Current studies have shown that serum γ-glutamyltranspeptidase (γ-GGT) can be used as a predictor of metabolic syndrome, and its level can reflect the severity of oxidative damage to cells by oxygen free radicals in the body, which is an early sensitive index to evaluate the degree of oxidative stress[11]. Meanwhile, serum γ-GGT levels can reflect liver function, and an increase in its level is an independent risk factor for multiple CVDs[12]. Recent studies have linked its elevated serum levels to many pro-atherosclerotic factors, such as insulin resistance, obesity, elevated plasma cholesterol levels, hypertension, and myocardial infarction[13-15]. Therefore, γ-GGT levels can be detected to understand whether ACS occurs in T2DM patients in a timely manner, providing a clinical basis for diagnosis and treatment. Bilirubin (BIL) is a natural antioxidant factor in the body and is a kind of bile pigment formed by the metabolism of the end products after ferroheme decomposition. Total bilirubin (TBIL) is the decomposition product of senescent red blood cells[16], with a potent antioxidant capacity, which can remove harmful substances produced by oxidative stress and protect various organs of the body from damage[17]. Elevated serum TBIL levels are a potential protective factor for the onset of T2DM [18]. The human body has an antioxidant protection system. Peroxidation and antioxidant are in a dynamic balance under normal circumstances that cause no damage to the human body, but an imbalance between them can lead to increased lipid peroxidation[19]. With the deepening of research on serum BIL, BIL has been found to be a member of the oxygenation system, which can protect lipid peroxidation[17] and protect lipids and lipoproteins from oxidation,

reducing arterial damage and atherosclerotic plaques[20] and lowering the possibility of developing coronary heart disease or ACS[21]. Heme oxygenase (HO) is the initiating enzyme and rate-limiting enzyme for BIL production and is available in HO-1 and HO-2 forms. Studies have shown that myocardial HO-1 activity in patients with acute myocardial infarction is obviously upregulated, and the serum TBIL level is strongly correlated with the occurrence of coronary artery disease[22].

However, whether serum TBIL and γ-GGT levels can be used as predictors of ACS in patients with T2DM and whether they are related to the incidence of major adverse cardiovascular events (MACEs) in T2DM + ACS patients has not been confirmed. Based on this, by studying changes in serum TBIL and γ-GGT levels in diabetes patients complicated with ACS and exploring their correlations with MACEs, this study provides more valuable information for the prognostic assessment of such patients and provides a theoretical basis for early clinical intervention in the future.

MATERIALS AND METHODS

Study subjects

The clinical data of inpatients in The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University from January 2022 to December 2022 were analyzed retrospectively. According to different conditions, patients were divided into the T2DM complicated with ACS group (T2DM + ACS, n = 96), simple T2DM group (T2DM, n = 85), and simple ACS group (ACS, n = 90). Inclusion criteria were as follows: (1) T2DM patients all met the diagnostic criteria for T2DM formulated by the WHO, namely, fasting blood glucose ≥ 7.0 mmol/L or 2-hour postprandial blood glucose ≥ 11.1 mmol/L, and glycosylated hemoglobin (HbA1c) ≥ 6.5% (48 mmol/mol); (2) ACS patients had unstable angina, non-ST-segment and ST-segment elevation myocardial infarction confirmed by coronary angiography (CAG), electrocardiogram (ECG), dynamic ECG, and troponin level detection, with a stenosis ≥ 50% of at least one vessel; (3) the T2DM group received CAG and ECG after admission to confirm no coronary lesions; (4) patients in the T2DM + ACS group were confirmed by CAG and ECG to meet the above diagnostic criteria for T2DM and ACS; (5) patients had a normal mental state and cognitive function; and (6) patients had complete clinical data and follow-up information. The exclusion criteria were as follows: (1) Secondary diabetes caused by type 1 diabetes, gestational diabetes and other endocrine diseases; (2) constitutional jaundice, old myocardial infarction, symptomatic heart failure, pulmonary hypertension, and pulmonary heart disease; (3) digestive system diseases, abnormal liver function, lung diseases and malignant tumors; (4) renal dysfunction with an estimated glomerular filtration rate < 30 mL/(min·1.73 m^2) or a history of renal replacement therapy, severe hepatic insufficiency with alanine transaminase or aspartate transaminase ≥ 5 times the upper limit of normal; (5) previous use of steroids, statins, etc. that affect liver or kidney function; and (6) incomplete clinical data and follow-up information. This study was approved by the Ethics Committee of The Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University.

Outcome measures

(1) General data: Age, sex, body mass index (BMI), smoking status, alcohol consumption status and other basic information of all subjects were collected; (2) Laboratory examination: Fasting venous blood was collected from all patients the morning after patient admission and after 12 h of fasting and uniformly submitted for examination. Using a Hitachi 7600 automatic biochemical analyzer (Hitachi, Japan), TBIL, serum γ-GGT, total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), and HbA1c levels were quantified; and (3) Cardiac function tests: Echocardiography was performed using color doppler ultrasonography (Hewlett-Packard, United States) within 72 h of admission, mainly measuring left ventricular ejection fraction (LVEF).

Follow-up

All T2DM + ACS patients received a 90-day follow-up after discharge and were divided into either the event or nonevent group according to the occurrence of MACEs: Recurrent myocardial infarction, revascularization, heart failure, and cardiogenic death.

Statistical analyses

All analyses were performed in SPSS 25.0 at a significance level of $\alpha = 0.05$. Measurement data are expressed as the mean ± SD; t test was used for intergroup comparisons, one-way analysis of variance for multigroup comparisons, and the lysergic acid diethylamide method for pairwise comparisons. Count data are expressed as percentages and were analyzed by the χ^2 test. Pearson linear correlation analysis was performed to determine the correlations of serum TBIL and γ-GGT levels with other indicators in T2DM patients with ACS. The influencing factors were analyzed by logistic regression.

RESULTS

General information

We collected the clinical data of each group for comparative analysis. No marked differences were identified in sex, age, BMI, drinking history, smoking history, or family history of coronary heart disease ($P > 0.05$), but the proportion of hypertension history was significantly different ($P < 0.05$), as shown in Table 1.

Table 1 Comparison of clinical data among groups

	T2DM + ACS (*n* = 96)	T2DM (*n* = 85)	ACS (*n* = 90)	*F* value	*P* value
Sex (male/female)	58/38	55/30	67/23	4.262	0.1187
Average age (yr)	53.6 ± 6.3	54.9 ± 4.7	53.0 ± 5.7	2.584	0.0773
BMI (kg/m^2)	22.6 ± 2.2	22.0 ± 1.7	22.2 ± 2.4	1.882	0.1542
Smoking history	37	30	37	0.6272	0.7308
Drinking history	29	26	34	1.4922	0.4743
Family history of coronary heart disease	18	13	15	0.3911	0.8224
Hypertension	32	20	38	6.8872	0.0319

T2DM: Type-2 diabetes mellitus; ACS: Acute coronary syndrome; BMI: Body mass index.

Laboratory indicators

Serum indices were significantly different among the three groups ($P < 0.05$). Specifically, the T2DM + ACS group exhibited notably higher γ-GGT, TC, LDL-C and HbAlc and lower left ventricular ejection fraction (LEVF), TBIL and HDL-C levels than the T2DM and ACS groups ($P < 0.05$), as shown in Table 2.

Correlation between TBIL level and other indices in the T2DM + ACS group

According to Pearson correlation analysis, serum TBIL was negatively correlated with γ-GGT, TC, LDL-C and HbAlc ($P < 0.05$) but was not related to TG, HDL-C and LEVF ($P > 0.05$), as shown in Table 3.

Correlations of γ-GGT level with other indices in patients in the T2DM + ACS group

As indicated by Pearson correlation analysis, serum γ-GGT was inversely associated with TBIL ($P < 0.05$) and positively correlated with TG and LDL-C ($P < 0.05$), but it had no correlation with TC, HDL-C, HbAlc or LEVF ($P > 0.05$), as shown in Table 4.

Univariate analysis of MACEs

Fifteen patients in the T2DM + ACS group developed MACEs, including 3 recurrent myocardial infarction, 6 revascularization, 3 heart failure and 3 cardiogenic deaths. No patients died in the nonevent group. Univariate analysis showed that age ($t = 3.3612$, $P = 0.0011$), TBIL levels ($t = 3.0742$, $P = 0.0028$), γ-GGT levels ($t = 2.6887$, $P = 0.0085$), LDL-C levels ($t = 2.0816$, $P = 0.0401$), HbA1c levels ($t = 2.7862$, $P = 0.0065$), and LEVF levels ($t = 3.2047$, $P = 0.0018$) were significantly different between the event and nonevent groups ($P < 0.05$), as shown in Table 5.

Multivariate logistic regression analysis

Multivariate logistic regression analysis based on univariate results showed that age, serum TBIL, serum γ-GGT and LEVF were independent influencing factors of ACS in T2DM patients ($P < 0.05$), in which the TBIL level and LEVF was a protective factor, while age and γ-GGT levels were risk factors, as shown in Table 6.

DISCUSSION

T2DM is an important factor causing atherosclerotic thrombosis. Insulin resistance and increased blood sugar can accelerate the development of atherosclerosis and increase the risk of ACS[23]. The mechanism of T2DM-induced atherosclerosis and thrombosis is complicated and is mainly due to lipid metabolism disorders induced by persistent hyperglycemia that promotes lipid deposition, leading to a microinflammatory state in the body and promoting thrombosis[24]. At present, there is still a lack of reliable indicators to evaluate the progression of T2DM complicated with ACS, and a great breakthrough has not been made in improving patient prognosis. Thus, it is necessary to seek ideal markers to provide a basis for clinical diagnosis and treatment.

This study explored serum TBIL and γ-GGT levels in T2DM patients with ACS and their correlations with MACEs. T2DM + ACS patients were found to have higher γ-GGT, TC, LDL-C and HbAlc levels than simple T2DM and ACS patients, while their levels of TBIL and HDL-C were significantly lower. In addition, serum TBIL was found to be inversely associated with γ-GGT, TC, LDL-C and HbAlc, but it had no correlation with TG and HDL-C. Serum γ-GGT was negatively correlated with TBIL and positively correlated with TG and LDL-C but not with TC, HDL-C or HbAlc. Both serum TBIL and HbA1c are risk factors for coronary heart disease. The former is a natural endogenous strong antioxidant with the functions of inhibiting lipid peroxidation, anti-ischemia and eliminating free radicals, which can protect LDL-C in the human body from oxidation[25]. The latter is a common clinical index that plays a key role in the formation and development of atherosclerosis and is mainly formed by the (irreversible) enzymatic reaction of hemoglobin and glucose[26]. BIL has been shown to prolong the survival time of ventricular myocytes by resisting

Table 2 Comparison of laboratory indexes among group

	T2DM + ACS (*n* = 96)	T2DM (*n* = 85)	ACS (*n* = 90)	*F* value	*P* value
TBIL (μmol/L)	6.76 ± 0.70	10.72 ± 1.21[1]	9.53 ± 1.03[1,2]	384.2	< 0.0001
γ-GGT (U/L)	36.81 ± 2.98	32.22 ± 3.22[1]	30.09 ± 3.29[1]	110.2	< 0.0001
TC (mmol/L)	5.33 ± 0.43	4.24 ± 0.36[1]	4.33 ± 0.40[1]	213.1	< 0.0001
TG (mmol/L)	0.96 ± 0.33	1.02 ± 0.22	1.05 ± 0.20[1]	2.923	0.0555
LDL-C (mmol/L)	2.41 ± 0.26	1.87 ± 0.24[1]	2.28 ± 0.25[1,2]	112.0	< 0.0001
HDL-C (mmol/L)	1.16 ± 0.07	1.19 ± 0.12[1]	1.21 ± 0.11[1]	5.750	0.0036
HbA1c (%)	8.19 ± 0.64	7.61 ± 0.60[1]	5.33 ± 0.69[1,2]	501.8	< 0.0001
LEVF (%)	44.92 ± 3.80	57.27 ± 6.39[1]	52.19 ± 4.64[1]	139.8	< 0.0001

[1]*P* < 0.05 *vs*. T2DM + ACS.
[2]*P* < 0.05 *vs*. T2DM.
TBIL: Total bilirubin; γ-GGT: γ-glutamyltranspeptidase; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; LEVF: Left ventricular ejection fraction; T2DM: Type-2 diabetes mellitus; ACS: Acute coronary syndrome.

Table 3 Correlation between serum total bilirubin level and laboratory indexes

	γ-GGT	TC	TG	LDL-C	HDL-C	HbA1c	LEVF
r value	-0.3275	-0.2906	-0.0822	-0.3086	-0.03255	-0.3140	0.0765
P value	0.0011	0.0041	0.4260	0.0022	0.7529	0.0018	0.4587

γ-GGT: γ-glutamyltranspeptidase; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; LEVF: Left ventricular ejection fraction.

Table 4 Correlation between serum γ-glutamyltranspeptidase level and laboratory indexes

	TBIL	TC	TG	LDL-C	HDL-C	HbA1c	LEVF
r value	-0.3275	0.1074	0.2281	0.2030	-0.0074	0.0225	-0.1250
P value	0.0011	0.2975	0.0254	0.0473	0.9430	0.8276	0.2248

TBIL: Total bilirubin; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; LEVF: Left ventricular ejection fraction.

oxygen free radical-induced damage. Gullu *et al*[27] showed that high BIL levels can protect coronary blood flow reserve and coronary microvascular endothelial function. BIL, with antioxidant and anti-inflammatory activities, has been reported to reduce atherosclerosis *in vivo*[28]. Atherosclerosis is known to be the pathological basis of CVD[29]. Erdogan *et al*[30] reported that increasing TBIL levels promote collateral angiogenesis in chronic total occlusion of the coronary artery. Furthermore, Bil can improve the activity of HO, which has an antiatherosclerotic effect[31]. The isozyme HO-1 participates in the anti-stress ability of cardiovascular system tissues and cells under pathological conditions and maintains the integrity and constancy of cardiovascular system function[32]. At the same time, Bil can increase cholesterol dissolution, promote cholesterol excretion from bile, reduce plasma cholesterol concentration, and prevent the development of atherosclerosis[33]. This study found that the level of TBIL was negatively correlated with TC and LDL-C, which also suggests that low Bil can be related to the occurrence of coronary heart disease through the increase in blood lipids. In patients with T2DM, glucose abnormalities are often accompanied by dyslipidemia, which together promote the formation of atherosclerotic plaques[34]. Our experimental results also prove that the influence of elevated serum γ-GGT levels on ACS in T2DM patients is related to abnormal lipid metabolism. γ-GGT levels are highest in people with ACS and lowest in healthy people[35]. Increased γ-GGT levels contribute to the development and progression of ACS caused by T2DM. In this study, Pearson analysis showed that γ-GGT was positively correlated with LDL-C and TG. It can be concluded that an increase in γ-GGT levels will cause abnormal lipid metabolism, a decrease in lipid peroxidation, the release of inflammatory factors, and damage to the vascular endothelium, which will affect the stability of plaques, causing damage to the plaques, increasing the plaque size, and inducing ACS. Moreover, LEVF levels were found to be significantly different between patients with MACEs and those without. Coronary artery microcirculatory

Table 5 Univariate analysis of major adverse cardiovascular events

	Event group (*n* = 15)	Non-event group (*n* = 81)	χ^2/t value	*P* value
Sex (male/female)	10/5	48/33	0.2904	0.5900
Average age (yr)	58.33 ± 7.68	52.68 ± 5.63	3.3612	0.0011
BMI (kg/m^2)	21.9 ± 2.6	22.7 ± 2.1	1.3045	0.1953
TBIL (μmol/L)	6.27 ± 0.87	6.85 ± 0.63	3.0742	0.0028
γ-GGT (U/L)	38.65 ± 2.91	36.47 ± 2.88	2.6887	0.0085
TC (mmol/L)	5.51 ± 0.26	5.30 ± 0.45	1.7492	0.0835
TG (mmol/L)	1.08 ± 0.25	0.94 ± 0.34	1.5177	0.1324
LDL-C (mmol/L)	2.53 ± 0.29	2.38 ± 0.25	2.0816	0.0401
HDL-C (mmol/L)	1.15 ± 0.08	1.16 ± 0.07	0.4970	0.6203
HbA1c (%)	8.59 ± 0.73	8.11 ± 0.59	2.7862	0.0065
LEVF (%)	42.13 ± 2.83	45.39 ± 3.74	3.2047	0.0018

BMI: Body mass index; TBIL: Total bilirubin; γ-GGT: γ-glutamyltranspeptidase; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; LEVF: Left ventricular ejection fraction.

Table 6 Logistic analysis of major adverse cardiovascular events

	Regression coefficient	Standard error	Wald	*P* value	HR	95%CI
Constant	-8.390	14.396	0.340	0.560	-	-
Age	0.251	0.086	8.578	0.003	1.285	1.086-1.519
TBIL	-1.706	0.764	4.992	0.025	0.182	0.041-0.811
γ-GGT	0.422	0.195	4.656	0.031	1.525	1.039-2.236
LDL-C	-1.156	1.785	0.042	0.517	0.315	0.010-10.397
HbA1c	1.082	0.742	2.123	0.145	2.950	0.688-12.644
LEVF (%)	-0.415	0.157	7.027	0.008	0.660	0.486-0.897

TBIL: Total bilirubin; γ-GGT: γ-glutamyltranspeptidase; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin; LEVF: Left ventricular ejection fraction; HR: Hazard ratio; 95%CI: 95% confidence interval.

disorders may cause myocardial fibrosis and even cardiac insufficiency[36]. Myocardial ischemia, hypoxia, and reperfusion injury ultimately lead to diastolic dysfunction[37]. The measurement of LVEF can sensitively and specifically reflect changes in left ventricular function in left ventricular dysfunction and secondary ventricular remodeling[38]. Studies have shown that the severity of coronary artery disease, myocardial ischemia, and myocardial cell injury or apoptosis are associated with decreased LVEF[39]. Finally, through univariate and multivariate logistic analyses, age, LEVF, serum TBIL levels, and serum γ-GGT levels were confirmed as independent influencing factors of ACS in T2DM patients, of which the TBIL level and LVEF levels were protective factors, while age and γ-GGT levels were risk factors.

However, some limitations of this study need to be addressed. First, due to the fact that this is a single-center retrospective analysis, the research materials and subjects were limited, warranting a larger sample size for further analysis. Second, other risk factors for ACS such as socioeconomic status, dietary patterns, physical activity, hormone levels and medication were not included in our study. In addition, lipids, which are risk factors for ACS, were not discussed in this study. Third, the samples studied were hospital-based and may not be representative of the general T2DM patient population. Fourth, the sample size of this study was relatively small. Therefore, further studies with larger sample sizes and more measures are needed.

CONCLUSION

In summary, serum TBIL levels and serum γ-GGT levels can be used as indicators to assess patients' condition and predict long-term adverse cardiovascular events in diabetes patients with ACS. However, as this is a retrospective, single-center study with a small sample size, it is necessary to increase the sample size for more in-depth clinical research on

whether patients with ACS complicated with diabetes can be accurately evaluated and predicted based on the detection of serum TBIL and γ-GGT levels.

ARTICLE HIGHLIGHTS

Research background

To provide more credible clinical evidence for the prognostic assessment of type-2 diabetes mellitus (T2DM) patients complicated with acute coronary syndrome (ACS) and a theoretical basis for early clinical intervention in the future.

Research motivation

Serum total bilirubin (TBIL) levels and γ-glutamyltranspeptidase (γ-GGT) levels can be used as indicators to assess patients' condition and predict long-term adverse cardiovascular events in T2DM patients with ACS.

Research objectives

T2DM + ACS patients were found to have higher γ-GGT, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and glycosylated hemoglobin (HbA1c) levels than patients with T2DM or ACS alone, with significantly lower levels of TBIL and high-density lipoprotein cholesterol (HDL-C). In addition, serum TBIL was found to be inversely associated with γ-GGT, TC, LDL-C and HbAlc, but it had no correlation with triacylglycerol and HDL-C. Through univariate and multivariate logistic analyses, age, left ventricular ejection fraction (LEVF), serum TBIL levels, and serum γ-GGT levels were confirmed as independent influencing factors of ACS in T2DM patients, of which TBIL and LVEF levels were protective factors, and age and γ-GGT levels were risk factors.

Research methods

The clinical data of inpatients were analyzed retrospectively. According to different conditions, they were divided into the T2DM complicated with ACS group (T2DM + ACS, n = 96), simple T2DM group (T2DM, n = 85), and simple ACS group (ACS, n = 90). The general data and laboratory indexes were compared among the three groups, and the correlations of serum TBIL and γ-GGT levels with other indicators were evaluated. T2DM + ACS patients received a 90-day follow-up after discharge and were further divided into event (n = 15) and nonevent (n = 81) groups according to the occurrence of major adverse cardiovascular events (MACEs). Univariate and multivariate analyses were further used to screen the independent influencing factors of MACEs in patients.

Research results

There is still a lack of reliable indicators to evaluate the progression of T2DM complicated with ACS, and a great breakthrough has not been made in improving patient prognosis. Therefore, it is necessary to seek ideal markers to provide a basis for clinical diagnosis and treatment.

Research conclusions

By studying changes in serum TBIL and γ-GGT levels in T2DM patients complicated with ACS and exploring their correlations with MACEs, it is confirmed that serum levels of TBIL and γ-GGT can be used to assess patients' condition and predict long-term MACEs in such patients.

Research perspectives

Cardiovascular disease is one of the major complications of diabetes, causing 50%-80% of early deaths. According to related research, ACS often occurs in patients with diabetes rather than nondiabetic patients.

FOOTNOTES

Co-first authors: Jie Chen and Wan-Chao Zhang.

Author contributions: Chen J and Zhang WC conceived and designed the experiments; Chen J, Zhang WC, Tang XQ, Yin RH, Wang T and Pan CJ collected and analyzed the data; Wei XY and Pan CJ contributed to the data collection; Chen J and Zhang WC overall supervise the study. All authors have approved the manuscript. Chen J and Zhang WC contributed equally to this work and are co-first authors, including design of the study, acquiring and analyzing data from experiments, and writing of the actual manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Clinical study of different prediction models in predicting diabetic nephropathy in patients with type 2 diabetes mellitus

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Abstract

BACKGROUND

Among older adults, type 2 diabetes mellitus (T2DM) is widely recognized as one of the most prevalent diseases. Diabetic nephropathy (DN) is a frequent complication of DM, mainly characterized by renal microvascular damage. Early detection, aggressive prevention, and cure of DN are key to improving prognosis. Establishing a diagnostic and predictive model for DN is crucial in auxiliary diagnosis.

AIM

To investigate the factors that impact T2DM complicated with DN and utilize this information to develop a predictive model.

METHODS

The clinical data of 210 patients diagnosed with T2DM and admitted to the First People's Hospital of Wenling between August 2019 and August 2022 were retrospectively analyzed. According to whether the patients had DN, they were divided into the DN group (complicated with DN) and the non-DN group (without DN). Multivariate logistic regression analysis was used to explore factors affecting DN in patients with T2DM. The data were randomly split into a training set (n = 147) and a test set (n = 63) in a 7:3 ratio using a random function. The training set was used to construct the nomogram, decision tree, and random forest models, and the test set was used to evaluate the prediction performance of the model by comparing the sensitivity, specificity, accuracy, recall, precision, and area under the receiver operating characteristic curve.

RESULTS

Among the 210 patients with T2DM, 74 (35.34%) had DN. The validation dataset showed that the accuracies of the nomogram, decision tree, and random forest models in predicting DN in patients with T2DM were 0.746, 0.714, and 0.730,

respectively. The sensitivities were 0.710, 0.710, and 0.806, respectively; the specificities were 0.844, 0.875, and 0.844, respectively; the area under the receiver operating characteristic curve (AUC) of the patients were 0.811, 0.735, and 0.850, respectively. The Delong test results revealed that the AUC values of the decision tree model were lower than those of the random forest and nomogram models ($P < 0.05$), whereas the difference in AUC values of the random forest and column-line graph models was not statistically significant ($P > 0.05$).

CONCLUSION
Among the three prediction models, random forest performs best and can help identify patients with T2DM at high risk of DN.

Key Words: Type 2 diabetes mellitus; Diabetic nephropathy; Random forest; Decision-making tree; Nomogram; Forecast

Core Tip: Machine learning is widely used in medical prediction models. Logistic regression (nomogram), decision tree, and random forest models are three important machine learning techniques. However, few studies have compared the predictive efficacies of these three models in patients with type 2 diabetes mellitus and diabetic nephropathy. Here, we established three risk prediction models-nomogram, decision tree, and random forest-for comparison and found that random forest has the strongest combined predictive power.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the most common diseases affecting the older population. However, its incidence among children, adolescents, and young adults is increasing because of obesity, physical inactivity, and poor dietary habits. According to the International Diabetes Federation, approximately 537 million people (20-79 years old) worldwide currently have diabetes, with more than 90% of cases being T2DM[1]. Approximately 4.2 million people died of diabetes and its complications in 2019. Diabetic nephropathy (DN) is a chronic kidney disease induced by DM and a frequent microvascular complication of DM[2]. Relevant research data show that approximately 1/100 patients with diabetes develop end-stage renal disease yearly, and approximately 3/50 patients with massive albuminuria eventually develop end-stage renal disease yearly[3]. Patients with DN have a higher risk of death than those with diabetes alone or without comorbid DN. However, recent studies have shown that the prevalence of DN in patients with T2DM ranges between 20% and 40%[4,5]. To reduce the death rate of patients, early identification, prevention, and slowing down the development of DN are important. However, random urine measurement of the urinary albumin/creatinine ratio and 24 h urinary albumin quantification have shortcomings in the diagnosis of DN. Renal biopsy is the gold standard for the diagnosis of DN, but the acceptance of the examination is often low, and the economic cost is high[6]. Therefore, constructing a diagnostic and predictive model of DN plays a significant role in auxiliary diagnosis. At present, machine learning has been widely used in medical prediction models. Logistic regression (nomogram), decision tree, and random forest models are three important techniques in machine learning, all of which can quickly mine effective information from data; However, their application effects differ for different data types[7]. Little research has compared the predictive efficacies of the three models for DN in patients with T2DM. Therefore, this study established a prediction model for DN in patients with T2DM based on a nomogram, decision tree, and random forest and compared the prediction efficacy of the three models, providing a basis for the clinical identification of high-risk populations.

MATERIALS AND METHODS

Data sources

First, this was a retrospective study. A total of 210 patients admitted to the First People's Hospital of Wenling with a clear diagnosis of T2DM between August 2019 and August 2022 were selected for this study. According to the diagnostic information, 74 of the 210 patients with T2DM complicated by DN were defined as the DN group, and the remaining 136 patients with T2DM without concurrent DN were defined as the non-DN group. The inclusion criteria were as follows: (1) Age 18 to 75 years; (2) T2DM diagnosed according to the diagnostic criteria; and (3) Complete clinical data, including demographic data and laboratory test results. The exclusion criteria were as follows: (1) Definite diagnosis of primary kidney disease or secondary kidney disease of the immune system, blood system, or drug; (2) Complication with severe

primary diseases of the digestive, respiratory, cardiovascular, hematological, and nervous systems, accompanied by more than one malignant tumor; and (3) Rapidly progressive hypertension or diseases other than cerebrovascular diseases within the last 3 mo.

Diagnostic criteria

Standards criteria for DM: Based on "Standards for the Diagnosis and Treatment of Diabetes (2023 Edition)" of ADA[8] and "Guidelines for the Diagnosis, Prevention and Treatment of Type 2 Diabetes"[9]. Diagnostic standards were developed as follows: (1) Common symptoms of diabetes such as polydipsia, polyuria, polyphagia, unexplained weight loss, and random blood sugar ≥ 11.1 mmol/L; (2) Fasting blood sugar ≥ 7.0 mmol/L; or (3) Blood glucose ≥ 11.1 mmol/L 2 h after dextrose load. Patients with no typical symptoms of diabetes need to be reexamined on another day to confirm. The fasting state refers to not eating any calories for > 8 h; random blood glucose levels were measured at any time of the day, regardless of the time of the last meal.

Diagnostic criteria for DN: The diagnostic criteria were established based on the ADA "Standards for the Diagnosis and Treatment of Diabetes (2023 Edition)", Guidelines for the Diagnosis, Prevention and Treatment of Type 2 Diabetes", and "Guidelines for primary management of diabetic kidney disease in China"[10]. That is, patients with diabetes with renal impairment and urinary microalbumin/creatinine ratio (ACR) ≥ 30 mg/g (or ≥ 3 mg/mmol) or glomerular filtration rate < 60 mL/min/1.73 m^2 for a total duration of > 3 mo can be diagnosed with DN.

Indicators of observation

General information: Age, sex, body mass index (kg/m^2), diabetes duration, and history of high blood pressure, diabetic retinopathy (DR), and coronary heart disease. Laboratory indicators (collected within 24 h after admission): Fasting blood glucose (FBG, mmol/L), serum creatinine (Scr, μmol/L), glycosylated hemoglobin (HbAlc, %, 7% = 53 mmol/mol), blood urea nitrogen (BUN, mmol/L), total cholesterol (mmol/L), triglyceride (mmol/L), high-density lipoprotein cholesterol (mmol/L), and low-density lipoprotein cholesterol (mmol/L).

Statistical analysis

Data were analyzed and processed using the SPSS software (version 23.0). Quantitative data in accordance with the normal distribution were expressed as mean ± SD, and the *t*-test was used for comparison among groups. Count data were expressed as (%), and the χ^2 test was used for comparison among groups. The DN and non-DN groups were subjected to univariate analysis, and variables with statistically significant variances were incorporated into a multifactor logistic regression analysis model to screen for highly relevant predictive variables. The prediction model was constructed using R language, and the data were randomly split into training and validation sets at a ratio of 7:3 for model construction and validation, respectively. A nomogram model was constructed using the "rms" package; in this model, multiple predictors were integrated, and line segments with a certain scale were drawn on the same plane to express the relationship between variables. A decision tree was constructed using the "rpart" package. In the construction of the decision tree, the Gini coefficient minimization criterion was adopted to select features, and a binary tree including root, internal, and leaf nodes was generated. The estimation error identification complexity parameter of cross-validation was automatically calculated, and the dataset was classified through multiple conditional discrimination processes to finally obtain the required results. A random forest was constructed using the "Random Forest" package, which is used to construct multiple decision trees. When a certain sample needs to be predicted, the predicted results of each tree in the forest are counted, and then the final result is selected from these predicted results by voting method. The sensitivity, specificity, accuracy, recall, precision, and area under the receiver operating characteristic curve (AUC) were used to compare the effectiveness of the models, and the best prediction effect of the model was selected. The DeLong test was used for AUC comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Concurrent DN condition

A total of 210 patients with T2DM were included in this study, of whom 87 were men and 123 were women. Seventy-four patients had T2DM complicated with DN, and the incidence of DN was 35.24%. There were 74 patients in the DN group, with a mean age of 56.01 ± 9.41 years. There were 136 patients in the non-DN group, with a mean age of 57.42 ± 8.15 years.

Univariate analysis of DN in patients with T2DM

The clinical data of the patients in the DN and non-DN groups were compared. The duration of diabetes, FBG, Scr, HbAlc, and BUN levels were higher in the DN group than in the non-DN group, and the proportion of patients with DR was also higher in the DN group than in the non-DN group ($P < 0.05$). More details are shown in Table 1.

Multivariate analysis of DN in patients with T2DM

Regression analysis was performed by taking the patients with T2DM complicated with DN to be the dependent variables and taking the duration of diabetes, FBG, Scr, HbAlc, BUN, and DR as the independent variables (there was no collinearity problem between the diagnosed variables), as shown in Tables 2 and 3. Multivariate analysis showed that the duration of diabetes, FBG, Scr, HbAlc, and DR were factors influencing DN in patients with T2DM ($P < 0.05$).

Table 1 Results of univariate analysis of diabetic nephropathy in patients with type 2 diabetes [*n* (%)/mean ± SD]

Indicators	DN group (*n* = 74)	Non-DN group (*n* = 136)	t/χ^2	*P* value
Age	56.01 ± 9.41	57.42 ± 8.15	-1.129	0.260
Sex			1.150	0.284
Male	27 (36.49)	60 (44.12)		
Female	47 (63.51)	76 (55.88)		
BMI (kg/m^2)	24.60 ± 3.92	23.73 ± 2.94	1.673	0.097
Duration of diabetes (yr)	5.28 ± 1.34	4.86 ± 0.76	2.507	0.014
History of hypertension			0.465	0.495
Yes	24 (32.43)	38 (27.94)		
No	50 (67.57)	98 (72.06)		
DR			8.761	0.003
Yes	34 (45.95)	24 (17.65)		
No	40 (54.05)	112 (82.35)		
Coronary heart disease			0.350	0.554
Yes	19 (25.68)	30 (22.06)		
No	55 (74.32)	106 (77.94)		
FBG (mmol/L)	8.18 ± 1.67	7.71 ± 1.14	2.160	0.033
Scr (μmol/L)	91.25 ± 14.72	84.61 ± 9.80	3.485	0.001
HbAlc (%)	7.04 ± 1.33	6.29 ± 1.05	4.239	< 0.001
BUN (mmol/L)	7.10 ± 0.96	6.78 ± 1.13	2.111	0.036
TC (mmol/L)	4.81 ± 0.89	4.83 ± 0.97	−0.189	0.851
TG (mmol/L)	1.78 ± 0.4	1.72 ± 0.38	1.090	0.277
HDL-C (mmol/L)	1.41 ± 0.31	1.45 ± 0.32	−0.972	0.332
LDL-C (mmol/L)	3.31 ± 0.57	3.33 ± 0.86	−0.185	0.853

BMI: Body mass index; FBG: Fasting blood glucose; Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; BUN: Blood urea nitrogen; DR: Diabetic retinopathy; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Nomogram model

Based on the results of the multifactor logistic regression analysis, the obtained independent predictors (FBG, Scr, HbAlc, DR, and duration of diabetes) were used to construct a nomogram model for predicting DN in patients with T2DM (Figure 1).

Decision tree model

A decision-tree prediction model of DN in patients with T2DM was constructed, and four explanatory variables were screened: FBG, HbAlc, Scr, and duration of diabetes. The results showed that the duration of diabetes was the first factor influencing DN in patients with T2DM. The model identified six judgment rules, of which three judged concurrent DN and three judged no concurrent DN. The incidence of DN in patients with T2DM with duration of diabetes ≥ 7 years was 6%. The incidence of DN in patients with T2DM with duration of diabetes < 7 years, HbAlc ≥ 7.6%, and Scr ≥ 89 μmol/L was 5%. The incidence of DN in patients with T2DM with duration of diabetes < 7 years, HbAlc < 7.6%, FBG ≥ 7.5 mmol/L, and Scr ≥ 98 umol/L was 5% (Figure 2).

Random forest model

Based on the overall change in the prediction precision of the constructed random forest model, the variables affecting DN in patients with T2DM were HbAlc, Scr, FBG, duration of diabetes, and DR (Figure 3).

Evaluation of prediction effect of three models

In the validation set, the overall evaluation of the efficacy of the nomogram in predicting concurrent DN in patients with T2DM was not significantly different from that of the random forest model. In contrast, the overall efficacy of the decision tree model was significantly lower than that of the nomogram and random forest model, with significant differences (all

Table 2 Variable assignment

Factors	Variables of interest	Assignment of value
Concurrent DN or not	Y	Yes = 1, No = 0
Duration of diabetes (yr)	× 1	Numerical value
FBG (mmol/L)	× 2	Numerical value
Scr (μmol/L)	× 3	Numerical value
HbAlc (%)	× 4	Numerical value
BUN (mmol/L)	× 5	Numerical value
DR	× 6	Yes = 1, No = 0

FBG: Fasting blood glucose; Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; BUN: Blood urea nitrogen; DR: Diabetic retinopathy.

Table 3 Results of multivariate analysis of diabetic nephropathy in patients with type 2 diabetes

Factors	*B*	*SE*	Wald	*P* value	OR	95%CI
Duration of diabetes (yr)	0.352	0.164	4.619	0.032	1.421	1.031-1.959
FBG (mmol/L)	0.272	0.128	4.518	0.034	1.312	1.021-1.685
Scr (μmol/L)	0.063	0.015	16.745	< 0.001	1.065	1.033-1.097
HbAlc (%)	0.707	0.161	19.31	< 0.001	2.029	1.480-2.781
BUN (mmol/L)	0.250	0.158	2.507	0.113	1.283	0.942-1.748
DR	0.883	0.360	6.035	0.014	2.419	1.196-4.894

FBG: Fasting blood glucose; Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; BUN: Blood urea nitrogen; DR: Diabetic retinopathy; OR: Odds ratio; CI: Confidence interval.

$P > 0.05$) (Table 4 and Figure 4).

DISCUSSION

According to the National Kidney Foundation, persistent proteinuria is the primary indicator of kidney injury. ACR > 30 μg/mg in random urine samples was defined as renal injury, of which 30-300 μg/mg was the microalbuminuria stage, suggesting that the kidney had increased capillary permeability, which was the earliest sign of renal injury in patients with diabetes. In patients with T2DM, abnormal blood glucose levels frequently exist prior to diagnosis, and patients with T2DM may already have microalbuminuria (or clinical albuminuria) at the time of initial diagnosis[11,12]. Nephropathy is one of the most frequent complications of diabetes, and its insidious characteristics provide an intervention node for studying DN. Interventions for early detection, early detection diagnosis, and treatment can prevent the occurrence of DN and reduce or delay the occurrence of end-stage renal disease[13]. Therefore, identifying and intervening in clinically modifiable factors for the occurrence and progression of DN remains the primary strategy for its prevention and cure of DN.

The incidence of DN complications in patients with T2DM in this study was 35.24%. Wagnew *et al*[14] revealed that the incidence of DN was 35.3%, which is consistent with the results of this study. However, Zhang *et al*[15] revealed that the incidence of DN was only 21.8%, which was significantly lower than that reported in the present study. The incidence of DN varies greatly among studies and may be related to factors such as population characteristics and regions. The findings of this study revealed that FBG, Scr, HbAlc, DR, and duration of diabetes were factors affecting DN in patients with T2DM. The levels of FBG, Scr, HbAlc, and duration of diabetes in the DN group were higher than those in the non-DN group, and the proportion of DR was higher than that in the non-DN group, which was similar to the results of previous studies[16-18]. This suggests that patients with DN have worse glycemic control and poorer renal function[19,20]. Moreover, compared with patients with T2DM without DR, patients with DR are more likely to develop DN.

Nagel *et al*[21] followed high-risk populations for > 20 years and found that fasting hyperglycemia was a predictor of high albumin leakage rate. Hyperglycemia causes renal damage through the activation of multiple pathways, including the formation of glycosylated complexes by the interaction of glucose with proteins outside the cell, metabolism to sorbitol through the polyol pathway, and metabolism to glucosamine through the hexosamine biosynthesis process, thereby mediating hyperglucose-induced renal damage. HbAlc can cause microvascular damage. In a tissue environment

Table 4 Efficacy of three model validation sets in predicting concurrent diabetic nephropathy in patients with type 2 diabetes

Model	Accuracy	Sensitivity	Specificity	Rate of recall	Rate of precision	AUC (95%CI)
Nomogram	0.746	0.710	0.844	0.906	0.690	0.811 (0.700-0.923)
Decision tree	0.714	0.710	0.875	0.906	0.659	0.735 (0.602-0.869)
Random forest	0.730	0.806	0.844	0.906	0.674	0.850 (0.750-0.950)

AUC: Area under the receiver operating characteristic curve; 95%CI: 95% confidence interval.

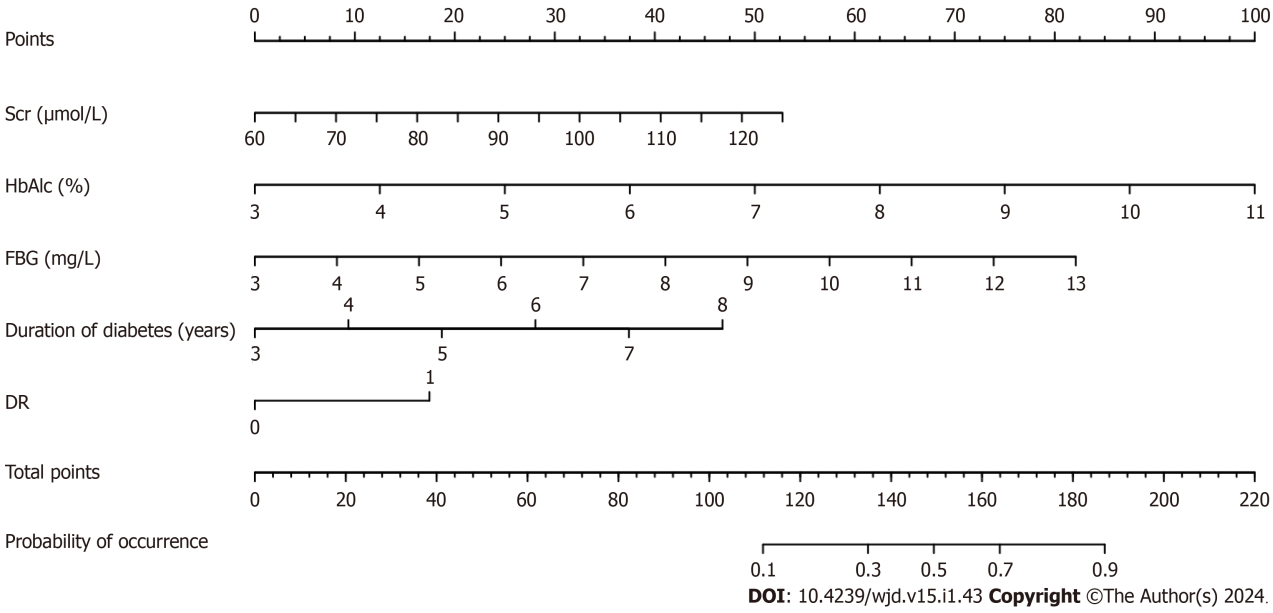

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Figure 1 Nomogram prediction model for diabetic nephropathy in patients with type 2 diabetes mellitus. Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; DR: Diabetic retinopathy; FBG: Fasting blood glucose.

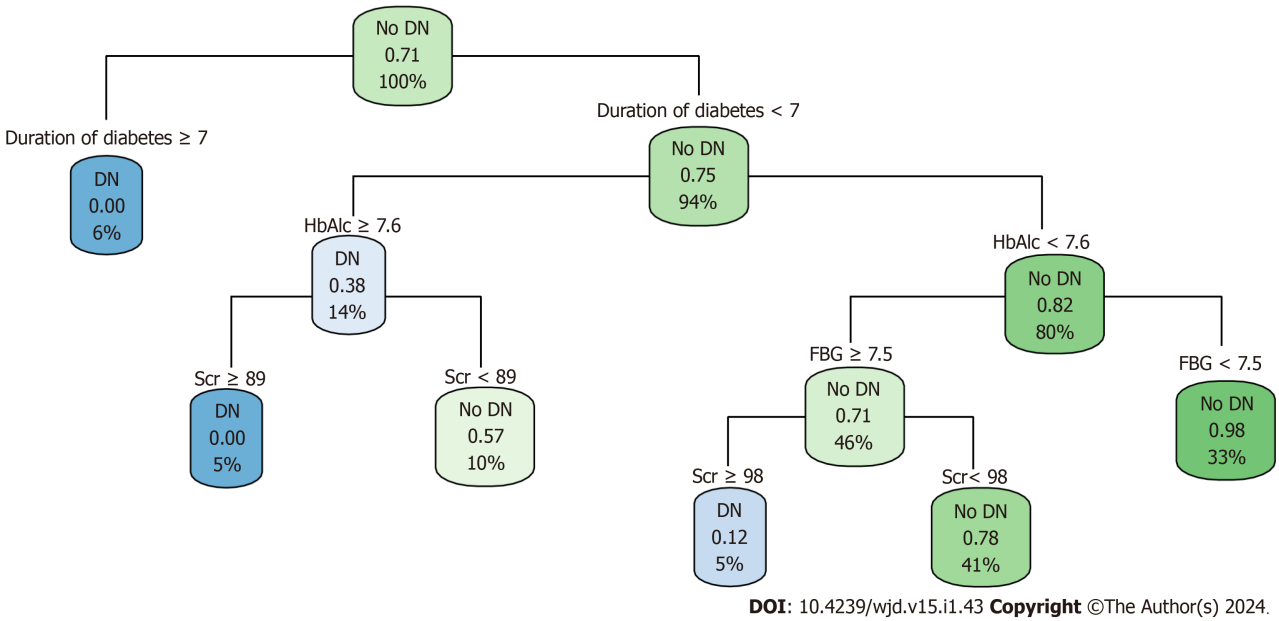

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Figure 2 Decision tree model of diabetic nephropathy in patients type 2 diabetes. Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; FBG: Fasting blood glucose.

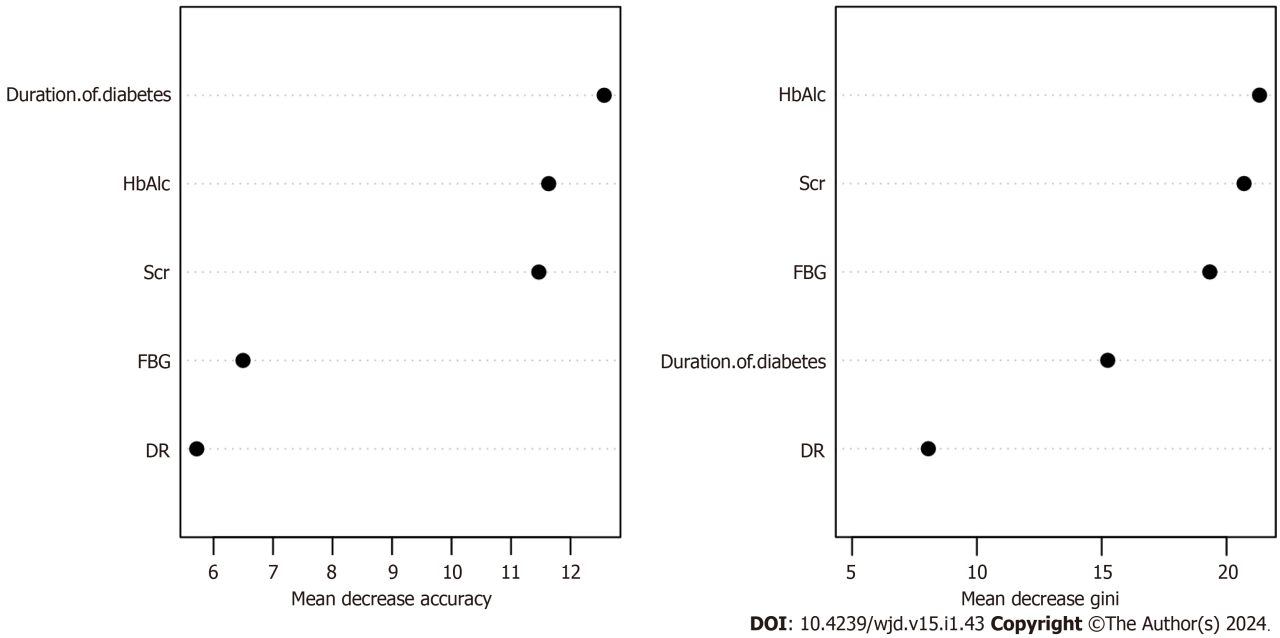

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Figure 3 Random forest model of diabetic nephropathy in patients with type 2 diabetes. Scr: Serum creatinine; HbAlc: Glycosylated hemoglobin; DR: Diabetic retinopathy; FBG: Fasting blood glucose.

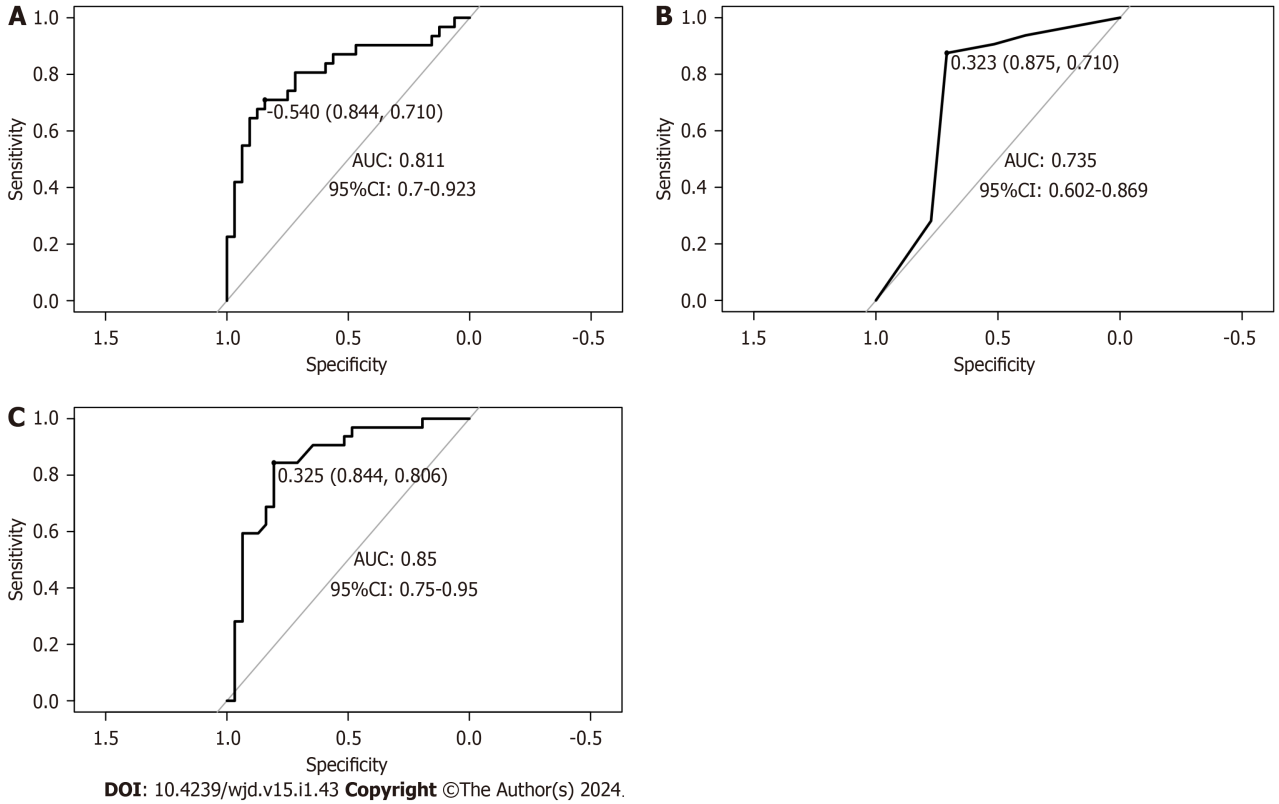

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Figure 4 Receiver operating characteristic curves for the validation sets of the three models. A: Nomogram; B: Decision-making tree; C: Random forest. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval.

with high glucose levels, the non-enzymatic catalytic process of the glycation reaction is accelerated, manifesting as a continuous increase in HbAlc. After glycation, abnormal hemoglobin levels cause chronic damage to the microcirculation vessels, damaging the basement membrane charge barrier and urine proteins. This indicates that microvascular damage to diabetic kidney tissues is a risk factor for microvascular or macrovascular lesions in patients with diabetes[22]. This study also confirmed that high blood glucose and HbAlc levels are associated with DN. Since Scr is mainly excreted in urine *via* glomerular filtration from the blood and is almost not reabsorbed by renal tubules, the output of creatinine is constant when renal function is normal or slightly damaged, and an increase in Scr indicates that renal function is

damaged[23]. Diabetes duration is the main risk factor for various complications in patients with T2DM, especially the primary risk factor for renal complications. If the duration of diabetes is > 5 years, microalbuminuria can occur. Without active intervention, it can progress to DN[24]. Studies have shown that DN and DR have the same risk factors, such as diabetes course and FBG[25]. Diabetic retinal abnormalities are associated with glomerular injury. Studies have shown that changes in retinal arteriole and venule diameter are associated with renal histological changes, such as basement membrane thickness and mesangial matrix volume increase[26]. In patients with diabetes with macroalbuminuria, retinopathy is rare; Therefore, DR combined with macroalbuminuria is more likely to be diagnosed as DN. For patients with DR, early intervention of risk factors and renal pathology examination should be performed to control the risk of DN.

Nomograms, decision trees, and random forest prediction models were established based on the above indicators. The results showed that the random forest model performed better than the nomogram and decision tree models in terms of AUC, sensitivity, and other evaluation indicators. In contrast, the overall evaluation indicators of the nomogram model were better than those of the decision tree model. The statistical difference between the AUC of the random forest and nomogram model was not significant; However, the statistical difference between the AUC of the decision tree and nomogram and the random forest model was significant. In general, the comprehensive predictive abilities of the three prediction models for concurrent DN in patients with T2DM constructed in this study were as follows: Decision tree < nomogram < random forest. This may be because the decision tree model prefers variables with higher values; however, these variables are not necessarily the best predictive variables, which reduces the predictive ability of the decision tree model. In addition, eliminating some candidate variables in the pruning process of the decision-tree model reduces its predictive ability to a certain extent[27]. A nomogram is a model that integrates multiple related factors to predict the probability of an event; it is intuitive, visual, and has good accuracy[28]. However, owing to its easy form (very much like a linear model), it is difficult to capture complex relationships and handle the problem of data imbalance and is sensitive to multicollinear data. Therefore, the prediction efficiency was slightly lower than that of the random forest model. However, random forest integrates the output of an individual decision tree to produce the final prediction result, which has the characteristics of robust operation. It is not easily affected by collinearity between variables and has a significant effect on reducing the variance of the model[29]. Tseng *et al*[30] found that the nomogram and random forest models outperformed the decision tree model in the diagnosis of postoperative cardiac patients with acute kidney injury. Hu *et al*[31] showed that the random forest model has better predictive power than the nomogram model and can effectively predict the risk of cognitive impairment in older adults. These results further demonstrate that the random forest model has a strong comprehensive prediction ability. As this was a retrospective study, all data were obtained from the same hospital, and the sample representation was limited.

CONCLUSION

This study established nomogram, random forest, and decision tree prediction models for T2DM complicated by DN using a machine learning algorithm. The result showed that the random forest model had good prediction and stability, thus providing a reference for the clinical identification of T2DM complicated by DN. Future studies need to further validate the model through prospective multi-center data and include more variables and samples to further improve the predictive ability of the model to better guide clinical practice.

ARTICLE HIGHLIGHTS

Research background

Hyperglycemia is the main pathophysiological feature of diabetes, and its complications are the key factors of death and disability in patients with diabetes. Diabetic nephropathy (DN) is a microvascular complication and is one of the main complications of diabetes. The initial prediction of DN is beneficial for taking measures to prevent and delay the occurrence and progression of corresponding complications. Machine learning has been widely used to construct predictive models for diabetic complications.

Research motivation

Patients with type 2 diabetes mellitus (T2DM) complicated by DN are at high risk of mortality. We explored the factors affecting the complications of DN to establish three prediction models commonly used in medicine, compared the prediction effects, and selected the optimal model to provide a basis for clinical identification of patients with T2DM complicated with DN.

Research objectives

This study aimed to explore the factors influencing T2DM complicated with DN and use these factors to construct a prediction model for DN. The prediction effect of random forest is the best among the three models of nomogram, decision tree, and random forest and may become a useful tool for the early recognition of the risk of DN.

Research methods

We retrospectively analyzed the clinical data of 210 patients with T2DM treated at our hospital between August 2019 and August 2022. Factors influencing DN were analyzed, and nomograms, decision trees, and random forest prediction models were established to compare their prediction efficiency. These three prediction methods are widely used in the medical field and have advantages and limitations. At the same time, through research, we can select a more suitable model to predict the complication risk of DN.

Research results

Fasting blood glucose, serum creatinine, glycosylated hemoglobin, diabetic retinopathy, and the duration of diabetes were independent factors influencing DN. Among the established nomograms, decision trees, and random forest prediction models, random forest has the best predictive ability and can be applied to the prevention and early screening of DN. Future studies should validate the model using prospective and multi-center data and include more samples and variables to further improve the prediction ability of the model. In addition, existing algorithms should be further improved, and a combination of multiple algorithms should be considered to improve the prediction accuracy.

Research conclusions

In this study, the predictive performances of three models were compared. The random forest model performed best in predicting the risk of DN in patients with T2DM and may be a useful alternative tool for diagnosing T2DM.

Research perspectives

Future studies should include larger and more comprehensive samples, conduct multi-center studies, further improve existing algorithms, and consider the combination of multiple algorithms to construct a more complete and accurate prediction model.

FOOTNOTES

Author contributions: Cai SS contributed to the conception and design of this study; Zheng TY and Wang KY participated in the administrative support; Zhu HP took part in the provision of study materials or patients; and all authors approved the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Heterogeneously elevated branched-chain/aromatic amino acids among new-onset type-2 diabetes mellitus patients are potentially skewed diabetes predictors

Min Wang, Yang Ou, Xiang-Lian Yuan, Xiu-Fang Zhu, Ben Niu, Zhuang Kang, Bing Zhang, Anwar Ahmed, Guo-Qiang Xing, Heng Su

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Abstract

BACKGROUND

The lack of specific predictors for type-2 diabetes mellitus (T2DM) severely impacts early intervention/prevention efforts. Elevated branched-chain amino acids (BCAAs: Isoleucine, leucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan, phenylalanine)) show high sensitivity and specificity in predicting diabetes in animals and predict T2DM 10-19 years before T2DM onset in clinical studies. However, improvement is needed to support its clinical utility.

AIM

To evaluate the effects of body mass index (BMI) and sex on BCAAs/AAAs in new-onset T2DM individuals with varying body weight.

METHODS
Ninety-seven new-onset T2DM patients (< 12 mo) differing in BMI [normal weight (NW), n = 33, BMI = 22.23 ± 1.60; overweight, n = 42, BMI = 25.9 ± 1.07; obesity (OB), n = 22, BMI = 31.23 ± 2.31] from the First People's Hospital of Yunnan Province, Kunming, China, were studied. One-way and 2-way ANOVAs were conducted to determine the effects of BMI and sex on BCAAs/AAAs.

RESULTS
Fasting serum AAAs, BCAAs, glutamate, and alanine were greater and high-density lipoprotein (HDL) was lower (P < 0.05, each) in OB-T2DM patients than in NW-T2DM patients, especially in male OB-T2DM patients. Arginine, histidine, leucine, methionine, and lysine were greater in male patients than in female patients. Moreover, histidine, alanine, glutamate, lysine, valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan were significantly correlated with abdominal adiposity, body weight and BMI, whereas isoleucine, leucine and phenylalanine were negatively correlated with HDL.

CONCLUSION
Heterogeneously elevated amino acids, especially BCAAs/AAAs, across new-onset T2DM patients in differing BMI categories revealed a potentially skewed prediction of T2DM development. The higher BCAA/AAA levels in obese T2DM patients would support T2DM prediction in obese individuals, whereas the lower levels of BCAAs/AAAs in NW-T2DM individuals may underestimate T2DM risk in NW individuals. This potentially skewed T2DM prediction should be considered when BCAAs/AAAs are to be used as the T2DM predictor.

Key Words: Hyperaminoacidemia; Branched-chain/aromatic amino acids; New-onset type-2 diabetes; Predictor; Obesity; Sex

Core Tip: Elevated branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) predict diabetes in animals with high sensitivity and specificity (both > 97%) and predict type-2 diabetes mellitus (T2DM) 10-20 years before T2DM onset. However, our results indicate that heterogeneously elevated BCAAs/AAAs among new-onset T2DM patients in differing BMI categories and sex may skew BCAA/AAA prediction of T2DM development among the general population: the greater BCAA/AAA elevation in obese individuals, especially males, would support T2DM prediction in these individuals, whereas the lack of or reduced BCAA/AAA elevation in NW and reproductive-aged females may compromise BCAA/AAA prediction of T2DM in these individuals. Potential nutritional, metabolic and molecular mechanisms are discussed.

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INTRODUCTION

According to the World Health Organization (WHO) (https://www.who.int/news-room/fact-sheets/detail/diabetes) and other global surveys[1], more than 465 million people worldwide will have type-2 diabetes mellitus (T2DM) in 2023, which is projected to double by 2050. T2DM was responsible for more than 4.2 million annual deaths in 2019. The global economic burden of T2DM in 2015 was 1.3 trillion United States dollars, which will double by 2030. Obesity (OB) is a major risk factor for T2DM and cardiovascular diseases (CVDs)[2]. More than 2.1 billion adults worldwide (39% of the adult population) are overweight (OW) or obese (700 million, 13% of the adult population) and are responsible for approximately 2.8 million deaths each year. China's prevalence of OW and OB in 2019 was 34.3% and 16.4% for adults (≥ 18 years), respectively[3]. The prevalence of diabetes among Chinese adults increased from 10.9% in 2013 to 12.4% in 2018 [3,4]. Moreover, 45.2% of the obese individuals had metabolically unhealthy OB (MUO) with comorbid diabetes (18.5%) or prediabetes (26.7%), whereas the other 55% of the obese individuals were metabolically healthy OW (MHO). Similarly, 32.7% of OW people have diabetes (12.8%) or prediabetes (19.9%), compared to 20.7% of normal weight (NW) people who have diabetes (7.6%) or prediabetes (13.1%)[3]. A 4-year follow-up study of 6748 nondiabetic middle-aged subjects (average 43 years old) showed that 55% of the population is metabolically healthy with a low risk of T2DM development, whereas 45% of the population is metabolically unhealthy and has a higher risk of diabetes development[5].

Early T2DM prediction could prevent or minimize the global impact of T2DM. T2DM onset can be delayed or prevented if intervened preclinically[6,7]. However, more than two-thirds of T2DM patients are not aware of having T2DM until their diagnosis. Elevated blood branched-chain amino acids (BCAAs: Leucine, isoleucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan and phenylalanine) are promising T2DM predictors, as their

elevations have successfully predicted prediabetes[7-9], homeostasis model assessment-insulin resistance[10-12], and T2DM 10-20 years ahead of their onset[13-16]. However, these findings have not been replicated in heterogeneous populations or with an established cutoff for standard diagnosis. In fact, most of the BCAAs/AAAs findings were tentative and reported as odds ratios, hazard ratios (HRs) or relative risks (RRs), or the results of comparing the highest quartile *vs* lowest quartile individuals were reported without mentioning the sensitivity, specificity, accuracy, and positive and negative predictive values of standardized diagnosis criteria.

Furthermore, most of the T2DM BCAAs/AAAs findings are based on obese individuals. Significant confounding effects of OB on BCAA/AAA elevation in T2DM were reported[17]. Greater baseline body mass index (BMI) and metabolic syndromes (MetS) often coexist in individuals who subsequently develop T2DM than in those without T2DM, suggesting a confounding effect of baseline BMI/OB and MetS on BCAA/AAA elevation, CVD and T2DM development [18-20]. In an 8.5-year follow-up study of 6134 nondiabetic individuals resulting in 306 new T2DM cases, the HR of the highest *vs* lowest quartile dropped from 12.07 to 3.20 (nearly 4-fold) after adjustment for BMI, family history of T2DM, alcohol consumption, and MetS[10]. Furthermore, T2DM prevalence/incident rates in these BCAAs/AAAs studies (3%-5%) were often much lower than the population-based epidemiological data (approximately 10% for diabetes and approximately 20% for prediabetes) (WHO: https://www.who.int/news-room/fact-sheets/detail/diabetes, CDC: https://www.cdc.gov/diabetes/prevention/about-prediabetes.html)[21].

Although the exact mechanisms remain unknown, publication bias, systematic exclusions of T2DM comorbid conditions such cardiovascular disorders, cancer or other diseases, or selective inclusion of healthier control individuals or male subjects may have caused the discrepancies. One 19-year follow-up study of 1279 nondiabetic European and 1007 nondiabetic South Asian male individuals reported a 35% prevalence of T2DM in South Asian men and a 14% prevalence of T2DM in European men without reporting women's data[14], which may compromise the results due to the sex-dependent elevation in BCAAs/AAAs[17,22,23].

BCAA/AAA elevation is further complicated by interactions of body composition, age, sex, genetics and dietary protein, fat, and energy intake. Higher dietary BCAA intake and elevated blood BCAAs are associated with increased risk of OB and IR in men but reduced risk in reproductive-aged women[24-26]. Higher animal protein but not plant protein intake is associated with higher longitudinal insulin resistance and risk of T2DM[27]. However, five years of consumption of a low-fat Mediterranean diet normalized BCAA levels and promoted T2DM remission[28].

The metabolic impact of BCAA supplementation is affected by BMI and/or adiposity status. A higher percentage intake of BCAAs in terms of total protein was associated with a significantly decreased risk of diabetes in lean/NW middle-aged Japanese men and women (BMI = 22)[29]. A higher dietary BCAA intake/ratio and elevated blood BCAAs were inversely associated with the risk/prevalence of OB in lean individuals (BMI < 24)[30,31]. Replacing animal protein with plant protein is associated with decreased T2DM risk in adult males[32]. Because a higher intake of animal protein is often associated with increased consumption of saturated fats and increased body fat/weight gain, a body fat/weight-dependent effect of BCAAs may be associated with T2DM risk/onset. In support of that, BCAA supplementation significantly increased hepatic gluconeogenesis, plasma lipid and muscular and renal lipid accumulation and reduced hepatic lipid accumulation in high-fat-diet-induced obese mice[33], whereas BCAA supplementation attenuated the severity of streptozotocin-induced diabetes in lean rats[34].

This study aimed to evaluate the effects of BMI and sex on BCAAs/AAAs in new-onset T2DM individuals in differing BMI categories.

MATERIALS AND METHODS

T2DM patients

New-onset T2DM patients were diagnosed at the Department of Endocrinology, the First People's Hospital of Yunnan Province, Kunming, China, from December 2016 to June 2018. Ninety-seven T2DM patients diagnosed with T2DM within 1 year were included in the analysis (53 male/44 female, 43.3 ± 11.2 years of age). The diagnosis and classification of T2DM were based on 1999 WHO standards[35]: (1) Fasting plasma glucose concentration ≥ 7.0 mmol (or ≥ 126 mg/dL); (2) ≥ 11.1 mmol (or ≥ 200 mg/dL) 2 h after a 75 g oral glucose load; and (3) HbA1c ≥ 6.5%. The exclusion criteria were as follows: (1) A history of diabetes that was diagnosed more than 12 mo prior; and (2) acute complications of diabetes and severe liver and/or kidney dysfunction or other serious health conditions. The study procedures were conducted in accordance with the Helsinki Declaration of 1975 and were approved by the Medical Ethics Review Committee of the First People's Hospital of Yunnan Province [No. 2016(001)].

Body weight classifications and abdominal fat area measures

Anthropometric measures of height, body weight and waist circumference (WC) were used to determine BMI and body weight status. The 2004 WHO classifications for the Asian/Chinese population were used for this study: (1) NW (BMI < 24); (2) OW (BMI ≥ 24 and < 28); and (3) OB (BMI ≥ 28), which differ from the standards of United States and European populations (NW: BMI < 25 kg/m^2, OW: BMI ≥ 25, OB: ≥ 30). Visceral adipose tissue (VAT) at the level of the umbilicus was measured *via* an abdominal dual BIA machine following the manufacturer's protocol (DUALSCAN HDS-2000, Omron Health care Co., Kyoto, Japan).

Blood sample collection and serum amino acid analysis

Fasting blood samples of the T2DM patients were collected and analyzed following the standard operation protocol. Briefly, overnight fasting (> 8 h) venous blood samples were collected and centrifuged immediately to separate the

serum.

The frozen serum samples were thawed on ice and at 4°C followed by deproteinization by the addition of acetonitrile (1:3 ratio of serum to acetonitrile). The sample was then vortexed for 2 min followed by centrifugation at 12000 rpm for 10 min at 4°C to remove any precipitate from the supernatant before ultra-performance liquid chromatography (UPLC)/triple stage quadrupole mass spectrometer (TSQ/MS) analysis. A quality control sample consisting of 6 reference standards (isoleucine, leucine, valine, tyrosine, tryptophan, phenylalanine) was prepared and run after each of the 15 serum samples.

A 5 μL aliquot of extracted serum sample was injected into the UPLC column (DionexUltiMate3000-UPLC, United States) TSQ/MS (Thermo TSQ Endura, United States). Separation was achieved on a Luna Omega Polar C18 column (2.1 mm × 100 mm, 1.6 μm, Phenomenex) held at 40°C. The samples were eluted with A (water with 0.1% formic acid) and B (acetonitrile), and the gradient program was 1% B over 0-0.5 min, 1%-20% B over 0.5-9 min, 20%-75% B over 9-11 min, and 75%-99% B over 11-16 min. The composition was held at 99% B for 0.5 min and finally returned to 1% B at 20 min. The flow rate was 0.3 mL/min. Mass spectrometry was performed in positive ion electrospray (ESI +) mode. The temperature for the ion transfer tube and vaporizer was set at 350°C and 300°C, respectively. The pressures for the sheath gas, aux gas and sweep gas were set at 40, 15 and 1 Arb, respectively. The positive ion voltage was set to 3.5 kV. All the compounds were detected in selective reaction monitoring mode.

Statistical analysis

The data are presented as the mean ± standard deviation or median [25% quartile range (QR), 75% QR]. The effects of BMI and sex were analyzed using ANOVA for quantitative variables with a normal distribution or using the Wilcoxon rank sum test for nonnormally distributed parameters (SPSS 24, IBM). Post hoc multiple comparisons were assessed using Bonferroni correction. Pearson's correlation coefficients were calculated among the variables. A 2-sided P≤0.05 was considered indicative of statistical significance.

RESULTS

Demographic and anthropometric characteristics

Ninety-seven (97) T2DM patients, including 57 males (59%) and 40 females (41%), all within 1 year of T2DM diagnosis, were identified and included in this study. Of them, 33 (34%) were NW-T2DM (BMI = 22.23 ± 1.60), 42 (43.2%) were OW-T2DM (BMI = 25.90 ± 1.08), and 22 (22.8%) were OB-T2DM (BMI = 30.96 ± 2.59), based on the WHO body weight classification (Table 1).

No group differences were found in T2DM diagnosis, sex distribution, height, medication, or systolic and diastolic blood pressures (Table 1). The OB-T2DM group was significantly younger (36.77 ± 10.32 years) than the NW-T2DM and OW-T2DM groups (43.64 ± 6.95 and 42.21 ± 9.76 years, respectively, $P < 0.05$, each). The OB-T2DM group also showed greater values than the NW-T2DM and OW-T2DM groups in body weight, WC, WC-to-height ratio (WHR), and BMI ($P < 0.01$, each, Table 1).

The male and female patients showed similarities in T2DM onset age (30.4 ± 77.0 *vs* 43.3 ± 91.7 d), BMI (25.9 ± 4.2 *vs* 25.8 ± 2.9), WHR (0.535 ± 0.05 *vs* 0.555 ± 0.05), systolic blood pressure (125.46 ± 16.4 *vs* 125.88 ± 16.1 mmHg), diastolic blood pressure (84.0 ± 14.3 *vs* 81.2 ± 7.3 mmHg), and antidiabetic medication. The males were, however, significantly younger (39.7 ± 8.8 *vs* 44.0 ± 9.6 year), heavier (76.7 ± 13.7 *vs* 64.9 ± 8.2 kg), and taller (171.6 ± 6.3 *vs* 159.2 ± 4.9 cm) with greater WC (91.7 ± 8.7 *vs* 88.3 ± 7.7 cm, $P < 0.05$, all) than the females.

Effects of BMI and sex on the serum amino acid profile

Two-way ANOVA of the effects of BMI and sex showed that among the 23 serum amino acids measured by using UPLC/TSQ-MS, 8 amino acids were significantly greater in obese than in NW and/or OW subjects (alanine, asparagine, carnosine, glutamate, hydroxyproline, proline, tyrosine, and tryptophan, $P < 0.05$, each) (Figure 1, Table 2). Valine, isoleucine, and phenylalanine were marginally greater in obese than in NW and/or OW subjects ($P \leq 0.1$, each). Ten amino acids were significantly greater in male subjects than in female subjects (serine, asparagine, glutamate, lysine, hydroxyproline, citrulline, isoleucine, leucine, tyrosine, and tryptophan, $P < 0.05$, each). Histidine, methionine, phenylalanine, and threonine were marginally greater in male subjects than in female subjects ($P < 0.1$, each). Significant BMI and sex interactions were found for proline only.

Further 1-way ANOVA of BMI effects largely confirmed the 2-way ANOVA results. Within-sex 1-way ANOVA showed that most of the BMI differences were present among the male subjects only (alanine, asparagine, glutamate, valine, isoleucine, leucine, tyrosine, and tryptophan) ($P \leq 0.05$, each) (Figure 1, Table 2), except that carnosine was significantly greater in the female obese group and proline was significantly lower in the female OW group than in the other two groups. Covariance analysis showed no difference in serum amino acids after controlling for day of T2DM diagnosis or controlling for metformin equivalent dose. of T2DM medicine. However, covariance analysis showed significant effects of BMI on taurine, citrulline and proline after controlling for age ($P \leq 0.05$, each, Table 2).

Effects of BMI and sex on lipid profile

Two-way ANOVA showed no significant effects of BMI and sex on triglycerides, total cholesterol, and low-density lipoprotein. However, high-density lipoprotein (HDL) was significantly affected by BMI, with significantly lower HDL levels found in obese T2DM patients than in their NW and OW counterparts ($P < 0.01$, $P < 0.05$, respectively), especially

Table 1 Characteristics of 97 new-onset type 2 diabetes patients of normal weight, overweight and obesity

	NW (33)	OW (42)	OB (22)	*F* value	*P* value
Sex: M/F [*n* (%)]	20/13 (33)	24/18 (42)	13/9 (22)	0.095	0.954
Age (yr, mean ± SD)	43.64 ± 6.95	42.21 ± 9.76	36.77 ± 10.32[a]	4.054	0.020[a]
T2DM onset (d)	46.1 ± 96.8	41.8 ± 90.3	8.5 ± 20.2	1.569	0.214
Medication (Y/N)	13/20	13/29	6/16	1.016	0.602
Metformin equiv dose (g, mean ± SD)	176 ± 221	214 ± 225	546 ± 34	1.384	0.256
Weight (kg)	60.53 ± 6.87	73.49 ± 9.71[b,c]	85.64 ± 11.35[b,c]	49.593	0.000[b]
Height (cm)	165.3 ± 8.26	168.5 ± 9.05	164.4 ± 6.91	1.407	0.250
BMI index	22.23 ± 1.60	25.88 ± 1.07[b,c]	31.23 ± 2.31[b,c]	208.086	0.000[b]
Waist circumference (cm)	84.85 ± 6.21	89.76 ± 6.07[b,c]	99.61 ± 7.05[b,c]	35.920	0.000[b]
Waist: Height ratio	0.514 ± 0.042	0.535 ± 0.034[a]	0.604 ± 0.041[b,c]	37.279	0.000[b]
Systolic blood pressure (mmHg)	122.1 ± 17.6	125.6 ± 13.2	130.7 ± 18.2	12.68	0.07
Diastolic blood pressure (mmHg)	81.9 ± 12.8	83.8 ± 8.7	82.5 ± 15.7	0.509	0.602

[a]$P < 0.05$ compared with normal weight.
[b]$P < 0.01$, compared with normal weight.
[c]$P < 0.01$, compared with overweight.
Data are numbers (*n*) of individuals (%) unless otherwise indicated. NW: Normal weight (body mass index: 18.5-23.9); OB: Obesity (body mass index: ≥ 28.0); OW: overweight (body mass index: 24.0-27.9); BMI: Body mass index; M: Male; F: Female.

among male patients (Table 2).

Associations of serum amino acids with VAT, body weight, BMI, and lipid profile

Although multiple amino acids were significantly correlated with VAT, body weight and BMI and negatively correlated with HDL, in pooled samples (Table 3), the correlations with VAT were the strongest (tyrosine, $r = 0.524$, $P < 0.0001$; phenylalanine, $r = 0.508$, $P < 0.0001$; tryptophan $r = 0.373$, $P < 0.01$; isoleucine, $r = 0.443$, $P = 0.002$; leucine, $r = 0.396$, $P = 0.006$, valine, $r = 0.375$, $P < 0.01$; methionine, $r = 0.379$, $P < 0.01$; alanine, $r = 0.429$, $P = 0.003$; glutamate, $r = 0.398$, $P < 0.01$; lysine, $r = 0.39$, $P < 0.01$; hydroxyproline, $r = 0293$; $P < 0.05$, threonine, $r = 0.388$, $P < 0.01$; proline, $r = 0.314$, $P < 0.05$; and histidine, $r = 0.278$, $P < 0.06$), followed by body weight, BMI and HDL (Table 3). Taurine was negatively correlated with VAT ($r = -0.367$, $P = 0.01$), and HDL was negatively correlated with isoleucine ($r = -0.309$, $P < 0.01$), leucine ($r = -0.276$, $P < 0.01$) and phenylalanine ($r = -0.307$ $P < 0.01$). Within-BMI category correlation analysis showed no significant correlations in the NW group (Table 3). For the OW group, phenylalanine was highly correlated with VAT ($r = 0.607$, $P = 0.002$), and BCAAs and AAAs were significantly or marginally correlated with body weight (tyrosine, $r = 0.343$, $P < 0.05$; tryptophan $r = 0.339$, $P < 0.05$; isoleucine, $r = 0.289$, $P = 0.063$; leucine, $r = 0.32$, $P = 0.039$, valine, $r = 0.286$, $P = 0.067$, respectively), and HDL was negatively correlated with isoleucine ($r = -0.417$, $P < 0.01$), leucine ($r = -0.345$, $P < 0.05$) and phenylalanine ($r = -0.323$, $P < 0.05$) (Table 3). For the obese group, alanine was significantly correlated with VAT ($r = 0.64$, $P < 0.05$); homocysteine was significantly correlated with body weight ($r = 0.475$, $P < 0.05$) and BMI ($r = 0.43$, $P < 0.05$)); HDL was negatively correlated with asparagine ($r = -0.478$, $P < 0.01$) and hydroxyproline ($r = -0.436$, $P < 0.05$); and BCAAs and AAAs were negatively correlated with waist-to-height ratio (isoleucine, $r = -0.54$, $P < 0.01$; leucine, $r = -0.51$, $P < 0.05$; and tryptophan, $r = -0.512$, $P < 0.05$). These observations support VAT as the major source of elevated BCAAs/AAAs in obese T2DM patients[36].

DISCUSSION

The global T2DM epidemic could be better managed or even prevented if potential T2DM candidates were identified and treated at the preclinical stage. BCAAs and AAAs are promising T2DM predictors because of their high diagnostic sensitivity and specificity (both > 97%) in predicting and discriminating diabetic rats from nondiabetic rats[37]. Such diagnostic sensitivity and accuracy, however, have not been demonstrated in T2DM prediction, possibly due to the confounding effects of unknown factors. Both OB and sex are implicated, although the exact mechanism remains unknown.

In this study, fasting serum levels of alanine, asparagine, carnosine, glutamate, hydroxyproline, proline, tyrosine, and tryptophan were significantly greater, whereas HDL was significantly lower in obese T2DM patients than in normal T2DM patients. Valine, isoleucine, leucine, and phenylalanine trended toward higher levels in obese than in NW-T2DM patients. The amino acid levels of the OW-T2DM patients were intermediate between those of the OB and NW groups. Ten amino acids (serine, asparagine, glutamate, lysine, hydroxyproline, citrulline, isoleucine, leucine, tyrosine, and

Table 2 One-way/two-way ANOVA of serum amino acids in newly onset type-2 diabetes mellitus patients of normal weight, overweight and obesity (mean ± SD)

		NW	OW	OB	Obesity, F, P (1-way ANOVA)	Obesity, F, P (2-way ANOVA)	Sex, *F* value, *P* value	Obesity × sex interaction, *F* value, *P* value	Age-adjusted *F* value, *P* value	Onset-adjusted, *F* value, *P* value	Metf. dose-adjusted, *F* value, *P* value
HbA1c (%)	Pooled	10.96 ± 2.73	9.91 ± 2.69	9.09 ± 2.40[a]	3.471, 0.035[a]	2.888, 0.061[d]	1.603, 0.209	0.376, 0.688	NS	NS	NS
	Male	11.51 ± 2.93	10.07 ± 2.78	9.25 ± 2.16	3.012, 0.058[d]						
	Female	10.12 ± 2.24	9.70 ± 2.64	8.85 ± 2.82	0.661, 0.522						
TC (mmol/L)	Pooled	4.82 ± 1.36	4.68 ± 1.65	4.55 ± 1.10	0.229, 0.796	0.239, 0.788	1.020, 0.315	0.035, 0.965	NS	NS	NS
	Male	4.91 ± 1.24	4.80 ± 1.96	4.73 ± 1.14	0.053, 0.948						
	Female	4.68 ± 1.58	4.52 ± 1.15	4.29 ± 1.04	0.243, 0.786						
TG (mmol/L)	Pooled	3.12 ± 6.77	2.73 ± 3.18	4.66 ± 4.20	1.161, 0.318	0.992, 0.375	1.758, 0.188	0.253, 0.777	NS	NS	NS
	Male	3.85 ± 8.61	2.91 ± 3.94	5.45 ± 4.99	0.711, 0.496						
	Female	2.00 ± 1.64	2.49 ± 1.80	3.53 ± 2.54	1.683, 0.200						
HDL (mmol/L)	Pooled	1.07 ± 0.27	1.03 ± 0.27	0.86 ± 0.22	4.744, 0.011[a]	4.478, 0.014[a]	0.998, 0.320	0.337, 0.715	NS	NS	NS
	Male	1.07 ± 0.26	0.98 ± 0.23	0.84 ± 0.24	3.602, 0.034[a]						
	Female	1.08 ± 0.31	1.09 ± 0.32	0.88 ± 0.19	1.635, 0.209						
LDL (mmol/L)	Pooled	2.82 ± 1.10	2.81 ± 0.87	2.34 ± 1.02	1.922, 0.152	1.670, 0.194	0.009, 0.925	0.121, 0.887	NS	NS	NS
	Male	2.83 ± 0.96	2.87 ± 0.84	2.29 ± 0.99	1.870, 0.164						
Histidine (μg/L)	Pooled	188.42 ± 40.62	197.28 ± 57.63	219.62 ± 53.34	2.488, 0.089	2.010, 0.140	3.614, 0.060[d]	0.651, 0.524	NS	NS	NS
	Male	190.30 ± 45.33	205.95 ± 62.41	234.87 ± 49.68	2.680, 0.078[d]						
	Female	185.53 ± 33.68	185.72 ± 49.92	197.59 ± 53.32	0.234, 0.792						
Arginine (μg/L)	Pooled	458.53 ± 180.93	480.08 ± 198.77	507.06 ± 127.36	0.487, 0.616	0.423, 0.656	0.219, 0.641	0.044, 0.957	NS	NS	NS
	Male	459.85 ± 163.56	492.30 ± 231.02	516.31 ± 131.56	0.369, 0.693						
	Female	456.51 ± 211.98	463.80 ± 150.31	493.70 ± 127.59	0.140, 0.870						
β-Alanine (μg/L)	Pooled	436.45 ± 188.57	445.91, 188.70	394.88, 202.89	0.530, 0.591	0.402, 0.670	0.569, 0.452	0.942, 0.394	NS	NS	NS
	Male	454.86 ± 200.68	425.40 ± 177.60	357.16 ± 178.59	1.100, 0.340						
	Female	408.11 ± 172.11	473.25 ± 204.50	449.37 ± 233.59	0.395, 0.676						
Carnosine (μg/L)	Pooled	5.99 ± 1.37	5.98 ± 2.06	6.95 ± 1.50	2.671, 0.074	3.122, 0.049	0.013, 0.908	1.193, 0.308	NS	NS	NS

	Male	6.27 ± 1.41	5.93 ± 2.36	6.65 ± 1.34	0.632, 0.535						
	Female	5.55 ± 1.22	6.04 ± 1.63	7.39 ± 1.69[a]	4.035, 0.026[a]						
Serine (μg/L)	Pooled	268.73 ± 91.46	239.17 ± 83.33	283.56 ± 79.43	2.258, 0.110	2.182, 0.119	7.451, 0.008[b]	0.567, 0.569	NS	NS	NS
	Male	297.99 ± 77.49	256.57 ± 87.30	295.74 ± 64.80	1.817, 0.172						
	Female	223.72 ± 95.80	215.97 ± 73.74	265.98 ± 98.35	1.038, 0.364						
Taurine (μg/L)	Pooled	71.97 ± 18.22	78.96 ± 30.38	75.98 ± 22.23	0.721, 0.489	1.054, 0.353	1.714, 0.194	0.685, 0.507	9.335, 0.003[b]	NS	NS
	Male	72.61 ± 17.85	74.52 ± 30.24	76.10 ± 21.65	0.083, 0.921						
	Female	71.00 ± 19.47	84.88 ± 30.39	75.81 ± 24.38	1.131, 0.334						
Alanine (μg/L)	Pooled	130.77 ± 49.32	139.06 ± 40.66	168.94 ± 56.03[a]	4.531, 0.013[a]	3.967, 0.022[a]	0.007, 0.934	0.812, 0.447	NS	NS	NS
	Male	123.80 ± 46.98	143.52 ± 40.18	170.88 ± 56.72[a]	4.013, 0.024[a]						
	Female	141.51 ± 52.77	133.10 ± 41.67	166.14 ± 58.32	1.357, 0.270						
Asparagine (μg/L)	Pooled	36.73 ± 13.82	30.17 ± 16.07	40.40 ± 13.67[c]	3.900, 0.024[a]	3.817, 0.026[a]	7.684, 0.007[b]	0.586, 0.559	NS	NS	NS
	Male	38.32 ± 15.37	34.05 ± 18.30	45.52 ± 11.83	2.161, 0.125						
	Female	34.28 ± 11.16	25.01 ± 10.95	33.01 ± 13.30	2.865, 0.070[d]						
Glutamine (μg/L)	Pooled	464.62 ± 156.05	450.21 ± 188.81	489.96 ± 120.18	0.422, 0.657	0.530, 0.590	0.652, 0.421	0.857, 0.428	NS	NS	NS
	Male	459.31 ± 156.09	486.60 ± 219.88	495.68 ± 135.66	0.192, 0.826						
	Female	472.80 ± 162.00	401.70 ± 127.29	481.70 ± 100.88	1.534, 0.229						
Glutamate (μg/L)	Pooled	2812.36 ± 1104.11	2768.39 ± 1153.78	3588.65 ± 1138.22[a,e]	4.259, 0.017[a]	4.028, 0.021[a]	5.188, 0.025[a]	0.176, 0.839	NS	NS	NS
	Male	2949.39 ± 1011.65	3028.47 ± 1234.29	3866.48 ± 1088.44	3.039, 0.056[d]						
	Female	2601.55 ± 1245.38	2421.62 ± 962.57	3187.33 ± 1147.67	1.470, 0.243						
Lysine (μg/L)	Pooled	341.06 ± 128.62	365.48 ± 152.71	423.72 ± 142.42	2.262, 0.110	2.087, 0.130	6.688, 0.011[a]	0.376, 0.687	NS	NS	NS
	Male	357.60 ± 124.07	406.28 ± 172.85	462.00 ± 157.70	1.831, 0.170						
	Female	315.62 ± 136.32	311.08 ± 101.65	368.42 ± 100.58	0.833, 0.443						
Hydroxyproline (μg/L)	Pooled	21.09 ± 11.77	14.33 ± 11.68[a]	21.41 ± 12.09	4.050, 0.021[a]	3.901, 0.024[a]	9.074, 0.003[b]	0.614, 0.543	NS	NS	NS
	Male	22.84 ± 12.69	17.00 ± 12.59	26.07 ± 12.48	2.472, 0.094						
	Female	18.39 ± 10.06	10.78 ± 9.55	14.67 ± 7.98	2.490, 0.097						
Threonine (μg/L)	Pooled	201.17 ± 88.38	208.93 ± 86.51	236.53 ± 82.51	1.173, 0.314	0.950, 0.390	3.035, 0.085[d]	0.293, 0.746	NS	NS	NS

	Male	209.66 ± 92.11	217.95 ± 89.86	258.54 ± 87.84	1.259, 0.292						
	Female	188.12 ± 84.22	196.90 ± 82.80	204.73 ± 66.12	0.118, 0.889						
Citrulline (μg/L)	Pooled	95.53 ± 32.87	90.68 ± 34.25	94.71 ± 27.50	0.237, 0.790	0.527, 0.592	6.414, 0.013[a]	0.001, 0.999	6.232, 0.014[a]	NS	NS
	Male	101.68 ± 34.73	96.78 ± 38.17	98.59 ± 30.95	0.105, 0.901						
	Female	86.06 ± 28.48	82.54 ± 27.12	89.11 ± 22.12	0.195, 0.824						
Proline (μg/L)	Pooled	2313.70 ± 644.63	2244.77 ± 716.36	2561.43 ± 555.47	1.707, 0.187	3.138, 0.048[a]	0.941, 0.335	3.279, 0.042[a]	6.754, 0.011[a]	NS	NS
	Male	2176.16 ± 572.03	2404.58 ± 839.03	2604.08 ± 534.61	1.560, 0.220						
	Female	2525.30 ± 714.13	2031.68 ± 448.42[a]	2499.81 ± 611.56	3.418, 0.043[a]						
Homocysteine (μg/L)	Pooled	2.50 ± 1.71	2.68 ± 1.64	2.27 ± 1.33	0.481, 0.620	0.800, 0.453	0.194, 0.661	1.812, 0.169	NS	NS	NS
	Male	2.26 ± 1.66	2.39 ± 1.43	2.61 ± 1.52	0.205, 0.815						
	Female	2.88 ± 1.78	3.06 ± 1.85	1.77 ± 0.82	1.916, 0.161						
Valine (μg/L)	Pooled	3839.99 ± 883.13	3718.96 ± 851.75	4259.02 ± 820.20	2.938, 0.058[d]	2.627, 0.078[d]	1.547, 0.217	1.385, 0.256	NS	NS	NS
	Male	3763.81 ± 926.69	3876.21 ± 837.88	4465.80 ± 583.75	3.144, 0.051[d]						
	Female	3957.18 ± 833.97	3509.28 ± 847.57	3960.35 ± 1041.61	1.264, 0.294						
Methionine (μg/L)	Pooled	2.44 ± 1.86	2.48 ± 1.97	3.21 ± 2.24	1.201, 0.306	0.754, 0.473	3.091, 0.082[d]	2.568, 0.082[d]	NS	NS	NS
	Male	2.23 ± 1.92	2.84 ± 2.11	3.99 ± 2.50	2.658, 0.079[d]						
	Female	2.76 ± 1.78	2.01 ± 1.70	2.09 ± 1.15	0.879, 0.424						
Isoleucine (μg/L)	Pooled	306.68 ± 75.83	326.23 ± 81.56	357.51 ± 75.72	2.778, 0.067	2.325, 0.104	6.977, 0.010[b]	0.786, 0.459	NS	NS	NS
	Male	313.67 ± 73.98	343.49 ± 87.07	386.75 ± 75.14[a]	3.283, 0.045[a]						
	Female	295.93 ± 80.37	303.21 ± 69.32	315.27 ± 56.43	0.200, 0.820						
Leucine(μg/L)	Pooled	492.26 ± 136.62	518.94 ± 147.01	577.40 ± 153.91	2.301, 0.106	1.909, 0.154	8.751, 0.004[b]	0.856, 0.428	NS	NS	NS
	Male	511.46 ± 137.96	548.74 ± 163.13	638.14 ± 137.20	2.896, 0.064[d]						
	Female	462.73 ± 134.45	479.21 ± 114.87	489.66 ± 138.87	0.129, 0.879						
Tyrosine (μg/L)	Pooled	155.13 ± 57.40	166.00 ± 63.14	203.68 ± 74.79[a]	3.998, 0.022[a]	3.370, 0.039[a]	5.255, 0.024[a]	1.448, 0.240	NS	NS	NS
	Male	154.79 ± 57.19	182.95 ± 70.70	225.47 ± 58.95[b]	4.857, 0.011[a]						
	Female	155.65 ± 60.07	143.39 ± 43.65	172.21 ± 87.10	0.685, 0.511						
Phenylalanine (μg/L)	Pooled	664.47 ± 163.17	699.89 ± 172.50	785.74 ± 197.37[a]	3.223, 0.044[a]	2.909, 0.060[d]	2.806, 0.097[d]	0.384, 0.682	NS	NS	NS
	Male	688.44 ±	709.87 ±	828.45 ±	2.534,						

		154.62	191.09	208.14	0.089[d]						
	Female	627.58 ± 175.24	686.60 ± 148.44	724.03 ± 173.28	1.002, 0.377						
Tryptophan(μg/L)	Pooled	658.00 ± 150.01	694.36 ± 188.43	790.15 ± 162.27[a]	4.082, 0.020[a]	4.277, 0.017[a]	13.121, 0.000[b]	0.051, 0.951	NS	NS	NS
	Male	702.38 ± 151.16	746.40 ± 193.72	847.94 ± 127.90	3.062, 0.055[d]						
	Female	589.72 ± 124.48	624.97 ± 161.06	706.66 ± 176.95	1.567, 0.222						

[a]$P < 0.05$ *vs* normal weight (or male *vs* female).
[b]$P < 0.01$, *vs* normal weight (or male *vs* female).
[c]$P < 0.1$ *vs* overweight.
[d]$P < 0.1$ *vs* normal weight.
[e]$P < 0.01$, *vs* overweight.
NS: Not significant, NW: Normal weight; OB: Obesity; OW: Overweight; HbA1c: Hemoglobin A1c; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

tryptophan) were significantly greater in male patients than in female patients. Histidine, methionine, phenylalanine, and threonine also trended toward being greater in male patients. Serum AAAs, BCAAs and several other amino acids were significantly correlated with abdominal adiposity and less so with body weight or BMI, whereas BCAAs/AAAs and other amino acids were negatively correlated with HDL.

Our results are in agreement with previous reports that BMI-/abdominal adiposity-dependent BCAA/AAA elevations are associated with BMI, insulin resistance and T2DM development[38-40]. A 15-year metabolomics follow-up study of 11896 non-T2DM Finnish individuals [baseline age 24-45 years, 392 incident T2DM cases identified (3.2% T2DM of the study population *vs* 8% T2DM of the general Finnish population)] showed that BCAAs/AAAs are the strongest predictor of diabetes along with triacylglycerol, linoleic n-6 fatty acid and HDL-cholesterol[41]. Simultaneous hyperaminoacidemia and dyslipidemia precede prediabetes and T2DM onset in MUO individuals[42], whereas amino acid and lipid homeostasis in MHO individuals is intermediate between lean health and MUO individuals[43,44]. A meta-analysis shows significant confounding effects of OB and metabolic health on T2DM development in that MUO individuals pose 10 times higher risk, metabolically unhealthy OW (MUOW) pose 7 times higher risk, metabolically unhealthy NW (MUNW) pose 4 times higher risk, MHO group pose 3 times higher risk ratio and MHO individuals (MHOW) pose 2 times higher risk ratio for T2DM development than metabolically healthy NW (MHNW) individuals, respectively[45]. However, MUO poses a 3.5 times higher risk, MUOW poses a 4 times higher risk and MUNW poses a 4 times higher risk for T2DM development than its metabolically healthy counterparts of the same BMI categories, *i.e.*, than MHO, MHOW, and MHNW, respectively[45]. Another 3-year follow-up study of 9623 non-T2DM Chinese adults showed decreased diabetes RR in the MHOW phenotype (0.65), no change in the MHO phenotype (0.99), and increased RR in the MUNW (1.81), MUOW (2.02) and MUO (2.48) phenotypes compared to MHNW[46].

The mechanism by which BCAAs/AAAs trigger T2DM development remains largely unknown and complex. Abnormal BCAA catabolism in muscle or loss of skeletal muscle mass may play a key role in the pathogenesis of elevated BCAAs in MetS, IR, liver cirrhosis, and T2DM, as skeletal muscle is the major site of BCAA catabolism due to the high activity of BCAA aminotransferase, which is absent in the liver[47-49]. Low skeletal muscle mass is associated with insulin resistance, diabetes, and MetS[50]. Furthermore, recent studies show that altered body composition, such as increased body fat percentage, abdominal fat mass and reduced lean muscle mass rather than BMI/body weight per se, could determine metabolic phenotypes in both obese and lean/NW children[51-54], adolescents and adults (including pre- and postmenopausal women)[55-59].

Both early-onset T2DM and typical T2DM show impaired expression of genes involved in branched-chain amino acid metabolism in muscle[60] but high circulating BCAAs and leucine treatment enhanced myotube lipid accumulation and oxidative stress in myotubes[61]. Increased BCAA catabolic flux may promote gluconeogenesis and glucose intolerance *via* glutamate transamination to alanine or trigger T2DM incidence by overstimulation of beta cell secretion and subsequent impairment of glucose-stimulated insulin secretion. Others show that 3-hydroxyisobutyrate, a catabolic intermediate of valine secreted from muscle cells, stimulates muscle fatty acid uptake and promotes lipid accumulation in muscle, causing insulin resistance in mice and in T2DM patients[62].

A direct association between tissue-specific alteration of BCAA-catabolizing enzymes and OB-related elevation in plasma BCAAs/branched-chain alpha-keto acids (BCKAs) has been demonstrated in rodent models of OB (OB/OB mice and Zucker rats) and in obese human subjects who underwent surgical weight loss intervention[63]. Plasma concentrations of BCAAs were significantly higher (56%-84%) in randomly fed obese mice and rats (OB/OB mice and Zucker rats) than in lean controls, and BCAA elevation diminished after overnight fasting due to reduced BCAA elevation (by 30%) in obese mice[63]. Therefore, fasting plasma BCAA levels did not differ between lean and obese animals. BCAA metabolism was altered in liver and adipose tissue but not in muscle in fed obese mice, which contributed to elevated plasma BCAA levels. In comparison with lean controls, obese rodents (OB/OB mice and Zucker rats) show decreased expression and activity of BCATm and BCKD E1α in liver and epididymal fat along with increased decreasing branched-chain α-keto acid dehydrogenase (BCKD) kinase (BCKDK) expression, whereas no such changes were found in skeletal

Table 3 Correlation coefficients between serum amino acids and adiposity, body weight and body mass index in 97 type 2 diabetes patients differing in body mass index categories

Amino acids	Pooled (*n* = 97)				Obese (*n* = 22)				Overweight (*n* = 42)				Normal weight (*n* = 33)			
	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL	VAT	Body weight	BMI	HDL
Valine	0.375[b]	0.236[a]	0.212[a]	NS	0.311	0.217	0.009	NS	0.140	0.286[c]	0.223	NS	0.150	0.008	0.167	NS
Isoleucine	0.443[b]	0.287[b]	0.255[a]	-0.309[b]	0.109	0.106	-0.095	NS	0.347[c]	0.289[c]	0.301[d]	-0.417[b]	0.351	0.047	0.114	NS
Leucine	0.396[b]	0.315[b]	0.249[a]	-.276[b]	0.120	0.201	-0.068	NS	0.312	0.320[a]	0.340[a]	-0.345[a]	0.133	0.159	0.173	NS
Tyrosine	0.524[b]	0.341[b]	0.301[b]	NS	0.447	0.136	0.002	NS	0.251	0.343[a]	0.094	NS	-0.036	0.154	0.347[a]	NS
Phenylalanine	0.508[b]	0.264[b]	0.262[a]	-0.307[b]	0.177	0.176	0.027	NS	0.607[b]	0.096	0.171	-0.323[a]	0.185	0.156	0.085	NS
Tryptophan	0.373[a]	0.362[b]	0.236[a]	NS	0.382	0.076	-0.230	NS	0.107	0.339[a]	0.114	NS	-0.042	0.280	-0.012	NS
Methionine	0.379[b]	0.251[a]	0.228[a]	NS	0.229	0.223	0.105	NS	0.377[c]	0.327[a]	0.357[a]	NS	-0.263	0.050	0.296[c]	NS
Taurine	-0.367[a]	-0.001	0.047	NS	-0.479[c]	-0.043	-0.164	NS	-0.451[a]	-0.153	0.142	NS	-0.345	0.083	-0.082	NS
Alanine	0.429[b]	0.194[d]	0.268[b]	NS	0.640[a]	0.178	0.078	NS	-0.027	-0.041	-0.095	NS	0.304	-0.171	0.043	NS
Asparagine	0.278[d]	0.218[a]	0.079	-0.222[a]	0.200	0.333	0.049	-0.478[a]	0.129	0.287[c]	0.008	NS	-0.192	0.206	-0.052	NS
Glutamine	0.253[c]	0.157	0.052	NS	0.422	0.075	0.042	NS	-0.021	0.282[c]	0.043	NS	0.000	0.070	-0.072	NS
Glutamate	0.398[b]	0.263[b]	0.234[a]	NS	0.147	0.172	0.133	NS	0.291	0.181	0.021	NS	-0.024	0.113	-0.053	NS
Lysine	0.390[b]	0.272[b]	0.203[a]	NS	0.372	0.124	0.053	NS	0.237	0.277[c]	0.090	NS	-0.246	0.059	-0.054	NS
Hydroxyproline	0.293[a]	0.025	-0.008	NS	-0.428	0.080	0.015	-0.436[a]	0.276	0.172	0.056	NS	0.192	-0.105	-0.171	NS
Threonine	0.388[b]	0.237[a]	0.190[c]	NS	0.573[a]	0.322	0.233	NS	0.108	0.190	0.177	NS	0.012	0.088	0.005	NS
Citrulline	0.118	0.035	-0.046	NS	-0.146	-0.114	-0.043	NS	0.068	0.231	0.015	NS	-0.120	-0.052	-0.233	NS
Proline	0.314[a]	0.099	0.104	NS	0.295	-0.015	-0.162	NS	0.059	0.205	-0.061	NS	0.018	-0.310[c]	0.047	NS
Homocysteine	-0.132	0.007	-0.011	NS	0.232	0.475[a]	0.431	NS	-0.201	-0.109	-0.061	NS	0.251	-0.015	-0.020	NS
Histidine	0.278[d]	0.331[b]	0.250[a]	NS	0.316	0.358	0.123	NS	0.085	0.275[c]	0.231	NS	-0.274	0.100	0.012	NS
Arginine	0.167	0.042	0.042	NS	0.097	-0.189	-0.226	NS	0.122	0.078	-0.100	NS	-0.215	-0.182	-0.088	NS
β-Alanine	-0.134	-0.073	-0.108	NS	0.241	-0.108	0.069	NS	-0.360[c]	-0.175	-0.329[a]	NS	0.193	0.306[c]	-0.037	NS
Carnosine	0.278[d]	0.162	0.157	NS	0.113	-0.121	-0.143	NS	0.056	0.062	0.054	NS	-0.649[a]	0.200	-0.156	NS
Serine	0.149	0.151	0.039	-0.240[a]	-0.157	-0.031	-0.154	NS	0.121	0.159	-0.121	NS	-0.220	0.465[b]	0.111	NS

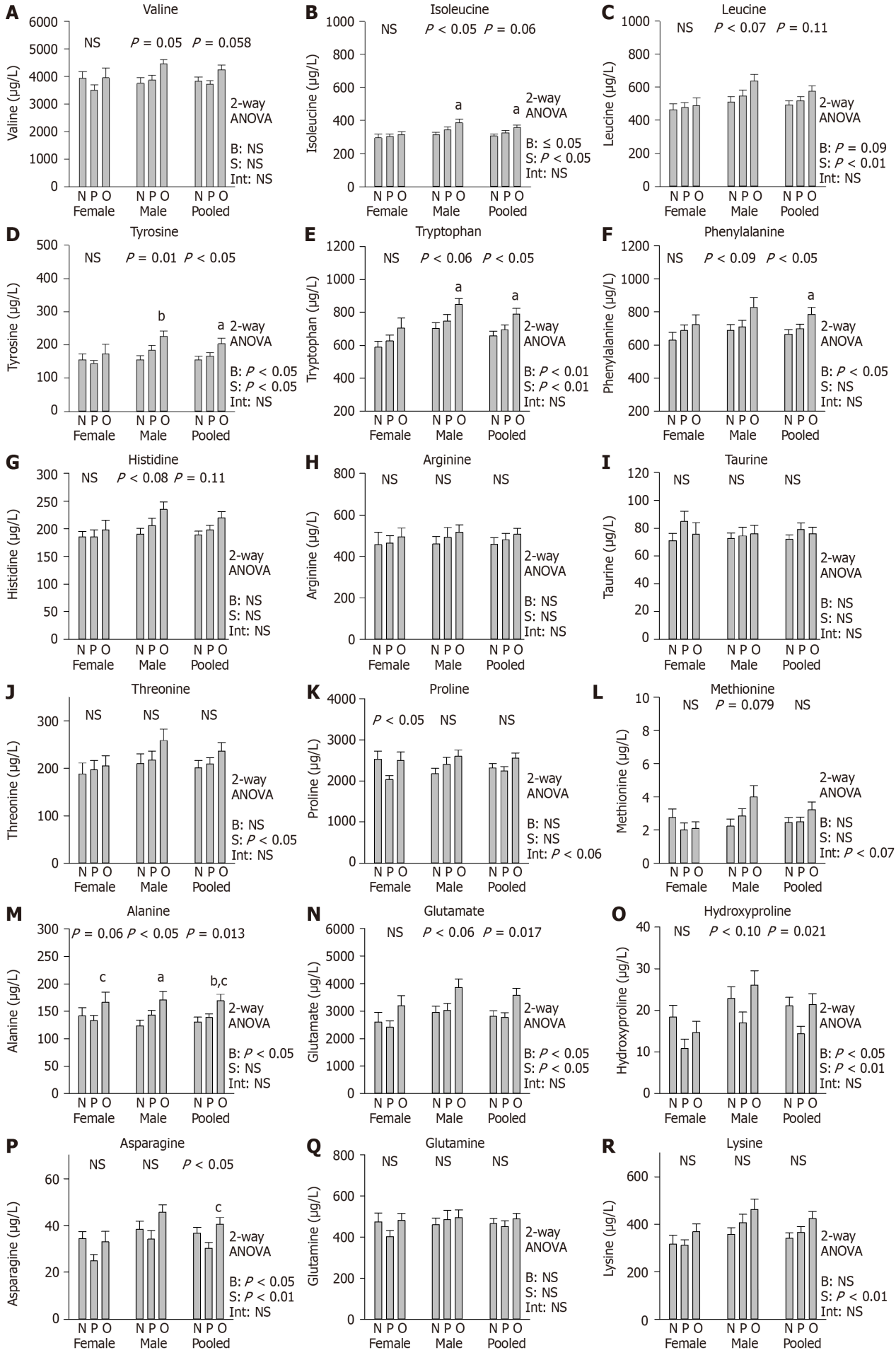
[a]$P < 0.05$.

[b]$P < 0.01$.
[c]$P < 0.1$.
[d]$P < 0.06$.
BMI: Body mass index; HDL: High density lipoprotein; VAT: Visceral adipose tissue; NS: Not significant.

muscle[63]. Because the postprandial elevation in BCAAs was more sensitive to fasting-induced BCAA reduction in obese rodents than in lean controls, this dynamic change in serum BCAAs suggests that postprandial samples rather than fasting samples could be better for analysis of potential T2DM predictors. The greater alteration in postprandial BCAAs/BCKAs in OB also indicates deficient BCAA/BCKA metabolic or disposal capabilities in the obese population. Furthermore, BCKAs are a more sensitive metabolic marker than BCAAs for OB[64]. Compared to wild-type animals, young male obese Zucker rats (with mutated leptin) show decreased BCKD activity in the kidney, heart, gastrocnemius and liver (-66% to -47%), increased plasma BCAAs (45%-69%) and BCKAs (100%) and hepatic BCKAs (193%-418%), leucine oxidation (23%), proteolysis (35%), urinary marker of proteolysis (183%-766%), increased dietary intake (23%), whole body protein synthesis (23%-29%), body weight (53%), liver weight (107%) and adiposity (300%)[64].

Among our findings, the male subjects exhibited higher levels of serum amino acids (including BCAAs and AAAs) and greater differences in amino acids between the obese and NW T2DM subjects than the female subjects. These sex differences are consistent with previous reports[65,66]. Although the underlying mechanism is unknown, animal studies have shown that the female steroid hormone 17β-estradiol stimulates BCAA catabolism by increasing the activity of the BCKD and by BCKDK, which is an inhibitor of BCKD activity[67]. As most of the female subjects in our study were of reproductive age, a potential confounding effect of estrogen may account for the sex difference in serum BCAA. Nevertheless, the moderate BMI-dependent BCAA elevation in the female subjects in our study may become significant if a large sample size or more postmenopausal women were included. Indeed, one cohort study of 2204 (most postmenopausal) women, including 115 T2DM patients, 192 individuals with impaired fasting glucose (IFG) and 1897 control individuals, who differed significantly in BMI (30.6 *vs* 27.9, *vs* 25.4, respectively) and age (63 *vs* 60 *vs* 50 years, respectively), showed significant BCAA elevation in subjects with T2DM and IFG compared with normal control individuals[68]. Moreover, among the elevated BCAA/BCKA metabolites, 3-methyl-2-oxovalerate was the strongest predictive biomarker for IFG with moderate heritability (h^2 = 0.20), based on the single-nucleotide polymorphism rs1440581 of the gene encoding for protein phosphatase (PP2Cm), which is needed for maintaining the activity of BCKD, the rate-limiting enzyme for BCKA catabolism[68]. However, that study may be confounded by a potentially higher estrogen level in the younger peri-postmenopausal control women (< 50 years) than in the postmenopausal women of the T2DM and IFG groups (both ≥ 60 years). Another study showed that higher diet and plasma BCAA concentrations were associated with increased T2DM risk among women with gestational diabetes, independent of BMI and other risk factors [69].

In the present study, BCAAs and AAAs were more closely correlated with abdominal adiposity than with body weight or BMI, indicating an important role of adiposity in hyperaminoacidemia/BCAA/AAA elevation. Other studies showed that the association of dietary BCAAs with T2DM risk/remission was dependent on the type of dietary fat, baseline triglycerides and body weight, as a Mediterranean diet rich in extravirgin olive oil (Med-diet) significantly reduced blood BCAAs and attenuated the association between plasma BCAA levels and T2DM incidence after a 3.8-year follow-up of 945 people compared to a control low-fat diet (LF)[70], and the Med-diet was associated with T2DM remission and BCAA reduction measured after an oral glucose tolerance test[28]. In addition, baseline plasma BCAAs indicated whether the LF or Med diet was capable of inducing T2DM remission. In diet-induced obese mice, high dietary fat increased the circulating BCAA pool, BCAA catabolism and OB by altering the gut microbiota[71,72]. BCAA supplementation to a


A
Valine
Valine (μg/L)
NS P = 0.05 P = 0.058
2-way ANOVA
B: NS
S: NS
Int: NS
B
Isoleucine
Isoleucine (μg/L)
NS P < 0.05 P = 0.06
a
a
2-way ANOVA
B: ≤ 0.05
S: P < 0.05
Int: NS
C
Leucine
Leucine (μg/L)
NS P < 0.07 P = 0.11
2-way ANOVA
B: P = 0.09
S: P < 0.01
Int: NS
D
Tyrosine
Tyrosine (μg/L)
NS P = 0.01 P < 0.05
b
a
2-way ANOVA
B: P < 0.05
S: P < 0.05
Int: NS
E
Tryptophan
Tryptophan (μg/L)
NS P < 0.06 P < 0.05
a
a
2-way ANOVA
B: P < 0.01
S: P < 0.01
Int: NS
F
Phenylalanine
Phenylalanine (μg/L)
NS P < 0.09 P < 0.05
a
2-way ANOVA
B: P < 0.05
S: NS
Int: NS
G
Histidine
Histidine (μg/L)
NS P < 0.08 P = 0.11
2-way ANOVA
B: NS
S: NS
Int: NS
H
Arginine
Arginine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
I
Taurine
Taurine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
J
Threonine
Threonine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: P < 0.05
Int: NS
K
Proline
Proline (μg/L)
P < 0.05 NS NS
2-way ANOVA
B: NS
S: NS
Int: P < 0.06
L
Methionine
Methionine (μg/L)
NS P = 0.079 NS
2-way ANOVA
B: NS
S: NS
Int: P < 0.07
M
Alanine
Alanine (μg/L)
P = 0.06 P < 0.05 P = 0.013
c
a
b,c
2-way ANOVA
B: P < 0.05
S: NS
Int: NS
N
Glutamate
Glutamate (μg/L)
NS P < 0.06 P = 0.017
2-way ANOVA
B: P < 0.05
S: P < 0.05
Int: NS
O
Hydroxyproline
Hydroxyproline (μg/L)
NS P < 0.10 P = 0.021
2-way ANOVA
B: P < 0.05
S: P < 0.01
Int: NS
P
Asparagine
Asparagine (μg/L)
NS NS P < 0.05
c
2-way ANOVA
B: P < 0.05
S: P < 0.01
Int: NS
Q
Glutamine
Glutamine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: NS
Int: NS
R
Lysine
Lysine (μg/L)
NS NS NS
2-way ANOVA
B: NS
S: P < 0.01
Int: NS
N P O
Female
Male
Pooled

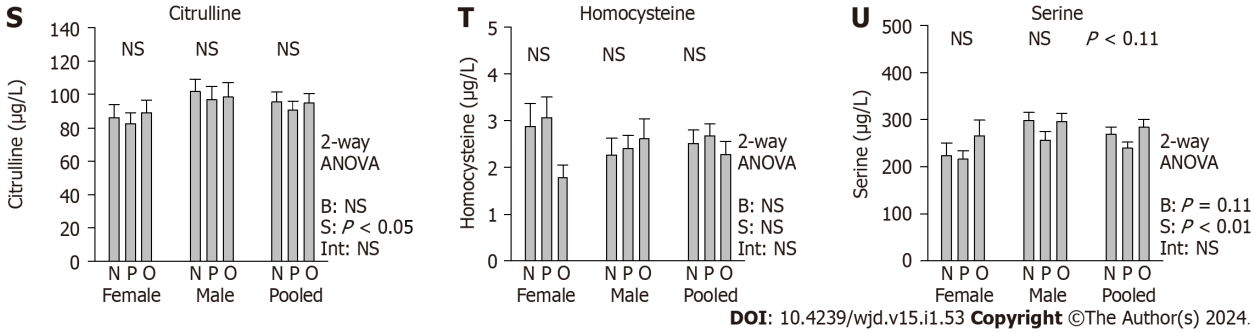


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Figure 1 One-way and two-way ANOVA of the effects of body mass index and sex on serum amino acid concentrations in male and female new-onset type-2 diabetes patients in differing body mass index categories. A: Valine; B: Isoleucine; C: Leucine; D: Tyrosine; E: Tryptophan; F: Phenylalanine; G: Histidine; H: Arginine; I: Taurine; J: Threonine; K: Proline; L: Methionine; M: Alanine; N: Glutamate; O: Hydroxyproline; P: Asparagine; Q: Glutamine; R: Lysine; S: Citrulline; T: Homocysteine; U: Serine. N: Normal weight; P: Overweight; O: Obese group. [a]$P < 0.05$ *vs* normal weight; [b]$P < 0.01$ *vs* normal weight; [c]$P < 0.05$ *vs* overweight; NS: Not significantly different ($P > 0.05$); B: Body mass index; S: Sex/sex; Int: BMI × sex interaction.

high-fat diet increased mTOR activity, whereas BCAA supplementation to a normal diet did not affect mTOR activity in animals[73].

Limitations and prospective use of BCAAs/AAAs in predicting different subtypes of T2DM

While elevated BCAAs/BCKAs may induce and interact with FFA accumulation in obese T2DM candidates, T2DM development in lean/NW populations may arise from different mechanisms. Unlike the gold standard for predicting different subtypes of T2DM diagnosis (*i.e.*, fasting glucose and HbA1c), so far, there is no established standard for T2DM prediction. Although BCAAs/AAAs is a promising predictor, its utility may be affected by a range of factors including race/ethnicity, age, sex, body weight/BMI, and subtypes of T2DM. The heterogeneous elevation of BCAAs/AAAs among new-onset T2DM patients indicates limitations and restricted utility of BCAAs/AAAs as the predictor for different subtypes of T2DM. While the greater BCAAs/AAAs elevation in obese T2DM patients would support its' prediction in individuals with OB(-propensity) which account for a large portion of T2DM population, the lower level, or a lack of BCAAs/AAAs elevation in normal-weight and reproductive-aged females would diminish its' predicting power in these individuals.

A longitudinal 12-year follow up study of old adults (56 ± 8 years) showed that when BMI-matched obese non-T2DM individuals (n = 189/group, BMI = 30) were compared, the highest quartile of individual with elevated baseline BCAAs/AAAs had a 2- to 3.5-fold higher odds of risk of developing diabetes per SD increment over a 12-year follow-up period based on individual BCAAs/AAAs, or a 5- to 7-fold higher odds of developing diabetes if all BCAAs/AAAs were combined, in comparison with those individuals whose plasma amino acid levels were in the lowest quartile[16]. However, such increments in odds of risk were reduced to 1.3 and 2.0, respectively if the obese T2D candidates were compared with NW controls (n = 400, BMI = 25) randomly selected form a larger pool. Thus, how the controls are selected can lead to very different outcomes.

Similarly, a meta-analysis show that MUO, MUOW and MUNW individuals would show similar 4-fold risk increase of developing T2DM if each of them is compared with healthy counterparts of their corresponding BMI category (*vs* MHO, MHOW, and MHNW, respectively), but MUO and MUOW would have 2-3-fold higher risks than the MU-NW group if each of them is compared with MH-NW[45]. Thus, BCAAs/AAAs elevation would be greater in obese T2DM candidates than in NW T2DM candidates if all were compared with NW controls, but similar BCAAs/AAAs elevations across different BMI categories if obese, OW and NW T2DM candidates were compared with same BMI categories controls, respectively.

T2DM is a highly heterogeneous disease that include latent autoimmune diabetes in adults (LADA, defined by the presence of glutamic acid decarboxylase antibodies (GADA), maturity onset diabetes of the young (MODY, defined by gene mutations that disrupt insulin production) and neonatal diabetes, in addition to insulin resistant and BMI-related subgroups.

A recent data-driven cluster analysis of 14755 European T2DM patients using six variables (GADA, age at diagnosis, BMI, HbA1c, β-cell function and insulin resistance) resulted in 5 well-separated novel subgroups of adult-onset diabetes with distinct outcomes: A cluster of more severe insulin resistant individuals associated with higher risk of diabetic kidney disease; insulin deficiency cluster associated with highest risk of retinopathy; relatively young insulin deficient individuals with poor glycemic control (high HbA1c) and; a larger group of elderly patients with benign disease course [74]. That finding has been confirmed and extended by another cluster analysis of 2316 Chinese T2D patients and 685 United States T2D patients using five variables (age at diagnosis, BMI, HbA1c/glucose, β-cell function and insulin resistance) that resulted in 4 clusters: Half of the patients were elders with milder metabolic derangements; 25% of the patients had the highest BMI values but average blood glucose, β-cell function and insulin resistance; 14% of the patients had severe insulin deficiency and highest blood glucose; 8% of the patients were elders with severe insulin resistance and β-cell dysfunction[75]. Similar results of cluster analysis were reported in 55777 individuals with prediabetes[76]. However, none of these studies included BCAAs/AAAs as the study variable. Thus, the contributions of BCAAs/AAAs to different clusters or subtypes of T2DM remain unknown.

Nevertheless, BCAAs/AAAs elevation, if standardized based on different age and BMI sub-groups, could be useful in screening future prediabetes and OB-related diabetes in infants and adolescents as blood BCAAs/AAAs were found significantly correlated with BMI standard deviation score, fasting glucose, HbA1c, triglycerides, cystatin C and creatinine in 2191 healthy participants aged 3 months to 18 years[77]. A 7.5-year longitudinal study of 396 nondiabetic Finnish girls showed that serum BCAA profile in childhood (11.2 ± 0.8 years at baseline) were associated with insulin resistance during pubertal development (significant both before and after menarche) independent of adiposity, and it predicted dysregulated glycemic and triglyceride levels in adulthood[78-80]. Blood BCAA/AAA were also found significantly elevated in OW and obese prepubertal children than in healthy controls[39-41,81].

The global prevalence of prediabetes in children and adults have reached alarming levels[81], with an annualized diabetes conversion rate of 5%–10%[82]. And 500 million adults in China (50% of China's adult population) and 98 million adult Americans (38.0% of the United States adult population) have prediabetes (https://www.cdc.gov/diabetes/data/statistics-report/index.html#anchor_23827), the world T2DM population could double by 2050. In the United States, the total cost of diagnosed diabetes in 2022 was $413 billion, including $106 billion in indirect costs (https://www.cdc.gov/diabetes/health-equity/diabetes-by-the-numbers.html). Because preventive lifestyle modification can reduce the risk of diabetes by up to 70%[83], identification of "at-risk" individuals 10-20 years prior to T2DM onset based on BCAAs/AAAs elevation would offer plenty time for lifestyle modifications.

While BCAAs/AAAs elevation alone may not predict all subtypes of T2DM, its combined use with other anthropogenic, metabolic, and genetic biomarkers such as visceral adiposity index, muscle mass index[47-50], fasting glucose, GADA (associated with LADA), genetic polymorphisms (associated with MODY), and metabolic parameters associated with insulin deficiency, diabetic kidney disease and retinopathy should be evaluated in future studies.

Study limitations

This study has limitations. The lack of BMI-matched healthy control individuals makes it impossible to evaluate a potential low grade hyperaminoacidemia/BCAA/AAA elevation that may exist in NW-T2DM patients. Only fasting samples, not postprandial/oral glucose tolerance test samples or BCKAs, were studied, which may have missed the dynamic changes in BCAA catabolism. The moderate sample size of this cross-sectional study could not discern the causality of the findings. The lack of lifestyle data limits exploration of the influences of diet and social, psychological, and physical activities on BCAA/AAA. Anti-diabetic medication taken by some T2DM patients may have compromised the results[84,85]. In addition, no genetic, race/ ethnicity influences can be derived from this study, although sex differences and significant correlations between BCAAs/AAAs and anthropometric parameters were demonstrated.

Future studies should overcome the above limitations of this study. To date, most of the published BCAAs/AAAs findings were based on comparisons between obese T2DM patients and nonobese individuals, whereas the BCAAs/AAAs data of the lean/NW-T2DM groups were largely unreported. This publication bias should be addressed by stratified analyses for age, BMI, adiposity, sex, and genotype as discussed above and by comparisons between metabolically healthy *vs* metabolically unhealthy individuals of different BMI categories.

CONCLUSION

In summary, heterogeneous elevation of BCAAs/AAAs is found in new-onset T2DM patients. While the greater BCAA/AAA elevation in obese and male T2DM patients would support BCAA/AAA prediction of T2DM development in these individuals, the lower or lack of elevated BCAAs/AAAs in NW and reproductive-aged female T2DM patients could compromise its prediction in these people. This potentially skewed T2DM prediction should be considered when BCAAs/AAAs are to be used as the T2DM predictor. As BCAAs/AAAs can predict T2DM as early as from childhood to early adulthood and 1-2 decades prior to T2DM onset[86-89], and because BCAA/BCKA elevation can be effectively normalized through diet, exercise, weight control interventions and pharmacogenetic therapy[84-86], study and normalization of BCAA/AAA elevation could provide a novel opportunity for curbing global T2DM epidemic.

ARTICLE HIGHLIGHTS

Research background

Type-2 diabetes mellitus (T2DM) is a major cause of comorbidity and mortality in society and was responsible for more than 4.2 million annual deaths in 2019 alone. The current world population of T2DM (approximately 450 million) is expected to double to 1 billion soon after 2050. This T2DM pandemic, however, can be curbed or prevented if the population at risk of T2DM can be identified and prophylactic actions be taken long before the onset of T2DM.

Research motivation

Research over the past decades has indicated that elevated branched-chain amino acids (BCAAs: Isoleucine, leucine, valine) and aromatic amino acids (AAAs: Tyrosine, tryptophan, phenylalanine) show high sensitivity and specificity (both > 97%) in predicting diabetes in animals and can successfully predict T2DM nearly 20 years before T2DM onset in select human populations. However, these findings have not been widely translated into clinical utilization due to unidentified factors.

Research objectives

We hypothesized that body weight and sex are potential confounding factors that could affect BCAAs/AAAs as general T2DM predictors. As the first step, the aim of our study was to determine the effects of body weight and sex on BCAAs/AAAs in new-onset T2DM individuals.

Research methods

Fasting blood samples were collected from 97 new-onset T2DM patients (53 male/44 female, 43.3 ± 11.2 years of age, differing in body mass index (BMI): Normal weight (NW), n = 33, BMI = 22.23 ± 1.60; overweight, n = 42, BMI = 25.9 ± 1.07; and obesity, n = 22, BMI = 31.23 ± 2.31). All T2DM cases were diagnosed within 12 mo at the First People's Hospital of Yunnan Province, Kunming, China. Serum amino acids were analyzed using ultra-performance liquid chromatography/triple stage quadrupole mass spectrometry.

Research results

Fasting serum AAAs, BCAAs, glutamate, and alanine levels were significantly greater and high-density lipoprotein levels were significantly lower in obese T2DM patients than in NW-T2DM patients, especially among male patients. Arginine, histidine, leucine, methionine, and lysine were greater in male patients than in female patients. Moreover, histidine, alanine, glutamate, lysine, valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, and tryptophan were significantly correlated with abdominal adiposity, body weight and BMI, respectively.

Research conclusions

Heterogeneously elevated amino acids, especially BCAAs/AAAs, are found in new-onset T2DM patients in differing BMI categories, which may indicate a potentially skewed prediction of T2DM development by BCAA/AAAs, *i.e.*, more accurate and reliable prediction in obese and male individuals than in NW individuals and females. This skewness may limit the universal application of this T2DM predictor.

Research perspectives

This study has limitations. The lack of BMI-matched healthy control individuals makes it impossible to determine whether a potential low grade hyperaminoacidemia/BCAA/AAA elevation exists in normal-weight T2DM patients. The moderate sample size and lack of lifestyle and genetic data of this cross-sectional study could not discern the causality and/or mechanisms of the heterogeneity. Further studies should include both metabolically healthy and metabolically unhealthy individuals in differing BMI categories to overcome the abovementioned limitations.

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FOOTNOTES

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Author contributions: Wang M, Xing GQ and Su H conceptualized and designed the research; Ou Y, Niu B and Kang Z screened patients and acquired clinical data; Wang M, Yuan XL, Zhang B and Zhu XF collected blood specimen and performed laboratory analysis; Wang M, Ahmed A, Yuan XL and Xing GQ performed Data analysis; Wang M, Su H and Xing GQ wrote the paper. All the authors have read and approved the final manuscript. Wang M proposed, designed and conducted serum amino acids analysis, performed data analysis and prepared the first draft of the manuscript. Ou Y was responsible for patient screening, enrollment, collection of clinical data and blood specimens. Both authors have made crucial and indispensable contributions towards the completion of the project and thus qualified as the co-first authors of the paper. Both Su H and Xing GQ have played important and indispensable roles in the experimental design, data interpretation and manuscript preparation as the co-corresponding authors. Su H applied for and obtained the funds for this research project. Su H conceptualized, designed, and supervised the whole process of the project. He searched the literature, revised and submitted the early version of the manuscript with the focus on the association between visceral adipose tissue (VAT) and BCAA/AAA. Xing GQ was instrumental and responsible for data re-analysis and re-interpretation, figure plotting, comprehensive literature search, preparation and submission of the current version of the manuscript with a new focus on BCAAs/AAAs as the predictors of diabetes and on potential underlying mechanisms. This collaboration between Su H and Xing GQ is crucial for the publication of this manuscript and other manuscripts still in preparation.

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ORIGINAL ARTICLE

Retrospective Study

Investigating the relationship between intracranial atherosclerotic plaque remodelling and diabetes using high-resolution vessel wall imaging

Yong-Qian Mo, Hai-Yu Luo, Han-Wen Zhang, Yu-Feng Liu, Kan Deng, Xiao-Lei Liu, Biao Huang, Fan Lin

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Abstract

BACKGROUND

Intracranial atherosclerosis, a leading cause of stroke, involves arterial plaque formation. This study explores the link between plaque remodelling patterns and diabetes using high-resolution vessel wall imaging (HR-VWI).

AIM

To investigate the factors of intracranial atherosclerotic remodelling patterns and the relationship between intracranial atherosclerotic remodelling and diabetes mellitus using HR-VWI.

METHODS

Ninety-four patients diagnosed with middle cerebral artery or basilar artery

atherosclerosis were enrolled. Their basic clinical data were collected, and HR-VWI was performed. The vascular area at the plaque (VA_{MLN}) and normal reference vessel ($VA_{reference}$) were delineated and measured using image postprocessing software, and the Remodelling index (RI) was calculated. According to the value of the RI, the patients were divided into a positive remodelling (PR) group, intermediate remodelling (IR) group, negative remodelling (NR) group, PR group and non-PR (N-PR) group.

RESULTS

The PR group exhibited a higher prevalence of diabetes and serum cholesterol levels than the IR and NR groups [45.2%, 4.54 (4.16, 5.93) *vs* 25%, 4.80 ± 1.22 and 16.4%, 4.14 (3.53, 4.75), respectively, $P < 0.05$]. The diabetes incidence was also significantly greater in the PR group than in the N-PR group (45.2% *vs* 17.5%, $P < 0.05$). Furthermore, the PR group displayed elevated serum triglyceride and cholesterol levels compared to the N-PR group [1.64 (1.23, 2.33) and 4.54 (4.16, 5.93) *vs* 4.54 (4.16, 5.93) and 4.24 (3.53, 4.89), $P < 0.05$]. Logistic regression analysis revealed diabetes mellitus as an independent influencing factor in plaque-PR [odds ratio (95% confidence interval): 3.718 (1.207-11.454), $P < 0.05$].

CONCLUSION

HR-VWI can clearly show the morphology and signal characteristics of intracranial vascular walls and plaques. Intracranial atherosclerotic plaques in diabetic patients are more likely to show PR, suggesting poor plaque stability and a greater risk of stroke.

Key Words: High-resolution vessel wall imaging; Intracranial atherosclerosis; Vascular remodelling; Magnetic resonance imaging

Core Tip: High-resolution vessel wall imaging provides clear visualization of intracranial vascular walls and plaques. Diabetic patients with intracranial atherosclerotic plaques are more likely to display positive remodelling, indicating unstable plaques and a heightened risk of stroke. These findings contribute to the basis for preventing ischaemic stroke.

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INTRODUCTION

Intracranial atherosclerotic disease is one of the main causes of ischaemic stroke in the world, accounting for approximately 10% of transient ischaemic attacks and 30%-50% of ischaemic strokes[1]. It is the most common factor among Asian people[2]. The adaptive changes in the structure and function of blood vessels that can adapt to changes in the internal and external environment are called vascular remodelling, which is a common and important pathological mechanism in atherosclerotic diseases, and the remodelling mode of atherosclerotic plaques is closely related to the occurrence of stroke. Positive remodelling (PR) is an outwards compensatory remodelling where the arterial wall grows outwards in an attempt to maintain a constant lumen diameter. For a long time, it was believed that the degree of stenosis can accurately reflect the risk of ischaemic stroke[3-5]. Previous studies have revealed that lesions without significant luminal stenosis can also lead to acute events[6,7], as summarized in a recent meta-analysis study in which approximately 50% of acute/subacute ischaemic events were due to this type of lesion[6]. Research[8,9] has pointed out that the PR of plaques is more dangerous and more likely to cause acute ischaemic stroke.

Previous studies[10-13] have found that there are specific vascular remodelling phenomena in the coronary and carotid arteries of diabetic patients. However, due to the deep location and small lumen of intracranial arteries and limitations of imaging techniques, the relationship between intracranial arterial remodelling and diabetes is still unclear. In recent years, with the development of magnetic resonance technology and the emergence of high-resolution (HR) vascular wall imaging, a clear and multidimensional display of the intracranial vascular wall has been achieved.

Therefore, in this study, HR wall imaging (HR-VWI) was used to display the remodelling characteristics of bilateral middle cerebral arteries and basilar arteries and to explore the factors of intracranial vascular remodelling and its relationship with diabetes.

MATERIALS AND METHODS

Subjects

This is a retrospective study. Patients were recruited from Shenzhen Second People's Hospital from December 2019 to March 2022. All patients met the following inclusion and exclusion criteria.

Inclusion criteria: The patient had a preliminary clinical diagnosis of ischaemic stroke or transient ischaemic attack in the corresponding blood supply areas of the bilateral middle cerebral arteries or basilar arteries and had a diagnosis of cerebral atherosclerosis on computed tomography angiography or magnetic resonance angiography (MRA). The patient had stable vital signs, was conscious, had no obvious restlessness and was expected to cooperate well with the HR magnetic resonance imaging (HR-MRI) examination.

Exclusion criteria: Nonatherosclerotic vascular diseases, such as arteritis, moyamoya disease and dissection, were present; the patient had contraindications for magnetic resonance examination (such as claustrophobia, cardiac pacemaker, nerve stimulator, drug pump, electronic cochlea, defibrillator, heart stent, artificial heart valve, metal clip after aneurysm surgery and other metal implants); the image quality was degraded due to motion or flow artefacts and could not be used for diagnosis; the basic clinical data of the patients were collected and included sex, age, blood pressure, history of diabetes, total cholesterol, triglycerides, *etc.* The study was approved by the local institutional review board.

MRI protocol

All patient imaging tests were performed using a Siemens Prisma 3.0 T (Siemens, Germany) magnetic resonance scanner with a 32-channel head coil.

The scanning protocols comprised conventional brain and cerebrovascular imaging. Conventional brain imaging encompassed T1-weighted imaging (T1WI), T2-weighted imaging (T2WI), and fluid-attenuated inversion-recovery (FLAIR). Cerebrovascular imaging involved 3-dimensional time-of-flight MRA (3D TOF MRA) and HR vascular wall imaging technology, specifically the three-dimensional sampling perfection with application-optimized contrasts using different flip angle evolution (3D-SPACE) sequence for HR-VWI. The contrast medium was meglumine gadolinium pyrospermate (Gd-DTPA) and was given *via* a bolus in an elbow vein at an amount of 0.2 mL/kg body weight. The imaging parameters of these sequences were as follows: (1) T1WI: Repetition time (TR)/ echo time (TE): 2000/7.4 ms, field of view (FOV) 220 × 220; slice thickness 5 mm, and slice number 20; (2) T2WI: TR/TE: 4000/117 ms, FOV 220 × 220; slice thickness 5 mm, and slice number 20; (3) FLAIR: TR/TE: 9000/81 ms, FOV 220 × 220; slice thickness 5 mm, and slice number 20; (4) 3D-TOF MRA: TR/TE: 21/3.4 ms, FOV 200 × 200; slice thickness 0.7 mm, and slice number 40; and (5) 3D-SPACE: Sagittal imaging orientation, TR/TE: 900/14 ms, FOV 240 × 240; slice thickness 0.6 mm, and slice number 224.

MRI image analysis

The 3D SPACE data (DICOM format) were imported into an image postprocessing workstation (Siemens Syngo Via workstation), and all parameter measurements were performed on the platform.

The images were analysed in the workstation by two senior neuroimaging physicians jointly, without knowledge of the patient information, and disagreements in conclusions were discussed and agreed upon.

The image quality was first evaluated to exclude patients with poor image quality that could not meet the diagnostic criteria. By curved planar reformation or multi-planar reconstruction reconstruction, the most stenotic site of the lumen at the plaque was selected as the target site in the transverse axis perpendicular to the vessel alignment, and the reference site was selected at the normal segment proximal to the target site (if the proximal reference site was not available, the adjacent distal site was used). The images of the narrowest site of the lumen and the reference site were magnified to 300%, and the vascular boundaries were manually traced by using the drawing tool according to the specific morphology of the narrowed vessel to obtain the vascular area at the narrowest point of the vessel (VA_{MLN}) and the vascular area of the reference vessel ($VA_{reference}$), as shown in Figure 1. Then, the remodelling index (RI) was calculated according to the formula: $RI = VA_{MLN}/VA_{reference}$. Based on the values of the RI, the plaque remodelling pattern was classified as follows: $RI > 1.05$ for PR, $0.95 < RI \leq 1.05$ for intermediate remodelling (IR), and $RI \leq 0.95$ for negative remodelling (NR).

Statistical analysis

All data were analysed by using the SPSS 26.0 package (Chicago, IL, United States). Variables underwent normality testing *via* the Kolmogorov-Smirnov test. Quantitative data are expressed as the mean ± SD, while nonnormally distributed data are presented as the median with interquartile range. Independent sample *t* tests were employed for normally distributed and homogenous variance quantitative data comparisons; otherwise, the Mann-Whitney *U* test was used.

Categorical values were summarized by counts and percentages. The chi-square test (or Fisher's exact test when appropriate) was applied for categorical data comparisons. Multivariate logistic regression was conducted to identify independent factors associated with plaque-PR. Statistical significance was defined as $P < 0.05$.

RESULTS

According to the inclusion criteria, we collected 113 patients. However, 13 patients were excluded due to quality reasons,

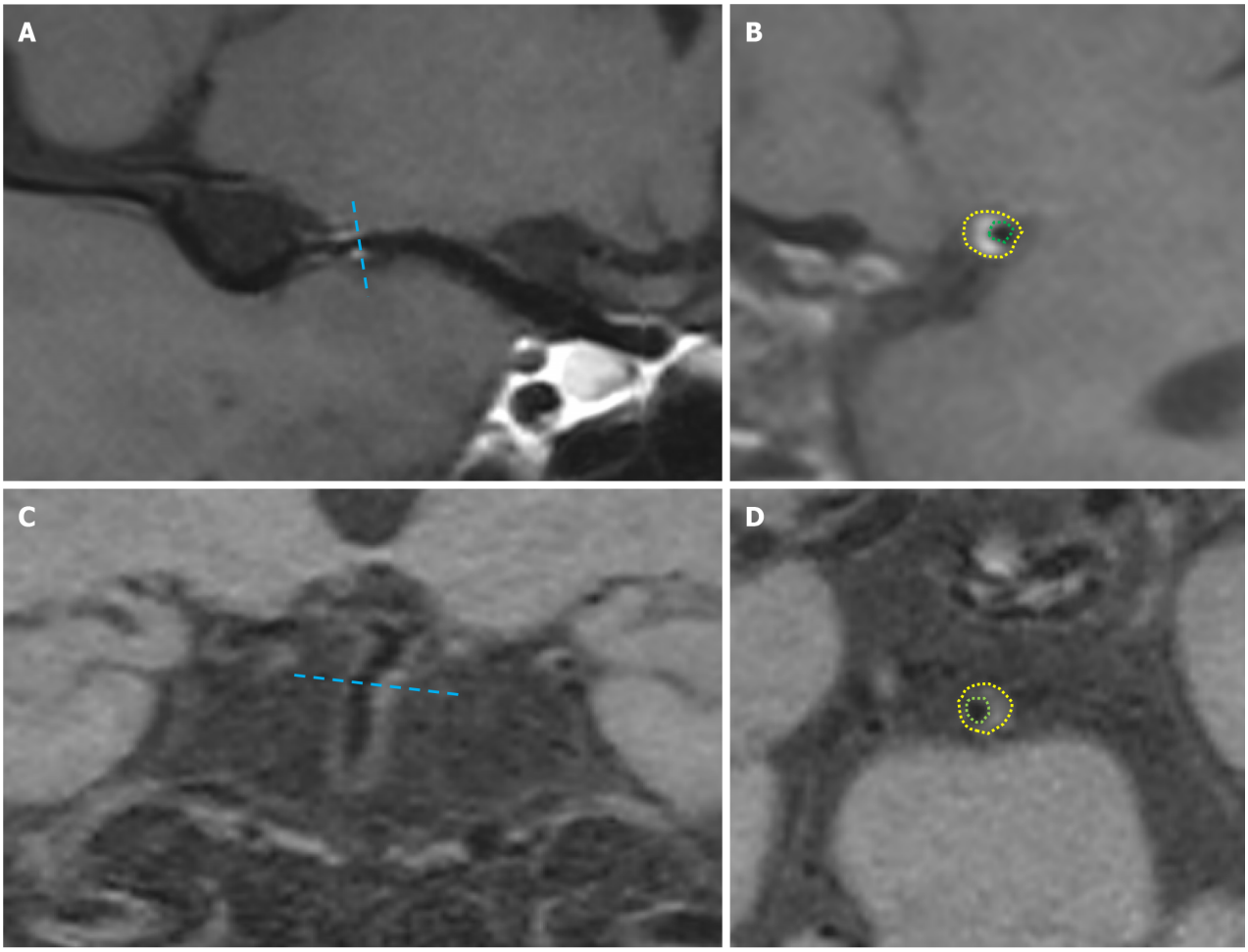

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Figure 1 Schematic of the measurement of the plaque. A: The long-axis diagram of the vessel for the first case; B: The cross-section of the vessel at the plaque for the first case; C: The long-axis diagram of the vessel; D: The cross-section of the vessel at the plaque. The green circle indicates the boundary of the inner vessel wall, while the yellow circle signifies the boundary of the outer vessel wall.

and 6 patients were also excluded that were diagnosed with arteritis or arterial dissection. Finally, a total of 94 patients were enrolled in this study (Figure 2), and they had an age range of 29 to 82 years and a mean age of 55.56 ± 12.168 years. Among them, there were 65 males, with an age range of 29 to 82 years, and the average age was 52.72 ± 11.905 years. There were 29 female patients, with an age range of 31 to 82 years, and the average age was 61.93 ± 10.347 years. There were 31 patients in the PR group, 8 patients in the IR group, and 55 patients in the NR group, in which the IR group and NR group were merged into the non-PR (N-PR) group with a total of 63 patients.

Comparison of clinical data between the PR group, IR group and NR group

The univariate analysis showed that there were statistically significant differences in the prevalence of diabetes and serum cholesterol levels among the three groups [14 (45.2%) and 4.54 (4.16, 5.93) *vs* 2 (25%) and 4.80 ± 1.22 *vs* 9 (16.4%) and 4.14 (3.53,4.75), respectively, $P < 0.05$]. There was no significant difference in age, sex, hypertension history or triglycerides among the three groups. Table 1 for the comparison of clinical data characteristics between the three groups of patients.

Comparison of clinical data between the PR group and the N-PR group

The univariate analysis showed that there were significant differences in the prevalence of diabetes, triglycerides and cholesterol between the two groups. The number of patients with diabetes in the PR group was greater than that in the N-PR group [14 (45.2%) *vs* 11 (17.5%), $P < 0.05$]. Compared with the N-PR group, the patients in the PR group had higher serum triglyceride and cholesterol values [1.64 (1.23, 2.33) and 4.54 (4.16, 5.93) *vs* 1.55 (1.06, 1.67) and 4.24 (3.53, 4.89), respectively, $P < 0.05$]. There was no significant difference in age, sex or hypertension history between the PR group and the N-PR group. Table 2 for the comparison of the clinical data characteristics and Figures 3 and 4 for the comparison of the image features between the two groups of patients.

Analysis of independent factors associated with plaque-PR

The indicators that had statistical significance in the univariate analysis and that may affect the vascular remodelling

Table 1 Comparison of clinical data between positive remodelling group, intermediate remodelling group and negative remodelling group

	PR group (*n* = 31)	IR group (*n* = 8)	NR group (*n* = 55)	*F*/*H*/χ^2 value	*P* value
Age	57.61 ± 12.77	56.00 ± 14.99	54.35 ± 11.46	0.716	0.491
Sex (%)	20 (64.5)	5 (62.5)	40 (72.7)	0.808	0.668
Hypertension (%)	22 (71)	4 (50)	37 (67.3)	1.269	0.530
Diabetes (%)	14 (45.2)	2 (25)	9 (16.4)	8.433	0.015
Triglyceride	1.88 ± 0.97	1.61 (1.10, 1.67)	1.64 (1.09, 2.33)	5.029	0.081
Cholesterol	4.54 (4.16, 5.93)	4.80 ± 1.22	4.14 (3.53, 4.75)	3.159	0.019

PR: Positive remodelling; IR: Intermediate remodelling; NR: Negative remodelling.

Table 2 Comparison of clinical data between the positive remodelling group and the non positive remodelling group

	PR group (*n* = 31)	N-PR group (*n* = 63)	*t*/*z*/χ^2 value	*P* value
Age	57.61 ± 12.77	54.56 ± 11.83	1.147	0.254
Sex (%)	20 (64.5)	45 (71.4)	0.465	0.495
Hypertension (%)	22 (71)	41 (65.1)	0.326	0.568
Diabetes (%)	14 (45.2)	11 (17.5)	8.166	0.004
Triglyceride	1.64 (1.23, 2.33)	1.55 (1.06, 1.67)	2.191	0.028
Cholesterol	4.54 (4.16, 5.93)	4.24 (3.53, 4.89)	2.452	0.014

PR: Positive remodelling; N-PR: Non positive remodelling.

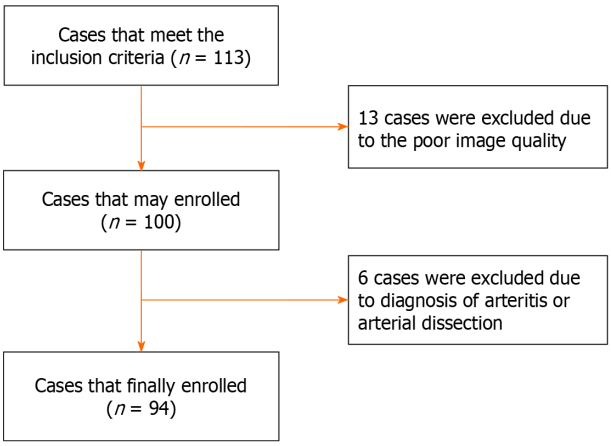

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Figure 2 The flow chart of selecting patients.

pattern, including age, sex, diabetes, hypertension and triglycerides, were included in the binary logistic regression analysis to explore the important factors affecting the remodelling pattern.

The results showed that diabetes was an independent feature of plaque-PR [odds ratio (95% confidence interval): 3.718 (1.207-11.454), $P < 0.05$]. There was no significant correlation between hypertension, triglycerides, sex and age and PR of plaque ($P > 0.05$). The independent factor analysis of patient characteristics and plaque-PR is shown in Table 3.

DISCUSSION

In our study, we used HR-VWI to explore the remodelling patterns of the responsible plaques of intracranial arteries and

Table 3 Analysis of independent factors associated with plaque positive remodelling

	OR (95%CI)	*P* value
Diabetes	3.718 (1.207-11.454)	0.022
Hypertension	1.077 (0.377-3.079)	0.890
Sex	0.558 (0.189-1.644)	0.290
Age	0.996 (0.952-1.042)	0.860
Triglyceride	1.977 (0.979-3.995)	0.057

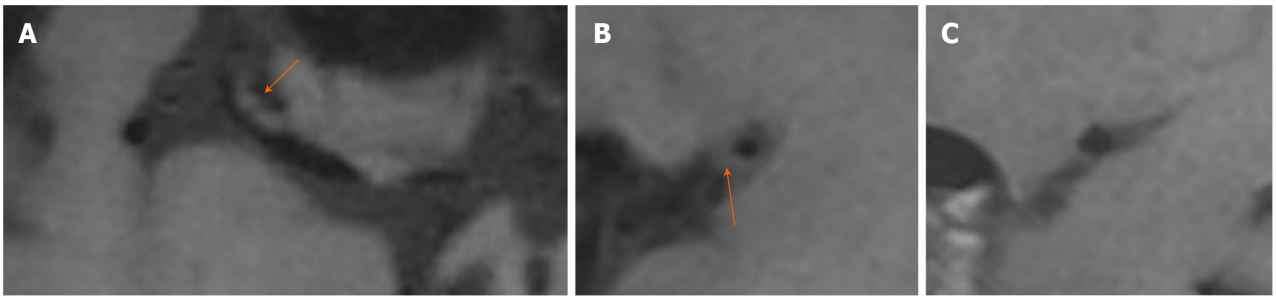

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Figure 3 The image of the positive remodelling. A and B: The M1 segment of the right middle cerebral artery plaque with eccentric thickening of the wall, mild narrowing of the lumen and a remodeling index of 1.87 (orange arrow); C: Its proximal normal wall.

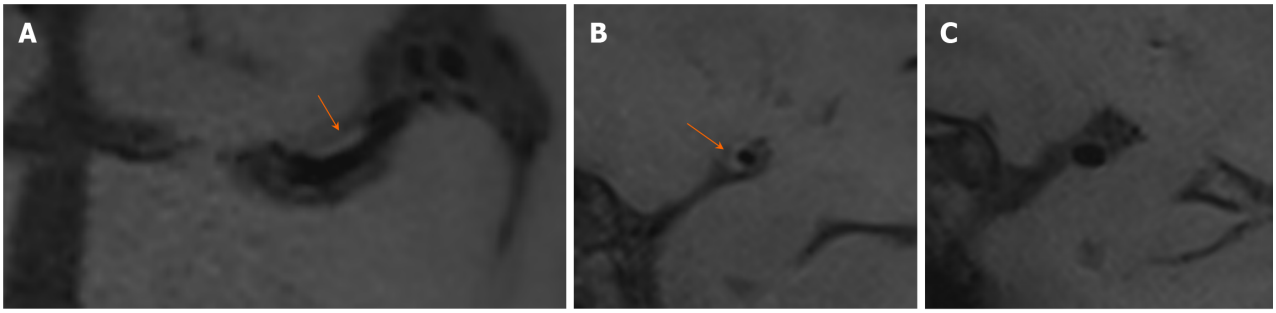

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Figure 4 The image of the negative remodelling. A and B: The M1 segment of the left middle cerebral artery plaque with eccentric thickening of the wall, mild narrowing of the lumen and a remodeling index of 0.58 (orange arrow); C: Its proximal normal wall.

their influencing factors in patients with ischaemic stroke or transient ischaemic attack. This proved that HR-VWI can noninvasively and accurately show the morphological characteristics and signal characteristics of intracranial artery plaques and can be used as an effective means to evaluate the vessel walls of intracranial atherosclerosis patients. The results showed that the prevalence of diabetes was higher in the group with PR of intracranial responsible plaques, and diabetes was an independent factor affecting the PR of plaques, which revealed that hyperglycaemia might promote the formation of PR of plaques.

An increasing number of studies have proven that arterial remodelling is an important pathological mechanism commonly present in atherosclerotic diseases. Arterial remodelling can occur in coronary arteries, renal arteries, femoral arteries, carotid arteries, *etc*. However, different arteries have different abilities and methods of remodelling. Arterial remodelling includes both PR (arterial dilation) and negative remodelling (artery constriction). PR is outwards compensatory remodelling, in which the arterial wall grows outwards in an attempt to maintain a constant lumen diameter, while negative remodelling is defined as local contraction of the vessel size[14].

Previous studies have often evaluated the severity of intracranial atherosclerotic plaques or the probability of ischaemic stroke by the degree or number of vascular stenoses. In recent years, studies have shown that the stability of the plaque itself is an important factor affecting the occurrence of stroke[15]. Intracranial atherosclerosis should be evaluated not only according to the degree of lumen stenosis but also according to the characteristics of the vascular wall. Plaques that do not cause lumen stenosis can also lead to stroke. The pattern of plaque remodelling is closely related to plaque instability. PR of arteries is more dangerous than negative remodelling and is more likely to cause acute ischaemic stroke[16,17].

In diabetes, vascular remodelling can be caused by irritation of the vascular wall due to hyperglycaemia, insulin resistance, endothelial cell dysfunction, nonenzymatic glycosylation and other factors. Watase *et al*[13] examined 557 plaques of common carotid arteries and internal carotid arteries and found that carotid artery plaques in diabetic patients showed more PR than those in nondiabetic patients. Terashima *et al*[12] also found that PR in the coronary artery is associated with diabetes. The results of our study also indicated that the PR of intracranial arterial plaques was related to diabetes, and the prevalence of diabetes in the PR group was higher than that in the nonPR group. Moreover, our study also shows that diabetes is an independent factor in the PR of intracranial plaques. It is suggested that hyperglycaemia may promote the formation of plaque-PR. Pathological studies have confirmed that positively remodelled plaques have a large lipid core and accumulate macrophages and inflammatory cells[18,19]. We speculate that hyperglycaemia may promote severe vascular wall inflammatory infiltration or the formation of a lipid core, thus increasing the risk of plaque rupture. This helps prompt clinicians to intervene early regarding blood glucose management in diabetic patients to prevent the occurrence of stroke.

Contrary to the results of our study, Zhang *et al*[16] found that the remodelling pattern of the middle cerebral artery was not associated with diabetes. The reasons why our study differed from their study may be related to the different inclusion criteria of patients because their study included asymptomatic patients with suspected middle cerebral atherosclerosis.

Although some studies have shown that[20] hypertension is related to damage to structural and functional cerebrovascular health and can promote an increase in the thickness of the middle layer of intracranial arteries, plaque and stenosis, the relationship between intracranial plaque remodelling and hypertension is still controversial. Guo *et al*[21] found that there was no statistically significant difference in the blood pressure indicators of the basilar artery between the PR group and the N-PR group, which was consistent with the results of this study, indicating that hypertension did not necessarily promote the PR of plaques. Our study also included the middle cerebral artery in addition to the basilar artery, both of which are prone to the development of intracranial plaques. However, Zhang *et al*[16] studied arterial plaques in the middle cerebral in patients with atherosclerosis and found that the PR group had a higher prevalence of hypertension than the N-PR group, but his study only included patients with moderate or severe MCA stenosis (n = 33) and did not include patients with mild MCA stenosis (stenosis degree < 30%). Our study also included patients with mild stenosis, and our study had a larger sample size (n = 67) and more types.

There are still some deficiencies in this study. First, the sample size in this study was small, and there was a lack of pathological controls. Therefore, in future research, the sample size needs to be expanded, and the possible highest number of pathological samples need to be obtained. Second, the parameters of the blood vessel wall and plaque were evaluated manually, and measurement error was inevitable. In addition, we did not conduct a follow-up study to evaluate whether the treatment effect of the PR group was better than that of the non-PR group, which will be explored in further studies in the future.

CONCLUSION

In conclusion, HR-VWI can clearly show the morphology and signal characteristics of intracranial vascular canal walls and plaques. Patients with diabetes mellitus are more likely to experience PR of intracranial atherosclerotic plaques, suggesting poor plaque stability and a greater risk of stroke, and the results of this study may provide a basis for the prevention of ischaemic stroke and may help reduce the incidence and severity of ischaemic stroke by predicting dangerous plaques.

ARTICLE HIGHLIGHTS

Research background

Intracranial atherosclerosis, a leading cause of stroke, involves arterial plaque formation.

Research motivation

This study explores the link between plaque remodelling patterns and diabetes using high-resolution vessel wall imaging (HR-VWI).

Research objectives

To investigate the factors of intracranial atherosclerotic remodelling patterns and the relationship between intracranial atherosclerotic remodelling and diabetes mellitus using HR-VWI.

Research methods

Exploratory techniques were employed in this investigation, focusing on unraveling the intricate relationship between intracranial atherosclerosis and diabetes. A cohort of 94 individuals diagnosed with atherosclerosis in the middle cerebral artery or basilar artery was assembled for scrutiny. Rigorous data collection ensued, supplemented by HR-VWI. Employing sophisticated image postprocessing, the vascular area at the plaque and normal reference vessel were meticulously measured. The remodelling index (RI) served as a pivotal metric, categorizing patients into distinct remodelling

groups. Statistical analyses illuminated pronounced associations between positive remodelling (PR) and heightened diabetes prevalence, substantiating the critical role of HR-VWI in delineating vascular nuances.

Research results

The study's findings illuminate compelling insights into the intricate dynamics of intracranial atherosclerosis. Notably, the PR group, identified through the RI, exhibited a significantly higher diabetes prevalence (45.2%) compared to intermediate remodelling and negative remodelling groups. This statistical distinction underscores the intimate connection between diabetes and atherosclerotic plaque characteristics. Additionally, the PR group demonstrated elevated serum cholesterol and triglyceride levels, reinforcing the correlation. Logistic regression analysis identified diabetes mellitus as an independent influencer in plaque-PR, further emphasizing its significance. The conclusive link between diabetic status and increased PR highlights the potential vulnerability and heightened stroke risk associated with intracranial atherosclerotic plaques in diabetic patients.

Research conclusions

In summation, our research underscores the pivotal role of HR-VWI in elucidating the intricate dynamics of intracranial atherosclerosis. The PR observed in diabetic patients accentuates a concerning association, indicative of heightened plaque instability and an augmented risk of stroke. The distinct patterns unveiled through the RI delineate varying degrees of atherosclerotic changes, with the PR group exhibiting a pronounced correlation with diabetes mellitus. These findings not only enhance our understanding of plaque characteristics but also emphasize the critical importance of HR-VWI in identifying at-risk individuals. This study contributes valuable insights that may inform targeted interventions for diabetic patients with intracranial atherosclerosis, potentially mitigating the risk of stroke.

Research perspectives

Our research opens avenues for further exploration and clinical implications. The identified correlation between PR and diabetes in intracranial atherosclerosis prompts future investigations into the underlying mechanisms. Understanding the intricate interplay between diabetes and plaque dynamics can inform tailored preventive strategies. Additionally, this study underscores the significance of HR-VWI as a diagnostic tool, suggesting its potential integration into routine clinical assessments for at-risk populations. Exploring therapeutic interventions that target plaque stability in diabetic patients may emerge as a crucial research direction, aiming to mitigate the heightened stroke risk. Overall, our findings pave the way for multidisciplinary collaborations and advancements in both diagnostic approaches and preventive measures for individuals with intracranial atherosclerosis and diabetes.

FOOTNOTES

Co-corresponding authors: Fan Lin and Biao Huang.

Author contributions: Mo YQ, Luo HY, and Zhang HW performed equally in conducting the data analyses and played pivotal roles in drafting and refining the manuscript; Deng K, Zhang HW, and Liu YF also made substantial contributions to the data analysis and manuscript preparation, enriching the intellectual content of the work; Liu XL helped perform the analysis with constructive discussions. Lin F and Huang B have been designated as co-corresponding authors due to their instrumental roles in conceiving, developing, and supporting the study. Beyond offering critical insights and design perspectives, both authors provided substantial project support, underscoring their commitment to the research's success. Their collaborative leadership ensured the study's robust conceptualization, and their shared responsibilities reflect a deep involvement in securing resources and facilitating the project's progression. Recognized for their equal and significant contributions, Lin F and Huang B's roles as co-corresponding authors highlight not only their intellectual input but also their tangible support, reinforcing the importance of their involvement in every aspect of the study. Luo HY contributed equally to this article as Mo YQ, for which our team expresses gratitude.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Peking University Shenzhen Hospital.

Informed consent statement: This study was retrospective, and the patient information was anonymized. The patients were therefore exempted from signing informed consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Observational Study

Body composition and metabolic syndrome in patients with type 1 diabetes

Qiong Zeng, Xiao-Jing Chen, Yi-Ting He, Ze-Ming Ma, Yi-Xi Wu, Kun Lin

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Abstract

BACKGROUND

In recent years, the prevalence of obesity and metabolic syndrome in type 1 diabetes (T1DM) patients has gradually increased. Insulin resistance in T1DM deserves attention. It is necessary to clarify the relationship between body composition, metabolic syndrome and insulin resistance in T1DM to guide clinical treatment and intervention.

AIM

To assess body composition (BC) in T1DM patients and evaluate the relationship between BC, metabolic syndrome (MS), and insulin resistance in these individuals.

METHODS

A total of 101 subjects with T1DM, aged 10 years or older, and with a disease duration of over 1 year were included. Bioelectrical impedance analysis using the Tsinghua-Tongfang BC Analyzer BCA-1B was employed to measure various BC parameters. Clinical and laboratory data were collected, and insulin resistance was calculated using the estimated glucose disposal rate (eGDR).

RESULTS

MS was diagnosed in 16/101 patients (15.84%), overweight in 16/101 patients (15.84%), obesity in 4/101 (3.96%), hypertension in 34/101 (33.66%%) and dyslipidemia in 16/101 patients (15.84%). Visceral fat index (VFI) and trunk fat mass were significantly and negatively correlated with eGDR (both $P < 0.001$). Female

patients exhibited higher body fat percentage and visceral fat ratio compared to male patients. Binary logistic regression analysis revealed that significant factors for MS included eGDR [P = 0.017, odds ratio (OR) = 0.109], VFI (P = 0.030, OR = 3.529), and a family history of diabetes (P = 0.004, OR = 0.228). Significant factors for hypertension included eGDR (P < 0.001, OR = 0.488) and skeletal muscle mass (P = 0.003, OR = 1.111). Significant factors for dyslipidemia included trunk fat mass (P = 0.033, OR = 1.202) and eGDR (P = 0.037, OR = 0.708).

CONCLUSION

Visceral fat was found to be a superior predictor of MS compared to conventional measures such as body mass index and waist-to-hip ratio in Chinese individuals with T1DM. BC analysis, specifically identifying visceral fat (trunk fat), may play an important role in identifying the increased risk of MS in non-obese patients with T1DM.

Key Words: Body composition; Metabolic syndrome; Insulin resistance; Visceral fat; Estimated glucose disposal rate

Core Tip: Visceral fat was found to be a superior predictor of metabolic syndrome (MS) compared to conventional measures such as body mass index and waist-to-hip ratio in Chinese individuals with type 1 diabetes (T1DM). Visceral fat index, estimated glucose disposal rate, and a family history of diabetes were identified as independent risk factors for MS in Chinese individuals with T1DM. Skeletal muscle mass showed a significant positive correlation with blood pressure and emerged as an independent risk factor for hypertension in Chinese individuals with T1DM. Body composition analysis, specifically identifying visceral fat, may be important in identifying the increased risk of MS in patients T1DM, particularly those who are non-obese.

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INTRODUCTION

Individuals with type 1 diabetes (T1DM) are particularly susceptible to cardiovascular (CV) metabolic risk factors[1], including overweight or obesity, hypertension, dyslipidemia, and insulin resistance, which, over time, contribute to additional CV complications[2,3]. CV disease (CVD) accounts for a substantial proportion of the increased mortality rate in T1DM patients, exceeding three times the rate observed in the general population[4]. Notably, T1DM patients experience a higher incidence of CVD at a younger age compared to their non-diabetic counterparts[5,6]. Recent research has shed light on the escalating prevalence of overweight and insulin resistance within the T1DM population[7], resulting in the coexistence of T1DM and metabolic syndrome (MS)-a condition also known as "double diabetes"[8]. This dual diagnosis has been associated with heightened CV risk[9] and renal disease[10]. Regrettably, MS in T1DM has not received commensurate research attention as its counterpart in type 2 diabetes (T2DM). Considering that CVD remains the primary cause of decreased life expectancy in T1DM[11], urgent consideration is warranted for the implementation of strategies targeting insulin resistance-related characteristics in T1DM management. A comprehensive understanding of the insulin resistance status among T1DM patients holds paramount clinical significance as it can guide treatment interventions effectively.

The body mass index (BMI) is commonly used to assess obesity; however, it does not accurately measure adiposity and fails to capture the distribution of body composition (BC), including fat mass and non-fat mass[12]. In individuals with diabetes, especially those with a normal BMI, the accumulation of adipose tissue, particularly in the abdominal region, is closely associated with insulin resistance and MS. Muscle mass also plays a significant role in overall health and metabolic regulation, Skeletal muscle accounts for up to 80% of glucose disposal[13]. Evaluating BC provides valuable insights into metabolic risks and facilitates effective disease management[14]. Dual-energy X-ray absorptiometry (DEXA) and bioelectrical impedance analysis (BIA) are commonly used techniques for BC assessment. While DEXA is considered the gold standard for measuring fat and lean mass, its use is limited to research settings due to equipment costs, radiation exposure, and lack of portability. In contrast, BIA is a practical, non-invasive, and easily applicable tool. BIA demonstrates comparable accuracy to magnetic resonance imaging scanning in predicting MS and offers the advantages of radiation-free measurement, cost-effectiveness, and wider applicability[15,16]. In this study, we employed BIA to assess BC in patients with T1DM and investigate the relationship between BC, MS, and insulin resistance.

MATERIALS AND METHODS

Study population

This cross-sectional study included patients diagnosed with T1DM from July 2021 to June 2023. The participants were recruited from the Department of Endocrinology and Metabolism at the First Affiliated Hospital of Shantou University Medical College. Some of the patients were originally part of our center's 3C follow-up cohort[17,18]. The inclusion criteria for this study were: (1) Clinical confirmation of T1DM; (2) age of 10 years or older; (3) informed consent; and (4) disease duration of more than 1 year. The exclusion criteria were: (1) Other types of diabetes; (2) severe hepatic or renal dysfunction; (3) acute stress conditions such as infection, inflammation, or tumors; and (4) pregnancy. This study was approved by the First Affiliated Hospital of Shantou University Medical College, and all participants provided signed informed consent. The study was conducted in accordance with the principles outlined in the Helsinki Declaration guidelines.

Data collection and physical examination

The demographic and clinical data of the enrolled patients were obtained through electronic medical record queries and on-site data collection. This included information such as gender, age, age of onset, family history of diabetes, smoking and alcohol history, daily insulin dosage, insulin treatment regimen, and presence of diabetes-related antibodies at the time of onset (GADA, IAA, ICA, and ZnT8A). The physical examination included measurements of height, weight, waist circumference, hip circumference, and blood pressure (BP).

Laboratory analyses

Laboratory analyses were conducted by collecting fasting blood samples to measure levels of glycated hemoglobin (HbA1c), fasting plasma glucose (FPG), lipid profile, creatinine, and uric acid (UA)[19]. HbA1c concentration was determined using a high-performance liquid chromatography technique (BIO-RAD D100, United States). High-density lipoprotein cholesterol (HDL-C) (CHOL), low-density lipoprotein (LDL) CHOL, total CHOL, and triglyceride (TG), creatinine, and UA were determined using an automatic biochemical analyzer (COULTER LX20; BECKMAN, United States).

BIA

BIA was conducted using the Tsinghua-Tongfang BC Analyzer BCA-1B to measure various BC parameters in all study participants[20]. Prior to the measurement, participants were instructed to clean their hands and feet and stand still on the device with their arms extended laterally at approximately a 30° angle. They were asked to tightly grip the handles, remain motionless, and refrain from speaking during the measurement. The test duration was 40 s.

The BIA measurement provided valuable information on BC indicators, including muscle mass, fat mass, protein mass, lean body weight, body water content, bone mass, muscle mass in different body regions, fat mass in different body regions, water content, body fat percentage, and visceral fat index (VFI). VFI represents the level of visceral fat and is calculated by the analysis software based on the scanning results of the size of the fat area around the visceral area. The calculation method for VFI is as follows: VFI = visceral fat area (cm^2) divided by $10cm^2$.

The definition of MS

According to specific Chinese Diabetes Society (CDS) criteria[21], MS is diagnosed when at least 2 out of 4 criteria are met: central obesity (a prerequisite for the age group < 18 years), hypertension, elevated TG levels, or decreased HDL-C levels.

In patients aged ≥ 18 years, overweight is defined as a BMI of 24-28 kg/m^2, and obesity is defined as a BMI > 28 kg/m^2. Central obesity is defined as a waist circumference ≥ 90 cm for men and ≥ 85 cm for women, regardless of BMI. Hypertension is defined as repeated BP measurements ≥ 130/85 mmHg or the use of antihypertensive medications for diagnosed hypertension. Dyslipidemia is characterized by TG levels ≥ 1.70 mmol/L, HDL-C levels < 1.04 mmol/L, or the use of specific treatments for these lipid abnormalities.

In patients aged < 18 years, overweight and obesity are defined as a BMI ≥ 85% and 95%, respectively, adjusted for age and sex. Central obesity is defined as a waist circumference ≥ the 90^{th} percentile for age and sex. According to the 2017 criteria, hypertension is defined as BP ≥ the 95^{th} percentile for age, sex, and height. Dyslipidemia is indicated by TG levels ≥ 1.47 mmol/L or HDL-C levels < 1.03 mmol/L.

The calculation of insulin resistance

The calculation of insulin resistance is determined by estimating the glucose disposal rate (eGDR) based on the Epidemiology of Diabetes Complications study conducted in Pittsburgh[22]. The eGDR is calculated using the formula: eGDR = 24.31 -[3.29 × hypertension status (1 if present; 0 if absent)] -[12.22 × waist-to-hip ratio] -[0.57 × HbA1c (%)]. A lower eGDR value indicates a higher level of insulin resistance.

Statistical analysis

Statistical analysis was performed using SPSS 19.0. The measurement data were presented as the mean ± standard deviation, and the numeration data were expressed as ratio or constituent ratio. Independent *t*-tests (for continuous variables) or chi-square tests (for categorical variables) were employed to compare differences between groups. The correlation between variables (for continuous variables) was assessed using the Pearson correlation test. Forward

conditional binary logistic regression analysis was used to find the independent risk factors for metabolic syndrome, hypertension and dyslipidemia. The logistic regression model included the following variables: Sex, age, duration, BMI, waist-hip ratio, family history of diabetes, eGDR, insulin dose, skeletal muscle mass, VFI and trunk fat mass. Statistical significance was set at $P < 0.05$.

RESULTS

The study included 101 Han Chinese individuals with T1DM, ranging in age from 11 to 62 years. The mean age of the participants was 30.97 ± 15.43 years, and the mean duration of T1DM was 11.35 ± 8.66 years. Among the participants, 16/101 (15.84%) were diagnosed with MS, 16/101 (15.84%) were overweight, 4/101 (3.96%) were obese, 34/101 (33.66%) had hypertension, and 16/101 (15.84%) had dyslipidemia.

The clinical characteristics of the subjects with and without MS are summarized in Table 1. There were no significant differences in age, duration of T1DM, sex, insulin regimen, insulin dosage, hypoglycemia frequency, FPG, and HbA1c between the groups. However, individuals with MS had significantly higher systolic BP (SBP), diastolic BP (DBP), BMI, waist circumference, HDL, LDL, TG, CHOL and UA levels compared to those without MS. The MS group also had a higher proportion of individuals with a positive family history of diabetes (68.75% *vs*. 20%, $P < 0.001$) and lower estimated glucose disposal rate (eGDR) (4.02 ± 0.87 *vs*. 8.42 ± 1.88, $P < 0.001$).

Gender-specific differences in BC were observed and classified into four groups based on gender and the presence or absence of MS (Table 2). Regardless of gender, individuals with MS had significantly higher fat, bone, protein, water and skeletal muscle mass, compared to those without MS. In male patients, there were higher values of lean body mass, bone mass, protein content, and water content compared to female patients. Conversely, female patients had higher values of VFI, fat mass, and trunk fat mass compared to male patients.

Correlation analyses among composition parameters and clinical variables are shown in Table 3 and Figure 1. Both VFI and trunk fat mass were significantly and negatively correlated with eGDR ($r = -0.486$, $P < 0.001$, and $r = -0.503$, $P < 0.001$, respectively). The VFI and trunk fat mass were positively correlated with age, duration of T1DM, LDL, TG, while the skeletal muscle mass was significantly positively correlated with age, SBP, DBP, HDL, and UA. In this study, no significant associations were found between VFI, trunk fat mass, skeletal muscle mass and HbA1c levels or insulin dosage.

To determine the independent factors associated with MS, hypertension, and dyslipidemia, a binary logistic regression analysis was performed using the forward conditional method, and the results are presented in Table 4. The analysis revealed that for MS, the significant factors included eGDR ($P = 0.017$, OR = 0.109), VFI ($P = 0.030$, OR = 3.529), and a family history of diabetes ($P = 0.004$, OR = 0.228). For hypertension, the significant factors were eGDR ($P < 0.001$, OR = 0.488) and skeletal muscle mass ($P = 0.003$, OR = 1.111). Regarding dyslipidemia, the significant factors were trunk fat mass ($P = 0.033$, OR = 1.202) and eGDR ($P = 0.037$, OR = 0.708).

DISCUSSION

This cross-sectional study focused on a population of Chinese individuals with T1DM, characterized by a relatively low BMI and an obesity rate of only 3.96%. The study findings revealed several noteworthy observations: Female patients exhibited higher body fat percentage and visceral fat ratio compared to male patients; VFI and trunk fat showed a significant negative correlation with eGDR; Notably, visceral fat emerged as a superior predictor of MS compared to conventional measures like BMI and waist-to-hip ratio; VFI, eGDR, and a family history of diabetes were identified as independent risk factors for MS; skeletal muscle mass showed a significant positive correlation with BP and emerged as an independent risk factor for hypertension. These findings contribute to our understanding of the relationship between BC, insulin resistance, and MS in individuals with T1DM, particularly in the Chinese population.

Over the past 15 years, there has been a growing interest in examining the impact of BC in patients with T1DM, particularly in studies conducted in Europe since 2003. During this time, there has also been an observed increase in obesity rates among individuals with T1DM[23]. Currently, studies consistently show an upward trend in obesity and the prevalence of MS among patients with T1DM. However, reported incidence rates vary across different regions, ranging from 3% to 50%[24,25]. In this study, we found a prevalence rate of 15.84% for MS and 3.96% for obesity among T1DM patients. The prevalence of MS in T1DM is lower than in the general Chinese population and the most Caucasus population[25,26]. Our findings align with similar studies conducted in Japan[27]. Importantly, while the prevalence of MS in the presented study is lower than in the general Chinese population, there is still a noticeable increase compared to data collected from our center a decade ago. Specifically, during our center's participation in the IDF-CDS 3C study in 2011-2012, the prevalence of MS among Chinese individuals with T1DM was reported as 10.1%[28].

The explanation for the increasing trend of obesity and MS in patients with T1DM is multifaceted. The rise in overweight and obesity rates can be attributed to factors such as the anabolic effects of insulin therapy or increased calorie intake due to hypoglycemia episodes[2,29]. Studies, such as the Diabetes Control and Complications Trial (DCCT), have shown that patients undergoing intensified insulin therapy experience weight gain over time compared to those on conventional treatment[30]. However, in our study, we did not find any significant associations between insulin dosage, treatment regimen, and BC variables such as weight, BMI, and visceral fat. Similarly, no correlations were observed between hypoglycemia frequency and measures of weight, BMI, or visceral fat. While the DCCT study suggested a trade-off between intensified glycemic control and insulin-induced adipogenesis[31], the Epidemiology of Diabetes

Table 1 Clinical characteristics of type 1 diabetes with and without metabolic syndrome

Parameter	MS (-), *n* = 85	MS (+), *n* = 16	*P* value
Age (yr)	30.87 ± 16.44	31.50 ± 8.63	0.823
Duration	11.04 ± 9.17	12.96 ± 5.08	0.421
Male (%)	38 (44.71)	7 (43.75)	0.944
Smoking (%)	2 (2.35)	0 (0)	0.535
Drinking (%)	3 (3.53)	2 (12.50)	0.129
Family history of Diabetes (presence) (%)	17 (20.00)	11 (68.75)	< 0.001
Insulin regimen			
Insulin pump	16	3	0.746
Basal-bolus insulin	66	13	
Pre-mix insulin	3	0	
Insulin dose (IU/kg)	0.86 ± 0.38	0.76 ± 0.27	0.775
Frequency of hypoglycemia (times/month)	2.89 ± 3.24	1.43 ± 1.46	0.082
SBP (mmHg)	120.92 ± 13.95	130.50 ± 11.49	0.011
DBP (mmHg)	79.45 ± 9.77	87.00 ± 6.71	0.004
BMI (kg/m^2)	19.82 ± 2.57	27.13 ± 4.07	<0.001
Waist (cm)	66.91 ± 7.26	85.63 ± 10.42	< 0.001
ZnT8 (+) (%)	18 (21.18)	0 (0)	0.042
IA2 (+) (%)	29 (34.12)	3 (18.75)	0.225
GAD (+) (%)	37 (43.53)	3 (18.75)	0.063
TPOAb (+) (%)	52 (61.18)	2 (12.50)	0.009
HDL (mmol/L)	1.81 ± 0.48	1.29 ± 0.42	< 0.001
LDL (mmol/L)	2.69 ± 0.99	3.50 ± 0.90	0.003
TG (mmol/L)	0.83 ± 0.46	1.85 ± 0.88	< 0.001
CHOL (mmol/L)	4.73 ± 1.15	5.44 ± 1.24	0.029
UA (μmol/L)	317.72 ± 90.24	417.63 ± 128.40	< 0.001
FPG (mmol/L)	8.35 ± 4.20	8.51 ± 5.29	0.894
HbA1c (%)	7.34 ± 1.42	7.69 ± 1.91	0.388
eGDR	8.42 ± 1.88	4.02 ± 0.87	< 0.001

SBP: Significantly higher systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; GAD: Glutamic acid decarboxylase antibody; TPOAb: Thyroid peroxidase antibody; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; UA: Uric acid; CHOL: Cholesterol; FPG: Fasting plasma glucose; HbA1c: Glycated hemoglobin; eGDR: Estimated glucose disposal rate.

Interventions and Complications (EDIC) study indicated that the negative effects of weight gain on CVD risk were minimal when considering the overall benefits of improved glycemic control[32]. Additionally, the EDIC study, with a follow-up period of 17 years, demonstrated that intensified insulin therapy significantly reduced the risk of CVD events and mortality[33]. Therefore, our perspective aligns with the notion that the impact of intensified glycemic control on factors like visceral fat is relatively modest. Considering the significant protective effects against microvascular complications and the substantial benefits for CVD, the potential risks associated with weight gain do not warrant significant changes in our clinical approach to intensified treatment strategies.

Our findings indicate that visceral fat content is an independent risk factor for MS in individuals with T1DMs. Visceral fat, as opposed to subcutaneous fat, plays a primary role in insulin resistance and related metabolic disorders[34]. Interestingly, in our study, BMI and waist-to-hip ratio did not prove to be optimal measures of insulin resistance in T1DMs patients. This may be due to their focus on weight-to-height ratio and waist and hip circumference without considering the specific quantity of abdominal fat or body muscle composition. Our research suggests that analyzing BC, specifically identifying visceral fat (trunk fat), may be crucial for identifying the increased risk of MS in T1DM patients, particularly those who are non-obese. Although routine assessment of BC was not recommended in diabetes care

Table 2 Comparison of body composition parameters between women and men in type 2 diabetes with and without metabolic syndrome

Parameter	Men with MS (*n* = 7)	Men without MS (*n* = 38)	Women with MS (*n* = 9)	Women without MS (*n* = 47)
Fat mass (kg)	20.50 ± 3.15	7.39 ± 3.25	25.47 ± 9.23	13.05 ± 4.24
bone mass (kg)	3.91 ± 0.26	2.94 ± 0.57	2.87 ± 0.40	2.49 ± 0.34
Protein mass (kg)	12.83 ± 1.01	9.05 ± 2.21	8.78 ± 1.58	7.32 ± 1.32
Water (kg)	45.53 ± 3.60	32.11 ± 7.87	31.16 ± 5.58	25.96 ± 4.65
Skeletal muscle mass (kg)	41.64 ± 3.30	29.38 ± 7.19	28.53 ± 5.09	23.74 ± 4.23
Fat-free mass (kg)	62.27 ± 4.88	44.12 ± 10.64	42.82 ± 7.54	35.78 ± 6.30
Body fat rate (%)	24.66 ± 2.38	14.06 ± 4.13	36.13 ± 5.83	26.26 ± 4.80
Visceral fat index	10.14 ± 1.13	5.46 ± 1.80	12.76 ± 2.86	8.13 ± 1.82
Trunk fat mass (kg)	10.26 ± 1.61	3.71 ± 1.61	12.76 ± 4.60	6.55 ± 2.13

All variables showed statistical differences between male and female groups and between groups with or without metabolic syndrome ($P < 0.05$). MS: Metabolic syndrome.

Table 3 Correlation analysis between body composition parameters and clinical variables in type 1 diabetes

	Visceral fat index		Trunk fat mass		Skeletal muscle mass	
	r	*P* value	*r*	*P* value	*r*	*P* value
Age	0.197	0.048	0.263	0.008	0.278	0.005
Duration	0.235	0.018	0.253	0.011	0.043	0.688
SBP	0.164	0.105	0.211	0.036	0.395	< 0.001
DBP	0.196	0.052	0.233	0.020	0.369	< 0.001
LDL	0.374	< 0.001	0.352	< 0.001	0.111	0.267
HDL	-0.103	0.305	-0.122	0.224	-0.229	0.021
TG	0.342	< 0.001	0.344	< 0.001	0.193	0.053
UA	0.008	0.934	0.032	0.753	0.332	0.001
HbA1c	0.044	0.666	0.056	0.579	-0.057	0.574
Insulin dose	-0.093	0.355	-0.244	0.014	-0.397	0.451

SBP: Significantly higher systolic blood pressure; DBP: Diastolic blood pressure; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride; UA: Uric acid; HbA1c: Glycated hemoglobin.

standards[35], analyzing BC may provide valuable information for disease management[14].

In our study, we observed that female patients with T1DM had higher rates of overweight, body fat percentage, and visceral fat ratio compared to male patients, which is consistent with findings from several studies conducted internationally. For instance, Krishnan *et al*[36] found that female adolescents with T1DM exhibited a more centralized fat distribution. Similarly, a Swedish study reported that girls with diabetes had higher body weight and BMI than boys[37]. Additionally, A Szadkowska *et al*'s research indicated that women with diabetes were more susceptible to developing abdominal obesity compared to women in the control group[38]. These gender disparities may be attributed to sexual dimorphism in insulin resistance and growth hormone levels[39]. Given these findings, it is crucial to prioritize and implement early interventions targeting obesity and metabolic issues in female patients with T1DM.

This study also identified a family history of diabetes as an independent risk factor for the development of MS in individuals with T1DM. It has been observed that individuals with both T1DM and MS have a higher prevalence of a family history of T2DM[40]. These individuals demonstrate a lower frequency of major histocompatibility complex genes and a stronger association with genes that contribute to the risk of T2DM[41-43]. The DCCT study found that individuals with a family history of T2DM had higher rates of T2DM, weight gain, central fat distribution, waist circumference, insulin dose, and severity of dyslipidemia compared to those without a family history of T2DM[44]. This may be due to the expression of T2DM susceptibility genes in this population. The study suggests that individuals with T1DM and a family history of T2DM or obesity may experience greater weight gain and the appearance of other features associated

Table 4 Risk factors for metabolic syndrome, hypertension and dyslipidemia in binary logistic regression (forward conditional)

	Factors	Regression coefficient	Standard error	Wald	*P* value	OR	95%CI for OR	
							Lower	Upper
Metabolic syndrome	eGDR	-2.219	0.925	5.748	0.017	0.109	0.018	0.667
	Visceral fat index	1.264	0.582	4.714	0.030	3.539	1.131	11.077
	Family history of Diabetes	1.479	0.508	8.490	0.004	4.390	1.623	11.874
Hypertension	eGDR	-0.717	0.166	18.534	< 0.001	0.488	0.352	0.677
	Skeletal muscle mass	0.105	0.035	8.939	0.003	1.111	1.037	1.190
Dyslipidemia	Trunk fat mass	0.184	0.102	3.214	0.073	1.202	0.983	1.469
	eGDR	-0.346	0.166	4.332	0.037	0.708	0.511	0.980

Wald: A chi square value equal to the square of regression coefficient divided by its standard error. OR: Odds ratio; eGDR: Estimated glucose disposal rate.

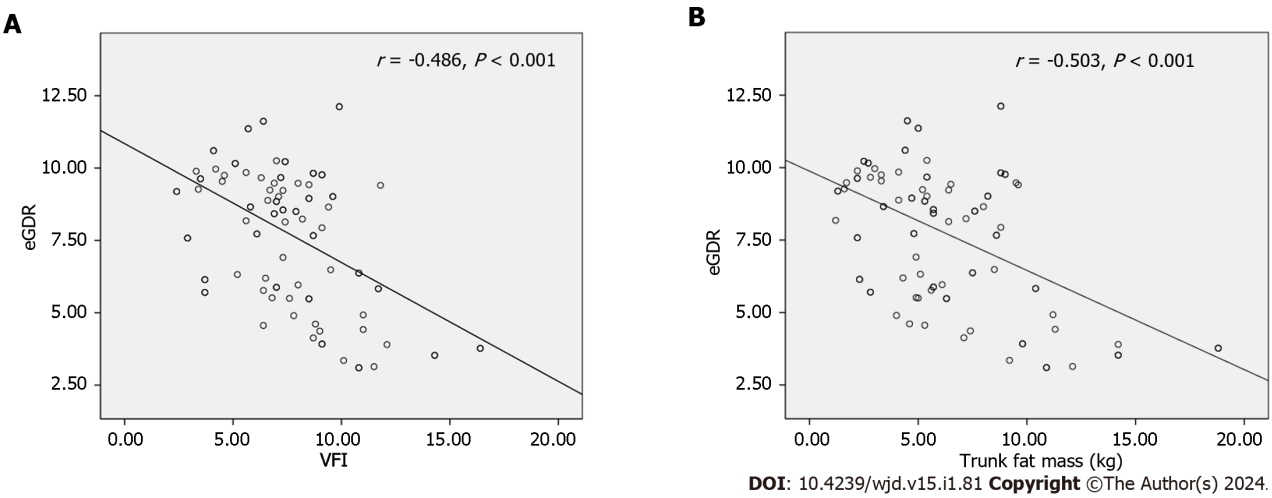

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Figure 1 Correlation between visceral fat index, trunk fat mass and estimated glucose disposal rate (*n* = 101). A: Visceral fat index were significantly and negatively correlated with estimated glucose disposal rate (r = -0.486, P < 0.001); B: Trunk fat masswere significantly and negatively correlated with estimated glucose disposal rate (r = -0.503, P < 0.001). eGDR: Estimated glucose disposal rate.

with MS when receiving intensified insulin therapy. Therefore, it is crucial to give increased attention to individuals with a family history of T2DM among those with T1DM in clinical practice. Close monitoring and stricter surveillance should be implemented to reduce their risk of developing MS or "dual diabetes".

An unexpected finding of this study is the positive correlation between skeletal muscle mass and BP in individuals with T1DM. Traditionally, the impact of diabetes on skeletal muscle has received less attention compared to adipose tissue. Studies have indicated structural and metabolic impairments in muscle mass, as well as decreased muscle function in individuals with T1DM. However, there is limited research on the correlation between skeletal muscle and BP. Data on whole-body DXA measurement from the National Health and Nutrition Examination Survey showed that total fat mass, total muscle mass, and trunk fat mass significantly and positively associated with BP. Among them, total muscle mass made relatively great contribution (35%-43%) to SBP[45]. Another study published in Hypertension in 2017 demonstrated a correlation between reduced skeletal muscle attenuation and incident hypertension in African American men[46]. The underlying mechanisms linking skeletal muscle and hypertension remain unclear, but it is speculated that infiltration of intramuscular adipose tissue may play a role. Insulin resistance is considered a direct regulator in the complex interplay between skeletal muscle obesity and hypertension[47], although other indirect mechanisms such as inflammation or oxidative stress may also be involved[47,48]. Skeletal muscle accounts for up to 80% of glucose disposal. Further research is needed to investigate the underlying mechanisms and conduct larger-scale population studies to explore this relationship in more detail.

This study has several limitations. Firstly, it is a cross-sectional study, which means that the results can only suggest correlation and not establish causation. Secondly, the sample size of the study is relatively small, which may introduce sample bias and limit the statistical power. Larger-scale studies are needed to further investigate these findings.

CONCLUSION

Visceral fat emerged as a superior predictor of MS compared to conventional measures like BMI and waist-to-hip ratio in Chinese T1DM patients. VFI, eGDR, and a family history of diabetes were identified as independent risk factors for MS. BC analysis, specifically identifying visceral fat (trunk fat), may be important in identifying the increased risk of MS in patients with T1DM, particularly those who are non-obese.

ARTICLE HIGHLIGHTS

Research background

At present, the mechanism of insulin resistance in patients with type 1 diabetes (T1DM) is not completely clear; The reasons for the increase in obesity and metabolic syndrome in T1DM patients are also unclear. Clarifying the relationship between body composition, metabolic syndrome and insulin resistance is of great significance for the implementation of strategies targeting insulin resistance-related characteristics in T1DM management.

Research motivation

In this study, we employed bioelectrical impedance analysis (BIA) to assess body composition (BC) in patients with T1DM and investigate the relationship between BC, metabolic syndrome (MS), and insulin resistance.

Our study contribute to our understanding of the relationship between BC, insulin resistance, and MS in individuals with T1DM, particularly in the Chinese population.

Another important significance of the study is to verify that BC studies, specifically detecting visceral fat (trunk fat), may be useful in recognizing the elevated risk of MS in non-obeseT1DM patients.

Research objectives

The objective of the research was to assess BC in T1DM patients and evaluate the relationship between BC, MS, and insulin resistance in these individuals. This study would contribute to identify the independent risk factors for MS in Chinese T1DM and verify that BC studies, specifically detecting visceral fat (trunk fat), may be useful in recognizing the elevated risk of MS in non-obese T1DM patients.

Research methods

A total of 101 subjects with T1DM, aged 10 years or older, and with a disease duration of over 1 year were included. BIA using the Tsinghua-Tongfang BC Analyzer BCA-1B was employed to measure various BC parameters. Clinical and laboratory data were collected, and insulin resistance was calculated using the estimated glucose disposal rate (eGDR).

The BIA measurement provided valuable analysis data such as muscle mass, fat mass, and visceral fat index (VFI). In this study, VFI represents visceral fat volumeas and was calculated as follows: VFI = visceral fat area (cm^2) divided by 10 cm^2.

Research results

Several important research achievements are as follows: Visceral fat was found to be a superior predictor of metabolic syndrome compared to conventional measures such as BMI and waist-to-hip ratio in Chinese individuals with T1DM; VFI, eGDR, and a family history of diabetes were identified as independent risk factors for metabolic syndrome in Chinese individuals with T1DM; skeletal muscle mass showed a significant positive correlation with blood pressure and emerged as an independent risk factor for hypertension in Chinese individuals with T1DM.

Research conclusions

Visceral fat, eGDR, and a family history of diabetes are important independent risk factors for metabolic syndrome while skeletal muscle mass acts as an independent risk factor for hypertension. Body composition analysis, specifically identifying visceral fat, has unique value in identifying metabolic syndrome in Chinese patients with T1DM.

Research perspectives

The future research direction is to evaluate the relationship between BC and MS, mortality through expanding sample size and cohort studies.

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FOOTNOTES

Author contributions: Lin K and Zeng Q designed the research study; Chen XJ, He YT, Ma ZM and Wu YX performed the research; Zeng Q and Chen XJ contributed new reagents and analytic tools; Lin K and Zeng Q analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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ORIGINAL ARTICLE

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Urinary exosomal microRNA-145-5p and microRNA-27a-3p act as noninvasive diagnostic biomarkers for diabetic kidney disease

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Abstract

BACKGROUND

Diabetic kidney disease (DKD), characterized by increased urinary microalbumin levels and decreased renal function, is the primary cause of end-stage renal disease. Its pathological mechanisms are complicated and multifactorial; Therefore, sensitive and specific biomarkers are needed. Urinary exosome originate from diverse renal cells in nephron segments and partially mirror the pathological changes in the kidney. The microRNAs (miRNAs) in urinary exosome are remarkably stable and highly tissue-specific for the kidney.

AIM

To determine if urinary exosomal miRNAs from diabetic patients can serve as noninvasive biomarkers for early DKD diagnosis.

METHODS

Type 2 diabetic mellitus (T2DM) patients were recruited from the Second Hospital of Hebei Medical University and were divided into two groups: DM, diabetic patients without albuminuria [urinary albumin to creatinine ratio (UACR) < 30 mg/g] and DKD, diabetic patients with albuminuria (UACR ≥ 30 mg/g). Healthy subjects were the normal control (NC) group. Urinary exosomal miR-145-5p, miR-27a-3p, and miR-29c-3p, were detected using real-time quantitative polymerase chain reaction. The correlation between exosomal miRNAs and the clinical indexes was evaluated. The diagnostic values of exosomal miR-145-5p and miR-27a-3p in DKD were determined using receiver operating characteristic (ROC) analysis. Biological functions of miR-145-5p were investigated by performing

Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment.

RESULTS

Urinary exosomal expression of miR-145-5p and miR-27a-3p was more upregulated in the DKD group than in the DM group (miR-145-5p: 4.54 ± 1.45 *vs* 1.95 ± 0.93, $P < 0.001$; miR-27a-3p: 2.33 ± 0.79 *vs* 1.71 ± 0.76, $P < 0.05$) and the NC group (miR-145-5p: 4.54 ± 1.45 *vs* 1.55 ± 0.83, $P < 0.001$; miR-27a-3p: 2.33 ± 0.79 *vs* 1.10 ± 0.51, $P < 0.001$). The exosomal miR-145-5p and miR-27a-3p positively correlated with albuminuria and serum creatinine and negatively correlated with the estimated glomerular filtration rate. miR-27a-3p was also closely related to blood glucose, glycosylated hemoglobin A1c, and low-density lipoprotein cholesterol. ROC analysis revealed that miR-145-5p had a better area under the curve of 0.88 [95% confidence interval (CI): 0.784-0.985, $P < 0.0001$] in diagnosing DKD than miR-27a-3p with 0.71 (95%CI: 0.547-0.871, $P = 0.0239$). Bioinformatics analysis revealed that the target genes of miR-145-5p were located in the actin filament, cytoskeleton, and extracellular exosome and were involved in the pathological processes of DKD, including apoptosis, inflammation, and fibrosis.

CONCLUSION

Urinary exosomal miR-145-5p and miR-27a-3p may serve as novel noninvasive diagnostic biomarkers or promising therapeutic targets for DKD.

Key Words: Urinary exosome; MicroRNA-145-5p; MicroRNA-27a-3p; Diabetic kidney disease; Diagnostic biomarkers

Core Tip: Diabetic kidney disease (DKD) is a serious complication of diabetes mellitus (DM). Novel biomarkers and effective therapeutic targets for DKD are needed in clinical settings. In this study, urinary exosomal microRNA-145-5p (miR-145-5p) and miR-27a-3p from patients with DKD were associated with kidney injury progression in type 2 DM patients. MiR-145-5p was highly specific and sensitive to DKD. It may be involved in the signaling pathways related to the pathological processes of DKD. Urinary exosomal miR-145-5p and miR-27a-3p may serve as novel noninvasive diagnostic biomarkers and therapy targets for DKD.

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INTRODUCTION

Diabetic kidney disease (DKD) is a serious complication of diabetes mellitus (DM). It is characterized by increased urinary microalbumin levels and decreased estimated glomerular filtration rate (eGFR), resulting in rapid progression to end-stage renal disease[1]. According to a recent report, the number of adults with diabetes worldwide is approximately 0.537 billion, and the total number may rise to 0.643 billion by 2030. Notably, approximately 20%-40% of DM patients progress to DKD[2]. The pathological mechanisms of DKD, including abnormal glucose metabolism, advanced glycation end product generation, inflammation, oxidative stress, and renal hemodynamic changes that eventually lead to renal injury, are complicated and multifactorial[3,4]. Podocytes are the key cell type damaged early in DKD due to their highly differentiated postmitotic phenotype with restricted abilities for self-repair and renewal. Hyperglycemia-induced podocyte injury can lead to glomerular filtration dysfunction and proteinuria[5]. Early diagnosis and specific treatment can prevent DKD progression; however, renal biopsy, the golden standard for DKD diagnosis, cannot be widely used because it is invasive, and microalbuminuria is often unable to reflect early renal injury or kidney dysfunction progression in DKD patients[6,7]. Sensitive biomarkers and effective therapeutic targets are needed for DKD in current clinical settings.

Exosome are lipid bilayer cup-shaped extracellular vesicles with 40-160 nm in diameter. Many cell types can excrete exosome; Hence, they can be detected in various body or tissue fluids, such as blood, urine, and saliva[8,9]. The bioactive cargo derived from parental cells, which includes proteins, metabolites, and genetic information, is delivered by the exosome to adjacent or distant cells that regulate the phenotypes or functions of recipient cells[10]. Circulating exosome cannot cross the glomerular filtration barrier. Urinary exosome are generally derived from diverse renal cells in nephron segments and are not easily confounded by circulating exosome. Urinary exosome have specific responses to the renal pathological changes[11]. Exosome can carry genetic information. MicroRNAs (miRNAs) are the most abundant exosomal RNAs, comprising roughly 22 nucleotides. miRNAs participate in post-transcriptional gene silencing or target mRNA degrading[12,13]. Several miRNAs play vital roles in the pathological processes of DKD[12]. MiR-145-5p, miRNA-27a, and miR-29c are associated with podocyte injury[14]. miRNAs contained in urinary exosome can avoid degradation by nuclease. They are remarkably stable, independent of urine, highly tissue-specific for the kidney, and can be collected

in large quantities noninvasively[15-17]. Therefore, urinary exosomal miRNAs are better candidates for diagnostic markers than free miRNAs[18]. Ghai *et al*[17] showed that miR-31-5p and miR-200c-3p were upregulated in urinary exosome from DKD patients compared with patients with normoalbuminuria. Park *et al*[19] identified 22 differentially expressed miRNAs in urinary exosome from DKD patients; of these, 14 differentially expressed miRNAs were associated with renal inflammation and glomerular injury. Zhao *et al*[20] found that the urinary exosomal miR-4534 was significantly increased and positively correlated with microalbuminuria in DKD patients with type 2 DM (T2DM). The exosomal miR-4534 can serve as a novel biomarker for early DKD diagnosis. Zang *et al*[21] also found that urinary exosomal miR-21-5p increased and miR-30b-5p decreased in T2DM patients with DKD compared with patients without DKD. These exosomal miRNAs were closely related to poor renal function and may serve as promising biomarkers for DKD prognosis.

In the present study, we extracted urinary exosome from T2DM patients to determine the expression of miR-145-5p, miR-27a-3p, and miR-29c-3p and evaluate their sensitivity and specificity in diagnosing DKD. Moreover, the molecular biological function of exosomal miR-145-5p was predicted by conducting a bioinformatics analysis to seek novel noninvasive diagnostic biomarkers and potential therapy targets for DKD.

MATERIALS AND METHODS

Characteristics of the study participants

The Ethics Committee of the Second Hospital of Hebei Medical University approved the trial protocols (approval number: 2022-R059). Written informed consent was obtained from each participant before the study. The diagnosis of T2DM and DKD was based on the criteria of the American Diabetes Association[22,23]. All T2DM patients were recruited from the Endocrinology Department of the Second Hospital of Hebei Medical University from February 2022 to May 2022. The age of the enrolled patients was 18-75 year, and their eGFR was ≥ 60 mL/min/1.73 m^2. T2DM patients with normoalbuminuria [urinary albumin to creatinine ratio (UACR) < 30 mg/g] were included in the DM group (n = 20), and T2DM patients with albuminuria (UACR ≥ 30 mg/g) were included in the DKD group (n = 20). In the DKD group, five patients were confirmed *via* kidney biopsy. Twenty healthy volunteers from the Medical Examination Department were included in the normal control (NC) group.

The exclusion criteria of the patients were as follows: (1) Those with T1DM and other specific types of diabetes; (2) Those with severe metabolic disorder or infectious disease within the last 1 mo; (3) Those with severe cardiovascular and cerebrovascular diseases within the last 3 mo; (4) Those with proliferative retinopathy or diabetic foot; (5) Those with nondiabetic renal diseases, kidney stones, and urinary tract infection; and (6) Those with a malignant tumor or autoimmune and chronic liver diseases.

The general and clinical data, including age, gender, body mass index (BMI), fasting blood glucose (FBG), glycosylated hemoglobin A1c (HbA1c), fasting insulin (FINS), serum C-peptide (C-P), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), blood urea nitrogen (BUN), serum creatinine (Scr) and UACR of all participants were obtained from the medical record system. Fasting urine samples were collected for urinary exosome separation and exosomal miRNAs detection. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration 2021 formula[24].

Isolation of urinary exosome

Each participant provided 200 mL of fasting urine, which was centrifuged at 4 °C and 3000 × g for 20 min. The supernatant was filtered with a 0.22-μm filter to remove cell debris, bacteria, and impurities. The urinary exosome were separated in a sterile environment according to our previous experiments[25] and stored at -80 °C for subsequent testing.

Nanoparticle tracking analysis

The urinary exosome particle sizes were measured *via* nanoparticle tracking analysis (NTA) using ZetaView PMX 110 (Particle Metrix, Germany). An exosome suspension of 50 μL was appropriately diluted using 1× phosphate buffer saline. NTA was recorded and analyzed at pH 7.0. The entered conductivity was 15000 μS/cm sensed.

Western blotting analysis

Total proteins were separated from exosome using radioimmunoprecipitation assay lysis buffer (Solarbio, China). Protein concentrations were estimated using a bicinchoninic acid protein analysis kit (Solarbio, China) according to our previous study[25]. Denatured proteins, 30 μg from each sample, were separated *via* 10% SDS-polyacrylamide gel electrophoresis (Epizyme, China) and transferred to polyvinylidene fluoride membranes (Millipore, United States). The membranes were blocked with 5% skim milk (BioFroxx, Germany) for 2 h at 24 °C and then incubated with the primary antibodies CD63 (1:1000, Abcam), CD9 (1:1000, Abcam), and TSG101 (1:1000, Abcam) at 4 °C overnight. The next day, the membranes were incubated with the secondary antibody (1:5000, Affinity) for 1 h at 24 °C. The protein bands were detected using chemiluminescence reagents (Sharebio, China) and quantified using ImageJ software (Bio-Rad, United States).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed according to a previous study[26]. Total RNA in the exosome was isolated using an RNA-easy isolation reagent (Vazyme, China). Complementary DNA (cDNA) was reverse transcribed from 1 μg total RNA using miRNA 1st Strand cDNA Synthesis Kit (Vazyme, China). RT-qPCR

was performed on a CFX96 PCR system (Bio-Rad, United States) using GoTaq® qPCR Master Mix (Promega, United States). The relative expression of miRNAs were normalized to that of the internal reference U6 and then calculated using the $2^{-\Delta\Delta Ct}$ method.

The following primers were used for RT-qPCR: hsa-miR-145-5p: F-5′-AAGCGACCGTCCAGTTTTCCC-3′, R-5′-ATCCAGTGCAGGGTCCGAGG-3′; hsa-miR-27a-3p: F-5′-AATCGGCGTTCACAGTGGCTAA-3′, R-5′-ATCCAGTGCAGGGTCCGAGG-3′; hsa-miR-29c-3p: F-5′-CGCGGCATAGCACCATTTGAAA-3′, R-5′-ATCCAGTGCAGGGTCCGAGG-3′; U6: F-5′-CTCGCTTCGGCAGCACA-3′, R-5′-AACGCTTCACGAATTTGCGT-3′.

Bioinformatics analysis

The potential target genes of miR-145-5p were predicted using three different gene databases: TargetScan7.2 (http://wwtargetscan.org/vert_72/), miRDB (https://mirdb.org/mirdb/index.html), and miRTarBase (https://mirtarbase.cuhk.edu.cn). Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were implemented on the Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov/). GO analysis elucidated detailed biological functions of potential target genes of miR-145-5p from the molecular function (MF), biological process (BP), and cellular component (CC) aspects. A *P*-value and a false discovery rate (FDR) of < 0.05 were considered statistically different. -Log10 (*P*-value) and FDR also represented the enrichment degree of gene function and signaling pathway.

Statistical analyses

All data were processed by GraphPad Prism 8.0 software (La Jolla, United States). A normality test was first performed in each group. Normally distributed data are presented as mean ± SD and analyzed using one-way analysis of variance (ANOVA), followed by the Tukey test. The heterogeneity of the variance data was detected using Welch's ANOVA test. Pearson correlation analysis was used to analyze the correlation between two normally distributed parameters. Receiver operating characteristic (ROC) curves were used to assess the diagnostic efficiency of urinary exosomal miRNAs in DKD. A *P* value of < 0.05 was considered statistically significant.

RESULTS

General and clinical data

The general and clinical data of participants are shown in Table 1. There were no differences in age, gender, BMI, and C-P levels among the three groups ($P > 0.05$). FBG, HbA1c, FINS, TC, and LDL-C in the DM and DKD groups were higher than those in the NC group ($P < 0.05$), but there were no differences in these indexes between the DM and DKD groups ($P > 0.05$). Scr and UACR were higher and eGFR was lower in the DKD group than in the DM group ($P < 0.001$). Patients with DKD exhibited mesangial cell proliferation, extracellular matrix accumulation, and basement membrane thickening (Figure 1A).

Urinary exosome characteristics

Western blot analysis verified the expression of exosome marker proteins, including CD9, CD63, and TSG101, in the particles from the NC, DM, and DKD groups (Figure 1B). NTA screen capture showed that abundant nanoparticles existed in the urine specimens of the three groups (Figure 1C). The particle diameters at peak concentrations were 120.8 nm (91.9% of the total), 126.6 nm (95.5% of the total), and 122.6 nm (85.4% of the total) for the NC, DM, and DKD groups, respectively. The average diameter sizes of exosome were 135.3 ± 1.59 nm, 149.2 ± 1.81 nm, and 147.7 ± 10.55 nm, respectively (Figure 1D). These results indicated that urinary exosome were successfully extracted from the urine samples.

Expression of urinary exosomal miR-145-5p, miR-27a-3p and miR-29c-3p

The relative expression of the urinary exosomal miR-145-5p, miR-27a-3p, and miR-29c-3p in the three groups was measured using RT-qPCR. The expression of exosomal miR-145-5p and miR-27a-3p was remarkably increased in the DKD group compared with the DM group (miR-145-5p: 4.54 ± 1.45 *vs* 1.95 ± 0.93, $P < 0.001$; miR-27a-3p: 2.33 ± 0.79 *vs* 1.71 ± 0.76, $P < 0.05$) and the NC group (miR-145-5p: 4.54 ± 1.45 *vs* 1.55 ± 0.83, $P < 0.001$; miR-27a-3p: 2.33 ± 0.79 *vs* 1.10 ± 0.51, $P < 0.001$). The expression of exosomal miR-27a-3p in the DM group was higher than that in the NC group (1.71 ± 0.76 *vs* 1.10 ± 0.51, $P < 0.05$), but no significant difference was found in the expression of exosomal miR-145-5p between the DM and NC groups (1.95 ± 0.93 *vs* 1.55 ± 0.83, $P > 0.05$). Similarly, the expression of exosomal miR-29c-3p did not differ among the three groups ($P > 0.05$) (Figure 2A).

Correlations between urinary exosomal miR-145-5p and miR-27a-3p and renal function in T2DM patients

Exosomal miR-145-5p was found to positively correlate with Scr ($r = 0.781$, $P < 0.0001$) and UACR ($r = 0.801$, $P < 0.0001$) and negatively correlate with eGFR ($r = -0.784$, $P < 0.0001$) (Figure 2B). Similarly, miR-27a-3p positively correlated with Scr ($r = 0.380$, $P = 0.016$) and UACR ($r = 0.439$, $P = 0.005$) and negatively correlated with eGFR ($r = -0.477$, $P = 0.002$) (Figure 2C). Moreover, miR-27a-3p positively correlated with glycolipid metabolism indexes, including FBG, HbA1c, and LDL-C (Figure 2D).

Diagnostic efficiency of exosomal miR-145-5p and miR-27a-3p in DKD

ROC analyses defined the diagnostic potential of urinary exosomal miR-145-5p and miR-27a-3p in DKD. MiR-145-5p had

Table 1 General and clinical data in participants

Biochemical and clinical data	NC (*n* = 20)	DM (*n* = 20)	DKD (*n* = 20)
Age (yr)	45.80 ± 14.69	50.15 ± 13.50	48.79 ± 15.14
Gender (female/male)	12/8	9/11	7/13
BMI (kg/m^2)	22.94 ± 2.32	24.95 ± 2.47	24.81 ± 3.9
FBG (mmol/L)	5.30 ± 0.38	8.90 ± 2.28[c]	11.78 ± 5.12[c]
HbA1c (%)	5.33 ± 0.24	8.74 ± 2.23[c]	8.95 ± 1.58[c]
FINS (μIU/mL)	9.58 ± 3.01	14.43 ± 8.12[a]	19.63 ± 13.13[b]
C-P (ng/mL)	1.98 ± 0.57	2.27 ± 1.07	2.80 ± 1.60
TC (mmol/L)	3.12 ± 0.50	4.53 ± 0.94[c]	5.14 ± 1.49[c]
TG (mmol/L)	1.43 ± 0.81	2.24 ± 1.45	3.49 ± 2.24[b]
LDL-C (mmol/L)	2.71 ± 0.75	3.64 ± 1.15[a]	4.32 ± 1.57[c]
BUN (mmol/L)	4.61 ± 1.39	5.33 ± 1.47	6.05 ± 2.31[a]
Scr (μmol/L)	61.50 ± 6.623	67.69 ± 7.76	97.02 ± 15.46[c,d]
eGFR (mL/min/1.73m²)	114.10 ± 13.05	108.00 ± 10.59	76.94 ± 18.06[c,d]
UACR (mg/g)	9.45 ± 4.76	13.37 ± 7.20	389.50 ± 311.70[c,d]

[a]$P < 0.05$ *vs* normal control group.
[b]$P < 0.01$ *vs* normal control group.
[c]$P < 0.001$ *vs* normal control group.
[d]$P < 0.001$ *vs* diabetes mellitus group.
The data are expressed as the mean ± SD, unless the gender. NC: Normal control; DM: Diabetes mellitus; DKD: Diabetic kidney disease; BMI: Body mass index; FBG: Fasting blood glucose; HbA1c: Glycosylated hemoglobin A1c; FINS: Fasting insulin; C-P: Serum C-peptide; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; BUN: Blood urea nitrogen; Scr: Serum creatinine; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin to creatinine ratio.

a better area under the curve (AUC) of 0.88 [95% confidence interval (CI): 0.784-0.985, $P < 0.0001$] than miR-27a-3p with an AUC of 0.71 (95%CI: 0.547-0.871, $P = 0.0239$) in DKD patients (Figure 3). For DKD diagnosis, exosomal miR-145-5p exhibited a higher sensitivity of 90% and a specificity of 75% at the best cutoff value of 2.67 than miR-27a-3p with a sensitivity of 65% and a specificity of 70% at the optimal cutoff value of 2.12. The combination of miR-145-5p and miR-27a-3p contributed to an increased AUC of 0.97 (95%CI: 0.927-1.000, $P < 0.0001$) with a sensitivity of 95% and a specificity of 90% for DKD diagnosis. Urinary exosomal miR-145-5p and miR-27a-3p may serve as potential biomarkers of early DKD diagnosis, especially miR-145-5p.

Bioinformatics analysis of miR-145-5p

Since urinary exosomal miR-145-5p has the potential to serve as a promising noninvasive diagnostic biomarker of DKD, its biological function was further explored using bioinformatics analysis. In total, 907, 909, and 248 potential target mRNAs of miR-145-5p were predicted using TargetScan, miRDB, and miRTarBase, respectively. A total of 77 mRNAs were detected simultaneously using the three gene databases (Figure 4A). We listed some gene names according to the target score, such as SMAD3, SOX9, and SRGAP2, which may be involved in the pathophysiological processes of DM and DKD[27-29] (Figure 4B).

GO analysis classified and described these target genes on CC, MF, and BP aspects. The CC catalog contained various cell locations, including the cytosol (count: 42, FDR = 1.97^{E-05}), nucleoplasm (count: 28, FDR = 0.009056116), SMAD protein complex (count: 3, FDR = 0.009056116), extracellular exosome (count: 19, FDR = 0.012172645), actin cytoskeleton (count: 6, FDR = 0.029013556), and actin filament (count: 5, FDR = 0.009056116) (Figure 5A). The terms including actin binding, protein binding, sequence-specific DNA binding, SMAD binding, mitogen-activated protein kinase (MAPK) binding, and small GTPase binding were enriched in the MF catalog (Figure 5B). Regarding the biological regulatory processes, terms such as transforming growth factor β (TGF-β) receptor signaling pathway, cellular response to TGF-β stimulus, response to hypoxia, positive regulation of cell proliferation, cell differentiation, cell motility, and actin filament organization were listed in the BP catalog (Figure 5C).

KEGG pathway enrichment analysis manifested the target genes of miR-145-5p. These genes were mainly enriched in 11 signaling pathways (FDR < 0.05), such as the MAPK signaling pathway (FDR = 0.002477523), TGF-β signaling pathway (FDR = 0.00784279), forkhead box O (FOXO) signaling pathway (FDR = 0.018431373), Ras signaling pathway (FDR= 0.036227759), and advanced glycosylation end products-the accumulation of their receptors (AGE-RAGE) signaling pathway in diabetic complications (FDR = 0.036960173), which may be involved in the pathological processes of DKD[3, 30]. Besides, the pathways related to adherens junction, cellular senescence, and cancer were included (Figure 5D).

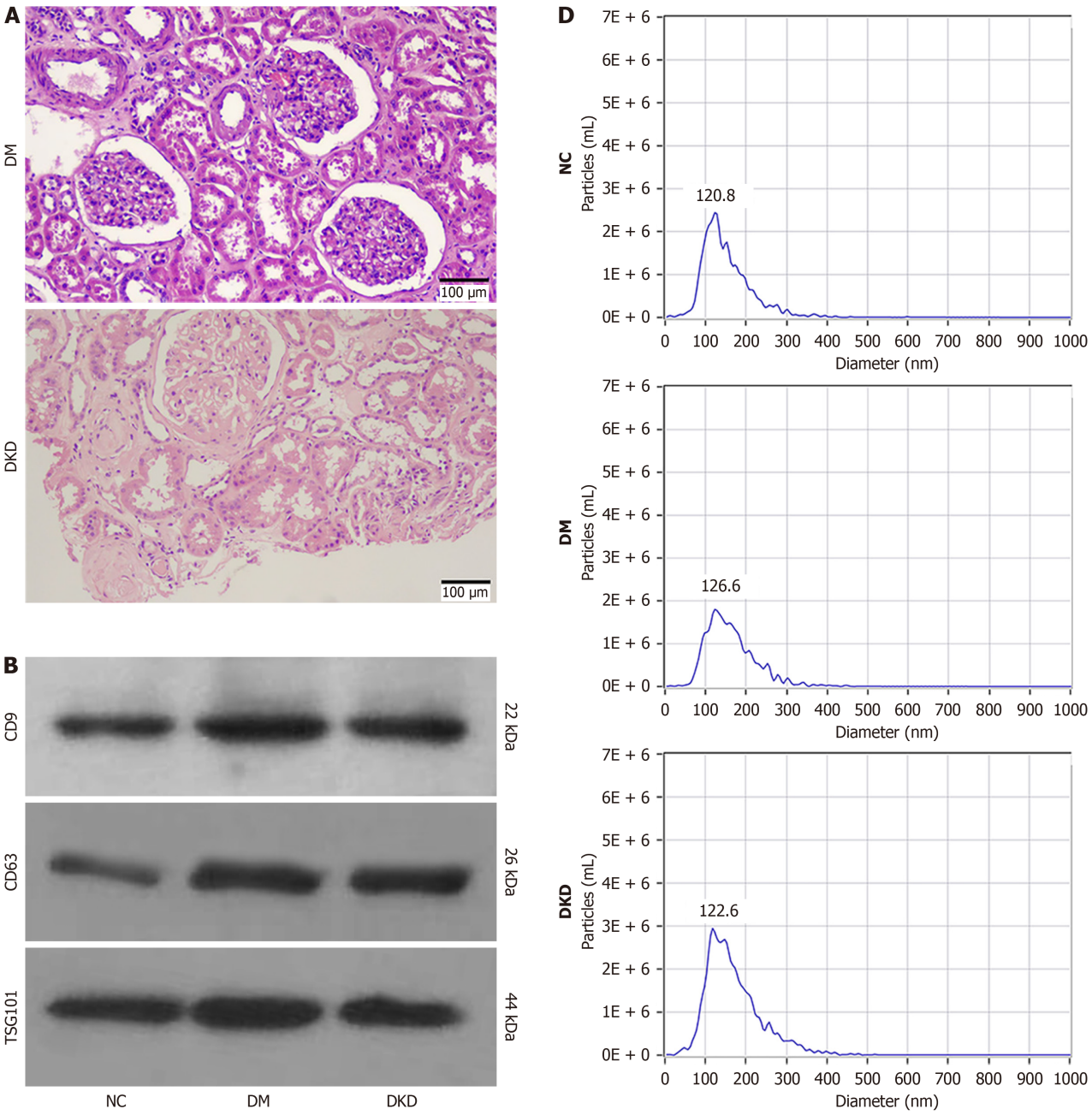

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Figure 1 The characterization of urinary exosome. A: The glomeruli histological features of diabetic kidney disease patients (Periodic acid-Schiff staining), Bar = 100 μm; B: The exosomal surface markers CD9, CD63 and TSG101 were detected by western blotting; C: The particle screenshots of nanoparticle tracking

analysis (NTA) in the urine samples from participants; D: The diameter sizes and diameter concentration distributions of the particles were measured by NTA. NC: Normal control; DM: Diabetes mellitus; DKD: Diabetic kidney disease.

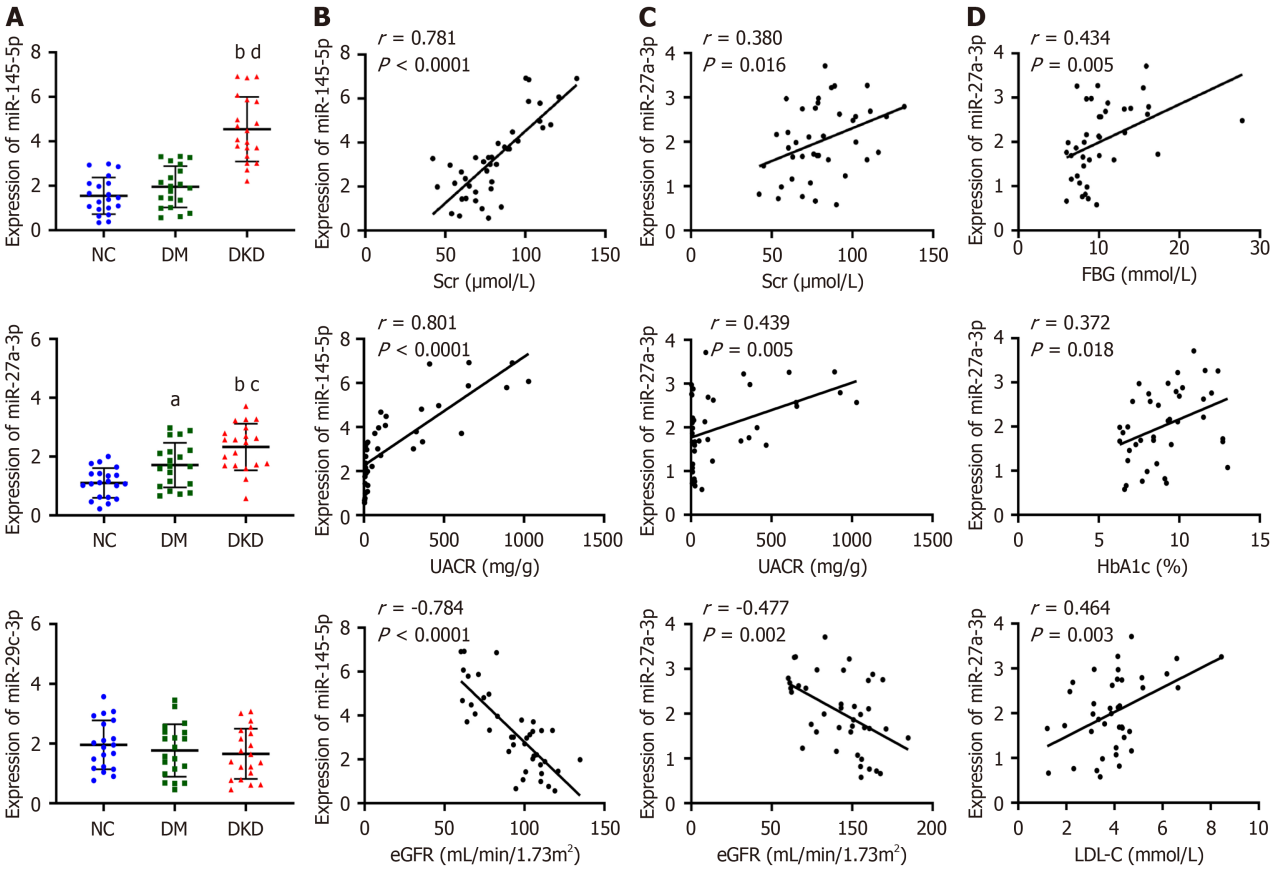

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Figure 2 The expressions of urinary exosomal microRNAs in three groups and the correlations between urinary exosomal miR-145-5p, miR-27a-3p and clinical data of type 2 diabetes mellitus patients. A: The exosomal miR-145-5p and miR-27a-3p were evidently up-regulated in diabetic kidney disease group compared with normal control (NC) and diabetes mellitus (DM) groups. The exosomal miR-27a-3p in the DM group was higher than that of the NC group. There were no differences in the expression of miR-145-5p between DM and NC groups, and miR-29c-3p among the three groups; B and C: Exosomal miR-145-5p and miR-27a-3p were positively correlated with serum creatinine and urinary albumin to creatinine ratio, negatively correlated with estimated glomerular filtration rate in type 2 DM (T2DM) patients; D: Exosomal miR-27a-3p was positively correlated with fasting blood glucose, glycosylated hemoglobin A1c and low-density lipoprotein cholesterol in T2DM patients. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* normal control group; $^{c}P < 0.05$, $^{d}P < 0.001$ *vs* diabetes mellitus group. NC: Normal control; DM: Diabetes mellitus; T2DM: Type 2 diabetes mellitus; DKD: Diabetic kidney disease; Scr: Serum creatinine; FBG: Fasting blood glucose; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin to creatinine ratio; HbA1c: Glycosylated hemoglobin A1c; LDL-C: Low-density lipoprotein cholesterol.

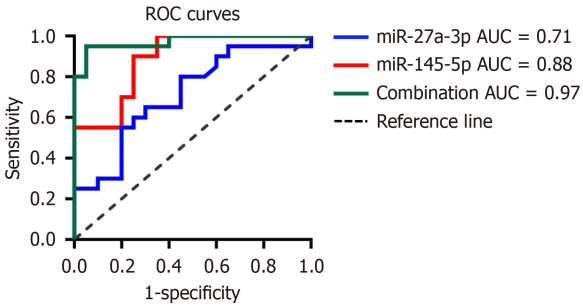

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Figure 3 Receiver operating characteristic curves of urinary exosomal miR-145-5p, miR-27a-3p and their combination to discriminate diabetic kidney disease from type 2 diabetes mellitus patients. The area under the curve for miR-27a-3p was 0.71 [95% confidence interval (CI): 0.547-0.871, $P = 0.0239$], 0.88 (95%CI: 0.784-0.985, $P < 0.0001$) for miR-145-5p, and 0.97 (95%CI: 0.927-1.000, $P < 0.0001$) for their combination. ROC: Receiver operating characteristic; AUC: Area under the curve.

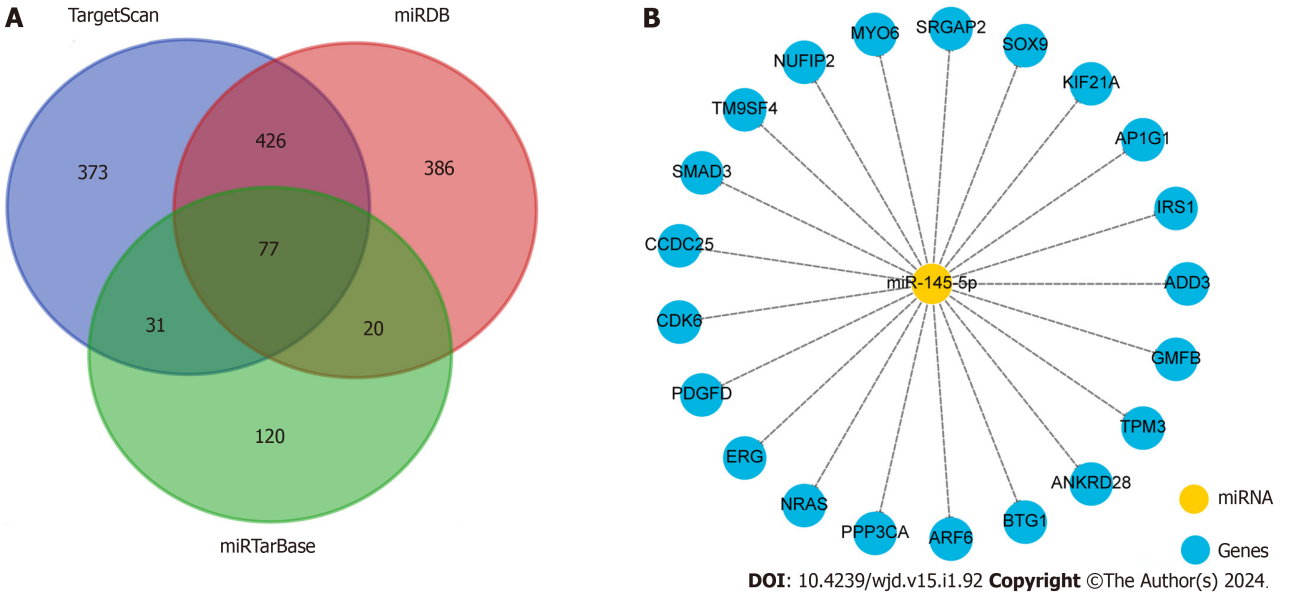

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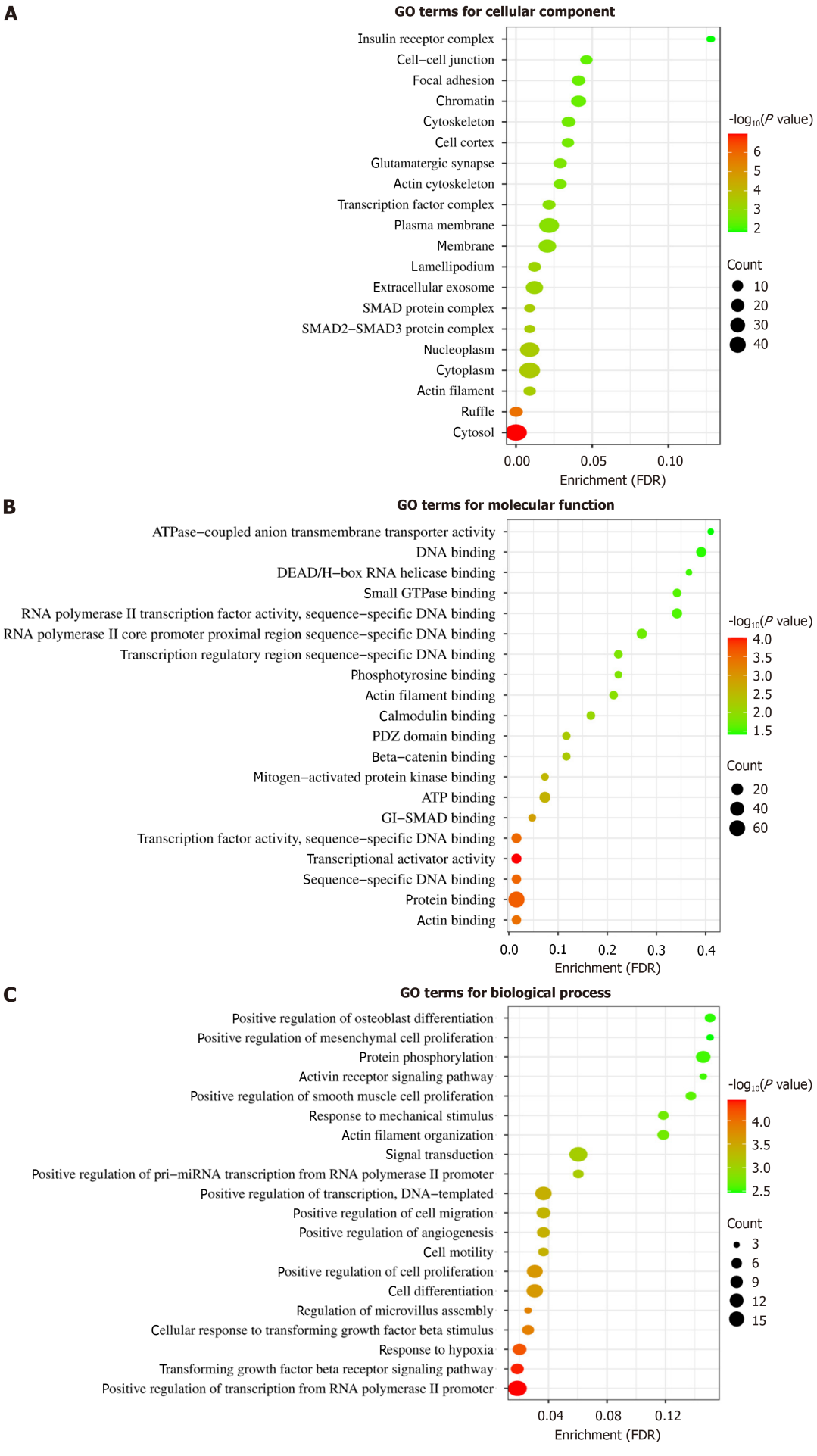
Figure 4 The potential target genes of exosomal miR-145-5p were predicted by three different gene databases. A: The Venn diagram showed that 907 genes were detected by TargetScan, 909 genes were tested by miRDB and 248 genes were predicted by miRTarBase. A total of 77 items gene were simultaneously predicted by the three gene databases; B: Among the 77 target genes, we listed some gene names according to the target score.

DISCUSSION

DKD is the primary cause of end-stage renal disease and seriously threatens the lives of patients with DM due to its irreversible and progressive evolution[1]. Early identification of high-risk patients may prevent DKD progression; However, sensitive and noninvasive diagnostic biomarkers for DKD are scarce.

miRNA dysregulation participates in various pathological processes of diabetic kidney injury and possesses great potential in the early diagnosis of DKD. A previous study showed that serum miR-145-5p was significantly lower in T1DM patients with nephropathy than T1DM controls[31]. MiR-145-5p overexpression suppressed podocyte apoptosis under high glucose (HG) conditions by inhibiting the Notch signaling pathway[32]. MiR-27a-3p was reported to be higher in the serum of T2DM patients than in the serum of non-diabetic individuals[33]. Upregulation of miR-27a accelerated renal tubular epithelial-mesenchymal transition and apoptosis of podocytes exposed to HG by activating the β-catenin signaling pathway[34]. MiRNA-29c was increased in the serum of DKD patients and HG-stimulated podocytes and induced a renal inflammatory response by downregulating tristetraprolin[35]. Exosome are found in almost all body fluids. Abundant miRNAs are cornered in exosome stably and specifically[35]. Urinary exosome are primarily generated from the kidney cells in almost all nephron segments; urinary exosomal miRNAs may reflect pathophysiological events of the kidney and are proposed as noninvasive biomarkers for DKD progression[36]. Cho *et al*[18] identified 21 differentially expressed urinary exosomal miRNAs between T2DM patients taking dipeptidyl peptidase-4 inhibitor + metformin and patients taking sulfonylurea + metformin using miRNA sequencing. Delić *et al*[37] also showed that the urinary exosomal miR-29b and miR-29c expression decreased in 5/6 nephrectomized rats compared with the sham group. Telmisartan or linagliptin treatment could significantly restore the levels of urinary exosomal miR-29c, which negatively correlated with an increase in UACR in rats. Thus, urinary exosomal miRNAs may serve as future indicators for DKD clinical therapeutic evaluation.

In the present study, urinary exosomal miR-145-5p was remarkably upregulated in T2DM patients with DKD. Barutta *et al*[38] demonstrated that urinary exosomal miR-145 was higher in T1DM patients with microalbuminuria than in normoalbuminuric and nondiabetic subjects. Moreover, miR-145 was enriched in both urinary exosome and their glomeruli tissues in DKD mice. Similarly, miR-145 was rapidly increased in HG-stimulated mesangial cells and their exosome *in vitro*, indicating that miR-145 might serve as a diagnostic biomarker for DKD. Zhang *et al*[39] reported that miR-145-5p enriched in exosome could lead to albuminuria and podocyte injury in healthy mice. These results suggest exosomal miR-145-5p is involved in kidney damage in DKD. The correlation analysis performed in the present study showed that exosomal miR-145-5p was positively correlated with albuminuria and Scr and negatively correlated with eGFR, suggesting that miR-145-5p can reflect the occurrence and development of DKD. Therefore, miR-145-5p can be regarded as a noninvasive biomarker for DKD. Castaño *et al*[40] confirmed that the circulating exosomal miR-27a-3p was overexpressed in high-fat diet-induced obese and prediabetic mouse models. Compared with control, the expression of miR-27a-3p was markedly decreased in the epididymal white adipose tissue of obese mice. The author highlighted that miR-27a-3p was one of the obesity-associated miRNAs and that the exosomal miR-27a-3p may play vital roles in the pathological processes of dyslipidemia and insulin resistance[40]. Our data revealed an increased level of exosomal miR-27a-3p in DKD patients. Upregulated miR-27a-3p was related to impaired renal function and was positively associated with glycolipid metabolism factors, including FBG, HbA1c, and LDL-C. Thus, exosomal miR-27a-3p is a valuable candidate to respond to the dysregulated glycolipid metabolism and kidney damage in T2DM. Urinary exosomal miR-29c has


A
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Cell–cell junction
Focal adhesion
Chromatin
Cytoskeleton
Cell cortex
Glutamatergic synapse
Actin cytoskeleton
Transcription factor complex
Plasma membrane
Membrane
Lamellipodium
Extracellular exosome
SMAD protein complex
SMAD2–SMAD3 protein complex
Nucleoplasm
Cytoplasm
Actin filament
Ruffle
Cytosol
0.00
0.05
0.10
Enrichment (FDR)
-log10(P value)
6
5
4
3
2
Count
10
20
30
40
B
GO terms for molecular function
ATPase–coupled anion transmembrane transporter activity
DNA binding
DEAD/H–box RNA helicase binding
Small GTPase binding
RNA polymerase II transcription factor activity, sequence–specific DNA binding
RNA polymerase II core promoter proximal region sequence–specific DNA binding
Transcription regulatory region sequence–specific DNA binding
Phosphotyrosine binding
Actin filament binding
Calmodulin binding
PDZ domain binding
Beta–catenin binding
Mitogen–activated protein kinase binding
ATP binding
GI–SMAD binding
Transcription factor activity, sequence–specific DNA binding
Transcriptional activator activity
Sequence–specific DNA binding
Protein binding
Actin binding
0.0
0.1
0.2
0.3
0.4
Enrichment (FDR)
-log10(P value)
4.0
3.5
3.0
2.5
2.0
1.5
Count
20
40
60
C
GO terms for biological process
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Positive regulation of mesenchymal cell proliferation
Protein phosphorylation
Activin receptor signaling pathway
Positive regulation of smooth muscle cell proliferation
Response to mechanical stimulus
Actin filament organization
Signal transduction
Positive regulation of pri–miRNA transcription from RNA polymerase II promoter
Positive regulation of transcription, DNA–templated
Positive regulation of cell migration
Positive regulation of angiogenesis
Cell motility
Positive regulation of cell proliferation
Cell differentiation
Regulation of microvillus assembly
Cellular response to transforming growth factor beta stimulus
Response to hypoxia
Transforming growth factor beta receptor signaling pathway
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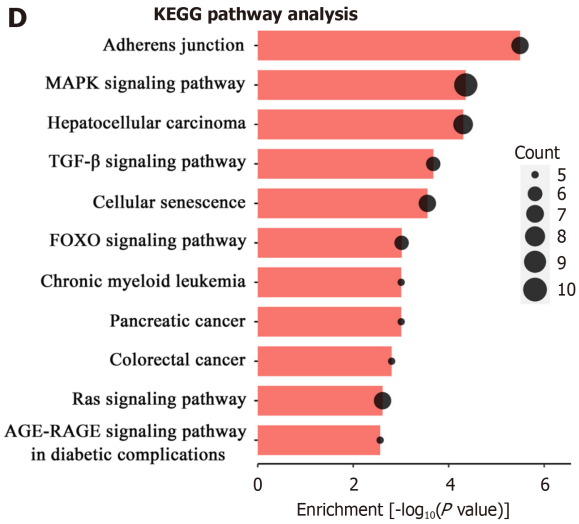

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Figure 5 Bioinformatics analysis of the genes of miR-145-5p potential target. A-C: Biological functions of the 77 intersectant target genes of miR-145-5p were described by Gene Ontology on cellular component, molecular function, and biological process aspect respectively; D: Kyoto Encyclopedia of Genes and Genomes pathways analysis predicted a total of 11 enriched signal pathways of the potential target genes of miR-145-5p. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: False discovery rate.

been reported to decrease in DKD and nondiabetic nephropathy patients, positively correlate with eGFR, and negatively correlate with the fibrosis score[41]. Our results exhibited a downregulated trend of exosomal miR-29c-3p in DKD; However, this trend was not statistically significant.

ROC analysis revealed that exosomal miR-145-5p had a better AUC with higher sensitivity and specificity than miR-27a-3p in determining diabetic kidney damage in T2DM patients. Combining the two exosomal miRNAs led to the improvement of diagnostic efficiency and were expected to serve as novel markers for early identification and diagnosis of DKD. The prediction and biological functional analysis of the target genes of miR-145-5p can guide future research on their pathological effects on DKD. GO analysis revealed the cell locations where the genes acted. The terms "actin filament", "actin cytoskeleton", and "cytoskeleton" were often related to the structure injury of podocytes[42]. The term "extracellular exosome" supported the biological regulatory roles of miR-145-5p in exosome. Consistent with this, our previous study confirmed that urinary exosomal miR-145-5p from patients with DKD induced podocyte apoptosis by inhibiting Srgap2 and activating the RhoA/Rho kinase (ROCK) pathway[25]. The term "insulin receptor complex" suggested that miR-145-5p may be involved in the pathophysiological process of glucose metabolism. MF suggested that miR-145-5p participates in actin binding, protein binding, GI-SMAD binding, MAPK binding and small GTPase binding, which were involved in the BP, including TGF-β receptor signaling pathway; cellular response to TGF-β stimulus; response to hypoxia; and cell proliferation, motility, or migration. Exposure of renal cells to HG can activate various intracellular signaling pathways, such as TGF-β-Smad-MAPK and small GTPase-related pathways. Complex perturbation and reciprocal modulation between these signalings pathways directly accelerate the pathological process of DKD[43]. The target genes of miR-145-5p were mainly involved in 11 signal pathways, including the MAPK pathway, TGF-β pathway, FOXO pathway, Ras pathway, and AGE-RAGE pathway in diabetic complications. Our previous research indicated that RhoA is a member of the Ras superfamily of GTP-binding proteins; ROCK is the downstream molecule of RhoA; and the RhoA/ROCK pathway is closely implicated in pathological processes such as inflammation, apoptosis, and fibrosis of DKD[44]. TGF-β and MAPK signaling pathways have been considered central to extracellular matrix accumulation and renal fibrosis in DKD[43,45]. The FOXO pathway is known to focus on oxidative stress and inflammatory responses in the pathological process of DKD[20]. The activation of the AGE-RAGE pathway can increase the release of reactive oxygen species and trigger various intracellular signaling cascades, including TGF-β, PKC, MAPK, nuclear factor-kappaB, and GTP-binding protein pathways. Thus, the AGE-RAGE pathway plays a central role in the multiple pathogenesis of DKD[43,46].

This study had some limitations. First, it was a cross-sectional, observational study with a small sample size without clinical follow-up and evaluation of these biomarkers in the middle or long term. Second, data on global miRNA sequencing of urinary exosome of participants in the three groups were lacking. Thus, a further prospective data analysis with a larger sample is needed to confirm the clinical applicability of these urinary exosomal miRNAs.

CONCLUSION

Our results show that urinary exosomal miR-145-5p and miR-27a-3p were markedly increased in DKD patients and were associated with the progression of kidney injury in T2DM patients. These findings imply that urinary exosomal miR-145-5p and miR-27a-3p are promising noninvasive biomarkers for DKD diagnosis. In particular, miR-145-5p was highly specific and sensitive to DKD. MiR-145-5p may be involved in signaling pathways, including MAPK, TGF-β, FOXO, Ras,

and AGE-RAGE pathways, which are related to the pathological processes, including inflammation, apoptosis, and fibrosis of DKD. These hint that miR-145-5p is a potential therapeutic target for DKD.

ARTICLE HIGHLIGHTS

Research background

Diabetic kidney disease (DKD) is the primary cause of end-stage renal disease due to its irreversible and rapidly progressive evolution. DKD remains a serious threat to the lives of diabetic patients.

Research motivation

Early diagnosis and specific treatment can prevent DKD progression. Urinary exosomal microRNAs (miRNAs) are generally derived from renal cells and directly mirror the pathological changes in the kidney. Urinary exosomal miRNAs are remarkably stable and highly tissue-specific for the kidney and may act as promising biomarkers for DKD.

Research objectives

To explore whether urinary exosomal miRNAs from diabetic patients can serve as noninvasive biomarkers for the early diagnosis of DKD.

Research methods

Patients with type 2 diabetes mellitus (T2DM) were enrolled and divided into a DM group, diabetic patients without albuminuria, and a DKD group, diabetic patients with a urinary albumin to creatinine ratio of ≥ 30 mg/g. Healthy subjects were included in the normal control group. The relative expressions of urinary exosomal miR-145-5p, miR-27a-3p, and miR-29c-3p were detected using real-time quantitative polymerase chain reaction. Correlation analysis, receiver operating characteristic analysis, and bioinformatics analysis were used to explore the potential of urinary exosomal miR-145-5p and miR-27a-3p as DKD biomarkers.

Research results

The expression of urinary exosomal miR-145-5p and miR-27a-3p was significantly upregulated in the DKD group. They were closely related to kidney damage and abnormal glycolipid metabolism in T2DM patients. Exosomal miR-145-5p had higher sensitivity and specificity for diagnosing DKD; combining miR-145-5p and miR-27a-3p increased their diagnostic efficiency. Bioinformatics analysis suggested that miR-145-5p regulated various molecular biological functions and signaling pathways involved in the pathological processes of DKD, including apoptosis, inflammation, and fibrosis.

Research conclusions

Urinary exosomal miR-145-5p and miR-27a-3p may serve as novel noninvasive diagnostic biomarkers for DKD.

Research perspectives

Urinary exosomal miR-145-5p and miR-27a-3p may complement traditional DKD diagnostic methods. They may also be effective therapeutic targets for DKD cell-free therapy in the future.

FOOTNOTES

Co-first authors: Lu-Lu Han and Sheng-Hai Wang.

Author contributions: Zhou H contributed to the conceptualization, methodology, resources, writing-reviewing and editing of this manuscript; Han LL participated in the visualization, writing-original draft preparation of this study; Wang SH took part in the investigation and data curation of this article; Yao MY was major in the interpretation of data, manuscript revision, language and format editing; and all authors were responsible for the content and proposed critical comments on the manuscript, as well as approving final version submission.

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Institutional review board statement: The study was conducted in accordance with the Declaration of Helsinki, all research protocols were approved by Ethical Committee of the Second Hospital of Hebei Medical University (approval number: 2022-R059, 28 February 2022) and the Chinese Clinical Trial Registry (approval number: ChiCTR2200066055). A written informed consent was demanded from each participant before they joined this study.

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ORIGINAL ARTICLE

Basic Study

Myricetin induces M2 macrophage polarization to alleviate renal tubulointerstitial fibrosis in diabetic nephropathy *via* PI3K/Akt pathway

Wei-Long Xu, Pei-Pei Zhou, Xu Yu, Ting Tian, Jin-Jing Bao, Chang-Rong Ni, Min Zha, Xiao Wu, Jiang-Yi Yu

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Abstract

BACKGROUND

Development of end-stage renal disease is predominantly attributed to diabetic nephropathy (DN). Previous studies have indicated that myricetin possesses the potential to mitigate the pathological alterations observed in renal tissue. Nevertheless, the precise molecular mechanism through which myricetin influences the progression of DN remains uncertain.

AIM

To investigate the effects of myricetin on DN and explore its potential therapeutic mechanism.

METHODS

Db/db mice were administered myricetin intragastrically on a daily basis at doses of 50 mg/kg or 100 mg/kg for a duration of 12 wk. Subsequently, blood and urine indexes were assessed, along with examination of renal tissue pathology. Kidney morphology and fibrosis were evaluated using various staining techniques including hematoxylin and eosin, periodic acid–Schiff, Masson's trichrome, and Sirius-red. Additionally, high-glucose culturing was conducted on the RAW 264.7

cell line, treated with 25 mM myricetin or co-administered with the PI3K/Akt inhibitor LY294002 for a period of 24 h. In both *in vivo* and *in vitro* settings, quantification of inflammation factor levels was conducted using western blotting, real-time qPCR and ELISA.

RESULTS

In db/db mice, administration of myricetin led to a mitigating effect on DN-induced renal dysfunction and fibrosis. Notably, we observed a significant reduction in expressions of the kidney injury markers kidney injury molecule-1 and neutrophil gelatinase associated lipocalin, along with a decrease in expressions of inflammatory cytokine-related factors. Furthermore, myricetin treatment effectively inhibited the up-regulation of tumor necrosis factor-alpha, interleukin-6, and interluekin-1β induced by high glucose in RAW 264.7 cells. Additionally, myricetin modulated the M1-type polarization of the RAW 264.7 cells. Molecular docking and bioinformatic analyses revealed Akt as the target of myricetin. The protective effect of myricetin was nullified upon blocking the polarization of RAW 264.7 *via* inhibition of PI3K/Akt activation using LY294002.

CONCLUSION

This study demonstrated that myricetin effectively mitigates kidney injury in DN mice through the regulation of macrophage polarization *via* the PI3K/Akt signaling pathway.

Key Words: Myricetin; Diabetic nephropathy; PI3K/Akt pathway; Renal tubulointerstitial fibrosis; Macrophage; Polarization

Core Tip: Myricetin, a flavonoid, has been extensively utilized in the domains of anti-inflammatory and anti-cancer research. However, the precise mechanism by which myricetin influences the onset and development of diabetic nephropathy (DN) remains a mystery. To address this knowledge gap, we conducted a study wherein we induced DN in db/db mice and subsequently administered myricetin as a treatment. Our findings demonstrated that high concentrations of myricetin effectively mitigated renal injury, inflammation, fibrosis, and other related factors in DN mice. Furthermore, we conducted *in vitro* experiments to confirm that myricetin activated the PI3K/Akt signaling pathway, thereby inhibiting the polarity shift of macrophages.

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INTRODUCTION

Diabetes mellitus (DM) is a major global public health concern that continues to increase in prevalence. Reportedly, there is an estimated 12.8% prevalence of diabetes in China, with a concerning trend of affecting younger generations[1,2]. DM ranks as the third most detrimental chronic non-communicable disease to human health, trailing behind cancer and cardiovascular diseases. Moreover, DM patients commonly experience chronic hyperglycemia, a metabolic disorder that adversely affects multiple kidney cell types, ultimately leading to progressive kidney failure[3,4]. Diabetic nephropathy (DN) is frequently observed as a chronic microvascular complication linked to end-stage renal disease (ESRD), and it constitutes a significant contributor to both disability and mortality[5]. The clinical characteristics of DN include persistent proteinuria and the gradual decline of glomerular filtration rate. Additionally, DN patients commonly exhibit pathological alterations such as tubulointerstitial fibrosis and progressive glomerular damage[6,7].

The precise pathogenesis and progression of DN remain unclear. Existing evidence suggests that the metabolic disorder induced by hyperglycemia may initiate the excessive activation of multiple pathways, potentially leading to mechanical damage of renal tissue[4,8,9]. Notably, inflammatory responses and the immune system play a crucial role in the progression of DN[10-12]. Macrophages, famous for their pluripotency and plasticity, differentiate into classically activated (M1) cells and alternatively activated (M2) cells, playing opposing roles in the regulation of inflammation[13]. M1 cells are closely associated with the proinflammatory response, and increased expressions of CD86, tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) represent the phenotype transformation of M1; whereas, M2 cells have increased expression of CD206, arginase-1 (Arg-1) and interleukin (IL)-10[14,15]. More importantly, the above two distinct cell subsets exist in a dynamic balanced state. Indeed, one study has indicated that the regulation of macrophage polarization could inhibit renal inflammation in mice with DM[16].

The PI3K-Akt pathway serves a crucial function in the advancement of DN, not only in regulating cell survival and proliferation but also in facilitating the progression of DN. Recent findings have indicated that, in the presence of diabetic conditions, the inhibitory effect of the transcription coregulator YAP on PTEN leads to activation of the PI3K/Akt pathway. Consequently, this activation results in accumulation of nuclear YAP, thereby enhancing the proliferation of

glomerular mesangial cells and contributing to the formation of DN[17]. It has been reported that the wogonin flavonoid exhibited inhibitory effects on tubulointerstitial fibrosis and renal tubular cell injury in mouse models of streptozotocin (STZ)-induced diabetes *via* PI3K/Akt/NF-κB signaling[18].

Currently, there is a limited availability of curative therapies for DN, and the effective prevention of renal failure progression caused by DN remains challenging. A significant proportion of ESRD patients who undergo long-term dialysis experience a high mortality rate, with approximately 20% of the population succumbing to this condition annually[19]. The primary strategies for DN patients to delay renal injury involve the control of blood glucose levels, blood pressure, and lifestyle modifications. However, the efficacy of these interventions is notably restricted[20,21]. As a result, there is an urgent need for new effective treatments to counter DM-associated kidney damage.

Abelmoschus Manihot capsule, an important Chinese patent medicine and widely used in treating kidney diseases, consists of seven flavonoids, including rutin, hyperoside, quercetin, myricetin, hibifolin, isoquercetin, and quercetin-3-o-robinobioside[22]. Our studies have been devoted to examining the roles of the total flavones of Abelmoschus manihot [23], hyperoside[24] and quercetin[25] in animal models of DN. Myricetin, present in dicotyledonous plants, has demonstrated a wide range of medicinal properties including anti-inflammatory, anti-cancer, and hepatoprotective[26,27]. For example, Park *et al*[28] found that 30 Mm of myricetin suppresses NF-κB activation and attenuates the secretion of TNF-α and IL-6. Liao *et al*[29] found that myricetin prevented diabetic-associated cardiac injury in STZ-induced mice and in high glucose-challenged neonatal rat cardiomyocytes. These investigators also found that myricetin possesses a potential protective effect by inhibiting IκBα/NF-κB pathways and enhancing Nrf2/HO-1. Kandasamy and Ashokkumar [30] found that STZ-induced diabetic nephrotoxic rats treated with myricetin were protected from glomerular injury, further suggesting its potential as an anti-hyperglycemic agent. These data collectively indicate that myricetin has effects on inhibiting the secretion of inflammatory factors and its potential therapeutic functions on diabetic-related disease as well. However, the mechanism underlying how myricetin inhibits the progress of DN remains a mystery.

For the current study, db/db mice were used to explore the effects of myricetin in the progression of DN. The mouse RAW 264.7 cell line was then used to study the mechanism by which myricetin intervenes in high glucose-induced macrophage injury. Our results indicate that myricetin regulates the polarization of macrophages through mediating the phosphorylation of Akt, and thus participates in the progression of DN.

MATERIALS AND METHODS

Cell culture

In vitro studies were carried out in the mouse RAW 264.7 cell line (American Type Culture Collection, Manassas, VA, United States), which was maintained in low glucose-Dulbecco's modified Eagle media supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum inactivated by heat. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. For experimentation, the cells were plated on a dish or in a microplate after trypsinization and incubated for 24 h before use. For the high-glucose treatments, glucose concentrations were adjusted to 5.5 mM, 25 mM or 33.3 mM (the latter designated as 'HG'). For the myricetin treatments, 12.5 μM, 25 μM or 50 μM were applied for 48 h before further analysis. LY294002 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), an Akt phosphorylation inhibitor, was administered for selective inhibition of Akt activation.

Experimental animal models

Mice (6 wk of age) of the db/m and db/db genotypes were obtained from the Hangzhou Ziyuan experimental animal facility [SCXK (Zhe) 2019–0004]. For the study period, the Experimental Animal Center of Jiangsu Provincial Hospital of Traditional Chinese Medicine (Jiangsu, China) housed all mice at 22°C with 12 h/12 h light/dark cycle, without restrictions on food or water intake. A 2-wk adaptation period with normal diet was allowed to all mice before experimental procedures were initiated. After another 4 wk of adaptive feeding, myricetin was administered to the db/db mice intragastrically at dosages of 50 mg/kg and 100 mg/kg every day. Positive controls included six db/db mice given the angiotensin II receptor blocker irbesartan (Sigma-Aldrich, Merck KGaA). All animals were subjected to weighing and serum and urine collection every 4 wk. At week 24, the mice were sacrificed for renal tissue collection.

In all experiments, guidelines provided by the National Institutes of Health (NIH, Bethesda, MD, United States) were followed. The study was carried out with approval by the Ethical Committee of Jiangsu Provincial Hospital of Traditional Chinese Medicine, in compliance with the guidelines for the Care and Use of Laboratory Animals [QK-20200408-001].

Histological examination

Collected renal tissue was initially fixed with a 4% paraformaldehyde solution for a duration of 24 h, after which it was embedded in paraffin. Subsequently, the tissue samples were sliced into sections with a thickness of 4 mm and subjected to staining using hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Masson's trichrome, and Sirius-red, respectively. Ten sections were chosen from every mouse and each was examined under light microscope at 100 × optical magnification. Histological changes were assessed at 200 × optical magnification. Brightfield images were acquired using an IX83 microscope (Olympus, Tokyo, Japan), and these images were subsequently analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, United States). Semi-quantitative analysis was performed to compare the samples from each group and a histogram was made. Representative renal images are presented from each group.

Flow cytometry

The cultured cells were washed and subsequently treated with CD16/CD32 antibodies to inhibit the activity of cell surface Fc receptors. Following this, the cells were stained using fluorescence-conjugated monoclonal antibodies, specifically APC-anti-CD206, PE-anti-CD86, and FITC-anti-F4/80 respectively (BioLegend, San Diego, CA, United States). The staining procedure was conducted for a duration of 20 min at room temperature in a dark environment, after which the samples were washed and analyzed using flow cytometry equipment from BD Biosciences (Franklin Lakes, NJ, United States). The data were analyzed using the FlowJo v10.8 software (Ashland, OR, United States).

Urine albumin-to-creatinine ratio measurement

Urinary albumin and creatinine levels were determined utilizing urinary albumin and creatinine testing kits, following the manufacturer's instructions (Jiancheng Bioengineering, Nanjing, Jiangsu, China). Urinary albumin was measured by immunoturbidimetry and creatinine by the sarcosine oxidase method. The urine albumin-to-creatinine ratio (uACR) measurement was calculated by dividing urinary albumin by urinary creatinine (μg/mg), which could be applied for detection, diagnosis and monitoring. The calibration of urine albumin by creatinine can effectively avoid the interference of other baseline factors such as body weight (BW) and food intake, and ensure comparability of the results.

Immunohistochemistry

A 30-min incubation of 5% bovine serum albumin was conducted on 5-mm sections of the kidney. Subsequently, the sections were incubated with primary antibodies overnight at 4°C. After washing, the slides were incubated with goat anti-rabbit IgG H&L antibody (Abcam, Cambridge, United Kingdom) for 60 min. Following this, a 1-min hematoxylin staining procedure was performed. Finally, the slides were mounted and observed under a microscope.

ELISA analysis

The concentration of each predicted mediator was quantified using commercially available ELISA kits obtained from Beyotime Biotech (Beijing, China), strictly following the manufacturer's instructions.

Western blotting analysis

Cell lysates were extracted and separated by SDS-PAGE, and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, United States) following protein concentration measurement. The blotted membranes were first blocked using fat-free milk and incubated overnight at 4°C with primary antibodies. Afterward, the membranes were washed with Tris-buffered saline-Tween and incubated with secondary antibodies. Results were detected *via* enhanced chemiluminescence (Applygen Technologies Inc, Beijing, China), and the densitometric data (based on the immunoreactive signals) were analyzed using open-source ImageJ software (https://imagej.net/ij/download.html). Protein levels were determined by calculating induction folds using the density ratio of the target protein to β-actin. The antibodies targeting kidney injury molecule-1 (Kim-1; Catalog No. sc-518008), neutrophil gelatinase associated lipocalin (NGAL; sc-515876), collagen-1a1 (Col1a1; sc-59772), alpha-smooth muscle actin (α-SMA; sc-53142), iNOS (sc-7271), Arg-1 (sc-166920), and others were purchased from Santa Cruz Biotechnology (Dallas, TX, United States).

qRT-PCR analysis

To determine the concentration of total RNA, Trizol reagent (Sigma-Aldrich, St Louis, MO, United States) was used following the manufacturer's instructions. The first strand of cDNA was synthesized using a reverse transcriptase enzyme (Life Technologies, Waltham, MA, United States). The relative levels of target genes were determined through reverse transcription-qPCR, as previously reported[31]. Normalization to β-actin was carried out, and the $2^{-\Delta\Delta CT}$ method was utilized to calculate the relative levels of target genes. The primers used for real-time PCR (5′-3′) can be found in Table 1.

Network pharmacology analysis

SwissTargetPrediction (http://www.SwissTargetPrediction.ch) was utilized for the purpose of predicting the structural similarity of anticipated targets based on the acquired formula. Concurrently, our disease-associated targets were obtained from GeneCards (https://www.genecards.org/) and Online Mendelian Inheritance in Man compendium (OMIM; https://www.OMIM.org/), with a specific focus on identifying targets related to "diabetic nephropathy". The target gene's name was matched by configuring the subject as "human" and employing the "Vlookup" function to filter genes that intersect with drugs and diseases.

DN and myricetin share numerous common targets, as evidenced by a Venn diagram generated using an online bioinformatics tool (http://www.bioinformatics.com.cn). To identify protein-protein interactions (PPIs), potential targets were entered into the STRING database (https://cn.string-db.org/), resulting in a visual PPI network constructed using Cytoscape 3.7.2. The degree value was then calculated to determine the key protein within the PPI network. Nodes within the network were color-coded and sized proportionally to their respective degree values, with larger and darker nodes indicating higher degree values.

Pathway enrichment analysis

The clusterProfiler, Stringin, DOSE, and Pathview programs were executed in the R language utilizing the bioinformatics open source platform Bioconductor (http://www.Bioconductor.org/). The visualization display was conducted through the WeChat letter platform. Gene Ontology (GO) bioinformatic analysis was used to elucidate the role of target proteins in drug therapy gene function, encompassing molecular function, cellular component, and biological process. In addition,

Table 1 Primers used in real-time PCR, 5-3

Gene	Forward primer	Reverse primer
Kim-1	ACATATCGTGGAATCACAACGAC	ACAAGCAGAAGATGGGCATTG
Ngal	TGGCCCTGAGTGTCATGTG	CTCTTGTAGCTCATAGATGGTGC
Col1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
α-Sma	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
iNos	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Tnf-a	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC

α-Sma: Alpha-smooth muscle actin; *Arg-1*: Arginase-1; *Col1a1*: Collagen-1a1; *Il*: Interleukin; *iNos*: Inducible nitric oxide synthase; *Kim-1*: Kidney injury molecule-1; *Ngal*: Neutrophil gelatinase associated lipocalin; *Tnf-a*: Tumor necrosis factor-alpha.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted as a component of signal pathway enrichment analysis to identify drug therapy targets.

Macromolecular docking

Myricetin was subjected to docking with Akt and PI3K, respectively. The crystal structure file of the protein target was obtained from the Protein Data Bank (PDB) database in PDB format. In order to facilitate docking, the compounds of myricetin in SDF format were downloaded from PubChem (https://PubChem.ncbi.nlm.nih.gov/). Subsequently, virtual docking experiments were conducted using Autodock 4.2.6. It is important to note that docking solely alters the conformation of the ligand, while preserving the conformational changes of the protein and leaving all other parameters unchanged. The outcomes of the docking process were visualized using PyMOL 2.2.0 software (https://pymol.org/2/) and Discovery Studio Client v19.1.0 (https://discover.3ds.com/discovery-studio-visualizer-download).

Statistical analysis

A total of three repetitions of each experiment were performed, and the results were summarized as means and standard deviations. The statistical software SPSS 22.0 (IBM Corp, Armonk, NY, United States) was utilized to conduct a one-way ANOVA with the aim of assessing between-group variation. A probability level of $P < 0.05$ was deemed as statistically significant.

RESULTS

Myricetin alleviated kidney injuries of DN mice

We first assessed the protective effects of myricetin in DN mice, with irbesartan serving as a positive control. As shown in Figure 1A, DN (db/db) mice exhibited significantly elevated BWs, blood glucose levels, and 24-h microalbumin concentrations ($P < 0.01$) compared to control (db/m) mice at week 12, confirming the successful establishment of the diabetic mouse model. During the entirety of the treatment period, the db/db + myricetin group (both 100 mg/kg and 50 mg/kg subgroups) exhibited a significant decrease in BW, blood glucose level, and 24-h microalbumin compared to the db/db group ($P < 0.01$) (Figure 1B-D).

Microalbuminuria is recognized as the earliest clinical indicator in the initial stages of DN. Therefore, we assessed the kidney/BW index, serum creatinine, creatinine clearance, and uACR following 12 wk of myricetin treatment. In comparison to db/m diabetic mice, the db/db diabetic mice exhibited significantly higher levels of serum creatinine, kidney/BW index, and uACR at 24 wk ($P < 0.01$; Figure 1E-G). The db/db mice demonstrated a reduced clearance of creatinine compared to the control mice ($P < 0.01$; Figure 1H), indicating further evidence of diabetic renal pathological damages. Following a 12-wk treatment with either 100 mg/kg or 50 mg/kg myricetin, the db/db + myricetin group displayed significant decreases in kidney/BW, serum creatinine and uACR, and a notable enhancement in the clearance of creatinine compared to the db/db group ($P < 0.01$; Figure 1E-H). Thus, these data indicated that myricetin treatment alleviated the kidney injuries of DN mice.

Myricetin ameliorated the renal histopathological changes of DN mice

We conducted histological analyses of the kidney using HE staining and PAS staining to further evaluate the therapeutic effect of myricetin in DN mice. Notably, DN mice exhibited distinct pathological kidney alterations, including glomerular

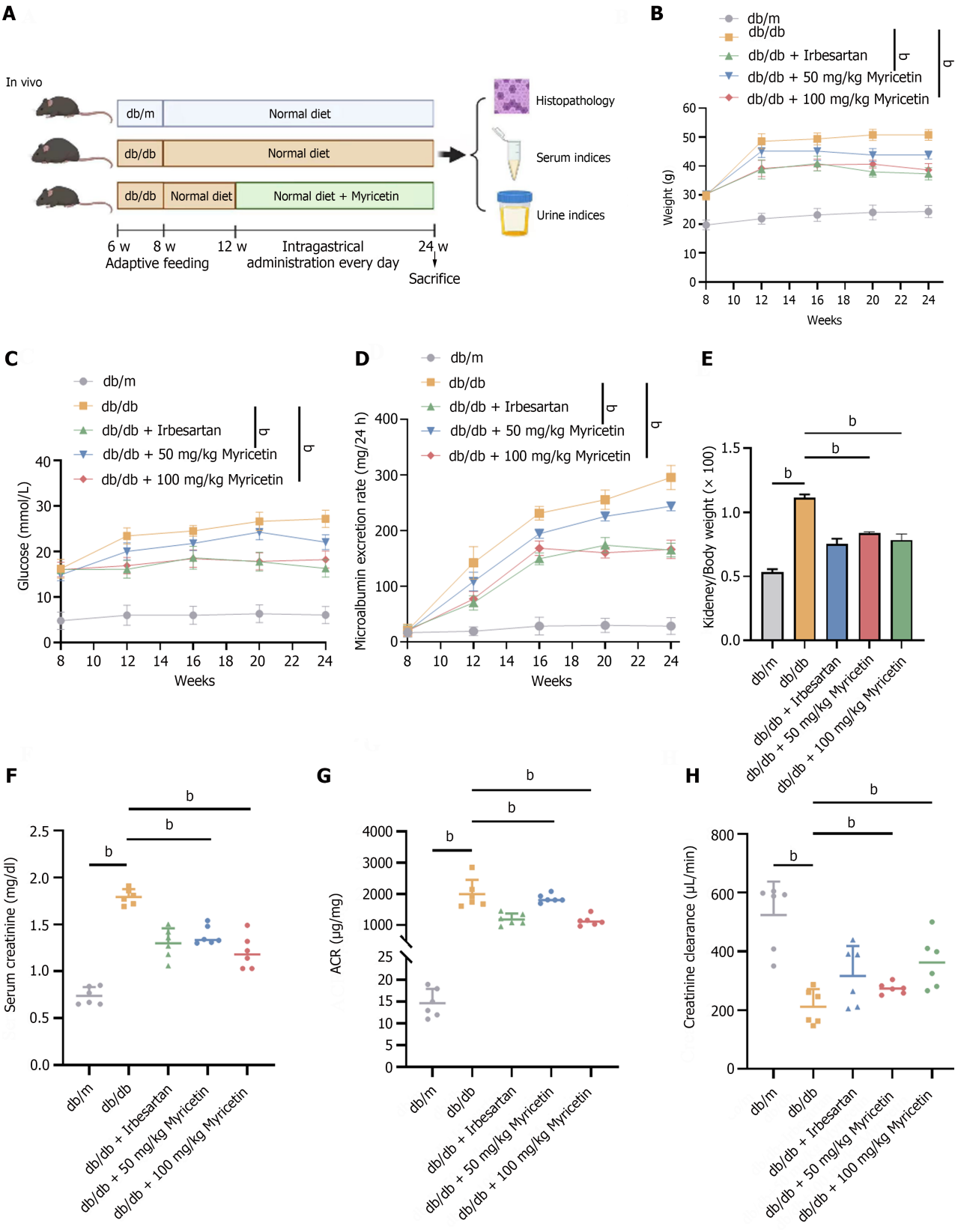

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Figure 1 Myricetin alleviated the kidney injuries of diabetic nephropathy mice. A: Timeline of *in vivo* assay; B: Body weights; C: Blood glucose levels; D: 24 h-microalbumin levels; E: Kidney/body weight ratios; F: Serum creatinine levels; G: Albumin-to-creatinine ratios; H: Creatinine clearance values. ACR: Albumin-to-creatinine ratio. $^{b}P < 0.05$.

mesangial cell proliferation, thickening of capillary basement membranes, and increased vacuolation of renal tubules (Figure 2A and B). Importantly, the renal tubular damage and expansion of mesangial matrix were significantly more serious in DN mice than in the control group ($P < 0.001$). The administration of myricetin resulted in a significant decrease in the renal tubular damage score and a reduction in the area of the mesangial matrix in db/db mice compared to the control group. Additionally, there was a notable increase in the expressions of KIM-1 and NGAL in the kidney tissues of db/db mice, which was reversed in the db/db + myricetin mice, particularly in the high-dose subgroup (Figure 2C and D). Taken together, these findings suggested that myricetin treatment effectively ameliorated the renal histopathological alterations in db/db mice.

Myricetin ameliorated inflammatory infiltration and renal fibrosis in DN mice

We used F4/80, a surface marker of M1-type macrophages, to stain the renal tissues to investigate the potential of myricetin in regulating renal function. Notably, an obvious infiltration of macrophage inflammation was observed in the kidney tissues of db/db mice when compared to the control group. However, the administration of myricetin significantly mitigated the infiltration of inflammation in the renal tissues (Figure 3A). Furthermore, the serum levels of M1 inflammatory factors were found to be up-regulated in db/db mice in comparison to db/m mice. Conversely, there was a significant decrease in the levels of IL-10, an M2 inflammatory factor, in db/db mice (Figure 3B and C). The administration of myricetin, particularly at a dosage of 100 mg/kg, resulted in a significant reduction in M1 inflammatory factors at both the mRNA and protein levels. Additionally, there was a notable increase in the expression of IL-10 following the administration of myricetin.

Given that prolonged inflammatory infiltration triggers the activation of immune cells such as fibroblasts and myofibroblasts, which are involved in collagen synthesis and deposition and ultimately lead to fibrosis, we assessed the extent of renal fibrosis by using Col1a1 antibody staining of renal tissues. It was observed that db/db mice exhibited a higher percentage of Col1a1-positive region compared to db/m mice. Furthermore, the administration of myricetin resulted in a significant decrease in the percentage of the Col1a1-positive region ($P < 0.001$; Figure 3D). Additionally, we measured the levels of Col1a1 and α-SMA in the renal tissues. It was found that the fibrosis marker genes Col1a1 and α-SMA were significantly up-regulated in db/db mice, but not in those treated with myricetin, particularly in the high-dose subgroup (Figure 3E and F). Compared with db/m mice, db/db mice had significantly more fibrosis based on Masson's trichrome staining and Sirius-red staining, while db/db mice treated with myricetin showed reduced fibrosis in their renal tissues (Figure 3G and H). Collectively, these data indicated that the myricetin treatment efficiently reduced inflammatory factor infiltration and occurrence of renal fibrosis in the db/db mice.

Myricetin switched the macrophage phenotype in the renal tissue of db/db mice

As shown in Figure 4, CD86 and CD206 were used as surface markers to identify the distinct polarized phenotypes of macrophages. The analysis of the polarized phenotypes of renal macrophages across the various groups of mice revealed a significant increase in $CD86^+$ macrophages in the kidneys of db/db mice compared to the control mice. Conversely, the number of $CD206^+$ macrophages was significantly reduced, indicating a bias towards M1 macrophage polarization in the kidneys of DN mice fed a high-fat diet. Following the administration of myricetin, a notable decrease in macrophage polarization of $CD86^+$ and an increase in macrophage polarization of $CD206^+$ were observed. This effect was particularly significant at higher doses and indicated that myricetin promoted the transition of macrophages from the M1 to the M2 phenotype, thereby mitigating the inflammatory effects. Notably, no significant disparity in the expressions of $CD86^+$ and $CD206^+$ macrophages was observed between the db/m group and the db/db + myricetin group. Thus, our results suggested that myricetin may have switched the phenotypes of macrophages in the renal tissue of db/db mice.

High glucose induced M1-type macrophage polarization of RAW 264.7 cells

In order to validate the regulation of myricetin against kidney injury of DN, we built a cell model using RAW 264.7 macrophages exposed to high glucose concentrations. Flow cytometric analysis was conducted to assess the expression of CD86 and CD206. The results revealed a higher prevalence of M1 macrophages in cultures with high glucose concentrations (25 mM and HG) as compared to M2 macrophages, while no significant difference was observed in the low-glucose (5.5 mM) culture condition (Figure 5A). Under the stimulation of HG, it was observed that the abundance of M1 macrophages peaked at 24 h, and their polarization decreased with prolonged exposure time (Figure 5B). Similarly, when RAW 264.7 cells were induced with HG, the highest level of nitric oxide synthase (NOS) activity was observed at 24 h (Figure 5C and D). Furthermore, a significant increase in the expression and secretion of M1 inflammatory factors was observed in cells stimulated with HG, while the levels of IL-10 and Arg-1 were significantly reduced (Figure 5E and F).

Myricetin regulated polarization of RAW 264.7 cells under the HG condition

As shown in Figure 6A, a concentration gradient of myricetin at 12.5 μM, 25 μM, and 50 μM was administered to the cells for 24 h. Flow cytometry analysis revealed that 25 μM myricetin exhibited the most potent inhibitory effect on M1-type polarization of the RAW 264.7 cells. The average percentage of M1-type macrophages was 6.56%, whereas the control group exhibited a percentage of 7.72% (Figure 6B). Furthermore, 25 μM myricetin significantly suppressed the NOS activity of RAW 264.7 induced by HG ($P < 0.001$; Figure 6C). Furthermore, our results revealed that treatment with 25 μM myricetin significantly decreased the expressions of iNOS, TNF-α, IL-6, and IL-1β and increased the levels of IL-10 and Arg-1 (Figure 6D and E). Collectively, these data demonstrated that myricetin effectively modulated the polarization of RAW 264.7 macrophages from M1 to M2 under HG stimulation.

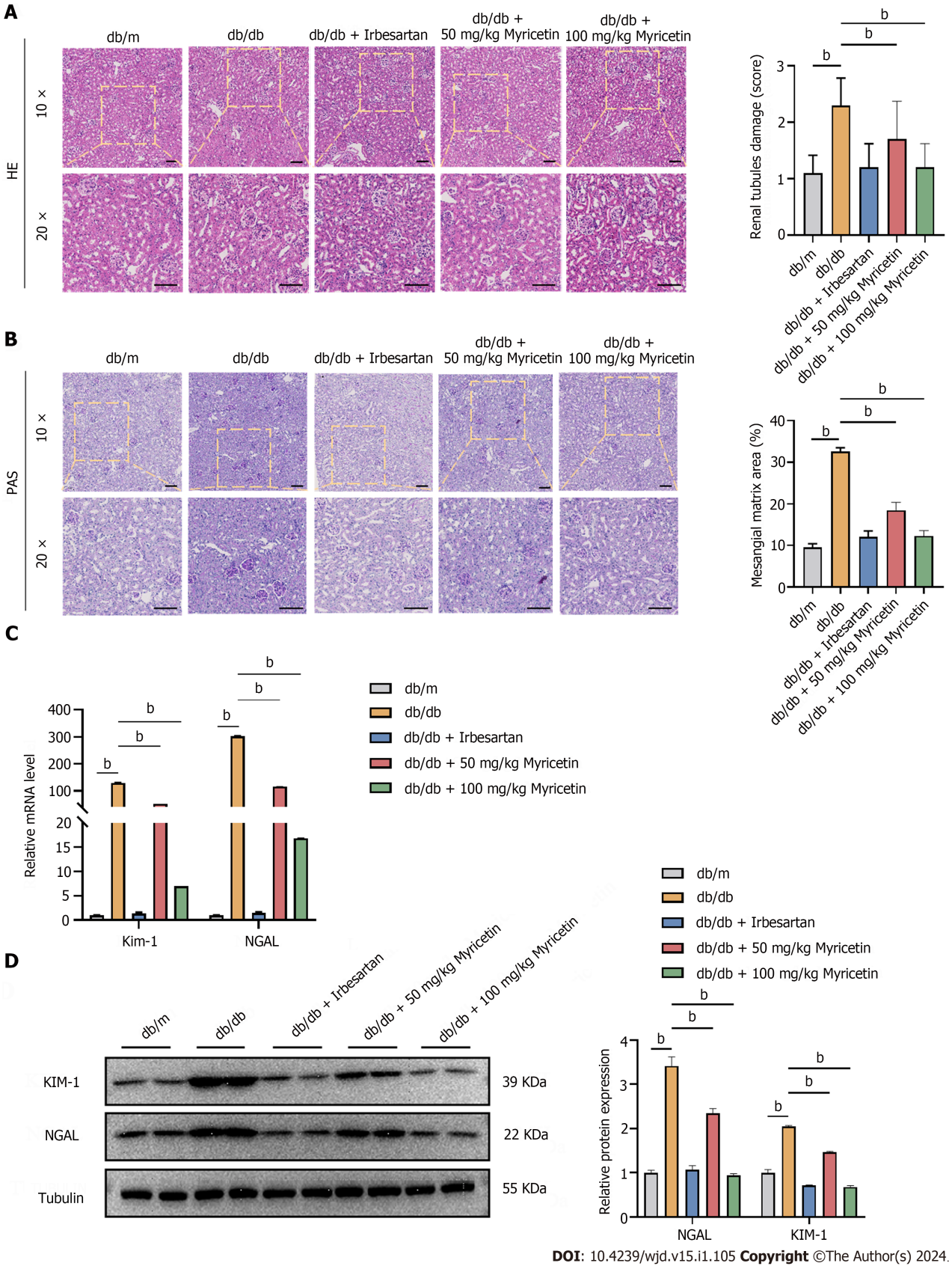

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Figure 2 Myricetin ameliorated the renal histopathological changes of diabetic nephropathy mice. A: Hematoxylin and eosin staining showing renal tubule damage analysis (left and right) of kidneys; B: Periodic acid-Schiff staining (left) and mesangial matrix analysis (right); C: Kidney injury molecule-1 (KIM-1) and neutrophil gelatinase associated lipocalin (NGAL) levels by reverse transcription-PCR; D: NGAL and KIM-1 Levels determined by western blotting. Scale bar: 100 μm. HE: Hematoxylin and eosin; PAS: Periodic acid-Schiff; KIM-1: Kidney injury molecule-1; NGAL: Neutrophil gelatinase associated lipocalin. $^{b}P < 0.05$.

Bioinformatics analysis of potential targets and pathways related with DN and myricetin

As shown in Figure 7A, a total of 3699 genes associated with DN were obtained from GeneCards and OMIM. The 43 genes that were linked to both myricetin and DN are presented in Venn diagram (Figure 7A). Subsequently, a PPI network comprised of these 43 potential targets was generated using the STRING database and visualized using Cytoscape 3.7.2 (Figure 7B). The PPI network revealed that Akt, TNF, and EGFR were the top three protein targets in immune and inflammatory signaling (Figure 7C). GO enrichment analysis revealed that myricetin treatment was associated with cellular response to oxidative stress, chemical stimulation, and protein kinases (Figure 7D). Additionally, KEGG pathway analysis indicated that myricetin primarily improved DN through the PI3K-Akt signaling pathway (Figure 7E and F). To further investigate this interaction, we used Autodock 4.2.6 to dock myricetin with Akt and PI3K, and subsequently performed a docking simulation using PyMOL 2.2.0 and Discovery Studio Client v19.1.0 (Figure 7G-H). Binding energy analysis indicated that myricetin exhibited a robust binding ability with Akt (-6.31 kcal/mol) and PI3K (-8.31 kcal/mol) (Figure 7I). Taken together, these results implied that the PI3K-Akt pathway served as a potential target in the protective mechanism of myricetin against kidney injury of DN mice.

Myricetin regulated polarization of RAW 264.7 cells through the PI3K-Akt pathway

We conducted additional experiments to validate the role of myricetin in regulating Akt kinase activity, thereby influencing macrophage polarization and cytokine secretion. Expression of the M1-type marker CD86 was significantly increased while the expression of the M2-type marker CD206 was significantly decreased compared to RAW 264.7 cells treated with myricetin alone ($P < 0.001$; Figure 8A). Our results indicated that myricetin induced a significant increase in Akt phosphorylation, while both LY294002 treatment and the HG condition inhibited Akt phosphorylation (Figure 8B). In accordance with prior data, we observed the up-regulation of M1 inflammatory factor-related genes in response to the HG condition and the administration of myricetin induced a notable decrease in the expression of these factors. However, the co-administration of LY294002 and myricetin to RAW 264.7 cells did not trigger the down-regulation of M1-related inflammation factors' expression (Figure 8C and D). Thus, our data indicated that myricetin may have regulated the macrophage polarization *via* the PI3K-Akt signaling pathway.

DISCUSSION

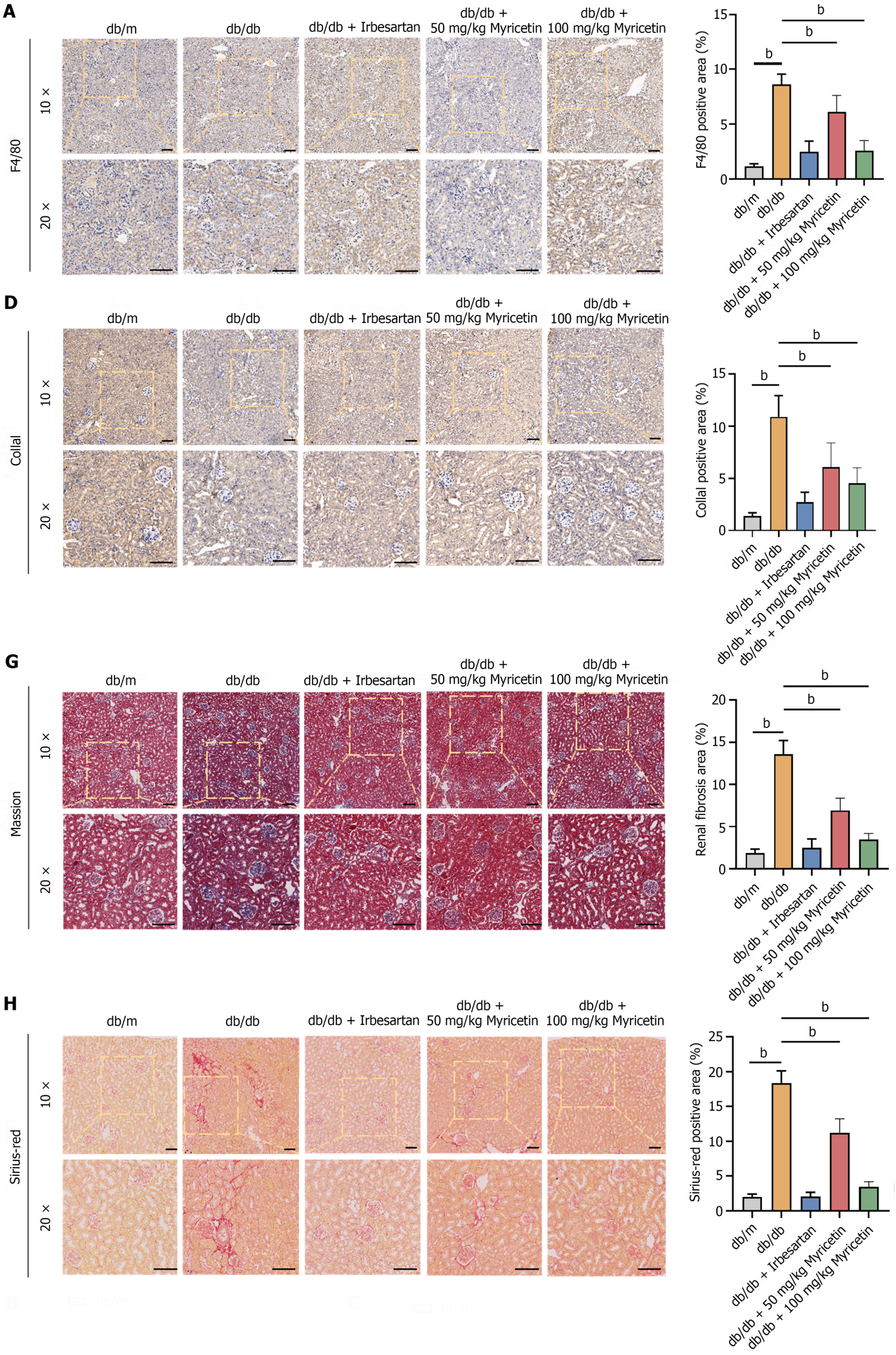
The worldwide prevalence of DM constitutes a pervasive metabolic disorder that is accompanied by a wide range of public health challenges. Projections indicate that by the year 2035, the number of individuals affected by this condition will reach 600 million[32,33]. The sustained elevation of blood glucose levels over an extended period of time gives rise to various well-established chronic complications affecting multiple organs, such as the heart, kidneys, nerves, and retinas. Among these complications, DN is the most frequently occurring chronic microvascular complication, and it has been identified as the primary cause of ESRD, disability, and mortality[5,19]. According to research, chronic hyperglycemia has been found to impact various kidney cell types and lead to progressive renal failure.

Currently, the only effective treatments available for ESRD patients are dialysis and transplantation. However, dialysis does not halt progression of the disease and the availability of donor kidneys is limited. Strategies for DN primarily focus on the control of blood glucose, with few effective therapies available for DN patients. Thus, there is an urgent need to develop novel and efficacious therapies. The molecular mechanism underlying DN is highly intricate, involving various metabolic disorders and pathways such as ferroptosis[34], oxidative stress[35], apoptosis[35], immune response and inflammatory-related pathways[36-38]. It is widely acknowledged that inflammation plays a pivotal role in the progression of DN. Notably, renal inflammation has been demonstrated to contribute to the development of DN. Additionally, abnormal levels of IL-6, IL-18, and IL-1 have been identified as significant points to the development of DN [39,40]. Thus, the investigation of novel therapeutic approaches for DN now places significant emphasis on the pharmaceutical agents that specifically target inflammation.

The observed anti-diabetic activity of flavonoids has boosted their potential as therapeutic agents for DM and its complications. Indeed, it has been demonstrated that application of quercetin led to a reduction in blood glucose levels in a diabetes animal model induced by STZ[41]. More recently, it has been reported that kaempferol and myricetin combination treatment is promising in diabetes rats, due to their modulation of levels of glucose, inflammation, lipids and liver enzymes[42]. Another study has further demonstrated that DM could be alleviated by myricetin alone *via* its effects on normalizing the profile of intestinal flora[43]. The application of compounds in these contexts has collectively demonstrated the ability of a natural product to improve glucose levels and inflammatory cytokine levels in diabetic rats.

Our current findings indicate that the administration of myricetin at doses of 50 mg/kg or 100 mg/kg partially improved glucose levels, kidney/BW index, serum creatinine, creatinine clearance, and uACR in db/db mice. Furthermore, histopathological analysis revealed that myricetin significantly alleviated the DN pathological injury of renal tissue in mice. The administration of myricetin resulted in a reduction in inflammatory factors' infiltration of kidney tissues and a decrease in the proportion of type M1 macrophages. Additionally, the renal fibrosis of DN mice was improved, as evidenced by a significant decrease in the accumulation of Col1a1 and α-SMA in DN mice treated with myricetin. These findings align with previous research and provide further validation of the role of myricetin in inhibiting the pathological progression of DN mice.

Importantly, myricetin not only plays a vital role in DN but also decreases migration of retinal pericytes[44], restores impaired motor and sensory functions[45], and enhances diabetic wound repair[46]. To our knowledge, only one cross-sectional population clinical study (consisting of 24138 subjects, among which 1357 had type 2 DM) has shown that myricetin intake might lower the prevalence type 2 DM and extend the period until other clinical treatments become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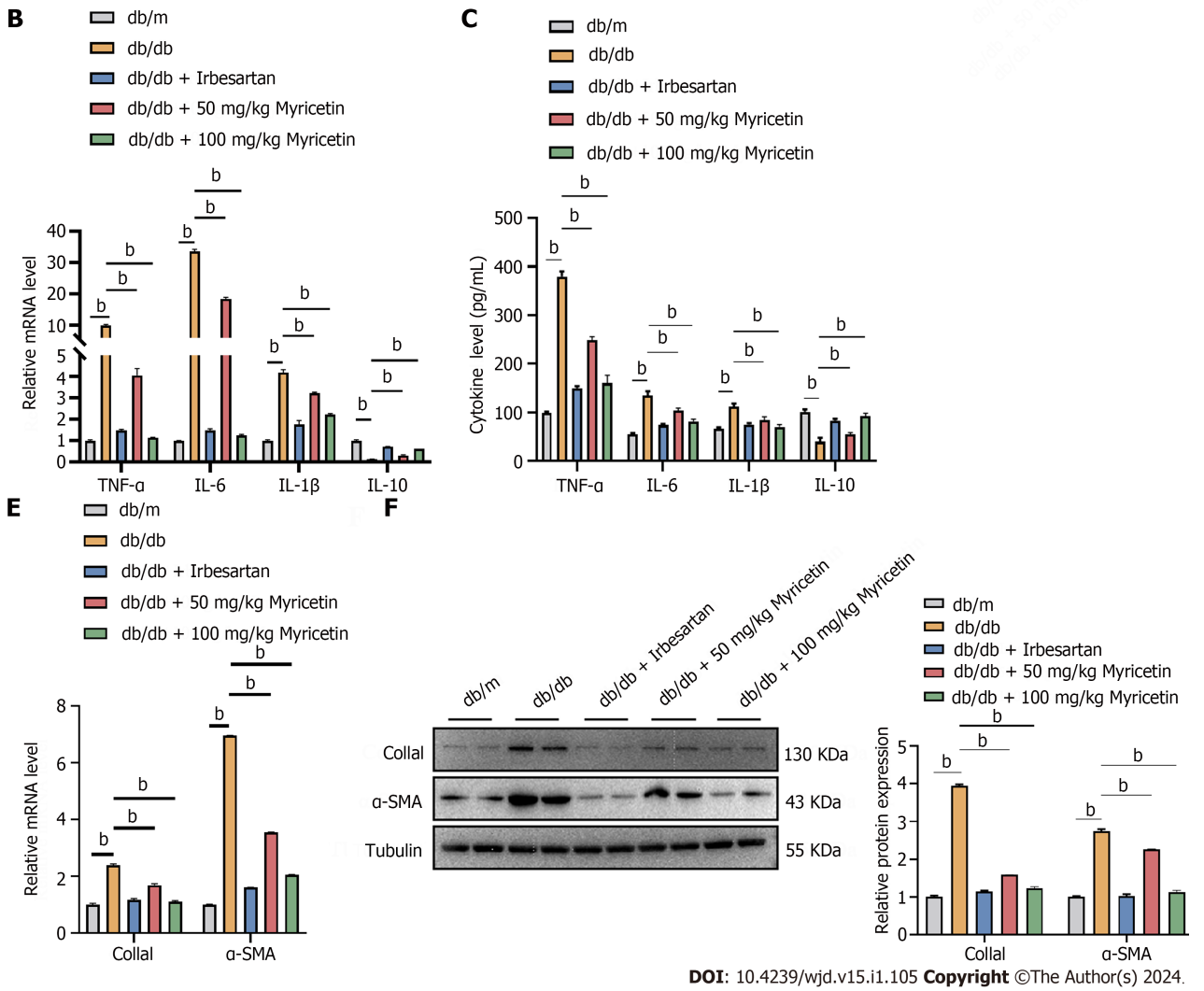

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Figure 3 Myricetin inhibited inflammation factors and fibrosis in the renal tissue of diabetic nephropathy mice. A: Immunofluorescent staining of F4/80 protein; B: Tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, and IL-10 mRNA levels; C: Serum TNF-α, IL-6, IL-1β, and IL-10 Levels; D: Immunofluorescent staining of collagen-1a1 (Col1a1) protein; E: Reverse transcription-PCR analysis of Col1a1 and alpha-smooth muscle actin (α-SMA) mRNAs; F: Western blotting analysis of Col1a1 and α-SMA proteins; G: Masson's trichrome staining (left) and renal fibrosis analysis (right); H: Sirius-red staining (left) and fibrosis analysis (right). TNF-α: Tumor necrosis factor-alpha; α-SMA: Alpha-smooth muscle actin; IL: Interleukin; Col1a1: Collagen-1a1. $^{b}P < 0.05$.

necessary[47]. Other studies have shown that *Abelmoschus manihot* capsule containing myricetin could be useful in decreasing proteinuria, blood creatinine and blood urea nitrogen in kidney patients[48,49].

Given the emphasis on the role of inflammation in the development of DM and its complications, our study aimed to investigate the mechanism by which myricetin regulates the serum levels of TNF-α, IL-6, and IL-1β in DN mice. We observed that myricetin exhibited anti-inflammatory effects by reducing inflammatory factors associated with M1. To further understand this mechanism, we performed experiments using RAW 264.7 cells and found that treatment with 25 μM myricetin effectively mitigated cell injury induced by the HG condition (specifically 33.3 mM glucose). The results indicated that myricetin exerted an inhibitory effect on the polarization of RAW 264.7 macrophages towards the M1-type and significantly suppressed the iNOS activity induced by HG. Furthermore, treatment with myricetin led to a significant downregulation of M1-dependent inflammatory factors, as evidenced by decreased IL-10 and Arg-1 expression and secretion. These findings suggested that myricetin modulated the polarization of RAW 264.7 macrophages towards the M2-type, which was implicated in the progression of DN.

Several proteins and pathways have been documented as capable of inducing alterations in the polarization of RAW 264.7 macrophages, such as the PI3K-Akt, Notch1, NF-κB, MAPKs, and JNK/STAT3 signaling pathways[50-53]. In the context of a diet-induced non-alcoholic steatohepatitis model in mice, myricetin was able to mitigate inflammatory hepatitis and fibrosis by modulating macrophage polarization. This was achieved through the inhibition of NF-κB signaling and STAT3 activation, as well as the phosphorylation of the signal transducer[54]. Furthermore, the administration of flavonoids showed ability to mitigate inflammation in lipopolysaccharide-stimulated RAW 264.7 cells through involvement of the NF-κB and MAPK pathways[55]. These data in the literature stimulated our interest to investigate the mechanism by which myricetin modulates macrophage polarization.

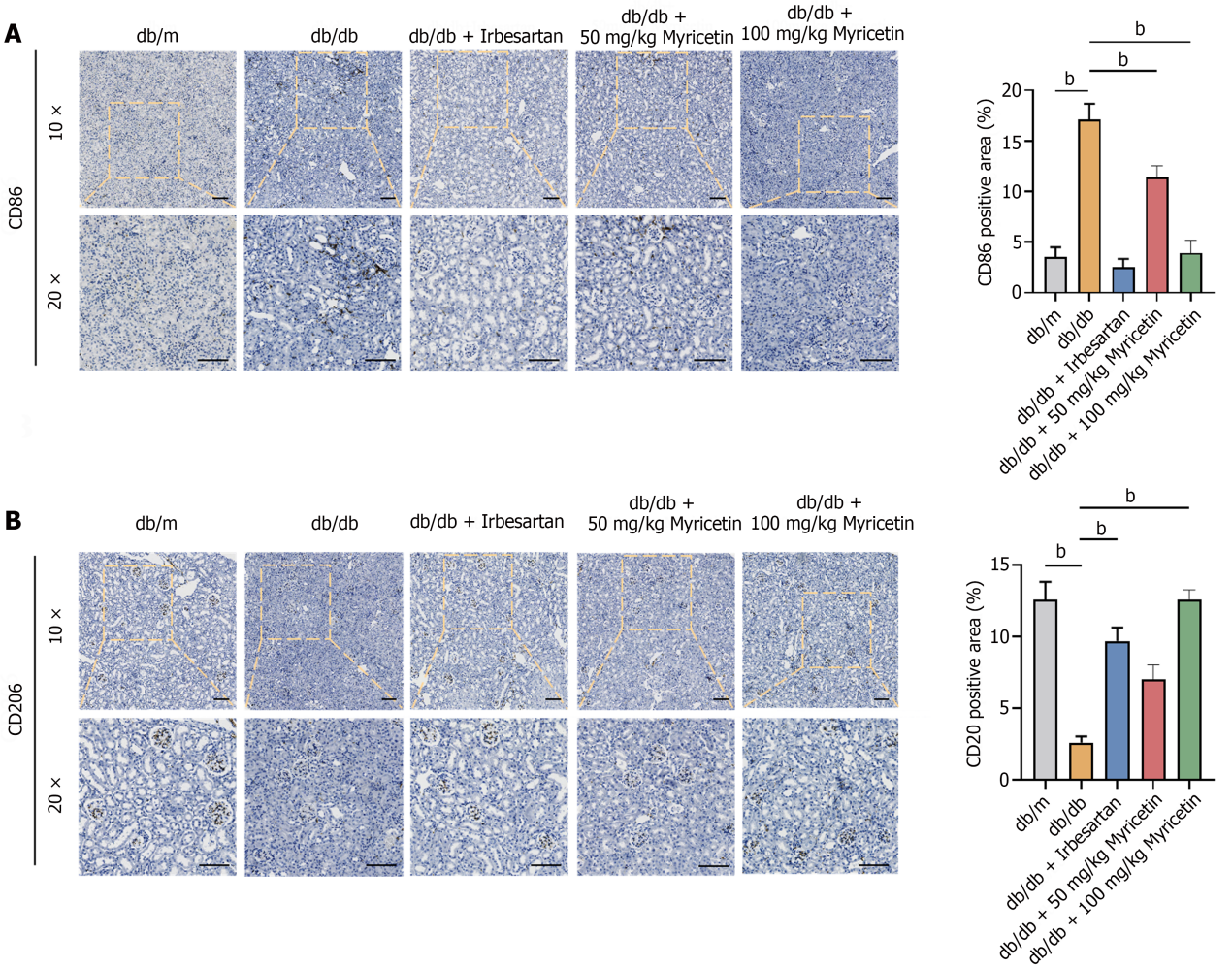

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Figure 4 Myricetin switched the phenotype of macrophages in renal tissues of db/db mice. A and B: Immunofluorescent staining of CD86 protein (A) and CD206 protein. Scale bar: 100 μm. $^{b}P < 0.05$.

Our bioinformatics analysis revealed that Akt was the primary target protein associated with immune response and inflammatory signaling pathways in DN mice and cultured cells. Additionally, our results indicated that myricetin had significant binding affinity with both Akt and PI3K. It has been reported that the PI3K/Akt pathway activates NF-κB through Akt phosphorylation, leading to the transcription of numerous inflammatory genes and receptors for advanced glycation products, and thereby promoting the production of cytokines that induce inflammation[56]. The association between up-regulated cytokines, including TNF-α, IL-1β, IL-1, IL-18, and DN and other diseases has been extensively documented[11,12]. Furthermore, the activation of the PI3K/Akt pathway has been identified as a critical factor in the polarization, migration, proliferation, and survival of macrophages[57]. The PI3K/Akt signaling pathway, through mTORC1, can regulate a macrophage's effector response, thereby modulating innate immune responses and macrophage polarization[58]. Genes related to PI3K/Akt signaling pathway deletion, including SHIP and PTEN, significantly inhibit the production of pro-inflammatory cytokines by enhancing the M2 macrophage phenotype[59]. Activation of the PTEN/PI3k/Akt pathway was reported to mediate the polarization of M2 macrophages among RAW 264.7 cells and in emphysematous mice[60].

In this study, we specifically inhibited the phosphorylation of Akt in myricetin-treated HG-induced RAW 264.7 cells, and in agreement with previous findings we found that the polarization of RAW 264.7 cells to M2-type was blocked; this indicated that myricetin can regulate the polarization of macrophages through the PI3K/Akt pathway and thus affect the secretion of cytokines (Figure 9). However, the PI3K/Akt pathway is well known for its ability to modulate various proteins and pathways, prompting the next question of interest: whether or not the function of myricetin in DN is due to its activation of Akt or other yet-unidentified proteins?

CONCLUSION

The results from this study suggest that high concentrations of myricetin have the potential to impede M1-type polarization of macrophages through the PI3K-AKT signaling pathway. Simultaneously, it exhibits promising efficacy in

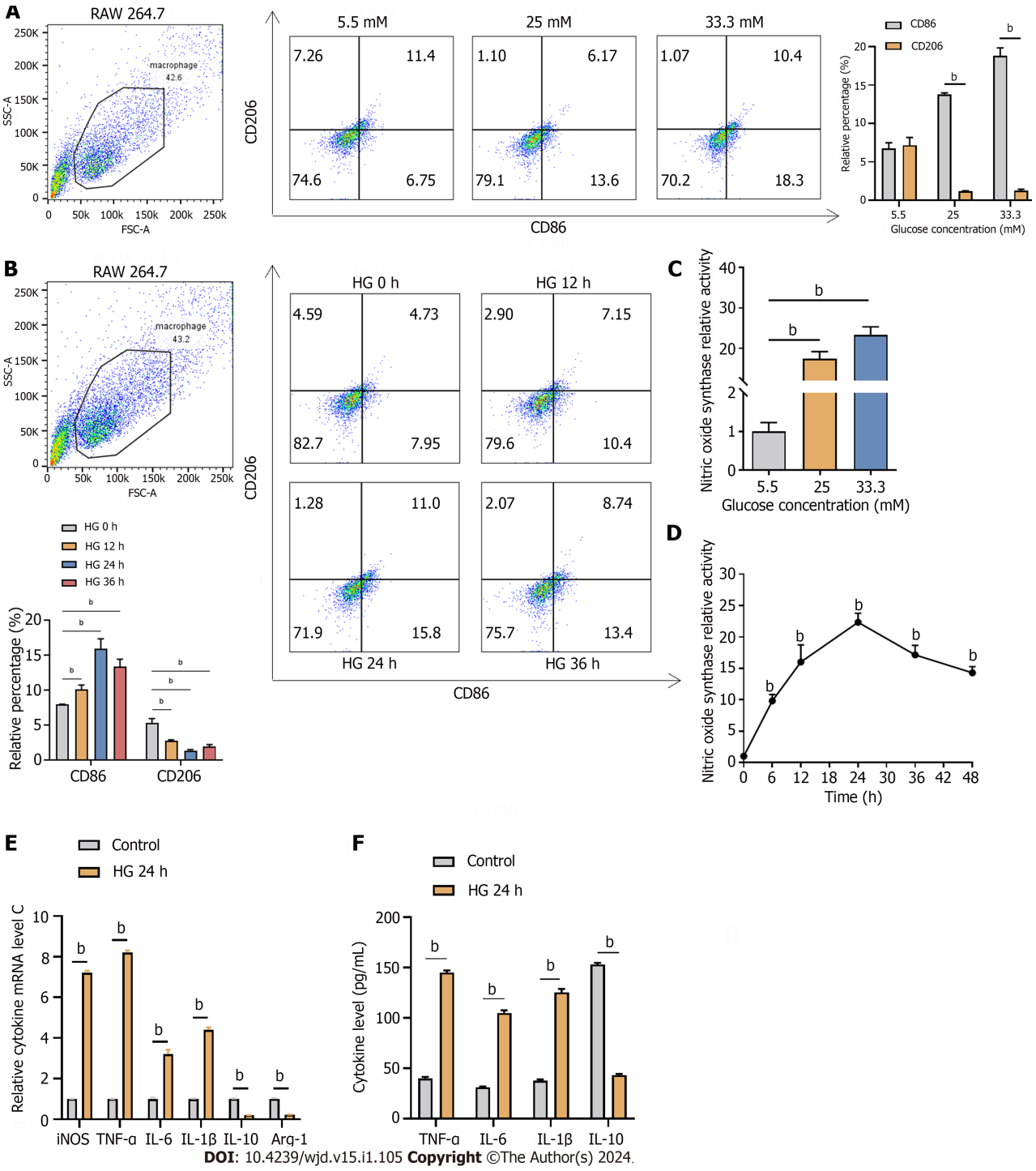

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Figure 5 High glucose induced the M1 macrophage polarization of RAW 264.7 cells. A and B: Flow cytometry analysis of RAW 264.7 macrophages labeled with CD86 and CD206 exposed to different concentrations of glucose (A) and to 33.3 mmol/L glucose for different times (B); C and D: Inducible nitric oxide synthase (iNOS) levels induced by different concentrations of glucose (C) and by 33.3 mmol/L glucose for different times (D); E and F: Relative cytokine mRNA (E) and protein (F) levels of iNOS, tumor necrosis factor-alpha, interleukin (IL)-6, IL-1β, IL-10, and arginase-1 after exposure to 33.3 mmol/L glucose for 24 h detected by reverse transcription-PCR (E) and western blotting (F). Arg-1: Arginase-1; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; iNOS: Inducible nitric oxide synthase; HG: High-glucose. $^{b}P < 0.05$.

the treatment of renal injury, inflammation, and fibrosis in mice with DN. Our study provides a proof-of-concept of the function of myricetin against the progress of kidney injury induced by DM and provides more fundamental data for the development of myricetin as a bona fide treatment for diabetics.

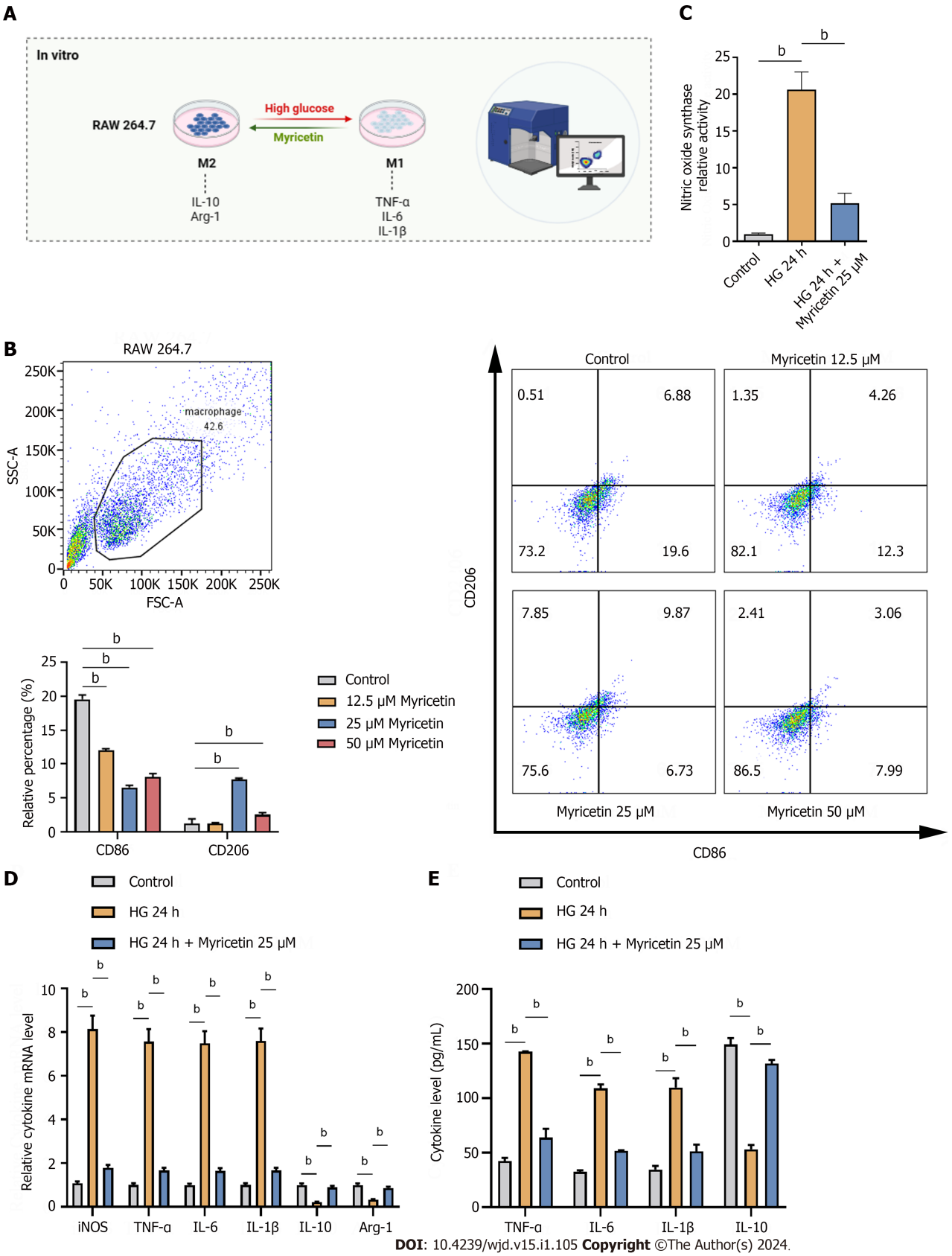

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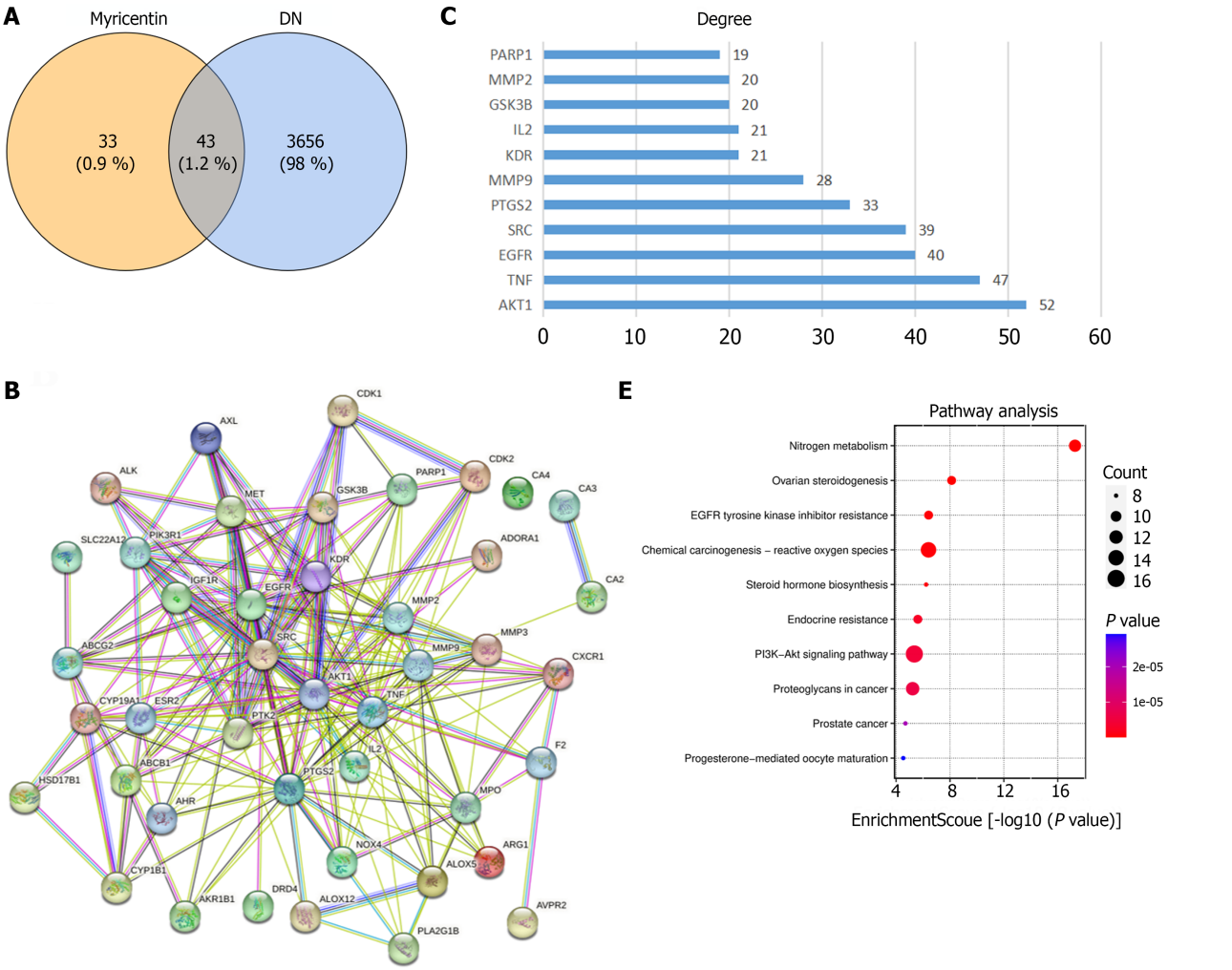
Figure 6 Myricetin regulated the polarization of RAW 264.7 cells to M2-type induced by high glucose. A: *In vitro* assay; B: Flow cytometry analysis of CD86-labeled and CD206-labeled RAW 264.7 macrophages induced with 33.3 mmol/L glucose and different concentration of myricetin; C: Levels of inducible nitric oxide synthase (iNOS) induced by 33.3 mmol/L glucose and after treatment with 25 μM of myricetin detected by ELISA; D and E: Levels of iNOS, tumor necrosis factor-alpha, interleukin (IL)-6, IL-1β, IL-10 and arginase-1 mRNAs (D) and proteins (E) induced with 33.3 mmol/L glucose and after treatment with 25 μM of myricetin detected by reverse transcription-PCR (D) and western blotting (E). Arg-1: Arginase-1; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; iNOS: Inducible nitric oxide synthase; HG: High-glucose. $^{b}P < 0.05$.


A
Myricentin
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33
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Degree
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IL2 21
KDR 21
MMP9 28
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CDK1
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ALK
MET
GSK3B
PARP1
CDK2
CA4
CA3
SLC22A12
PIK3R1
ADORA1
KDR
IGF1R
EGFR
CA2
MMP2
ABCG2
SRC
MMP3
MMP9
CXCR1
AKT1
CYP19A1
ESR2
TNF
PTK2
F2
IL2
ABCB1
HSD17B1
PTGS2
MPO
AHR
NOX4
ARG1
ALOX5
CYP1B1
AKR1B1
DRD4
ALOX12
AVPR2
PLA2G1B
E
Pathway analysis
Nitrogen metabolism
Ovarian steroidogenesis
EGFR tyrosine kinase inhibitor resistance
Chemical carcinogenesis – reactive oxygen species
Steroid hormone biosynthesis
Endocrine resistance
PI3K–Akt signaling pathway
Proteoglycans in cancer
Prostate cancer
Progesterone–mediated oocyte maturation
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bicarbonate transport
peptidyl–serine modification
response to amyloid–beta
peptidyl–serine phosphorylation
aminoglycoside antibiotic metabolic process
transferase complex, transferring phosphorus–containing groups
protein kinase complex
apical part of cell
basolateral plasma membrane
membrane raft
membrane microdomain
membrane region
chromosomal region
apical plasma membrane
chromosome, telomeric region
carbonate dehydratase activity
hydro–lyase activity
protein serine/threonine kinase activity
alditol:NADP+ 1–oxidoreductase activity
carbon–oxygen lyase activity
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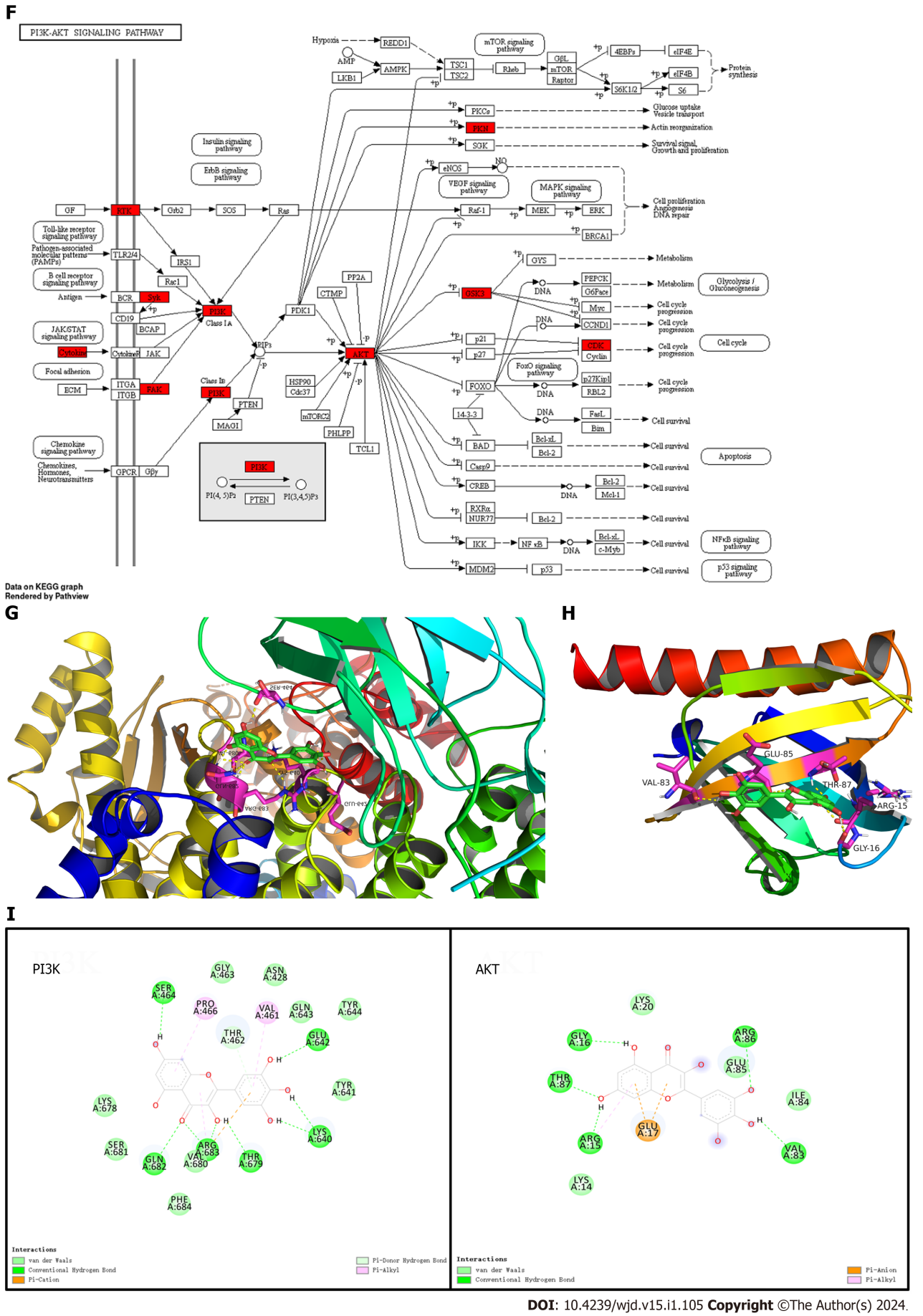

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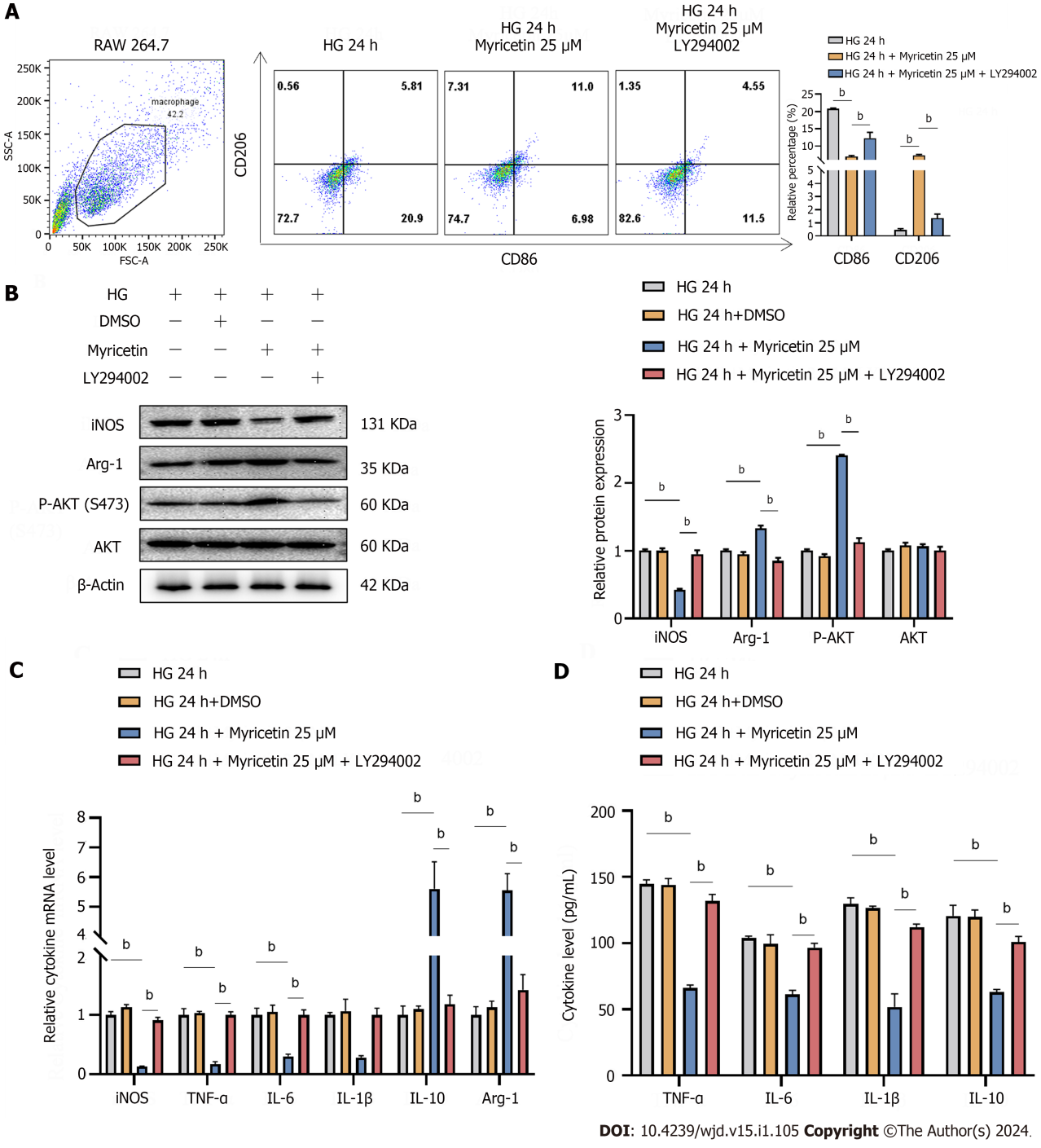
Figure 7 Identification of potential targets of diabetic nephropathy and myricetin using bioinformatics analysis. A: Venn diagrams; B: Protein-

protein interaction network of myricetin and diabetic nephropathy targets, in which edge points represent protein interactions and line thickness indicates data strength; C: Myricetin's key target gene for treating diabetic nephropathy; D: Potential targets for myricetin treatment of diabetic nephropathy from the Gene Ontology algorithm; E: Myricetin treatment pathways from the Kyoto Encyclopedia of Genes and Genomes database; F: Signaling pathway of PI3K-Akt; G and H: The 3D structures of the docking mode of myricetin and PI3K (G) and myricetin and Akt (H); I: Visualization of the binding abilities of myricetin with PI3K and Akt. DN: Diabetic nephropathy.



DOI: 10.4239/wjd.v15.i1.105

Figure 8 Myricetin regulated the polarization of RAW 264.7 cells through the PI3K/Akt signaling pathway in diabetic nephropathy mice. A-D: CD86-labeled and CD206-labeled RAW 264.7 macrophages under the 33.3 mmol/L glucose condition and after 25 μM myricetin treatment and LY294002 administration assessed by flow cytometry (A) and for protein levels of inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1), phosphorylated-Akt (at S473) and Akt by western blotting (B) or mRNA levels of iNOS, tumor necrosis factor-alpha, interleukin (IL)-6, IL-1β, IL-10, and Arg-1 by reverse transcription PCR (C), and for secreted levels of TNF-α,IL-6, IL-1β, and IL-10 by ELISA. Arg-1: Arginase-1; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; iNOS: Inducible nitric oxide synthase; HG: High-glucose. $^{b}P < 0.05$.

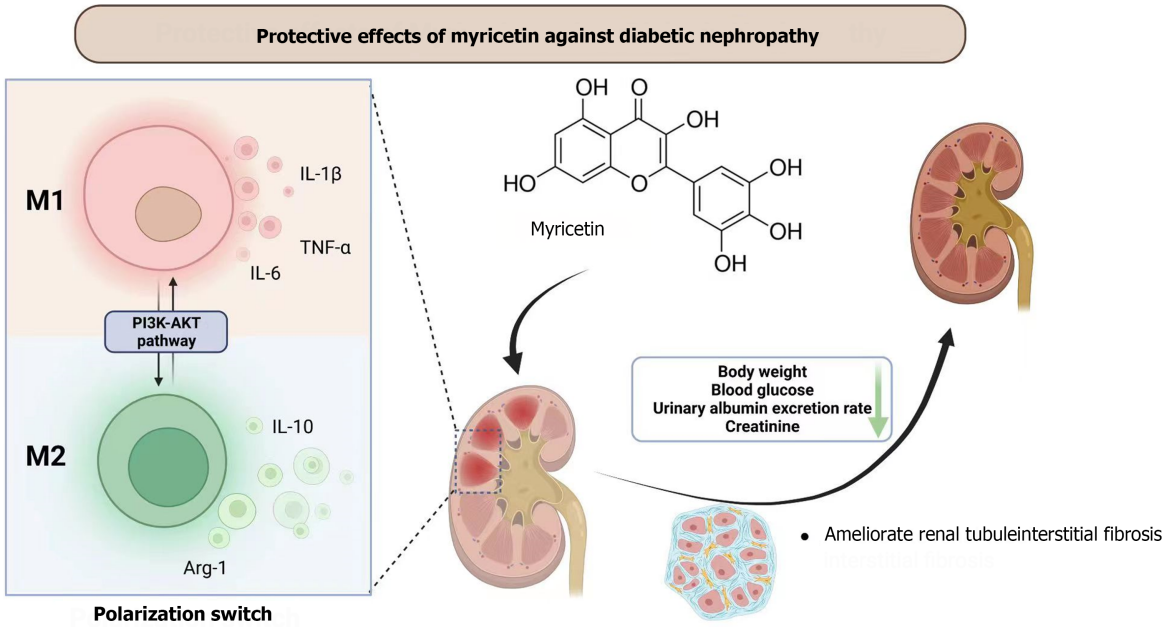


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Figure 9 Overview of protective effects of myricetin against diabetic nephropathy. Myricetin can regulate the polarization of macrophages through the PI3K/Akt signaling pathway to ameliorate the injuries of kidneys in mice with diabetic nephropathy. Arg-1: Arginase-1; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin.

ARTICLE HIGHLIGHTS

Research background

Diabetic nephropathy (DN) is frequently observed as a chronic microvascular complication linked to end-stage renal disease, and it constitutes a significant contributor to both disability and mortality worldwide. Current therapies merely delay renal injury by controlling metabolic disturbances that occur in the early stage and, as such, there remains an urgent need to seek out and develop new drugs for clinical use. To this end, we have performed focused research on the Chinese patent medicine *Abelmoschus manihot* for its ability to decrease proteinuria in patients with DN.

Research motivation

Previous studies have indicated that myricetin possesses the potential to mitigate the pathological alterations observed in renal tissues of DN patients and models. Nevertheless, the precise molecular mechanism through which myricetin influences the progression of DN remains uncertain.

Research objectives

To investigate the effects of myricetin on DN and explore the underlying mechanisms of its potential therapeutic effects.

Research methods

Db/db diabetic mice were administered myricetin and effects on blood and urine indexes and renal tissue pathology were assessed. Additionally, the RAW 264.7 cell line was cultured in high glucose conditions and then exposed to the PI3K/Akt inhibitor LY294002. In both the *in vivo* and *in vitro* settings, quantification of various inflammation factors' levels was conducted using western blotting, real-time qPCR and ELISA.

Research results

In the db/db mice, myricetin had a mitigating effect on renal dysfunction and fibrosis, including kidney injury markers kidney injury molecule-1 and neutrophil gelatinase associated lipocalin and inflammatory cytokine-related factors. In the RAW 264.7 cells, myricetin treatment effectively inhibited the up-regulation of tumor necrosis factor-alpha, interleukin (IL)-6, and IL-1β and modulated M1-type polarization. Molecular docking and bioinformatic analyses revealed that Akt was the target of myricetin. The protective effect of myricetin was nullified upon blocking the polarization of RAW 264.7 *via* inhibition of PI3K/Akt activation using LY294002.

Research conclusions

Myricetin effectively mitigates kidney injury in DN mice through the regulation of macrophage polarization *via* the PI3K/Akt signaling pathway.

Research perspectives

Myricetin represents a promising therapy in treating DN.

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FOOTNOTES

Co-first authors: Wei-Long Xu and Pei-Pei Zhou.

Co-corresponding authors: Xiao Wu and Jiang-Yi Yu.

Author contributions: Xu WL and Yu JY designed the study; Xu WL and Zhou PP carried out the experiments; Tian T, Yu X, and Bao JJ contributed experiment assistance; Yu X, Bao JJ, and Zha M analyzed the data; Xu WL and Zhou PP generated the figures; Ni CR donated the myricetin natural product; Xu WL, Zhou PP, Yu JY, and Wu X drafted and revised the manuscript; Yu JY and Wu X conceived and supervised the study; All authors approved the final version of the article. Xu WL and Zhou PP contributed equally to this work as co-first authors. Xu WL and Zhou PP together completed the chief experiments, formation of figures, and writing of initial manuscript, which were the most important and indispensable part of this study. Especially, Xu WL designed the study. Yu JY and Wu X contributed equally to this work as co-corresponding authors. Yu JY and Wu X revised the manuscript, conceived and supervised the study to make the study better presented. Especially, Yu JY provided enough financial support in the progress of experiments and ensured all the journal's administrative requirements.

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Institutional animal care and use committee statement: Pathogen-free environments and *ad libitum* feeding were ensured for all animals. In accordance with its Ethics Committee, Jiangsu Province Hospital of Chinese Medicine approved the procedures for care and use of animals [QK-20200408-001]. Full compliance with all applicable institutional and governmental regulations regarding animal ethics was maintained throughout the study.

Conflict-of-interest statement: The authors declare that there were no commercial nor financial relationships that could be considered as potential conflicts of interest in the research.

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ARRIVE guidelines statement: The authors have read and the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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LETTER TO THE EDITOR

Nutrition interventions and clinical outcomes of pregnant women with gestational diabetes mellitus: More than meets the eye

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Abstract

In the retrospective study by Luo *et al* regarding clinical outcomes in gestational diabetes mellitus (GDM), the results are statistically significant in favour of the benefits of individualized nutrition interventions enumerated therein. The study has provided important evidence to improve maternal and child health in the Asian population. The methods, however, appear to have considerable limitations, wherein the time point of diagnosis of GDM, severity of GDM, selection bias, compliance to therapy, important maternal covariates, observable microvascular abnormalities and the confounding effect of added insulin have not been considered. We have provided suggestions to improve the external validity of the study, including the use of Equator Network reporting guidelines and inclusion of overweight and obese patients in future studies.

Key Words: Glucose intolerance; Hyperglycemia; Obesity; Pregnancy; Research methodology

Core Tip: In the retrospective study by Luo *et al* regarding clinical outcomes in gestational diabetes mellitus (GDM), the results are statistically significant in favour of the benefits of individualized nutrition interventions enumerated therein. The study has provided important evidence to improve maternal and child health in the Asian population. The methods, however, appear to have considerable limitations, wherein the time point of diagnosis of GDM, severity of GDM, selection bias, compliance to therapy, important maternal covariates, observable microvascular abnormalities and the confounding effect of added insulin have not been considered. We have provided suggestions to improve the external validity of the study, including the use of Equator Network reporting guidelines and inclusion of overweight and obese patients in future studies.

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TO THE EDITOR

We read with great interest the retrospective study by Luo *et al*[1] and congratulate the authors on their effort to establish the role of individualized nutrition interventions on clinical outcomes in gestational diabetes mellitus (GDM).

The authors have chosen a very important and relevant contemporary topic affecting the health of pregnant women and their newborns, and have examined their concerns for different complications and effects in detail. They have well highlighted the importance of individualized nutrition intervention programmes to tackle these concerns.

The authors have analyzed and compared intervention and control group data to arrive at a well-formed conclusion about the role of individualized nutrition interventions. Their conclusion, that pregnancy weight gain in GDM could be considerably controlled by careful modulation of diet and exercise, which can, in turn, improve glycolipid metabolism and reduce perinatal complications, is in concurrence with previous research from the Asian population[2].

The authors have described the limitations of the study honestly, and have overall contributed to providing significant evidence for the benefit of maternal and child health of the Chinese population. However, we find it pertinent to emphasize the following, the consideration of which could have further strengthened the evidence generated by the study.

GDM is glucose intolerance that is first diagnosed during pregnancy most commonly at 24–28 wk gestation[2]. However, the sample in the study was not stratified at the outset with respect to the gestational age at which gestational diabetes was detected, or the severity of GDM at diagnosis. Hence, the time point of initiation of therapy for the subjects in each of the groups may have been further elucidated.

It remains unclear how this study followed a retrospective data collection timeline, and the group size and method of selection of patients in the two groups is also not explained. Equator Network guidelines for reporting in the description of methodology, in our view, improve the external validity of the study.

The interventions in the study seem subject to selection bias, given that the authors have not specified the protocol of individualization. For example, in patients with inflammatory bowel disease, a range of exercises have been shown to be safe including moderate intensity aerobic exercise, resistance training and high intensity interval training[3]. It would be clinically useful to know what intensity and duration of dietary and exercise-related interventions would benefit what levels of blood sugar in patients with GDM.

The authors have not explored whether those following an individualized regimen had a higher level of compliance compared to the other group. If the authors obtained patient records retrospectively, it is also not apparent as to how the compliance to therapy was cross-verified.

Complications reported by the authors fall into a debatable circumstance – it appears that in a study of over 400 patients, no patient had more than one complication. Caesarean section can be indicated in patients with premature rupture of membranes if induced labour does not progress, as well as in advanced cases of pregnancy-induced hypertension. Factors such as cephalopelvic disproportion that could have led to more cesarean sections in the conventional group could have been detailed further.

The most important biochemical parameters in the study were fasting blood sugar of 5.1 mmol/L and post-prandial blood sugar of 6.7 mmol/L. One of the important complications of high blood sugar levels in pregnancy is the increased risk of microvascular disturbances. Some authors have suggested that there is no risk of ocular complications in gestational diabetes[4]. However, others have emphasized that GDM is a significant risk factor for long-term ophthalmic morbidity as patients with history of GDM had significantly higher incidence of glaucoma, diabetic retinopathy and retinal detachment compared to controls[5]. A series of retinal arteriolar abnormalities, including narrower caliber, reduced fractal dimension and larger branching angle have been observed in GDM at 26-28 wk of gestation. These are attributable to a relatively hypoxic state of the retina, although the causal relationship needs further elucidation[6]. A significant proportion of women develop diabetes mellitus years following GDM. Diabetic retinopathy will affect about one-fifth of them[7]. It would have been useful if ophthalmoscopic findings could also have been included in the study as an outcome measure, as the state of the retinal vasculature is a true reflection of small blood vessels elsewhere in the body [6].

The authors were ethically correct in starting insulin in addition to nutritional intervention therapy if the blood glucose remained uncontrolled after an interval of 2 wk of individualised nutrition intervention. However, we feel that this appears to be too short an interval for the intervention to have any significant impact on blood glucose. The authors have not specified what proportion of patients required insulin, and thus the confounding effect of insulin on outcomes of the intervention group cannot be ruled out.

In addition, the study makes no mention of important maternal covariates such as pre-pregnancy weight, past history of GDM, and family history of diabetes. Blood pressure at baseline has not been mentioned and it is unclear if differences in predisposition to pregnancy-induced hypertension existed between the two groups at the beginning of the study. Obesity is itself a risk factor for GDM[8]. It is also not clear whether the weight or BMI of subjects in the two groups at the time of diagnosis of GDM has been considered which would again influence the outcome of the study. As the pre-pregnancy BMI is normal in both groups, we believe that validation of the study outcomes through a prospective study with a carefully stratified, overweight and obese GDM cohort is required for it to be more clinically useful in day-to-day practice[9].

Once again, we congratulate the authors on providing important suggestions about non-pharmacologic management of GDM to improve maternal-fetal outcomes, and hope that future research in this direction includes the aforementioned considerations to further strengthen the evidence.

FOOTNOTES

Author contributions: Morya AK designed and formulated the research; Nishant P, Sinha S and Prasad R performed research; Sinha S and Sinha RK analyzed data and wrote the letter; and Nishant P revised the letter; All the authors have read and approved the final manuscript; Sinha S, Nishant P and Sinha RK analyzed the existing data and performed extensive literature search to justify the analysis presented in the manuscript.

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