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ABOUT COVER

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EDITORIAL

Potential mechanism of teneligliptin in the treatment of diabetic cardiomyopathy

Jing Guo, Yi Cao, Qing-Yuan Wu, Lu-Sha Cen

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Abstract

Diabetic cardiomyopathy (DCM), a complication of diabetes, poses a significant threat to public health, both its diagnosis and treatment presents challenges. Teneligliptin has promising applications and research implications in the treatment of diabetes mellitus. Zhang *et al* observed the therapeutic effect of teneligliptin on cardiac function in mice with DCM. They validated that teneligliptin's mechanism of action in treating DCM involves cardiomyocyte protection and inhibition of *NLRP3* inflammasome activity. Given that the *NLRP3* inflammasome plays a crucial role in the onset and progression of DCM, it presents a promising therapeutic target. Nevertheless, further clinical validation is required to ascertain the preventive and therapeutic efficacy of teneligliptin in DCM.

Key Words: Teneligliptin; *NLRP3* inflammasome; Diabetes; Diabetic cardiomyopathy; Diabetes complications

Core Tip: Diabetic cardiomyopathy (DCM) is a complication of diabetes, presenting significant challenges in both diagnosis and treatment of DCM. Zhang *et al* observed the therapeutic effect of teneligliptin on cardiac function in mice with DCM. They confirmed that teneligliptin functions by protecting cardiomyocytes and mitigating inflammation by inhibiting *NLRP3* inflammasome activity. This discovery offers clinical management of DCM patients; however, its clinical application necessitates further clinical verification and discussion.

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INTRODUCTION

The number of patients with diabetes mellitus (DM) is expected to reach 783 million by 2045 worldwide[1]. Diabetic cardiomyopathy (DCM), a distinct diabetes-associated cardiac complication, ranks among the primary causes of death in patients with diabetes. DCM is characterized by structural alterations and functional irregularities of the heart, coronary atherosclerosis, significant valvular heart disease, and the absence of hypertension[2]. The pathophysiology of DCM involves various molecular processes, such as hyperglycemia, insulin resistance, accelerated fatty acid oxidation, oxidative stress, mitochondrial dysfunction, and endothelial dysfunction[3]. Given that the progression of DCM correlates with chronic inflammation and cardiomyocyte demise, ultimately leading to heart failure[4], its prevention and treatment merit urgent attention.

DCM AND *NLRP3* INFLAMMASOME

Cardiac inflammation manifests in the early stages of diabetes, with the development of DCM being mainly attributable to the nod-like receptor (NLR) family pyrin domain containing 3 (*NLRP3*) inflammasome. In DCM, activation of the *NLRP3* inflammasome in cardiomyocytes triggers pyroptosis of the heart cells, aggravating the cardiac condition[5]. Using a diabetic mouse model, Song *et al*[6] revealed that sirtuin 3 deficiency aggravated hyperglycemia-induced mitochondrial damage, increased reactive oxygen species accumulation, activated the *NLRP3* inflammasome, and ultimately aggravated DCM. Zhang *et al*[7] discovered that high glucose stimulation in a diabetic cell model activates the *NLRP3* inflammasome, leading to increased secretion of interleukin-1β by neonatal rat ventricular myocytes, and subsequent induction of myocardial injury[8]. Moreover, inhibition or silencing of the *NLRP3* inflammasome gene has shown potential therapeutic effects in DCM. Gao *et al*[9] found that inhibiting the *NLRP3* inflammasome could effectively suppress the pyrodeath of cardiomyocytes. Yang *et al*[10] discovered that metformin demonstrates cardioprotective and anti-inflammatory effects in DCM by activating adenosine 5′-monophosphate-activated protein kinase/autophagy and subsequently inhibiting the *NLRP3* inflammasome. In conclusion, these findings suggest that the *NLRP3* inflammasome represents a promising molecular target in DCM, emphasizing the significance of interventions that can target the activity of this immune system complex for effectively managing the cardiac complications[11].

In clinical practice, DCM treatment includes conventional cardiovascular and anti-glycemic drugs, as well as new therapies such as coenzyme Q10, MicroRNA, and stem cell therapy[12]. However, each method has its limitations: For example, traditional cardiovascular drugs are applied only at the heart of DCM development and have more obvious symptoms when applicable. Conventional hypoglycemic drugs have insignificant efficacy, and sodium-glucose cotransporter-2 inhibitors is the only first-line drug recommended for DCM[13]. Teneligliptin, a dipeptidyl peptidase-4 inhibitor, is a newer drug used in the management of type 2 DM (T2DM). Teneligliptin has promising applications in the treatment of DM and its associated complications and thus warrants further research. Because it can be used in patients with T2DM with renal and/or mild-to-moderate hepatic impairment, it has a unique place in therapy[14]. This drug has the advantages of being inexpensive and safe, improving blood glucose consistently (decreasing the glycated hemoglobin A1c value), and being available to patients with mild to moderate hepatic impairment. More reassuringly, patients with mild, moderate, or severe renal impairment or end-stage kidney disease can safely take the drug without dose adjustment [15,16]. Wang and Zhang[17] showed that teneligliptin attenuated diabetes-related cognitive impairment by inhibiting endoplasmic reticulum stress and the *NLRP3* inflammasome in diabetic mice. Few studies have investigated the effect and mechanism of action of teneligliptin on *NLRP3* inflammatory vesicles. Although the study by Zhang *et al*[7] introduced the concept of applying teneligliptin to treat DCM in patients with kidney damage, the model in that study is more similar to type 1 DM, and the suggested clinical application of this drug requires further research and discussion.

Ultimately, the incidence of cardiac issues correlates closely with the severity of diabetes, and the utilization of hypoglycemic drugs may exert dual effects on both the preventing and treating DCM. The study by Zhang *et al*[7] introduces a novel concept for the clinical management of DCM patients with kidney damage, offering a promising avenue for therapeutic intervention. However, the preventive and therapeutic effects of teneligliptin on DCM require further validation through large-scale clinical trials. Additionally, the question of whether it is covered by medical insurance warrants consideration.

CONCLUSION

Cardiac inflammation contributes to the onset and progression of DCM, which is often associated with *NLRP3* inflammasome activation. Traditional drugs for heart treatment can only be administered when cardiac symptoms are evident, underscoring the importance of identifying preventive measures for DCM. Through in vivo and in vitro experiments, teneligliptin has been shown to inhibit *NLRP3* inflammasome activity and exert anti-inflammatory and protective effects in cardiomyocytes. Although large-scale clinical studies are still needed, the *NLRP3* inflammasome represents a novel target of teneligliptin for the clinical treatment of DCM.

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EDITORIAL

Utilising continuous glucose monitoring for glycemic control in diabetic kidney disease

Vamsidhar Veeranki, Narayan Prasad

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Abstract

In this editorial, we comment on the article by Zhang *et al*. Chronic kidney disease (CKD) presents a significant challenge in managing glycemic control, especially in diabetic patients with diabetic kidney disease undergoing dialysis or kidney transplantation. Conventional markers like glycated haemoglobin (HbA1c) may not accurately reflect glycemic fluctuations in these populations due to factors such as anaemia and kidney dysfunction. This comprehensive review discusses the limitations of HbA1c and explores alternative methods, such as continuous glucose monitoring (CGM) in CKD patients. CGM emerges as a promising technology offering real-time or retrospective glucose concentration measurements and overcoming the limitations of HbA1c. Key studies demonstrate the utility of CGM in different CKD settings, including hemodialysis and peritoneal dialysis patients, as well as kidney transplant recipients. Despite challenges like sensor accuracy fluctuation, CGM proves valuable in monitoring glycemic trends and mitigating the risk of hypo- and hyperglycemia, to which CKD patients are prone. The review also addresses the limitations of CGM in CKD patients, emphasizing the need for further research to optimize its utilization in clinical practice. Altogether, this review advocates for integrating CGM into managing glycemia in CKD patients, highlighting its superiority over traditional markers and urging clinicians to consider CGM a valuable tool in their armamentarium.

Key Words: Chronic kidney disease; Diabetic kidney disease; Glycemic control; Continuous glucose monitoring; Glycated hemoglobin; Glycemic variability

Core Tip: Continuous glucose monitoring (CGM) emerges as a transformative tool, offering real-time insights into glycemic variability among diabetic patients with chronic kidney disease (CKD), particularly during dialysis and post-transplantation phases. Innovations include CGM's ability to accurately detect hyper- and hypoglycemic events, aiding in timely therapeutic adjustments to mitigate risks. Studies demonstrate CGM's superiority over traditional markers like glycated haemoglobin in capturing acute glycemic fluctuations, particularly in dialysis patients, mainly due to the shorter life span of red blood cells, besides maintaining accuracy across all CKD stages, including those on peritoneal dialysis. CGM has substantive potential in individualized glycaemic management of CKD.

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INTRODUCTION

Chronic kidney disease (CKD) poses a significant challenge in managing glycemic control, particularly in diabetic patients who develop diabetic kidney disease (DKD). DKD is a prevalent complication of diabetes mellitus (DM), affecting 20%-25% of patients with type-2 DM[1,2]. DKD patients may experience frequent episodes of hypoglycemia with the progression of kidney disease. This is mainly due to impaired renal gluconeogenesis, defective renal clearance of insulin, elevated insulin resistance, and diminished β-cell function. With limitations of glycated haemoglobin (HbA1c), glycated albumin and fructosamine in CKD, the application of continuous glucose monitoring (CGM) in patients with diabetes is promising. In this comprehensive review, Zhang *et al*[3] have reviewed how glycemic control in these populations is crucial, yet conventional markers may not accurately reflect glycemic fluctuations.

LIMITATIONS OF CONVENTIONAL GLYCEMIC MARKERS

Although HbA1c is widely used as the primary method for monitoring blood sugar levels, its accuracy can be compromised by conditions such as anemia and renal dysfunction. Factors like reduced lifespan of red blood cells, anemia, blood transfusions, and the use of drugs that stimulate red blood cell production or iron supplements can falsely show lower HbA1c levels[4,5]. The risk of hypoglycemia and hyperglycemia is particularly heightened in patients in advanced stages of CKD and those undergoing dialysis. With the progression of renal decline and initiation of dialysis, glycemic variability (GV) can be further affected due to changes in glucose content in dialysates and the effects on insulin metabolism in failing kidney tubules. Monitoring glycemia is essential for effective management of DKD. Glycated albumin and fructosamine are suggested as alternatives for long-term blood sugar monitoring. These markers indicate blood sugar levels over a shorter period (2–4 weeks) compared to HbA1c, as they have a shorter lifespan in the bloodstream. Nonetheless, unlike direct blood glucose measurements, the glycated albumin and fructosamine assays can be affected by low albumin levels, which is often seen in nutritionally deprived CKD patients[6,7]. Hence, exploring alternative methods, such as CGM, is currently a necessity.

CGM AND ITS ADDED ADVANTAGE IN GLYCEMIC MANAGEMENT

CGM devices employ minimally invasive sensors that penetrate subcutaneous tissue to measure interstitial glucose. Interstitial glucose diffuses into the sensor's filament *via* capillary action, where it undergoes electrochemical reactions to determine its concentration. Real-time interstitial glucose readings are then transmitted to a mobile device for continuous monitoring. CGM offers a more reliable glycemic evaluation for patients with diabetes and CKD, including ESKD, by providing continuous, real-time glucose readings without frequent finger-pricking. CGM systems can be classified into professional, real-time (rt-CGM), and intermittently scanned (flash CGM) devices. The performance of CGM sensors in advanced CKD can be affected by factors like oxygen levels, uric acid, and exogenous substances, although CGM generally provides accurate monitoring. This makes CGM a valuable tool in managing diabetes in CKD patients, improving glycemic control and patient quality of life. Studies have shown that the correlation between HbA1c and mean sensor glucose decreases in advanced CKD stages, with HbA1c being less reliable as the CKD progresses. CGM-derived metrics, such as the glucose management index, have been proposed as alternatives for glycemic evaluation in CKD patients[8]. The 2020 Kidney Disease: Improving Global Outcomes guidelines suggest using glucose management indicator in advanced CKD or dialysis patients[9]. Time-in-range (TIR) metrics are also recommended for managing glycemia, though their validity and prognostic value in advanced CKD need further clinical trials.

GV and the role of CGM in the management of DM in progressive kidney disease

High GV is correlated with the pathogenesis and progression of diabetic-related complications, heightened risk of

hypoglycemia, and reduced patient quality of life[10,11]. GV is increasingly recognized as a pivotal parameter in glycemic management. CGM has become crucial in diabetes management because it continuously tracks glucose levels and offers a detailed picture of GV. Unlike traditional methods like self-monitoring of blood glucose (SMBG), which only provide snapshot measurements, CGM captures real-time data, enabling better management of hyperglycemia and hypoglycemia. GV is linked to diabetic complications, including microvascular issues like retinopathy, nephropathy, and neuropathy, as well as macrovascular complications like cardiovascular disease[12]. High GV increases the production of reactive oxygen species (ROS), activating pathogenic mechanisms such as the polyol pathway, advanced glycation end-products, protein kinase C, and the hexosamine pathway. This oxidative stress contributes to endothelial dysfunction and thereby increasing the risk of micro and macrovascular complications[13].

Long-term GV (variations over weeks to months), assessed by HbA1c and fasting/postprandial glucose levels, is associated with vascular complications and mortality. On the other hand, short-term GV (within-day and between-day glycemic fluctuations), measured by CGM indices like standard deviation, coefficient of variation, and TIR, is linked to diabetic retinopathy, kidney disease, peripheral neuropathy, and cardiovascular issues[14]. Various combinations of therapeutics, including dipeptidyl peptidase-4 inhibitors, sodium-glucose cotransporter-2 inhibitors, GLP-1 receptor agonists along with specific insulin preparations were found to have an improved GV and TIR across various studies[15-17]. However, whether this will benefit the end-organ damage is yet to be seen. CGM's detailed monitoring offers significant advantages over SMBG, highlighting the need for comprehensive glycemic control beyond HbA1c.

CGM in diabetic patients on various modalities of renal replacement therapy

In patients on hemodialysis, CGM has shown promise in improving glucose control and reducing the incidence of hypoglycemic events[18]. Despite challenges such as sensor accuracy fluctuation over dialysis sessions, CGM emerges as a valuable tool for clinicians to monitor glycemic trends and mitigate the risk of asymptomatic hypoglycemia in patients on hemodialysis. Similarly, in peritoneal dialysis patients, CGM emerges as a valuable adjunct in detecting and managing glucose variations induced by dialysate glucose absorption[19]. Notably, CGM accuracy remains unaffected by factors such as acidosis, urea levels, or volume overload, providing consistent monitoring across a wide range of glucose levels.

Nonetheless, there are certain limitations of CGM among patients with kidney disease. For instance, CGM sensors demonstrate variable accuracy, with mean absolute relative differences (MARD) values ranging from 11.3% to 36.1%, surpassing recommended thresholds[20]. Factors like inflammation post-sensor insertion, dialysis fluid loss, and interdialytic weight changes compromise CGM accuracy, especially in later dialysis sessions, weakening interstitial-capillary glucose correlation. Though MARD values are relatively stable in peritoneal dialysis patients, further research is needed in both cohorts to enhance CGM precision. The application of CGM in kidney transplant recipients is crucial, particularly its potential in managing perioperative and post-transplant hyperglycemia. Studies indicate that CGM offers valuable insights into glycemic control post-transplantation, aiding in the prevention of de novo post-transplant diabetes and complications related to pre-existing diabetes[21].

CONCLUSION

In conclusion, this article advocates for integrating CGM into the management of glycemia in CKD patients, emphasising its superiority over traditional markers in capturing dynamic glucose fluctuations and acute incidents of hypo- and hyperglycemia. However, challenges such as sensor accuracy, durability, and standardisation of application protocols persist. Future research should focus on addressing these challenges to optimise CGM utilisation in clinical practice. Furthermore, further research is essential to standardise and optimise CGM use in this population. Overall, this review serves as a comprehensive guide for clinicians navigating the complexities of glycemic management in CKD, urging them to consider CGM as a valuable tool in their armamentarium.

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EDITORIAL

Potential prospects of Chinese medicine application in diabetic retinopathy

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Abstract

Current treatment strategies for diabetic retinopathy (DR), an eye condition that can lead to blindness, have mainly focused on proliferative DR, including vitreous injection, retinal photocoagulation, and vitrectomy. Vitreous injections mainly depend on anti-vascular endothelial growth factor therapy. In this editorial, we comment on the article by Sun *et al*. We focus specifically on the mechanisms of the protective effect of genipin on the retina. Genipin is a gardenia extract used in traditional Chinese medicine (TCM). In their study, the authors suggest that controlling advanced glycosylation by the intraocular injection of genipin may be a strategy for preventing retinopathy. The innovative use of a Chinese medicine extract injected into the eye to achieve a curative effect has attracted our attention. Although TCM is effective in treating DR, the topical application of DR, especially intraocular injections, is not yet feasible. Herein, we present a brief analysis of effective Chinese medicines for the treatment of DR. The effectiveness of local injections of TCM applied directly into the eyes holds promise as an effective treatment approach for DR.

Key Words: Diabetic retinopathy; Traditional Chinese medicine; Genipin; Topical application; Advanced glycation end products

Core Tip: In this study, *in vitro* experiments showed that genipin can reverse high glucose-induced damage in cell proliferation and apoptosis, while reducing energy metabolism, oxidative stress, and inflammatory injury induced by high glucose. The *in vitro* results showed that intravitreal injection with genipin reduced the expression of CHGA, UCP2, and glucose transporter 1 (GLUT1), and the CHGA/UCP2/GLUT1 signalling pathway may play an important role in this process. This study innovatively treated streptozotocin-induced mice with an intraocular injection of genipin, and concluded that genipin ameliorates diabetic retinopathy by downregulating advanced glycation end products, thereby protecting human retinal microvascular endothelial cells.

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INTRODUCTION

Diabetic retinopathy (DR) is a leading cause of vision loss in the working-age population and one of the most common and serious microvascular complications of diabetes mellitus. The estimated prevalence of DR exceeds 40% after 5 years of diabetes onset, further increasing to 87% after > 20 years[1]. The International Clinical Classification Criteria classifies DR into the following five stages based on the increasing risk of retinopathy: (1) Stage 1, characterized by the absence of apparent DR; (2) Stage 2, defined as mild non-proliferative DR (NPDR) with only microaneurysms; (3) Stage 3, moderate NPDR between mild and severe NPDR; (4) Stage 4, severe NPDR, encompassing lesions that adhere to the "4:2:1 principle"; and (5) Stage 5, proliferative DR (PDR), which encompasses all lesions that display clear neovascularization [2]. The development of DR lesions in the macula can result in varying degrees of vision loss[3]. DR is a multifactorial disease with a complex pathogenesis, with inflammation, persistent hyperglycemia, angiogenesis, apoptosis, and advanced glycosylation end products as its primary pathogenic mechanisms. However, at present, the role of oxidative stress and the interactions between these factors remain unclear[4,5].

At present, clinical strategies for the treatment of DR focus mainly on the modes of drugs, lasers, and surgery. In fact, intravitreal anti-vascular endothelial growth factor (VEGF) drugs have emerged as the first line of treatment for severe NPDR and PDR[6]. However, these treatments have limitations and complications. Owing to the need for frequent injections, financial burden, and poor patient compliance, the use of anti-VEGF drugs is limited[7]. Furthermore, frequent intravitreal injections of anti-VEGF drugs, such as ranibizumab and bevacizumab, can have adverse effects, such as endophthalmitis and traumatic cataracts[8], while laser and surgical treatments can lead to uveitis and worsening macular edema[9]. Therefore, there is a need to identify new anti-DR drugs or complementary and alternative therapies with improved efficacy and fewer adverse effects.

Genipin, a traditional Chinese medicine (TCM) that is isolated from the fruits of Gardenia jasminoides, has been extensively studied for its antidiabetic and neuroprotective activities[10]. Sun *et al*[11], in 2023, in their study, have provided new insights into the molecular dynamics and therapeutic modalities of the potential effects of genipin on diabetic microangiopathy. Their findings indicated that the intravitreal injection of genipin protects the retina of diabetic mice from high-glucose-induced damage *in vivo*. Furthermore, the CHGA/UCP2/glucose transporter protein 1 signaling pathway plays an important role in this process. In addition, *in vitro* studies support these findings, indicating that genipin reverses AGE-induced cell proliferation and apoptotic damage in vitro, while reducing high glucose-induced energy metabolism, oxidative stress, and inflammatory damage. Based on these experimental findings, the authors propose that regulating advanced glycosylation through intraocular injection of genipin may be a potential strategy to mitigate severe retinopathy and prevent vision loss (Figure 1).

APPLICATION OF TRADITIONAL CHINESE MEDICINE IN DIABETES RETINOPATHY

The prevention and treatment of DR using TCM has recently gained considerable attention in research on fundus diseases. The active ingredients of TCM, which are derived by activating blood circulation, removing blood stasis, tonifying qi, and nourishing blood, have significant antioxidant, anti-inflammatory, anti-apoptotic, and other pharmacological effects that can block DR progression through various mechanisms[12]. Chinese medicine has amassed a substantial body of clinical evidence and research data for the prevention and treatment of DR. Many TCM extracts have been extensively studied in this context. Polyphenolic compounds, such as curcumin, puerarin, and resveratrol prevent and treat DR through various mechanisms. Additionally, herbal single-drug extracts, including Fructus arctii, Dendrobium, Scutellaria, Gypenoside, and Radix trichosanthis, are commonly utilized in research on diseases associated with diabetes-related vascular complications[13].

Among these drugs, curcumin, puerarin, Fructus arctii, and resveratrol have been used in animal experiments and *in vitro* studies. They regulate various pathogenic mechanisms of DR and inhibit its progression. This provides an experimental foundation for their potential clinical use in treating DR. For instance, curcumin treatment decreases leukocyte adhesion and vascular leakage in the retinas of diabetic rats and reduces the expression of pro-inflammatory mediators in

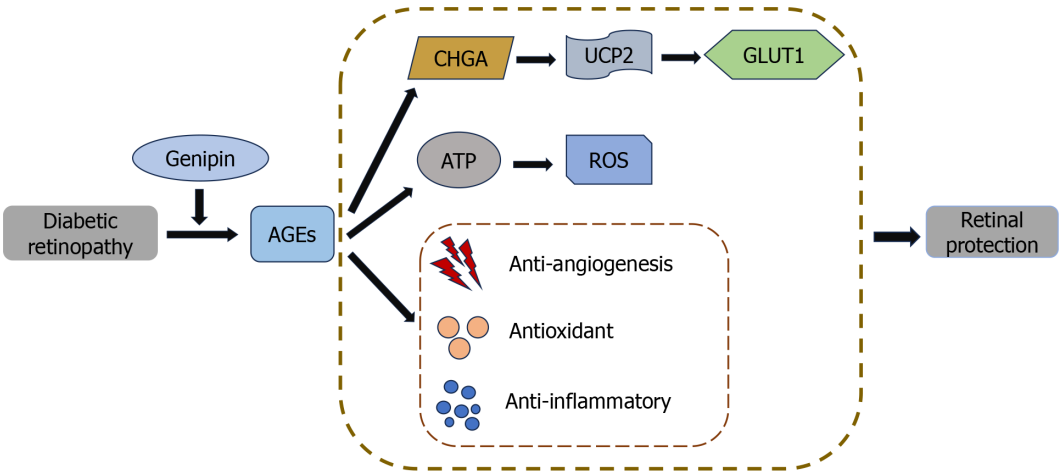

Figure 1 The retinal protection mechanism of genipin. AGEs: Advanced glycation end products; CHGA: Chromogranin-A; UCP2: Uncoupling protein 2; GLUT1: Glucose transporters type 1; ATP: Adenosine-triphosphate; ROS: Reactive oxygen species.

retinal tissues. Furthermore, curcumin regulates VEGF-mediated angiogenesis and improves glucose and lipid metabolism[14]. In addition to its anti-inflammatory and anti-angiogenic effects, puerarin treatment reduces the expression of Bax and caspase-3 and inhibits the production of reactive oxygen species, as well as NMDA-induced iNOS and nNOS release, reflecting its anti-apoptotic and anti-oxidative stress capacity[15]. Resveratrol has been experimentally demonstrated to exert antioxidant effects by increasing the levels of naturally occurring antioxidants, enzymes, and molecular defenses in various cell types in the eye. In addition, resveratrol exerts an anti-VEGF effect and inhibits the proliferation and migration of vascular endothelial cells, exerting a combined anti-PDR effect[16].

TCM treatment for DR has demonstrated certain advantages. Previous studies have elucidated the molecular mechanisms and potential of TCM against DR from the perspective of signaling pathways. The results showed that the key signaling pathways of TCM for the treatment of DR include anti-inflammatory, anti-angiogenic, and anti-oxidative stress-based pathways, such as NF-κB, MAPK/NF-κB, TLR4/NF-κB, VEGF/VEGFR2, HIF-1α/VEGF, STAT3, and Nrf2/HO-1. Relevant Chinese medicines can regulate the interactions between signaling pathways through multitarget synergistic effects to inhibit DR progression. Many herbal medicines counteract DR by affecting various pathological processes. For example, curcumin can inhibit retinal inflammation *via* the p38 MAPK/NF-κB signaling pathway and angiogenesis *via* the VEGF/VEGFR2 signaling pathway, thereby exerting beneficial effects on DR[17]. Considering the complexity of the pathological mechanisms of DR, multi-component, multi-target, and multi-pathway Chinese medicines are promising drug candidates for the treatment of DR. However, a lack of documentation on the corresponding side effects is a major limitation of TCM therapeutic studies in relation to DR. With the increasing popularity of herbal medicines, their side effects have become more common. For example, some animal studies have indicated that the mega-dose administration of curcumin causes a decrease in testosterone levels and may affect reproductive function[18]. Wang *et al*[19] reported 33 cases of hemolytic reactions due to puerarin injection. These findings highlight the need to collate and analyze the adverse effects of TCM treatment, implement appropriate control measures, and leverage its pleiotropic effects to identify TCM as a promising candidate for the prevention and treatment of eye diseases.

However, at present, the majority of TCM treatments are administered orally, and the use of intraocular injections remains rare[14]. With substantial advances in macromolecular biologics for ophthalmic purposes, vitreous cavity injections have become a crucial treatment option for a broad range of fundus diseases. Patients with diabetes often have various underlying diseases and require various systemic medications[20]. Topical ocular medications can reduce the systemic drug burden in patients, especially their impact on liver and kidney functions. In their article, the authors propose an innovative conclusion: Controlling advanced glycation through the intraocular injection of genipin to mitigate severe retinopathy and prevent vision loss. However, this clinical application has room for improvement, including a need to enhance the drug transduction of genipin[12]. As such, a gap exists between the experimental and clinical applications.

SAFETY OF OPHTHALMIC INJECTIONS

In the pursuit of clinical efficacy, considerable attention should be paid to drug safety. In this respect, quality standards and standardized management operations for ophthalmic injections should be emphasized. The international quality standards for ophthalmic injections include pH, osmolality, molar concentration, and cytotoxicity. Among these factors, the special route of administration limits sterility, and the number of insoluble particles is a key factor affecting the quality standards of ophthalmic injections. Notably, owing to the physiological and anatomical characteristics of the ophthalmic site, its tolerance to particles may be lower. Therefore, the United States Pharmacopoeia has established more stringent standards for insoluble particles in ophthalmic injections than those for small-volume intravenous injections [21]. Monoclonal antibody injections have the characteristics of biological products. Biological and immunological

methods must be applied to complete the identification and consistency analysis and to assess purity, impurities, potency, and other relevant factors. Moreover, conducting inspections for "substances related to TCM injection" and "residues of heavy metals and harmful elements" is mandatory for TCM formulations used as general injections.

At present, the available dosage forms of ophthalmic preparations in TCM are fewer than those of chemical medicines, particularly ophthalmic injections[22]. This could be attributed to the inherent complexity of TCM and the rigorous quality control standards for ophthalmic preparations. When developing drugs, in addition to ensuring that the original herbs meet legal standards, attention should be paid to the method of crushing the herbs and the particle size of the powder. In addition, the selection of additives, sterility checks, and eye irritation tests must be standardized and monitored. In addition to safety concerns, ensuring the quality of ophthalmic preparations requires consideration of the specific drug varieties used in clinical practice, and a comprehensive evaluation of raw materials, excipients, processes, and other factors. Nevertheless, certain critical considerations regarding ophthalmic preparations of TCM need to be addressed, including its maximum frequency of daily use, specifying indications, and evaluating efficacy by incorporating the unique characteristics of TCM ophthalmic preparations. We believe that the local application of TCM can be anticipated to emerge as a new trend and holds promise as an effective approach for the treatment of DR.

CONCLUSION

The intraocular injection of genipin to control advanced glycation may represent an effective strategy for the prevention of severe retinopathy and vision loss. More generally, the prevention and treatment of DR using TCM is gradually becoming an important focus in advancing research on fundus diseases. Given the stringent quality standards required for intraocular injectable drugs, conducting high-quality pilot studies is imperative. Comprehensive studies on topical intraocular injections of herbal medicines may also facilitate further advances in therapeutic approaches for the treatment of fundus diseases.

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FOOTNOTES

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EDITORIAL

Don´t give up on mitochondria as a target for the treatment of diabetes and its complications

Christian Cortés-Rojo, Manuel Alejandro Vargas-Vargas

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Abstract

In this editorial, we discuss an article by Wang *et al*, focusing on the role of mitochondria in peripheral insulin resistance and insulin secretion. Despite numerous *in vitro* and pre-clinical studies supporting the involvement of mitochondrial dysfunction and oxidative stress in the pathogenesis of diabetes and its complications, efforts to target mitochondria for glycemic control in diabetes using mitochondria-targeted antioxidants have produced inconsistent results. The intricate functionality of mitochondria is summarized to underscore the challenges it poses as a therapeutic target. While mitochondria-targeted antioxidants have demonstrated improvement in mitochondrial function and oxidative stress in pre-clinical diabetes models, the results regarding glycemic control have been mixed, and no studies have evaluated their hypoglycemic effects in diabetic patients. Nonetheless, pre-clinical trials have shown promising outcomes in ameliorating diabetes-related complications. Here, we review some reasons why mitochondria-targeted antioxidants may not function effectively in the context of mitochondrial dysfunction. We also highlight several alternative approaches under development that may enhance the targeting of mitochondria for diabetes treatment.

Key Words: Diabetes mellitus; Insulin resistance; Mitochondrion; Mitochondrial dysfunction; MitoQ; MitoTEMPO; SkQ; Elamipretide; Mitochondria transplantation; Glycemic control

Core Tip: Mitochondrial dysfunction and oxidative stress are closely linked to the development of diabetes and its complications. This has motivated the targeting of antioxidants to the mitochondria for diabetes treatment, which has generated in pre-clinical trials some encouraging results in diabetic complications, but inconsistent results in glycemic control. Moreover, there are very few studies with these molecules and only in healthy patients, with no encouraging results. There are several challenges to overcome to make mitochondria an efficient pharmacological target against diabetes, but recent developments such as mitochondrial transplantation, bioactive small peptides, and atomistic simulations could help to achieve this goal.

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INTRODUCTION

The conventional view of mitochondria as solely energy-producing organelles is evolving, as these structures are now recognized to perform a diverse array of functions (Figure 1). These functions include the regulation of intracellular ion levels, which can be detrimental if concentrations exceed certain thresholds, such as iron[1], calcium[2], and copper[3]. Mitochondria are also implicated in thermoregulation through the activation of uncoupling proteins (UCPs), in the programmed cell death of defective cells *via* apoptosis, in the regulation of various physiological processes through the production of reactive oxygen species (ROS), and in the retrograde regulation of gene expression[4] (Figure 1). To integrate and regulate these functions, mitochondria respond to cellular energy levels by sensing the redox state of pyridine nucleotides (*i.e.*, NADH/NAD$^+$ ratios)[5] and the phosphorylation state of adenine nucleotides (*i.e.*, ATP/AMP ratios)[6]. Similar to cytosolic processes, mitochondrial proteins involved in ATP synthesis, mitochondrial DNA (mtDNA) transcription and translation, the Krebs cycle, respiratory uncoupling, protein import, and apoptosis are believed to be regulated by phosphorylation/dephosphorylation cycles of kinases and phosphatases within the mitochondrion[7]. Furthermore, mitochondria are considered signaling organelles that modulate extracellular protein functions by activating cytosolic kinases through ROS or direct physical contact with other structures, such as the endoplasmic reticulum or cytoskeleton elements[8]. Additionally, the release of mtDNA from the matrix into the cytosol and extracellular space can induce inflammation[9] (Figure 1).

The complexity of mitochondrial function increases when considering that mitochondria are not isolated organelles but form dynamic associations with other mitochondria through fusion and fission processes, known as mitochondrial dynamics. Mitochondrial dynamics significantly influence mitochondrial function, as mitochondria undergoing predominant fission and fragmentation exhibit impaired function, loss of membrane potential ($\Delta\Psi$), and increased ROS production, whereas the opposite occurs with predominant fusion and mitochondrial network formation[10]. Mitophagy is another process interconnected with mitochondrial dynamics and mitochondrial energization. Mitophagy serves as a quality control system that eliminates dysfunctional mitochondria in response to decreased mitochondrial $\Delta\Psi$, activating a series of proteins responsible for engulfing dysfunctional mitochondria in autophagosomal membranes for subsequent lysosomal degradation (Figure 1). This process is linked to mitochondrial dynamics, as mitochondrial fusion proteins Mitofusin 1 and 2 are degraded during mitophagy, preventing dysfunctional mitochondria from fusing and propagating defects, such as mtDNA mutations, to healthy mitochondria[11].

In a recent issue of the *World Journal of Diabetes*, Wang *et al*[12] reviewed evidence from animal and human models indicating that ATP production by oxidative phosphorylation and tight coupling of oxidative phosphorylation are crucial for maintaining insulin secretion in the pancreas and insulin sensitivity in peripheral tissues. They highlighted human studies showing that in obese patients with type 2 diabetes or elderly individuals with insulin resistance, there is an accumulation of fat in skeletal muscle and liver, decreased oxidative phosphorylation, reduced oxidative functions of mitochondria (*e.g.*, NADH oxidation), and decreased size and number of mitochondria. In preclinical models of metabolic syndrome, these dysfunctions contribute to inefficient fatty acid oxidation and the accumulation of incomplete lipid oxidation products, such as diacylglycerol, which disrupts insulin receptor activation and leads to insulin resistance[12].

Correct functioning of the electron transport chain (ETC) is essential for the efficient oxidation of fatty acids and metabolic fuels, as NADH produced during mitochondrial β-oxidation is reoxidized in complex I of the ETC. If NADH is not reoxidized, fatty acid oxidation slows, leading to the accumulation of lipids such as diacylglycerol and ceramide, and resulting in reductive stress. This causes an increase in the NADH/NAD$^+$ ratio and dysregulation of catabolic and antioxidant enzyme activity due to increased acetylation[13]. Slowing of electron transport in the ETC also increases the formation of ROS, which play a crucial role in the development of diabetic complications by activating processes of cell death, inflammation, and fibrosis (Figure 1), ultimately damaging target tissues such as the kidney and liver[14].

The hypothesis that mitochondrial ROS are responsible for the development of diabetic complications led to clinical trials with antioxidants to reduce these complications[15,16]. However, these results largely discouraged the idea that ROS is involved in the pathogenesis and complications of diabetes. The explanation for these disappointing results was that common antioxidants were unable to reach the sites of ROS production in the mitochondria and neutralize them. This is due to the large molecular size of most antioxidants and the relative impermeability of the inner mitochondrial membrane, preventing their passage into the mitochondrial matrix[17]. This led to the idea of conjugating antioxidants

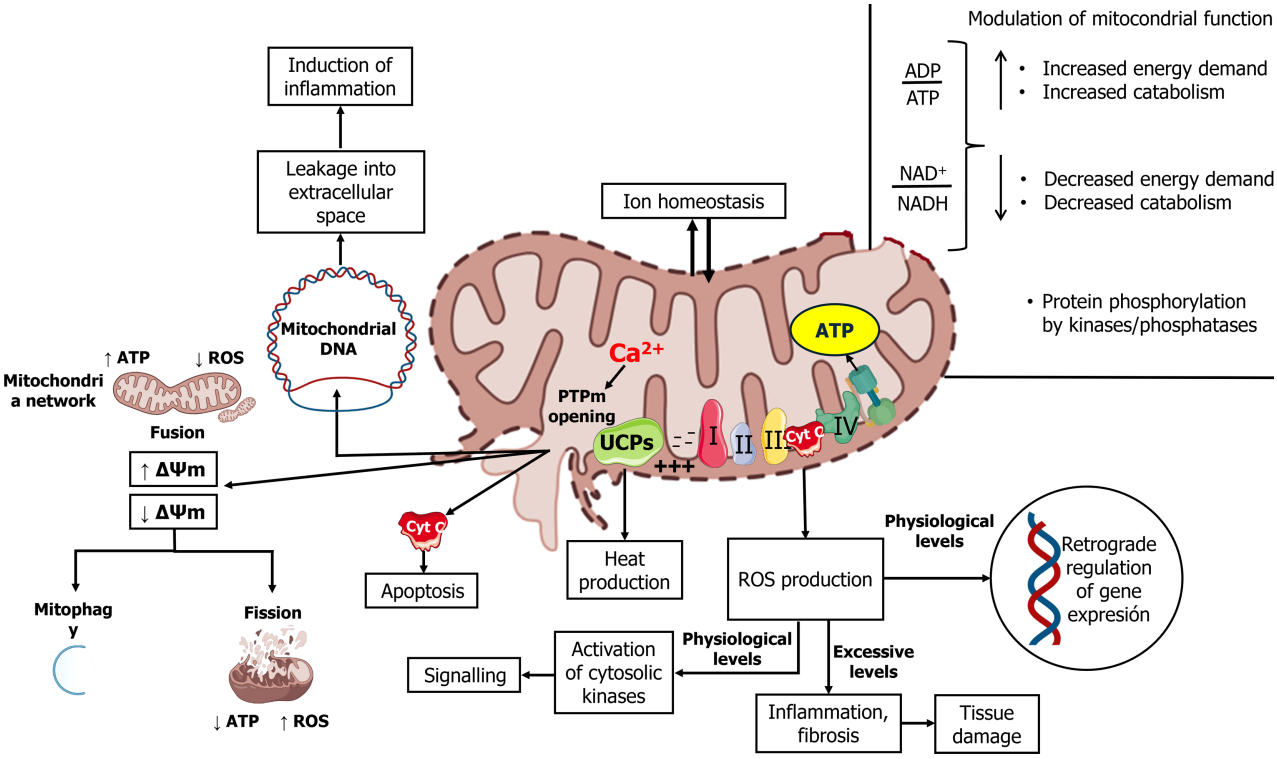

Figure 1 Complexity of mitochondrial functioning. Conventionally, mitochondria are recognized as central to oxidative metabolism for ATP production *via* the electron transport chain, regulated by mitochondrial energy levels and kinase/phosphatase systems. However, mitochondria also play roles in maintaining ionic homeostasis and various physiological functions. The physiological production of mitochondrial reactive oxygen species (ROS) modulates cell signaling and gene expression, while excessive ROS production can lead to detrimental processes such as inflammation and fibrosis. Uncontrolled opening of the mitochondrial permeability transition pore releases cytochrome c and mitochondrial DNA, promoting apoptosis and inflammation, respectively. These events are mitigated through processes like cell fission and mitophagy to prevent the transmission of mitochondrial DNA mutations to healthy mitochondria. Conversely, mitochondrial fusion into networks enhances mitochondrial function and reduces ROS production. ROS: Reactive oxygen species; Cyt C: Cytochrome c; UCPs: Uncoupling proteins.

with Skulachev ions to promote their accumulation within mitochondria[18]. Skulachev ions, originally designed by Skulachev[19] in Russia to test the chemiosmotic hypothesis[19], are hydrophobic compounds that generally contain a positively charged phosphorus or nitrogen atom, allowing their penetration into the negatively charged mitochondrial matrix[19].

Thus, Skulachev[19] pioneered the study of the potential clinical utility of targeting antioxidants to the mitochondria for treating diseases involving excess ROS production. This research led to the design and synthesis of various mitochondria-targeted antioxidants, some of which have been tested for treating diabetes and its complications in both pre-clinical and clinical studies with mixed results. However, as discussed in the following sections, this marks only the beginning of an era of "mitochondrial medicine," which considers mitochondrial targets beyond ROS and even explores mitochondrial replacement strategies.

ANTIOXIDANTS CONJUGATED WITH TRIPHENYLPHOSPHONIUM CATIONS AND THEIR EFFECTS ON DIABETES

One of the most studied antioxidants derived from the conjugation of a triphenylphosphonium cation (*i.e.*, Skulachev ion) to an antioxidant is mitoquinone (MitoQ). MitoQ contains a triphenylphosphonium cation attached to ubiquinone-10 *via* a hydrophobic linker. MitoQ has shown mixed results in lowering blood hyperglycemia in preclinical models of diabetes or obesity. In a model of accelerated metabolic syndrome development, MitoQ decreased fasting blood glucose, insulin, cholesterol, and triglyceride levels and normalized glucose tolerance during a 7-week administration following a high-fat diet[20]. MitoQ has been shown to increase insulin secretion in pancreatic beta cells grown in hyperglycemic media, simulating hyperglycemic conditions in humans[21]. However, in two recent studies in rats with type 2 diabetes induced by a high-fat diet and streptozotocin, MitoQ failed to lower blood glucose levels. The rationale for this result is that diabetes-induced in this manner is a more severe and advanced form of diabetes than in humans[22,23]. Similarly, using Akita ($Ins2^{+}/^{-Akita}$) mice, a model of type 1 diabetes, it was found that MitoQ administration did not lower blood glucose levels, which reached values of 681.78 mg/dL[24]. Despite these discouraging results regarding blood glucose, MitoQ showed promising results by protecting against diabetic kidney damage in at least two studies[24,25], improving diabetic neuropathy[23], and protecting against the development of hepatic steatosis[20,22].

Another mitochondria-targeted antioxidant tested in pre-clinical trials against hyperglycemia is SkQ1. SkQ1 is similar to MitoQ, except that the antioxidant portion containing ubiquinone-10 is replaced by a plastoquinone molecule, which is present in chloroplasts and provides SkQ1 with a wider antioxidant window than MitoQ[26]. In rats with alloxan-induced type 1 diabetes, administration of SkQ1 seven days before diabetes induction normalized blood glucose levels [27]. In contrast, in a study using *db/db* mice, a model of type 2 diabetes, administration of SkQ1 for 12 weeks did not reduce blood glucose or glycosylated hemoglobin levels[28]. In the case of alloxan-induced diabetes, it can be inferred that SkQ1 inhibits the development of diabetes due to its antioxidant properties, as alloxan induces diabetes by destroying pancreatic beta cells *via* oxidative damage[29]. Therefore, to demonstrate that SkQ1 has a hypoglycemic effect once diabetes is established, it is necessary to test the effect of SkQ1 once diabetes is established with alloxan or to test its effects in a model of diabetes that better mimics the development of diabetes in humans. Despite the mixed results of SkQ1 in hyperglycemia, it is noteworthy that its administration in diabetic rats significantly improved wound healing regardless of the absence of a hypoglycemic effect. This is a promising result given the relatively high incidence of defective wound healing in diabetic patients, the high proportion of patients suffering from diabetic foot ulceration, and the very high costs and dramatic impairment to the quality of life of those who suffer amputations[30].

Regarding human studies on antioxidants targeting mitochondria *via* a triphenylphosphonium cation, a recent systematic review and meta-analysis analyzed the results of 19 randomized controlled trials (RCTs). Some of these studies used MitoQ and MitoTEMPO, in which the ubiquinone-10 portion of MitoQ was substituted with the antioxidant piperidine nitroxide (TEMPO). This analysis revealed that only two studies in healthy patients showed no effect of MitoQ supplementation for 4 or 6 weeks on glycosylated hemoglobin and fasting blood glucose levels[31]. Importantly, there are no RCTs on the effects of mitochondria-targeted antioxidants in patients with impaired glucose metabolism. Therefore, it was concluded that there is limited evidence from RCTs to support the use of mitochondria-targeted antioxidants for the management of glycemic control[31].

SOME CONSIDERATIONS ON THE INCONSISTENCY OF THE OUTCOMES OF MITOCHONDRIA-TARGETED ANTIOXIDANTS IN THE CONTROL OF HYPERGLYCEMIA IN DIABETES

The inconsistency in the results obtained with antioxidants targeting mitochondria with a triphenylphosphonium cation could be due to several reasons. If the entry of conjugated antioxidants to a positively charged triphenylphosphonium cation is dependent on mitochondrial $\Delta\psi$, will the accumulation of antioxidants in the mitochondrial matrix be possible when there is a dissipation of $\Delta\psi$? The $\Delta\psi$ can decrease for different reasons, including an increase in UCP expression, uncontrolled opening of the mitochondrial permeability transition pore (mPTP) by an increase in calcium levels, or mitochondrial fatty acid transport[32]. These events also occur in diabetes, with increased UCP2 expression in pancreatic beta cells in type 2 diabetes[33], increased fatty acid transport in the mitochondria due to increased blood levels of free fatty acids in obesity and diabetes[34], and increased induction of mPTP in the liver[35]. Therefore, one or several of these events occurring simultaneously could limit the entry of MitoQ, MitoTEMPO, or SkQ1 into the matrix by dissipating the $\Delta\psi$, thus decreasing their efficacy in diabetes. It has also been argued that MitoQ has a narrow window of anti- and pro-oxidant concentrations, which limits its efficacy in human clinical trials of neurodegenerative diseases[36]. Other considerations that could limit the action of mitochondria-targeted antioxidants include their lack of specificity for a particular oxidizing species, the influence of the physicochemical environment surrounding the mitochondrial inner membrane on the reactivity of the antioxidants with their targets, among other factors previously discussed[37].

DON'T GIVE UP ON MITOCHONDRIA FOR THE TREATMENT OF DIABETES AND ITS COMPLICATIONS!

Based on the information presented in the previous section, it appears that treatment with mitochondria-targeted antioxidants is not promising for the management of glycemic control in patients with diabetes. However, these studies have demonstrated the need, as mentioned by Wang *et al*[12] in their article in the *World Journal of Diabetes*[12], to develop new models of diabetes that more closely mimic the features of human diabetes and to foster a collaborative venture involving academia and industry to enable better and faster development of drugs for diabetes management[12], including mitochondria-targeted molecules such as MitoQ, MitoTEMPO, and SkQ. Notably, these molecules already show promising beneficial effects on diabetes complications independent of a decrease in hyperglycemia[20,22-25,30].

In addition to mitochondria-targeted antioxidants, other therapeutic strategies do not depend on the mitochondrial membrane potential to act within this organelle. For example, Elamipretide is a tetrapeptide (H-D-Arg-Dmt-Lys-Phe-NH_2) that selectively binds cardiolipin. This phospholipid is essential for the structure and function of several mitochondrial inner membrane proteins, including the ETC complexes. Elamipretide increases electron flow through complex IV, which enhances mitochondrial respiration, ATP synthesis, $\Delta\psi$, and decreases ROS formation. Additionally, it inhibits the interaction of cytochrome *c* with cardiolipin that activates its peroxidase function, preventing peroxidative damage to cardiolipin and detachment of cytochrome *c* from the mitochondria, thus preventing apoptosis[38]. Similar to antioxidants containing a triphenylphosphonium cation, Elamipretide protects against the development of diabetic nephropathy in diabetic *db/db* mice without improving glycemic control[39].

Finally, mitochondrial transfer between different cells has attracted significant attention. This phenomenon has been found to occur actively *in vivo via* extracellular vesicles and nanotubes and is postulated to play physiological and pathological roles[40]. This inspired the development of therapeutic strategies involving the transplantation of healthy

mitochondria into diseased tissues with dysfunctional mitochondria. In the case of diabetes, the transfer of mitochondria from mesenchymal stromal cells to damaged pancreatic beta cells has been successfully tested in cell culture, improving the bioenergetics of damaged cells and, thus, insulin secretion[41]. Therefore, further research is warranted to develop the technology required for mitochondrial transplantation to either increase insulin production in the pancreas or decrease insulin resistance in peripheral tissues.

CONCLUSION

The mitochondrion is an extraordinarily complex organelle that functions as a hub for integrating oxidative metabolism, cell signaling, and death signals, protecting surrounding cells from the spread of cellular defects. Cells can transfer mitochondria to each other to improve the function of neighboring cells that are damaged or exhibit mitochondrial dysfunction. Moreover, mitochondria can fuse to enhance their function and undergo fission when mitochondrial function is defective. Under these circumstances, mitochondria undergo a process of self-destruction by mitophagy to discard those with defects in their mtDNA or bioenergetics. Additionally, the mitochondrion is a signaling organelle that, through the emission of different stimuli, regulates gene expression in the nucleus, kinase activity in the cytosol, as well as immunity and inflammation. Given their multiple layers of complexity, it is not surprising that mitochondria are a difficult therapeutic target, which is reflected by inconsistent results regarding glycemic control in pre-clinical and, in some cases, human clinical trials with mitochondria-targeted antioxidants or peptides. However, these molecules have been successfully used in pre-clinical models of diabetic complications, including diabetic nephropathy, diabetic wound healing, hepatic steatosis, and diabetic neuropathy. This suggests that mitochondria remain a promising therapeutic target for treating diabetic complications and highlights the need for further development of molecules targeting other aspects of mitochondria, such as mitophagy, mitochondrial dynamics, cation overload, and channels such as mPTP or UCPs. Computational developments such as molecular docking and molecular dynamics simulations could improve the design and selection of highly specific molecules for their targets. As discussed by Wang *et al*[12], a collaborative venture involving academia and industry is necessary for better and faster development of new drugs targeting mitochondria for treating diabetes and its complications or improving existing drugs.

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Abstract

Since the discovery of insulin over 100 years ago, the focus of research in the management of type 1 diabetes (T1D) has centered around glycemic control and management of complications rather than the prevention of autoimmune destruction of pancreatic β cells. Fortunately, in recent years, there has been significant advancement in immune-targeted pharmacotherapy to halt the natural progression of T1D. The immune-targeted intervention aims to alter the underlying pathogenesis of T1D by targeting different aspects of the immune system. The immunotherapy can either antagonize the immune mediators like T cells, B cells or cytokines (antibody-based therapy), or reinduce self-tolerance to pancreatic β cells (antigen-based therapy) or stem-cell treatment. Recently, the US Food and Drug Administration approved the first immunotherapy teplizumab to be used only in stage 2 of T1D. However, the window of opportunity to practically implement this approved molecule in the selected target population is limited. In this Editorial, we briefly discuss the various promising recent developments in the field of immunotherapy research in T1D. However, further studies of these newer therapeutic agents are needed to explore their true potential for prevention or cure of T1D.

Key Words: Type 1 diabetes; Immunotherapy; Teplizumab; Antigen based therapy; Stem cell immunotherapy

Core tip: There has been a paradigm shift in research on type 1 diabetes (T1D) in the last decade. From managing the consequences of β cell death to prevention of β cell destruction, immunotherapy is showing the path forward. Recent regulatory approval of teplizumab in stage 2 of T1D marks the first significant advance in research of immunotherapy. In this Editorial, we briefly explore the recent developments and prospects in the field of immunotherapy in T1D encompassing antibody-based therapy, antigen-based therapy, and stem-cell-based immunotherapy.

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INTRODUCTION

The basic pathophysiology behind the development of type 1 diabetes (T1D) is immune-mediated destruction of insulin-producing pancreatic β cells[1]. The insulin-producing capacity of the endocrine pancreas depends upon its functional β cell number and size, collectively known as β cell mass. The body's aberrant immune system attacks and self-destroys the normal host tissue in autoimmune disease. Like any other autoimmune disease, progressive immune-mediated destruction of pancreatic β cells leads to a reduction of functional β cell mass in T1D. Once this β cell mass falls below the critical level, the onset of T1D ensues. However, the treatment of T1D primarily focuses on the exogenous replacement of insulin. Recently, studies have focused on exploring the possibilities of immune modulating therapy in T1D to protect pancreatic β cell mass from autoimmune destruction. Various molecules targeting mediators like T cells, B cells, cytokines and antigen-based therapies are now being evaluated to prevent or delay β cell destruction in T1D. Initial results in a few studies were encouraging in maintaining β cell function (as measured by c-peptide level) when used in the early stages of disease development[2]. In this Editorial, we briefly explore the potential of these novel immunomodulatory therapies in managing patients with T1D.

IMMUNOTHERAPY IN T1D

The pathophysiological stages of development of T1D are now being classified into three distinct stages: stage 1-positive antibody status with normoglycemia; stage 2-positive antibody status with dysglycemia or prediabetes; and stage 3-positive antibody status with frank diabetes or overt hyperglycemia[3]. Stage 3 of T1D development marks the onset of significant destruction of β cells. Stage 3 of disease development has been further subclassified into: stage 3a (not insulin requiring); stage 3b (insulin-requiring but with residual clinically relevant β cell mass); and stage 3c (insulin requiring without any clinically relevant β cell mass)[4]. The immune system plays a pivotal role in the de-velopment of T1D. Although it was classically thought that autoimmune destruction of β cells is mainly due to T cells, B cells also play a critical role[5]. Once self-tolerance is lost, and with subsequent environmental triggers, autoreactive T cells are developed. When the self-antigens from β cells are presented by antigen-presenting cells (APCs) to these autoreactive T cells, cytotoxic T cells are activated, destroying pancreatic β cells. The main target of immunotherapy in T1D is to either prevent or at least delay the immune-mediated destruction of β cells[6,7]. The immunotherapy in T1D can be either-antibody-based therapy, antigen-based therapy, or stem cell therapy. In the following sections, we will briefly discuss the recent status of immunotherapy in T1D.

ANTIBODY-BASED IMMUNOTHERAPY

Antibody-based therapies against different target antigens have been tried to halt the autoimmune destruction of pancreatic β cells. These antibody-based therapies (monoclonal and polyclonal) are directed mainly against T cells or B cells or cytokine signaling (Figure 1). Most of these studies that evaluated antibody-based immunotherapies in T1D were done in stage 2 or early stage 3 of T1D. The details of the important antibody-based immunotherapies are given in the next section.

T-cell-directed antibody therapy

Teplizumab: Teplizumab is a humanized monoclonal antibody [immunoglobulin G (IgG) 1 kappa] directed against the CD3 portion of T-cell receptors[8]. This molecule is non-Fc binding, thus reducing the risk of cytokine release syndrome (CRS) compared to previous generations of anti-CD3 molecules. Teplizumab produces early suppression of cellular immune response by preventing the binding of $CD4^+$ T helper cells to APCs by inhibiting CD3 of T-cell receptors. Prolonged and sustained binding of this molecule exerts a state of $CD8^+$ T-cell exhaustion and thus induces chronic immunosuppression[9]. In 2022, teplizumab obtained US Food and Drug Administration (FDA) approval for delaying or prevention the onset of stage 3 of T1D in patients currently in stage 2 of T1D. It was approved for use in adults and

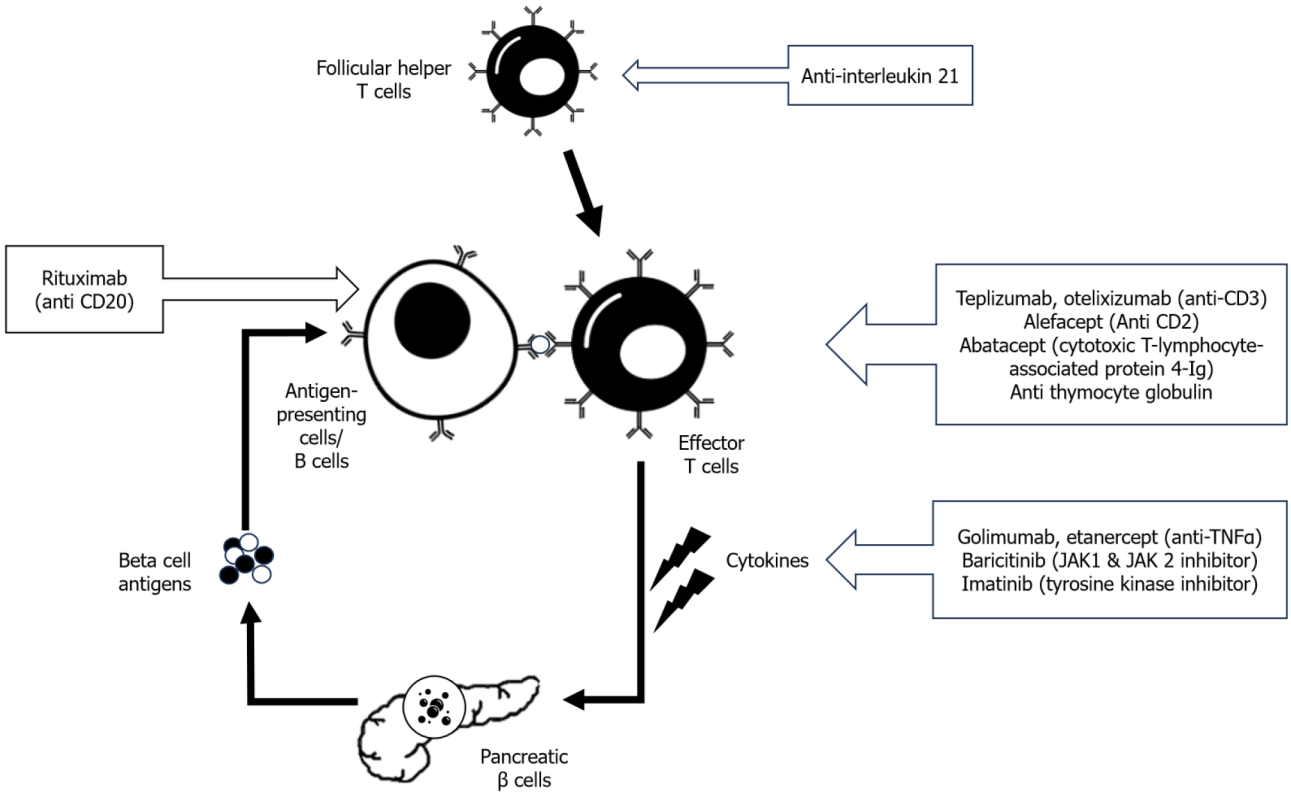

Figure 1 Outline of antibody-based immunotherapy in type 1 diabetes.

children aged > 8 years[10].

The landmark trials that evaluated the safety and efficacy of teplizumab are summarized in Table 1[11-15]. All of these studies are done in stage 3 of the T1D except the TrialNet study, which was done in stage 2 of T1D[12]. These trials showed a more favorable c-peptide response in teplizumab than in the placebo arm. In the meta-analysis by Kamrul-Hasan *et al*[16], 834 subjects from six studies that evaluated the efficacy and safety of teplizumab as a disease-modifying therapy in T1D, were included. The authors reported greater preservation of area under the curve (AUC) of c-peptide in the teplizumab arm through 6-24 mo of follow-up [mean difference 0.07 nmol/L (95% confidence interval: 0.01-0.14, P = 0.03)]. Moreover, fewer patients reported reduced c-peptide response after 2 years of follow-up in the teplizumab arm (odds ratio 0.12). However, the authors also reported an increased risk of grade 3 or higher adverse events, nausea, rash, lymphopenia, and discontinuation of the study drug in the teplizumab arm than placebo. Adverse events are commonly associated with any monoclonal-antibody-based therapy. In the case of teplizumab, most adverse drug reactions occurred during the treatment period and were mild to moderate and manageable. Similarly, other recent meta-analyses also reported that patients in the teplizumab arm had higher AUC of c-peptide and lower exogenous insulin requirement but similar glycosylated hemoglobin (HbA1c) levels in comparison to placebo[17,18]. In another meta-analysis, Liu *et al*[19] included both the anti-CD3 monoclonal antibodies, teplizumab (seven studies) and otelixizumab (five studies). The authors also reported greater AUC of c-peptide and decreased exogenous insulin requirement in the anti-CD3 antibody arm. They found no significant difference in HbA1c and serious adverse events between the study and placebo arm[19]. Moreover, in the follow-up study of the AbATE trial, prolonged immunological response was reported even after 7 years of diagnosis of T1D in patients who initially responded to teplizumab[20].

Predictors of therapeutic response: The studies done with teplizumab also tried to find out possible predictors of response to therapy. If we can establish predictors for therapeutic response for this expensive therapy, it can be used cost-effectively in selective patients who are more likely to respond. The authors of the Protégé Trial reported that the recently diagnosed patients (< 6 wk) had the highest response. Moreover, patients living in the USA, patients with lower HbA1c, higher c-peptide, and lower insulin requirement at baseline were more likely to respond[14]. As younger patients exhibit stronger immune reactions than adult patients, they have a higher chance to respond[14,15]. Better glycemic control as measured by lower HbA1c level was also reported as a favorable predictor in trials done in stage 3 T1D[15,21]. This may be due to higher preserved β cell mass or increased insulin sensitivity in the treatment responders. The relationship of treatment outcome with baseline β cell reserve as measured by AUC of c-peptide is heterogeneous and may be related to the stage of T1D. Patients in stage 2 T1D are likely to respond when the baseline c-peptide level is lower, whereas a higher baseline c-peptide is a response predictor for patients in early stage 3 disease[12,14,21]. This paradoxical finding can be explained by the hypothesis that prior to significant immune mediated destruction in early stage of disease (stage 2), patients with stronger immune reaction are more likely to respond to immunomodulatory therapy. On the contrary, in patients with a later stage of disease (stage 3), when immune-mediated destruction is already significant, patients with higher residual functioning β cell mass respond better to therapy. The TrialNet study group also reported that patients

Table 1 Major studies evaluating the efficacy and safety of teplizumab

Ref.	Study design	Intervention	Population	Major outcome
Ramos *et al* [11], 2023	PROTECT study	Phase 3, randomized, placebo-controlled trial with teplizumab or placebo for two 12-day courses	The 328 participants, stage 3 T1D, age 8-17 years, within 6 weeks of diagnosis	Higher stimulated c-peptide levels (teplizumab *vs* placebo) (least squares mean difference, 0.13 pmol per mL; 95%CI: 0.09-0.17; $P < 0.001$); no significant difference in HbA1c level, insulin requirement or hypoglycemia; ADR: headache, gastrointestinal symptoms, rash, lymphopenia, and mild cytokine release syndrome
Herold *et al* [12], 2019	TrialNet study	Phase 2, randomized, placebo-controlled, double-blind trial of teplizumab (single 14-day course)	The 76 participants, relatives of T1D, stage 2, age > 8 years	Low-risk diagnosis of T1D (teplizumab *vs* placebo) (hazard ratio 0.41; 95%CI: 0.22-0.78; $P = 0.006$); longer median time to diagnose T1D (teplizumab *vs* placebo) (48.4 months *vs* 24.4 months); ADR of lymphopenia and rash
Herold *et al* [13], 2013	AbATE Study	An open-label, randomized, controlled trial with teplizumab (two of 14-day course, one year apart)	The 83 participants, stage 3 T1D, age 8-30 years, within 8 weeks of diagnosis	Reduced decline in c-peptide at 2 years (-0.28 nmol/L; 95%CI: 0.36-0.20) *vs* control (-0.46 nmol/L; 95%CI: 0.57-0.35; $P = 0.002$); ADR: Rash, transient upper respiratory infections, headache, and nausea
Hagopian *et al*[14], 2013	Protégé study	Phase 3, randomized, double-blind, parallel, placebo-controlled 2-years with teplizumab (3 dosing regimens, two of 14 days course, 26 weeks apart)	The 462 of 516 participants completed 2 years follow up, stage 3 T1D, age 8-35 years, within 12 weeks of diagnosis	Reduced the loss of area under curve mean c-peptide at 2 years (teplizumab *vs* placebo) ($P = 0.027$); ADR: lymphopenia; no differences in adverse events or serious adverse events among groups at 2 years
Herold *et al* [15], 2013		Randomized placebo-controlled trial	The 63 participants, stage 3 T1D, within 4-12 months of diagnosis	The 21.7% higher c-peptide response (teplizumab *vs* placebo) [0.45 *vs* 0.371; difference, 0.059 nmol/L (95%CI: 0.006-0.115 nmol/L)] ($P = 0.03$); the teplizumab group required less exogenous insulin ($P < 0.001$) with no significant difference in HbA1c level; ADR: rash, lymphopenia and nausea

ADR: Adverse drug reaction; CI: Confidence interval; HbA1c: Glycosylated hemoglobin; T1D: Type 1 diabetes.

who were anti-zinc transporter 8 (ZnT8) antibody negative, HLA-DR3 negative, and HLA-DR4 positive responded better to teplizumab than placebo[12]. Increased CD8+ T cell (cytotoxic and memory) and decreased $CD4^+$ T cell (helper and memory) cells were observed in treatment responders in different trials[12,13,15,22]. However, none of these metabolic or immunologic predictors were consistently reported across all the studies and thus needed further validation in future studies.

Cost-effectiveness: One of the significant limitations of the broader use of this novel molecule is its premium cost. Teplizumab costs around $193000 for a single course of 14 d therapy. Mital *et al*[23] tried to analyze the cost-effectiveness of teplizumab depending on the HLA-DR3, HLA-DR4, and ZnT8 antibody status. They predicted if the cost of therapy is more than $100000, treating only a quarter of the patients at risk will be cost-effective. If we consider current annual cost of management of T1D patients and cost of teplizumab therapy, it may be cost-effective only if the prospective patient fulfills all the favorable criteria of therapeutic response- HLA-DR3 negative, HLA-DR4 positive and negative anti ZnT8 antibody status[23,24]. However, cost cannot be the sole deciding factor for restricting the benefit of this drug to whom it can be effective. We hope that in future this drug will be more affordable for the larger number of T1D patients. Guidelines for practical clinical use, screening, and proper patient selection for this molecule are now being formulated [25].

Otelixizumab: Otelixizumab is another anti-CD3 monoclonal chimeric and humanized antibody that had been evaluated in T1D. In a dose-finding, safety, and tolerability assessment randomized control trial (RCT), a 6-d course of otelixizumab in four different dosages was given to 30 T1D patients within 32 d of diagnosis[26]. A sustained metabolic response of preserved c-peptide level was found for up to 18 mo following a 9 mg dose of otelixizumab. However, not all the studies showed significant improvement in c-peptide level when compared to placebo, specifically when it is used in lower dosage[27,28]. The presence of a positive insulin autoantibody is reported to be a predictor of therapeutic response[29].

Alefacept: Alefacept is a fusion protein that antagonizes CD2 costimulatory receptors, inhibiting T cell proliferation and action. In T1DAL RCT, two 12-d courses of alefacept were given at 12 wk apart in 49 newly diagnosed (< 100 d) T1D patients[30]. After 24 mo, patients in the alefacept arm showed significantly higher AUC for c-peptide response for 2 and 4 h ($P = 0.015$ and $P = 0.002$, respectively). There was also a significant reduction of exogenous insulin requirement and hypoglycemia event in the alefacept arm. Alefacept also induced favorable immunological response by depleting $CD4^+$ and $CD8^+$ T cells[30]. However, at 12 mo follow-up, there was no significant difference in c-peptide AUC compared to the placebo[31].

Abatacept: Abatacept is a fusion protein of the Fc portion of IgG1 and cytotoxic T-lymphocyte-associated antigen 4, which blocks the costimulatory signal by blocking the CD28 T-cell receptor. In a multicenter RCT, 112 newly diagnosed T1D patients (between 6 and 45 years of age) were included and intravenous abatacept was given for 27 infusions over 2

years. The authors reported significant preservation of β cell function as measured by higher AUC of c-peptide at 2 years of follow-up in patients who received abatacept (P = 0.0029)[32]. In the follow-up study, the authors also reported persistent beneficial effects in the abatacept arm even after 1 year of cessation of treatment (P = 0.046)[33]. However, in a phase 2 RCT where 101 participants of stage 1 T1D patients were included, monthly infusion of abatacept for one year failed to significantly delay the progression of T1D (P = 0.11)[34].

Antithymocyte globulin: Anti-thymocyte globulin (ATG) is a rabbit polyclonal IgG antibody that acts against multiple T-cell antigens. It may act through various mechanisms like T-cell depletion, induction of anergy in T cells, and selective induction of regulatory T (Treg) cells[35]. In the START trial, high-dose ATG (6.5 mg/kg) failed to show any significant preservation of c-peptide response in recent onset T1D patients[36]. ATG showed acute T-cell depletion sparing effector memory T cells. Higher adverse events, including CRS, were reported in the ATG arm. To decrease the risk of these adverse events, later studies used a lower dosage of ATG. In the TrialNet ATG-granulocyte colony-stimulating factor (G-CSF) study, low-dose ATG (2.5 mg/kg) or low-dose ATG with pegylated G-CSF were studied in recent onset (< 100 d) T1D patients[37]. The authors reported significant HbA1c reduction and slowing of c-peptide decline after 1 year of follow-up in the low-dose ATG group without any extra benefit with the addition of GCSF. In the 2-year follow-up of the same trial, although reduction in HbA1c and T-cell depletion with preservation of Treg cells were reported in both ATG as well as ATG with G-CSF arm, higher AUC of c-peptide in comparison to placebo was seen only in the low-dose ATG arm but not in the ATG with G-CSF arm[38]. However, low-dose ATG with GSF had been reported to preserve the AUC of c-peptide following mixed meal tolerance tests in other studies[39,40]. In a cost-effectiveness analysis study, low-dose ATG was found to be more cost-effective than other immunotherapies (including teplizumab) or no therapy in patients with recent onset T1D[41].

Anti-interleukin 21 and liraglutide: Researchers have also explored the role of combination therapy in immunomodulatory therapy for T1D. Anti-interleukin (IL)-21 antibody antagonizes IL-21-mediated autoreactive T-cell trafficking to pancreatic islets as well as proliferation of effector and follicular helper T cells[42,43]. Glucagon-like peptide-1 agonists like liraglutide have been reported to improve β cell survival[44,45]. In the proof of concept animal study in the T1D mouse model, Rydén *et al*[46] reported that the combined anti-IL-21 and liraglutide therapy can reverse diabetes. In a phase 2, multicenter, parallel-group, placebo-controlled RCT, 308 T1D patients were randomized to four arms – anti-IL-21 only, liraglutide only, combined anti-IL-21 with liraglutide, or placebo. The decline of post-mixed meal tolerance test c-peptide level at 52 wk was significantly smaller (P = 0.0017) in the combined group in comparison to the placebo, but not in the anti-IL-21 only (P = 0.093) or liraglutide only (P = 0.38) groups[47]. Although the authors also reported a reasonable safety profile for this combination therapy, it should be further confirmed in future phase 3 trials.

B-cell-directed antibody therapy

Rituximab: Like various T-cell depletion therapies, depletion of B cells using anti-CD20 monoclonal antibody rituximab has also been studied in T1D. In a placebo-controlled RCT (TrialNet Anti-CD20), 87 recently diagnosed T1D patients were randomized to four different dosage infusions of rituximab or placebo[48]. The patients in the rituximab arm showed significantly higher AUC of c-peptide than placebo during the mixed meal tolerance test at 1-year follow-up. The patients in rituximab also had lower HbA1c and required less exogenous insulin. Patients who responded to rituximab showed greater T-cell proliferative response to islet cell antigens[49]. However, at 30 mo follow-up of the same study, there was no significant difference in the AUC of c-peptide between the rituximab and placebo arms[50]. The effect on B-cell depletion by rituximab also weaned off by 18 mo. The authors concluded that although rituximab can delay the decline of c-peptide in T1D, it cannot prevent the inevitable β cell loss[50]. In a recent RCT, combined therapy of autologous CD4+ $CD25^{high}CD127^{-}$ Treg cells and rituximab was found to be superior in maintaining remission in recently diagnosed T1D patients in comparison to either the monotherapy or control[51].

Antibody therapy against cytokine signaling

Golimumab: The proinflammatory cytokine, tumor necrosis factor (TNF)-α plays an essential role in the pathogenesis of various autoimmune diseases[52]. In animal model studies, antagonizing TNF-α has been shown to prevent the development of autoimmune diabetes[53,54]. In the phase 2 T1GER study, the efficacy and safety of golimumab (anti TNF-α monoclonal antibody) were evaluated in recently diagnosed T1D patients (stage 3 of T1D)[55]. Patients were randomized to every fortnightly subcutaneous injection of golimumab (56 patients) or placebo (28 patients) for 52 wk. The authors reported higher 4-h mixed meal tolerance test AUC of c-peptide (0.64 pmol/mL *vs* 0.43 pmol/mL, P < 0.001) and lower exogenous insulin requirement (0.51 U/kg/day *vs* 0.69 U/kg/day) in golimumab arm than placebo. In the 2-year follow-up study (52 wk of therapy and 52 wk of off-therapy) of the same trial, patients in the golimumab arm showed persistently lower reductions in AUC of c- peptide at 78 and 104 wk compared to placebo[56]. The adverse events were reported to be similar in both arms of the study.

Etanercept: Etanercept, the recombinant soluble TNF-α receptor protein, blocks the activity of proinflammatory cytokine TNF-α and thus can be helpful in autoimmune diseases. In a pilot RCT, 18 patients with recently diagnosed T1D were given subcutaneous twice weekly etanercept or placebo for 24 wk[57]. The patients in the etanercept arm showed better HbA1C levels (5.9% *vs* 6.8%; P < 0.05) and AUC of c-peptide (+ 39% *vs* -20%; P < 0.05) than placebo, suggesting β cell preservation. In a recent study, etanercept was combined with glutamic acid decarboxylase (GAD-alum) and vitamin D (etanercept diamyd combination regimen) to evaluate the efficacy in newly diagnosed anti-GAD antibody-positive T1D patients[58]. However, this combination failed to show any significant beneficial effect in this trial.

Baricitinib: Baricitinib is a Janus kinase (JAK1 and JAK2) cytokine inhibitor which had been used successfully in autoimmune disorders[59,60]. In the phase 2 multicenter RCT (BANDIT study), 91 recently diagnosed T1D patients were randomized to either oral baricitinib (4 mg/d) or placebo for 48 wk[61]. Following the mixed meal tolerance test, the c-peptide level was significantly higher in the baricitinib group than placebo (P = 0.001) at 48 wk follow-up. Exogenous insulin requirement was also considerably low in the baricitinib group, with no significant difference in HbA1C level compared to the placebo. The adverse events were reported to be similar in both groups.

Imatinib: The role of small tyrosine kinase inhibitors like imatinib also had been evaluated in T1D. In a nonobese diabetic mouse study, imatinib had been reported to induce durable remission[62]. Imatinib has been postulated to act through both immunological and metabolic pathways involving endoplasmic reticulum stress in β cells[63]. In a phase 2 multicenter RCT, patients with recent onset T1D were randomized to receive either oral imatinib (400 mg/d) or placebo for 26 wk[64]. The patients in the imatinib arm showed higher AUC of c-peptide compared to the placebo at 12 mo (P = 0.048) follow-up. However, the effect was not sustainable at 24 mo. Future studies are needed to find the ideal dose and duration as well as to monitor the adverse effects of this exploratory therapy.

Apart from the pharmaceutical agents mentioned above, other molecules like verapamil, ladarixin, canakinumab and anakinra were also studied in recent onset T1D with variable success[65-67]. In a recent meta-analysis, which evaluated the effect of monoclonal antibody-based immunotherapy on c-peptide level in patients with recent onset T1D, 11 studies of four antibody-based immunotherapy (teplizumab, rituximab, otelixizumab and abatacept) were included[68]. The authors reported favorable c-peptide response in favor of β cell protection with all these four molecules. In a comparative study, the results (AUC of c-peptide response) from the primary studies of various immunotherapies (teplizumab, alefacept, abatacept, rituximab, high dose ATG, and low dose ATG ± G-CSF) in T1D were evaluated to rank them according to their effectiveness[69]. The authors reported that low-dose ATG and teplizumab showed maximum impact in preserving β cell function among the molecules studied. However, when these immunotherapies are used, we must be careful about the potential risk of adverse events like lymphopenia, viral infections, and CRS.

We should also remember that the sole FDA-approved immunotherapy, teplizumab, is indicated to be used only in stage 2 of T1D and thus practically has minimal application unless the disease is diagnosed early in the dysglycemia stage. More widespread screening programs like TrialNET, ASK, Sanford Population-Level Estimation of T1D Risk GEnes in Children, and T1 Detect JDRF are needed to find suitable candidates who are at the window of opportunity for this therapy[70]. The durability of the immunotherapies and frequency of dosage to maintain a state of disease remission should also be evaluated in longitudinal follow-up studies. Another concern is the safety of the long-term use of these immunomodulatory therapies due to the risk of CRS, reactivation of viral infections, *etc.* Thus, further well-designed RCTs with longer follow-up are needed to evaluate the efficacy, durability of therapeutic response, and safety of these immunotherapies in T1D. Moreover, the drugs should be affordable and cost-effective to ensure the access of newer therapies to the target populations.

ANTIGEN-BASED IMMUNOTHERAPY

Although several nonspecific approaches of immunoregulation have been trialed for T1D, antigen-based immunotherapy is thought to be a more favorable approach owing to its specificity and possible long-lived effects, without broad immunosuppression being needed[71]. The basis of antigen-specific immunotherapy is either inactivation of the pathogenic effector T (Teff) cells functionally, transforming them into Treg cells, or both. An ideal antigen-based immunotherapy could be administered multiple times (nonimmunogenic by itself) and would induce Treg cells that could repress islet-specific Teff cells of various specificities and lead to long-standing tolerance[72]. However, many questions are to be considered, along with the duration of the treatment and cost when designing antigen-specific immunotherapy.

Antigen targets and delivery strategies

For antigen-based immunotherapy to be fruitful, the subject getting treatment must have a T-cell population specific for the therapy-delivered antigen. Therefore, when developing an immunotherapy for individuals with T1D, the major specificities of circulating autoreactive T cells are essential factors to consider. In T1D individuals, the isolated $CD4^+$ and $CD8^+$ T-cell clones with specificity for a range of autoantigens have been identified. These include insulin, pre-proinsulin, GAD, islet antigen-2, islet glucose-6-phosphatase catalytic subunit related protein, and chromogranin A. Recently, proinsulin (c-peptide region) has been a hotspot for responsiveness of T cells in patients with T1D[73,74]. Furthermore, islet inflammation along with endoplasmic reticulum stress can cause further islet-derived antigen release, thereby inducing epitope spreading, post-translational modification of peptides, and fusion of peptide fragments originating from different proteins with subsequent generation of hybrid peptides[75]. Therefore, an in-depth knowledge of the critical antigenic targets and their kinetics during the progression of disease is crucial for choosing the most fitting targets for antigen-based therapy. Disease heterogeneity adds to the complexity of antigen-specific immunotherapy in T1D because of a subgroup of patients depending on their HLA alleles or disease endotypes[76]. Heterogeneity and HLA haplotype can influence the presentation of epitopes from islet antigens to the autoreactive T cells as well as the response of T cells [77,78]. Considerations and requirements for antigen-based immunotherapy in T1D are illustrated in Figure 2. Experts have used different delivery strategies for antigen-specific immunotherapy, for example, proteins and peptides, plasmid DNA, cell-based strategies, antigen-loaded nanoparticles, and liposome-based approaches (Table 2). The key question is which approach is more likely to restore β cell tolerance?

Table 2 Antigen-specific immunotherapies- strengths and weaknesses

Strategies	Immunological target	Advantages	Disadvantages
Autoantigenic peptides and proteins	APCs	Biocompatible; possibility to conjugate to a vehicle	Short half-life; adjuvant required
Autoantigen-encoding plasmid DNA	APCs	Long-lived effect	Gene therapy
Antigen-loaded cell-based strategies	Autoreactive T cells	Powerful immunoregulatory effect	Leukapheresis required; personalized medicine
Antigen-loaded nanoparticles and liposomes	APCs and T cells	Customizable; powerful immunoregulatory effect; might act by biomimicry	Synthetic; preclinical developmental phase

APCs: Antigen-presenting cells.

Preclinical and human studies

The antigen-specific approach involves the delivery of β cell autoantigens through a route and regimen that induces immune tolerance. Several antigen-specific approaches have entered into trials in the last decade to explore their safety, feasibility, and efficacy using different delivery strategies[79]. Recently completed and ongoing immunotherapy trials using antigen-specific strategies in T1D are reviewed elsewhere[80]. Single-peptide immunotherapy using a proinsulin sequence showed hints of efficacy and immunomodulation and was well tolerated[81]. Gibson *et al*[82] used a preclinical, humanized model of peptide immunotherapy. They showed that combining numerous different β cell peptides into a single injectable may produce a significantly increased effect compared with a single peptide in generating immune regulation. A recent study has addressed whether therapies delivering several antigens concurrently are efficacious and any safety issues that can arise from administration of multiple antigens. A mixture of six β cell peptides from two islet autoantigens was administered to patients with recent-onset T1D[83]. Multiple-peptide immunotherapy showed the potential to rectify immune regulatory defects central to the pathobiology of T1D in this first-in-human study. No serious adverse effects were observed in groups that received drug treatment. Together with the observations in pre-clinical models that delivery of multiple peptides from more than one antigen may have more impact than a monopeptide[82], these recent findings justify future well-designed clinical trials.

The last decade has seen the knowledge translated into definite antigen-based immunotherapies, promising to restore the breach of immunological tolerance to β cell autoantigens selectively. However, in both prevention and reversion trials in T1D, suboptimal effects have been obtained so far. Consequently, there is still a need to optimize those immunotherapies and their associated factors, such as patient and disease heterogeneity; choice of antigen (peptide or whole molecule, conventional or unconventional, single antigen or cocktail); posology; administration patterns, route, timing and use of adjuvants; biomarkers for stratification and therapeutic outcome[80].

STEM-CELL-BASED IMMUNOTHERAPY

T1D can be reversed by transplantation of pancreas or islet cells, which serves as proof of principle for cell-based therapy [84]. However, several issues limit its widespread use, particularly the insufficient supply of highly functional β cells. Yet, the sourcing problem could be circumvented by differentiating stem cells (SCs) into insulin-producing cells, and it has garnered the most enthusiasm for creating functional β cells[85,86]. These SC-derived islets could be derived from a single-cell source using a standardized process. The resulting cell product could be well characterized, allowing for more predictable transplant outcomes.

SCs [embryonic SCs (ESCs), induced pluripotent SCs (iPSCs)], and adult SCs are being widely explored for T1D therapeutics[87]. In preclinical studies, ESC-derived β cells have shown favorable results by insulin production in response to glucose stimulation and restoration of normoglycemia[87]. iPSCs offer a practical alternative to ESCs. They can be derived from adult somatic cells, thus eliminating ethical concerns. In diabetic mouse models, iPSC-derived β cells have also exhibited the ability to secrete insulin and restore normoglycemia. Besides iPSCs, adult SCs, including mesenchymal SCs and hematopoietic SCs, have been investigated for T1D as well[88].

Recent technological advances have made human clinical trials utilizing SC-derived pancreatic endoderm cells (PECs) possible. An initial 2014 human clinical trial used the ViaCyte Inc. device (VC-01) to immunoprotect the cells using a cell-impermeable membrane entirely. Although some endocrine cells were found, fibrosis around the capsule led to graft loss, and insulin secretion was not detected from the device[89,90]. To circumvent this issue, a clinical trial (NCT03163511) was initiated in 2017 to evaluate the newer PEC-Direct device (PEC-01 cells implanted subcutaneously in VC-02 devices) that contained membrane openings allowing vascularization to develop nutrient exchange and promote survival of cells. Total immunosuppression was required after transplantation. In 63% of units, insulin expression within β cells was observed at 3-12 mo post-transplantation, with a preponderance of α cells reflecting the immature graft state. The recently published reports from this ongoing trial demonstrated detectable levels of c-peptide in peripheral blood by 6-9 mo post-transplantation[91,92]. Vertex pharmaceuticals embarked on a clinical trial with T1D patients in 2021, where an ESC-

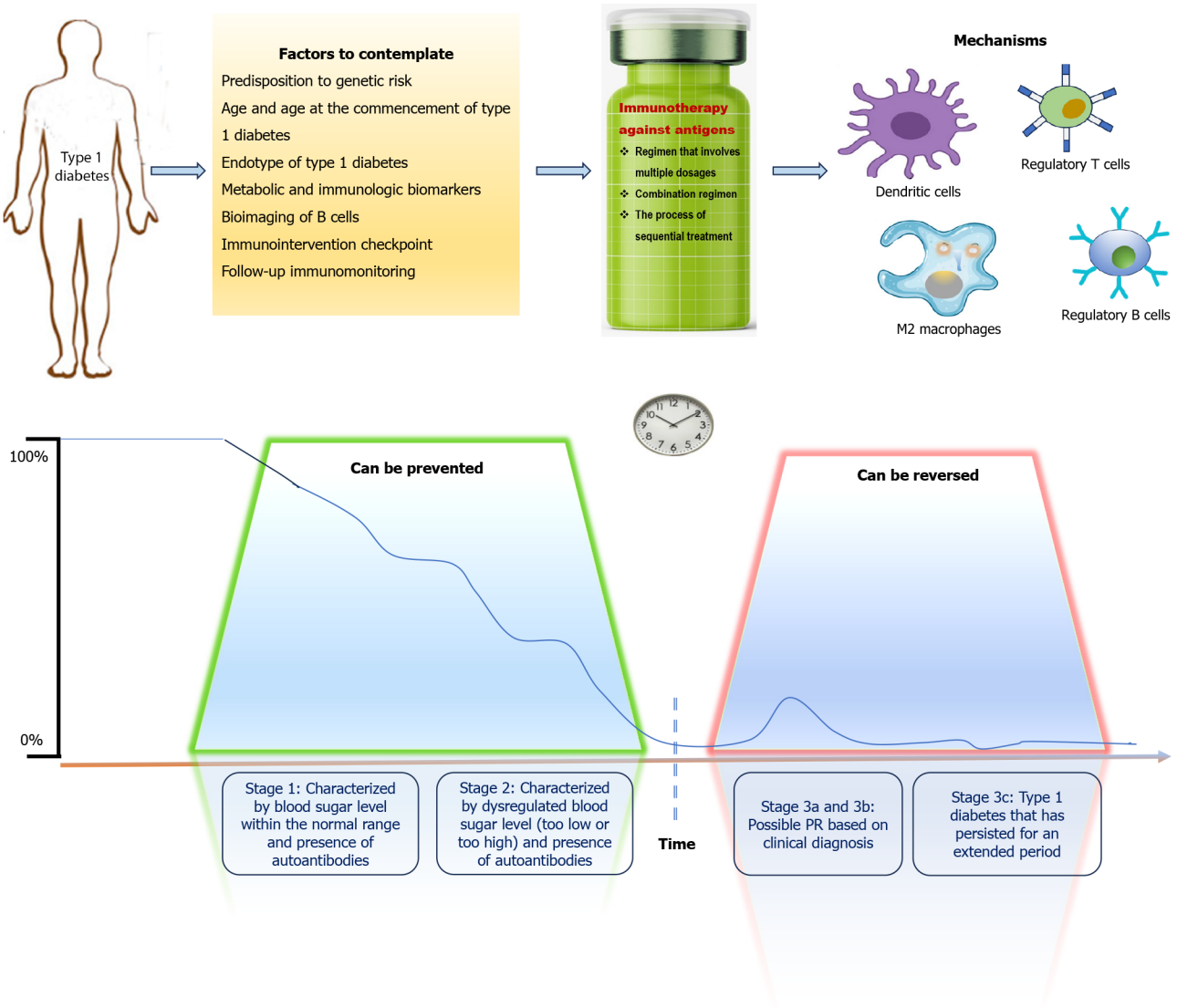

Figure 2 Considerations and requirements for successful immunotherapy using antigen-specific approaches in type 1 diabetes. If the antigen-specific therapy is administered before the clinical diagnosis of type 1 diabetes (T1D) (stage 1 and 2), the development of T1D could be revoked; otherwise, if T1D has already been diagnosed (stage 3), the intervention should be accompanied by a β cell regenerative agent to restore the β cell mass and its functionality. One should consider the genetic risk susceptibility of the patient, age and age at T1D onset, autoreactivity profile and T1D endotype, metabolic and immunologic biomarkers, and ideal antigen-specific therapy time point for the treatment to be as personalized as possible. On this basis, the partial remission (PR) – when β cell tolerance is thought to be restored transiently – and exploring subsequent but milder PR phases could be of value as immune intervention checkpoints (in stage 3a and 3b). In addition, the antigen-specific immunotherapy should enforce the generation of tolerogenic dendritic cells, M2 macrophages, regulatory T (Treg) cells and regulatory B cells, thus reeducating the immune response against β cell autoantigens and re-establishing β cell immunological tolerance.

derived islet, VX-880, was transplanted without an immunoprotective device under immunosuppressive coverage. Initial findings seem promising[93]. An ongoing Vertex trial (NCT04786262) will determine whether the success can be replicated and investigate the safety of implantation of SC-derived islets in a site such as the liver. In addition to these critical advances, several organizations intend to conduct clinical trials of functional SC-derived islets[94]. It is important to note that although freed from reliance on exogenous insulin, the Vertex result presently is based on a single patient and that ViaCyte's strategy, established on differentiation of PEC to adequate numbers of functional β cells within porous devices, has not yet been shown to work. The requirement for long-term immunosuppression may restrict the clinical application of both the ViaCyte and Vertex approaches.

As things stand, the clinical trial results highlight the great promise SC-islets hold for treating T1D. The last few years have been notable for game-changing early progress in clinical trials with SC-based therapies for T1D[95]. Nevertheless, several remaining challenges need to be addressed before this SC therapy can be converted into a routine procedure. The central pillars of a successful SC-islet therapy for T1D are illustrated in Figure 3.

CONCLUSION

T1D involves the autoimmune destruction of insulin-producing β cells in the pancreas. Over 100 years since the discovery

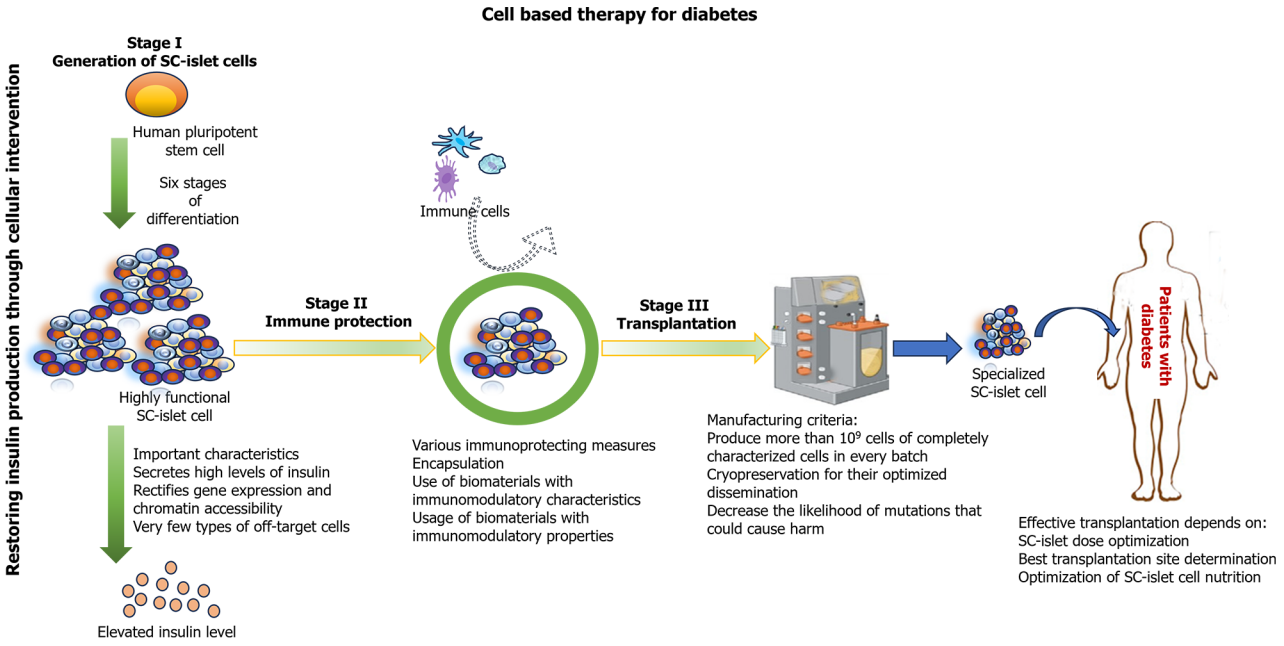

Figure 3 Key components of successful stem cell derived islet therapy for treating type 1 diabetes.

of insulin, there is still no cure for T1D. However, therapeutic options for T1D are again at a turning point. Years of effort to develop immune interventions are ultimately starting to pay off, with hints of progress in both new onset and preventative settings. We discussed the recently completed and ongoing clinical trials that have studied the efficacy and safety of several immunotherapeutic strategies targeting various mechanisms of autoimmunity, which are considered significant in disease pathogenesis. While more targeted immunotherapies with potentially fewer adverse effects get closer to the translation into clinical practice, new challenges may need to be faced. A better understanding of disease endotypes may facilitate the stratification of individuals to different treatment options. While moving forward, success lies in selecting which interventions are best suitable for which stage of the disease. Therefore, the timing and benefit/risk profile of candidate approaches should be considered carefully. It is also essential to conduct more clinical trials at T1D diagnosis to compare interventions. With the increasing interest in combination approaches, immunotherapeutic strategies targeting different aspects of the immune system are likely to be essential contributors to the future therapeutic landscape, together with the practice of individualized patient-tailored approaches, a change towards early intervention, and an emphasis on outcome measures.

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FOOTNOTES

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EDITORIAL

Surgical or medical treatment of obesity-associated type 2 diabetes-an increasing clinical conundrum

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Abstract

In this editorial, we comment on the article by He *et al*, specifically in relation to the efficacy of bariatric surgery *vs* glucagon-like peptide-1 receptor agonist (GLP-1RA) therapy in the management of type 2 diabetes (T2D) associated with obesity. Bariatric surgery has now also been shown to be safe and effective in pre-teens and teenagers with obesity and T2D, but information on newer GLP-1RAs in these groups is predictably limited. In older individuals (age > 65 years), both bariatric surgery and GLP-1RA therapy improve cardiovascular outcomes. Bariatric surgery is not infrequently associated with post-operative postprandial hypoglycemia, which is not the case with GLP-1RAs and, paradoxically, there is evidence that GLP-1RAs may reduce both the frequency and severity of postprandial hypoglycemia. Comparative trials of the long-term efficacy of bariatric surgery and GLP-1RAs are indicated.

Key Words: Glucagon-like peptide-1; Glucagon-like peptide-1 receptor agonist; Obesity; Diabetes; Weight loss; Bariatric surgery; Metabolic surgery; Hypoglycemia

Core Tip: Glucagon-like peptide-1 receptor agonist-based therapy should be considered for the remission of type 2 diabetes and weight loss as an alternative to bariatric surgery.

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INTRODUCTION

The informative review by He *et al*[1], addresses the relevance of bariatric surgery to the management of type 2 diabetes (T2D) in adults with obesity and its potential application to other cohorts–older people (> 65-70 years) and children (< 16 years) who have T2D, obesity in type 1 diabetes (T1D) and in T2D when body weight is normal. They also addressed the long-term adverse sequelae of bariatric surgery, particularly post-bariatric surgery hypoglycemia (PBSH)[2]. A recent study[3] has confirmed the efficacy of different forms of bariatric surgery [predominantly Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy] compared with traditional medical/lifestyle management in producing sustained improvements in glycemic control (including T2D remission) associated with the maintenance of substantial weight loss and amelioration of diabetic microvascular complications. It can no longer be disputed that bariatric surgery (now often referred to as 'metabolic surgery') is the most effective approach to the treatment of T2D associated with obesity[4]. That lifestyle approaches usually fail in the long-term, so that weight loss is often modest and weight regain is very common, is not surprising and, at least in part, attributable to potent neural/hormonal mechanisms that favor an increased energy intake and diminished energy expenditure[5]. Limitations of bariatric surgery are, for the main part, predictable–it is an invasive procedure, costly, not applicable on a population basis, requires life-long follow up (particularly to monitor for nutritional deficiencies) and is usually associated with adverse effects that are, not infrequently, major. It is also appreciated that the improvement in glycemic control in T2D occurs very soon after bariatric surgery and is, accordingly, not solely attributable to weight loss per se[6]. After most forms of bariatric surgery, but particularly RYGB, gastric emptying and small intestinal transit are accelerated markedly[7] to increase secretion of the 'incretin' hormone, glucagon-like peptide-1 (GLP-1)[6]. The latter is likely to be central to the improvements in glycemic control and reduction in body weight induced by bariatric surgery, but also pivotal to the etiology of PBSH[8]. It is, therefore, not surprising that the advent of agonists of GLP-1, used widely in the management of T2D and obesity with and without diabetes, now provides a therapeutic alternative to bariatric surgery for the first time.

DEVELOPMENT OF GLP-1 RECEPTOR AGONISTS FOR THE MANAGEMENT OF OBESITY

The development of GLP-1 receptor agonists (RAs) followed the recognition that GLP-1, released from the small intestine in response to the presence of nutrients, was an 'incretin' hormone, so that it stimulated insulin (and also, suppressed glucagon) potently and in a glucose-dependent manner. It was shown subsequently, that even in low concentrations, GLP-1 also slowed the rate of gastric emptying substantially, thereby contributing to the reduction in postprandial glucose excursions[9]. Because of its short plasma half-life (about 2 minutes), GLP-1RAs resistant to proteolytic degradation were developed for the management of T2D. With 'first generation' compounds (*e.g.* exenatide twice daily), lowering of glucose in T2D was also found to be associated with modest effects to reduce appetite and weight–the latter on average by approximately 10%[10]. This observation, coupled with the demonstration of cardiovascular and renal protective effects, stimulated the evaluation of 'second generation' GLP-1RAs (*e.g.* liraglutide) in higher doses than those used in T2D, for the management of obesity without T2D, with a resulting increased weight loss of approximately 15% [11]. More potent GLP-1RAs, such as subcutaneous semaglutide, were then developed. Recently, co-agonists where a 'third generation' GLP-1RA has been combined with another hormone(s) [glucose-dependent insulinotropic polypeptide (GIP), amylin and glucagon] have been developed. Tirzepatide, a co-agonist of GLP-1 and GIP receptors, is now widely available and induces weight loss of > 20%[12], albeit a little less in individuals with T2D compared to those with obesity without diabetes. Triple agonists (*e.g.* GLP-1, GIP and glucagon) are currently in development[13]. The majority of GLP-1RAs, alone or in combination, are usually administered subcutaneously once a week. However, an oral formulation of semaglutide, administered daily, has recently become available for the management of T2D and is currently being evaluated in a higher dose for use in obesity management[14]. Furthermore, non-peptide small molecule GLP-1RAs (*e.g.* orforglipron) are in late development[15]. It should be appreciated that adherence with GLP-1RAs in the longer term is suboptimal and their use is not infrequently compromised by gastrointestinal adverse effects and cost[16]. However, while future studies are required to determine their safety and long-term efficacy, it is clear that GLP-1RAs may now represent an alternative to bariatric surgery for the management of obesity with, or without, concomitant T2D.

USE OF GLP-1RA IN PEDIATRIC AND OLDER POPULATIONS

He *et al*[1] review the evidence relating to the safety and efficacy of bariatric surgery in youths (< 18 years) and older individuals (> 65-70 years)[1]. While there may be a role of bariatric surgery in the management of obesity in pre-teens and teens with severe obesity (body mass index > 40)[17], long-term, prospective studies are required to guide the management of childhood obesity. In older individuals, bariatric surgery has now been shown to be safe and effective at reducing weight, with concomitant improvements in cardiovascular health and quality of life[1].

As with bariatric surgery, there is much less information about the effect of GLP-1RAs in individuals < 18 years or > 65-70 years of age. Currently, there are three subcutaneous GLP-1RAs approved for treatment of youths aged 10–18 years with T2D that have been shown to improve glycemic control: Liraglutide, extended release exenatide and dulaglutide [18]. In youths with obesity (without T2D), aged 12–18 years, GLP-1RAs can now be used for weight management, although the observed reductions in mean weight were modest (4.9 kg), apart from semaglutide, which showed a mean weight loss of 17.7 kg in one study[19]. The long-term efficacy and safety of GLP-1RA therapy in youths requires further study.

While in older people, GLP-1RA therapy has been associated with improved cardiovascular outcomes[20], their use must be balanced against potential deleterious effects. Although with liraglutide, no decline in bone mineral density was observed after 2 years of treatment[21], subcutaneous semaglutide was associated with a reduction in bone mineral density after 1 year of treatment[22]. However, the combination of GLP-1RA therapy with exercise may potentially preserve bone mineral density[23]. Studies are required to address this issue more comprehensively and to also determine whether there is an increase in fracture risk. Muscle loss is an inevitable sequelae of weight loss and there is appropriate concern that due to their effects to reduce energy intake and muscle mass significantly[24,25]. GLP-1RAs have the potential to exacerbate sarcopenia and/or malnutrition. Accordingly, as is the case with bariatric surgery, judicious use of GLP-1RAs in this population is essential at present

In the elderly, there is a high rate of cessation of therapy after 2 years which is likely to reflect, at least in part, adverse effects or cost[16]. Symptoms such as constipation may occur with more prolonged treatment[26]. There is also a suggestion that possible effects such as small bowel obstruction, albeit rare, may occur more frequently with prolonged treatment, with evidence that the highest risk is after 1.6 years of therapy[27].

GLP-1 AND POST BARIATRIC SURGERY HYPOGLYCEMIA

In prospective studies[28,29], about a quarter of patients following RYGB experience PBSH and approximately 7% required hospital admission because of severe hypoglycemia (< 3.1 mmol/L)[28]. GLP-1RA therapy, in isolation, is not associated with hypoglycemia[30,31], which is not surprising given the glucose-dependency of insulin stimulation. However, there is a risk of hypoglycemia when GLP-1RAs are used concurrently with insulin or sulphonylureas, which may, potentially, be increased[32,33]. The underlying mechanism of PBSH is complex and incompletely defined but may, in part, relate to rapid delivery of nutrients to the small intestine to result in exaggerated GLP-1 and insulin responses [34]. We have reported that in individuals with PBSH following RYGB, the postprandial GLP-1 and insulin responses are greater than in those without PBSH[8]. It is, accordingly, not surprising that a GLP-1 receptor antagonist, avexitide, has been associated with reductions in postprandial hypoglycemia and is in development for its management[35]. Small trials and case reports also indicate of a reduction in the frequency of PBSH with GLP-1RA therapy, which is intuitively surprising[36]. The mechanism(s) underlying the apparently paradoxical reduction in the frequency of hypoglycemia after both agonism or antagonism of the GLP-1 receptor remain to be determined but may relate to a slowing of small intestinal transit and/or suppression of the postprandial rise in glucose, to thereby attenuate the insulinemic response.

CONCLUSION

GLP-1RAs, particularly newly developed drugs, clearly have the potential to represent an alternative to surgery for the long-term management of T2D associated with obesity in adults < 65 years. In individuals with substantial comorbidity or those who wish to avoid an invasive procedure, they now represent a reasonable alternative to surgery. Bariatric surgery and GLP-1RAs may both be effective options for the management of diabetes and obesity in youths and older individuals. Further studies are, however, required to determine the safety and efficacy of both treatments in childhood obesity.

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REVIEW

Role of cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes pathway in diabetes and its complications

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Abstract

Diabetes mellitus (DM) is one of the major causes of mortality worldwide, with inflammation being an important factor in its onset and development. This review summarizes the specific mechanisms of the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)-stimulator of interferon genes (STING) pathway in mediating inflammatory responses. Furthermore, it comprehensively presents related research progress and the subsequent involvement of this pathway in the pathogenesis of early-stage DM, diabetic gastroenteropathy, diabetic cardiomyopathy, non-alcoholic fatty liver disease, and other complications. Additionally, the role of cGAS-STING in autonomic dysfunction and intestinal dysregulation, which can lead to digestive complications, has been discussed. Altogether, this study provides a comprehensive analysis of the research advances regarding the cGAS-STING pathway-targeted therapeutic agents and the prospects for their application in the precision treatment of DM.

Key Words: Cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes; Diabetes mellitus; Inflammation; Glycolipid metabolism; Diabetes gastroenteropathy; Nonalcoholic fatty liver disease; Diabetes cardiovascular disease; Diabetes nephropathy

Core Tip: Inflammation mediated by the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway is closely related to the occurrence and development of diabetes and its complications. This article focuses on the specific mechanism of cGAS-STING signaling pathway in mediating inflammatory response as well as the role of cGAS-STING signaling in complications such as diabetes, diabetic gastroenteropathy, diabetic cardiomyopathy, and non-alcoholic fatty liver disease, along with the role of transmission pathways and the related research progress.

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia caused by multiple etiological factors. Long-term disorders of glucose and lipid metabolism can cause chronic progressive lesions, functional decline and failure of multiple systems, tissues and organs. The latest statistics in 2021, the number of people with diabetes worldwide is about 529 million, accounting for about 6.1% of the total population. Type 2 DM (T2DM) accounts for 96% of the cases, of which more than 50% of T2DM can be attributed to obesity, and lack of exercise. Complications such as diabetes cardiovascular disease, diabetes nephropathy (DNe), blindness, limb loss, disability, and chronic pain significantly reduce the quality of life of patients, and place a huge burden on public health[1].

Traditional hypoglycemic drugs including biguanides, sulfonylureas, thiazolidinediones, and gliclazones do not meet the clinical needs, with some of them exhibiting poor or unsustainable efficacy or adverse drug reactions such as hypoglycemia, weight gain, and gastrointestinal reactions. Some Food and Drug Administration-approved drugs, such as glucagon-like peptide-1 receptor agonists (such as selegiline and liraglutide), dipeptidyl peptidase-4 inhibitors (such as selegiline and viglitin), and sodium-dependent glucose transporter 2 inhibitors (such as dagliflozin), have already been applied in clinical settings. However, some drugs such as glucokinase activators, peroxisome proliferator-activated receptor (PPAR) agonists, free fatty acid (FFA) receptor 1 agonists, and menin inhibitors are still in clinical trials; and have in treatment of DM and delaying β-cell damage have greater potential. Siehler *et al*[2] identified the insulin inhibitory receptor Inceptor, a new potential target for treating DM, which increases the sensitivity of the insulin signaling pathway in pancreatic β cells and promotes their protection and regeneration. Teplizumab delays type 1 DM (T1DM) progression by binding to cluster of differentiation CD3 on effector T cells and inhibiting their action on pancreatic β cells[3]. Presently available therapeutic agents mainly slow down the damage to islet β cells and reduce lipid cell metabolism. The development of medicines and the understanding of disease pathogenesis are closely linked. Recent studies have shown that the cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS)-stimulator of interferon genes (STING) pathway plays an important role in the early and progressive stages of diabetes as well as in multiorgan complications. It is expected to be a new therapeutic target with great potential[4].

cGAS, a natural immune receptor, recognizes various double-stranded DNA (dsDNA) in the cytoplasm, including those from exogenous viruses, bacteria, endogenous mitochondria, nuclei, and reverse-transcribed DNA[5]. Following dsDNA binding, the cGAS enzyme is activated, which then catalyzes the synthesis of the second messenger cGAMP from adenosine triphosphate (ATP) and guanosine triphosphate (Figure 1). Next, cGAMP binds to the dimeric protein STING on the endoplasmic reticulum (ER) membrane, altering its conformation, triggering STING oligomerization, and its transfer to the Golgi apparatus. In the Golgi apparatus, the two cysteine residues of STING (namely Cys88 and Cys91) are palmitoylated. STING then recruits and interacts with TANK-binding kinase 1 (TBK1), which phosphorylates interferon (IFN) regulatory factor 3 (IRF3), triggering dimerization, nuclear translocation, and induction of IRF3-related target genes, thereby affecting IFN production. Recently, the cGAS-STING pathway has been reported to be involved in the pathogenesis of metabolic diseases, as cGAS can recognize dsDNA from endogenous mitochondria and induce metabolic inflammation[6].

Reportedly, the cGAS-STING pathway plays an important role in both early and progressive stages of DM and in associated multiorgan complications, and it has been proposed as a novel, potential therapeutic target. Hence, this study aims to discuss the role of the cGAS-STING pathway in DM and its subsequent complications. Additionally, the regulatory roles of the cGAS-STING pathway in DM, diabetic gastroenteropathy (DG), non-alcoholic fatty liver disease (NAFLD), diabetic cardiomyopathy (DCM), DNe, diabetic retinopathy (DR), and diabetic wound (DW) healing have been discussed. This review highlights the implications of the cGAS-STING pathway in distinct disease processes and may provide insights into the systemic management of DM. A summary of additional literature related to this research has been provided in Table 1.

CGAS-STING IN DM PATHOGENESIS

DM is a multiorgan metabolic disorder, mainly caused by absolute or relative insulin deficiency and characterized by impaired glucose tolerance and hyperglycemia. Reportedly, inflammatory pathways can lead to obesity, insulin resistance (IR), and subsequent DM-associated metabolic disorders and complications. T1DM is an autoimmune disease characterized by the autoimmune elimination of pancreatic islet β cells, resulting in insulin deficiency. In animal models, immune cells have been implicated in T1DM progression. T cells can induce an inflammatory infiltration around the islet β cells and DM, and macrophages mediate islet inflammation and secretion of inflammatory factors interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which are associated with the synergistic effects of IFN-γ that result in apoptosis of pancreatic β cells[7]. T2DM is characterized by a relative insulin deficiency because of the development of IR in organs such as the liver, muscle, and other major organs that are not sensitive to insulin. Fat accumulation in the liver and muscle tissues is a precursor to IR, and related adipokines (such as leptin and adiponectin) mediate inflammatory responses, allowing immune cell infiltration in adipose tissues. Altogether, these factors substantially increase the inflammatory response in the body, ultimately leading to DM-associated organ damage and dysfunction[8], suggesting that regulating local inflammatory cytokine production may control the development of DM.

cGAS-STING in T1DM pathogenesis

T1DM is an autoimmune disease characterized by β cell destruction, mainly by free radical and oxidant production leading to DNA damage and activation of an apoptotic cascade triggered by polyadenosine diphosphate-ribose polymer (PARP) activation[9]. PARP is involved in various cellular processes related to DNA repair and programmed cell death. In DM, following glycolysis, the tricarboxylic acid cycle and polyol pathway are activated, which severely disturbs the ratio of nicotinamide adenine dinucleotide forms (NADH:NAD+), resulting in excessive NADH production and disruption of the redox balance[10]. Autoimmune stimulation triggers the infiltration of monocytes and macrophages into the pancreas, and free radicals and oxidants produced by monocytes and pancreatic cells, in combination, lead to intracellular DNA single-strand breaks and PARP activation. Moreover, depletion of cellular NAD+ leads to inhibition of cellular ATP production, resulting in cellular dysfunction and cell death. The marked loss of β cells decreases glucose tolerance and introduces hyperglycemia[11]. Hyperglycemia triggers the intracellular release of oxidative mediators from the mitochondrial electron transport chain, NADH/nicotinamide adenine dinucleotide phosphate hydrogen oxidases, and other sources, which then induce DNA single-strand breaks, thereby reactivating PARP. Furthermore, the effects of increased glucose levels are exacerbated by an increase in aldose reductase activity, leading to nicotinamide adenine dinucleotide phosphate hydrogen depletion and the production of reactive oxidants. PARP activation promotes the activation of activator protein 1, mitogen-activated protein kinases, and nuclear factor (NF)-κB, along with the expression of proinflammatory mediators, adhesion molecules, and inducible nitric oxide synthase (NOS)[12]. Ultimately, both PARP activation and mitochondrial oxidative stress form a positive feedback loop.

cGAS-STING in T2DM pathogenesis

Mitochondrial apoptotic pathway- and ER stress-induced lipotoxic injury in pancreatic β cells is an important pathological feature of T2DM[13]. The ER is the site of FFA esterification, and prolonged exposure to high-fat environments overloads its esterification capacity, leading to impaired ER function and enhanced ER stress. Intracellular oxidants affect mitochondria and lead to DNA single-strand breaks and cGAS-STING-IRF3 pathway activation[14]. Hu *et al*[15] reported that blocking the STING-IRF3 pathway ameliorated lipotoxicity-induced islet damage. Additionally, Wang *et al*[16] showed that the cGAS-STING pathway activated protein kinase B (Akt) to promote the inflammatory response. According to the studies, the specific mechanism is speculated as follows. Insulin regulates the production of glucose, lipids, and proteins through the phosphatidylinositol 3-kinase (PI3K) pathway. The substrate protein of the insulin receptor is phosphorylated, leading to the binding and activation of PI3K, which upregulates Akt, which is involved in insulin signaling, metabolism, cell growth, and cell cycle. Akt induces glucose transporter-4-mediated glucose transport into cells, promoting glucose metabolism in an insulin-dependent manner, and regulates the mammalian target of rapamycin (mTOR) *via* direct and indirect pathways. Reportedly, Akt phosphorylates mTOR and inhibits Ras homolog enriched in brain (Rheb) activation, a positive regulatory protein for mTOR activation, by inactivating tubulin sclerosis complex (TSC) 2 to enhance mTOR activation. Under normal circumstances, TSC-1 and TSC-2 form a dimeric complex that inhibits Rheb, thereby inhibiting the mTOR function. However, Akt can phosphorylate TSC-2 and inhibit the formation of the TSC-1/TSC-2 complex, thereby releasing the inhibitory effect on Rheb and activating mTOR[17]. Reportedly, mTOR mediates the association of Akt with the cGAS-STING pathway, and TBK1 can phosphorylate the S2159 site of mTOR to increase IFN-β levels[18]. TBK1 activation inhibits the activity of mTOR complex 1 (mTORC1)[19]. Altogether, these findings establish a relationship between the Akt protein family and the cGAS-STING pathway.

To explore the relationship between Akt and cGAS-STING pathway in DM pathogenesis, gene databases (www.genecards.org, https://omim.org/) were used, and "diabetes" and "cGAS-STING" were searched as keywords, which provided 18798 and 410 genes, respectively, along with 290 intersecting genes. Next, a protein-protein interaction network was formed using the STRING tool (version 11.5) to select the potential proteins in Akt-related pathways. The resulting data were imported into Cytoscape (version 3.9.1) for visualization (Figure 2A). The larger the circle area and the darker the color, the closer the association with other proteins. Figure 2B presents the 20 targets with the highest degree value. The higher the degree value, the closer the association with other proteins. Finally, the protein-protein interaction map was simplified, and TSC-1, TBK1, IRF3, and mTOR were found to be the interacting proteins between Akt-associated and cGAS-STING pathways (Figure 2C).

Table 1 Associations of the cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes pathway with diabetes and complications

Types of complications of diabetes	Link to cGAS-STING pathway	Ref.
Diabetic gastroenteropathy	Activation of PI3K/AKT/mTOR and AMPK/mTOR signaling pathways leads to apoptosis of gastric smooth muscle cells	Zhang *et al*[26]
	Phosphorylation of S2481 site on mTORC2 can promote glucose metabolism in gastrointestinal smooth muscle cells	Yan *et al*[27]
	cGAS STING regulates mTORC1 mediated cell apoptosis through TBK1 signaling	Bodur *et al*[28]
	cGAS STING regulates mTORC1 mediated cell apoptosis through TREX1 signaling	Hasan *et al*[29]
	cGAS/STING/IRF3/NF-κB/INF pathway participates in mitochondrial autophagy in the stomach and duodenum	Puthanmadhom Narayanan *et al*[32]
Nonalcoholic fatty liver disease	Activation of the STING signaling pathway enhances hepatic steatosis and inflammatory response, exacerbating hepatic stellate cell fibrosis	Wang *et al*[48], Yu *et al*[49]
	STING promotes macrophage induced liver cell fat deposition and pro-inflammatory response through the NFB and JNK pathways	Luo *et al*[47]
	STING and IRF3 activation promote lipid accumulation in stem cells	Qiao *et al*[50]
	Mitochondrial autophagy mediated mtDNA/cGAS/STING signaling plays a broad regulatory mechanism in different aseptic inflammatory responses	Su *et al*[51]
	Pink1 can inhibit cGAS/STING activation and reduce mitochondrial autophagy	Zhong *et al*[52]
Diabetic cardiomyopathy	The use of STING inhibitors in both the lipotoxic H9C2 cell model and the DCM mouse model can significantly inhibit myocardial cell inflammation and apoptosis	Ma *et al*[54]
	cGAS/STING pathway initiates NLRP3 inflammasome induced cardiomyocyte pyroptosis and chronic inflammation	Yan *et al*[55]
	cGAS/STING signaling activates the autophagy pathway LKB1/AMPK/ULK1 in cardiomyocytes, leading to hypertrophy, apoptosis, and oxidative damage in primary neonatal rat cardiomyocytes. The cardiac specific overexpression of Metrnl can improve the cardiac injury in diabetes mice	Lu *et al*[56]
	MtDNA activates the cGAS STING pathway, promoting epithelial mesenchymal transition in vascular endothelium	Liu *et al*[58]
	cGAS exacerbates the inflammatory cascade and participates in the formation of atherosclerosis through the synergistic signaling of IRF and IFN	Lu *et al*[59], Pham *et al*[60]
	TDP43 serves as an upstream regulatory factor in AS, triggering inflammatory responses by inducing the release of mtDNA and activating the cGAS STING pathway	Huangfu *et al*[61]
Diabetes nephropathy	The cGAS STING signaling pathway of renal macrophages is activated, and macrophages are activated towards M1 type through NF-κB signaling protein leads to TNF-α and IL-1β release increase	Han *et al*[67]
	Damage to autophagy in podocytes leads to the accumulation of damaged mitochondria, and TBK1 is an important downstream molecule of the cGAS-STING pathway in podocytes	Zang[68], Myakala *et al*[69]
	Sacubitril/valsartan can repair mtDNA damage, inhibit cGAS/STING pathway activation, and protect renal function	Myakala *et al*[70]
	DsbA-L can antagonize cGAS/STING pathway activation and improve high glucose induced renal tubular injury	Yang *et al*[71]
Diabetic retinopathy	The levels of STING and p-TBK1 protein in retinal endothelial cells of diabetes mice were significantly increased	Wen *et al*[74]
	STING influences PPAR by α plays a key role in the degeneration of retinal glial cells and vascular damage	Yuan *et al*[75], Dong *et al*[79]
	TGR5 blocks the IP3R1-GRP75-VDAC1 axis mediated efflux of Ca^{2+} from the endoplasmic reticulum to mitochondria	Li *et al*[76]
	Upregulation of *ARPE-19* gene expression and STIGN-NF-κB pathway	Chen *et al*[77]

	activation related	
	JQ-1cGAS-STING inhibitor can alleviate retinopathy caused by oxidative stress in diabetes	Zou *et al*[78]
Diabetic wound	ROS induces macrophage polarization through mtDNA/STING signaling, exacerbating endothelial cell dysfunction	Geng *et al*[81]
	STING inhibitors can inhibit inflammation and promote wound healing	Feng *et al*[82]
	IRF3 regulates Hippo YAP pathway to inhibit wound healing	Yuan *et al*[83]
	STING leads to an increase in JMJD3 in macrophages, limiting wound repair and enhancing inflammatory response	Audu *et al*[84]

cGAS: Cyclic guanosine monophosphate-adenosine monophosphate synthase; STING: Stimulator of interferon gene; PI3K: Phosphatidylinositol 3-kinase; AKT: Activated protein kinase B; mTOR: Mammalian target of rapamycin; mTORC2: Mammalian target of rapamycin complex 2; TBK1: TANK-binding kinase 1; TREX1: 3-prime repair exonuclease 1; IRF3: Interferon regulatory factor 3; NF-κB: Nuclear factor-κB; IFN: Interferon; JNK: c-Jun NH2-terminal kinase; mtDNA: Mitochondrial DNA; NLRP3: Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing protein 3; Metrnl: Meteorin-like; AS: Atherosclerosis; TNF: Tumor necrosis factor; IL: Interleukin; PPAR: Peroxisome proliferator-activated receptor; TGR5: Takeda G protein-coupled receptor 5; ROS: Reactive oxygen species; YAP: Yes-associated protein; JMJD3: Jumonji domain-containing protein-3.

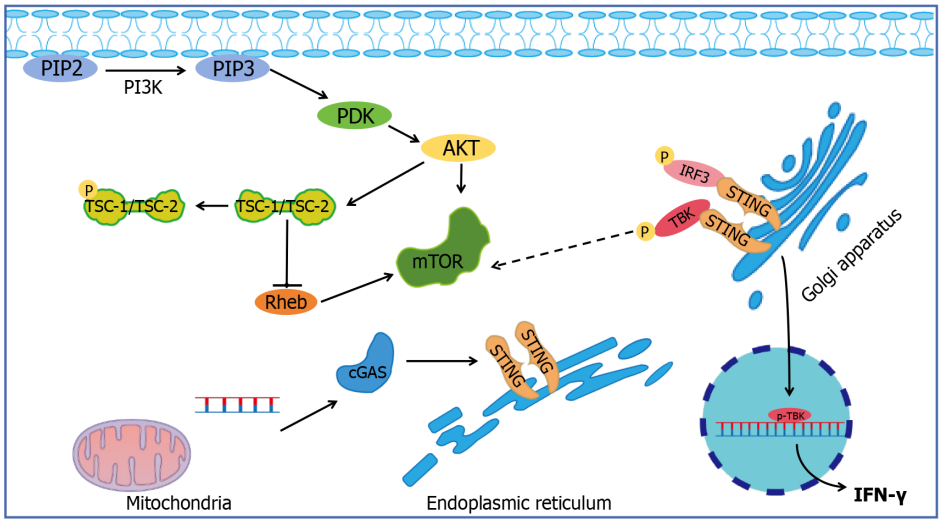
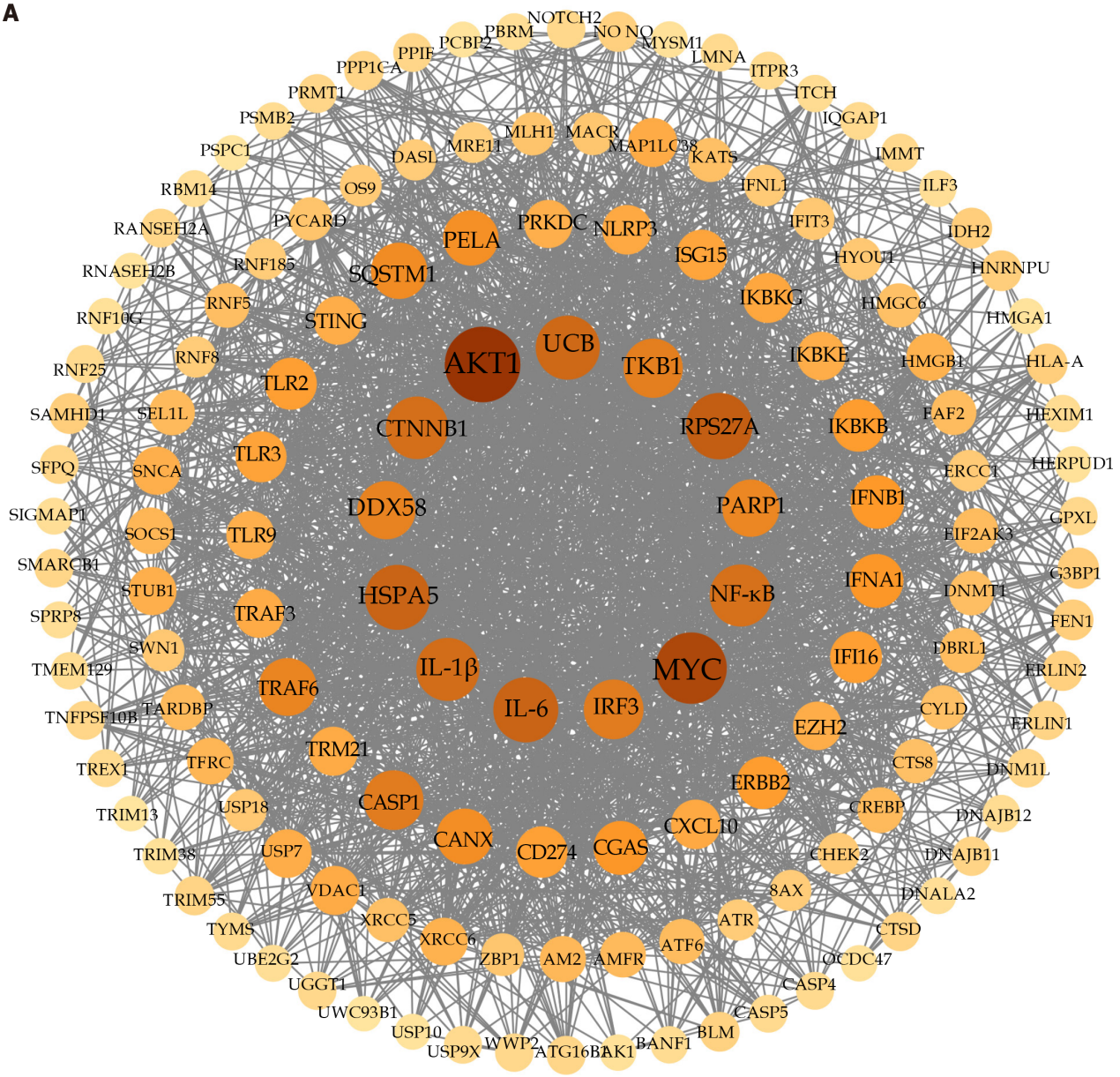


Figure 1 The binding of cyclic guanosine monophosphate-adenosine monophosphate synthase to double-stranded DNA results in its activation as a secondary messenger, leading to the production of cyclic guanosine monophosphate-adenosine monophosphate. Cyclic guanosine monophosphate-adenosine monophosphate then binds to interferon gene stimulating factor (STING), which is bound to the endoplasmic reticulum (ER) membrane, thereby causing its activation. STING conformational change and transfer to the Golgi apparatus. In the Golgi apparatus, the two cysteine residues of STING (Cys88 and Cys91) are palmitoylated. Subsequently, STING will recruit TANK binding kinase 1 (TBK1) and interact with it. TBK1 phosphorylates interferon regulatory factor 3 (IRF3), triggering dimerization, nuclear translocation, and induction of target genes in IRF3, thereby affecting interferon production. Activated protein kinase B can directly phosphorylate mammalian target of rapamycin (mTOR); or inhibit the activation of Ras homolog enriched in brain by inactivating tuberous sclerosis complex 2, and then enhance the activation of mTOR. TBK1 increase the activation of mTOR, on the contrary, phosphorylation of TBK1 inhibits the activity of mTOR. PIP2: Phosphatidylinositol-(3,4)-P2; PIP3: Phosphatidylinositol-(3,4,5)-P3; PI3K: Phosphatidylinositol 3-kinase; PDK: Phosphoinositide-dependent kinase; mTOR: Mammalian target of rapamycin; cGAS: Cyclic guanosine monophosphate-adenosine monophosphate synthase; STING: Stimulator of interferon gene; TSC: Tuberous sclerosis complex; Akt: Activated protein kinase B; TBK: TANK binding kinase; IRF: Interferon regulatory factor 3; IFN: Interferon; Rheb: Ras homolog enriched in brain.

CGAS-STING IN DG PATHOGENESIS

DG includes all gastrointestinal manifestations of DM. Reportedly, up to 50% of patients with T1DM, T2DM, or poor glycaemic control exhibit delayed gastric emptying (GE)[20]. Owing to poor understanding of the disease by clinicians and unclear early symptoms, DG is often misdiagnosed. Scintigraphy or capsule magnetic resonance endoscopy is the main diagnostic approaches for DG. Many patients can present with gastroparesis, a characteristic syndrome of moderate-to-severe upper gastrointestinal symptoms, or delayed gastroparesis, but without gastric outlet obstruction. Gastroparesis can significantly affect the quality of life, with up to 50% of patients experiencing severe symptoms of anxiety or depression. Diabetic gastroparesis is generally used to describe the upper gastrointestinal manifestations of DM, but not all gastrointestinal symptoms originate from the stomach, some also originate from the small intestine[21]. Therefore, DG is used, as a broader term, to describe all the gastrointestinal manifestations of DM including gastroparesis and diabetic dyspepsia. Diabetic dyspepsia is characterized by upper gastrointestinal symptoms, along with normal,

A



B

No.	Protein	Degree	No.	Protein	Degree
1	AKT1	93	11	mTOR	60
2	MYC	84	12	TBK1	59
3	RPS27A	74	13	DDX58	57
4	HSPA5	71	14	TRAF6	56
5	IL6	71	15	PARP1	55
6	UBC	69	16	SQSTM1	53
7	IL1B	67	17	CASP1	52
8	CTNNB1	66	18	RELA	51
9	NFKB1	65	19	CGAS	48
10	IRF3	60	20	IFNA1	48

C

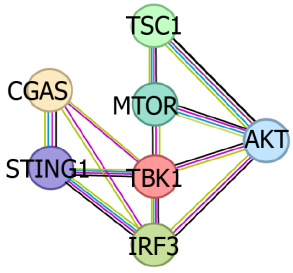


Figure 2 Gene database-based target analysis of the cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes pathway in diabetes mellitus. A: Combination between "diabetes" and "cGAS-STING" predicted by String database (version 11.5); B: The 20 targets with the highest degree value; C: The protein interaction diagram of the interaction between "Akt" and "cGAS-STING". mTOR: Mammalian target of rapamycin; cGAS: Cyclic guanosine monophosphate-adenosine monophosphate synthase; STING: Stimulator of interferon gene; TSC: Tuberous sclerosis complex; Akt: Activated protein kinase B; TBK: TANK binding kinase; IRF: Interferon regulatory factor 3; IFN: Interferon; IL: Interleukin; NF-κB: Nuclear factor-κB; PARP: Polyadenosine diphosphate-ribose polymer.

mild, or asymptomatic delayed GE. The latter accounts for 40% of patients with DM and delayed GE[22]. The main symptoms of diabetic gastroparesis include postprandial satiety, nausea, vomiting, abdominal distension, epigastric pain, and weight loss. In T2DM, the age at which symptoms appear is later than that in people with T1DM or idiopathic gastroparesis. Approximately 14% of people with both T1DM and T2DM present symptoms of infection before they become ill. Furthermore, approximately 33% of patients with idiopathic and diabetic gastroparesis present intermittent worsening of symptoms[23].

In recent years, diabetes gastroparesis has been related to many factors, including disorders of autonomic nervous structure and function (sympathetic and parasympathetic imbalance), intestinal neuromuscular dysfunction, and glucose and hormone metabolism disorders. These pathological and physiological changes can lead to abnormal gastric electrophysiology and gastrointestinal sensory, as well as motor dysfunction. The gastrointestinal nervous system contains approximately 100 million neurons, which are organized into different ganglia, including movement-regulating intermuscular plexus, and absorption- and secretion-regulating submucosal plexus[24]. The interstitial cells of Cajal (ICC) play a role in the pacemaker and information transmission. In patients with DM and laboratory diabetic rats, cells in the motor vagus nerve and sympathetic ganglion have been reported to reduce, and structural changes such as segmental demyelination and axonal degeneration have been observed in vagus nerve fibers in the myenteric plexus, submucosal plexus, and outside of the gastrointestinal tract. The loss of nerve fibers is usually multifocal, indicating ischaemic injury. Mainly, a decrease in intestinal neurons or ICC induces dysfunction of the intestinal neuromuscular function. Consequently, the number of macrophages associated with immune macrophage inhibitory neurons and neuronal NOS expression decreases. Studies have shown that the interaction between macrophages, ICC, and neuromuscular cells may play an important role in DM-induced gastroparesis. Alternating M2 macrophages that express cytoprotective markers such as hem oxygenase-1 have been reported in the gastric mucosal layer of normal mice. In mice with delayed GE, the intrinsic macrophages predominantly comprise classically activated M1 macrophages that produce cytokines inducing ICC apoptosis[25].

cGAS-STING and autophagy mechanism in diabetic cells

ICC apoptosis in the gastrointestinal tract is one of the pathogenic mechanisms of gastroparesis. Studies have reported the involvement of two mTOR, a protein kinase associated with apoptosis, energy metabolism, and DM, pathways in ICC apoptosis, namely the PI3K/Akt/mTOR inhibitory apoptotic pathway and the adenosine monophosphate-activated protein kinase (AMPK)/mTOR pro-apoptotic pathway. In early diabetic gastroparesis, ICC increases growth factor secretion upstream of the PI3K/Akt pathway by autocrine or paracrine mechanisms in response to an initial high glucose (HG) stimulus, activating the anti-apoptotic effects to maintain cell function. Continuous and prolonged hyperglycemic stimuli result in the inhibition of the apoptotic pathway, which is difficult to compensate for. At this point, the pro-apoptotic AMPK/mTOR pathway becomes dominant. AMPK phosphorylates and activates TSC-2 upstream of mTOR, promoting TSC-1/TSC-2 complex formation, decreasing mTOR activity, and thus, leading to ICC apoptosis[26]. Yan *et al*[27] found that phosphorylating the S2481 site on mTORC2 can promote glucose uptake, glucose metabolism, and ATP synthesis in the gastrointestinal smooth muscle. Furthermore, Bodur *et al*[28] showed that the innate immune kinase TBK1 regulates anti-inflammatory effects by stimulating type I IFN (IFN-I) production. TBK1 directly activates mTORC1 in the cells through specific mTOR phosphorylation (S2159 phosphorylation site), revealing the stimulus-selective role of TBK1 in mTORC1 regulation. A study on macrophages isolated from genome-edited mTOR S2159A-knockout mice revealed that mTOR S2159 phosphorylation promoted mTORC1 signaling, IRF3 nuclear translocation, and IFN-β production, indicating a mechanistic link between the cGAS-STING-TBK1 signaling pathway and the mTORC1 function in cell apoptosis. Hasan *et al*[29] found that 3-prime repair exonuclease 1 (TREX1), which exhibits DNA enzyme activity, can sense cytoplasmic DNA and activate IFN responses through the cGAS-STING pathway. The significant decrease in the mTORC1 activity in TREX1-/- mouse tissues suggested that the cGAS-STING pathway may play an important role in mTORC1 regulation and metabolism. These findings suggest the cGAS signaling pathway as a new target for the treatment of gastrointestinal neurological function; however, further research is needed to fully elucidate the complex cGAS-STING signaling-gastrointestinal neuromodulation interplay.

cGAS-STING and macrophages in DM and polarization mechanism

The intestinal neuronal apoptosis is associated with ICC and macrophages, and neuronal NOS-expressing neurons are the first to be eliminated. Reportedly, the interactions among macrophages, ICC, and the neuromuscular system may mediate gastroparesis in mice and humans with DM. The muscular layer of the gastric mucosa of normal GE mice is filled with alternatively activated M2 macrophages that express cytoprotective markers, including hem oxygenase-1. In mice with delayed GE, classically activated M1 macrophages are predominant and produce cytokines leading to ICC apoptosis. Macrophages are necessary for the development of delayed GE in diabetic mice[30]. Deng *et al*[31] reported that high-fat diet (HFD)-induced aseptic neuritis is related to the polarization of astrocytes and M1 microglia. Microglia are macrophage-like cells in the central nervous system, and their aggregation can be symptomatic of neurodegenerative diseases such as Alzheimer's and Parkinson's, which can cause GE disorders in the gastrointestinal tract. A HFD can damage mitochondrial DNA (mtDNA) in the stomach and duodenum through oxidative stress[32]. High blood glucose increases pyruvate production, leading to mitochondrial membrane hyperpolarization and free radical production, which then oxidatively damage susceptible mtDNA with limited repair capacity. This damage increases mitochondrial autophagy, and as both mtDNA and nuclear DNA in cells can activate the cGAS-STING-IRF3-NF-κB-INF pathway, chronic neuroinflammatory responses are triggered. Although the mechanism of cGAS-STING signaling in the gastrointestinal nervous system remains unelucidated, its important role in central chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and ischaemic brain injury is well known[33]. Overall, these findings suggest the potential of cGAS as a target for glial cell polarization and cGAS-STING as a potential

therapeutic target in the gastrointestinal nervous system, warranting further research.

cGAS-STING and the mechanism of gut microbiota dysbiosis

Dysbiosis is both a cause and a consequence of gastrointestinal dysfunction in DM. The gastrointestinal tract is exposed to microorganisms, and hence, is an important defense barrier for the body. In the classical pathway, IFN-I play a crucial role in intestinal defense and is associated with intestinal immune dysfunction. The cGAS-STING signaling pathway induces IFN-I in the presence of exogenous DNA and plays a crucial role in gastrointestinal homeostasis[34]. In the intestinal mucosa, mainly dendritic cells and monocytes located in the lamina propria release IFN-I during homeostasis. Similar findings have been observed in both mouse and human intestines, where IFN-I directly bound STING to bacterial cyclic dinucleotides, which act as secondary messengers in bacteria, establishing STING as an independent pattern recognition receptor. The gut microbiota maintains IFN-I signaling and is critical for the immune recognition of dendritic cells[35]. The absence of STING is associated with a higher susceptibility to inflammation in the gut microbiota. In non-classical pathways, STING binds to nuclear transcription factors. In 2020, Obata *et al*[36] reported the biosensor activity of aromatic hydrocarbon receptor (AHR) in the intestinal neural network and the close association of its functional expression to the gut microbiota in regulating the excitability of intestinal neurons and intestinal physiological functions. In 2023, Zhang *et al*[37] showed that AHR activation is driven by STING1, a nuclear protein, and it controls the composition of intestinal microflora, but this function was not dependent on DNA sensing and autophagy and competed with cGAS-STING signaling inhibition. They compared the differences between wild-type and STING1Gt/Gt mice (lack of functional STING1 expression). STING1 knockout attenuated the protective effects of AHR ligands on gut microbiota and innate immunity. Nuclear STING1 exhibits various independent functions of IFN, which are essential for regulating intestinal immunity and microbial homeostasis. Collectively, these findings suggest that STING-mediated microbial signaling is critical in the surveillance of gut microbiota and that STING overexpression leads to disease progression and tissue dysfunction *via* IFN-I[38].

Extracellular vesicles (EVs) can transport various biomolecules, including RNA, DNA, proteins, and lipids, within living organisms[39]. Disrupted gut barrier in patients with DM results in the leakage of microbiota-derived products into the circulatory system of the host, and thus, in distant organs. In diabetic mice, gut microbiota-derived EVs encasing microbial DNA were shown to be captured by pancreatic CD11c+ islets, subsequently initiating a cellular inflammatory response *via* the cGAS-STING signaling pathway, which promoted pancreatic islet inflammation and β cell abnormalities [40]. In the liver, cGAS-STING activation following the capture of EVs by Vsig4+ macrophages has been shown to exacerbate the development of NAFLD and liver fibrosis[41]. Additionally, Enterobacteriaceae fragilis EVs can promote macrophage M1/M2 polarization and induce vascular complications in individuals with T2DM[42]. Although validated in animal models, the efficacy of EV-mediated cGAS-related signaling pathway activation remains unelucidated in humans. The cGAS-STING pathway recognizes bacterial cyclic dinucleotides, sustains the growth of probiotics, and maintains gut homeostasis[43]. Decreased probiotic populations can cause immune dysregulation in the host, increasing the risk of pathogenic invasion. Lactobacillus, the major probiotic genus in the gut, stimulates IFN-I expression through the cGAS-STING pathway and induces macrophage-specific immune responses. Reportedly, STING can promote the production of short-chain fatty acids by utilizing intestinal bacteria and induce mucosal immunity in a G-protein coupled receptor 43-dependent manner, which reduces bacterial translocation by preserving the integrity of the intestinal barrier [44]. Overall, the interaction of intestinal probiotics with the cGAS-STING pathway facilitates the maintenance of intestinal homeostasis, whereas STING deficiency can increase intestinal susceptibility to inflammation and alterations in the intestinal flora.

CGAS-STING IN NAFLD PATHOGENESIS

NAFLD, a clinical-pathological syndrome, refers to the accumulation of excessive fat in liver cells because of factors other than alcohol consumption, including simple fatty liver, non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma. DM-driven dyslipidemia, inflammatory response, IR, and other mechanisms affect the progression of NAFLD. Approximately 75% of patients with T2DM have NAFLD[45].

NAFLD pathogenesis has been related to innate immune-mediated aseptic inflammation, and IFN-I has played an important role in its development[46]. Luo *et al*[47] reported that cGAS, STING, and IRF3 levels were increased in the liver of NAFLD or NASH mice and activated the pro-inflammatory response of liver macrophages. They investigated the role of STING in NAFLD using wild-type C57BL/6J mice fed with HFD and a low-fat diet for 12 weeks. STING knockout inhibited the production of several inflammatory cytokines induced by HFD. The experimental results showed that D played a detrimental role. Furthermore, the macrophage liver cell co-culture experiment showed that STING promoted macrophage-induced fat deposition and pro-inflammatory response in liver cells through the NF-κB and Jun N-terminal kinase pathways.

Wang *et al*[48] showed the crucial role of STING in NAFLD progression based on the analyses of liver samples from 98 patients with NAFLD and 8 controls. STING and phosphorylated TBK1 (p-TBK1) expression in non-parenchymal liver cells increases with the severity of inflammation and fibrosis, particularly in hepatic portal vein macrophages of patients with fibrotic NASH. Activation of the STING pathway in macrophages enhances hepatic steatosis and inflammatory response, thereby exacerbating hepatic stellate cell fibrosis. These findings suggest the involvement of the cGAS-STING pathway in NAFLD or NASH pathogenesis. Yu *et al*[49] reported an increase in mtDNA in liver cells of mice fed with a methionine- and choline-deficient diet and an HFD; furthermore, the STING signaling pathway was induced in cultured Kupffer cells. Notably, STING deficiency alleviated hepatic steatosis, fibrosis, and inflammation in the NASH mouse

model, along with serum cholesterol, triglyceride, and low-density lipoprotein low-density lipoprotein (LDL) levels. Qiao *et al*[50] reported upregulated STING and IRF3 in the liver of HFD-fed mice and FFA-induced L-O2 liver cells by regulating NF-κB signaling pathways, inflammatory cytokines, and apoptotic signaling pathways, which increase fat deposition. STING or IRF3 knockdown significantly reduces FFA-induced liver inflammation, lipid accumulation, and cell apoptosis, and increases glycogen storage, which is associated with reduced expression of gluconeogenesis- and lipid synthesis-associated liver enzymes. Su *et al*[51] found that mitochondrial autophagy-mediated mtDNA/cGAS/STING signaling plays a broad regulatory mechanism in various aseptic inflammatory responses and macrophage STING signaling notably promoted aseptic inflammatory liver injury in aged mice. STING knockout in liver injury models can significantly alleviate liver injury in aged mice. Analyses of the STING/TBK1 signaling pathway, *TNF-α*, and *IL-6* gene showed that STING knockout ameliorated the age-dependent increase in the pro-inflammatory response of the liver. This suggests that STING deficiency may protect older mice from various types of sterile inflammatory liver injury. Zhong *et al*[52] reported similar observations and confirmed that aging damages macrophage mitochondria, leading to the activation of mitochondrial autophagy. Phosphatase and tensin homolog deleted on chromosome ten-induced kinase 1 overexpression and Torin1 treatment can restore mitochondrial autophagy and inhibit cGAS-STING activation in aging macrophages.

CGAS-STING IN DCM PATHOGENESIS

DCM is a serious cardiac complication of DM that can lead to heart failure even without valvular disease, hypertension, and coronary artery disease. Risk factors for coronary heart disease in DM are high blood glucose, blood pressure, cholesterol, and LDL levels, along with decreased high-density lipoprotein, age, sex, smoking, and family history. DM negatively affects the heart, leading to changes in gene expression, abnormal energy metabolism, reduced left ventricular function, oxidative stress, aseptic inflammation, lipid accumulation, and mitochondrial dysfunction, which can result in the onset and development of cardiac dysfunction, myocardial hypertrophy, and myocardial remodeling[53]. The clinical symptoms of DCM include heart failure, angina pectoris, and arrhythmia among other symptoms, which is the greatest risk factor for death in patients with diabetes. Presently, there are no specific treatments for DCM. In recent years, research on DCM pathogenesis has gradually increased, suggesting an association with the cGAS-STING pathway.

Ma *et al*[54] reported that in HFD-fed T2DM mice, mtDNA in the cytoplasm of mouse cardiomyocytes increased and the cGAS-STING pathway was activated, along with the increased expression of downstream molecules IRF3, NF-κB, IL-18, and IL-1β. Further validation using palmitic acid (PA) to cultivate H9C2 in lipophilic rat cardiomyocytes showed that the intracellular cGAS-STING pathway was activated, resulting in an increase in cytoplasmic mtDNA. PA induced changes in mitochondrial homeostasis, resulting in an excessive production of mitochondrial reactive oxygen species and oxidative damage to mtDNA. STING inhibitors significantly inhibited myocardial cell inflammation and apoptosis in both lipid-toxic H9C2 cell models and DCM mouse models. Yan *et al*[55] induced DM in STING-knockout mice by injecting streptozotocin (STZ) and HFD and found that STING knockout reduced myocardial cell scorch and inflammatory response, preventing DM-induced cardiac hypertrophy and improving cardiac function. Mitochondrial oxidative damage and FFAs induce mtDNA escape, stimulating the cGAS-STING pathway to initiate nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) inflammasome-induced cardiomyocyte pyroptosis and chronic inflammation. In hyperlipidemia or hyperglycemia, mitochondrial dysfunction is the main reason for the increase in mtDNA. These findings suggest the importance of the mtDNA-activated cGAS-STING pathway in the pathogenesis of DCM and that STING is a potential target for treating DCM. Meteorin-like (Metrnl) is an important secretory adipocyte factor discovered recently and plays an important role in regulating glycolipid metabolic diseases such as dyslipidemia, obesity, T2DM, coronary heart disease, and NAFLD. Lu *et al*[56] found that plasma Metrnl, myocardial Metrnl protein, and mRNA levels were significantly downregulated in STZ-induced T1DM and leptin receptor-deficient (db/db) T2DM mice. cGAS-STING signaling activates the liver kinase B1/AMPK/Unc-51-like kinase 1 autophagy pathway in cardiomyocytes, leading to hypertrophy, apoptosis, and oxidative damage in primary neonatal rat cardiomyocytes. Overall, cardiac-specific overexpression of Metrnl can ameliorate cardiac injury and dysfunction in T1DM and T2DM mice.

MACROVASCULAR LESION AND VASCULAR ENDOTHELIAL INJURY IN DM

cGAS-STING contributes to both macrovascular and microvascular complications in DM, with major risk factors including long duration of DM, poor glycemic control, hypertension, hyperlipidemia, and IR. DM-induced vascular atherosclerosis is the basis of coronary heart disease, cerebrovascular disease, and peripheral arterial disease in DM. Additionally, the combination of these diseases and other cardiovascular events caused by atherosclerosis is collectively referred to as atherosclerotic cardiovascular disease, which is responsible for the death of approximately 45% of patients with DM[57]. The cGAS-STING-mediated inflammatory response mainly involves LDL accumulation in the vascular intima, activating the expression of leukocyte adhesion molecules and chemokines in endothelial cells, promoting the recruitment of macrophages and T cells, and promoting local inflammation and plaque growth by secreting pro-inflammatory cytokines. Liu *et al*[58] exposed human aortic endothelial cells to different concentrations of PA. Notably, the cGAS-STING pathway was selectively activated by mtDNA and promoted epithelial-mesenchymal transition in vascular endothelium. cGAS knockdown attenuated PA-induced activities, suggesting the involvement of the mtDNA-induced cGAS-STING pathway in endothelial dysfunction. Reportedly, mtDNA triggers primary autoimmune activation by

evading self-clearance (DNA degradation and autophagy), thereby exacerbating the formation of atherosclerosis lesions. Lu *et al*[59] reported that cGAS exacerbates the inflammatory cascade through the synergistic signaling of IRF and IFN, triggering the transformation of macrophage phenotype to M1 (pro-inflammatory phenotype) and increasing lipid deposition by upregulating the uptake of cholesterol-related molecules, thereby leading to atherosclerosis. Pham *et al*[60] found that the STING pathway promotes atherosclerosis through pro-inflammatory activation of macrophages. They found that lipids and macrophages accumulated in atherosclerotic plaques of the mouse aorta, and the expression of STING, cGAMP, and IFN-I in macrophages was increased. Furthermore, in STING-knockdown mice, atherosclerosis lesions in the aortic arch, lipid and macrophage accumulation in plaques, and expression of inflammatory molecules in the aorta were reduced. Huang *et al*[61] showed that the transactive response DNA binding protein of 43 acts as an upstream regulator in atherosclerosis, activating the cGAS-STING pathway by inducing the release of mtDNA and triggering inflammatory responses. Increasing evidence suggests a close association between NLRP3 inflammasomes and atherosclerosis, warranting the investigation of the role of NLRP3 inflammasome regulation and activation mechanisms in atherosclerosis. Altogether, these studies show that cGAS-STING-mediated inflammatory response plays an important role in developing diabetic macroangiopathy, suggesting a new direction for research on treatment.

CGAS-STING IN DNE PATHOGENESIS

DNe is a microvascular complication characterized by the deterioration of renal function. Despite strict blood pressure control, approximately 40% of patients with DM develop DNe because of the use of statins and renin-angiotensin system inhibitors. The main symptoms include overall renal dysfunction, thickening of the glomerular basement membrane, reduction of podocytes in the glomerulus, expansion of mesangial volume, nodular lesions, and proliferation of hyaline substance[62]. Owing to the death of numerous glomeruli, the glomerular filtration rate and the production of large amounts of proteinuria decreases, ultimately leading to total renal failure. Inflammation plays an important role in the pathogenesis of podocyte injury. Saito *et al*[63] found that TNF-α could reduce the expression of the podocyte structural protein nephrin. Pedigo *et al*[64] reported that TNF-α could inhibit podocyte cholesterol efflux, leading to cholesterol accumulation and podocyte apoptosis. Gutwein *et al*[65] showed that TNF-α and IFN-γ induced CXC chemokine ligand 16 expression in podocytes, ultimately disrupting the lipid metabolism in podocytes. Herder *et al*[66] found that IL-6 could increase signal transducer and activator of transcription 3 expression leading to fusion and disappearance of foot processes in podocytes. The cGAS-STING pathway plays an important role in mediating metabolic inflammation. Reportedly, the cGAS-STING pathway is involved in DNe pathogenesis. In an HG environment, mature macrophages are activated into M1 macrophages, leading to chronic kidney inflammation. Han *et al*[67] found that numerous CD86+ M1 macrophages infiltrated the kidney tissue of patients with DNe, and STING expression was significantly increased, indicating the upregulation of the cGAS-STING pathway. Mouse macrophage RAW264.7 *in vitro* analysis under HG stimulation showed activation of the macrophage cGAS-STING signaling pathway, transformation to the M1 type, phosphorylated p65 and NF-κB upregulation, and increased release of TNF-α and IL-1β. The addition of STING inhibitor C-176 markedly inhibited the activation of M1 macrophages and the expression of downstream inflammatory proteins and cytokines. These findings confirm the activation of the macrophage cGAS-STING signaling pathway under HG conditions. Zang[68] used a podocyte line PA injury model of db/db mice and MPC5 mice as a model of DNe. The renal pathology showed a hypertrophic glomerulus, slightly dilated mesangium, reduced expression of the podocyte marker protein nephrin, widely fused podocytes, irregularly thickened glomerular basement membrane, and increased levels of podocyte apoptosis. Activation of the cGAS-STING pathway was detected mainly in renal cortical podocytes, with increased cGAS and STING expression, and increased TBKI phosphorylation, but unchanged levels of IRF3 phosphorylation and IFN-β. The protective effect of C-176 or STING knockout in damaged lipotoxic podocytes is exhibited through cellular autonomy, consistent with animal experiments. The mode of activation of the cGAS-STING pathway in MPC5 cells was through STING-TBK1-p65-IRF3 was not activated-consistent with animal experiments. GSK8612 inhibition of TBK1 is sufficient to induce cellular self-protection, suggesting that TBK1 is an important downstream molecule of the cGAS-STING pathway in podocytes. Damage to podocyte autophagy leads to the accumulation of damaged mitochondria, and mtDNA leaks into the cytoplasm through Bcl-2 associated X-protein-mediated macropores, activating the cGAS-STING/TBK1/p65 pathway, thus, resulting in the production of inflammatory factors and podocyte damage. Myakala *et al*[69] reported that cGAS activation induces nephritis in db/db and KKAy mice. DNA damage or mitochondrial dysfunction can release DNA into the cytoplasm to activate cGAS, leading to the production of the second messenger cGAMP. Subsequently, STING induces the IFN-I response or NF-κB activation, thereby inducing the expression of inflammatory factors. Myakala *et al*[70] reported that sakubatrox/valsartan can repair mtDNA damage, inhibit the activation of the cGAS-STING pathway, and reduce proteinuria, mesangial dilation, and podocyte loss in db/db and KKAy mice, and thus, exhibit a protective effect on renal function in T2DM mice. Disulfide bond-forming oxidoreductase A-like protein overexpression can antagonize mitochondrial stress-induced mtDNA release and activation of the cGAS-STING pathway in adipose tissue, ameliorate HG-induced renal tubular injury, and prevent ectopic fat deposition and lipid-related kidney injury in DNe[71]. Additionally, mitochondrial dysfunction and tubular inflammation contribute to the pathogenesis of acute kidney injury and subsequent chronic kidney disease. Reportedly, activating the cGAS-STING pathway in the kidneys of patients with acute kidney injury resulted in cisplatin-induced tubular inflammation, whereas STING knockout ameliorated the acute kidney injury phenotype. Furthermore, inhibition of STING can alleviate folate-induced nephritis, tubular injury, renal fibrosis, and mitochondrial dysfunction in mice. Altogether, activation of the cGAS-STING pathway can lead to kidney injury, whereas its inhibition can delay the progression of kidney diseases.

CGAS-STING IN DR PATHOGENESIS

DR is a microvascular complication of DM. Currently, 8 out of the world's adult population have DM. 5% of the adult population have DM, and approximately 30% of patients with DM can develop DR[72]. DR is one of the retinal inflammatory diseases that can lead to loss of tight junctions, increased permeability, thickening of the basement membrane, and loss of peripheral cells in the retina. Hyperglycemia can affect normal glucose metabolism *via* the polyol pathway, hexosamine pathway, advanced glycation end products, and protein kinase C[73], leading to oxidative stress, cytokine release, mitochondrial dysfunction, and immune system activation. Therefore, targeting cGAS and STING expression in various retinal cell types may be a potential therapeutic approach for DR.

Wen *et al*[74] reported significantly increased levels of STING and p-TBK1 in retinal endothelial cells of HFD-fed diabetic mice. *In vitro*, PA treatment can induce mtDNA leakage into the cytoplasm of human retinal vascular endothelial cells and increase p-TBK1 protein and *IFN-β* mRNA levels. The STING pathway alleviates endothelial inflammation and provides an optional therapeutic target for treating DR and other microvascular complications of DM. Yuan *et al*[75] showed that the STING pathway was activated in DR by affecting PPARs in glial cells cultured with diabetes Prara-/- mice and diabetic stressor 4-hydroxynonenal cytokines. Glucose metabolism in retinal glial cells plays a crucial role in microglioma, neurodegeneration, and vascular damage. Li *et al*[76] reported that mitochondrial Ca^{2+} overload led to the opening of mitochondrial permeability transition pores and mtDNA leakage into the cytoplasm in rats, activating cytoplasmic mtDNA, cGAS, and stim-mediated inflammatory responses. Takeda G protein-coupled receptor 5 agonists can alleviate mitochondrial Ca^{2+} overload and mitochondrial dysfunction. Takeda G protein-coupled receptor 5 blocks the efflux of Ca^{2+} from the ER to the mitochondria, which is mediated by the inositol 1,4,5-triphosphate receptor-75-kDA glucose-regulated protein-voltage-dependent anion channel 1 axis. Chen *et al*[77] found that the adult retinal pigment epithelial cell line-19 gene was upregulated in oxidative stress-induced retinal aging, and vascular endothelial growth factor and its key mediator hypoxia-inducible factor-1 was involved. The expression of STING was increased, and the specific mechanism may be related to DNA clearance disorders and STING-NF-κB pathway activation. Similarly, Zou *et al*[78] used JQ-1cGAS-STING inhibitor to ameliorate oxidative stress-induced DR. Dong *et al*[79] reported that monocyte activation plays an important role in DR and other DM complications. PPARα is significantly downregulated in monocytes derived from animals and patients with DM, impairing mitochondrial function, increasing monocyte glycolysis, increasing mtDNA release in the cytoplasm of diabetic monocytes, and activating the cGAS-STING pathway. Notably, STING knockout or STING inhibitor can attenuate DM or PPARα knockout-induced monocyte activation.

CGAS-STING IN DW PATHOGENESIS

DW is a chronic complication that affects wound closure in patients with DM. DW pathogenesis is complex and involves numerous different pathways related to the local hyperglycemic environment, including accumulation of advanced glycosylation end products, oxidative stress injury, and chronic inflammation. Presently, DW is considered a persistent chronic low-grade inflammation. Reportedly, cellular aging and immune damage play a crucial role in DW healing. In wound healing, aging fibroblasts and endothelial cells induce myofibroblast differentiation by secreting platelet-derived growth factor AA, thereby accelerating wound healing. However, high blood glucose interferes with this process, leading to immune cell infiltration and low-grade inflammation of the wound, thus, resulting in delayed wound healing. The cGAS-STING signaling is involved in innate immunity and cytoplasmic DNA can induce STING-dependent inflammatory responses, which play an important role in DW[80]. Geng *et al*[81] reported an increase in STING levels and M1 macrophages in DW tissue from patients and mice. The high reactive oxygen species content released in an HG environment induced mtDNA leakage to the cytoplasm, activated STING signaling, released pro-inflammatory cytokines, induced macrophage polarization to a pro-inflammatory phenotype, and exacerbated endothelial cell dysfunction. Feng *et al*[82] showed that in diabetic mice, STING activation promoted inflammatory response and delayed skin wound healing. STING knockdown and STING inhibitors in the STZ-induced DM mouse model and db/db mouse model inhibited inflammation and promoted wound healing. Yuan *et al*[83] reported that PA-induced inhibition of endothelial angiogenesis was mediated through the dysregulation of the Hippo-Yes-associated protein (YAP) pathway, an important signaling pathway regulating tissue repair and regeneration. PA inhibited endothelial cell proliferation, migration, and tube formation, which was associated with increased macrophage stimulating 1 (MST1) expression, YAP phosphorylation/inactivation, and nuclear repulsion. YAP overexpression or MST1 knockdown could prevent PA-induced inhibition of angiogenesis. PA treatment in vascular endothelium induced mtDNA leakage into the cytoplasm, activating the cytoplasmic DNA sensor cGAS-STING-IRF3 signaling to regulate the Hippo-YAP pathway, thereby inducing MST1 expression, YAP inactivation, and neovascularization inhibition. Audu *et al*[84] investigated the mechanism of macrophages in DW repair and found that increased Jumonji domain-containing protein D3 (JMJD3) in DW macrophages increased the expression of inflammatory genes. RNA sequencing of DW macrophages isolated from the bone marrow cells of JMJD3-deficient (JMJD3 f/fLyz2 Cre+) mice revealed that JMJD3 regulated the STING gene (Tmem173). These findings show the association of STING with chronic inflammation and its role in limiting wound repair and increasing the inflammatory response in diabetic mice. Furthermore, they suggest that cGAS STING plays an important role in the healing of DW.

Table 2 Mechanistic and physiologic effects of cyclic guanosine monophosphate-adenosine monophosphate synthase inhibitors associated with diabetes and its complications

Inhibitor	Mechanism	Physiologic effects
Compound 18	Small molecule inhibitors break the molecular structure of cGAS by binding to hydrogen bonds[91]	Compound 18 improves glucose tolerance in high fat diet mice[94]
RU.521, RU.356, RU332	Competes with ATP and GTP for enzyme binding sites by virtue of its own structural advantages[95]	RU521 attenuates cGAS-STING-mediated cardiac dysfunction in BRG1 knockout diabetic cardiac mice[96]
PF-06928215	Competes with cGMP for the cGAS binding site[97]	PF-06928215 attenuates cGAS-STING-mediated cardiac dysfunction in double knockout of Akt2 and AMPK mice [98]
HCQ	Prevents cGAS from binding to DNA by occupying the DNA binding site[97]	Improvement of inflammation by decreasing IFN-β release from Th1 cells
Aspirin	Aspirin acetylates cGAS to block cGAS-STING signaling. Aspirin's metabolite salicylate may affect NF-κB nuclear translocation[89]	No relevant evidence. Aspirin is only a theoretical cGAS inhibitor because it is easily hydrolyzed in the body
Suramin	Similar to nucleic acid structure, competes for DNA and cGAS binding sites[99]	Suramin blocks dsDNA binding to cGAS and limit AIM2 inflammatory vesicle formation[100]

cGAS: Cyclic guanosine monophosphate-adenosine monophosphate synthase; STING: Stimulator of interferon gene; ATP: Adenosine triphosphate; GTP: Guanosine triphosphate; cGMP: Cyclic guanosine monophosphate; NF-κB: Nuclear factor-κB; Akt: Activated protein kinase B; AMPK: Adenosine monophosphate-activated protein kinase; IFN: Interferon; Th1: T helper type 1; dsDNA: Double-stranded DNA; AIM2: Absent in melanoma 2.

INHIBITORS OF THE CGAS-STING PATHWAY FOR DM AND RELATED COMPLICATIONS

Considerable evidence indicates the contribution of the cGAS-STING signaling pathway to the development of adverse inflammatory and autoimmune responses, which in turn, may exacerbate metabolic disorders. Therefore, the development of cGAS-STING pathway inhibitors is necessary. cGAS and STING inhibitors that have been developed for metabolic diseases are listed in Tables 2 and 3, respectively. However, their clinical application remains elusive, with some of the small molecule inhibitors still in the theoretical modeling stage without known effective doses and pharmacotoxicology, warranting studies to validate these compounds in clinical trials. For instance, the cGAS-STING pathway plays an important regulatory role in tumor development and suppresses tumorigenesis by promoting the cytotoxic effects of T cells and natural killer cells, inducing apoptosis and autophagy. Therefore, the STING signaling pathway exhibits a dual effect on the human body based on its up- or down-regulation[85]. Hence, designed drugs should balance the effects of the STING pathway, rather than completely blocking or activating the pathway. The cGAS-STING pathway can interact with NF-κB, Jun N-terminal kinase, pyroptosis, AMPK, toll-like receptor 4, and mTOR signaling pathways to regulate cellular inflammation and metabolism[86], making the precise designing of targeted drugs to specifically block the STING pathway challenging.

Drug discovery to clinical translation is a time-consuming and expensive process, with an estimated period of 10-15 years, and costs upwards of 1 billion USD before a drug is approved by regulatory agencies and commercialized. Presently, there are two different approaches to drug development, namely phenotype-based drug discovery (PBDD) and target-based drug discovery[87]. PBDD is often used empirically to validate the efficacy of pre-existing compounds against target diseases. For example, suramin, an important drug for treating river blindness and African sleeping sickness, was found to also act as a cGAS inhibitor that blocks the cGAS-to-DNA binding[88]. Elkon[89] reported that aspirin can inactivate cGAS by acetylating the Lys414, Lys384, and Lys39 sites. In PBDD, animal models play an important role because the effects of drugs are sequentially assessed first in cells, followed by tissues or animal models without knowing the specific molecular target. Animal models play an important role in disease pathophysiology, drug target identification, toxicity, pharmacokinetic, and efficacy assessments of novel therapeutic agents, providing substantial basis for transferring the drug from early preclinical studies to later human clinical trials. However, its drawback is that the success rate of drugs subjected to preclinical animal testing remains low in clinical studies. This may be because of the large gap between the preclinical data generated in various standardized animal models of the target disease and the clinical translation gap[90]. Although animal experimental data can help prevent further development of drugs with severe toxicity, they cannot predict subjective drug effects or specific activity, highlighting the importance of selecting a predictable and effective animal model for the overall success of drug discovery and development. The target-based drug discovery approach develops novel targeted drugs based on existing mechanisms. Various novel small molecule compounds have been developed using new molecular technologies utilizing chemical biology, proteomics, and network biology. For example, RU.521 and G150 are recently developed compounds based on the results of high-throughput screening, and the most potent cGA inhibitors. The inhibitor compounds S3, S2, and 18 were developed based on PF-06928215 by using database virtual screening techniques[91]. Additionally, the use of rodents humanized mouse models for preclinical drug safety and efficacy testing of new drugs is considered acceptable and may be more appropriate than that of standard rodents[92]. The establishment of transgenic immunodeficient mice has led to significant advances in these techniques over the past two decades. Transgenic animals have exogenous genes introduced into their genomes. They are typically produced by microinjecting DNA into the prokaryotic nucleus of a fertilized egg, which is subse-

Table 3 Mechanistic and physiologic effects of stimulator of interferon gene inhibitors associated with diabetes and its complications

Inhibitor	Mechanism	Physiologic effects
Nitro fatty acids	Inhibits palmitoylation by binding to STING[101]	Nitro fatty acids protect against mitochondrial damage in hepatocytes of mice with nonalcoholic fatty liver disease[102]
C-176	Covalent small molecule inhibitors. Inhibits STING palmitoylation [103]	C-176 attenuates cGAS-STING pathway-mediated diabetic cardiomyopathy[54]
UNC93B1	The mechanism of action involves the targeting of STING degradation *via* the autophagy-lysosome pathway[104]	Unc93b1 ameliorates neuronal apoptosis induced by high glucose through the TLR9 signaling pathway[105]
SP23	Hydrolysis STING by the ubiquitin-proteasome pathway[106]	Improvement of inflammation by decreasing IFN-β release from Th1 cells

cGAS: Cyclic guanosine monophosphate-adenosine monophosphate synthase; STING: Stimulator of interferon gene; IFN: Interferon; Th1: T helper type 1; TLR9: Toll-like receptor 9.

quently implanted in the fallopian tube of a surrogate mother. Transgenic animals have become a key tool in functional genomics for modeling human diseases and validating new drugs[93]. Overall, the role of experimental animals in the development of novel drugs is crucial, and the development of novel cGAS-STING-targeted drugs may promote the personalized and precision treatment of DM.

CONCLUSION

This review discusses the close relationship between the cGAS-STING pathway and DM and associated complications. The findings showed that inflammation and mitochondrial dysfunction can promote disease progression, and they may be associated with the cGAS-STING pathway. Overall, inhibiting the cGAS-STING pathway can improve disease status and delay the progression of diseases such as IR, NAFLD, DCM, and DNe. This study on the cGAS-STING pathway may provide new insights into the treatment of DM and its complications. This review shows the involvement of the cGAS-STING pathway in different organs, providing a theoretical basis for long-term holistic treatment of multiple organs affected by DM. Future studies may focus on inhibiting cGAS-STING pathway-induced inflammation as a potential therapeutic approach for treating DM and related complications.

FOOTNOTES

Author contributions: Fan MW and Tian JL contributed equally to this study as they are co-first authors of this manuscript. Fan MW, Zhang SH, and Chen Y discussed the data; Tian JL, Chen T, Zhang C, Liu XR, and Zhao ZJ drafted the manuscript and also took responsibility of the data analysis. Zhang SH and Chen Y contributed equally to this study as they are co- corresponding authors of this manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Relationship between hemoglobin glycation index and risk of hypoglycemia in type 2 diabetes with time-in-range in target

Bei-Si Lin, Zhi-Gu Liu, Dan-Rui Chen, Yan-Ling Yang, Dai-Zhi Yang, Jin-Hua Yan, Long-Yi Zeng, Xu-Bin Yang, Wen Xu

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Abstract

BACKGROUND

In patients with type 2 diabetes mellitus (T2DM), the risk of hypoglycemia also occurs in at a time-in-range (TIR) of > 70%. The hemoglobin glycation index (HGI) is considered the best single factor for predicting hypoglycemia, and offers new perspectives for the individualized treatment of patients with well-controlled blood glucose levels that are easily ignored in clinical settings.

AIM

To investigate the relationship between HGI and hypoglycemia and the implications of HGI on hypoglycemia in T2DM with TIR > 70%.

METHODS

All participants underwent a 7-days continuous glucose monitoring (CGM) using a retrospective CGM system. We obtained glycemic variability indices using the CGM system. We defined HGI as laboratory hemoglobin A1c minus the glucose management indicator. Patients were categorized into low HGI (HGI < 0.5) and high HGI groups (HGI ≥ 0.5) according to HGI median (0.5). Logistic regression and receiver operating characteristic curve analyses were used to determine the risk factors for hypoglycemia.

RESULTS

We included 129 subjects with T2DM (54.84 ± 12.56 years, 46% male) in the study. Median TIR score was 90%. The high HGI group exhibited lower TIR and greater time below range with higher hemoglobin A1c than the low HGI group; this suggests more glycemic excursions and an increased incidence of hypoglycemia in the high HGI group. Multivariate analyses revealed that mean blood glucose, standard deviation of blood glucose and HGI were independent risk factors for hypoglycemia. Receiver operating characteristic curve analysis indicated that the HGI was the best predictor of hypoglycemia. In addition, the optimal cut-off points for HGI, mean blood glucose, and standard deviation of blood glucose in predicting hypoglycemia were 0.5%, 7.2 mmol/L and 1.4 mmol/L respectively.

CONCLUSION

High HGI was significantly associated with greater glycemic excursions and increased hypoglycemia in patients with TIR > 70%. Our findings indicate that HGI is a reliable predictor of hypoglycemia in this population.

Key Words: Hemoglobin glycation index; Hypoglycemia; Type 2 diabetes mellitus; Continuous glucose monitoring; Time in range

Core Tip: Our study focused on the relationship between hemoglobin glycation index and hypoglycemia in patients with type 2 diabetes and time-in-range > 70%. Our findings suggest that a high hemoglobin glycation index was significantly associated with greater glycemic excursions and increased hypoglycemia and was the best predictor of the occurrence and severity of hypoglycemia in this population.

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INTRODUCTION

Hemoglobin A1C is widely recognized as the gold standard for evaluating glycemic control and is significantly associated with chronic complications of diabetes mellitus[1]. While hemoglobin A1C can provide an average blood glucose level over the past three months, it fails to accurately evaluate fluctuations in blood glucose levels and hypoglycemia episodes[2,3]. Patients with similar hemoglobin A1C values may experience variations in glucose fluctuations[4]. Therefore, different patients with similar hemoglobin A1C experience different complications. Therefore, a novel index is required to facilitate precise glucose management. With the increasing use of continuous glucose monitoring (CGM) system in recent years, several glucose control parameters other than hemoglobin A1C have been proposed[5]. Among these parameters, time in range (TIR) and glucose management indicator (GMI) are proven novel measures for evaluating glycemic control[6,7].

The TIR is an important metric for evaluating glycemic control and strongly correlates with diabetic complications[8]. Therefore, TIR is more precise than hemoglobin A1C for accessing glycemic control[9]. Patients who achieve TIR within the target range experience less glycemic fluctuation and episodes of hypoglycemia[10]. However, a recent study suggested that some patients who meet TIR target range still experience significant hypoglycemia[11]. Given that patients with diabetes are at a higher risk of mortality due to hypoglycemia, identifying the potential factors contributing to hypoglycemic episodes in patients who achieve targeted TIR is essential.

The GMI can be easily obtained from CGM data; it is a crucial indicator for evaluating glucose status[8]. However, hemoglobin A1C and GMI do not always align[12]. There is a significant disparity between GMI and actual hemoglobin A1C values in some patients, which inhibits the effective utilization of GMI in clinical practice[12]. Therefore, further investigation of this discrepancy between GMI and laboratory hemoglobin A1C levels is important so that clinicians can help their patients effectively manage glycemic control and establish personalized glycemic targets.

To illustrate the discordance between the predicted hemoglobin A1C and laboratory hemoglobin A1C, the hemoglobin glycation index (HGI), calculated as the measured hemoglobin A1C minus GMI, was derived[4]. High HGI value was associated with increased incidence of diabetes-related complications and comorbidities[13,14]. In addition, a high HGI has been proved to attribute to the occurrence of hypoglycemia in patients with poor glucose control[15]. Hence, glycemic hypoglycemia is considered a bridge between high HGI and diabetes-associated complications. However, previous studies investigating the relationship between HGI and hypoglycemia mainly focused on populations whose blood glucose level was beyond the target but not on those with well glycemic control. Patients with good glycemic control may still experience diabetic complications; notable, this population exhibited more episodes of hypoglycemia. Nevertheless, the potential association between HGI and hypoglycemia in this particular population remains elusive. Therefore, invest-

igating the relationship between the HGI and hypoglycemia in patients with TIR > 70% may help clinicians establish effective glycemic control and reduce the incidence of diabetes-associated complications in this population. Therefore, in this study, we explored the role of HGI in assessing glycemic status in patients with type 2 diabetes and a TIR > 70%.

MATERIALS AND METHODS

Study design and population

This single-center retrospective study was conducted at the Department of Endocrinology and Metabolic Diseases, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong Province, China, between June 2017 and December 2019 (Figure 1). The inclusion criteria were as follows: (1) Met the 1999 World Health Organization diagnostic criteria for type 2 diabetes mellitus (T2DM) and age > 18 years; (2) Antihyperglycemic treatment was stable for at least 3 months prior to admission; (3) Laboratory-measured hemoglobin A1c levels were measured and a 7-days CGM was simultaneously applied on the same day simultaneously; and (4) Patients without conditions that may interfere with hemoglobin A1C measurement, such as anemia, hemoglobinopathies, severe renal failure (estimated glomerular filtration rate < 30 mL/minutes/1.73 m^2), and pregnancy. The exclusion criteria included allergies to the CGM sensors and poor compliance with the use of CGM.

Anthropometric indices and laboratory examination

Body mass index was calculated using standard methods [body mass index = weight (kg)/ height^2 (m^2)]. Systolic blood pressure and diastolic blood pressure were measured thrice and averaged. Blood tests were performed after an overnight fast. Laboratory hemoglobin A1C levels were determined using high-performance liquid chromatography with an automated analyzer (Bio-Rad D10; Bio-Rad Laboratories, Hercules, CA, United States) with the whole blood samples. The reference range for laboratory hemoglobin A1C was 4.3%-6.1%, with intra- and inter-batch coefficients of variation of 0.46% and 0.99%, respectively. Hemoglobin, hematocrit, and red blood cell distribution widths in whole blood samples were measured using a fully automated analyzer (Maccura Biotechnology, Chengdu, China). Serum was obtained after centrifugation of whole blood at 3000 rpm for 10 minutes and subsequently analyzed for total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol using a Hitachi Model 7600 Series Automatic Analyzer.

CGM

All subjects underwent a 7-days CGM with a professional retrospective CGM system (iPro™2, Medtronic Minimed Inc., Northridge, CA, United States) following their admission. At least four times of capillary blood glucose monitoring per day were conducted during the CGM period, and capillary blood glucose values were entered into the CGM system monitor for calibration purposes. The CGM data were subsequently downloaded using Carelink iPro and analyzed using GlyCulator 2.0. Only the data sets with adequate glucose data (≥ 70% per day) were included. Data collected on the days of sensor insertion and detachment were considered unstable calibration data and were excluded[16]. Invalid data with evidence of CGM malfunction or sensor loss were excluded from further analysis. Glycemic variability indices adopted in this study included[8]: (1) Mean blood glucose (MBG) is the average BG concentration based on CGM data; (2) Standard deviation of blood glucose (SDBG) is the standard deviation of the total blood glucose within 7 days; (3) Coefficient of variability of glycemia (%CV: SDBG/MBG × 100); (4) The mean amplitude of glycemic excursions (MAGE): The arithmetic mean of the differences between consecutive peaks and nadirs with measurement in the peak-to-nadir direction by the first qualifying excursion; and (5) Absolute means of daily differences (MODD): The mean of absolute differences between glucose values at the same time on two consecutive days. TIR (3.9-10.0 mmol/L), time below range (TBR < 3.9 mmol/L) and time above range (TAR > 10.0 mmol/L) were also obtained from the CGM system. GMI was calculated by CGM-derived mean glucose using the published equation [GMI (%) = 3.31 + 0.02392 × mean glucose in mg/dL][17]. HGI was defined as laboratory hemoglobin A1C minus GMI, and the subjects were divided into two subgroups according to the median HGI (median: 0.5). Patients with HGI < 0.5 were designated as low HGI subgroup while those with HGI ≥ 0.5 were classified as high HGI subgroup.

Definition of hypoglycemia

In accordance with international consensus guidelines, a level 1 hypoglycemic episode was defined as ≥ 15 consecutive minutes of sensor glucose < 3.9 mmol/L, a level 2 hypoglycemic period was defined as sensor glucose < 3.0 mmol/L for ≥ 15 consecutive minutes, both ending at the start of the first 15-minutes period with a sensor glucose ≥ 3.9 mmol/L or 3.0 mmol/L[18,19]. Nocturnal hypoglycemia was defined as hypoglycemia occurring between 10 pm and 6 am[20]. Nocturnal hypoglycemia was further divided into two periods: Hypoglycemia at night (10 pm to 3 am) and early morning (3 am to 6 am).

Statistical analysis

All data analyses were performed using the SPSS software version 26.0 (SPSS IBM Inc., Chicago, IL, United States). Data are represented as mean ± SD for normally distributed variables and as median with interquartile range for non-normally distributed data variables. Continuous variables were compared with analysis using the Student's *t*-test or Mann-Whitney testing, and categorical variables were compared using the χ^2 test or Fisher's exact probabilities. Multiple logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CI) to evaluate the risk factors for

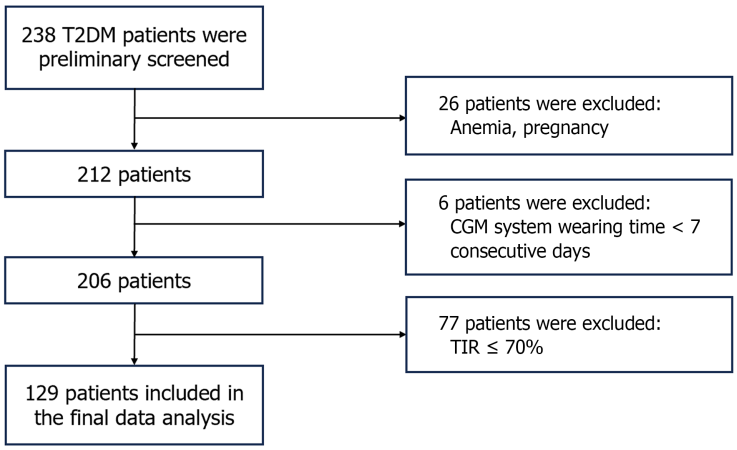

Figure 1 Flow chart of the trial. T2DM: Type 2 diabetes mellitus; CGM: Continuous glucose monitoring; TIR: Time in range.

hypoglycemia. Multivariate logistic regression analysis was used with a forward selection method, and only factors that showed *P* values of < 0.25 on univariate analysis were chosen in the multivariate model after excluding multicollinearity by Spearman's correlation analysis. The validity of CGM-derived metrics for hypoglycemia diagnosis was estimated using receiver operating characteristic (ROC) curves, optimal cutoff values, area under the curve, sensitivity, and specificity with 95%CI. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical characteristics and CGM parameters of subjects

A total of 129 patients with an average age of 54.84 ± 12.56 years and 54 males (41.9%) were enrolled into our study. There were no significant differences in the treatment between the high and low HGI groups, and the proportion of patients receiving sulfonylurea and insulin therapy, who might be potentially more prone to hypoglycemia, was relatively small. Although the rate of insulin use was higher in the high-HGI group than that in the low-HGI group, the difference was not statistically significant (35% *vs* 21%, $P = 0.071$). Additionally, the high-HGI group exhibited decreased hematocrit levels, whereas no variations in red cell distribution width were observed between the two groups. The mean laboratory hemoglobin A1C level in all subjects was 6.63% ± 1.27%. Moreover, laboratory hemoglobin A1C was significantly higher in the high HGI group than that in the low HGI group (7.43% ± 1.30 % *vs* 5.95% ± 0.71%, $P < 0.001$) (Table 1).

There were no significant differences in GMI values between high and low HGI groups (6.11% ± 0.70% *vs* 6.06% ± 0.63%, $P = 0.752$). Glycemic variability indices, including CV (21.41% ± 6.78% *vs* 24.76% ± 8.31%, $P = 0.017$), largest amplitude of glycemic excursions (LAGE) (8.26% ± 2.89% *vs* 9.63 ± 3.06%, $P = 0.005$) and MODD [1.30 (0.50) *vs* 1.50 (0.60), $P = 0.024$] were significantly higher in high HGI group than those in low HGI group, while no significant differences of SDBG, MBG and MAGE were observed between groups. The TIR was significantly lower in the high HGI group than in the low HGI group. An absolute difference in TBR was also observed between the two groups, whereas no statistically significant difference was observed in time above range between the groups (Table 2).

Hypoglycemia status and factors affecting hypoglycemia in type 2 diabetes

Despite higher hemoglobin A1C levels, the high HGI group exhibited a significantly higher incidence of hypoglycemia than the low HGI group (67% *vs* 14%, $P < 0.001$) (Table 1). The time, frequency, and severity of hypoglycemia were further compared between the high- and low-HGI groups. The frequencies of hypoglycemia in the early morning (from 3 am to 6 am) and at night (from 10 pm to 3 am) were significantly lower in the low-HGI group than in the high-HGI group (3.78% *vs* 10.14%, $P < 0.0001$ and 3.67% *vs* 10.96%, $P < 0.0001$, respectively). We next evaluated the duration of the glucose for level 1 hypoglycemia (below 3.9 mmol/L) or level 2 hypoglycemia (below 3.0 mmol/L) and found that the percentage of time for either was significantly higher in the high HGI group than those in the low HGI group (Figure 2). Logistic regression analyses revealed that low MBG, high SDBG, and high HGI were independent risk factors for the presence of hypoglycemia, whereas sex, age, laboratory hemoglobin A1c, hematocrit, LAGE, MAGE, MODD, and use of insulin and/or sulfonylurea treatment did not show a relationship with hypoglycemia in type 2 diabetes patients with TIR > 70% (Table 3).

Predicting factors for hypoglycemia

ROC curve analysis was conducted to evaluate the effects of HGI, MBG, and SDBG on hypoglycemia. The area under the ROC curve for HGI (0.79, 95%CI: 0.71-0.87, $P < 0.0001$) was superior to that for MBG (0.67, 95%CI: 0.58-0.75, $P = 0.0003$) and SDBG (0.67, 95%CI: 0.58-0.75, $P = 0.0004$) in predicting hypoglycemia in type 2 diabetes patients with TIR > 70%. ROC curve analysis showed that the optimal cutoff values of HGI, MBG, and SDBG for predicting hypoglycemia were 0.5%, 7.2 mmol/L and 1.4 mmol/L respectively (Figure 3A). We next conducted ROC curve analysis by combining the three

Table 1 Clinical characteristics of study participants

Characteristic	Total	HGI < 0.5	HGI ≥ 0.5	*P* value
Participants, *n* (%)	129	72 (56)	57 (44)	
Age (years), mean ± SD	54.84 ± 12.56	53.33 ± 12.28	56.75 ± 12.75	0.414
Female	70 (54)	35 (49)	35 (61)	0.148
BMI (kg/m^2)	24.52 ± 3.11	24.33 ± 12.14	24.76 ± 6.62	0.392
Duration of diabetes (months), medians (IQR)	73 (109.5)	73 (85.17)	97.33 (127.77)	0.287
Diabetes therapy, *n* (%)				
Diet only	25 (19)	18 (25)	7 (12)	0.077
Sulfonylurea	21 (16)	11 (15)	10 (18)	0.812
Metformin	58 (45)	27 (38)	31 (54)	0.075
α-glucosidase inhibitor	33 (26)	18 (25)	15 (26)	0.865
DPP4 inhibitor	28 (22)	18 (25)	10 (18)	0.391
SGLT-2 inhibitor	5 (4)	3 (4)	2 (4)	0.848
CSII/MDI	35 (27)	15 (21)	20 (35)	0.071
SBP (mmHg), mean ± SD	127.35 ± 14.09	126.22 ± 13.51	128.77 ± 14.79	0.375
DBP (mmHg), mean ± SD	80.84 ± 9.19	79.79 ± 8.06	82.18 ± 10.37	0.104
TC (mmol/L), mean ± SD	4.39 ± 1.07	4.33 ± 1.02	4.46 ± 1.13	0.790
TG (mmol/L), mean ± SD	1.63 ± 1.26	1.63 ± 1.48	1.64 ± 0.93	0.285
LDL-C (mmol/L), mean ± SD	2.68 ± 0.89	2.60 ± 0.86	2.78 ± 0.92	0.275
HDL-C (mmol/L), mean ± SD	1.13 ± 0.48	1.19 ± 0.58	1.07 ± 0.30	0.216
Laboratory HbA1c (%), mean ± SD	6.63 ± 1.27	5.95 ± 0.71	7.43 ± 1.30	< 0.001[a]
Hemoglobin (g/L), mean ± SD	133.84 ± 14.68	136.33 ± 13.21	131.70 ± 15.92	0.085
Hemotocrit (%), mean ± SD	0.40 ± 0.04	0.41 ± 0.04	0.39 ± 0.05	0.042[a]
Red cell distribution width (%), mean ± SD	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.556
Hypoglycemia, *n* (%)	49	10 (14)	39 (67)	< 0.001[a]

[a]*P* < 0.05, compared with the values from two groups.

HGI: Hemoglobin glycation index; BMI: Body mass index; DPP4: Dipeptidyl peptidase-4; SGLT-2: Sodium-glucose co-transporter type 2; CSII: Continuous subcutaneous insulin infusion; MDI: Multiple daily injection; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TC: Total cholesterol; TG: Triglyceride; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; HbA1c: Hemoglobin A1c; IQR: Interquartile range.

indicators, which suggested the area under the ROC curve for combination of high HGI, low MBG and high SDBG (0.91, 95%CI: 0.84-0.95, *P* < 0.0001) was higher than that for combination of high HGI and low MBG (0.83, 95%CI: 0.75-0.89, *P* < 0.0001) and combination of high HGI and high SDBG (0.82, 95%CI: 0.74-0.88, *P* < 0.0001) (Figure 3B). Based on these cutoff values, patients were divided into four groups according to the number of hypoglycemia predictors, in order to analyze the differences in the severity and occurrence time of hypoglycemia: Group 0 (no risk factors) (HGI < 0.5% and MBG ≥ 7.2 mmol/L and SDBG < 1.4 mmol/L); group 1 (one risk factor) (HGI ≥ 0.5% or MBG < 7.2 mmol/L or SDBG ≥ 1.4 mmol/L); group 2 (two risk factors) (HGI ≥ 0.5% and MBG < 7.2 mmol/L or HGI ≥ 0.5% and SDBG ≥ 1.4 mmol/L or MBG < 7.2 mmol/L and SDBG ≥ 1.4 mmol/L); group 3(three risk factors) (HGI ≥ 0.5% and MBG < 7.2 mmol/L and SDBG ≥ 1.4 mmol/L). The incidence of glucose < 3.9 mmol/L and < 3.0 mmol/L was near 0% for the group with none of the three predictors, 24.1% and 7.4% respectively for the groups with a single predictor, 85.1% and 25.5% respectively for the groups with two predictors, 100% and 73.3% respectively for the groups with three predictors, suggesting a linear relationship across the groups. It is noteworthy that when patients meet the criteria of HGI ≥ 0.5% and MBG < 7.2 mmol/L and SDBG ≥ 1.4 mmol/L, the incidence of glucose < 3.9 mmol/L was 100%, while the incidence of hypoglycemia (glucose < 3.0 mmol/L) is 0% when there were no risk factors. In addition, the incidence of hypoglycemia in the early morning and at night also showed a significant increase as the number of predictors increased. The frequency of hypoglycemia in the early morning and at night was 7.7% and 0%, respectively, in cases with 0 risk factors; 24.1% and 18.5%, respectively, in cases with 1 risk factor; 51.1% and 57.4%, respectively, in cases with 2 risk factors; and 80% and 93.3%, respectively, in cases with 3 risk factors (Figure 4).

Table 2 Continuous glucose monitoring parameters

Characteristic	Total	HGI < 0.5	HGI ≥ 0.5	*P* value
Participants, *n* (%)	129	72 (56)	57 (44)	
GMI (%), mean ± SD	6.09 ± 0.67	6.11 ± 0.70	6.06 ± 0.63	0.752
Average glucose (mmol/L), mean ± SD	7.11 ± 1.06	7.13 ± 1.10	7.08 ± 1.02	0.885
SDBG (mmol/L), mean ± SD	1.67 ± 0.64	1.59 ± 0.63	1.77 ± 0.64	0.961
CV (%), mean ± SD	22.89 ± 7.65	21.41 ± 6.78	24.76 ± 8.31	0.017[a]
MAGE (mmol/L), mean ± SD	4.08 ± 1.81	3.88 ± 1.72	4.35 ± 1.91	0.742
LAGE (mmol/L), mean ± SD	8.86 ± 3.03	8.26 ± 2.89	9.63 ± 3.06	0.005[a]
MODD (mmol/L), medians (IQR)	1.40 (0.70)	1.30 (0.50)	1.50 (0.60)	0.024[a]
TIR (% of time 39-10 mmol/L), medians (IQR)	90 (17)	93.50 (13)	86 (16)	0.002[a]
TBR (% of time < 3.9 mmol/L), medians (IQR)	1 (5)	0 (2)	2 (11)	< 0.001[a]
TAR (% of time > 10 mmol/L), medians (IQR)	6 (13)	4.50 (11)	8 (12)	0.068

[a]*P* < 0.05, compared with the values from two groups.

HGI: Hemoglobin glycation index; GMI: Glucose management indicator; SDBG: Standard deviation blood glucose; CV: Coefficient of variation; MAGE: Mean amplitude of glucose excursion; LAGE: Largest amplitude of glycemic excursions; MODD: Mean of daily differences; TIR: Time in range; TBR: Time below range; TAR: Time above range.

Table 3 Clinical markers of glycemia variables for hypoglycemia analyzed by univariate and multiple logistic regression analyses

	Univariate logistic regression			Multiple logistic regression		
	χ^2	*P* value	OR (95%CI)	Wald χ^2	*P* value	OR (95%CI)
Sex (female/male)	0.82	0.37	0.73 (0.36-1.45)			
Age	4.25	0.04[a]	0.42 (0.19-0.96)			
BMI	0.09	0.77	1.11 (0.55-2.25)			
Laboratory HbA1c	0.36	0.55	0.81 (0.41-1.62)			
Hemotocrit	1.81	0.18	1.75 (0.78-3.95)			
MBG	4.26	0.04[a]	0.45 (0.21-0.96)	9.05	< 0.01[a]	0.17 (0.05-0.54)
SDBG	9.74	< 0.01[a]	3.44 (1.58-7.47)	5.30	0.02[a]	5.37 (1.28-22.46)
LAGE	3.41	0.07	7.56 (0.88-64.67)			
MAGE	6.95	0.01[a]	2.62 (1.28-5.35)			
MODD	0.32	0.57	1.36 (0.46-4.01)			
HGI	6.98	< 0.01[a]	2.65 (1.29-5.44)	4.65	0.03[a]	2.46 (1.09-5.58)
Use of insulin and/or sulfonylurea	0.30	0.58	1.22 (0.60-2.51)			

[a]*P* < 0.05.

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; HbA1c: Hemoglobin A1c; MBG: Mean blood glucose; SDBG: Standard deviation blood glucose; LAGE: LAGE: Largest amplitude of glycemic excursions; MAGE: Mean amplitude of glucose excursion; MODD: Mean of daily differences; HGI: Hemoglobin glycation index.

DISCUSSION

In this observational study, we investigated the relationship between HGI and hypoglycemia in patients with type 2 diabetes and TIR > 70%. Our findings indicate that the high HGI group demonstrated more hypoglycemia episodes and more instances of glucose fluctuations than the low HGI group, even in patients with type 2 diabetes with a TIR > 70%. Our study also found that the best single predictor of hypoglycemia was HGI, and hypoglycemia should be of note when

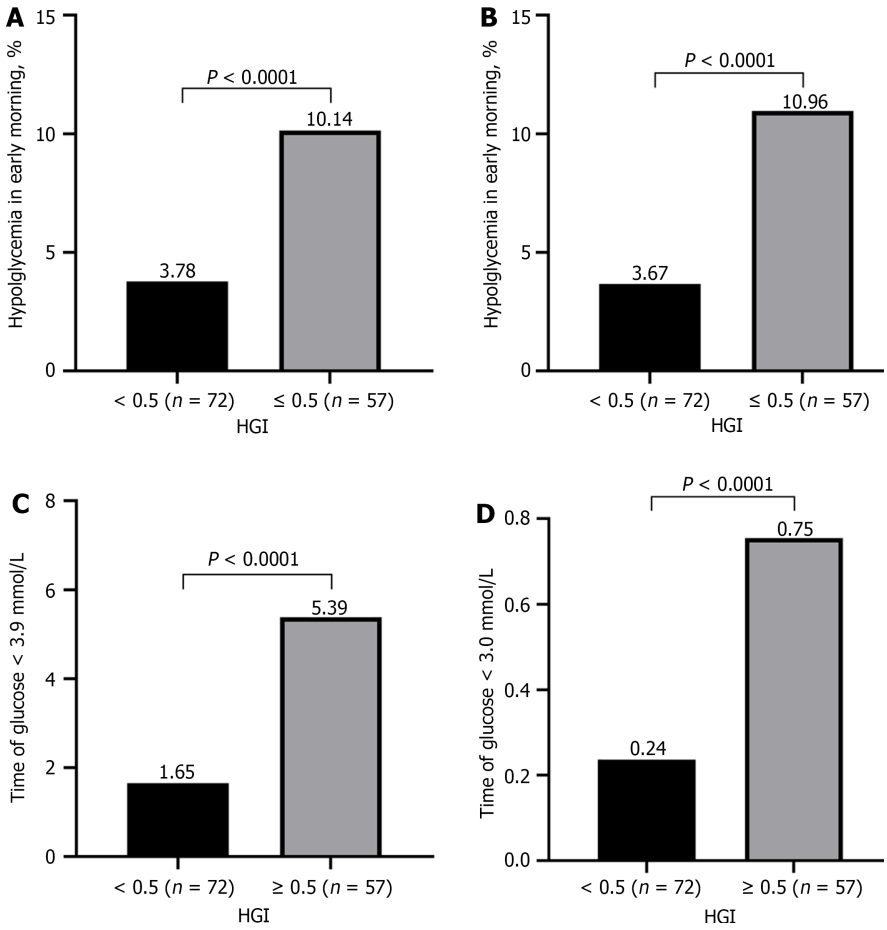

Figure 2 The differences in hypoglycemia between high hemoglobin glycation index and low hemoglobin glycation index groups. A and B: Frequency of hypoglycemia in early morning (A) and nighttime (B); C and D: Frequency of time of glucose < 3.9 mmol/L (C) and < 3.0 mmol/L (D). HGI: Hemoglobin glycation index.

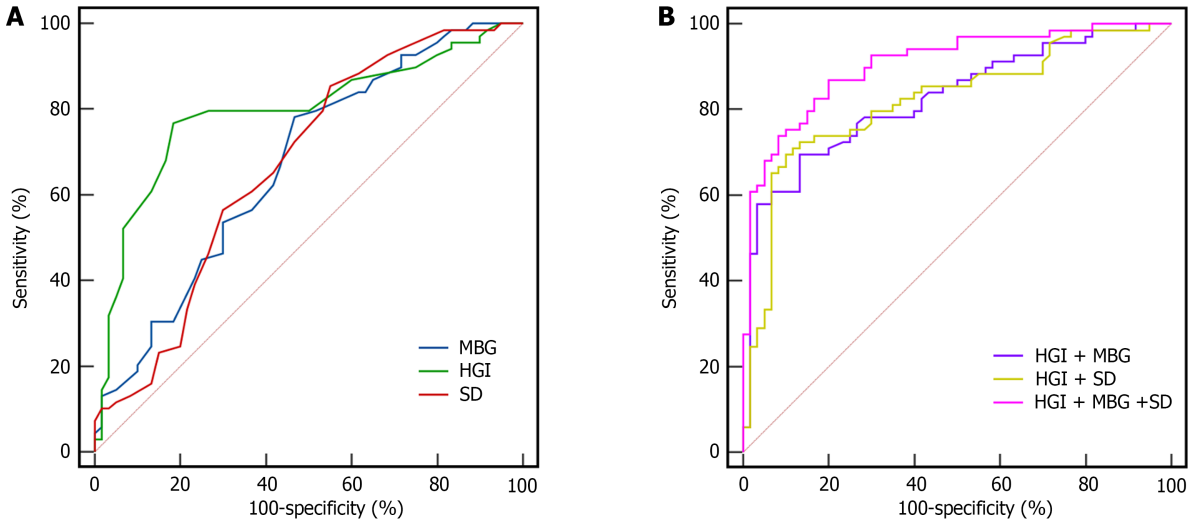

Figure 3 Receiver operator characteristic curve. A: Receiver operator characteristic curve of high hemoglobin glycation index, low mean blood glucose and high standard deviation blood glucose in assessing risk of hypoglycemia among people with type 2 diabetes mellitus; B: Receiver operator characteristic curve of combination of high hemoglobin glycation index, low mean blood glucose and high standard deviation blood glucose in assessing risk of hypoglycemia among people with type 2 diabetes mellitus. HGI: Hemoglobin glycation index; MBG: Mean blood glucose; SD: Standard deviation.

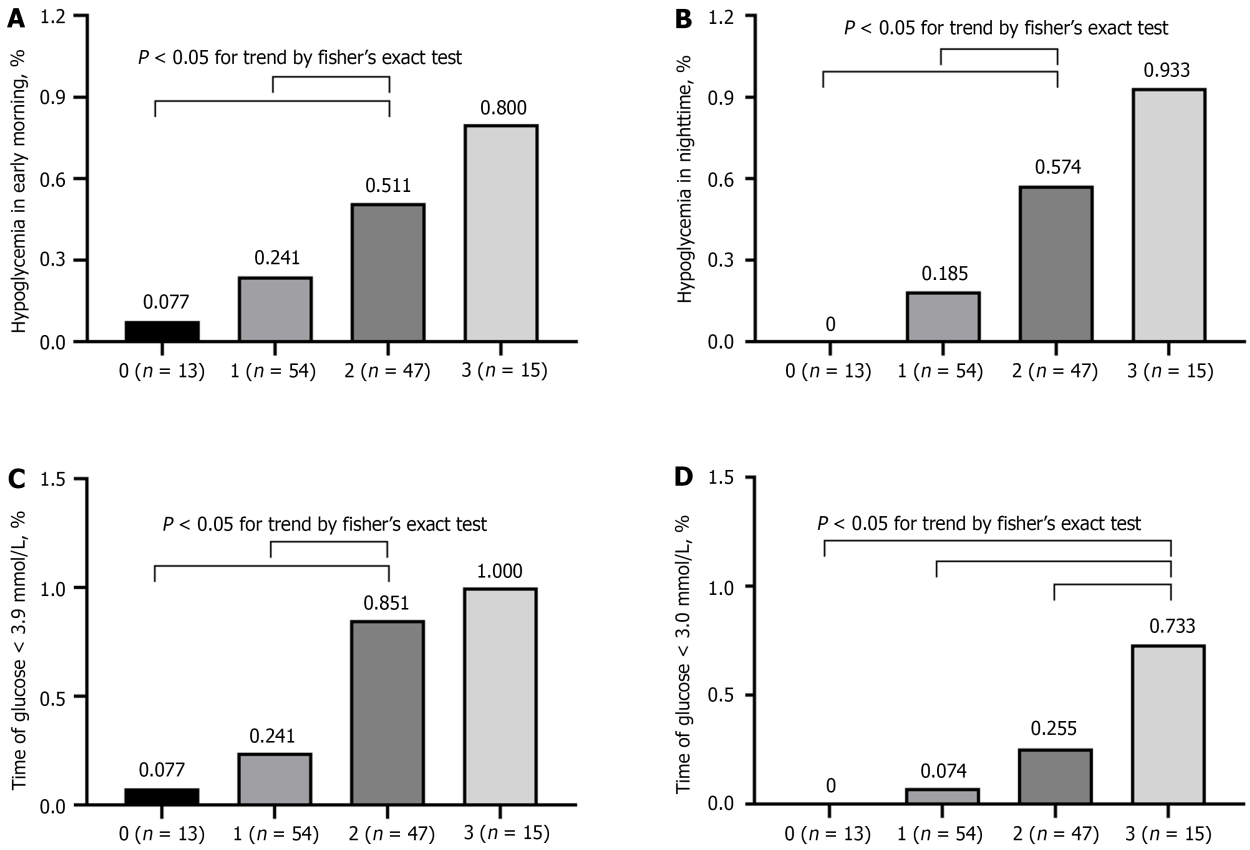

Figure 4 Relationship between the number of risk factors and hypoglycemia. A and B: Frequency of hypoglycemia in early morning (A) and nighttime (B); C and D: Frequency of time of glucose < 3.9 mmol/L (C) and < 3.0 mmol/L (D). Number of risk factors for hypoglycemia: 0 [hemoglobin glycation index (HGI) < 0.5% and mean blood glucose (MBG) ≥ 7.2 mmol/L and standard deviation blood glucose (SDBG) < 1.4 mmol/L]; 1 (HGI ≥ 0.5% or MBG < 7.2 mmol/L or SDBG ≥ 1.4 mmol/L); 2 (HGI ≥ 0.5% and MBG < 7.2 mmol/L or HGI ≥ 0.5% and SDBG ≥ 1.4 mmol/L or MBG < 7.2 mmol/L and SDBG ≥ 1.4 mmol/L); 3 (HGI ≥ 0.5% and MBG < 7.2 mmol/L and SDBG ≥ 1.4 mmol/L).

the laboratory hemoglobin A1C was greater than GMI by ≥ 0.5% in this population. Moreover, hypoglycemia was more likely to occur in patients with higher HGI (HGI ≥ 0.5%), lower MBG level (MBG < 7.2 mmol/L) and larger fluctuations in BG level (SDBG ≥ 1.4 mmol/L), suggesting that the combined assessment of these three variables was useful in detecting hypoglycemia in this population.

Recently, the HGI has emerged as a novel parameter to demonstrate the discordance between predicted hemoglobin A1C and laboratory hemoglobin A1C. Patients with high HGI experience higher incidence of diabetes-associated complications. Patients in the Diabetes Control and Complications Trial study were stratified into low-, moderate-, and high-HGI groups. After seven years of follow-up, patients in the high-HGI group had a significantly higher risk of developing retinopathy and nephropathy than those in the other groups[21]. Ahn *et al*[22] investigated the relationship between the HGI and cardiovascular diseases in patients with prediabetes and individuals with treatment-naïve diabetes and found that patients in the highest HGI tertile had a significantly increased risk of composite cardiovascular diseases. However, the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial suggested that the HGI may serve as a predictor of macrovascular and microvascular diseases, but not better than hemoglobin A1C, in patients with diabetes receiving intensive treatment[14]. Another ancillary study to Action to Control Cardiovascular Risk in Diabetes trial was conducted to investigate whether intensive hypoglycemic therapy (hemoglobin A1C goal < 6.0%) can reduce cardiovascular events in T2DM with high cardiovascular risk compared with standard treatment (hemoglobin A1C goal 7.0%-7.9%). However, the study was terminated after 3.7 years due to an increase in all-cause mortality among patients in the intensive treatment group. Interestingly, *post-hoc* subgroup analysis revealed that higher mortality in intensively treated patients with T2DM was observed only in the high HGI subgroup[23]. Patients who received intensive treatment in the ADVANCE and Action to Control Cardiovascular Risk in Diabetes trials experienced increased hypoglycemia. As a result, the discrepancy in the association between high HGI and diabetic complications in previous studies might be partly attributed to increased hypoglycemia during intensive treatment. However, research on the HGI has mainly focused on the relationship between the HGI and chronic complications and comorbidities in diabetes, whereas little attention has been paid to the relationship between the HGI and hypoglycemia in well-controlled patients.

As mentioned earlier, hemoglobin A1C may not precisely reflect blood glucose levels, even in well-controlled patients, which could lead to potential errors in clinical evaluation and treatment[24-26]. For patients with a high HGI and TIR in the target region, the actual glycosylated hemoglobin levels were higher than the predicted hemoglobin A1C. Thus, intensive hypoglycemic treatment may be administered in this population if glycosylated hemoglobin levels are used as

treatment targets. In contrast, for patients with low HGI and TIR in the target who were considered to have fewer chronic complications of diabetes, relaxation of the criteria for the control target may be considered. However, patients with strict glycemic control are prone to hypoglycemia. Hypoglycemia causes cognitive impairment and myocardial ischemia, which can result in sudden death[27]. Therefore, the relationship between the HGI and hypoglycemia needs to be clarified in patients with diabetes and good glycemic control. Interestingly, our study indicated that although with higher hemoglobin A1C and TIR in target, hypoglycemia occurred more frequently in the high HGI group than in the low HGI group.

In this study, we found no statistically significant difference in GMI values between the high and low HGI groups, but the high HGI group showed a significant increase in hemoglobin A1C, which points to the likelihood of greater blood glucose fluctuations in the high HGI group. Consistent with this, our results showed that the high HGI group had higher levels of CV, LAGE, and MODD, suggesting more glucose excursions. Additionally, higher hemoglobin A1C was more likely to lead to intensified hypoglycemic treatment by clinical physicians, which may be the reason for the higher incidence of hypoglycemia in the high HGI group. Moreover, we found that the frequency of nocturnal hypoglycemia and the severity of overall hypoglycemia were much higher in the high HGI group than in the low HGI group. These results suggest that the risk of hypoglycemia also occurred in patients with type 2 diabetes with TIR in target; thus, neither hemoglobin A1C nor GMI were proper predictors of hypoglycemia in this population. Our study showed that the HGI can serve as an effective indicator to compensate for the limitations of hemoglobin A1C in assessing glycemic control.

TIR has emerged as a significant metric in clinical studies of diabetes. Several investigations have demonstrated that TIR is closely associated with the risk of complications and mortality in patients with diabetes[28,29]. Consequently, clinical guidelines have incorporated TIR as a key objective for blood glucose management, recommending a target of > 70% for most non-pregnant adult patients with diabetes[8]. In our study, the median TIR of patients was 90%, leading to the assumption that patients had good glycemic control, potentially overlooking the occurrence of hypoglycemia. However, the single use of TIR has been criticized for not being adequately sensitive to hypoglycemia[30,31], and it has been reported that 7% of diabetic individuals with TIR greater than 70% were hospitalized for hypoglycemia or diabetic ketoacidosis[11]. Consequently, it is imperative to incorporate additional indicators to enhance the clinical glycemic management of patients with a TIR exceeding 70%. Our findings support and extend those of previous studies on glycemic control in well-controlled diabetes management. We found that HGI can be used as an indicator for assessing blood glucose to improve clinical outcomes, especially in patients with diabetes whose TIR is greater than 70%, which may be easily ignored in clinical settings.

In the present study, MBG, SDBG, and HGI were identified as risk factors for hypoglycemia, and HGI was suggested as the most effective individual measurement for predicting hypoglycemia. Previous studies have demonstrated that the occurrence of hypoglycemia increases with strict glycemic control[32]. Consistently, we found that patients with TIR > 70% were more likely to develop hypoglycemia with a higher HGI. Moreover, our study suggests that the HGI is the best predictor of the occurrence and severity of hypoglycemia. We further combined the indexes of HGI, MBG and SDBG, whose cutoff values were 0.5%, 7.2 mmol/L and 1.4 mmol/L respectively, with a sensitivity of 87% and a specificity of 80%. The results showed that patients with hemoglobin A1C higher than GMI by ≥ 0.5%, MBG < 7.2 mmol/L, and SDBG ≥ 1.4 mmol/L had a significantly higher risk for clinically hypoglycemia, no matter in early morning or nighttime. Previous studies have shown that using hemoglobin A1C alone as the glycemic control target might lead to irrational clinical use of hypoglycemic agents and setting unreasonable and unattainable blood glucose goals for patients to maintain good glycemic control[33-36]. Therefore, combinations of the HGI and other CGM indices, such as the HGI and hemoglobin A1C, provide individualized treatment for patients.

Our study had several strengths. First, we specifically targeted patients with a TIR exceeding 70%, a demographic that is frequently neglected regarding glycemic variability and the associated risk of hypoglycemia in clinical settings. Furthermore, our results indicate that the HGI may provide significant advantages for glucose management in patients with diabetes. Importantly, the integration of the HGI, MBG, and SDBG further improved the predictive sensitivity for hypoglycemia, thereby aiding clinicians in personalized glycemic management strategies for patients with a TIR greater than 70%. Our findings may help identify patients at a potentially greater risk of hypoglycemia and guide appropriate therapeutic schedules for patients with strict glycemic control.

Limitation

The limitations of our study include its retrospective design and the relatively small sample size. In addition, 7 days wearing time of the CGM device was considered shorter than 10-14 days in previous studies. However, minimal differences were observed between the 14- and 7-day GMI[37], suggesting that the variance around the estimation error for the CGM metrics is similar[17]. Further prospective studies with larger sample sizes and longer wearing time of iPro2 are warranted.

CONCLUSION

In this retrospective study, we found that a higher HGI was significantly associated with increased hypoglycemia, even in patients with TIR in target. The HGI was found to be the best predictor of hypoglycemia. Patients with a TIR exceeding 70% who exhibited lower MBG, greater glycemic excursions, and higher HGI were more likely to develop hypoglycemia.

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FOOTNOTES

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ORIGINAL ARTICLE

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Delayed treatment of diabetic foot ulcer in patients with type 2 diabetes and its prediction model

Hui Chen, Ying Xi

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Abstract

BACKGROUND

Diabetic foot (DF) is a serious complication of type 2 diabetes. This study aimed to investigate the factors associated with DF occurrence and the role of delayed medical care in a cohort of patients with type 2 diabetes.

AIM

To reveal the impact of delayed medical treatment on the development of DF in patients with type 2 diabetes and to establish a predictive model for DF.

METHODS

In this retrospective cohort study, 292 patients with type 2 diabetes who underwent examination at our hospital from January 2023 to December 2023 were selected and divided into the DF group (n = 82, DF) and nondiabetic foot group (n = 210, NDF). Differential and correlation analyses of demographic indicators, laboratory parameters, and delayed medical treatment were conducted for the two groups. Logistic regression was applied to determine influencing factors. Receiver operating characteristic (ROC) analysis was performed, and indicators with good predictive value were selected to establish a combined predictive model.

RESULTS

The DF group had significantly higher body mass index (BMI) ($P < 0.001$), disease duration ($P = 0.012$), plasma glucose levels ($P < 0.001$), and HbA1c ($P < 0.001$) than the NDF group. The NDF group had significantly higher Acute Thrombosis and Myocardial Infarction Health Service System (ATMHSS) scores ($P < 0.001$) and a significantly lower delayed medical treatment rate (72.38% *vs* 13.41%, $P < 0.001$). BMI, duration of diabetes, plasma glucose levels, HbA1c, diabetic peripheral neuropathy, and nephropathy were all positively correlated with DF occurrence. ATMHSS scores were negatively correlated with delayed time to seek medical treatment. The logistic regression model revealed that BMI, duration of diabetes, plasma glucose levels, HbA1c, presence of diabetic peripheral neuropathy and

nephropathy, ATMHSS scores, and delayed time to seek medical treatment were influencing factors for DF. ROC analysis indicated that plasma glucose levels, HbA1c, and delayed medical treatment had good predictive value with an area under the curve of 0.933 for the combined predictive model.

CONCLUSION

Delayed medical treatment significantly affects the probability of DF occurrence in patients with diabetes. Plasma glucose levels, HbA1c levels, and the combined predictive model of delayed medical treatment demonstrate good predictive value.

Key Words: Delayed treatment; Medical attention; Diabetic foot ulcer; Type 2 diabetes; Prediction model

Core Tip: This retrospective cohort study investigates factors influencing diabetic foot (DF) in type 2 diabetes patients. Key findings highlight that increased body mass index, longer diabetes duration, elevated plasma glucose and HbA1c levels, as well as complications like diabetic neuropathy, are positively associated with DF occurrence. Additionally, a low Attitudes Toward Medical Help Seeking Scale score and delayed medical care over 3 months correlate with DF. These insights underscore the importance of proactive diabetes management and timely medical intervention to prevent DF, with the study's predictive model demonstrating strong diagnostic potential (area under the curve = 0.933).

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INTRODUCTION

Type 2 diabetes is a long-term condition marked by the body's inability to properly use insulin or a reduced efficiency in insulin function, leading to persistently elevated blood sugar levels. Its epidemiological characteristics vary by region and population and are associated with genetic, lifestyle, and environmental factors[1,2]. Its typical manifestations include polyuria, polydipsia, polyphagia, and weight loss. Prolonged hyperglycemia can cause the occurrence and development of various complications[3,4].

Diabetic foot (DF) is a serious diabetes-related complication involving nerve damage in the lower extremities and various levels of vascular disease. This condition can cause infections, ulcers, and serious damage to deep tissues in patients. Severe symptoms can result in difficulty walking and even amputation, significantly influencing the quality of life of patients with diabetes[5,6]. At present, the cure rate for DF is improving and the amputation rate is gradually decreasing; however, its incidence is increasing year by year[7,8].

Delay in seeking medical care is defined as the behavior of individuals who do not seek timely medical care after discovering abnormal bodily symptoms due to objective or subjective reasons. This behavior occurs because the early symptoms of DF are not evident, and patients with diabetes do not actively seek foot examinations at hospitals to assess their risk of developing DF[9,10]. As a consequence, delayed medical care often leads to DF.

Although previous studies have analyzed the risk factors for DF[11-13], no research has focused on the impact of delayed medical care on the probability of DF occurrence in patients with diabetes. Therefore, investigating the impact of delayed medical care on DF occurrence in patients with diabetes is of utmost importance.

MATERIALS AND METHODS

Study design

This retrospective cohort study included 292 individuals diagnosed with type 2 diabetes who received examinations at our hospital over the period from January 2023 to December 2023. They were divided into the DF group (n = 82) and nondiabetic foot group (n = 210, NDF group). This study was approved by the institutional review board and ethics committee of Shaanxi Provincial People's Hospital. Given its retrospective design, this study only used data from unidentified patients and informed consent was waived.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) Diagnosis of type 2 diabetes as per World Health Organization guidelines[14]; (2) Aged between 18 and 80 years; and (3) Ineligibility for surgical revascularization.

The exclusion criteria were as follows: (1) Prior occurrence of acute coronary syndrome, myocardial infarction, or transient ischemic stroke within the last 6 months; (2) Presence of uncontrolled immune disorders or active severe systemic infections; (3) Severe hematologic disorders or coagulation abnormalities; (4) History of malignant tumors; (5) Participation in other clinical trials within the preceding 3 months; or (6) Other concerns identified by the investigators that may impede compliance or safety.

The patients were grouped based on the presence or absence of DF: DF group (n = 82) and NDF group (n = 210). The diagnostic criteria were based on the 2012 Infectious Diseases Society of America clinical practice guidelines for DF infections[14].

Data collection

Patient demographic data were acquired from the medical records system. Upon admission, 5 mL of fasting blood sample was obtained from the antecubital vein in the morning for blood testing. Hematological parameters such as hemoglobin (g/dL), hematocrit (%), white blood cell count (× 10^9/L), and platelet count (× 10^9/L) were measured using a fully automated coagulation analyzer (HC00608166, STA Compact, China).

Biochemical parameters and genomic and proteomic data were analyzed using venous blood samples of 5-7 mL (including whole blood, plasma, and serum). The measured biochemical markers included HbA1c, plasma glucose, total cholesterol (measured by the CHOD-PAP method), low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, urea, and creatinine. All quantitative measurements were performed using a BS 400 auto-analyzer in accordance with the procedures provided by Dia Sys Diagnostic Systems GmbH, Germany. The glomerular filtration rate (GFR) was computed from serum creatinine levels using the formula: GFR (mL/minute/1.73 m^2) = (140 - age) × weight (kg)/72 × serum creatinine (mg/dL).

Delay in seeking medical care

Delay in seeking medical care (patient delay) refers to the behavior of individuals who fail to seek timely medical attention after noticing abnormal symptoms due to objective or subjective reasons. The time of medical delay is defined as the interval between the time the patient first noticed the symptoms and their first visit to a healthcare facility, with a duration of over 3 months considered as medical delay.

Attitudes Toward Medical Help Seeking Scale (ATMHSS): The ATMHSS is a self-assessment scale consisting of 35 items divided into four dimensions: Behavioral intention (12 items), nonfatalism (11 items), medical trust (7 items), and nonavoidant attitudes (5 items). Each item is rated on a Likert 4-point scale, with “disagree” to “agree” corresponding to scores of 0-3. The total score ranges from 0 to 105, where a high score indicates a positive attitude toward seeking medical care. The scale has a Cronbach's α coefficient of 0.82.

Sample size and statistical power

The sample size was mainly depended on the number of patients included in the inclusion time frame complied with the events per variable > 10 principle. Using G*Power 3.1.9.7, we conducted a *post hoc* analysis based on the "Means: Difference between two independent means (two groups)" option for t-tests. We configured the analysis with a two-tailed mode, an effect size of d = 0.6, and a significance level (α err prob) of 0.05. After inputting the sample sizes for the two groups, we calculated the power (1 - β err prob), which yielded a result of 0.969.

Statistical analysis

Patient characteristics were compared between the two groups using independent t-tests for continuous variables and χ^2 tests for categorical variables. The normality of continuous variables was checked using the Shapiro-Wilk test. Normally distributed data were expressed as mean ± SD, and categorical variables were reported as counts and percentages. A P value of less than 0.05 was deemed statistically significant. All analyses were conducted with SPSS software, version 29.0 (SPSS Inc., Chicago, IL, United States).

Spearman correlation analysis was conducted. Indicators showing significant differences in the differential and correlation analyses were included as covariates in logistic regression analysis. The diagnostic efficiency of delayed medical care for DF was assessed using the area under the receiver operating characteristic (ROC) curve, and a combined predictive model was established by incorporating blood glucose levels, glycated protein levels, and delayed medical care.

RESULTS

Demographic characteristics

Between-group comparison showed no statistically significant differences in general demographic data such as age and gender in the presence of DF (P > 0.05; Table 1). However, the body mass index (BMI) of the DF group was significantly higher than that of the NDF group (23.51 ± 3.57 *vs* 25.17 ± 3.57, P < 0.001). This finding suggests that an increase in BMI may elevate the incidence of DF.

Table 1 Comparison of general demographic data, *n* (%)

Characteristic	NDF group (*n* = 210)	DF group (*n* = 82)	t/χ^2	*P* value
Age (years)	51.15 ± 9.32	52.69 ± 9.15	1.283	0.201
BMI (kg/m^2)	23.51 ± 3.57	25.17 ± 3.57	3.588	< 0.001
Sex	107 (50.95)	47 (57.32)	0.720	0.396
Smoking history	51 (24.29)	19 (23.17)	0.295	0.587
Alcohol history	65 (30.95)	24 (29.27)	0.060	0.807
Family history of diabetes	44 (20.95)	21 (25.61)	0.150	0.699
Hypertension	39 (18.57)	18 (21.95))	1.229	0.268
Hyperlipidemia	37 (17.62)	16 (19.51)		

BMI: Body mass index; DF: Diabetic foot; NDF: Nondiabetic foot group.

Diabetes-related indicators and complications

Differential analysis was conducted on the occurrence probabilities of diabetes duration, classification, staging, and complications between the two groups (Table 2). No significant differences were observed in classification, staging, diabetic retinopathy, and diabetic vascular disease ($P > 0.05$). The duration of diabetes in the DF group was significantly longer than that in the NDF group (10.12 ± 4.95 *vs* 11.74 ± 4.84, $P = 0.012$). In addition, the occurrence rates of diabetic neuropathy (22.86% *vs* 40.2%, $P = 0.005$) and diabetic nephropathy (0.48% *vs* 4.88%, $P = 0.035$) were markedly lower in the NDF group compared with those in the DF group. These findings suggest that an increase in diabetes duration and the occurrence of some complications may contribute to the increased incidence of DF.

Routine blood examination

Routine blood examination indicated no statistical differences in hemoglobin concentration, white blood cell count, red blood cell count, neutrophil count, and platelet count between the two groups ($P > 0.05$; Figure 1). This finding suggests that preoperative blood routine indicators have no impact on the research results.

Blood glucose and lipid levels

Examination of the patients' blood glucose and lipid levels indicated no statistical differences in total cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol between the two groups ($P > 0.05$; Figure 2). This finding suggests that preoperative blood routine indicators have no impact on the research results. However, the DF group exhibited significantly higher levels of plasma glucose (5.71 ± 0.61 *vs* 10.24 ± 4.72, $P < 0.001$) and HbA1c (7.41 ± 1.87 *vs* 9.12 ± 1.65, $P < 0.001$) than the NDF group. This result indicates that elevated blood glucose levels have a negative impact on DF occurrence.

Renal function levels

Differential analysis was conducted on the renal function indicators of the two groups, including plasma creatinine, plasma urea, and estimated GFR (Table 3). No significant differences in renal function indicators were found between the two groups ($P > 0.05$). This finding suggests that intraoperative indicators have no impact on the research results.

Delayed medical care

The patients' delayed medical care of more than 3 months was recorded, and their willingness to seek medical care was assessed using the ATMHSS (Table 4). The NDF group had significantly higher ATMHSS scores (68.71 ± 10.41 *vs* 59.84 ± 9.78, $P < 0.001$) and lower rate of delayed medical care (13.41% *vs* 72.38%) compared with the DF group. This finding indicates that low willingness to seek medical care and delayed medical care may lead to DF.

Correlation analysis

A significant correlation was observed between various indicators and DF occurrence (Table 5). On the one hand, BMI, duration of diabetes, plasma glucose, HbA1c, and presence of diabetic peripheral neuropathy and diabetic nephropathy were positively correlated with DF occurrence. On the other hand, the ATMHSS score and duration of medical delay were negatively correlated with DF occurrence. These results underscore the predictive potential of these indicators for DF occurrence.

Logistic regression analysis

Logistic regression analysis revealed a significant finding (Table 6). The multivariate regression model demonstrated that BMI, duration of diabetes, plasma glucose, HbA1c, presence of diabetic peripheral neuropathy and diabetic nephropathy, ATMHSS score, and medical delay time all had a strong diagnostic value for DF.

Table 2 Diabetes-related indicators and complications, *n* (%)

Characteristic		NDF group (*n* = 210)	DF group (*n* = 82)	t/χ^2	*P* value
Duration of diabetes (years)		10.12 ± 4.95	11.74 ± 4.84	2.554	0.012
Types	Insulin resistance	179 (85.24)	70 (85.37)	0.000	1.000
	Insufficient insulin secretion	31 (14.76)	12 (14.63)		
Stages	Impaired glucose tolerance	29 (13.81)	11 (13.41)	0.008	0.996
	Type 2 diabetes stage	120 (57.14)	47 (57.32)		
	Late stage of type 2 diabetes	61 (29.05)	24 (29.27)		
Diabetic retinopathy		43 (20.48)	24 (29.27)	2.105	0.147
Diabetic vascular disease		69 (32.86)	30 (36.59)	0.218	0.640
Diabetic peripheral neuropathy		48 (22.86)	33 (40.24)	8.048	0.005
Diabetic nephropathy		1 (0.48)	4 (4.88)	4.426	0.035

DF: Diabetic foot; NDF: Nondiabetic foot group.

Table 3 Renal function levels, *n* (%)

Characteristic	NDF group (*n* = 210)	DF group (*n* = 82)	t/χ^2	*P* value
Plasma creatinine (μmol/L)	49.7 ± 4.12	50.27 ± 4.34	1.024	0.307
Plasma urea (mmol/L)	1.35 ± 0.59	1.41 ± 0.67	0.687	0.493
eGFR, mL/min/1.73 m^2			0.048	0.827
≥ 60	153 (72.86)	58 (70.73)		
< 60	57 (27.14)	24 (29.27)		

DF: Diabetic foot; NDF: Nondiabetic foot group; eGFR: Estimated glomerular filtration rate.

Table 4 Delayed medical care, *n* (%)

Characteristic		NDF group (*n* = 210)	DF group (*n* = 82)	t/χ^2	*P* value
ATMHSS score		68.71 ± 10.41	59.84 ± 9.78	6.84	< 0.001
Medical delay time	Less than 3 months	152 (72.38)	11 (13.41)	80.773	< 0.001
	More than 3 months	58 (27.62)	71 (86.59)		

DF: Diabetic foot; NDF: Nondiabetic foot group; ATMHSS: Acute thrombosis and myocardial infarction health service system.

ROC analysis

A validation cohort of patients was used to establish a predictive model for DF occurrence based on factors such as BMI, duration of diabetes, blood glucose levels, complications, and medical delay (Table 7). The results demonstrated that plasma glucose [area under the curve (AUC) = 0.819], HbA1c (AUC = 0.804), and medical delay (AUC = 0.795) exhibited good predictive value. The combined predictive model yielded an AUC of 0.933 (Figure 3).

DISCUSSION

Diabetes represents an increasingly growing public health concern, with its associated complications drawing increasing attention[15,16]. Owing to the global prevalence of diabetes and the increased life expectancy of patients with diabetes, the incidence of DF has risen. Diabetes-related foot complications are one of the most common complications among patients with diabetes, constituting a significant healthcare burden[17-19].

Table 5 Correlation analysis

Characteristic	Rho	*P* value
BMI (kg/m^2)	0.206	< 0.001
Duration of diabetes (years)	0.147	0.012
Plasma glucose	0.626	< 0.001
HbA1c (%)	0.498	< 0.001
ATMHSS score	-0.364	< 0.001
Diabetic peripheral neuropathy	0.175	0.003
Diabetic nephropathy	0.152	0.009
Medical delay time	-0.534	< 0.001

BMI: Body mass index; ATMHSS: Acute thrombosis and myocardial infarction health service system.

Table 6 Logistic regression analysis

	Coef	Odds ratio	B	Beta	*P* value
BMI (kg/m^2)	0.130	1.139	3.440	0.130	< 0.001
Duration of diabetes (years)	0.067	1.069	2.481	0.067	0.013
Plasma glucose	0.775	2.171	6.459	0.775	< 0.001
HbA1c (%)	0.902	2.463	7.363	0.902	< 0.001
ATMHSS score	0.083	0.921	5.770	-0.083	< 0.001
Diabetic peripheral neuropathy	0.821	2.273	2.945	0.821	0.003
Diabetic nephropathy	2.372	10.718	2.107	2.372	0.035
Medical delay time	2.828	0.059	7.88	-2.828	< 0.001

BMI: Body mass index; ATMHSS: Acute thrombosis and myocardial infarction health service system.

Table 7 Receiver operating characteristic analysis

	Sensitivities	Specificities	AUC	Youden index
BMI (kg/m^2)	0.573	0.633	0.631	0.206
Duration of diabetes (years)	0.463	0.714	0.597	0.177
Plasma glucose	0.720	1.000	0.819	0.720
HbA1c (%)	0.646	0.867	0.804	0.513
ATMHSS score	0.646	0.700	0.731	0.346
Diabetic peripheral neuropathy	0.402	0.771	0.587	0.173
Diabetic nephropathy	0.049	0.995	0.522	0.044
Medical delay time	0.866	0.724	0.795	0.590

BMI: Body mass index; ATMHSS: Acute thrombosis and myocardial infarction health service system; AUC: Area under the curve.

Patient delay in seeking medical attention may result in the disease being in an advanced stage at the time of diagnosis. Medical delay can significantly reduce the clinical effectiveness of treatment, increase the treatment burden on patients, and even impact their short- and long-term prognoses.

The primary finding of this study is the impact of medical delay on the incidence of DF. Between-group comparison of the probability of medical delay and patients' willingness to seek medical care revealed that patients with medical delay and low willingness to seek medical care may have an increased likelihood of developing DF. The possible underlying mechanism is that patient delay in seeking medical attention may lead to the disease being in an advanced stage at the

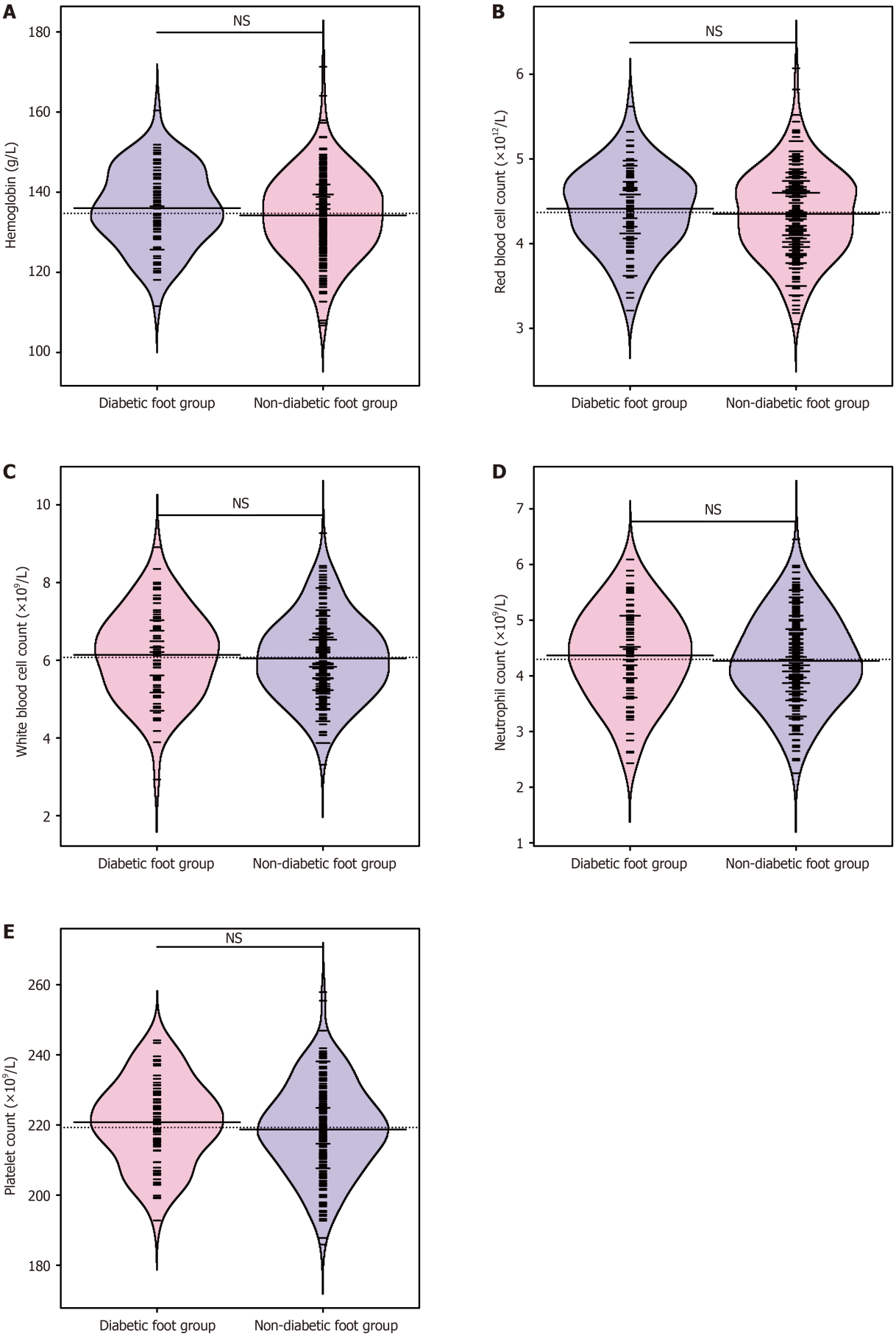

Figure 1 Blood Routine Examination. A: Hemoglobin concentration; B: Red blood cell count; C: White blood cell count; D: Neutrophil count; E: Platelet. NS: No significant difference.

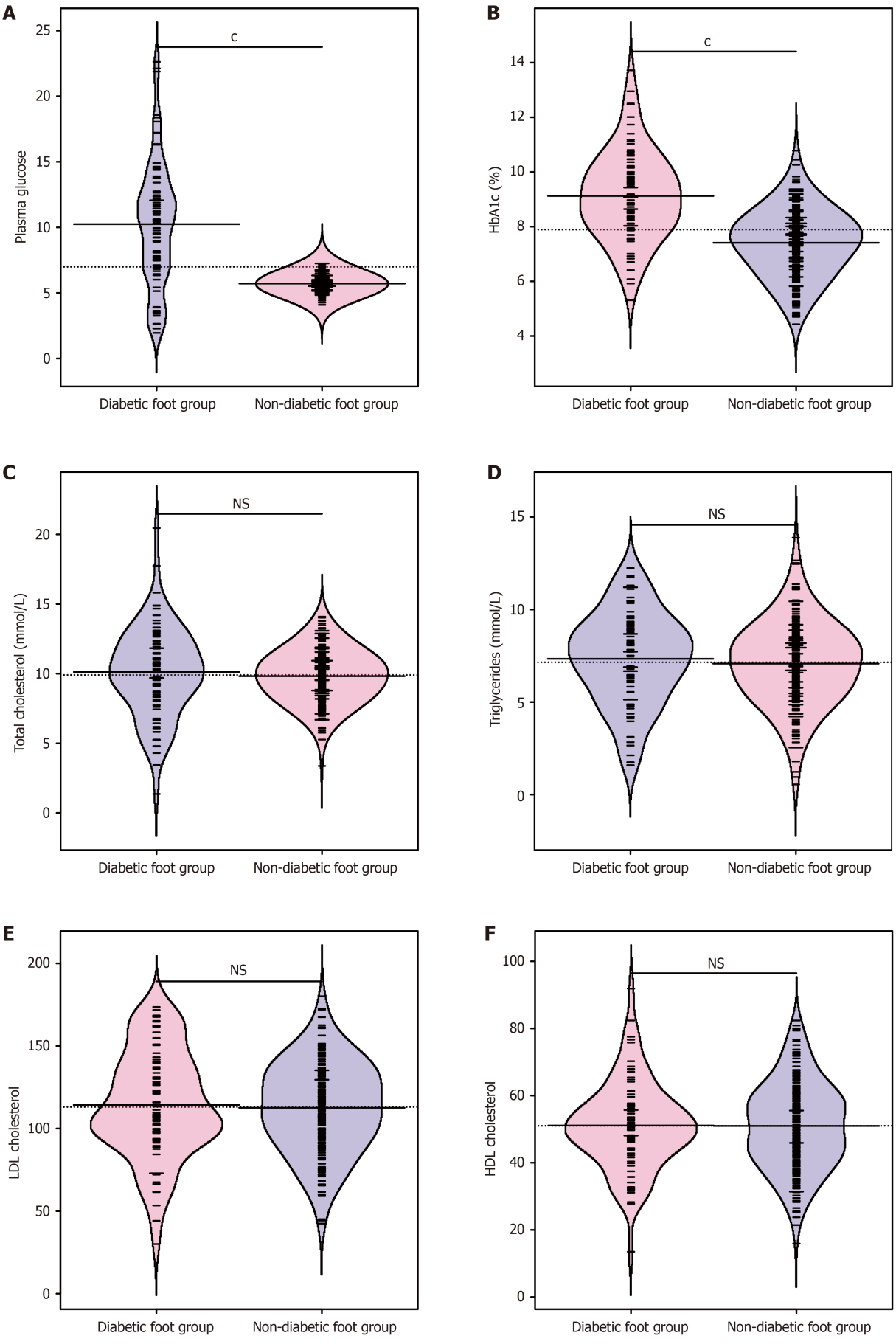

Figure 2 Blood glucose and lipid levels. A: Plasma glucose; B: HbA1c; C: Total cholesterol; D: Triglycerides; E: Low-density lipoprotein cholesterol; F: High-density lipoprotein cholesterol. $^{c}P < 0.001$; NS: No significant difference.

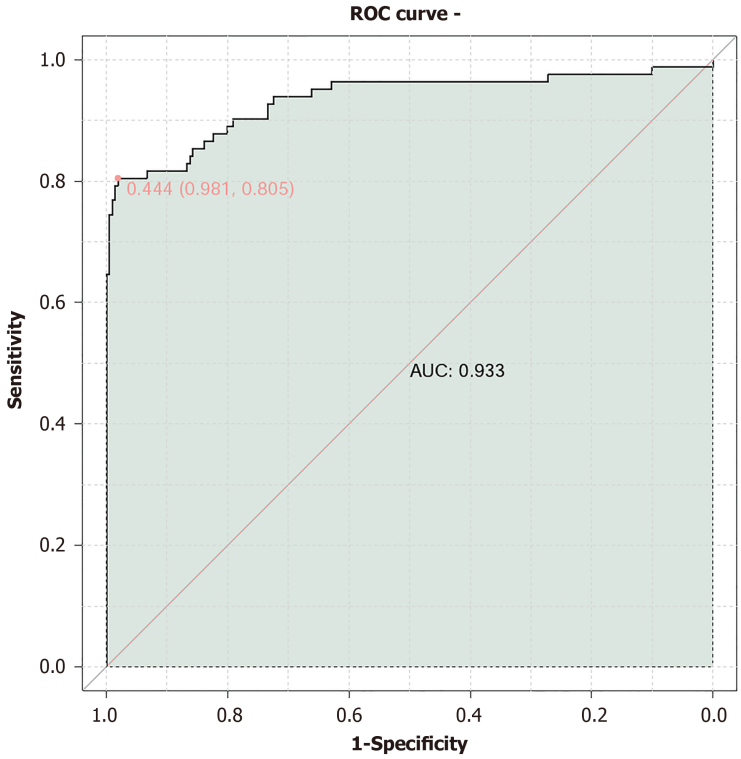

Figure 3 Analysis of the combined predictive model for plasma glucose, HbA1c, and medical delay. ROC: Receiver operating characteristic; AUC: Area under the curve.

time of diagnosis, significantly reducing the clinical effectiveness of treatment, increasing the treatment burden on patients, and possibly affecting their short- and long-term prognoses. The low willingness of patients to seek medical care may be due to inadequate education, insufficient awareness of DF prevention, and lack of habit of timely check-ups and medical care[20,21]. In addition, the early symptoms of DF, such as coolness in the soles of the feet and delayed sensation, are often subtle and easily overlooked by patients, leading to medical delay.

Furthermore, the results of this study indicate that BMI, plasma glucose, and HbA1c levels are associated with DF. This relationship may be related to poor control of blood glucose levels in patients, leading to vascular changes in the lower extremities, insufficient blood supply to the lower limbs, and ultimately the occurrence of DF[22-24]. Chen *et al*[25] also illustrated that uncontrolled blood glucose levels in patients with diabetes can lead to diabetic complications. Consistent with the present findings, high blood glucose levels are associated with severe DF ulcers[25].

Diabetic neuropathy can affect the central and peripheral nervous systems, with the latter being particularly common. In particular, distal sensory neuropathy is the most prevalent and accounts for over 50% of all diabetic neuropathies[26,27]. Diabetic nephropathy is one of the most significant complications in patients with diabetes and often concurrently involves microvascular disease in other organs or systems[28,29]. Although the correlation of certain complications such as diabetic retinopathy and DF with diabetes is relatively low, the occurrence of diabetic neuropathy and diabetic nephropathy is significantly associated with DF. As the duration of diabetes progresses, the incidence of DF also increases.

BMI, duration of diabetes, plasma glucose, HbA1c, and presence of diabetic peripheral neuropathy and diabetic nephropathy are all positively correlated with DF occurrence. Meanwhile, the ATMHSS score and delayed medical care are negatively correlated with DF occurrence. Logistic regression analysis indicates that these factors are risk factors for DF. ROC analysis demonstrates that the combined predictive model of blood glucose levels and delayed medical care has good predictive value.

This study investigates the predictive role of delayed medical care and other factors in patients with DF. However, it has certain limitations. First, the retrospective design imposes inherent constraints on causal inferences, as the observed correlation between personalized care interventions and postoperative outcomes does not establish a clear causal relationship. The reliance on retrospective data collection also introduces the possibility of information bias and confounding variables that may impact the observed results. In addition, the limited sample size may restrict the applicability of the results to a broad patient population. For future research endeavors, we aim to carry out multicenter prospective studies to comprehensively investigate the influence of delayed medical care on the incidence of DF.

CONCLUSION

Delayed medical care significantly influences the likelihood of DF occurrence in patients with diabetes. The combined predictive model of plasma glucose, HbA1c levels, and delayed medical care demonstrates good predictive value.

FOOTNOTES

Author contributions: Chen H designed the experiments and conducted clinical data collection; Xi Y performed postoperative follow-up and recorded the data; Chen H and Xi Y conducted the collation and statistical analysis, wrote the original manuscript and revised the paper; Both authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Association between sensitivity to thyroid hormones and non-high-density lipoprotein cholesterol levels in patients with type 2 diabetes mellitus

Xiao-Ye Duan, Jun-Ling Fu, Li-Na Sun, Zhi-Jing Mu, Shuang-Ling Xiu

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Abstract

BACKGROUND

Dyslipidemia and type 2 diabetes mellitus (T2DM) are chronic conditions with substantial public health implications. Effective management of lipid metabolism in patients with T2DM is critical. However, there has been insufficient attention given to the relationship between thyroid hormone sensitivity and dyslipidemia in the T2DM population, particularly concerning non-high-density lipoprotein cholesterol (non-HDL-C).

AIM

To clarify the association between thyroid hormone sensitivity and dyslipidemia in patients with T2DM.

METHODS

In this cross-sectional study, thyroid hormone sensitivity indices, the thyroid feedback quantile-based index (TFQI), the thyroid-stimulating hormone index (TSHI), the thyrotrophic T4 resistance index (TT4RI), and the free triiodothyronine (FT3)/free thyroxine (FT4) ratio were calculated. Logistic regression analysis was performed to determine the associations between those composite indices and non-HDL-C levels. Random forest variable importance and Shapley Additive Explanations (SHAP) summary plots were used to identify the strength and direction of the association between hyper-non-HDL-C and its major predictor.

RESULTS

Among the 994 participants, 389 (39.13%) had high non-HDL-C levels. Logistic regression analysis revealed that the risk of hyper-non-HDL-C was positively correlated with the TFQI (OR: 1.584; 95%CI: 1.088-2.304; P = 0.016), TSHI (OR: 1.238; 95%CI: 1.034-1.482; P = 0.02), and TT4RI (OR: 1.075; 95%CI: 1.006-1.149; P =

0.032) but was not significantly correlated with the FT3/FT4 ratio. The relationships between composite indices of the thyroid system and non-HDL-C levels differed according to sex. An increased risk of hyper-non-HDL-C was associated with elevated TSHI levels in men (OR: 1.331; 95%CI: 1.003-1.766; P = 0.048) but elevated TFQI levels in women (OR: 2.337; 95%CI: 1.4-3.901; P = 0.001). Among the analyzed variables, the average SHAP values were highest for TSHI, followed by TT4RI.

CONCLUSION

Impaired sensitivity to thyroid hormones was associated with high non-HDL-C levels in patients with T2DM.

Key Words: Non-high-density lipoprotein cholesterol; Sensitivity to thyroid hormones; Type 2 diabetes mellitus; Thyroid feedback quantile-based index; Free triiodothyronine/free thyroxine ratio

Core Tip: Reduced central thyroid hormone sensitivity was an independent risk factor of high non-high-density lipoprotein cholesterol (non-HDL-C), even after adjusting for multiple confounding factors. The patients with hyper-non-HDL-C were more susceptible to metabolic disorders and impaired sensitivity to thyroid hormones. Meanwhile, the relationships between thyroid hormone sensitivity and non-HDL-C levels were different in male and female, indicating a gender-related regulation of thyroid hormones on serum non-HDL-C levels. This study may provide new evidence for the role of reduced thyroid hormone sensitivity for non-HDL-C levels and lie the groundwork for future therapeutic strategies for diabetes-related cardiovascular disease risk.

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INTRODUCTION

Dyslipidemia and type 2 diabetes mellitus (T2DM) are chronic diseases with significant public health implications[1]. Atherogenic dyslipidemia is one of the major risk factors for atherosclerotic cardiovascular disease (ASCVD) in people with T2DM[2]. Patients with diabetes have approximately double the ASCVD risk of those without diabetes[3]. ASCVD, a vascular complication of T2DM, is a leading cause of mortality. Therefore, the management of lipid metabolism in patients with T2DM is crucial.

Thyroid hormones are not only essential determinants of overall energy expenditure but also important regulators of various aspects of lipid metabolism[4,5], including the synthesis, mobilization, and decomposition of fat and other processes through a complex regulatory mechanism[6]. Many studies have revealed a causal association between thyroid dysfunction and dyslipidemia[5,7,8]. However, previous studies have shown that thyroid hormone or thyroid-stimulating hormone (TSH) levels alone may not be sufficient to explain the relationship between the thyroid system and dyslipidemia[6-8], and the calculation of comprehensive indices can systematically reflect the regulation of thyroid hormone homeostasis[9]. The TSH index (TSHI), thyrotrophic T4 resistance index (TT4RI) and thyroid feedback quantile-based index (TFQI) have been well verified for evaluating central sensitivity to thyroid hormones, and the free triiodothyronine (FT3)/free thyroxine (FT4) ratio is an index that reflects the peripheral bioavailability of thyroid hormones[9,10]. An increasing number of studies have shown that higher values of these composite indices are associated with hyperuricemia, homocysteinemia, vitamin D deficiency, obesity, metabolic syndrome, diabetes, hypertension, reduced kidney function, and diabetes-related mortality, even in euthyroid populations[9-16]. These findings have led to new directions in research regarding the relationship between thyroid function and lipid metabolism. Liu *et al*[17] reported that the risk of dyslipidemia was positively correlated with the TFQI, TSHI, and TT4RI and negatively correlated with FT3/FT4 in patients with coronary heart disease. A recent study indicated that among euthyroid adults, reduced central and peripheral sensitivity to thyroid hormones was associated with high remnant cholesterol (RC) levels[10]. To our knowledge, no study has investigated the association between thyroid hormone sensitivity and dyslipidemia in the T2DM population; in particular, there is a lack of focus on non-high-density lipoprotein cholesterol (non-HDL-C)[17-21]. Non-HDL-C, calculated as total cholesterol (TC) minus high-density lipoprotein (HDL), includes all plasma lipoproteins, such as low-density lipoprotein cholesterol (LDL-C), triglyceride (TG)-rich lipoprotein (TRL), TRL-remnants, and lipoprotein(a)[22]. As non-HDL-C is a measure of all atherogenic cholesterol, it is not surprising that it strongly correlates with ASCVD risk and is also better at predicting ASCVD risk in patients on statin therapy and/or in those with T2DM [23]. Usually, maintaining the optimum level of LDL-C is the primary goal for dyslipidemia management in the general population. However, patients with T2DM who have extremely low LDL-C levels still remain at a very high risk of ASCVD[24]. In line with international guidelines, the 2020 Chinese Guideline on the Primary Prevention of Cardiovascular Diseases recommends non-HDL-C as an alternative treatment target to LDL-C[25]. Previous studies have

revealed that TSH levels within the reference range are positively associated with increased non-HDL-C. However, the relationship between thyroid hormone sensitivity and non-HDL-C has rarely been investigated. Thus, the effects of thyroid hormone and thyroid hormone sensitivity on non-HDL-C in individuals with T2DM remain unclear.

Therefore, the purpose of this study was to clarify the association between thyroid hormone sensitivity and non-HDL-C in patients with T2DM and to further explore these associations in different sexes in an attempt to provide new evidence for the role of impaired thyroid hormone sensitivity for serum atherogenic non-HDL-C levels.

MATERIALS AND METHODS

Study design and participants

A total of 1147 patients with T2DM were recruited from the Department of Endocrinology, Xuanwu Hospital, Capital Medical University, from January 2020 to December 2021. All participants met the 1999 World Health Organization diagnosis and classification criteria for T2DM. The exclusion criteria were as follows: (1) Age ≤ 35 years; (2) Oncological, infectious, serious liver or renal disease; (3) Lack of data on TSH, FT3, FT4, TC, or HDL cholesterol (HDL-C); and (4) A history of surgery for thyroid diseases, antithyroid therapy and hormone replacement. After exclusion, 994 participants were included in the final analysis (Figure 1).

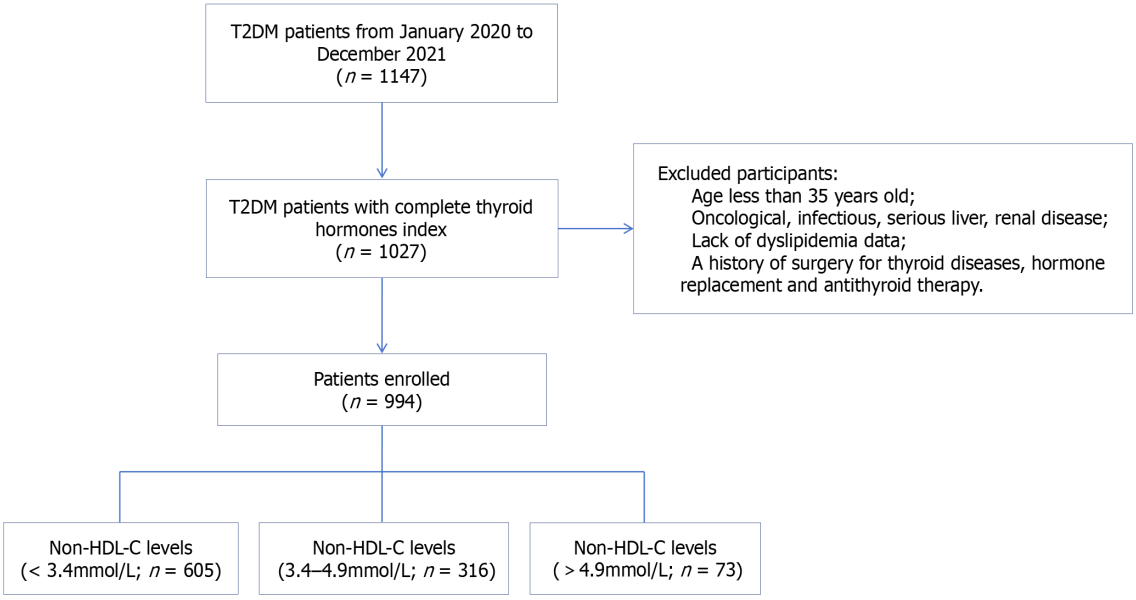

Figure 1 Flow chart of patient recruitment. Non-HDL-C: Non-high-density lipoprotein cholesterol; T2DM: Type 2 diabetes mellitus.

Clinical and biochemical measurements

Blood samples from the participants were obtained after overnight fasting and were measured in the biochemistry laboratory of Xuanwu Hospital of Capital Medical University. Biochemical parameters, including fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), TC, TG, HDL-C, low-density lipoprotein (LDL), uric acid, albumin, prealbumin, 25-hydroxyvitamin D [25(OH)D] and hemoglobin, were measured. Fasting blood insulin and fasting blood C-peptide levels were also measured.

All study subjects fasted for 10 h, and elbow venous blood was collected in the morning to determine FBG, TG, TC, LDL-C, and HDL-C levels. The HbA1c values were determined *via* liquid chromatography tandem mass spectrometry. Fasting insulin and C-peptide levels were measured *via* radioimmunoassay. The level of TSH was measured using a third-generation immunoassay. FT3 and FT4 levels were measured *via* a competitive immunoassay. The reference ranges for FT3, FT4, and TSH were 2.3-4.2 pg/mL, 0.89-1.76 ng/dL, and 0.55-4.78 mLU/L, respectively.

Hyper-non-HDL-C, hypertriglyceridemia, hypercholesterolemia, hypo-HDL cholesterolemia, and hyper-low-density lipoprotein cholesterolemia were defined as non-HDL-C ≥ 3.4 mmol/L, TG ≥ 1.7 mmol/L, TC ≥ 5.2 mmol/L, HDL-C < 1.0 mmol/L, and LDL-C ≥ 3.4 mmol/L[5]. Hypertension was defined as a systolic blood pressure (SBP) ≥ 130 mmHg, a diastolic blood pressure (DBP) ≥ 85 mmHg or specific treatment for previously diagnosed hypertension[13].

Definition of variables

Central indices of thyroid hormone sensitivity were calculated with the following formulas: TFQI = cumulative distribution function (CDF) FT4 - (1 - CDF TSH). TSHI = Ln TSH (μIU/mL) + 0.1345 × FT4 (pmol/L). TT4RI = FT4 (pmol/L) × TSH (μIU/mL). Central thyroid hormone sensitivity indicators reflect the response of the hypothalamus-pituitary-thyroid axis to changes in peripheral FT4. Negative values indicate higher central sensitivity, and positive values indicate lower

central sensitivity to changes in FT4. For TSHI, TT4RI, and TFQI, the higher the values, the lower is the central sensitivity to thyroid hormones[9].

The peripheral index of thyroid hormone sensitivity was calculated as follows: FT3/FT4 ratio = FT3 (pmol/L)/FT4 (pmol/L). Higher values indicate greater peripheral sensitivity to thyroid hormones.

The fasting RC level was calculated as TC - (HDL-C + LDL-C) (mmol/L)[10].

Non-HDL-C was calculated as TC - HDL-C (mmol/L). Homeostasis Model Assessment of insulin resistance (HOMA-IR) was calculated as the fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5.

Statistical analysis

For continuous variables, the data are presented as the means ± SDs or medians (upper and lower ranges). Data for categorical variables are expressed as numbers (%). One-way ANOVA, the Kruskal-Wallis H (K) test or the chi-square test was used for comparisons between variables where appropriate. The results of the logistic regression analysis are presented as ORs and 95%CIs. Pearson's and partial correlation coefficients were used to explore the associations of non-HDL-C with the thyroid-associated variables adjusted for sex, age, body mass index (BMI) and HbA1c. To further evaluate the potential associations of hyper-non-HDL-C with impaired thyroid hormone sensitivity, a logistic regression model was performed, and stratification was performed according to sex. IBM SPSS Statistics software, version 24.0 (IBM Corp., Armonk, NY, United States), and GraphPad 7.0 software were used for analyses of the data. Two-tailed *P* values < 0.05 were considered statistically significant. The Shapiro-Wilk test was used for the normality test. Random forest variable importance and Shapley Additive Explanations (SHAP) summary plots were used to identify the strength and direction of the association between hyper-non-HDL-C and its major predictor.

RESULTS

Baseline characteristics of the participants

A total of 994 adults included in this study were divided into three groups (non-HDL-C < 3.4 mmol/L, 3.4-4.9 mmol/L and > 4.9 mmol/L). The characteristics of the participants according to different non-HDL-C levels are shown in Table 1. The median age was 65.8 years (range 35.0-89.0), and 447 patients (44.9%) were males. Among all individuals with T2DM, 533 (54.1%) had fatty liver, 561 (56.8%) had hypertension, and 155 (27.5%) had cardiovascular disease.

Among them, 389 patients (39.13%) had high non-HDL-C levels (≥ 3.4 mmol/L). Compared with the normal non-HDL-C group (< 3.4 mmol/L), there was no significant difference in BMI, SBP, DBP or the incidence of hypertension. The levels of fasting plasma glucose (FPG), HbA1c, and HOMA-IR were significantly increased in the very high non-HDL-C level group (> 4.9 mmol/L) and decreased in the normal non-HDL-C group. These findings suggested poorer glycemic control in high non-HDL-C group. However, the levels of estimated glomerular filtration rate (eGFR) and 25(OH)D were significantly reduced in the very high non-HDL-C level group and significantly elevated in the normal non-HDL-C group ($P < 0.05$). TSH, TFQI, TSHI, TT4RI, TG, TC, LDL-C and RC levels tended to increase with increasing HDL level ($P < 0.05$), whereas the levels of FT3 and FT3/FT4 were significantly lower in the high non-HDL-C group than in the normal non-HDL-C group ($P < 0.05$; Table 1).

As shown in Figure 2, central thyroid hormone sensitivity indices, including the TFQI, TSHI, and TT4RI, were significantly elevated in patients in the high non-HDL-C group, whereas the peripheral thyroid hormone sensitivity index FT3/FT4 was significantly lower ($P < 0.05$).

Correlations between non-HDL-C levels and thyroid-associated variables

The correlations between non-HDL-C levels and thyroid-associated variables are presented in Table 2. TSH, TT4RI, TSHI, and the TFQI were positively associated with non-HDL-C levels, whereas the FT3/FT4 ratio was negatively associated with non-HDL-C levels in all participants. FT3 and FT4 were negatively correlated with the levels of non-HDL-C in men, whereas TSHI, TT4RI were positively correlated. However, non-HDL-C levels were negatively associated with the FT3/FT4 ratio and positively associated with the TFQI in women (all $P < 0.05$).

We also analyzed the correlations between lipid profiles such as TG, TC, HDL, LDL and RC and thyroid-associated variables (Table 3). Lipid profiles, especially TG, TC and RC, were significantly associated with thyroid hormone sensitivity indices before and after adjusting for age, sex, BMI, and HbA1c. LDL levels were negatively associated with FT3 and the FT3/FT4 ratio and positively associated with TSHI, whereas HDL levels were negatively associated with FT3, TSH, the FT3/TF4 ratio, the TT4RI and the TSHI.

Relationship between hyper-non-HDL-C and impaired sensitivity to thyroid hormones

To investigate the relationship between hyper-non-HDL-C and impaired sensitivity to thyroid hormones, we performed logistic regression analyses (Table 4). The risk of hyper-non-HDL-C was positively correlated with the TFQI (OR: 1.584; 95%CI: 1.088-2.304; $P = 0.016$), TSHI (OR: 1.238; 95%CI: 1.034-1.482; $P = 0.02$), and TT4RI (OR: 1.075; 95%CI: 1.006-1.149; $P = 0.032$) but was not significantly correlated with the FT3/FT4 ratio. Even after adjusting for age, sex, BMI, and HbA1c, the associations between hyper-non-HDL-C and impaired central sensitivity to thyroid hormones were significant. However, the relationships between composite indices of the thyroid system and non-HDL-C levels differed according to sex. An increased risk of hyper-non-HDL-C was associated with elevated TSHI levels in men (OR: 1.331; 95%CI: 1.003-1.766; $P = 0.048$) but elevated TFQI levels in women (OR: 2.337; 95%CI: 1.4-3.901; $P = 0.001$).

Table 1 Basic characteristics of the population

Characteristics	All (*n* = 994)	Non-HDL-C levels (mmol/L)			*P* value
		< 3.4 (*n* = 605)	3.4-4.9 (*n* = 316)	> 4.9 (*n* = 73)	
Age (years)	65.8 (35.0-89.0)	66.0 (36.0-89.0)	64.0 (35.0-86.0)	64.0 (36.0-83.6)	0.010
Sex (male/female)	447/547	278/327	140/176	29/44	0.576
Disease duration of T2DM (years)	13.0 (0.0-40.0)	13.0 (0.0-35.0)	13.0 (0.0-40.0)	11.0 (0.02-30.0)	0.336
BMI (kg/m^2)	25.6 (14.38-47.97)	25.6 (14.38-45.45)	25.6 (16.0-47.9)	25.6 (19.5-41.1)	0.743
SBP (mmHg)	130 (90-210)	130 (90-190)	130 (90-210)	130 (110-180)	0.294
DBP (mmHg)	80 (50-120)	80 (50-120)	80 (50-110)	80 (60-100)	0.162
FPG (mmol/L)	8.22 (2.88-24.52)	7.79 (3.12-24.17)	8.84 (2.88-23.49)	9.97 (3.25-24.52)	< 0.001
Fasting C peptide (ng/mL)	2.30 (0.01-16.34)	2.21 (0.01-13.35)	2.37 (0.17-16.34)	2.78 (0.27-6.93)	0.008
HOMA-IR	4.47 (0.04-279.63)	4.22 (0.05-243.20)	4.74 (0.47-279.63)	5.43 (0.04-34.27)	0.047
HbA1c (%)	8.1 (4.9-15.1)	7.8 (4.9-14.8)	8.7 (5.4-15.1)	9.2 (6.0-14.7)	< 0.001
Creatinine (μmol/L)	62 (28-266)	62 (30-241)	60 (28-230)	65 (30-266)	0.016
eGFR [mL/min (1.73 m^2)$^{-1}$]	101.1 (19.1-323.7)	100.7 (21.1-291.4)	103.5 (19.1-323.7)	95.0 (22.4-284.9)	0.009
UA (mmol/L)	324 (7-811)	324 (7-811)	321 (98-797)	338 (209-612)	0.126
UACR (mg/g)	4.0 (0.0-2404.2)	4.1 (0.0-1637.8)	2.6 (0.0-1796.2)	12.8 (0.1-2402.2)	0.013
25(OH)D (ng/mL)	16.80 (3.00-57.55)	17.52 (3.00-57.55)	16.57 (3.00-50.21)	14.73 (3.31-35.78)	0.039
Fatty liver, *n* (%)	533 (54.1)	304 (50.6)	180 (57.7)	49 (67.1)	0.002
Hypertension, *n* (%)	561 (56.8)	344 (57.2)	172 (54.8)	45 (61.6)	0.530
Cardiovascular disease, *n* (%)	155 (27.5)	111 (33.5)	34 (82.9)	10 (24.4)	0.002
Diabetic retinopathy, *n* (%)	238 (24.1)	142 (23.6)	68 (21.8)	28 (38.4)	0.011
Diabetic peripheral neuropathy, *n* (%)	411 (41.7)	248 (41.2)	123 (49.5)	40 (54.8)	0.055
Diabetic peripheral vascular disease, *n* (%)	157 (38.8)	89 (38.5)	55 (38.5)	13 (41.9)	0.931
Serum lipid level (mmol/L)					
TG	1.35 (0.24-21.80)	1.37 (0.24-8.59)	1.69 (0.39-9.99)	2.51 (0.63-21.80)	< 0.001
TC	4.25 (2.03-10.35)	3.74 (2.03-5.74)	5.07 (4.13-7.20)	6.53 (5.58-10.35)	< 0.001
LDL-C	2.51 (0.15-6.88)	2.08 (0.15-3.80)	3.28 (1.12-4.66)	4.32 (1.37-6.88)	< 0.001
HDL-C	1.10 (0.33-3.42)	1.10 (0.33-3.42)	1.10 (0.47-2.66)	1.09 (0.58-1.90)	0.896
non-HDL-C	3.10 (1.02-9.30)	2.56 (1.02-3.39)	3.95 (3.40-4.89)	5.3 (4.90-9.30)	< 0.001
RC	0.53 (-1.31-7.93)	0.45 (-1.31-2.40)	0.67 (-0.34-3.66)	1.13 (-0.33-7.93)	< 0.001
Thyroid function and indices of thyroid hormone sensitivity					
FT3 (pg/mL)	2.93 (0.91-4.49)	2.93 (0.91-4.19)	2.97 (1.01-4.49)	2.80 (1.15-3.97)	0.006
FT4 (ng/dL)	1.19 (0.72-1.59)	1.18 (0.72-1.92)	1.18 (0.75-1.96)	1.21 (0.76-1.59)	0.905
TSH (uIU/mL)	1.74 (0.01-9.89)	1.65 (0.07-9.89)	1.86 (0.07-9.48)	2.03 (0.16-9.12)	0.017
FT3/FT4	2.47 ± 0.47	2.47 ± 0.47	2.48 ± 0.45	2.34 ± 0.52	0.040
TT4RI	2.10 (0.01-12.23)	1.99 (0.10-9.89)	2.27 (0.1-12.23)	2.56 (0.20-8.43)	0.008
TSHI	0.72 (-4.43-2.42)	0.66 (-2.48-2.42)	0.79 (-4.43-2.42)	0.91 (-1.66-2.63)	0.010
TFQI	0.00 ± 0.35	-0.02 ± 0.36	0.03 ± 0.34	0.08 ± 0.34	0.026

The data are expressed as the means ± SDs, medians (upper and lower quartiles) or *n* (%). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; UA: uric acid; UACR: urinary albumin-creatinine ratio; FPG: fasting plasma glucose;

HbA1c: glycosylated hemoglobin; TG: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; non-HDL-C: Non-high-density lipoprotein cholesterol; RC: remnant cholesterol; T2DM: Type 2 diabetes mellitus; HOMA-IR: Homeostasis Model Assessment of insulin resistance; 25(OH)D: 25-hydroxyvitamin D; FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

Table 2 Correlations between non-high-density lipoprotein cholesterol levels and thyroid parameters in patients with type 2 diabetes mellitus

Variables		All		Male		Female	
		r	*P* value	r	*P* value	r	*P* value
FT3 (pg/mL)	Model 1	-0.090[a]	0.005	-0.153[a]	0.001	-0.062	0.147
	Model 2	-0.128[a]	< 0.001	-0.187[a]	< 0.001	-0.095	0.027
	Model 3	-0.113[a]	< 0.001	-0.168[a]	< 0.001	-0.082	0.059
FT4 (ng/dL)	Model 1	-0.008	0.795	-0.115[b]	0.015	0.014	0.737
	Model 2	-0.013	0.693	-0.118[b]	0.013	0.012	0.78
	Model 3	-0.024	0.451	-0.14[a]	0.003	0	0.991
TSH (uIU/mL)	Model 1	0.073[b]	0.022	0.141[a]	0.003	0.007	0.867
	Model 2	0.079[b]	0.014	0.143[a]	0.002	0.016	0.714
	Model 3	0.093[a]	0.004	0.154[a]	0.001	0.024	0.575
FT3/FT4	Model 1	-0.051	0.107	-0.019	0.694	-0.075	0.078
	Model 2	-0.076[b]	0.017	-0.037	0.441	-0.101[b]	0.019
	Model 3	-0.033	0.301	-0.003	0.942	-0.052	0.228
TT4RI	Model 1	0.076[b]	0.018	0.121[b]	0.01	0.037	0.394
	Model 2	0.079[b]	0.013	0.121[b]	0.011	0.044	0.308
	Model 3	0.088[a]	0.006	0.129[a]	0.007	0.046	0.286
TSHI	Model 1	0.085[a]	0.008	0.129[a]	0.006	0.052	0.224
	Model 2	0.087[a]	0.006	0.129[a]	0.007	0.058	0.175
	Model 3	0.103[a]	0.001	0.14[a]	0.003	0.069	0.111
TFQI	Model 1	0.078[b]	0.014	0.029	0.539	0.121[a]	0.004
	Model 2	0.074[b]	0.02	0.024	0.612	0.121[a]	0.005
	Model 3	0.065[b]	0.044	0.014	0.777	0.109[b]	0.011

[a]$P < 0.01$.
[b]$P < 0.05$.
Model 1 was an unadjusted analysis. Model 2 was adjusted for age and sex. Model 3 was adjusted for age, sex, body mass index, and glycosylated hemoglobin. FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

SHAP was employed to assess the importance and contribution of thyroid-related variables within the optimal random forest models for hyper-non-HDL-C, with a prioritized list vividly illustrating their respective impacts (Figure 3). Among the analyzed features, the average SHAP values were highest for TSHI, followed by TT4RI (Figure 3A). The distribution of the SHAP scores was also analyzed for each feature (Figure 3B). As shown in the SHAP summary plot, the red dots indicate high feature values; however, the blue dots represent low feature values. SHAP values above zero suggested higher non-HDL-C values, whereas values below zero indicated lower non-HDL-C values. For example, a higher TSHI, TT4RI and TFQI (depicted in red) correlated with higher SHAP values, suggesting that they were all risk factors for hyper-non-HDL-C. Regarding the TSHI in the different sex models (Figure 4), an increase in the TSHI was strongly associated with an increase in its contribution to the model predictions. In terms of contributors in the sex models, the TT4RI ranked second in the male model and third highest in the female model. Compared with these factors, the other features had smaller contributions. The rankings of these other features also varied between different sex models.

Table 3 Relationships between lipid profiles and thyroid-associated variables

Variables		TG		TC		LDL-C		HDL-C		RC	
		r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
FT3 (pg/mL)	Model 1	-0.092[a]	0.004	-0.116[a]	< 0.001	-0.086[a]	0.007	-0.093[a]	0.004	-0.037	0.246
	Model 2	-0.114[a]	< 0.001	-0.149[a]	< 0.001	-0.106[a]	0.001	-0.083[a]	0.009	-0.081[b]	0.011
	Model 3	-0.099[a]	0.002	-0.137[a]	< 0.001	-0.094[a]	0.003	-0.097[a]	0.003	-0.068[b]	0.035
FT4 (ng/dL)	Model 1	-0.007	0.838	0.001	0.971	0.008	0.8	0.03	0.352	-0.032	0.312
	Model 2	-0.01	0.764	-0.003	0.928	0.006	0.84	0.03	0.349	-0.039	0.224
	Model 3	-0.02	0.535	-0.012	0.717	-0.002	0.948	0.037	0.249	-0.048	0.139
TSH (uIU/mL)	Model 1	0.074[b]	0.021	0.045	0.157	0.04	0.206	-0.080[b]	0.013	0.083[a]	0.01
	Model 2	0.077[b]	0.016	0.05	0.12	0.043	0.177	-0.082[b]	0.01	0.09[a]	0.005
	Model 3	0.082[b]	0.01	0.063	0.05	0.056	0.084	-0.08[b]	0.012	0.095[a]	0.003
FT3/FT4	Model 1	-0.082[b]	0.01	-0.093[a]	0.003	-0.065[b]	0.041	-0.141[a]	< 0.001	0.008	0.802
	Model 2	-0.095[a]	0.003	-0.114[a]	< 0.001	-0.078[b]	0.015	-0.134[a]	< 0.001	-0.021	0.519
	Model 3	-0.061	0.06	-0.082[b]	0.011	-0.045	0.167	-0.163[a]	< 0.001	0.01	0.749
TT4RI	Model 1	0.079[b]	0.013	0.052	0.102	0.046	0.147	-0.066[b]	0.039	0.078[b]	0.014
	Model 2	0.081[b]	0.011	0.055	0.087	0.048	0.133	-0.068[b]	0.034	0.082[b]	0.01
	Model 3	0.081[b]	0.012	0.065[b]	0.043	0.057	0.075	-0.061	0.057	0.083[b]	0.01
TSHI	Model 1	0.099[a]	0.002	0.061	0.056	0.048	0.135	-0.066[b]	0.039	0.095[a]	0.003
	Model 2	0.1[a]	0.002	0.063[b]	0.049	0.049	0.126	-0.067[b]	0.036	0.098[a]	0.002
	Model 3	0.103[a]	0.001	0.079[b]	0.014	0.064[b]	0.047	-0.059	0.065	0.102[a]	0.002
TFQI	Model1	0.087[a]	0.006	0.074[b]	0.02	0.059	0.066	-0.003	0.922	0.061	0.054
	Model2	0.085[a]	0.008	0.071[b]	0.026	0.056	0.078	< 0.001	0.989	0.057	0.076
	Model3	0.068[b]	0.035	0.068[b]	0.035	0.052	0.106	0.02	0.529	0.043	0.181

[a]$P < 0.01$

[b]$P < 0.05$.

Model 1 was an unadjusted analysis. Model 2 was adjusted for age and sex. Model 3 was adjusted for age, sex, body mass index, and glycosylated hemoglobin. FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

DISCUSSION

The present study revealed that impaired sensitivity to thyroid hormones was significantly associated with non-HDL-C levels in the T2DM population. Our results indicated that reduced central thyroid hormone sensitivity (increased TSHI, TT4RI, and TFQI) was an independent risk factor for hyper-non-HDL-C, even after adjusting for multiple confounding factors. Moreover, the associations between thyroid hormone sensitivity indices and non-HDL-C levels differed between men and women, suggesting that sex-associated regulation of thyroid hormones impacted serum non-HDL-C levels.

Our study revealed that the levels of TSH, TFQI, TSHI, and TT4RI were significantly greater in individuals with hyper-non-HDL-C than in those with normal non-HDL-C, indicating the presence of central thyroid hormone resistance in participants with high non-HDL-C levels. Moreover, the FT3/FT4 ratio decreased, indicating impaired thyroid hormone sensitivity in peripheral organs. Furthermore, central thyroid hormone sensitivity indices (TFQI, TSHI, and TT4RI) were found to be independent risk factors for hyper-non-HDL-C in patients with T2DM. Moreover, we identified the strength and direction of the association between high non-HDL-C and its major predictor derived from random forest variable importance using SHAP and random forest model analyses. To our knowledge, no study has investigated the association between thyroid hormone sensitivity and non-HDL-C[4,5,17-19]. Non-HDL-C is a better marker of atherogenicity and represents the residual ASCVD risk in patients who have achieved target LDL-C goals[21-23]. Since non-HDL-C is known as 'bad cholesterol', it contains all atherogenic lipoproteins, which accumulate in the intima of the arteries, leading to the formation of atherosclerotic plaques[20]. According to international guidelines, the 2023 Chinese Guideline on the Primary Prevention of cardiovascular disease (CVD) recommends non-HDL-C as an alternative treatment target for LDL-C; however, clinicians often do not pay sufficient attention to this point[23]. Asvold *et al*[24] conducted a large sample cohort study with a follow-up of 11 years and reported that changes in TSH levels were associated with concomitant

Table 4 Logistic regression analysis of the relationship between non-high-density lipoprotein cholesterol and impaired sensitivity to thyroid hormones in patients with type 2 diabetes mellitus

Variables	All		Male		Female	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
FT3 (pg/mL)	0.91 (0.71-1.166)	0.456	0.698 (0.41-1.191)	0.187	1.002 (0.769-1.306)	0.987
FT4 (ng/dL)	0.919 (0.689-1.225)	0.564	0.194 (0.061-0.617)	0.005	1.012 (0.775-1.32)	0.932
TSH (uIU/mL)	1.091 (1.015-1.172)	0.018	1.136 (1.02-1.264)	0.02	1.037 (0.935-1.15)	0.49
FT3/FT4	1.01 (0.754-1.352)	0.949	1.369 (0.861-2.177)	0.184	0.846 (0.577-1.24)	0.392
TT4RI	1.075 (1.006-1.149)	0.032	1.086 (0.985-1.197)	0.097	1.055 (0.963-1.156)	0.252
TSHI	1.238 (1.034-1.482)	0.02	1.331 (1.003-1.766)	0.048	1.155 (0.918-1.452)	0.218
TFQI	1.584 (1.088-2.304)	0.016	0.961 (0.545-1.692)	0.89	2.337 (1.4-3.901)	0.001

Data were analyzed *via* logistic regression analysis adjusted for age, sex, body mass index and glycosylated hemoglobin. FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

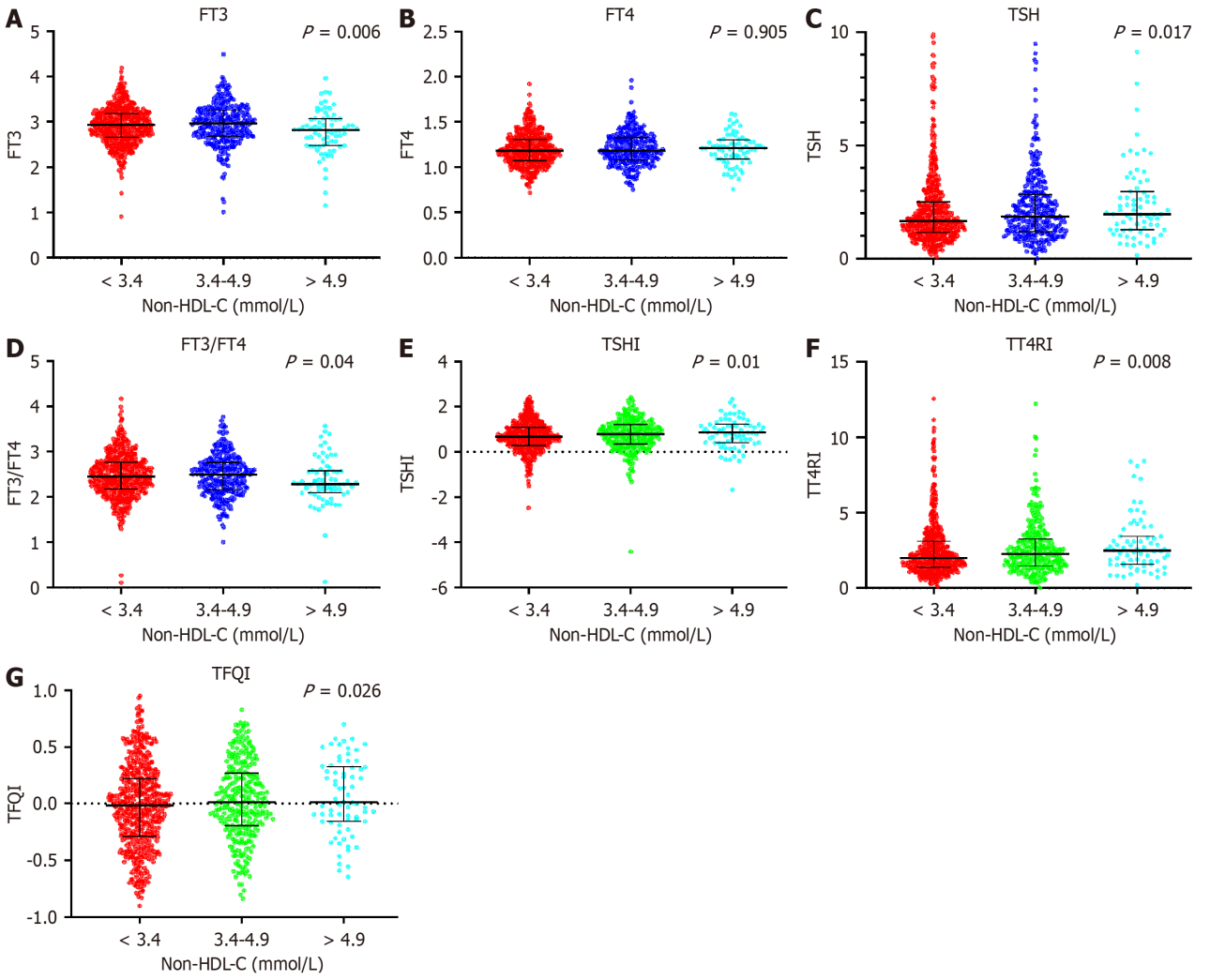

Figure 2 Comparison of thyroid-associated variables in different non-high-density lipoprotein cholesterol level groups. Data were analyzed by Student *t*-test or Mann-Whitney *U* test. A: Free triiodothyronine (FT3); B: Free thyrotropin (FT4); C: Thyroid-stimulating hormone (TSH); D: FT3/FT4; E: TSH index; F: Thyrotrophic T4 resistance index; G: Thyroid feedback quantile-based index. Non-HDL-C: Non-high-density lipoprotein cholesterol; FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

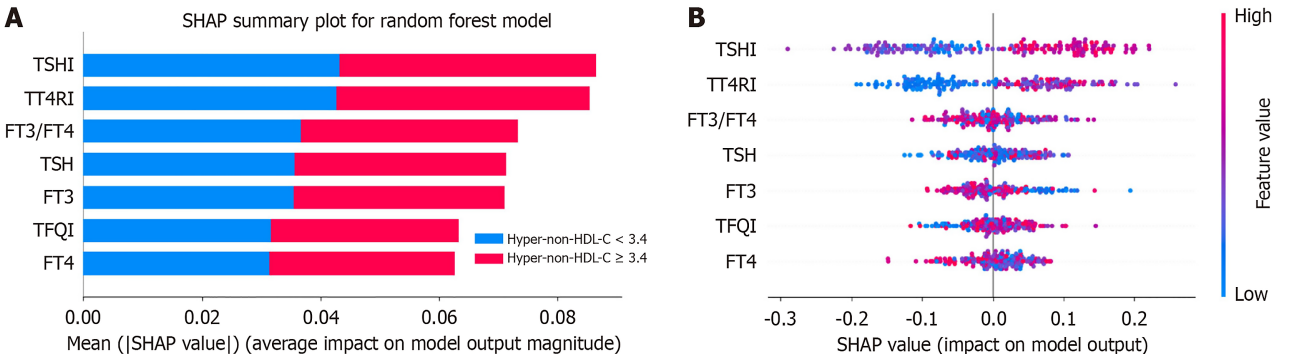

Figure 3 Feature importance of Shapley Additive Explanations values for the random forest model in detecting hyper-non-high-density lipoprotein cholesterol. A: Variables with the most significant impact on the prediction of hyper-non-high-density lipoprotein cholesterol (hyper-non-HDL-C) ranked in order of importance; B: Distribution of the influence of each variable on the prediction of hyper-non-HDL-C. The numerical characteristics of the variables are visually represented by colors, with larger values shown in red and smaller values shown in blue. Negative Shapley Additive Explanations values (spread to the left) suggest a decrease in the probability of hyper-non-HDL-C, whereas positive values (spread to the right) suggest an increase in probability. SHAP: Shapley Additive Explanations; FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index; Hyper-non-HDL-C: Non-high-density lipoprotein cholesterol.

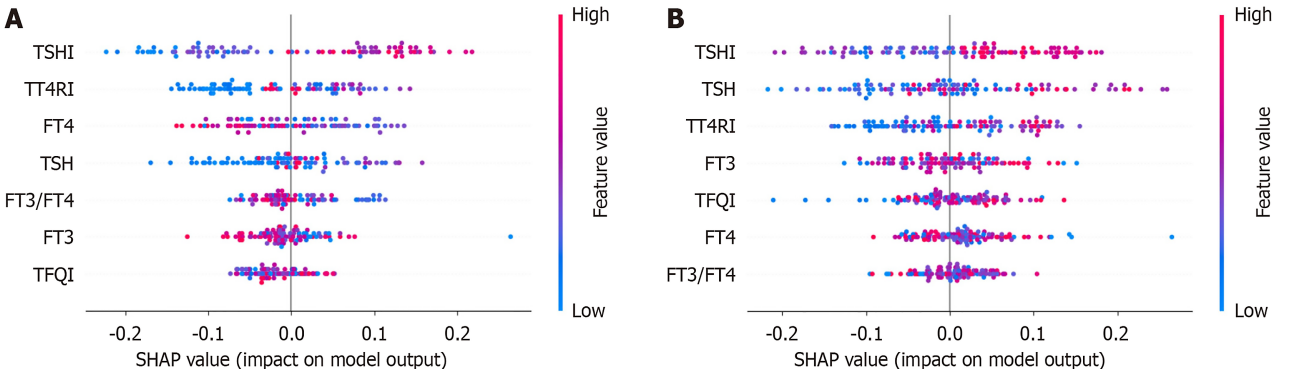

Figure 4 Feature importance of Shapley Additive Explanations values for the random forest model in detecting hyper-non-high-density lipoprotein cholesterol for men and women. A: The influence of each variable on the prediction of hyper-non-high-density lipoprotein cholesterol (hyper-non-HDL-C) in men; B: The influence of each variable on the prediction of hyper-non-HDL-C in women. The numerical characteristics of the variables are visually represented by colors, with larger values shown in red and smaller values shown in blue. Negative Shapley Additive Explanations values (spread to the left) suggest a decrease in the probability of hyper-non-HDL-C, whereas positive values (spread to the right) suggest an increase in the probability. SHAP: Shapley Additive Explanations; FT3: Free triiodothyronine; FT4: Free thyrotropin; TSH: Thyroid-stimulating hormone; TSHI: Thyroid-stimulating hormone index; TT4RI: Thyrotrophic T4 resistance index; TFQI: Thyroid feedback quantile-based index.

changes in non-HDL-C and TG levels (all $P < 0.001$) irrespective of sex. Several studies have implied a close association between higher normal-range TSH and concentrations of total serum non-HDL-C parameters[19,26]. Thyroid hormones within the reference range combined with elevated TSH have been shown to be associated with pronounced lipid disorders and consequently an increased risk of atherosclerotic vascular disease[7,8]. Physiologically, thyroid hormones and TSH are inversely correlated owing to a negative feedback loop. However, high thyroid hormones can coexist with high TSH in individuals with resistance to thyroid hormones[9]. Reduced sensitivity to thyroid hormone in the general population has been shown to be a vital risk factor for various metabolic diseases, such as diabetes and hypertension[5,13,15]. These discoveries have also led to new directions in research regarding the relationship between thyroid function and lipid metabolism. Our findings suggest that the composite thyroid hormone sensitivity indices are better indices than the absolute values of FT3, FT4, and TSH, which could provide more information on thyroid function and directly relate thyroid hormone resistance to lipid metabolism.

Our study demonstrated that lipid profiles, especially TG, TC and RC, were also significantly associated with thyroid hormone sensitivity indices. However, HDL-C levels were negatively associated with peripheral thyroid hormone resistance. In line with previous studies, Liu *et al*[17] analyzed the associations between thyroid system indices and lipid profiles (TC, TG, HDL-C, LDL-C) and reported that the risk of dyslipidemia was positively correlated with TFQI, TSHI, and TT4RI and negatively correlated with FT3/FT4 in patients with coronary heart disease. A recent large sample study indicated that among euthyroid adults, reduced sensitivity to thyroid hormones was associated with high RC levels[10]. Therefore, sensitivity to thyroid hormones is associated with dyslipidemia, indicating that periodic screening of thyroid hormones in the T2DM population is recommended to aid early prevention of dyslipidemia.

In addition, our findings also revealed that elevated TFQI levels were associated with an increased risk of hyper-non-HDL-C in women. The TFQI is a new index for detecting acquired thyroid hormone resistance that was first proposed in 2019 by Laclaustra *et al*[9]. The performance of the TFQI was shown to be more stable than that of the TSHI and TT4RI[9]. Lipid abnormalities are especially relevant in women because they escalate rapidly with biological aging and endocrine changes related to menopause[26]. The menopausal transition and loss of estrogen possibly explain this association between TFQI and non-HDL-C in females, which might act as a trigger factor and impede metabolic function[27].

In this study, the patients with high non-HDL-C levels had not only higher TG, TC, LDL-C and RC levels but also significantly higher levels of FPG, HbA1c, and HOMA-IR, suggesting a poorer balance of glucose-lipid metabolism in individuals with high non-HDL-C. Similar findings have also been reported by Vazirian *et al*[28], indicating that elevated non-HDL-C serves as a significant predictor of glucose-lipid metabolism. In addition, patients with high non-HDL-C levels had worse renal function (lower eGFRs and higher creatinine levels compared with those in the normal group, $P < 0.05$). Among the previous studies that investigated the association of non-HDL-C and renal function, conclusions have been inconsistent[29,30]. Saland *et al*[30] reported that longitudinal increases in proteinuria and decreases in eGFR were independently associated with significant concomitant increases in non-HDL-C in children with chronic kidney disease. In another study, the prevalence of hyper-non-HDL-C was not related to chronic kidney disease stage[29]. Moreover, our data revealed that the 25(OH)D level was significantly reduced in the high non-HDL-C group compared with the normal non-HDL-C group. Most previous studies have shown no associations between vitamin D deficiency and elevated non-HDL-C[31,32]. Nwosu *et al*[33] reported significant inverse correlations between 25(OH)D and non-HDL-C cholesterol. In brief, patients with high non-HDL-C levels could face multiple endocrine and metabolic disorders, which is worth exploring in larger sample studies.

The novelty of this study lies in providing another layer of evidence for resistance to thyroid hormones as an independent risk factor for hyper-non-HDL in the T2DM population, which would be highly important for the T2DM population with an increased risk of ASCVD. The limitations of this study should be acknowledged. First, this was a cross-sectional study that utilized blood sample data from only a single point, which means that the direct causal relationship between non-HDL-C levels and thyroid hormone sensitivity cannot be inferred. However, this study supports the important hypothesis that thyroid hormone sensitivity may be useful for assessing the risk of dyslipidemia. In the future, more studies are needed to demonstrate causality. Second, information on the medication history of the participants was lacking, which might affect the data for thyroid hormone and non-HDL levels. Third, because our analysis was restricted to patients with T2DM selected only from Xuanwu Hospital, it is uncertain whether our findings can be generalized to other populations.

CONCLUSION

In conclusion, this is the first study to demonstrate an association of high non-HDL-C levels with reduced sensitivity to thyroid hormone in the patients with T2DM, providing new evidence for the role of reduced thyroid hormone sensitivity for non-HDL-C levels. Future investigations are needed to explore the underlying mechanism of this phenomenon and to lay the groundwork for future therapeutic strategies for diabetes mellitus-related CVD risk.

FOOTNOTES

Author contributions: Duan XY analyzed the data and wrote the manuscript; Fu JL, Sun LN and Mu ZJ contributed to the data collection; Xiu SL contributed to the data interpretation and reviewed the manuscript; all the authors read and approved the submitted version of the manuscript.

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Abstract

BACKGROUND

Diabetic cardiomyopathy (DCM) is a multifaceted cardiovascular disorder in which immune dysregulation plays a pivotal role. The immunological molecular mechanisms underlying DCM are poorly understood.

AIM

To examine the immunological molecular mechanisms of DCM and construct diagnostic and prognostic models of DCM based on immune feature genes (IFGs).

METHODS

Weighted gene co-expression network analysis along with machine learning methods were employed to pinpoint IFGs within bulk RNA sequencing (RNA-seq) datasets. Single-sample gene set enrichment analysis (ssGSEA) facilitated the analysis of immune cell infiltration. Diagnostic and prognostic models for these IFGs were developed and assessed in a validation cohort. Gene expression in the DCM cell model was confirmed through real time-quantitative polymerase chain reaction and western blotting techniques. Additionally, single-cell RNA-seq data provided deeper insights into cellular profiles and interactions.

RESULTS

The overlap between 69 differentially expressed genes in the DCM-associated module and 2483 immune genes yielded 7 differentially expressed immune-related genes. Four IFGs showed good diagnostic and prognostic values in the validation cohort: Proenkephalin (Penk) and retinol binding protein 7 (Rbp7), which were highly expressed, and glucagon receptor and inhibin subunit alpha,

which were expressed at low levels in DCM patients (all area under the curves > 0.9). SsGSEA revealed that IFG-related immune cell infiltration primarily involved type 2 T helper cells. High expression of Penk ($P < 0.0001$) and Rbp7 ($P = 0.001$) was detected in cardiomyocytes and interstitial cells and further confirmed in a DCM cell model *in vitro*. Intercellular events and communication analysis revealed abnormal cellular phenotype transformation and signaling communication in DCM, especially between mesenchymal cells and macrophages.

CONCLUSION

The present study identified Penk and Rbp7 as potential DCM biomarkers, and aberrant mesenchymal-immune cell phenotype communication may be an important aspect of DCM pathogenesis.

Key Words: Diabetic cardiomyopathy; Immune feature genes; Proenkephalin; Retinol binding protein 7; Immune cell infiltration; Intercellular communication

Core Tip: In this study, we utilized bulk RNA sequencing (RNA-seq) data and machine learning techniques to identify and validate four immune feature genes associated with diabetic cardiomyopathy (DCM). Notably, retinol binding protein 7 and proenkephalin showed significantly elevated expression in cardiomyocytes and interstitial cells in DCM, as confirmed by single-cell RNA-seq and molecular experiments, highlighting their robust diagnostic potential. Furthermore, single-cell RNA-seq data revealed abnormal cellular phenotype transformations and communications in DCM, particularly between fibroblasts and macrophages.

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INTRODUCTION

Cardiovascular disease continues to be the leading cause of mortality globally, with diabetes mellitus representing a significant risk factor[1,2]. Diabetic cardiomyopathy (DCM), a severe chronic complication of diabetes that profoundly affects the cardiac structure and function, culminates in heart failure without the presence of common causes like coronary artery disease, hypertension, and valvular heart disease[3]. DCM manifests as alterations in heart size, shape, and function, alongside an increase in fibrous tissue (cardiac fibrosis)[4,5]. Jia *et al*[6] reported the occurrence of heart failure in individuals with diabetes ranges between 19% and 26% and found that nearly one-third of asymptomatic patients had evidence of subclinical DCM.

The development and progression of DCM involve complex and multifactorial mechanisms, including hyperglycemia, insulin resistance, inflammation, immune activation, oxidative stress, and mitochondrial dysfunction[6-9]. The heart consists of cardiomyocytes as well as various non-muscle cells, including fibroblasts, blood vessel cells, neurons, and immune cells[10]. A comprehensive understanding of the intricate adaptive processes triggered by cardiac injury remains a considerable challenge. However, inflammation and immune cell signaling are key components of this process. Recent research has highlighted the significant role of inflammation and immune cell signaling in the pathology of DCM. Accumulating evidence suggests the involvement of cardiac myocytes, immune cells, and endothelial cells in the development of cardiac fibrosis[11-13]. Cardiomyocyte death triggers immune pathways that, initiate inflammatory responses[14]. Perturbations in the innate and adaptive immune systems play roles in the occurrence and progression of DCM[13,15], which is evidenced by imbalances in immune cell populations, enhanced generation of pro-inflammatory molecules, and elevated oxidative stress[10,16,17].

Many questions are unresolved in the molecular study of DCM. Despite advances in our understanding of immune dysregulation in DCM, many molecular and cellular characteristics of this process are not clear. Investigating these aspects is crucial for elucidating the immunopathology of DCM and identifying potential therapeutic targets[13]. According to a review by Lorenzo-Almorós *et al*[18], the prevalence of DCM varies greatly, influenced by the specific population studied and the diagnostic criteria applied. Therefore, the present study investigated immune dysregulation in DCM and identified key molecular characteristics associated with immune-mediated pathology.

Machine learning algorithms have been used to analyze large-scale omics datasets, such as gene expression profiles, proteomics data, and metabolic profiles, to identify molecular signatures associated with DCM. For example, Hathaway *et al*[19] used machine learning techniques to identify key genes and pathways involved in the pathogenesis of DCM, which provided insights into potential therapeutic targets. Single-cell sequencing has facilitated the identification and characterization of various heart cell types, such as cardiomyocytes, fibroblasts, immune cells, and endothelial cells. This technology allows for the examination of gene expression profiles at the single-cell level, helping researchers uncover cell-specific transcriptional changes and dysregulated pathways in DCM. We used the above multidimensional approach to

analyze immune gene expression, profile immune cell populations, and assess intercellular communication patterns in DCM to advance our understanding of the intricate molecular and immunological mechanisms that drive the progression of DCM.

MATERIALS AND METHODS

Data sources and processing

Independent bulk RNA sequencing (RNA-seq) (GSE5606, GSE6880, GSE4745, and GSE197999) and single-cell RNA-seq (scRNA-seq) datasets (GSE213337) were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE5606 and GSE68803 were combined into a comprehensive dataset containing 20 samples as discovery cohorts, and GSE4745 and GSE197999 were merged as validation cohorts. Gene expression was adjusted for each sample using the "ComBat" method in the R (version 4.2.1) package "sva" to eliminate differences due to batch effects. For the scRNA-seq dataset, GSE213337 contained DCM and a healthy control sample, and low-quality cells were filtered out (cells with gene counts between 200 and 2500 were retained). Two samples were then combined into one integrated dataset and subjected to batch effect removal and dimensionality reduction using the "Harmony" algorithm in the “Seurat” package (version 4.3.0) for subsequent analysis. ProteomicsDB (https://www.proteomicsdb.org/) contains thousands of samples from a variety of biological sources, such as tissues, body fluids, or cell lines, and it was used to explore tissue and cellular expression patterns of target genes.

Weighted gene co-expression network analysis and identification of disease-related modules

Weighted gene co-expression network analysis (WGCNA) was performed using the "WGCNA" package (version 1.71). Initially, the scale-free network properties were evaluated to determine a soft threshold. The correlation matrix was then converted into an adjacency matrix using this power value, and this matrix provided the foundation for building a co-expression network. A topological overlap matrix (TOM) was created with a minModuleSize value of 30 and a mergeCutHeight value of 0.2 to quantify similarity. Dendrograms with hierarchical clustering were created to display genes as determined by TOM (1-TOM). Module eigengenes (MEs) were calculated as the first principal component to summarize the gene expression profile within each module. Correlations between MEs and clinical features revealed modules associated with the disease of interest. Strong relationships between the modules and the genes of interest were shown by the correlation between gene significance (GS) and module membership (MM). Subsequently, normalization of the gene expression microarray data and differentially expressed genes (DEGs) were screened through the "LIMMA" package. DEGs were defined as $P < 0.05$ and $|\text{LogFC}| > 1$. The "ggVolcano" package was utilized to create volcano graphs visualizing the DEGs.

Identification of immune feature genes using machine learning algorithms

A list containing 2483 immune-related genes (IRGs) was acquired from the ImmPort database (https://www.immport.org/). Intersections were taken between the DEGs in the disease-related modules identified by WGCNA and the list of IRGs to identify differential IRGs (DIRGs). Least absolute shrinkage and selection operator (LASSO) and support vector machine recursive feature elimination (SVM-RFE), were used to select the most disease-related identification of immune feature genes (IFGs) and determine their intersections. LASSO and SVM-RFE effectively reduce the number of features and select the most relevant ones for predictive modeling or data analysis. Feature genes with non-zero coefficients were selected by using lambda.min to identify optimal λ values, and the LASSO regression model was constructed using the R package "glmnet". The R package "caret", which includes the SVM-RFE algorithm, is a powerful tool for feature selection. The "rfe" function in this package allows us to construct RFE models based on the "svmRadial" method to select feature genes.

Construction of the gene expression regulatory network

The NetworkAnalyst program (https://www.networkanalyst.ca/) was utilized to generate transcription factor-gene and miRNA-gene regulatory networks for IFGs. The transcription factor network was constructed with JASPAR as the retrieval target dataset, and the miRNA regulatory network was constructed using miRTarBase 8.0. The nodes and edges contributed by both networks were unionized into a single network, which was modified by Cytoscape software (version 3.10.1).

Nomogram model construction, receiver operating characteristic analysis, and decision curve analysis

Using the "rms" R package, a prognostic nomogram was created to assess the expected risk of disease based on the feature gene expression levels. Harrell’s concordance index (C-index) was performed to assess the performance of the nomogram. The calibration curve compares the actual probability of occurrence with the predicted probability, which was constructed by repeating bootstrap self-sampling 100 times (B = 100) in the R package "PredictABEL" to obtain reliable estimates of calibration performance for validating the nomogram model. The R package "pROC" was applied to assess the diagnostic value of IFGs in DCM. This approach facilitated the determination of the optimal gene expression cut-off value, the generation of receiver operating characteristic (ROC) curves, and the calculation of the area under the curve (AUC). A multivariable logistic regression model was employed to construct a combined diagnostic ROC curve. Decision curve analysis (DCA) attempts to construct models that predict maximum net benefits. Both simple models with single feature genes as predictors and complex models with multiple genes were constructed using the "decision_curve"

function in the "rmda" package.

Single-sample gene set enrichment analysis

Single-sample gene set enrichment analysis (ssGSEA) assesses immune cell infiltration by comparing a gene expression to a reference immune cell gene set and was performed using the "GSVA" package (version 1.46.0), which outputs the ssGSEA score for each sample[20]. The correlation matrix was computed using the function rcorr in the package "Hmisc" for the feature genes and immune cell populations.

Human ventricular cardiomyocyte (AC16 cell) culture and high glucose exposure

AC16 cells were purchased from Procell (Procell, China) and cultured under standard conditions in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Procell, China) at 37 °C in an atmosphere of 5% CO_2 and 95% air. Cell passages ranging between 2 and 20 were utilized in the study. Before experimentation, the cells were cultured in DMEM with 5.5 mmol/L glucose for a minimum of four passages. The cells were exposed to high glucose (HG) conditions *via* treatment with 33 mmol/L D-glucose and 0.3 mmol/L palmitate (Sigma-Aldrich, New Zealand) for 24 to 48 hours to simulate an *in vitro* diabetic environment. Cells were plated at 3×10^5 cells per 60-mm dish for RNA and protein extraction. Samples were harvested 24 and 48 hours following HG conditions.

Real time-quantitative polymerase chain reaction

As directed by the manufacturer, total RNA was isolated using the TRNzol Universal Kit (DP424, TIANGEN, Beijing, China). The HiScript III All-in-One RT SuperMix Reagent (R333-01, Vazyme, Nanjing, China) was used to reverse-transcribe one microgram of RNA template following the manufacturer's instructions. Using certain primers and the ChamQ Universal SYBR quantitative polymerase chain reaction Master Mix (Q711-02, Vazyme, Nanjing, China), real-time polymerase chain reaction (PCR) was carried out. Using an Applied Biosystems 7500 real-time PCR machine, the samples were cycled under the following conditions: 42 cycles of denaturation at 95 °C for 20 seconds and extension at 65 °C for 1.5 minutes followed by an initial denaturation step at 95 °C for 3 minutes. Using GAPDH as a reference gene, the expression of target genes was calculated through the $2^{-\Delta Cq}$ method. The primer sequences below were specifically used: Proenkephalin (Penk): CGGTTCCTGACACTTTGCACT (forward primer) and CACATTCCATTACGCAAGCCA (reverse primer); retinol binding protein 7 (Rbp7): CTCAGCGGTACTTGGACCC (forward primer) and CGAGTGGCAAAGTCAATACCT (reverse primer); and GAPDH: GGAGCGAGATCCCTCCAAAAT (forward primer) and GGCTGTTGTCATACTTCTCATGG (reverse primer).

Western blotting assay

Lysed AC16 cell samples were placed in RIPA buffer with a cocktail of 4% protease inhibitors (HY-K0021, MedChemExpress, Shanghai, China). The protein concentration was estimated using a BCA Protein Assay Kit (PA115, TIANGEN, Beijing, China), and an equal amount of protein (30 μg) was loaded onto a 10%-15% SDS polyacrylamide gel and separated for 1.2 hours at 90 V. The membranes were blocked with 5% nonfat milk and then left to overnight incubation with primary antibodies. Using Pro-Light HRP chemiluminescent reagent (PA112, TIANGEN, Beijing, China), PVDF membranes were observed with a ChemiDoc XR-S system (Bio-Rad, United States) after being treated with the relevant horseradish peroxidase-conjugated secondary antibodies. The protein band density was determined using ImageJ software. A 1:100 dilution of Rbp7 primary antibody was purchased from Thermo Fischer Scientific (MA5-24514, United States), and a 1:10000 dilution of GAPDH (ab181602, Abcam, Shanghai, China) was applied as an internal reference for expression value normalization. A 1:200 dilution of the Penk primary antibody was purchased from ABclonal (A6302, Wuhan, China), and a 1:2000 dilution of β-tubulin (10068-1-AP, Wuhan, China) was used as an internal reference.

scRNA-seq computational pipelines and analysis

In the integrated dataset, linear principal component analysis targeted variable genes, using the top 20 principal components for UMAP-based nonlinear dimensionality reduction. These principal components were employed to build a shared nearest neighbor (SNN) graph based on the "FindNeighbors" function. Clusters were then formed using the "FindClusters" function applied to the SNN graph. Identification of cell types was achieved by detecting DEGs in each cluster with the "FindAllMarkers" function of Seurat. Verification of cluster labels was manually conducted using established databases, including CellMarker2.0[21] and PanglaoDB[22]. Gene expression data for each identified cell type were retrieved using the "Fetchdata" function, facilitating further statistical analysis and visual representation.

The cell-cell communication analysis relied on two R packages, "celltalker" (version 0.0.7.900) and "cellChat"[23] (version 1.6.1). The "celltalker" package provided access to intercellular communication networks *via* the Ramilowski pairs dataset. Circle plots were generated to display the top 3 significant interactions for each cell type, which were determined based on specified criteria (false discovery rate < 0.05). The "cellChat" package was further used for the analysis of communication patterns by setting the ligand-receptor set to CellChatDB.mouse. The function "computeCommunProb" inferred the cell-cell interactions, and "computeCommunProbPathway" further inferred the communication at the signaling pathway level. The aggregated cell-cell communication network was constructed using the "aggregateNet" function. Network centrality scores were computed using the "netAnalysis_computeCentrality" function, and the number of patterns of interaction was determined using the "selectK" function, which is presented as river plots by "netAnalysis_river".

Table 1 Detailed information on the datasets used in this study

Accession number	Method	Origin	Samples		Aim
			DCM	CTL	
GSE5606	Bulk RNA-seq	*Rattus norvegicus*	7	7	Merged as discovery cohorts
GSE6880	Bulk RNA-seq	*Rattus norvegicus*	3	3	
GSE4745	Bulk RNA-seq	*Rattus norvegicus*	12	12	Merged as validation cohorts
GSE197999	Bulk RNA-seq	*Rattus norvegicus*	5	5	
GSE213337	scRNA-seq	*Mus musculus*	1	1	Single cell analysis

RNA-seq: RNA sequencing; DCM: Diabetic cardiomyopathy; CTL: Control.

Table 2 Results of least absolute shrinkage and selection operator and support vector machine recursive feature elimination algorithms for the selection of immune-related feature genes

LASSO		SVM-RFE		
Variables	Coefficients	Variables	Accuracy	Kappa
Penk	4.895	Il12a	0.800	0.600
Il12a	0	Angptl4	0.900	0.800
Gcgr	-4.308	Rbp7	0.967	0.933
Rbp7	1.761	Sctr	0.900	0.800
Inha	-1.155	Penk	1.000	1.000
Sctr	0	Gcgr	1.000	1.000
Angptl4	2.382	Inha	1.000	1.000

LASSO: Least absolute shrinkage and selection operator; SVM-RFE: Support vector machine recursive feature elimination; Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

RESULTS

DCM-related gene modules

To identify DCM-related gene modules, we performed WGCNA on an integrated dataset of two independent bulk RNA-seq datasets (GSE5606 and GSE6880) (Table 1). The soft thresholding method emphasizes strong correlations between genes and penalizes weak correlations, which helps construct scale-free networks. We selected an optimal power value of 5 for the construction of DCM scale-free networks (Figure 1A). Gene clustering resulted in the identification of 17 distinct modules, represented in different colors, where each module is comprised of genes that strongly correlated with each other but not with genes in other modules (Figure 1B). After excluding the gray module (genes in the module typically do not align with well-defined biological functional categories), module correlation analysis showed that the green module had the most significant strong positive association with DCM (Figure 1C). Correlation analysis between GS and green MM (581 genes) showed a correlation coefficient of 0.96 ($P < 0.0001$) (Figure 1D). These findings indicate that the genes in the green module are crucially involved in the biological processes associated with DCM.

Penk, glucagon receptor, Rbp7, and inhibin subunit alpha as IFGs in DCM

To understand the immunological molecular pathology of DCM, we identified IFGs associated with this disease. First, we extracted the gene profile of the most correlated green module and calculated the DEGs (Figure 2A). We screened a total of 69 DEGs, including 37 up-regulated genes (Figure 2B) and 32 down-regulated genes in the green module. The 69 DEGs were intersected with the list of 2483 IRGs to obtain 7 DIRGs (Figure 3A). To further identify the most representative IFGs among the 7 DIRGs, machine learning models were constructed based on the LASSO and SVM-RFE algorithms. The LASSO regression model identified five genes with non-zero coefficients, which suggests that these genes [Penk, glucagon receptor (Gcgr), Rbp7, inhibin subunit alpha (Inha), and Angptl4] are strongly associated with DCM (Table 2). SVM-RFE is a feature selection technique that uses SVM to repeatedly construct a model and remove features with the smallest ranking criterion to identify the most critical features for disease classification or prediction. The SVM-RFE model optimized for the best predictive performance highlighted four genes (Penk, Inha, Gcgr, and Rbp7) as having the best prediction performance, where the accuracy and kappa coefficient of the model were 1.0 (Figure 3B and Table 2). Therefore, these four IFGs may play essential roles in the immunopathological characteristics of DCM. Among these

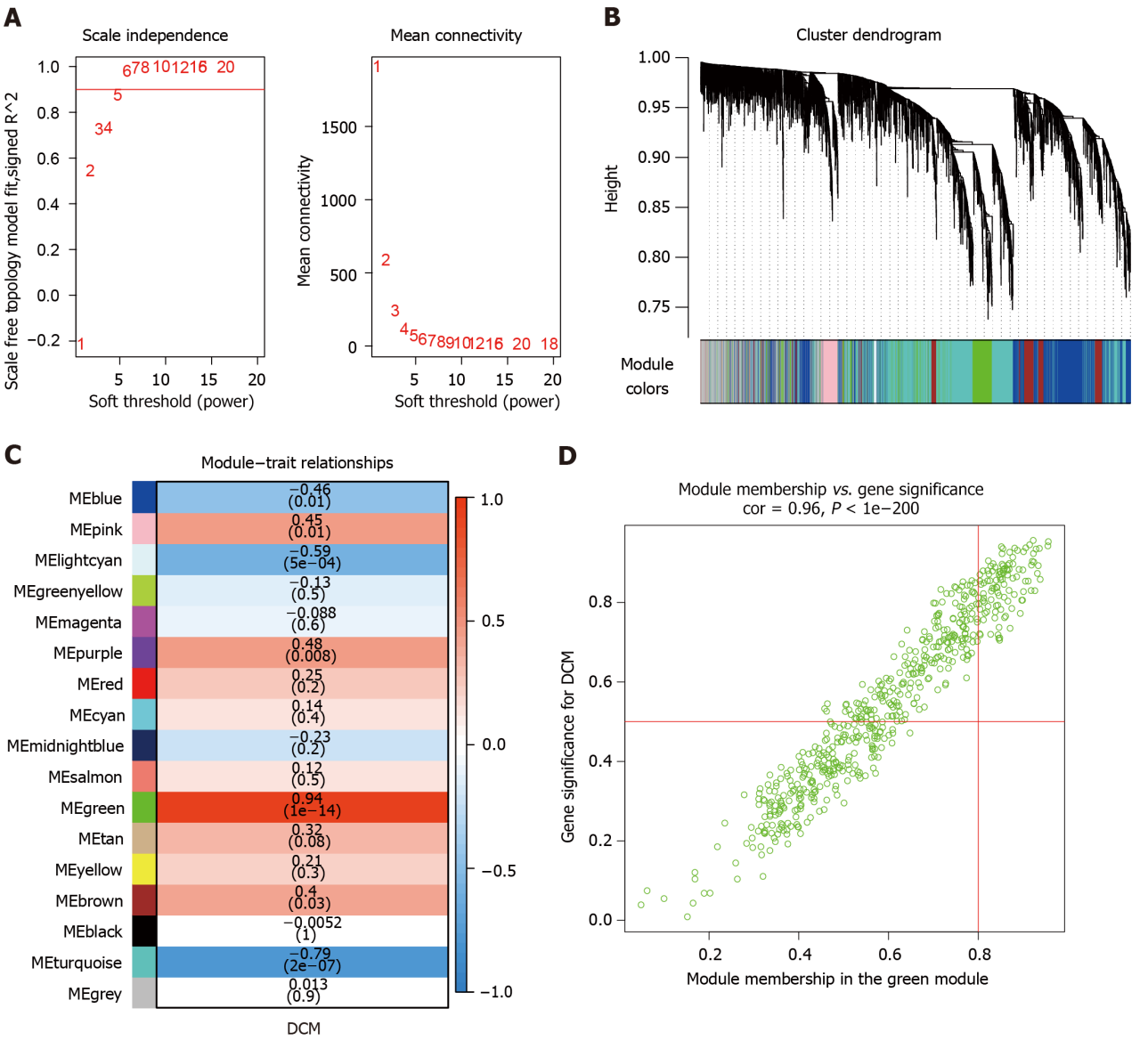

Figure 1 Weighted gene co-expression network analysis identified diabetic cardiomyopathy-related modules in the discovery cohort. A: Soft thresholding power analysis enabled the determination of the scale-free fit index for network topology; B: Cluster dendrogram and module assignment for modules from weighted gene co-expression network analysis in diabetic cardiomyopathy (DCM); C: Correlations of module eigengenes and DCM; D: Correlations between gene significance and green module membership. DCM: Diabetic cardiomyopathy.

characterized IFGs, the expression of Penk and Rbp7 was up-regulated, and Gcgr and Inha was down-regulated (Figure 3C).

Regulatory network of IFG expression

To characterize the *in vivo* expression regulation patterns of the identified IFGs, we constructed transcription factor and miRNA regulatory networks. The identified joint network consisted of a total of 27 nodes (IFGs with the transcription factors and miRNAs identified as their regulators) and 30 edges (regulatory interactions between the nodes) containing 20 transcription factors and 3 miRNAs. Among these transcription factors, TFAP2A is a co-transcription factor for Gcgr, Rbp7, and Inha. Two miRNAs, miR-215-5p and miR-192-5p, regulated Penk, and miR-26b-5p regulated Rbp7 expression (Figure 3D). Therefore, targeting TFAP2A or specific miRNAs could modulate the expression of multiple DCM-related genes simultaneously, which provides a strategic approach for therapeutic intervention.

IFGs as potential biomarkers for DCM

To evaluate the ability of the IFGs to distinguish between disease and control samples, we used predictive and diagnostic models to validate their ability to be extrapolated to RNA-seq validation cohorts (GSE4745 and GSE197999). A logistic regression nomogram model was constructed using Penk, Gcgr, Rbp7, and Inha as independent variables and DCM as the dependent variable. The model exhibited excellent discriminatory ability, as indicated by a C-index of 1.0 (Figure 4A). The nomogram model was further validated using a calibration curve, which demonstrated strong calibration with a mean absolute error of 0.008. This result indicated good agreement between the predicted probabilities and observed

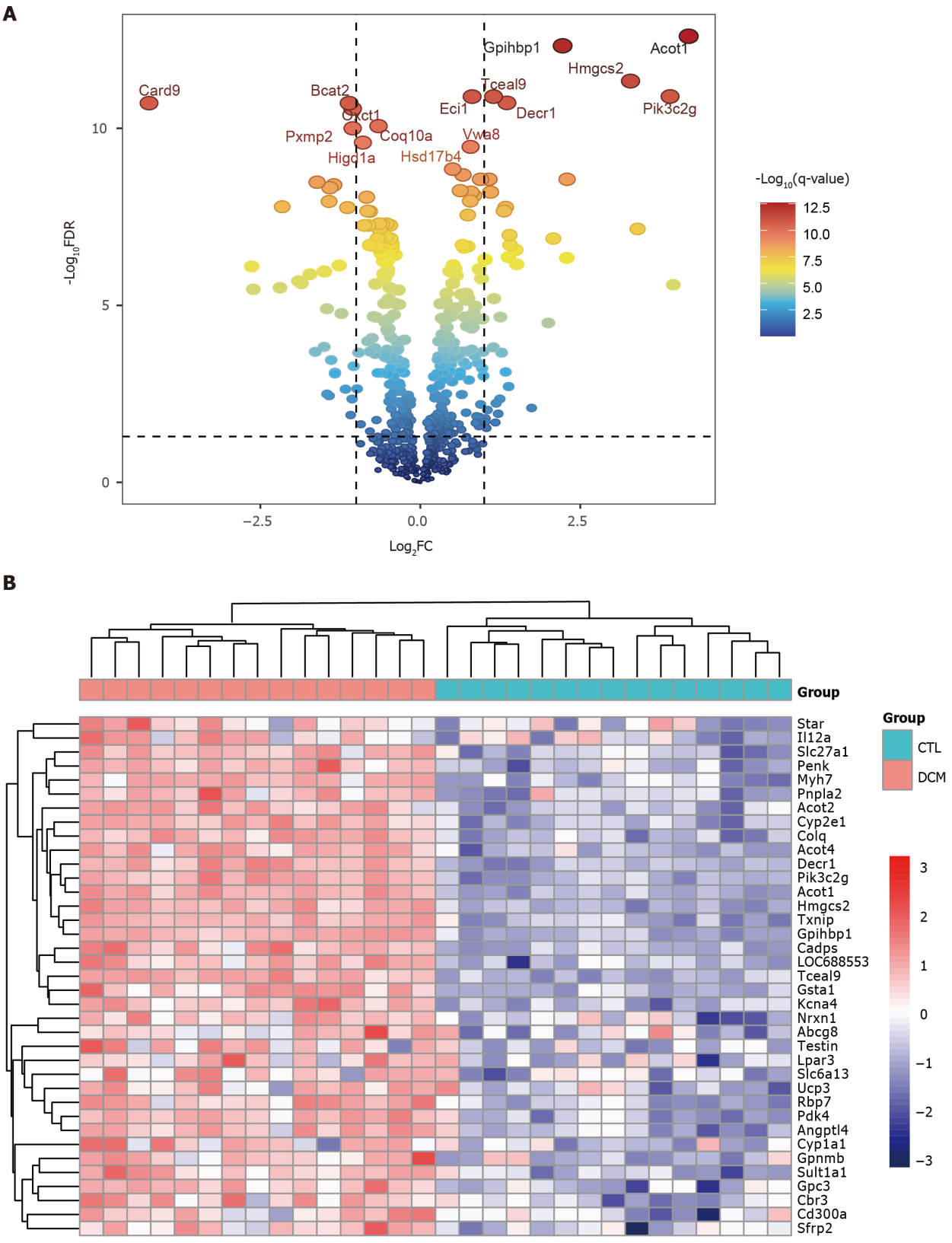

Figure 2 Identification of differentially expressed genes in the green module. A: Volcano plot of differentially expressed genes (DEGs) in the green module; B: Heatmap of the 37 upregulated DEGs in the green module.

outcomes (Figure 4B).

ROC curves assessed the discriminatory capacity of the four feature genes (Penk, Rbp7, Gcgr, and Inha), demonstrating their strong diagnostic potential with AUC values between 0.973 and 0.996 in DCM patients (Figure 4C). Logistic regression-based multivariable diagnoses also resulted in AUCs of 1.000 for the four genes and two up-regulated genes (Penk and Rbp7) in DCM (Figure 4D). DCA was employed to assess the clinical utility of various models in predicting disease outcomes. The decision curves for the four individual gene models and the complex model (Penk, Gcgr, Rbp7,

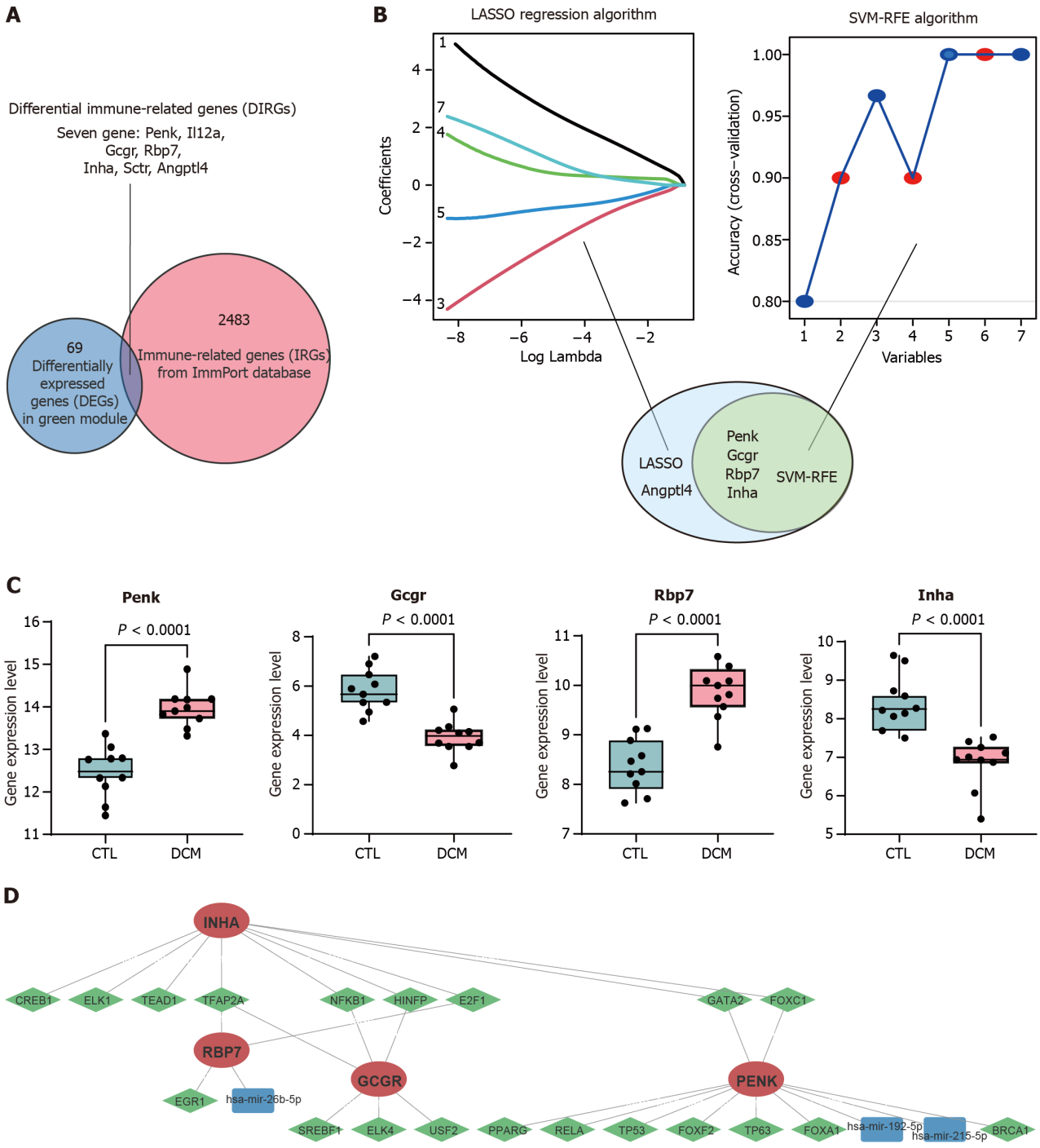

Figure 3 Identification of immune feature genes. A: The differentially expressed genes in the green module were intersected with the list of immune-related genes from the ImmPort database to obtain 7 differential immune-related genes; B: Machine learning algorithm based on least absolute shrinkage and selection operator (LASSO) and support vector machine recursive feature elimination (SVM-RFE) to select immune feature genes (IFGs) of diabetic cardiomyopathy (DCM). LASSO identified and selected 5 genes, whereas SVM-RFE identified and selected 4 genes; C: Box plot comparing the expression levels of the four common IFGs identified by LASSO and SVM-RFE between DCM patients and control patients; D: Gene expression regulatory networks. Red indicates target genes, green indicates transcription factors, and blue indicates miRNAs. LASSO: Least absolute shrinkage and selection operator; SVM-RFE: Support vector machine recursive feature elimination; DCM: Diabetic cardiomyopathy; CTL: Control; Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

and Inha) exhibited noticeable separation from the extreme curves representing "None" (no intervention) and "All" (treating all individuals) (Figure 4E). Within the threshold range of 0 to 1, the decision curve of the complex model consistently outperformed the curves of the four simple models and demonstrated a greater net benefit (Figure 4E).

Correlation between immune cell infiltration and IGF expression in DCM

SsGSEA allows for the estimation of the relative abundance of immune cell types in complex tissue samples based on gene expression data. To examine the immune cell infiltration characteristics in DCM, we used ssGSEA to obtain immune

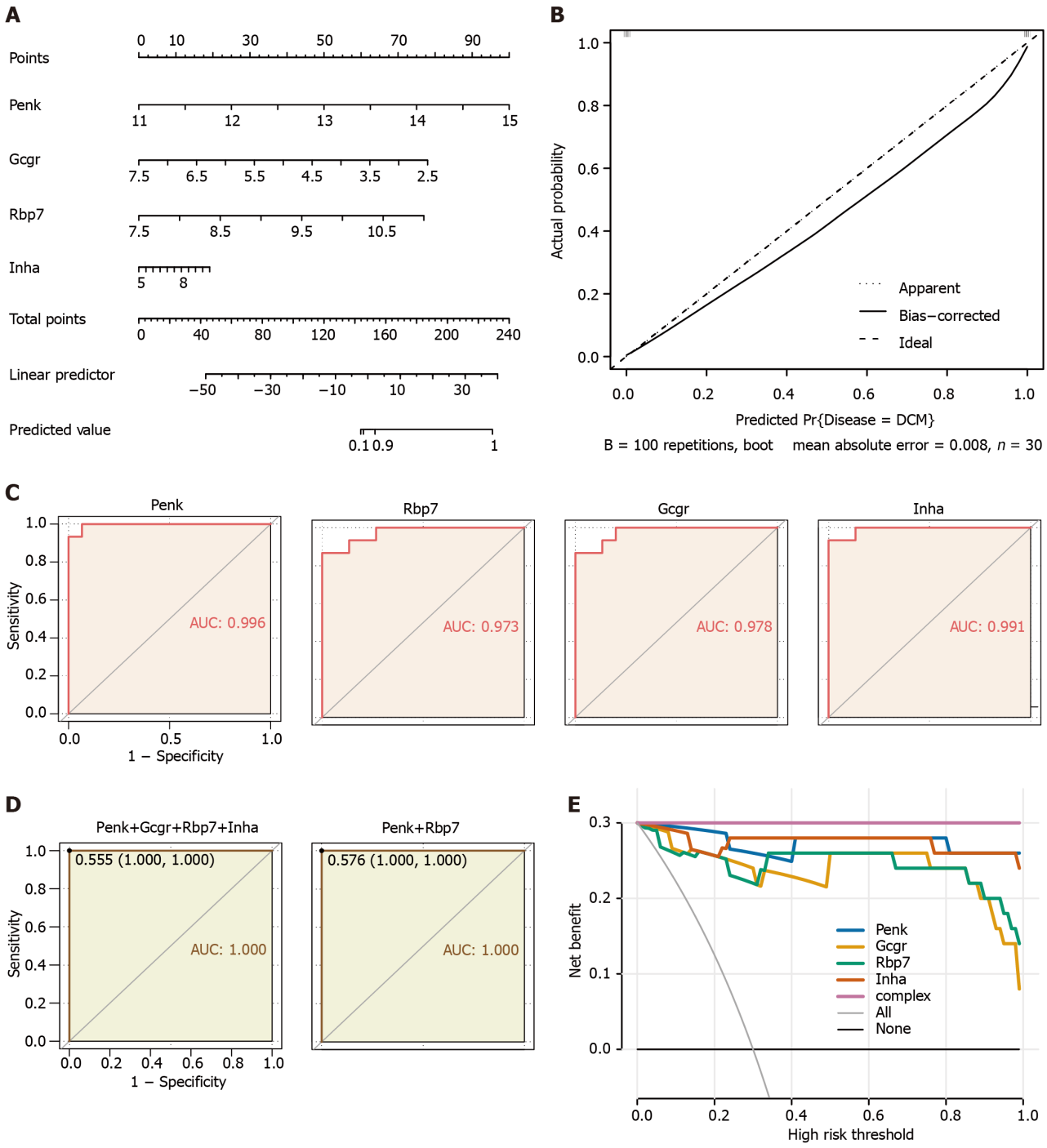

Figure 4 Clinical prediction models and diagnostic value for immune feature genes in the validation cohort. A: Nomogram predicting disease risk by proenkephalin (Penk), glucagon receptor (Gcgr), retinol binding protein 7(Rbp7), and inhibin subunit alpha (Inha); B: Calibration curves visually assessing the risk nomogram model calibration, a key aspect of model validity; C: Receiver operating characteristic (ROC) analysis of immune feature genes in diabetic cardiomyopathy (DCM) patients; D: ROC analysis of the diagnostic value of multivariable (four feature genes, unregulated Penk and Rbp7) in DCM patients; E: Decision curve analysis model construction was performed using a simple logistic regression model with Penk, Gcgr, Rbp7, and Inha as independent predictors and DCM as the outcome; a complex model (complex) was constructed by combining the four genes as predictors. Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

infiltration scores for disease and control samples. The ssGSEA results discovered statistically significant differences in the scores of five immune cell types, type 2 T helper cells, activated B cells, memory B cells, neutrophils, and myeloid-derived suppressor cells, between the disease and control samples. Type 2 T helper cells exhibited the most significant difference ($P < 0.001$), which indicated their potential involvement in DCM pathology (Figure 5A). Type 2 T helper cells are known for their role in humoral immunity and allergic responses and may contribute to inflammatory processes in DCM. Correlation analysis between the four selected IFGs and immune cells revealed significant positive correlations ($P < 0.01$) between Penk and Rbp7 and type 2 T helper cells, and Gcgr and Inha showed significant negative correlations ($P < 0.01$) with type 2 T helper cells (Figure 5B). These genes may be involved in pathways that regulate Th2 cell proliferation

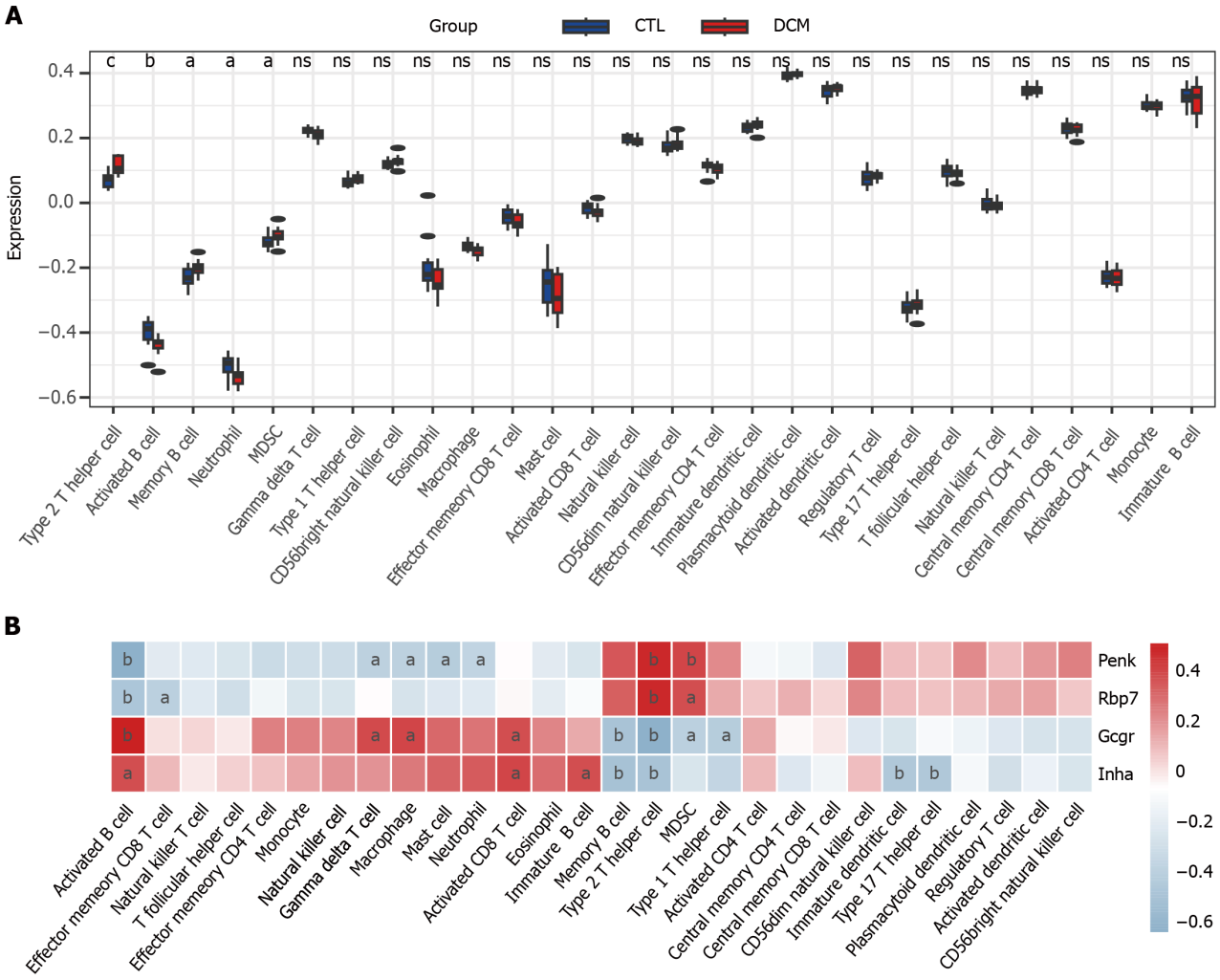

Figure 5 Single-sample gene set enrichment analysis was used to analyze immune cell infiltration in diabetic cardiomyopathy patients and its correlation with feature genes. A: Box plot of immune scores for diabetic cardiomyopathy patients and control; B: Heatmap of the correlation between feature genes and immune cell infiltration. [a]*P* < 0.05, [b]*P* < 0.01 [c]*P* < 0.001. DCM: Diabetic cardiomyopathy; CTL: Control; Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

or function, which suggests a potential mechanism for the influence of inflammation on the pathology of DCM.

Expression patterns of IFGs and intercellular profiles determined by scRNA-seq of diabetic mouse hearts

To explore the expression patterns of the four identified IFGs, scRNA-seq analysis was conducted on the hearts of diabetic mice (Table 1). The integrated dataset, which consisted of DCM and healthy control samples, contained a total of 30905 genes and 12593 cells and allowed for robust comparative analysis. The cellular analyses showed that Penk and Rbp7 significantly elevated in DCM, which suggested their active involvement in the disease process. Conversely, Inha and Gcgr were expressed at low levels or undetectable, which indicated their minimal direct involvement under the conditions studied (Figure 6A). Additional tissue expression data from the ProteomicsDB database confirmed the substantial expression of Penk and Rbp7 in the heart, particularly Rbp7, which reinforced the RNA-seq findings and highlighted the potential biological relevance of these genes in cardiac tissues (Figure 6B). Molecular experiments on AC16 cells demonstrated minimal Penk expression in untreated cells, with significant upregulation of Penk and Rbp7 after a 48 hours HG treatment (Figure 6C and D). This response was consistent with the increased cardiac stress and metabolic changes observed in DCM, which supported the disease relevance of Penk and Rbp7 expression observed in the scRNA-seq analysis.

We used UMAP dimensionality reduction and graph-based clustering and identified 14 cell clusters (resolution = 0.5) (Figure 7A) and 9 cell types (Figure 7B), which were annotated based on their highly expressed genes (DEGs) (Figure 7C). Analysis of feature gene expression patterns across these cell types revealed that Penk was primarily expressed in fibroblasts, and Rbp7 was enriched in endothelial cells and CMs. Notably, Rbp7, which is crucial for vascular function, was also detected in pericytes (Figure 7D). We also examined intercellular dysregulation. Using dimensionality reduction, we observed increased fibroblast numbers and reduced macrophage numbers (Figure 7E and F). DCM enhanced intercellular communication, primarily *via* increased fibroblast signaling (Figure 8A), which highlighted the strong interaction between fibroblasts and macrophages (Figure 8B). Signaling analysis revealed the involvement of the macrophage migration inhibitory factor (MIF) signaling pathway (Figure 8C). Weighted network analysis indicated that

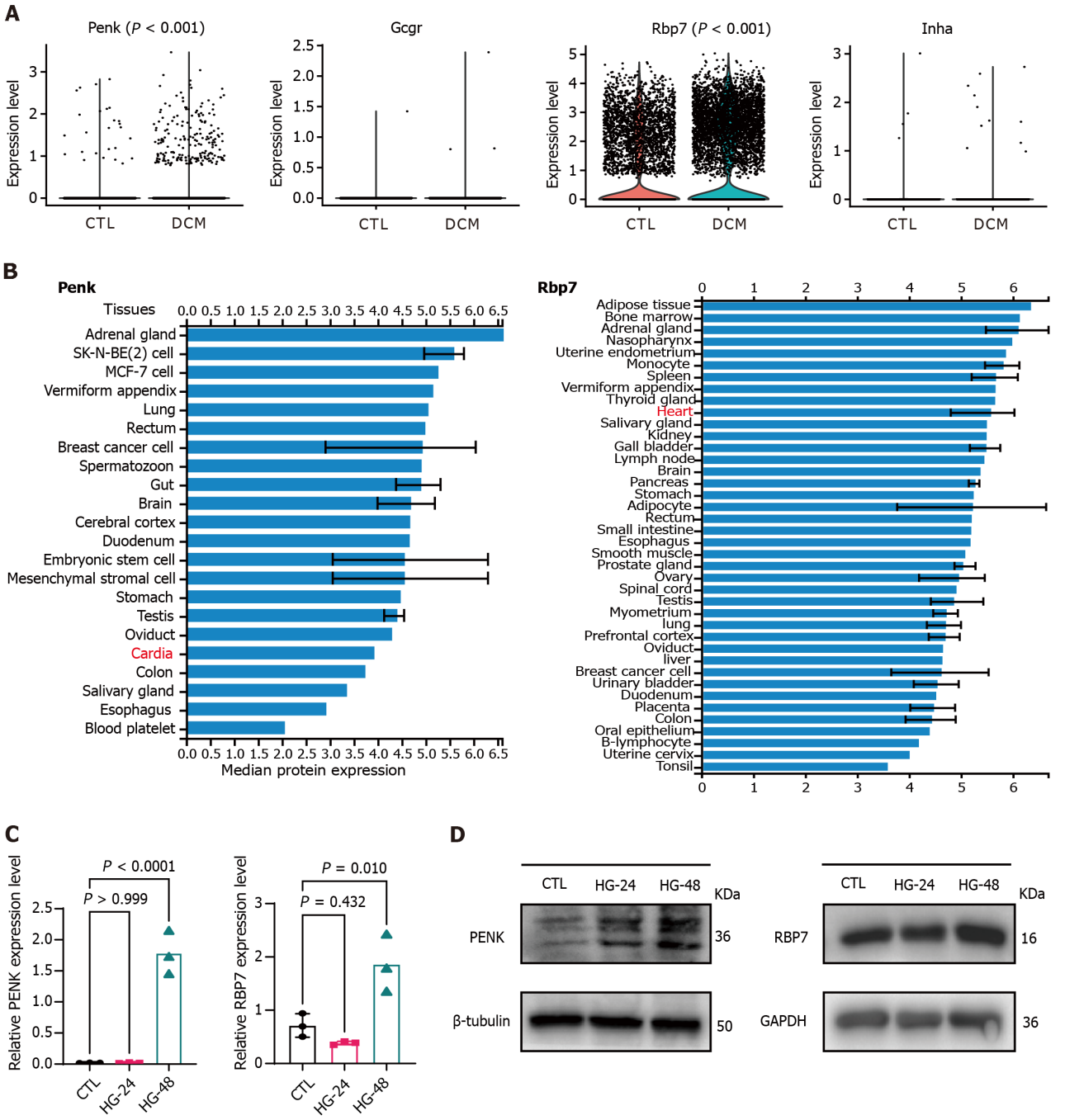

Figure 6 Expression levels of immune feature genes according to single-cell RNA sequencing data. A: Violin plots comparing the expression levels of immune feature genes in control (CTL) (5833 cells) and diabetic cardiomyopathy (6760 cells) samples, with each point representing one cell; B: Tissue-specific expression of proenkephalin and retinol binding protein 7 in the ProteomicsDB repository; C: Relative gene expression levels in AC16 cells were examined using real time-quantitative polymerase chain reaction. Each point represents one independent biological experiment. HG-24 and HG-48, AC16 cells exposed to high glucose for 24 hours and 48 hours, respectively; D: Western blot showing the protein expression levels of the two genes. β-Tubulin and GAPDH were used as endogenous CTLs. DCM: Diabetic cardiomyopathy; CTL: Control; Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

macrophages were significant MIF receivers, and pericytes were significant sender proteins (Figure 8D and E). Macrophages received MIF signals and communicated with fibroblasts *via* the growth arrest-specific pathway (Figure 8F). Global communication analysis identified four patterns that showed how sender (outgoing) and target (incoming) cells coordinated with specific signaling pathways, which may be crucial for understanding the pathological landscape of DCM (Figure 8G).

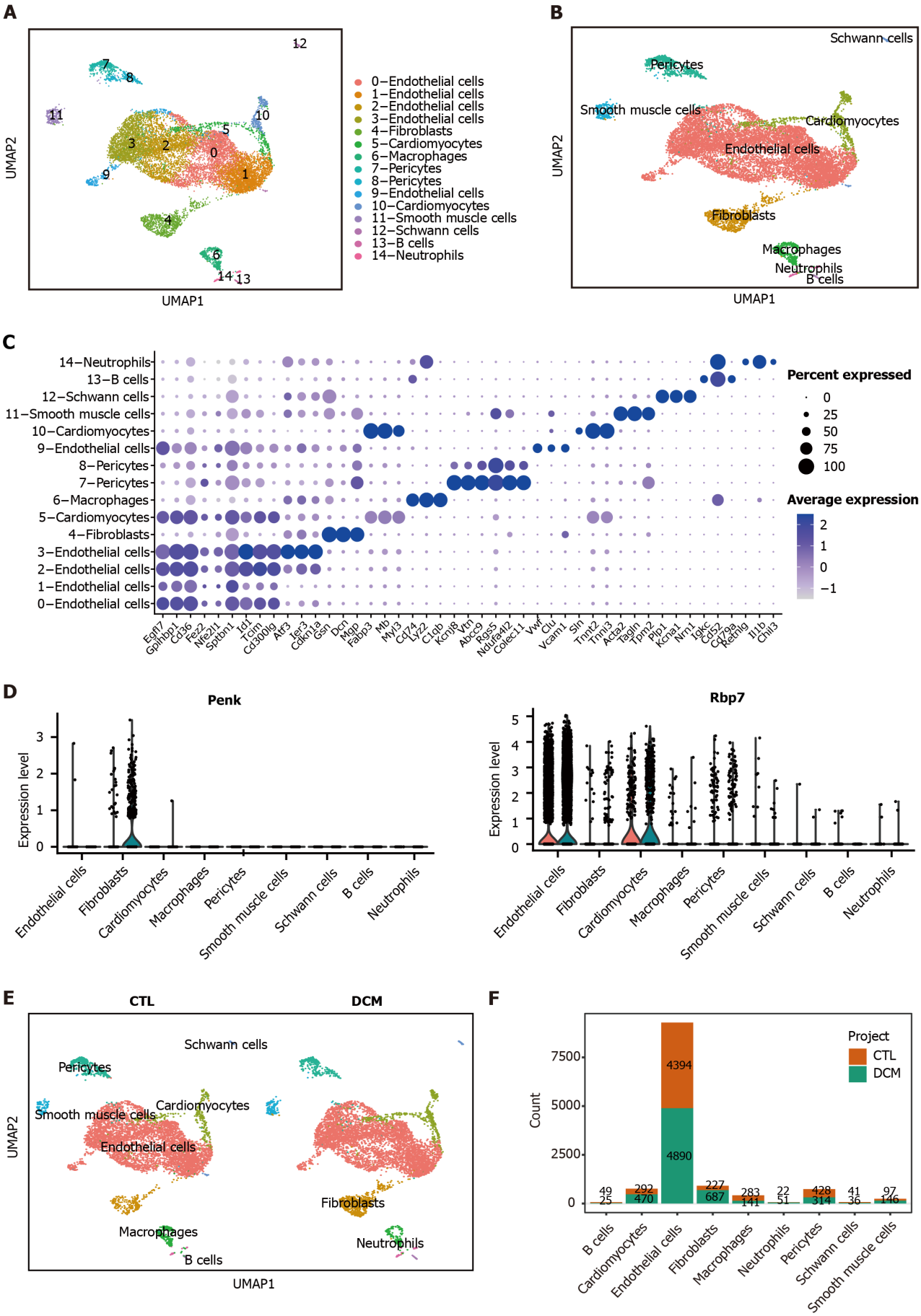

Figure 7 Analysis of the expression patterns of immune feature genes and cell-cell communication. A: UMAP dimensionality reduction

embedding was performed on the integrated dataset of single-cell RNA sequencing data from all profiled samples (n = 12593 cells). The embedding is color-coded by inferred cluster identity and annotated with manual cell type labels; B: Clusters with similar marker gene expression modules were merged and clustered into the same cell type; C: Dot plot showing cell-specific markers; D: Violin plots showing statistically significant feature gene (proenkephalin and retinol binding protein 7) expression values for each cell type, grouped by the donor of origin [control (CTL) and diabetic cardiomyopathy (DCM)]; E: UMAP for cell clusters grouped by the donor of origin (CTL or DCM); F: Bar chart representing the cell counts of different cell types in the two groups. DCM: Diabetic cardiomyopathy; CTL: Control; Penk: Proenkephalin; Gcgr: Glucagon receptor; Rbp7: Retinol binding protein 7; Inha: Inhibin subunit alpha.

DISCUSSION

The present study integrated transcriptome sequencing data and applied machine learning algorithms to analyze immune gene datasets and identified four IRGs as the most relevant to DCM. Analysis of single-cell sequencing data and *in vitro* modeling using AC16 human cardiomyocytes exposed to HG further validated the upregulation of Penk and Rbp7, which highlighted their potential importance in DCM immunopathology. Single-cell data also suggested a regulatory role for phenotypic transformation and signaling communication between the extracellular mesenchyme and immune cells outside of myocardial working cells, particularly fibroblasts and macrophages, in the pathomechanism of DCM. The workflow of this study is summarized in Figure 9.

We investigated dysregulated IRGs in DCM and identified the upregulation of Penk and Rbp7 as disease signatures. Single-cell expression pattern analysis did not reveal which class of immune cells was enriched based on the limited availability of current DCM single-cell data, which led to low-resolution heart cell clustering. Despite recognizing this limitation, the included single-cell datasets and exploration of public databases provided evidence that both genes were expressed in cardiomyocytes. Our molecular experiments further confirmed this result.

Penk encodes the precursor peptide enkephalin, which is an opioid-like peptide that is transiently expressed in immune cells and may regulate immune cell activity and immune responses[24]. An endogenous opioid peptide was also found within the central and autonomic nervous systems of various organs, such as the heart. Recent meta-analysis findings highlight a notable association between Penk and diabetes, indicating its potential as a predictive biomarker for the disease[25]. Our DCM diagnostic and predictive models constructed based on gene expression data consistently emphasized the value of these factors as biomarkers. Previous evidence also suggested that human bone marrow mesenchymal stromal cells expressed and secreted Penk under inflammatory conditions, which led to elevated levels of the anti-inflammatory and immunomodulatory factor IL-10[26]. Another molecule, Rbp7, is a target gene of PPARγ and plays a role in antioxidant activity in the vascular system, which suggests that it is a protective factor in cardiovascular health[27]. Rbp7 is a putative RNA-binding protein (RBP) implicated in cell cycle arrest and transmission competence [28]. RBPs play a key role as mediators and regulators of RNA-protein interactions, influencing a wide range of genetic, epigenetic, and metabolic activities in both immune and non-immune cells. They are essential for numerous cellular functions related to immunity and homeostasis[29]. However, the knowledge of these two factors in diabetes is limited, and this analysis did not address how gene dysregulation in cardiomyocytes mediated the pathological process of DCM. Characterization of the expression of the gene in the heart in a well-rounded set of immune cells and mechanistic exploration of its dysregulated expression in cardiomyocytes could provide fresh perspectives on the pathogenesis of DCM.

Recent studies highlighted the importance of immune dysregulation in DCM pathogenesis, which emphasized the role of immune cells and inflammatory mediators in driving cardiac dysfunction[30,31]. New research points to the role of non-immune cells, including endothelial and fibroblast cells, in controlling the inflammatory response and encouraging cardiac fibrosis in DCM[32,33]. These findings underscore the complexity of immune-mediated mechanisms in DCM and the need for comprehensive approaches to unravel its pathophysiology. Our study also revealed differences in immune cell infiltration in DCM, particularly alterations in T-helper (Th) 2 cells. Th cell subsets, including Th2 cells, play a crucial role in directing immune responses *via* the production of signature cytokines. In the context of diabetes, T lymphocytes, including Th and regulatory T cells, modulate cardiac inflammation[34,35]. Th1/Th2 cytokine imbalances are notable features of diabetes complications and emphasize the significant role of Th cells in DCM pathology[36-40].

The scRNA-seq analysis of diabetic mouse hearts provided insights into enhanced intercellular communication, particularly between fibroblasts and other cell populations, especially macrophages. The MIF signaling pathway plays an essential role in the communication patterns among these cells, and macrophages are the main targets of signaling and vascular pericytes are the source of signaling. These findings elucidate the complex interactions between different cell types and signaling pathways. Cardiac fibrosis, a key pathological aspect of DCM, results from complex interactions among immune cells, fibroblasts, and other host-derived cells[13,41,42]. Diverse cell types including cardiomyocytes, endothelial cells, and pericytes play roles in promoting fibrosis through various mechanisms, such as the production of proteases involved in matrix metabolism, the secretion of fibrotic mediators and matrix cell proteins, and the transition to a fibroblast phenotype[43]. Under the influence of various cytokines, monocytes/macrophages differentiate into myofibroblasts, which produce inflammatory mediators and pro-fibrotic factors to establish a detrimental pathological cycle[5,44,45]. Although not yet reported in DCM, similar phenomena have been observed in models of myocardial infarction and diabetic nephropathy[46,47]. The role of cardiac pericytes is not clear because these cells are not well characterized, and their bidirectional differentiation into myofibroblasts and endothelial cells is controversial[48]. Cardiac pericytes may contribute to diabetes-associated fibrosis *via* the secretion of inflammatory mediators and fibroblast-activating growth factors[42,49]. Macrophages activate TGF-β signaling in pericytes *via* the secretion of amphiregulin, which promotes pericyte differentiation into myofibroblasts and participates in tissue repair[50]. Further examination of the MIF signaling-mediated macrophage-pericyte axis in DCM may provide insights into how these cells contribute to

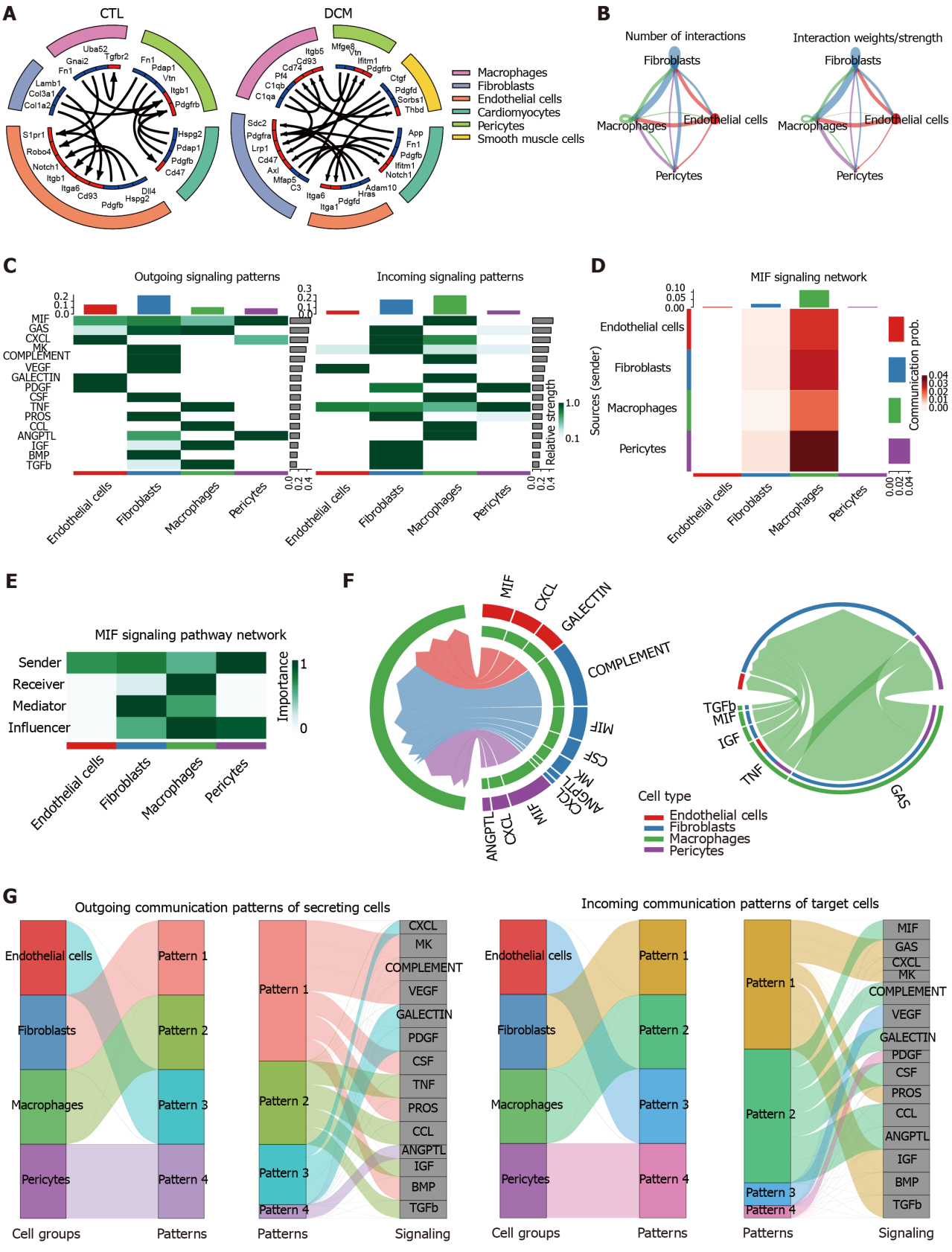

Figure 8 Analysis of cell-cell signaling pathway networks and communication patterns in diabetic cardiomyopathy. A: The circle plot shows the cell-cell communication analysis of the control (CTL) and diabetic cardiomyopathy (DCM) groups, with the ligand color in blue and the receptor color in red; B: The circle plot depicting the aggregated cell-cell communication network in DCM illustrates either the number of interactions or the total interaction strength (weights) between any two cell groups; C: A signaling role analysis was conducted on the aggregated cell-cell communication network across all signaling pathways to identify the signals contributing the most to outgoing or incoming signaling in specific cell groups. This was visualized using a combined heatmap, where each row corresponds to a signaling pathway; D: Heatmap visualizing the cell-cell communication network specifically based on the migration inhibitory factor (MIF) signaling pathways. The intensity of the color in each cell reflects the strength of the interaction between the corresponding cell groups. Each row corresponds to a sender cell

group, and each column represents a receiver cell group; E: Heatmap displaying the computed centrality scores (importance) of cell groups in the intercellular communication network (dominant senders, receivers, mediators, and influencers) inferred from the MIF signaling pathway; F: Chord diagram illustrating the significant signaling pathways between selected source cell groups and target cell groups (source: Endothelial cells, fibroblasts, and pericytes, target: Macrophages) (left panel) in the intercellular communication network. The chords connecting the source and target arcs represent the significant signaling pathways between the corresponding cell groups; G: River plot to visualize associations of latent patterns (outgoing and incoming) with cell groups and signaling pathways. The specific communication patterns are defined by different colors in the network. MIF: Migration inhibitory factor; DCM: Diabetic cardiomyopathy; CTL: Control.

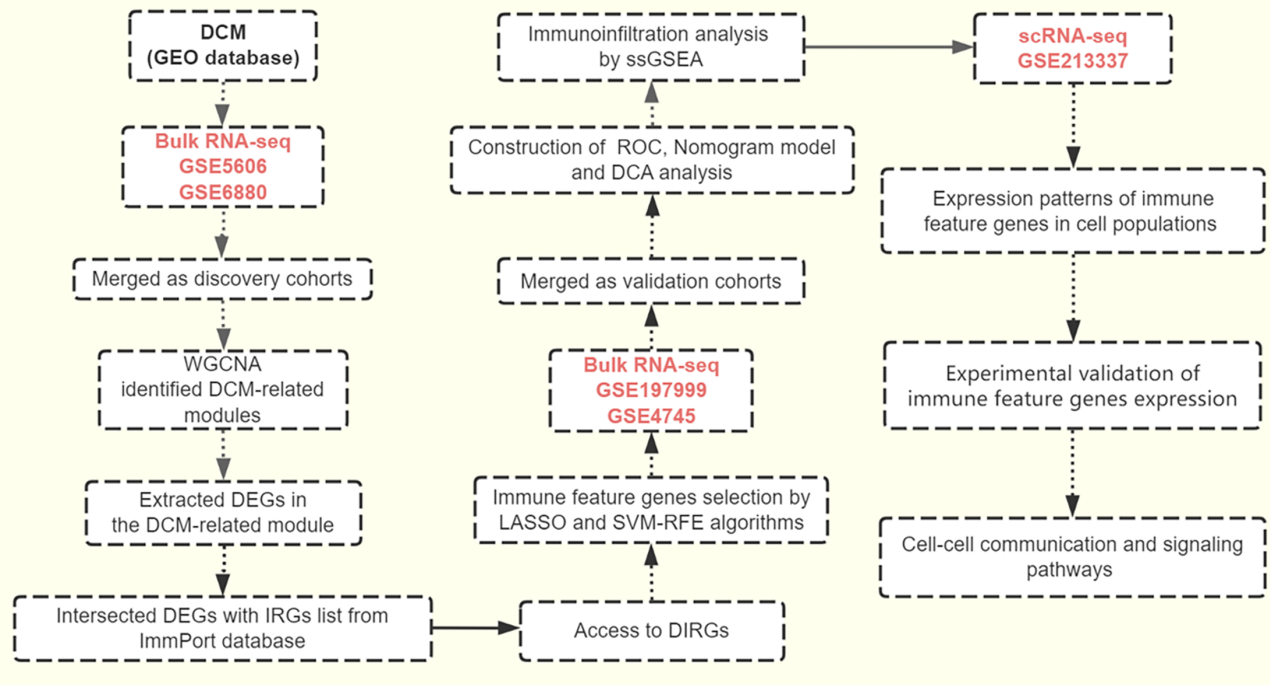

Figure 9 The workflow of this study. DCM: Diabetic cardiomyopathy; RNA-seq: RNA sequencing; WGCNA: Weighted gene co-expression network analysis; DEGs: differentially expressed genes; IRGs: Immune-related genes; DIRGs: Differential immune-related genes; LASSO: Least absolute shrinkage and selection operator; SVM-RFE: Support vector machine recursive feature elimination; ROC: Receiver operating characteristic; DCA: Decision curve analysis; ssGSEA: Single-sample gene set enrichment analysis.

immune surveillance and effector functions in this disease[51].

Overall, the present study identified dysregulated Penk and Rbp7 as potential DCM biomarkers, but their exact mechanism of action in DCM must be further examined. Aberrant mesenchymal-immune cell phenotypic transformation and communication may be essential for DCM pathogenesis. Our analysis was limited by the heterogeneity of single-cell data from mice, which led to low-resolution heart cell clustering and limited characterization of immune cell types enriched in Penk and Rbp7. Our study did not investigate the precise mechanisms by which dysregulated genes in cardiomyocytes mediate the pathological process of DCM in vivo. Future research should focus on elucidating the molecular mechanisms of the role of Penk and Rbp7 in DCM and further investigating the functional consequences of aberrant mesenchymal-immune cell communication in disease progression.

CONCLUSION

Our study elucidated the immunological molecular mechanisms underlying DCM and proposed potential diagnostic and prognostic biomarkers. WGCNA and machine learning identified Penk and Rbp7 as promising candidates with high diagnostic and prognostic value for DCM. Our findings also highlight the involvement of type 2 T helper cells in immune cell infiltration associated with DCM. scRNA-seq analysis suggested abnormal cellular phenotype transformation and signaling communication, particularly between mesenchymal cells and macrophages, which suggests a crucial role for aberrant cell communication in DCM pathogenesis. Overall, the present study offers valuable insights into the immunological mechanisms of DCM and suggests avenues for future research and potential therapeutic targets.

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FOOTNOTES

Author contributions: Song YF proposed and designed the study and revised the manuscript; Zheng ZQ and Cai DH performed the research, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Asiaticoside improves diabetic nephropathy by reducing inflammation, oxidative stress, and fibrosis: An *in vitro* and *in vivo* study

Lan-Gen Zhuang, Rong Zhang, Guo-Xi Jin, Xiao-Yan Pei, Qiong Wang, Xiao-Xu Ge

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Abstract

BACKGROUND

Diabetic nephropathy (DN) is a severe microvascular complication of diabetes characterized by inflammation, oxidative stress, and renal fibrosis. Asiaticoside (AC) exhibits anti-inflammatory, antioxidant, and anti-fibrotic properties, suggesting potential therapeutic benefits for DN. This study aimed to investigate the protective effects of AC against DN and elucidate the underlying mechanisms involving the nuclear factor erythroid 2-related factor 2 (NRF2)/heme oxygenase-1 (HO-1) antioxidant pathway.

AIM

To investigate the renoprotective effects of AC against DN and elucidate the role of the NRF2/HO-1 pathway.

METHODS

The effects of AC on high glucose (HG)-induced proliferation, inflammation, oxidative stress, and fibrosis were evaluated in rat glomerular mesangial cells (HBZY-1) *in vitro*. A streptozotocin-induced DN rat model was established to assess the *in vivo* impact of AC on renal injury, inflammation, oxidative stress, and fibrosis. The involvement of the NRF2/HO-1 pathway was examined using pharmacological inhibition studies in the cell model.

RESULTS
AC inhibited HG-induced HBZY-1 cell proliferation and significantly improved various indicators of DN in rats, including reduced body weight, and elevated blood glucose, serum creatinine, blood urea nitrogen, and 24-h urine protein. Both *in vitro* and *in vivo* studies demonstrated that AC decreased inflammation and oxidative stress by reducing interleukin (IL)-6, IL-8, tumor necrosis factor-alpha, reactive oxygen species, and malondialdehyde levels while increasing superoxide dismutase activity. Additionally, AC suppressed the expression of fibrogenic markers such as collagen I, collagen IV, and fibronectin. AC activated NRF2 expression in the nucleus and increased HO-1 and NAD(P)H dehydrogenase (Quinone) 1 protein expression in renal tissues and HG-induced HBZY-1 cells.

CONCLUSION
AC improves DN by reducing inflammation, oxidative stress, and fibrosis through the activation of the NRF2/HO-1 signaling pathway. These findings not only highlight AC as a promising therapeutic candidate for DN but also underscore the potential of targeting the NRF2/HO-1 pathway in developing novel treatments for other chronic kidney diseases characterized by oxidative stress and inflammation.

Key Words: Asiaticoside; Diabetic nephropathy; Inflammation; Renal fibrosis; Reactive oxygen species

Core Tip: This study investigated the protective effects of asiaticoside (AC) in diabetic nephropathy (DN) using *in vitro* and *in vivo* models. AC attenuated high glucose-induced proliferation, inflammation, oxidative stress, and fibrosis in rat glomerular mesangial cells. In a streptozotocin-induced DN rat model, AC ameliorated renal injury, and reduced inflammatory cytokines, oxidative stress markers, and fibrogenic markers. Notably, the renoprotective effects of AC were associated with the activation of the nuclear factor erythroid 2-related factor 2 (NRF2)/heme oxygenase-1 antioxidant signaling pathway, suggesting AC's therapeutic potential for DN by targeting inflammation, oxidative stress, and fibrosis through NRF2-associated mechanisms.

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INTRODUCTION

Diabetic nephropathy (DN), a severe microvascular complication of diabetes mellitus, is known for its low cure rate, low awareness rate, high morbidity, and high disability[1,2]. In the advanced stages of diabetes, DN transitions from mild renal inflammation to various phases of renal fibrosis, renal sclerosis, and ultimately end-stage renal disease (ESRD)[3]. Studies have shown that after 20 years of diabetes, the prevalence of DN could be as high as 30%-40%, with 5%-10% of patients progressing to ESRD. Projections suggest that DN may become the seventh leading cause of death worldwide by 2030[3-6]. While strict control of blood glucose levels and blood pressure is crucial in managing DN, some cases still progress to ESRD despite successful glucose control. Therefore, the development of new therapeutic modalities and drugs for DN is essential.

Before delving into therapeutic options, it is crucial to elucidate the complex pathogenesis of DN, which remains incompletely understood. Recent research efforts have suggested that hyperglycemia-induced oxidative stress may be a key factor in the development of renal complications in diabetes. Moreover, inflammation in renal tissues plays a vital role in the onset and progression of DN[7]. Oxidative stress typically arises from an overproduction of reactive oxygen species (ROS) or a decrease in antioxidant capacity. In diabetes, elevated ROS levels can disrupt the intracellular metabolism of DNA, proteins, and lipids through oxidative modifications, leading to renal dysfunction by activating various cellular signaling pathways[8,9]. The combination of hyperglycemia and excessive intracellular ROS can trigger renal cells to produce cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. These ROS and cytokines then interact, leading to the activation of inflammatory factors, adhesion molecules, and chemokines. The resulting inflammation contributes to glomerulosclerosis and tubulointerstitial fibrosis, which exacerbates renal damage and promotes DN progression. Therefore, reducing oxidative stress and mitigating inflammatory damages could be a beneficial therapeutic approach for managing DN.

Nuclear factor erythroid 2-related factor 2 (NRF2), a protein linked to oxidative stress, plays a significant role in DN inflammation. Evidence suggests the implication of NRF2 in DN progression, as it has been shown to regulate pro-inflammatory cytokine production, reduce inflammation, and counteract oxidative stress for renal protection[10]. NRF2 associates with Kelch-like ECH-associated protein 1 in the cytoplasm. When triggered by external factors, it translocates to the nucleus to bind to antioxidant response elements in the promoters of genes like heme oxygenase-1 (*HO-1*) and

NAD(P)H dehydrogenase (Quinone) 1 (*NQO-1*). This leads to augmented antioxidant capacity, aiding in the fight against oxidative stress[11].

Asiaticoside (AC), a primary compound of ursane-type triterpene glycoside derived from *Centella asiatica*, has a history of over 2000 years in traditional Chinese medicine for treating a variety of ailments. Multiple studies have shown that AC exhibits antioxidant, anti-inflammatory, anti-fibrotic, and other important pharmacological properties[12-14]. Additionally, AC has been found to activate NRF2 and suppress ROS production, making it a potent compound to boost antioxidant capacity[15]. The current study aimed to investigate the protective effects of AC against inflammation, oxidative stress, and fibrosis in a rat model of DN and high-glucose (HG) induced glomerular mesangial cells, and elucidate the potential underlying mechanisms involving the Nrf2/HO-1 antioxidant pathway. The impact of AC on HG induced proliferation of rat mesangial cells (HBZY-1) was initially examined. Furthermore, we established a streptozotocin (STZ)-induced DN rat model to evaluate the *in vivo* effects of AC on renal injury, inflammation, oxidative stress, and fibrosis. The role of the NRF2/HO-1 pathway in mediating the protective effects of AC was also explored using pharmacological inhibition of NRF2. The findings from this study will provide insights into the therapeutic potential of AC for DN and elucidate a possible mechanism involving the activation of the NRF2 antioxidant pathway.

MATERIALS AND METHODS

Cells and culture conditions

The rat mesangial cell line HBZY-1 was procured from Wuhan Boster Biotechnology Company in Wuhan, China. HBZY-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) from HyClone Laboratories Ltd. in Logan, United States, supplemented with 10% fetal bovine serum from Sangon Biotech in Shanghai, China, 100 μg/mL streptomycin, and 100 U/mL penicillin from HyClone, and maintained under specified conditions (50 mL/L CO_2 and 37 °C). The stock solution of AC ($C_{48}H_{78}O_{19}$; CAS: 16830-15-2; Figure 1A) was obtained from Yuan Ye Biotechnology Co. Ltd. in Shanghai, China. This solution was prepared by dissolving 95.90 mg of AC in 100 mL of dimethyl sulfoxide from Santa Cruz Biotechnology. Subsequently, the full culture medium was used to create a working solution of varying concentrations.

Cell Counting Kit assay

HBZY-1 cells in the logarithmic growth phase were harvested and plated at a density of 1 × 10^4 cells/well in 96-well plates for overnight incubation to ensure proper attachment. Subsequently, the cells were exposed to various treatments using AC solutions at different concentrations (0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mmol/L), or with AC solutions (0, 2, 4, and 8 mmol/L) in combination with glucose (5.5 mmol/L), mannitol (30.0 mmol/L), and HG (30.0 mmol/L). After 24 h, each well was supplemented with 10% Cell Counting Kit-8 (CCK-8) working reagent (CA1210, Beijing Solebo Science and Technology Co. Ltd., Beijing, China), followed by incubation at 37 °C for 2 h. Finally, the absorbance values were determined at a wavelength of 450 nm using a microplate reader. For cytotoxicity assay, cells were treated with AC or HG for 24 h, and cells were subjected to indicated treatment for 48 h in cell proliferation assay.

Animal model and treatment options

Male Sprague-Dawley rats (n = 40, 8-week-old) were purchased from Shanghai SLAC Laboratory Animal Co. All experimental protocols were approved by the Institutional Animal Ethics Committee of Bengbu Medical College (2022-117). The rats were randomly assigned to different treatment groups including control, DN model, AC drug, and DN + AC. A rat model was established based on a previously published study[16], which is a commonly used animal model for diabetes induction and the study of DN progression. Rats in the DN and DN + AC groups were given a high-fat diet for 8 wk, while those of the control and AC drug groups were given a normal diet. At week 5, these rats were injected intraperitoneally with 60 mg/kg of STZ (v900890-1 g; Sigma, St. Louis, MO, United States), while rats in the control and AC drug groups received normal saline injections. Changes in body weight, blood glucose, kidney weight/body weight ratio (mg/g), serum creatinine, blood urea nitrogen, and 24-h urine protein, as well as histological changes, were analyzed to confirm the onset of DN. After DN induction, rats in the AC and DN + AC groups were administered with AC (dissolved in normal saline) at 10 mg/kg body weight/d by gavage for 4 wk, while the DN and control groups were administered the same volume of normal saline alone.

Renal histology and electron microscopy

The samples for the renal histological studies were prepared following a specific protocol. The animals were euthanized by cervical dislocation. Following euthanasia, the right kidney of each rat was immediately dissected and fixed with 40 g/L paraformaldehyde at room temperature for over 24 h. Subsequently, the samples underwent standard histological analysis procedures and were stained with hematoxylin and eosin (H&E), Masson's, and Periodic acid-Schiff (PAS) stains. The stained tissue sections were then examined using confocal laser scanning microscopy for analysis. Additionally, to observe ultrastructural changes in the kidney, the prepared samples underwent transmission electron microscopy (TEM) following standard procedures. The resulting TEM images were recorded and analyzed.

Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from digested HBZY-1 cells using Trizol reagent (Invitrogen, Carlsbad, CA, United States). Subsequently, cDNA was synthesized from 2 μg of total RNA with a cDNA synthesis kit (Takara Co. Ltd., Dalian, China). The quantitative real-time polymerase chain reaction (qRT-PCR) analysis was carried out using the SYBR Premix Ex

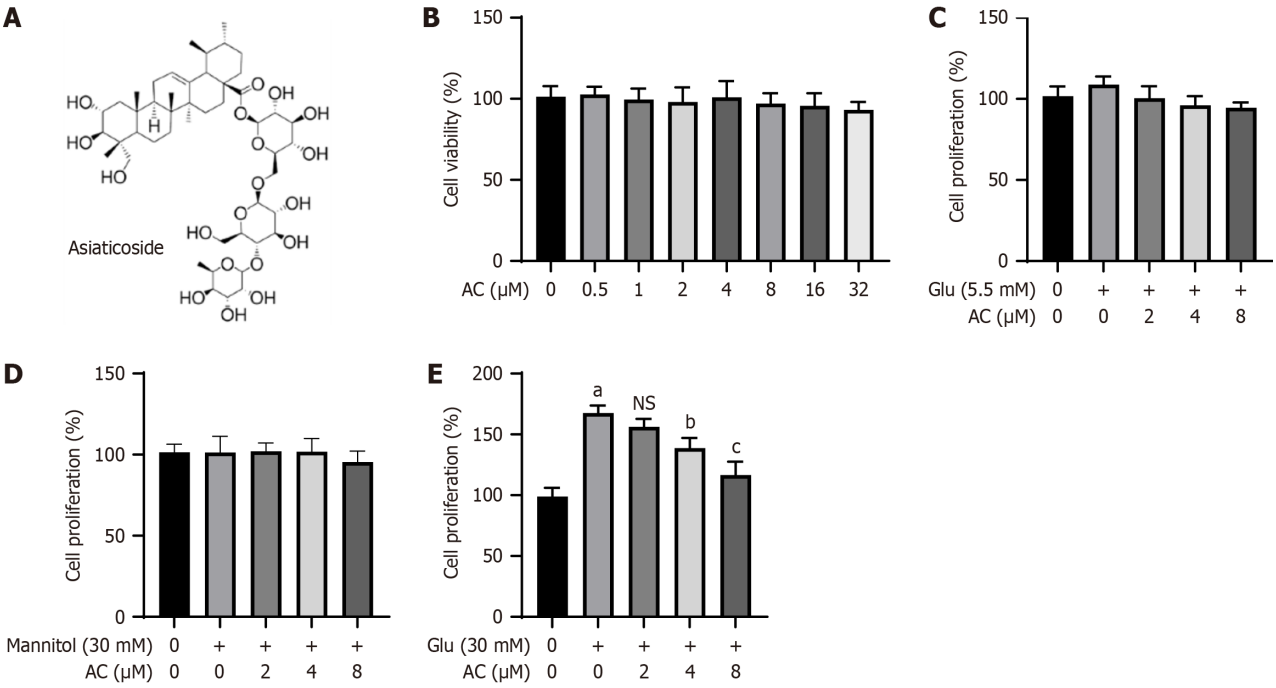


Figure 1 Effect of asiaticoside on high glucose-induced proliferation of rat glomerular mesangial cells. A: Chemical structure of asiaticoside (AC); B: Viability of rat glomerular mesangial cells (HBZY-1) treated with AC at different concentrations (0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μM) for 24 h detected by Cell Counting Kit-8 (CCK-8) assay; C: Proliferation levels of HBZY-1 cells after co-treatment of AC at different concentrations (0, 2, 4, and 8 μM) and glucose (5.5 mmol/L) for 48 h detected by CCK-8 assay; D: Proliferation of HBZY-1 cells after co-treatment of AC treatment at different concentrations (0, 2, 4, and 8 μM) and mannitol (30 mmol/L) detected by CCK-8 assay; E: Proliferation of HBZY-1 cells treated with AC at different concentrations (0, 2, 4, and 8 μM) and glucose (30 mmol/L) detected by CCK-8 assay. n = 3 experiments. $^{a}P < 0.001$ *vs* control, $^{b}P < 0.01$, and $^{c}P < 0.001$ *vs* (Glu, 30 mmol/L + AC, 0 μM); not significant ($P > 0.05$) *vs* (Glu, 30 mmol/L + AC 0 μM). AC: Asiaticoside; Glu: Glucose; NS: Not significant.

TAQTM II kit (Takara) to measure *IL-6, IL-8, TNF-α*, and collagen I levels in HBZY-1 cells from different groups (Con, HG, HG + 2 μM of AC, HG + 4 μM of AC, and HG + 8 μM of AC), along with mRNA levels of collagen IV and fibronectin. Finally, the relative fold change in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

The sequences for the forward (F) and reverse (R) primers are as follows: *IL-6*: F, 5′-CCAGTTGCCTTCTTGGGACT-3′ and R, 5′-TCTGACAGTGCATCATCGCT-3′; *IL-8*: F, 5′-GAGTTTGAAGGTGATGCCGC-3′ and R, 5′-CTTCTGAACCATGGGGGCTT-3′; *TNF-α*: F, 5′-ACTGAACTTCGGGGTGATCG-3′ and R, 5′-GCTTGGTGGTTTGCTACGAC-3′; Collagen I: F, 5′-ACATGTTCAGCTTTGTGGACC-3′ and R, 5′-CTTTGCATAGCACGCCATCG-3′; Collagen IV: F, 5′-GCCCGTGGATCCCATAGGT-3′ and R, 5′-GGAGCAGCAACAGGATAGGC-3′; Fibronectin: F, 5′-GGATCCCCTCCCAGAGAAGT-3′ and R, 5′-GGGTGTGGAAGGGTAACCAG-3′; and *GAPDH*: F, 5′-CCGCATCTTCTTGTGCAGTG-3′ and R, 5′-ACCAGCTTCCCATTCTCAGC-3′

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) tests were conducted using corresponding kits *as per* the standard procedure. Initially, the medium from the treated HBZY-1 cells was collected and centrifuged at 13000 × g for 10 min. Subsequently, the levels of IL-6, IL-8, and TNF-α in the isolated cell supernatants were determined using commercial ELISA Kits from Jikai Biotechnology, Shanghai, China, following the instructions provided by the manufacturer.

Redox state assessment

The redox state was assessed by quantifying the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and ROS. Specifically, MDA content, SOD activity, and ROS content in HBZY-1 cells or tissues were measured with an MDA assay kit (Nanjing Jiancheng Institute of Biological Engineering), total SOD activity assay kit (Nanjing Jiancheng Institute of Biological Engineering), and ROS assay kit (Beijing Solebo Technology Co. Ltd.), respectively, following the provided instructions.

Western blot analysis

HBZY-1 cells or renal tissues were subjected to lysis using Radio Immunoprecipitation Assay (RIPA) buffer containing protease inhibitor (Beyotime, Shanghai, China). Protein concentrations were determined with the bicinchoninic acid Protein Assay Kit (ThermoFisher Scientific, Waltham, United States). Subsequently, 30 μg of the lysate was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoresis was run at 80 V until the sample reached the separating gel, followed by separation at 120 V. Proteins were then transferred to polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, United States) membranes after electrophoresis. PVDF membranes were blocked with 5% skim milk for 1 h at room temperature, followed by overnight incubation at 4 °C with antibodies against NRF2, HO-1, NQO-1,

collagen I, collagen IV, and fibronectin (Proteintech, Wuhan, China). After washing with Tris-buffered saline with Tween 20 (TBST) buffer, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Proteintech) for 2 h at room temperature, with intermittent TBST buffer washes. Protein bands were visualized using a chemiluminescent reagent (Thermo Fisher Scientific) and quantified by densitometry analysis.

Statistical analysis

Data of all the experimental results are represented as the mean ± SD. Data analyses were conducted using a *t*-test or one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey's test to compare the differences between groups at a significance level of $P < 0.05$.

RESULTS

AC inhibits the proliferation of HG-induced HBZY-1 cells

Prior to validating the activity of AC in HG-induced cell model, we initially assessed the cytotoxic impact of various concentrations of AC (Figure 1A), within the gradient range of 0.5 μM to 32.0 μM on HBZY-1 cells (Figure 1B). CCK-8 results revealed that different doses of AC (ranging from 0.5 μM to 32.0 μM) did not exhibit significant cytotoxic effects on viability of HBZY-1 cells. Subsequently, we introduced normal glucose (5.5 mmol/L), mannitol (30.0 mmol/L), and high glucose (HG, 30.0 mmol/L) levels in the medium along with AC to examine their impact on HBZY-1 cell proliferation. As illustrated in Figure 1C-E, the addition of HG notably enhanced the proliferation of HBZY-1 cells ($P < 0.001$), while normal glucose and mannitol did not promote cell proliferation. AC at high doses (4 μM and 8 μM) could effectively inhibit HG-induced proliferation of HBZY-1 cells ($P < 0.01$). These data suggest the anti-proliferation effect of AC on HG-induced HBZY-1 cells.

AC reduces HG-induced inflammatory responses and oxidative stress

The impact of different concentrations of AC on HG-induced inflammatory responses and oxidative stress was examined in HBZY-1 cells. Figure 2A and B demonstrates that the mRNA levels of *IL-6*, *IL-8*, and *TNF-α* in HBZY-1 cells, as well as their contents in the supernatant, were notably elevated following HG treatment ($P < 0.001$) compared to those in control cells. Additionally, AC led to a dose-dependent decrease in the mRNA levels of *IL-6*, *IL-8*, and *TNF-α* in HG-induced cells, as well as the contents of these cytokines in the supernatants ($P < 0.01$ or $P < 0.001$). HG treatment induced significantly higher levels of ROS and MDA in HBZY-1 cells compared to the absence of HG treatment ($P < 0.001$), while SOD activity was markedly reduced post HG treatment. A gradual reduction in ROS and MDA levels, coupled with an increase in SOD activity, was observed with increasing AC concentrations in the presence of HG treatment ($P < 0.01$ or $P < 0.001$) (Figure 2C-E).

AC suppresses HG-induced fibrosis in HBZY-1 cells

The expression levels of collagen I, collagen IV, and fibronectin serve as markers for cellular fibrosis. As shown in Figure 3, results from qRT-PCR and WB analysis demonstrated that HG treatment led to significantly elevated mRNA and protein levels of collagen I, collagen IV, and fibronectin in HBZY-1 cells ($P < 0.001$) compared to the control group. Moreover, increasing AC concentrations resulted in a gradual decrease in the mRNA and protein levels of collagen I, collagen IV, and fibronectin in HG-induced HBZY-1 cells ($P < 0.01$ or $P < 0.001$). Therefore, AC suppresses HG-induced fibrosis in HBZY-1 cells.

AC regulates the HG effect by activating the NRF2/HO-1 pathway

We further observed a decrease in nuclear NRF2 protein expression and total HO-1 and NQO-1 protein levels in HBZY-1 cells following HG treatment. Conversely, AC treatment led to increased levels of nuclear NRF2, total HO-1, and NQO-1 proteins in a dose-dependent manner (Figure 4A). The requirement of NRF2 in the activity of AC was further elucidated by using the NRF2 inhibitor ML385. The combination treatment of HG + AC + ML385 reduced protein expression levels of nuclear NRF2, HO-1, and NQO-1 (Figure 4B). Moreover, the anti-inflammatory effect of AC on IL-6, IL-8, and TNF-α was abolished after ML385 inhibition of NRF2 activity (Figure 4C). Similar results were observed in the oxidative stress, as evidenced by the elevated ROS and MDA levels, as well as the decreased SOD activity upon ML385 treatment (Figure 4D). Besides, ML385 treatment also increased the levels of collagen I, collagen IV, and fibronectin proteins after HG + AC treatment (Figure 4E). These results suggest that AC may modulate the effects of HG by activating the NRF2/HO-1 pathway.

AC mitigates oxidative stress and fibrosis in a DN rat model

Subsequently, we evaluated the effect of AC administration on oxidative stress and fibrosis in a DN rat model. Compared with the sham group, no significant differences in the body weight, blood glucose, kidney weight/body weight ratio, serum creatinine, blood urea nitrogen, and 24-h urinary protein were observed in the AC treatment alone group. Compared to the sham group, the body weight, blood glucose, serum creatinine, blood urea nitrogen, and 24-h urinary protein were significantly elevated in the DN group. AC administration significantly reduced body weight and biochemical parameters in the DN model group, such as blood glucose, serum creatinine, blood urea nitrogen, and 24-h urinary protein levels (Figure 5A-F).

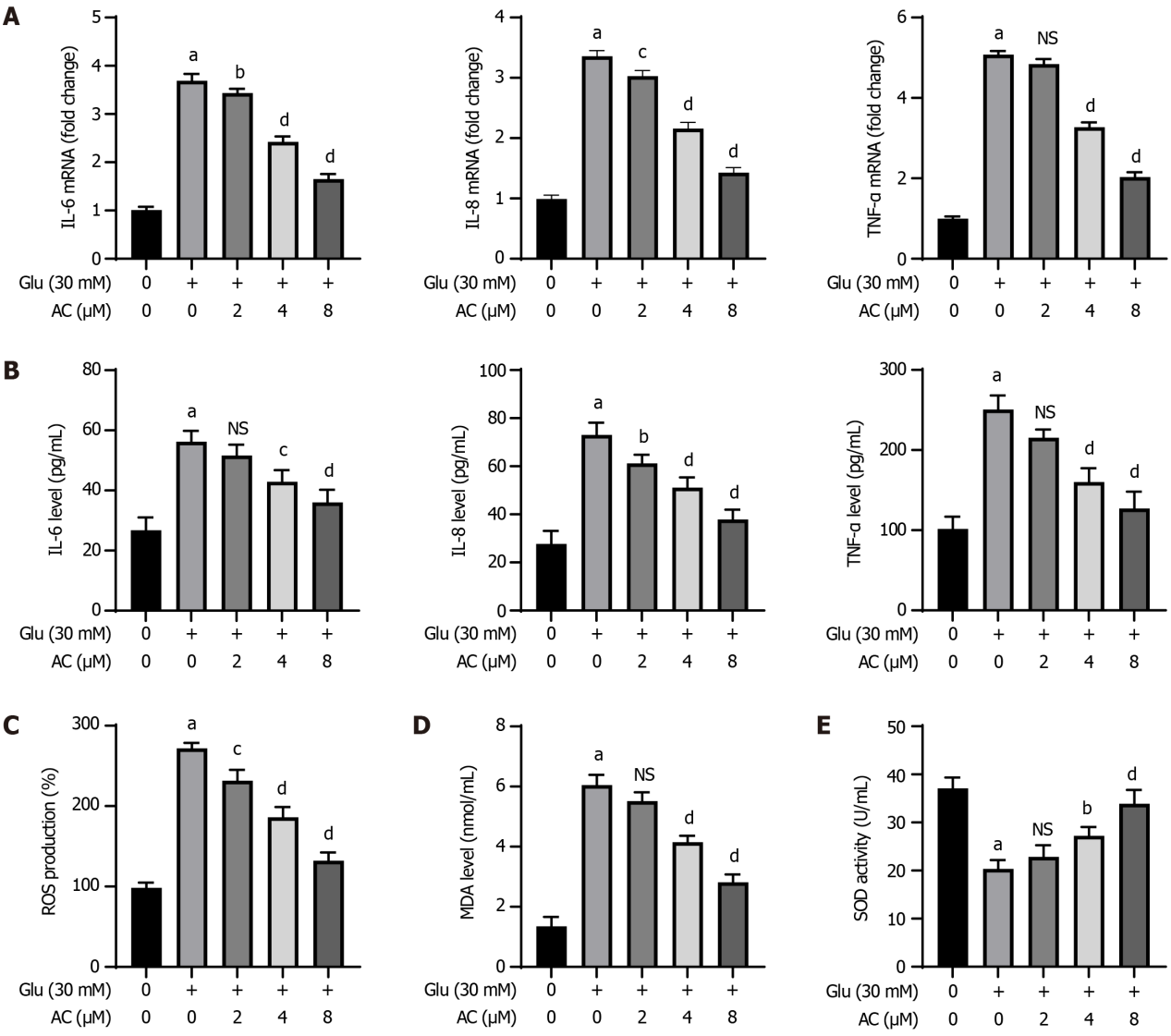


Figure 2 Effect of asiaticoside on high glucose-induced inflammatory response and oxidative stress in rat glomerular mesangial cells. A: Quantitative real-time polymerase chain reaction analysis of the mRNA levels of interleukin (IL)-6, *IL*-8, and tumor necrosis factor (TNF)-α in rat glomerular mesangial (HBZY-1) cells treated with asiaticoside at different concentrations (0, 2, 4, and 8 μM) and glucose (30 mmol/L); B: IL-6, IL-8, and TNF-α in the supernatant of HBZY-1 cells determined by ELISA; C-E: Reactive oxygen species, malondialdehyde, and superoxide dismutase levels in HBZY-1 cells determined by ELISA using corresponding kits. n = 3 experiments. $^{a}P < 0.001$ *vs* control, $^{b}P < 0.05$, $^{c}P < 0.01$, and $^{d}P < 0.001$ (Glu, 30 mmol/L + AC, 0 μM); not significant ($P > 0.05$) *vs* (Glu, 30 mmol/L + AC 0 μM). AC: Asiaticoside; Glu: Glucose; NS: Not significant.

The renal histological observations in the AC group revealed no significant changes in the renal tissues compared to those in the sham group (Figure 5G). However, HE staining showed an increase in renal inflammatory infiltration in the DN model group. Additionally, Masson's staining indicated severe renal interstitial fibrosis in the DN model group, PAS staining showed glomerular hypertrophy and mesangial matrix dilation, and TEM imaging revealed podocyte detachment and disappearance. These pathophysiological changes in the rat model were largely mitigated with AC treatment. Besides, there were no significant differences in the levels of IL-6, IL-8, TNF-α, MDA, and SOD in the serum of rats of the AC treatment group compared to the sham group. The DN group exhibited significantly increased levels of IL-6, IL-8, TNF-α, and MDA, with a considerable decrease in SOD activity. AC treatment resulted in a significant reversal of these changes compared to the DN group, indicating that AC effectively alleviated inflammatory and oxidative stress in the DN rats (Figure 5H and I).

We further analyzed the expression levels of proteins related to fibrosis in the renal tissues. Compared to the sham group, there were no significant changes in the levels of collagen I, collagen IV, and fibronectin proteins in the renal tissues of rats of the AC treatment alone group. The DN group showed significantly higher expression levels of these fibrogenic markers, while AC administration resulted in a notable decrease in the levels of collagen I, collagen IV, and fibronectin proteins in the renal tissues of rats of the DN model group (Figure 5J). Additionally, the DN group displayed decreased levels of nuclear NRF2 and total HO-1, as well as NQO-1 proteins in the renal tissues. However, AC treatment restored the expression of nuclear NRF2 and total HO-1, as well as NQO-1 protein levels, indicating activation of the NRF2 signaling pathway by AC (Figure 5K).

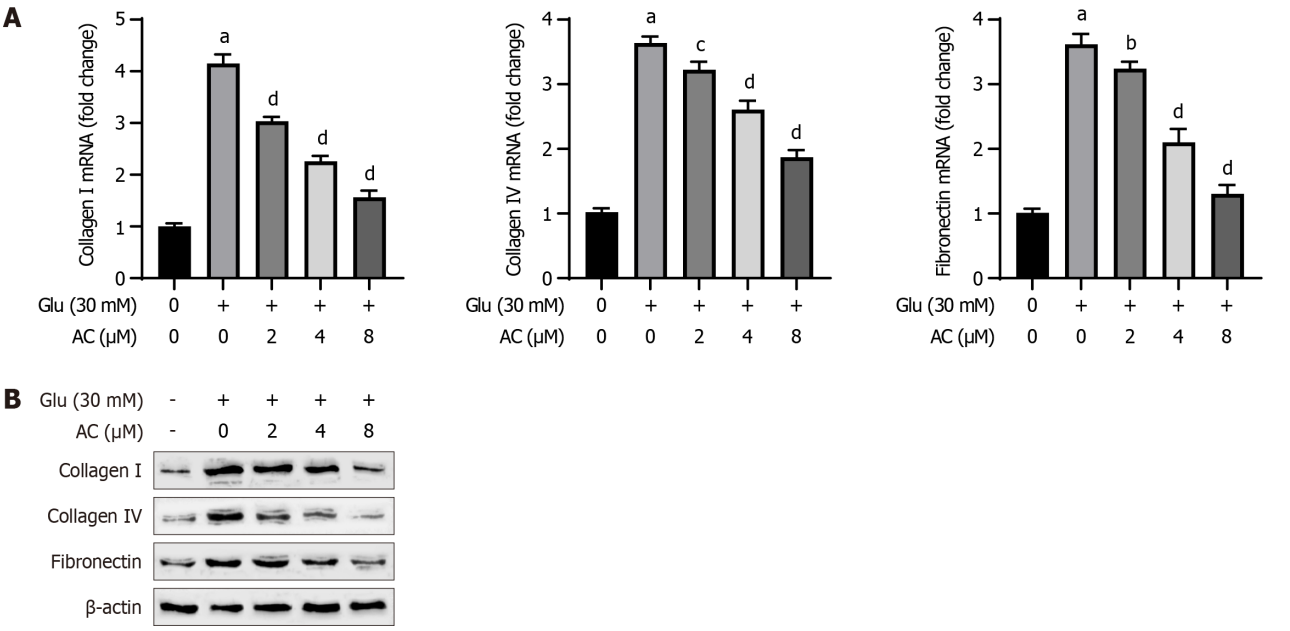


Figure 3 Effect of asiaticoside on high glucose-induced fibrosis in rat glomerular mesangial cells. A:Quantitative real-time polymerase chain reaction analysis of the mRNA levels of collagen I, collagen IV, and fibronectin in rat glomerular mesangial (HBZY-1) cells treated with asiaticoside at different concentrations (0, 2, 4, and 8 μM) and glucose (30 mmol/L); B: Western blot analysis of the protein levels of collagen I, collagen IV, and fibronectin in HBZY-1 cells. *n* = 3 experiments. $^{a}P < 0.001$ *vs* control; $^{b}P < 0.05$, $^{c}P < 0.01$, and $^{d}P < 0.001$ *vs* Glu (30 mmol/L). AC: Asiaticoside; Glu: Glucose.

DISCUSSION

This study demonstrated that AC inhibited HG-induced proliferation, inflammatory responses, oxidative stress, and fibrosis in HBZY-1 cells, and ameliorated renal injury in a streptozotocin-induced DN rat model. AC reduced levels of inflammatory cytokines, ROS, MDA, and fibrogenic markers while increasing superoxide dismutase (SOD) activity. Notably, the renoprotective effects of AC were associated with the activation of the NRF2/HO-1 signaling pathway, suggesting that AC may have therapeutic potential for DN by targeting inflammation, oxidative stress, and fibrosis through NRF2-associated antioxidant mechanisms.

HBZY-1 cells are one of the major cell types used in the study of renal defects such as DN[17]. The abnormal proliferation of mesangial cells is increasingly recognized as potentially playing a crucial role in the pathophysiological mechanism of early DN. A previous study has indicated that HG can significantly increase the proliferation of HBZY-1 cells, leading to renal interstitial fibrosis and ultimately chronic kidney failure[18]. Building on this foundation, HBZY-1 cells were exposed to normal glucose, high mannitol, and HG in this study. The results showed that HG notably enhanced the proliferation of HBZY-1 cells, whereas glucose or mannitol had no significant impact on their proliferation. Treatment with AC inhibited the HG-induced proliferation of HBZY-1 cells in a dose-dependent manner.

Prior research has established a strong correlation between heightened cellular oxidative stress triggered by hyperglycemia and the onset as well as progression of DN[19]. This increase in ROS levels could directly result from hyperglycemia-induced oxidative stress[20]. Among the products of oxidative stress, MDA signifies the end product of lipid peroxidation caused by free radicals, with higher MDA levels indicating a more severe oxidative stress response [21]. SOD serves as a crucial antioxidant marker that facilitates the conversion of superoxide to oxygen and hydrogen peroxide[22]. The levels of MDA and SOD are utilized to gauge the degree of oxidative stress in cell and renal tissues. In this study, a notable rise in ROS and MDA levels was observed in HG-treated HBZY-1 cells and in renal tissues of DN rats. Additionally, SOD activities were significantly decreased in the HG-induced cell model and in DN rats. Treatment with AC resulted in a marked reversal of these effects on SOD and MDA levels in both HBZY-1 cells and DN rats, indicating that AC could effectively mitigate oxidative stress in DN rats and mesangial cells induced by HG.

Inflammatory responses in DN are primarily triggered by oxidative stress, leading to accelerated renal injury in rats. Tashiro *et al*[23] found high levels of pro-inflammatory factors in early-stage DN patients. Similarly, our study observed significant increases in IL-6, IL-8, and TNF-α levels in the serum of DN rats, as well as in the mRNA levels of these cytokines in HBZY-1 cells treated with HG. Treatment with AC reversed these changes, demonstrating a strong anti-inflammatory effect which can be attributed to its effect to antagonize the oxidative stress.

The main pathological characteristics of DN involve thickening of the glomerular basement membrane, dilation of the mesangium, glomerulosclerosis, and tubulointerstitial fibrosis[24]. In this study, we utilized H&E, Masson's, and PAS staining techniques to assess the pathogenic changes in the renal tissues. Treatment with AC was found to significantly mitigate pathological renal damage in rats. Collagen IV and fibronectin are commonly used as fibrosis markers in DN clinical diagnosis[25,26]. Our findings revealed a notable increase in the expression levels of collagen I, collagen IV, and fibronectin in the renal tissues of DN rats induced by STZ, indicting a significant rise in renal interstitial fibrosis in DN rats. In line with this, both mRNA and protein levels of collagen I, collagen IV, and fibronectin were markedly elevated in

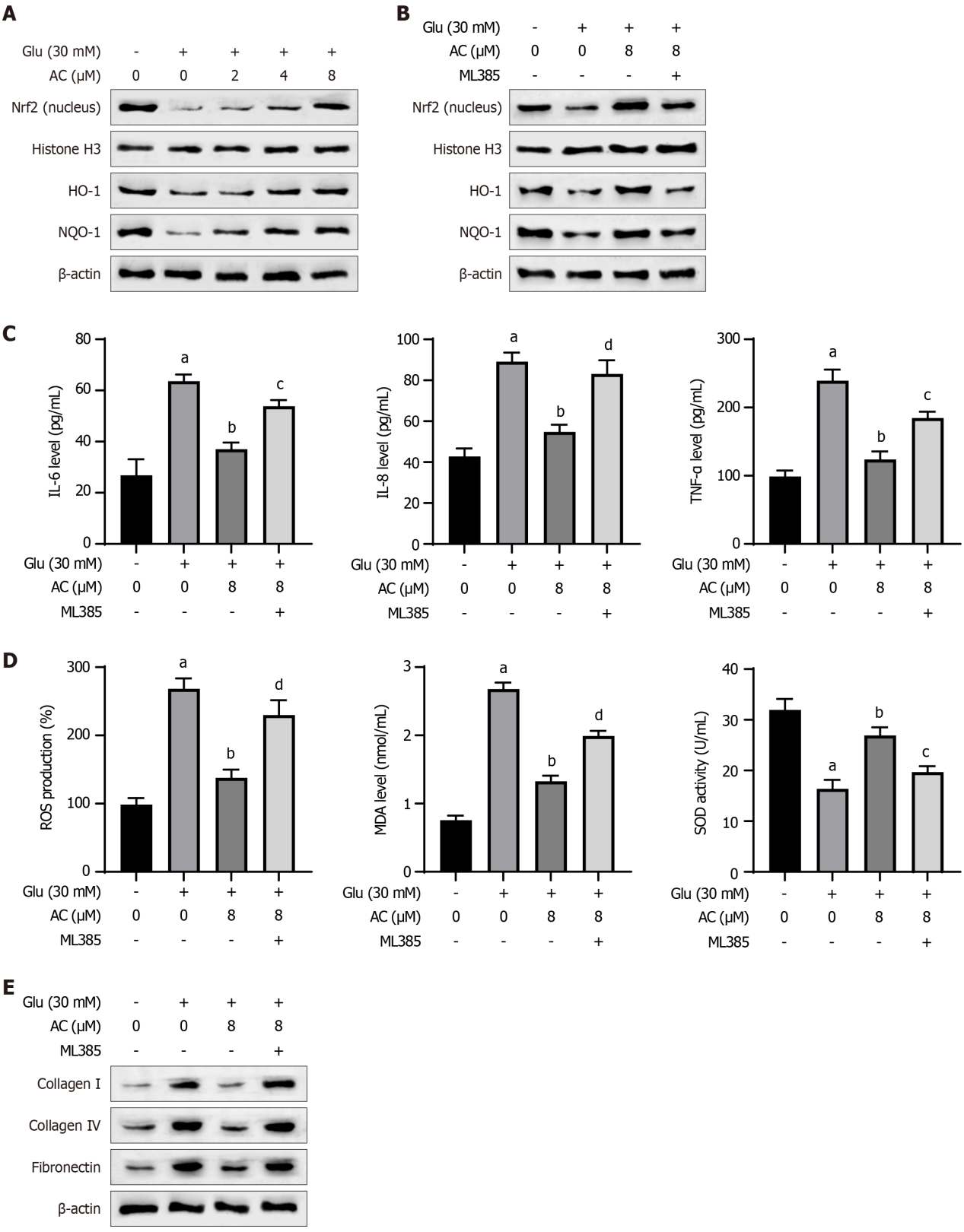
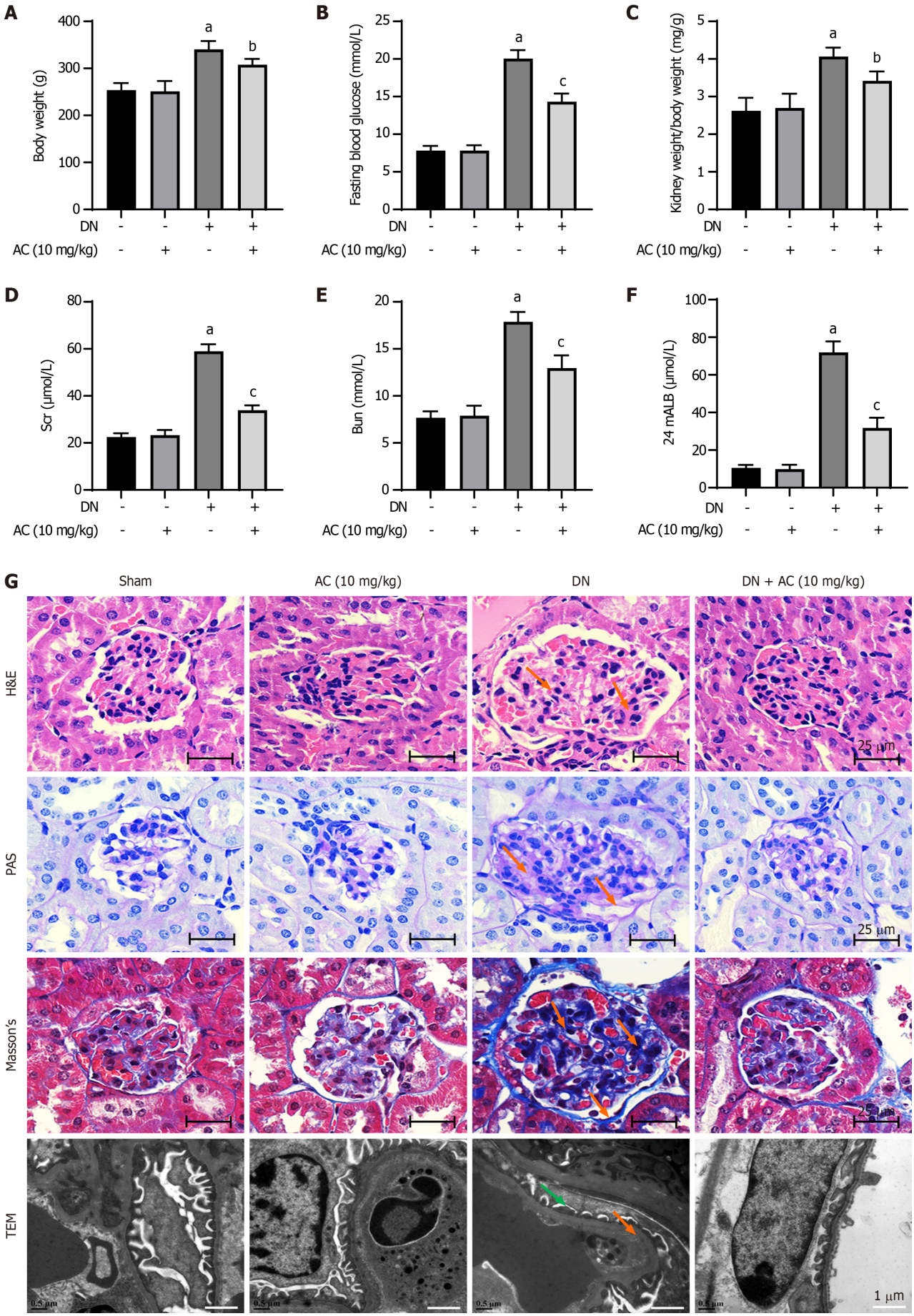


Figure 4 Asiaticoside regulates high glucose-induced effects on rat glomerular mesangial cells by activating the NRF2/ heme oxygenase-1 pathway. A: Western blot analysis of the NRF2 protein expression and total heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase (Quinone) 1 (NQO-1) protein expression in the nucleus of rat glomerular mesangial (HBZY-1) cells treated with asiaticoside (AC) at different concentrations (0, 2, 4, and 8 μM) and glucose (30 mmol/L); B: Western blot analysis of NRF2 protein expression and total HO-1 and NQO-1 protein expression in the nucleus of HBZY-1 cells treated with AC (8 μM), glucose (30 mmol/L), and ML385 (NRF2 inhibitor, 5 μM); C: Interleukin (IL)-6, IL-8, and tumor necrosis factor-α levels in the supernatant of HBZY-1 cells; D: Reactive oxygen species, malondialdehyde, and superoxide dismutase in HBZY-1 cells; E: Western blot analysis of the expression levels of collagen I, collagen IV, fibronectin, and β-actin in HBZY-1 cells. n = 3 experiments. $^{a}P < 0.001$ *vs* control, $^{b}P < 0.001$ *vs* Glu (30 mmol/L), $^{c}P < 0.01$, and $^{d}P < 0.001$ *vs* Glu (30 mmol/L) + AC (8 μM). AC: Asiaticoside; Glu: Glucose.


A
Body weight (g)
400
300
200
100
0
a
b
DN - - + +
AC (10 mg/kg) - + - +
B
Fasting blood glucose (mmol/L)
25
20
15
10
5
0
a
c
DN - - + +
AC (10 mg/kg) - + - +
C
Kidney weight/body weight (mg/g)
5
4
3
2
1
0
a
b
DN - - + +
AC (10 mg/kg) - + - +
D
Scr (μmol/L)
80
60
40
20
0
a
c
DN - - + +
AC (10 mg/kg) - + - +
E
Bun (mmol/L)
20
15
10
5
0
a
c
DN - - + +
AC (10 mg/kg) - + - +
F
24 mALB (μmol/L)
100
80
60
40
20
0
a
c
DN - - + +
AC (10 mg/kg) - + - +
G
Sham
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DN
DN + AC (10 mg/kg)
H&E
PAS
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25 μm
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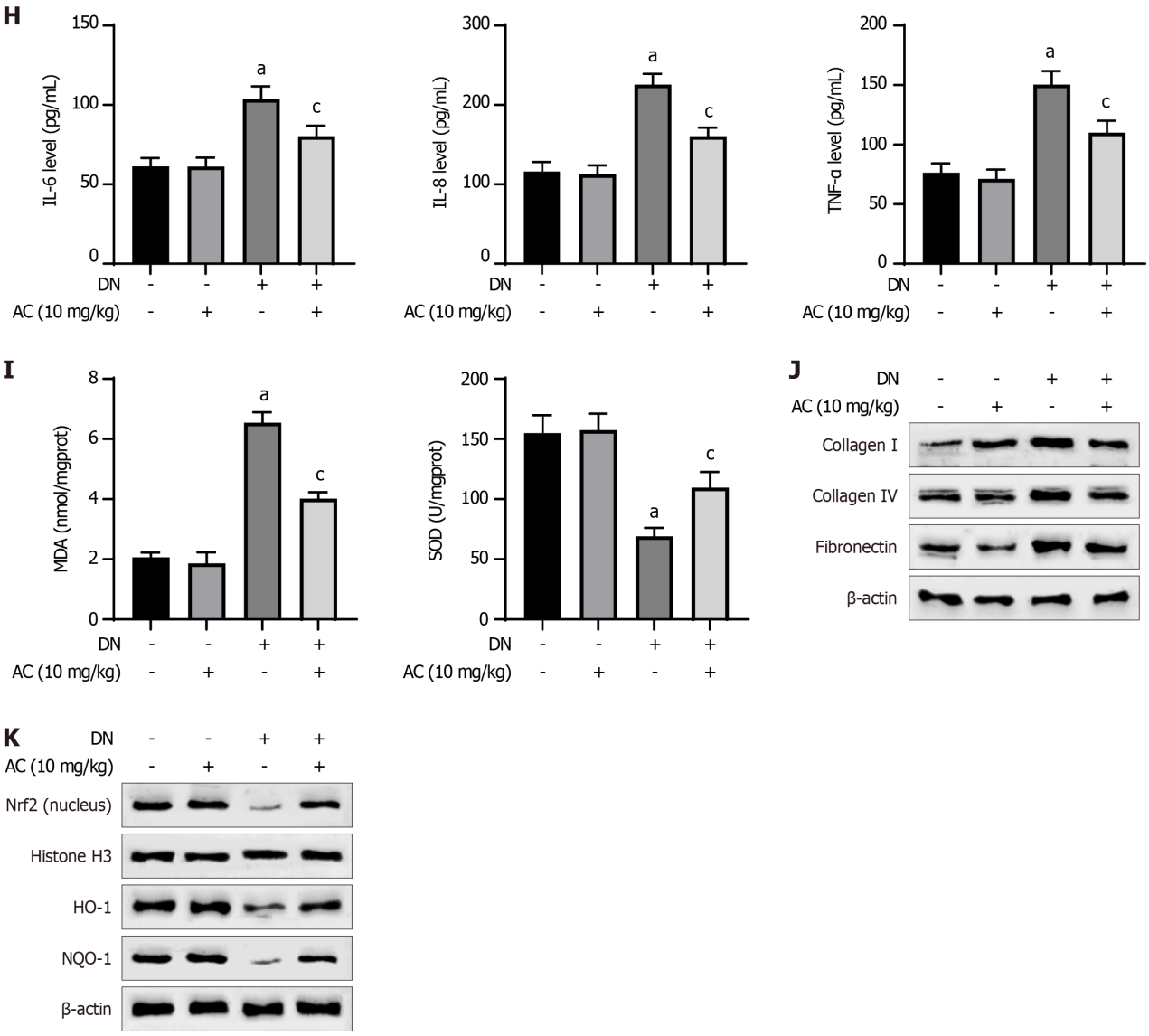

Figure 5 Asiaticoside attenuates oxidative stress and fibrosis in a diabetic nephropathy rat model. A-F: Graphical summary of the changes in body weight, blood glucose, kidney weight/body weight ratio, serum creatinine, blood urea nitrogen, and 24-h urine protein in control, diabetic nephropathy (DN) model, asiaticoside (AC) drug, and DN + AC groups; G: Results of hematoxylin and eosin (scale bar: 25 μm, orange arrows indicate mesangial hyperplasia), Masson's (scale bar: 25 μm, orange arrows mark collagen fiber deposition), and Periodic acid-Schiff staining (scale bar: 25 μm, orange arrows indicate glycogen deposition), as well as TEM (scale bar: 1 μm, orange arrow shows uniform thickening of the basement membrane, and green arrow indicates loss of the podocyte foot process); H and I: Graphical summary of (H) the contents of interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α, and (I) the contents of malondialdehyde and superoxide dismutase in rat serum of each experimental group; J: Western blot analysis of the protein expression of collagen I, collagen IV, and fibronectin; K: Protein expression of nuclear NRF2 and total heme oxygenase-1, as well as NAD(P)H dehydrogenase (Quinone) 1, in renal tissues. n = 6 animals in each group. $^{a}P < 0.001$ *vs* Sham, $^{b}P < 0.05$, and $^{c}P < 0.001$ *vs* diabetic nephropathy. DN: Diabetic nephropathy; AC: Asiaticoside; PAS: Periodic acid-Schiff; TEM: Transmission electron microscopy; HO-1: Heme oxygenase-1; NQO-1: NAD(P)H dehydrogenase (Quinone) 1; IL: Interleukin; TNF: Tumor necrosis factor; H&E: Hematoxylin and eosin.

HG-treated HBZY-1 cells. Treatment with AC effectively suppressed these changes, suggesting that AC could attenuate renal injury in diabetic rats by inhibiting renal interstitial fibrosis.

The NRF2/HO-1 signaling pathway plays a significant role in counteracting oxidative stress, and recent research has highlighted that enhancing NRF2/HO-1 signaling can mitigate renal injury in diabetic rats[27]. In response to oxidative stress, NRF2 translocates to the nucleus and triggers the expression of HO-1 and NQO-1. We observed a significant activation of nuclear NRF2 expression, total HO-1, and NQO-1 protein in kidney tissue of DN rats and HBZY-1 cells following AC treatment. These results suggest that the beneficial effects of AC on inflammation, oxidative stress, and renal fibrosis may be linked to the activation of the NRF2/HO-1 pathway. Furthermore, by using the NRF2 inhibitor ML385, the protective effect of AC on inflammatory, oxidative stress, and fibrogenic features in HBZY-1 cells was suppressed. Overall, these findings support the notion that the anti-inflammatory, anti-oxidative, and anti-fibrotic properties of AC are closely associated with the activation of the NRF2/HO-1 signaling pathway.

While this study provides valuable insights into the therapeutic potential of AC for DN, certain limitations should be acknowledged. First, the STZ-induced diabetes model used in this study may not fully recapitulate the complex pathophysiology of human DN, which often develops gradually over years in the context of chronic hyperglycemia and

metabolic dysregulation. The effect of AC should be further evaluated in genetically modified animal models. Additionally, the assessment of renal fibrosis in this study relied primarily on the evaluation of fibrogenic markers (collagen I, collagen IV, and fibronectin) and standard histological stains (Masson's trichrome and PAS). The incorporation of Picrosirius red staining, which specifically stains collagen fibrils and facilitates quantitative analysis of fibrosis, could have provided more comprehensive and quantitative insights into the extent of renal fibrosis and the mitigating effects of AC. Future studies are also warranted to elucidate how AC activates NRF2 to antagonize oxidative and inflammatory stress in DN models.

CONCLUSION

In summary, our study has demonstrated that AC could effectively ameliorate renal damages in a rat model of DN by attenuating oxidative stress, inflammatory responses, and renal fibrosis through activating the NRF2 pathway. These findings suggest that AC may be employed as a protective agent for ameliorating DN progression.

FOOTNOTES

Author contributions: Zhuang LG and Ge XX participated in literature search, study design, and manuscript writing and critical revision; Zhang R, Jin GX, Pei XY, and Wang Q participated in data collection, analysis, and interpretation; and all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Effect of cuproptosis on acute kidney injury after cardiopulmonary bypass in diabetic patients

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Abstract

BACKGROUND

Cardiopulmonary bypass (CPB) is a common procedure in cardiac surgery. CPB is a high-risk factor for acute kidney injury (AKI), and diabetes is also such a factor. Diabetes can lead to copper overload. It is currently unclear whether AKI after CPB in diabetic patients is related to copper overload.

AIM

To explore whether the occurrence of CPB-AKI in diabetic patients is associated with cuproptosis.

METHODS

Blood and urine were collected from clinical diabetic and non-diabetic patients before and after CPB. Levels of copper ion, lactate, glucose, heat shock protein-70 (HSP-70), and dihydrolipoamide dehydrogenase (DLAT) were determined. A diabetic rat model was established and CPB was performed. The rats were

assessed for the development of CPB-AKI, and for the association of AKI with cuproptosis by detecting copper levels, iron-sulfur cluster proteins and observation of mitochondrial structure by electron microscopy.

RESULTS
CPB resulted in elevations of copper, lactate, HSP-70 and DLAT in blood and urine in both diabetic and non-diabetic patients. CPB was associated with pathologic and mitochondrial damage in the kidneys of diabetic rats. Cuproptosis-related proteins also appeared to be significantly reduced.

CONCLUSION
CPB-AKI is associated with cuproptosis. Diabetes mellitus is an important factor aggravating CPB-AKI and cuproptosis.

Key Words: Cardiopulmonary bypass; Acute kidney injury; Cuproptosis; Diabetes; Copper overload; Iron-sulfur cluster proteins

Core Tip: This study found that cardiopulmonary bypass (CPB) resulted in elevations of copper, lactate, heat shock protein-70 and dihydrolipoamide dehydrogenase in blood and urine in both diabetic and non-diabetic patients. CPB was associated with pathologic and mitochondrial damage in the kidneys of diabetic rats. Cuproptosis-related proteins also appeared to be significantly reduced. CPB- acute kidney injury (AKI) is associated with cuproptosis. Diabetes mellitus is an important factor aggravating CPB-AKI and cuproptosis. We believe this manuscript is valuable for all the researchers who are interested in.

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INTRODUCTION

Cardiopulmonary bypass (CPB) has brought new opportunities for valve replacement and coronary artery bypass surgery and is a common procedure in cardiac surgery[1]. However, CPB can lead to a series of complications, the most common of which is acute kidney injury (AKI)[2,3]. One study found that 22.3% of patients with CPB worldwide develop AKI, with severe AKI leading to a mortality rate of more than 35%. Even mild AKI lead to a more than four-fold increase in the risk of death and prolonged hospital stay[4]. Moreover, the incidence of AKI is approximately two times greater with CPB heart bypass surgery compared with non-CPB bypass surgery[5]. Another study reported that 2%-5% of patients with CPB-AKI require renal replacement therapy[6], which seriously affects patient prognosis[6] and increases economic burden. There is also evidence that transient and mild kidney injury is associated with an increased risk of chronic kidney disease[4].

Diabetes mellitus (DM) is an independent risk factor for AKI[7,8]. Approximately 70%-80% of patients with heart disease have comorbid diabetes or abnormal glucose metabolism[9]. Additionally, 30%-40% of diabetic patients will have varying degrees of combined kidney damage[10,11]. CPB-AKI in diabetic patients is currently thought to have two causes. First, the long-term chronic inflammatory response, stress response, and immune imbalance state of the body induced by hyperglycemia, as well as the damage to micro-vessels caused by hyperglycemia can lead to an increased susceptibility to renal injury, causing an increased incidence of AKI. Second, cellular mechanical damage (release of protein hydrolyzing enzymes and metalloproteins) caused by *ex vivo* dilution of blood and exposure to non-physiologic surfaces during CPB, as well as dysregulation of organ flow and activation of the inflammatory cascade response caused by the non-pulsatile hemodynamics of CPB, can increase the incidence of or exacerbate renal injury. Our previous study found that perioperative hyperglycemia caused an increase in urinary toxic metabolites[12] and also suggested that patients with perioperative hyperglycemia can develop renal injury.

Clinical diagnostic indicators of perioperative AKI include creatinine levels and urine output. These indicators are imprecise and incomplete, which can lead to a delay in the diagnosis of AKI[13]. Therefore, the actual incidence of AKI may be higher than expected. Additionally, the pathogenesis of surgery-related AKI is unclear and may be related to ionic imbalances, immune dysregulation, inflammation, and stress[13]. Currently, there is no effective prevention or treatment for CPB-related AKIs[14,15].

More recently, the copper-dependent cell death driven by mitochondrial stress and damage, termed cuproptosis, has been described[16]. Cuproptosis is a new mode of cell death caused by the accumulation of copper ions and their binding to tricarboxylic acid cycle thioctylated proteins. The binding leads to aberrant oligomerization of the proteins and loss of iron-sulfur (Fe-S) cluster proteins[16]. This type of cell death depends on impaired mitochondrial respiration and

subsequent mitochondrial protein stress, rather than mitochondrial oxidative stress[17]. Generally, copper ions have very low intracellular concentrations[18], and mainly bind reversibly with metallothionein to participate in organismal physiological and pathological processes[19], such as antioxidant activity, mitochondrial respiration, and regulation of signaling pathways[20]. There are several isoforms of metallothionein. Metallothionein 2a is mainly found in renal cells. The proximal tubule, Henry's loop, distal tubule, and collecting ducts of the kidney can reabsorb metal ions, so the kidney is more susceptible to metal ion imbalance. However, it is not known what role(s) copper imbalance and cuproptosis play in AKI.

CPB causes the release of intracellular copper into the bloodstream[21]. Hemodilution involved in CPB in turn leads to a decrease in plasma copper concentration[22]. Additionally, diabetes results in a state of copper overload[23]. To date, it has not been reported domestically or internationally whether the post-CPB copper ion status and cuproptosis in patients with diabetes are involved in the development of diabetic CPB-AKI.

The aim of this study was to explore the effects of diabetes and CPB on copper, metabolism, and cuproptosis by collecting blood and urine from clinically CPB diabetic and non-diabetic patients. Furthermore, a rat model of DM was established using intraperitoneal injection of streptozotocin (STZ), followed by CPB to explore whether the occurrence of CPB-AKI in diabetic rats is associated with cuproptosis.

MATERIALS AND METHODS

Patients

All patients were treated at the First Hospital of Harbin Medical University. Please add IRB number and date in this section. The clinical trial was approved by the Ethics Committee of the Harbin Medical University on May 10, 2022, with the approval No. 202231. A total of 46 non-diabetic patients (CD group) and 58 diabetic patients (CZ group) who underwent CPB were included. Before and after CPB, the basic vital signs of the patients were measured, and blood and urine were collected (Figure 1). Blood samples were collected from patients before and after CPB in this study. Patients were enrolled for subsequent analysis based on the AKI criteria proposed in the kidney disease improving global outcomes guidelines: An increase in serum creatinine of > 0.3 mg/dL (> 26.5 μmol/L) within 48 hours.

Animals

Male Sprague-Dawley rats (500-600 g) obtained from Harbin Medical University were randomly divided into three groups: Control group (KB, n = 5), CPB group (CPB, n = 5), and diabetes CPB group (CPB + glucose, n = 5). The rats were housed individually in cages in a room maintained at 22°C-24°C with a 12-hour light/dark cycle. The animal experiments conformed to the guidelines of the National Institutes of Health on the Care and Use of Animals. And the study was approved by the Animal Management Committee of Harbin Medical University on December 25, 2022, with the approval No. 2022IIT037 (Figure 2).

Rat model of DM

The rats were fasted overnight but had free access to drinking water before induction of diabetes. Diabetes was induced by a single intraperitoneal injection of 60 mg/kg of STZ (S0130, Sigma-Aldrich) freshly dissolved in 0.1 moL/L citrate buffer at potential of hydrogen (PH) 4.5[24]. The presence of hyperglycemia was confirmed 72 hours after STZ administration. Blood samples were collected from the tail veins of rats after a 12-hour fast for blood glucose measurement. The model was considered successful if the blood glucose concentration was equal to or greater than 16.8 mmol/L. Age-matched rats were injected with an equal volume of citrate buffer to serve as non-diabetic normoglycemic controls.

CPB model

Before the induction of CPB, rats were fasted overnight with free access to drinking water. The rats were anesthetized with 6.0% sevoflurane, intubated using a 16-gauge intravenous catheter, and mechanically ventilated. The ventilator setting included a fraction of inspired oxygen of 0.21 and ventilatory rate of 60 cycles per minute. CPB in the rat was performed using the surgical techniques described previously[24,25]. Heart rate and rectal temperature were continuously monitored, and the rectal temperature was maintained at approximately 37.5 °C. After systemic heparinization using 400 IU/kg, the right femoral artery was cannulated with a 24-gauge angiocatheter. A 14-gauge multi-pore angiocatheter was introduced into the right internal jugular vein and advanced into the right atrium. Mechanical ventilation was terminated upon CPB. The CPB flow rate exceeded 80 mL/kg/minute. Circulation volume was adjusted to ensure the flow rate resulted in a flow time of 60 minutes. During CPB, an oxygen-air mixture in a 1:4 ratio passed through the membrane lung. Mechanical ventilation was restarted before the flow was reduced, and circulation volume was gradually decreased. After the rat resumed spontaneous breathing, tracheal intubation was removed, and the rat was fed in the laboratory for observation. Before and after CPB, 0.5 ml of blood was collected from the right femoral artery into a sterile EP tube. The sample was then centrifuged at 1000g for 15 minutes, and the supernatant was collected, aliquoted, and stored at -80 °C. By detecting the creatinine levels in rats and combining with kidney. Hematoxylin-eosin staining (HE) pathological changes, the establishment of the AKI model was verified. By detecting the serum creatinine levels in rats and combining with HE pathological changes with kidney, the establishment of the AKI model was verified.

Copper level test

Blood and urine were collected before and after CPB according to the actual situation of each surgery. Kidney tissues

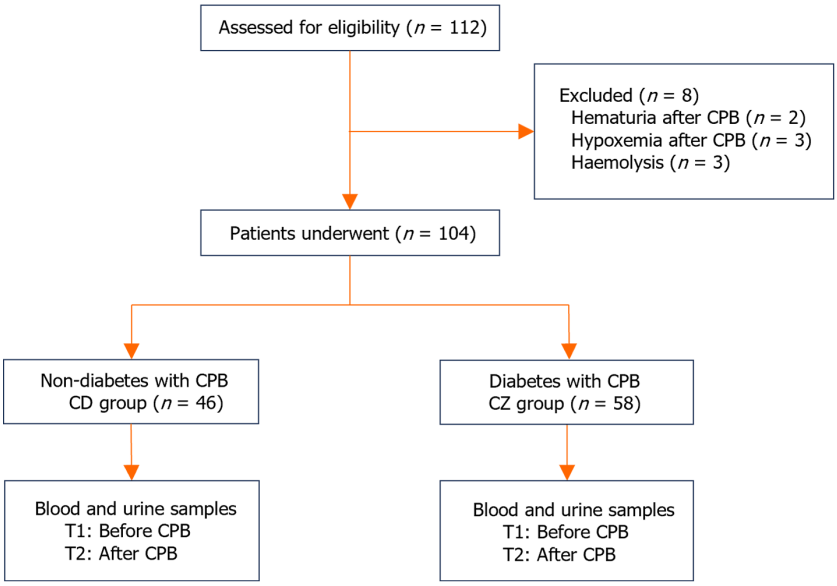

Figure 1 Patient recruitment flowchart. CPB: Cardiopulmonary bypass; CD group: Non-diabetic patients; CZ group: Diabetic patients.

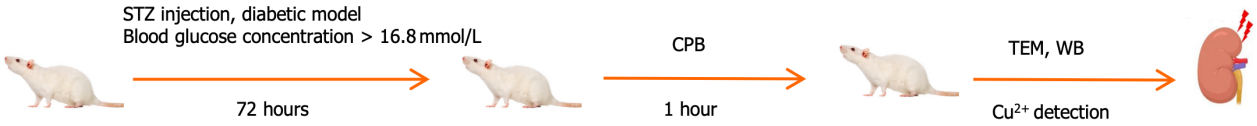

Figure 2 Experimental flow. STZ: Streptozotocin; CPB: Cardiopulmonary bypass; TEM: Transmission electron microscopy; WB: Western blotting.

from diabetic rats were collected at the end of CPB. Kidney tissue was milled in liquid nitrogen and lysed using radio immunoprecipitation assay lysis (RIPA) buffer (P0013B, Beyotime). The supernatant was collected for assay of total protein concentration using a BCA protein assay kit and the manufacturer's instructions. Copper levels in blood, urine, and kidney tissue supernatants were determined by inductively coupled plasma-mass spectrometry (ICP-MS). Briefly, exactly 0.20 mL of the sample to be tested was pipetted into a 10 mL transparent polytetrafluoroethylene centrifuge tube, followed by exactly 10 mL of concentrated highly pure nitric acid. Tube contents were mixed thoroughly for 1 minute. The sample was heated in a reactor at 120 °C until the mixture evaporated to approximately 1 mL and became clear. This nitrolyzed sample was diluted with ultrapure water to 5 mL, shaken for 1 minute, and filtered through a sterile filter membrane with a 0.22 μm pore size to remove impurities. The sample was diluted according to the concentration and analyzed by ICP-MS. (Shanghai Enzyme-linked Biotechnology Co., Ltd)[26].

Enzyme-linked immunosorbent assay

The levels of heat shock protein-70 (HSP-70) and dihydrolipoamide dehydrogenase (DLAT) in the plasma of clinical patients and rats before and after CPB were assessed using enzyme-linked immunosorbent assay kits (ab133060, Abcam and NOV-BG-HUM10729-96T, NEWBIOSCIENCE, respectively) following the manufacturers' instructions.

Western blotting

Renal samples obtained from rats were lysed in complete RIPA buffer (P0013B, Beyotime). Protein concentration of tissue homogenates was measured using the BCA protein assay kit (P0010S, Beyotime). Equal amounts of soluble protein were separated by 10% or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Each membrane was blocked for 30 minuites using QuickBlock blocking buffer and incubated overnight at 4 °C with antibodies to lipoic acid synthetase [lipoic acid synthase (LIAS); ab246917, Abcam], aconitase 2 (ACO-2; ab110321, Abcam), and succinate dehydrogenase (SDHB; ab175225, Abcam)[16]. This was followed by five washes using tris-buffered saline-tween (TBST) and incubation with secondary antibody for 50 minutes. After five washes with TBST, signals were visualized using an ECL luminescence kit (P0018S, Beyotime) and a gel imaging system.

Transmission electron microscopy

Renal tissues were fixed in 2% glutaraldehyde and 1% osmium tetroxide. Samples were processed and sectioned. The 70-80 nm thick and sections were placed on copper mesh grids. Sections were stained with uranyl acetate and lead citrate for contrast and viewed by transmission electron microscopy (TEM).

Statistical analysis

Statistical analysis was performed using statistical product and service solutions 26.0 software, and graphs were created using GraphPad Prism 9.5.1 software. Data are presented as mean ± SD. The Shapiro-Wilk normality test was used to assess normality, and Levene's test was applied to evaluate homogeneity of variance. For comparisons between two groups, an independent sample *t*-test was used for data that met the assumptions of normality and homogeneity of variance; a corrected Welch *t'* test was employed for data that conformed to normality but not homogeneity of variance. For comparisons among multiple groups, a One-way ANOVA test was conducted for data that satisfied the assumptions of normality and homogeneity of variance; a Kruskal-Wallis test was applied for data that did not meet the assumptions of normality or homogeneity of variance. The level of statistical significance was set at $P < 0.05$.

RESULTS

Comparison of basic information and vital signs between diabetic and non-diabetic patients

The age, height, weight, gender, and CPB time of the two groups of patients did not differ statistically significantly (Table 1). There were also no statistically significant differences in systolic blood pressure (SBP), diastolic blood pressure (DBP), and blood oxygen saturation (SPO_2) between the two groups of patients. Intragroup comparison showed that the SBP, DBP, SPO_2, hemoglobin (Hb), and heart rate of the two groups of patients before CPB (T1) were statistically significant ($P < 0.05$) compared to those after CPB (T2) (Table 2).

Verification of the model

Changes in creatinine concentrations before and after CPB in patients met the corresponding criteria for AKI (Figure 3A). In rats, creatinine levels significantly increased after CPB, and this elevation was further exacerbated in the CPB + glucose group, with statistically significant differences observed (Figure 3B). HE staining of rat kidney tissue revealed damage to renal tubular epithelial cells after CPB, which was more pronounced in the CPB + glucose group (Figure 4).

CPB and DM elevates copper in blood and urine

To verify whether CPB and diabetes cause disturbances in body copper, blood and urine were collected for determination of copper levels before and after CPB from diabetic and non-diabetic patients. Copper levels were significantly elevated in the blood of diabetic patients before and after CPB compared to non-diabetic patients (Figure 5). Diabetic patients had significantly higher copper levels in their blood after CPB (Figure 5). Copper levels in urine were determined. The levels reflect kidney function to some extent. The copper levels were significantly higher in diabetic patients before and after CPB (Figure 5). Copper was also significantly elevated in the urine of non-diabetic patients after CPB compared to before CPB (Figure 5). The results suggest that the metabolic disturbances of DM results in elevation of copper in blood and urine, and that these levels increase further following CPB.

CPB exacerbates metabolic disturbances caused by diabetes

One of the typical features of diabetes is metabolic disorders in the body. To investigate whether CPB exacerbates diabetes-induced metabolic disorders, lactate, glucose, and PH were assayed in diabetic and non-diabetic patients before and after CPB, and the variability in basal vital signs was analyzed. Before CPB, there was no difference in PH and lactate between diabetic and non-diabetic patients, whereas blood glucose was significantly higher in the diabetic group (Figure 6). Following CPB, PH was significantly lower in both diabetic and non-diabetic patients compared to prior to CPB. Following CPB, lactate levels were decreased in non-diabetic patients and increased in diabetic patients (Figure 6). Diabetic patients displayed a significant increase in blood glucose after CPB and non-diabetic patients tended to have higher blood glucose after CPB, with no statistically significant differences (Figure 6). The results suggest that CPB exacerbates metabolic disorders caused by diabetes.

Cuproptosis may occur after CPB in diabetic patients

Elevated copper ions cause heterodimerization of DLAT, an important protein in the tricarboxylic acid cycle (TCA) cycle, and increase DLAT deposition[16]. Induction of HSP-70 and reduction of Fe-S cluster proteins are feature of copper-dependent cuproptosis[25]. To verify whether the elevation of copper after CPB in diabetic patients causes an increase in DLAT deposition and HSP-70, which further causes cuproptosis, blood levels of DLAT and HSP-70 were determined. Before CPB, DLAT, and HSP-70 were elevated in the blood of diabetic patients. After CPB, DLAT and HSP-70 were significantly elevated in the blood of diabetic and non-diabetic patients, with more significant elevation in diabetic patients (Figure 7). The results suggest that cuproptosis may occur after CPB, while diabetes may be a risk factor for cuproptosis.

Copper-dependent cuproptosis occurs in kidneys of diabetic rats after CPB

AKI is a common complication after CPB. To investigate whether renal injury caused by CPB is associated with copper deposition and copper-dependent cuproptosis, copper levels and expressions of cuproptosis-related proteins in renal tissues were determined after CPB in diabetic rats. Copper was significantly increased in kidney tissues of diabetic rats after CPB compared with the CPB and KB groups (Figure 8). In addition, cuproptosis-related proteins, including LIAS, ACO-2 and SDHB, were significantly decreased in renal tissues of diabetic rats after CPB (Figure 9).

Table 1 Demographic characteristics, mean ± SD

Parameter	CD group	CZ group	F value	*P* value
Age (Years)	64.61 ± 7.43	60.07 ± 8.91	1.961	0.055
Height (cm)	164.08 ± 10.46	164.62 ± 8.99	-0.198	0.844
Weight (kg)	66.80 ± 12.27	64.00 ± 11.18	0.860	0.394
CPB time (min)	76.26 ± 12.44	80.10 ± 10.54	-1.205	0.234

CPB: Cardiopulmonary bypass; CD group: Non-diabetic patients; CZ group: Diabetic patients.

Table 2 Comparison of vital signs between diabetic and non-diabetic patients, mean ± SD

Sample	Group	T1	T2	*P* value
SBP	CD	148.09 ± 22.14	107.17 ± 12.88	0.000
	CZ	136.79 ± 22.04	103.69 ± 11.90	0.000
	P value	0.073	0.317	
DBP	CD	71.57 ± 13.85	56.56 ± 8.45	0.000
	CZ	72.48 ± 10.34	54.48 ± 10.27	0.000
	P value	0.786	0.437	
HR	CD	75.83 ± 10.41	99.13 ± 10.49	0.000
	CZ	79.34 ± 12.40	97.41 ± 12.52	0.000
	P value	0.281	0.601	
SPO_2	CD	97.30 ± 1.96	99.04 ± 1.33	0.001
	CZ	97.17 ± 2.47	99.07 ± 1.28	0.001
	P value	0.835	0.944	
Hb	CD	13.91 ± 1.60	9.80 ± 0.92	0.000
	CZ	13.62 ± 1.62	9.94 ± 0.80	0.000
	P value	0.531	0.548	

CD group: Non-diabetic patients; CZ group: Diabetic patients; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HR: Heart rate; SPO_2: Pulse oxygen saturation; Hb: Respiratory rate; T1: Before cardiopulmonary bypass; T2: After Cardiopulmonary bypass.

Renal mitochondrial damage after CPB in diabetic rats

Cuproptosis is associated with proteolipid acylation in the mitochondrial tricarboxylic acid cycle. Mitochondria are a key link in kidney injury. Histologically, mitochondrial swelling and fragmentation are observed after diverse insults to the kidney[26]. Based on these findings, TEM was used to explore the renal mitochondrial damage in diabetic rats after CPB. The mitochondria in renal tissues of diabetic rats in the KB group were oval and rod shaped, with intact mitochondrial membranes. The cristae were perpendicular to the long axis of the mitochondria, and were neatly aligned. In contrast, the mitochondria of kidneys in the CPB + glucose group were swollen, fragmented, and showed vacuolated structures. In addition, the mitochondrial cristae were disarranged (Figure 10).

DISCUSSION

Cuprotosis was first described in 2022[16]. Since then, copper death has become a hot topic medically. This study is the first clinical evidence of copper ion overload in blood and urine of diabetic patients after CPB. The evidence is consistent with the suggestion that CPB-AKI injury in patients with diabetes might be related to copper overload.

In the present study, marked differential changes in the blood levels of copper and metabolic disorders after CPB were observed in patients with diabetes. Significant mitochondrial damage was observed in renal tissues of diabetic rats after CPB. This damage was associated with high concentrations of copper as well as cuproptosis.

No statistically significant differences were evident in the basic information and vital signs (T1) before CPB between patients with and without diabetes. However, the vital signs of both groups of patients at the T1 were statistically

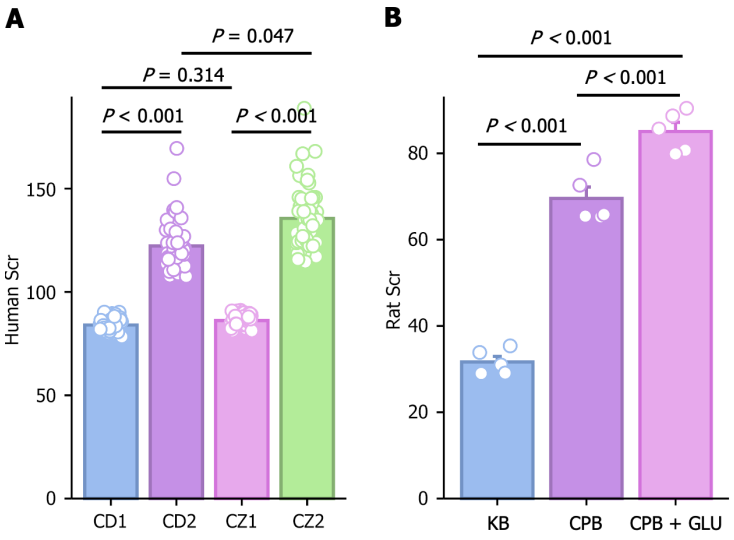

Figure 3 Serum creatinine levels (μmol/L) in different groups. A: Serum creatinine in rats; B: Serum creatinine in patients. $P < 0.05$ indicates statistical significance. CD: Non-diabetic patients; CZ: Diabetic patients; KB: Control group; CPB: Cardiopulmonary bypass; Glu: Glucose; Scr: Creatinine.

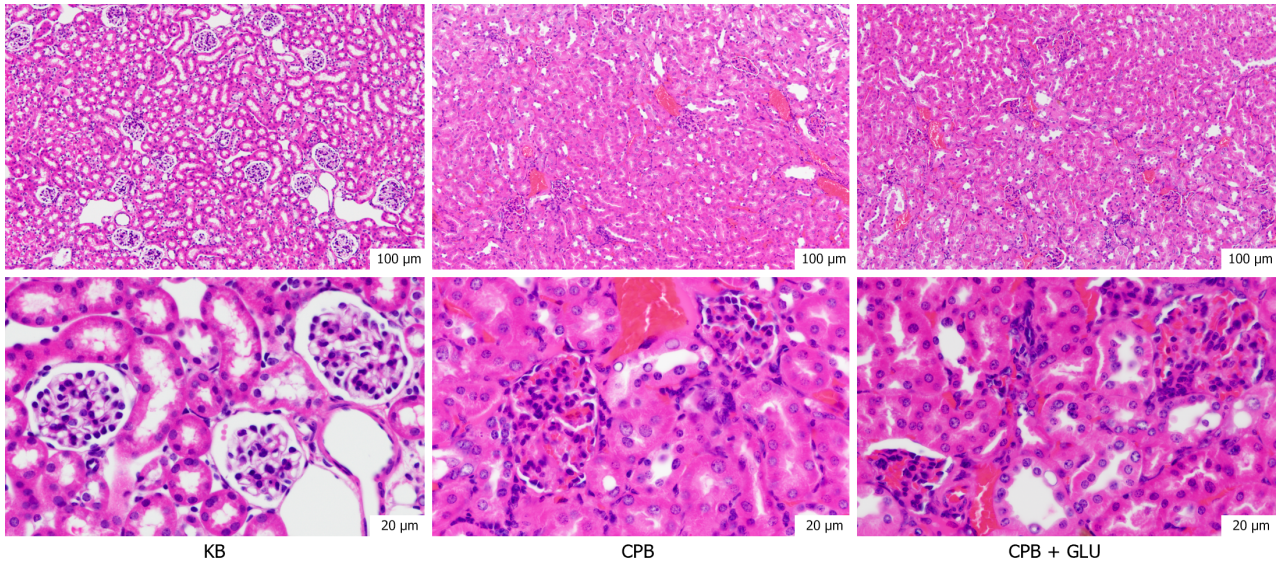

Figure 4 Hematoxylin-eosin staining in renal tissue of rats in each group. KB: Control group; CPB: Cardiopulmonary bypass; Glu: Glucose.

significant different at the T2 time point. This is because during the CPB process, the heart is not beating. When the surgery is over and the heart resumes beating with the assistance of vasoactive drugs. The heart rate is relatively fast and blood pressure is controlled within an appropriate range. The results are statistically significant changes in vital signs before and after CPB in both groups of patients. During the CPB process, to ensure the effective blood volume of the patient, a large amount of blood is diluted. Thus, after CPB, the patient's Hb is relatively reduced. However, organ perfusion during CPB is sufficient, so the differences in vital signs could not have affected the results of this study.

During CPB, the body's circulation is non-physiologic and non-pulsatile. The multitude of inflammatory humoral responses, coagulation, fibrinolysis, and kallikrein cascades occur upon exposure of blood to the artificial surfaces used in CPB[22]. In addition, CPB mechanically damages cells. In this study, plasma and urine copper levels were elevated after CPB in clinical patients, which is also evidence of cellular damage. The increased levels of copper ions in serum and urine in diabetes patients after CPB was even more significant. In animal experiments, compared with the KB group, the copper ions in the CPB group increased, albeit non-significantly, possibly due to the small number of cases. Most of the body's copper exists in bound form, and copper is one of the metals that is indispensable for mitochondrial function[27]. The most important copper-dependent enzyme in organisms is the cytochrome c multi-subunit enzyme-protein complex, which is found mainly in association with the inner mitochondrial membrane[28]. Mitochondria have a high affinity for copper entry, and thus are important organelles for copper entry and functioning[27]. Another cause of elevated copper in the organism is metabolic disorders, or restricted elimination. Diabetic patients who undergo CPB show significant lactic acid buildup and elevated blood glucose, consistent with elevated copper. This may also be one of the reasons for the disorder of copper ions in diabetes patients after CPB. We also found altered mitochondrial structure in renal tissues

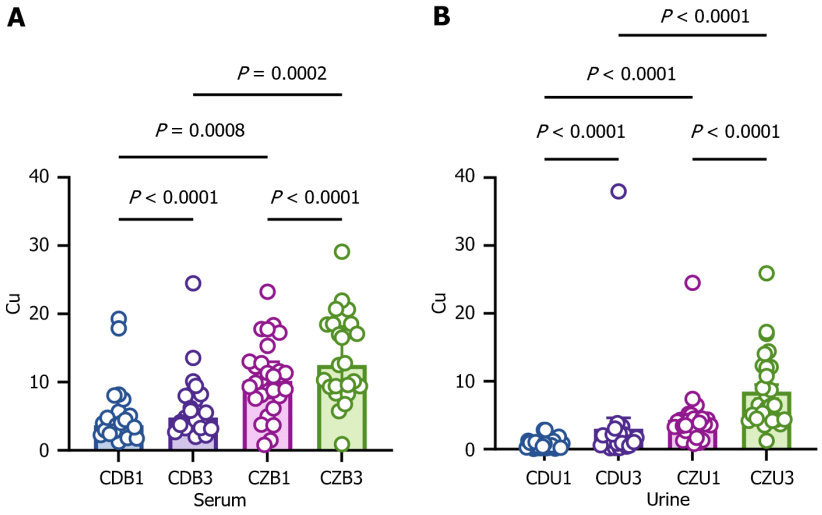

Figure 5 Comparison of copper ion levels between diabetic and non-diabetic patients. A: Copper ion levels in serum; B: Copper ion levels in urine. In two group, copper levels were significantly elevated in after cardiopulmonary bypass (CPB). But in diabetic patients, copper levels had significantly higher after CPB. Cu: Copper ion; CDB1 and CDU1: Patients without diabetes before cardiopulmonary bypass; CDB3 and CDU3: Patients without diabetes after cardiopulmonary bypass; CZB1 and CZU1: Patients with diabetes before cardiopulmonary bypass; CZB3 and CZU3: Patients with diabetes after cardiopulmonary bypass.

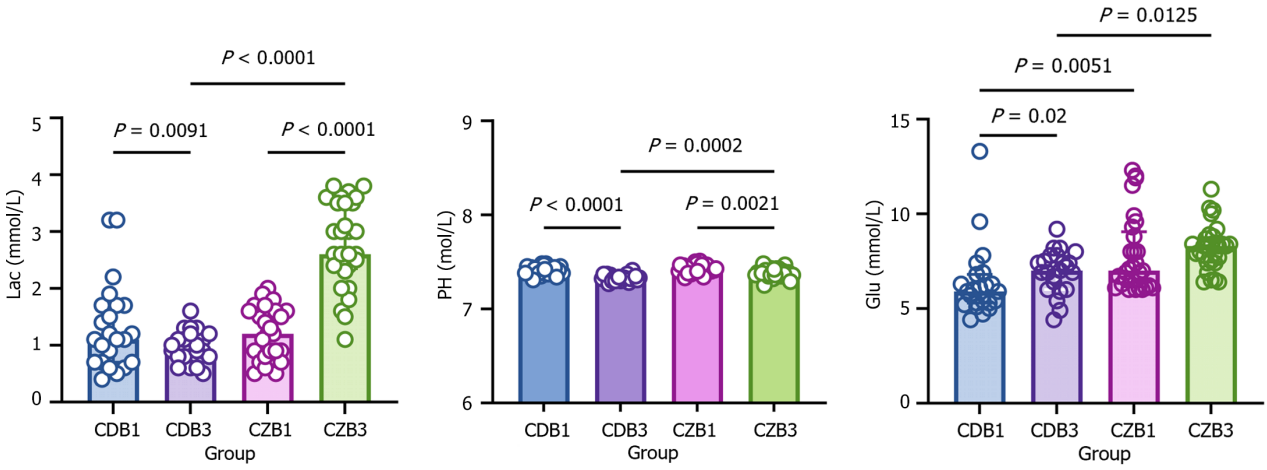

Figure 6 Serum expressions of serum lactic acid, potential of hydrogen, and glucose in diabetic and non-diabetic patients. In two group, potential of hydrogen (PH), lactic acid (Lac) and glucose were significantly elevated in after cardiopulmonary bypass (CPB). But in diabetic patients, PH, Lac and glucose had significantly higher after CPB. Lac: Lactic acid; PH: Potential of hydrogen; Glu: Glucose; CDB1: Patients without diabetes before cardiopulmonary bypass; CDB3: Patients without diabetes after cardiopulmonary bypass; CZB1: Patients with diabetes before cardiopulmonary bypass; CZB3: Patients with diabetes after cardiopulmonary bypass.

after CPB in diabetic rats, a phenomenon that may be due to mitochondrial copper overload and may be attenuated or reversed by copper chelators. These studies are beyond the scope of the present investigation.

The kidney requires many mitochondria to provide sufficient energy to enable removal of waste from the blood, reabsorption of nutrients, regulated balance of electrolytes and fluid, maintenance of acid–base homeostasis, and regulation of blood pressure. Apart from the heart, the kidneys are the most mitochondria-rich organ in the body. Mitochondrial damage occurs early in AKI and diabetic nephropathy, causing an imbalance in renal function. The disruption of mitochondrial homeostasis in the early stages of AKI is an important factor that drives tubular injury and persistent renal dysfunction[26]. A plethora of evidence suggests that mitochondrial dysfunction as an initiator of, and contributor to, AKI and is a therapeutic target[29]. In the present study, pathological and mitochondrial damage in the kidney was observed after CBP in diabetic rats. These results suggest that CPB directly or indirectly caused AKI in the diabetic rats. Adenosine triphosphate (ATP) is required for energy supply during tissue and cellular repair. Interruption of this energy supply due to mitochondrial damage delayed or prevents repair, which further exacerbates the damage. Therefore, the mitochondrial damage found in the kidneys in this study is a hallmark of AKI and also a cause of further aggravation of renal injury.

Copper is an essential component of various enzymes involved in the electron transport chain, cellular metabolism, and antioxidant system[30]. Both copper deficiency and excess can lead to abnormal cellular function and eventually cell death. Copper overload is cytotoxic[16]. In an animal study, CPB led to elevated copper in renal tissues of diabetic rats,

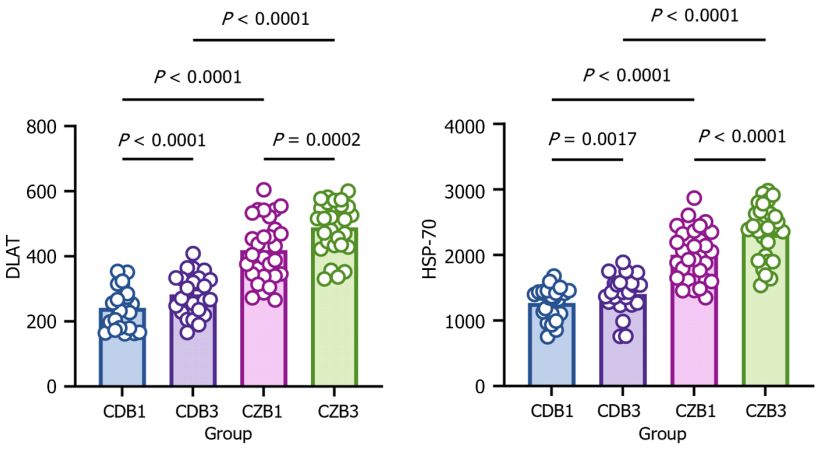

Figure 7 Serum expressions of dihydrolipoamide dehydrogenase and heat shock protein-70 in diabetic and non-diabetic patients. In two group, dihydrolipoamide dehydrogenase (DLAT) and heat shock protein-70 (HSP-70) were significantly elevated in after cardiopulmonary bypass (CPB). But in diabetic patients, DLAT and HSP-70 had significantly higher after CPB. DLAT: Dihydrolipoamide dehydrogenase; HSP-70: Heat shock protein-70; CDB1: Patients without diabetes before cardiopulmonary bypass; CDB3: Patients without diabetes after cardiopulmonary bypass; CZB1: Patients with diabetes before cardiopulmonary bypass; CZB3: Patients with diabetes after cardiopulmonary bypass.

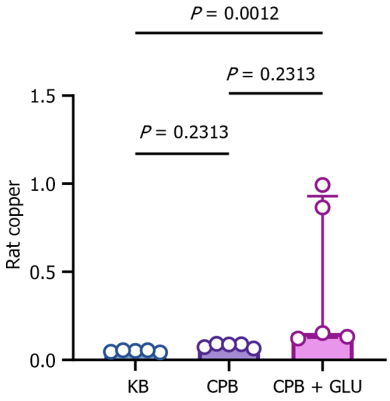

Figure 8 Expression level of copper ions in renal tissue of diabetic and non-diabetic rats. Copper was significantly increased in kidney tissues of diabetic rats after cardiopulmonary bypass (CPB) compared with the CPB and control groups. KB: Control group; CPB: Cardiopulmonary bypass; Glu: Glucose.

indicating that elevated copper is associated with renal injury. In addition, the level of Fe-S cluster proteins were decreased in the kidneys. Another function of mitochondria is the assembly of Fe-S cluster proteins, which is an important physiological process for human survival[31]. Cuproptosis is a type of cell death associated with Fe-S cluster proteins. High levels of copper can block Fe-S cluster formation by inhibiting the activity of relevant mitochondrial assembly proteins[32]. Increased cell death has been associated with the loss of Fe-S cluster proteins. It is also believed that cuproptosis is dependent on mitochondrial respiration; LIAS is a key protein for cuproptosis, and cuproptosis is increased when LIAS production is inhibited[16]. In the present clinical study, after CPB, urinary copper ions in diabetes patients were significantly increased, and DLAT and HSP-70 were both increased. Elevations of DLAT and HSP-70 are associated with cuproptosis[16]. Our previous research confirmed the change in the expression of copper ion related genes in diabetes with renal injury[33]. The prior and present evidence implicate CPB as the direct or indirect cause of cuproptosis in the kidney of diabetic rats.

The main factor in the occurrence of cuproptosis is the accumulation of copper, not the alteration of the associated chaperone proteins. In an organism, the organelle most associated with cuproptosis is the mitochondria. Mitochondria generate and transduce a redox signal that regulates the activity of cellular copper import and export machinery, thereby controlling total copper concentrations. The accumulation of copper in mitochondria impairs mitochondrial membrane integrity and aggravates oxidative stress-related injury[27]. In addition, the occurrence of cuproptosis is associated with mitochondrial respiration and with thioctylated proteins in the mitochondrial tricarboxylic acid cycle[16]. Therefore, we hypothesized that another phenomenon that characterizes the occurrence of copper-dependent cuproptosis is mitochondrial damage, which is mainly manifested as swelling and rupture of mitochondria. Copper ion transport is dependent on two homologous ATPases, *ATP7A* and *ATP7B*[30,34]. Whether the impaired ATP synthesis resulting from the damage to mitochondria, which was presently evident in the kidneys after CPB, also attenuates copper clearance and exacerbates copper accumulation, which in turn causes more severe cuproptosis, remains unknown.

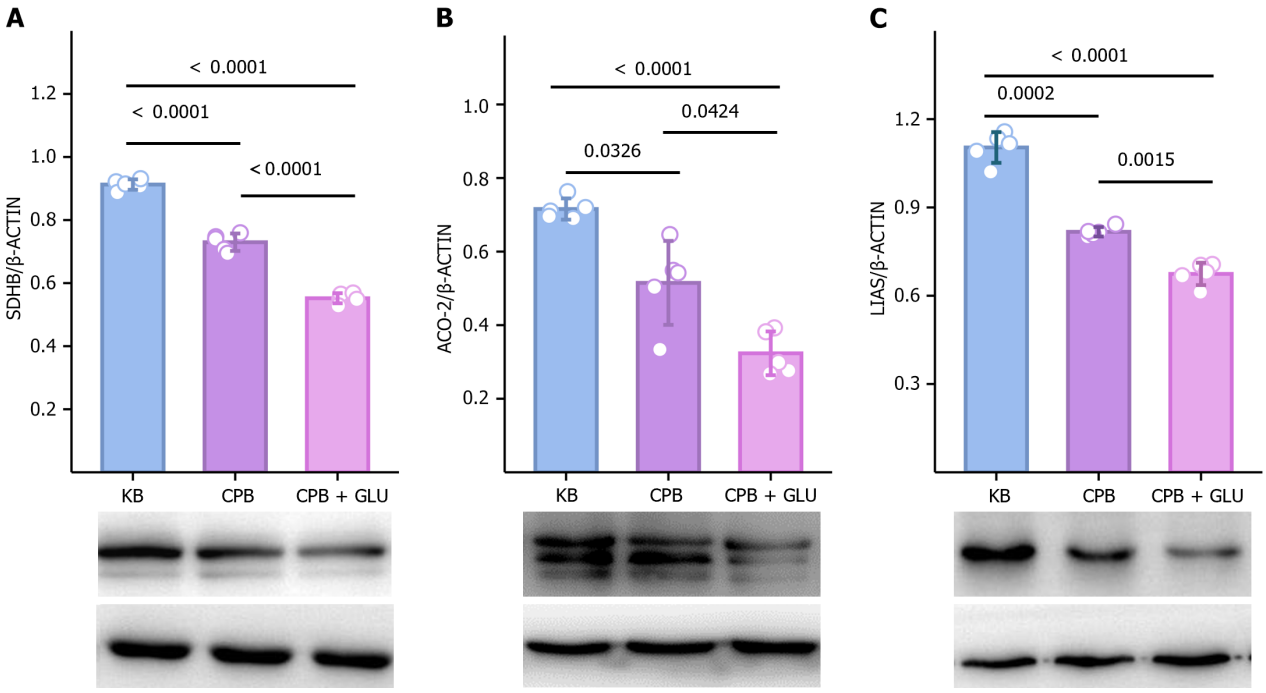

Figure 9 Expression of iron-sulfur cluster proteins in diabetic and non-diabetic rats. Cuproptosis-related proteins, including lipoic acid synthase, aconitase 2 and succinate dehydrogenase, were significantly decreased in renal tissues of diabetic rats after cardiopulmonary bypass. SDHB: Succinate dehydrogenase; ACO-2: Aconitase 2; LIAS: Lipoic acid synthase; KB: Control group; CPB: Cardiopulmonary bypass; Glu: Glucose.

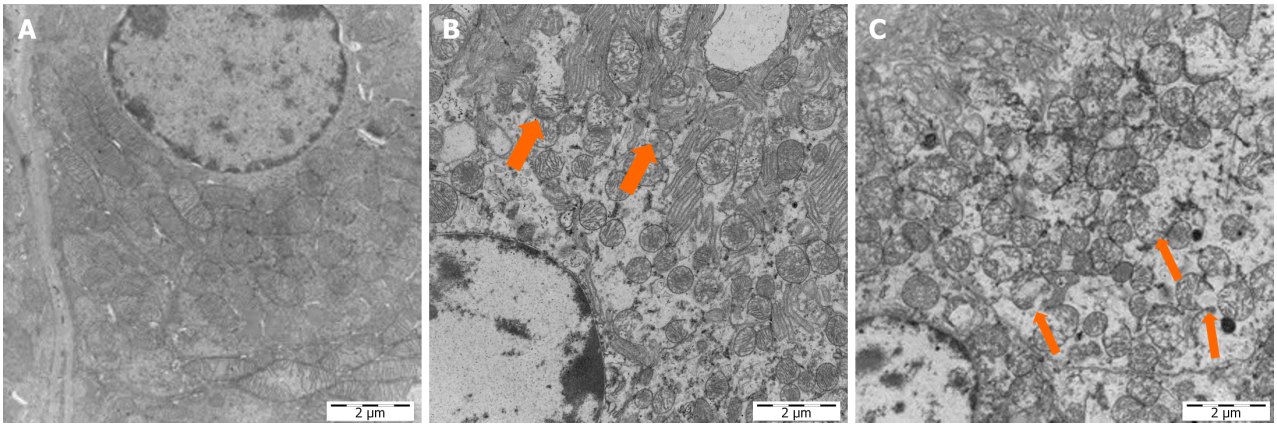

Figure 10 Transmission electron microscopy of rat mitochondria. A: In the control group, the mitochondrial envelope is intact and the cristae structure is clear; B: In the cardiopulmonary bypass (CPB) group, a small portion of cells showed mitochondrial damage; C: In the CPB + glucose group, most of the cells donated were damaged, contents had been released, and the crest was broken.

There are some limitations in the present study. We did not perform long-term observations after CPB to determine whether this acute-phase cuproptosis and alteration in renal pathology affects long-term renal function or translates to more severe chronic kidney disease.

CONCLUSION

CPB-AKI is associated with cuproptosis. DM is an important factor aggravating CPB-AKI and cuproptosis.

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FOOTNOTES

Author contributions: Sana SRGL designed the study, collected data, and wrote and revised the manuscript; Wang YN, Deng XJ, Lv CB, Qiu ZZ interpreted and analyzed the data; Zhu LX, Shi JH collected the data.

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SYSTEMATIC REVIEWS

Combining GLP-1 receptor agonists and SGLT-2 inhibitors for cardiovascular disease prevention in type 2 diabetes: A systematic review with multiple network meta-regressions

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Abstract

BACKGROUND

Glucagon-like peptide-1 receptor agonists (GLP-1RA) and sodium-glucose co-transporter-2 inhibitors (SGLT-2I) are associated with significant cardiovascular benefit in type 2 diabetes (T2D). However, GLP-1RA or SGLT-2I alone may not improve some cardiovascular outcomes in patients with prior cardiovascular co-morbidities.

AIM

To explore whether combining GLP-1RA and SGLT-2I can achieve additional benefit in preventing cardiovascular diseases in T2D.

METHODS

The systematic review was conducted according to PRISMA recommendations. The protocol was registered on PROSPERO (ID: 42022385007). A total of 107049 participants from eligible cardiovascular outcomes trials of GLP-1RA and SGLT-2I were included in network meta-regressions to estimate cardiovascular benefit of the combination treatment. Effect modification of prior myocardial infarction (MI) and heart failure (HF) was also explored to provide clinical insight as to when the

combination treatment should be considered.

RESULTS

The estimated hazard ratios (HR)$_{\text{GLP-1RA/SGLT-2I}}$ *vs* $_{\text{Placebo}}$ (0.75-0.98) and HR$_{\text{Combination}}$ *vs* $_{\text{GLP-1RA/SGLT-2I}}$ (0.26-0.86) for primary and secondary cardiovascular outcomes suggested that the combination treatment may achieve additional cardiovascular benefit compared with GLP-1RA or SGLT-2I alone. In patients with prior MI or HF, the monotherapies may not improve the overall cardiovascular outcomes, as the estimated HR$_{\text{MI+/HF+}}$ (0.57-1.52) suggested that GLP-1RA or SGLT-2I alone may be associated with lower risks of hospitalization for HF but not cardiovascular death.

CONCLUSION

Considering its greater cardiovascular benefit in T2D, the combination treatment of GLP-1RA and SGLT-2I might be prioritized in patients with prior MI or HF, where the monotherapies may not provide sufficient cardiovascular protection.

Key Words: Type 2 diabetes; Glucagon-like peptide-1 receptor agonist; Sodium-glucose co-transporter-2 inhibitor; Combination treatment; Cardiovascular outcome; Systematic review; Network meta-regression

Core Tip: Major cardiovascular outcome trials suggest that patients with prior cardiovascular co-morbidities may not gain sufficient cardiovascular protection from glucagon-like peptide-1 receptor agonists (GLP-1RA) or sodium-glucose co-transporter-2 inhibitors (SGLT-2I) alone. This systematic review with network meta-regression demonstrated that the combination treatment may provide greater cardiovascular benefit compared with GLP-1RA or SGLT-2I alone. In patients with prior myocardial infarction or heart failure, the monotherapies may not be associated with consistently improved cardiovascular outcomes, hence the combination treatment might be considered for cardiovascular disease prevention.

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INTRODUCTION

The macro- and micro-vascular benefits of glucagon-like peptide-1 receptor agonists (GLP-1RA) and sodium-glucose co-transporter-2 inhibitors (SGLT-2I) are independent of their glucose-lowering effects[1]. In patients with type 2 diabetes (T2D), the major cardiovascular outcome trials (CVOT) showed that dipeptidyl peptidase-4 inhibitors (DPP-4I) did not improve cardiovascular outcomes[2], whereas cardiovascular benefit of GLP-1RA or SGLT-2I was significant[3,4]. Further subgroup analyses indicated that the background cardiovascular risk should be considered when examining the cardiovascular outcomes of these newer glucose-lowering medications. For instance, prevention of major adverse cardiovascular events (MACE) was only seen in those patients with baseline atherosclerotic cardiovascular disease[3,4]. Moreover, a series of CVOT conducted in patients with heart failure (HF) have demonstrated that (compared with placebo) SGLT-2I significantly reduced risk of hospitalization for HF or cardiovascular death, irrespective of their history of T2D[5-8]. However, similar cardiovascular benefits were not observed in those with myocardial infarction (MI)[9,10]. Cardiovascular co-morbidities are not only approximately twice as common but are also associated with disproportionately worse cardiovascular outcomes in patients with T2D, compared to the general population[11]. Therefore, it is of clinical importance to investigate whether the combination treatment of GLP-1RA and SGLT-2I could achieve greater cardiovascular benefit, particularly when considering patients with cardiovascular co-morbidities who may not gain sufficient cardiovascular protection from the monotherapies.

This systematic review with multiple network meta-regressions was mainly aimed to explore whether combining GLP-1RA and SGLT-2I can provide additional cardiovascular benefit in T2D. Cardiovascular outcomes of these newer antidiabetic medications were also estimated under effect modification of prior cardiovascular diseases. This was to provide clinical insight as to when the combination treatment might be prioritized.

MATERIALS AND METHODS

Study search and inclusion

We conducted a comprehensive systematic review with multiple network meta-regressions (and parallel network meta-analyses) according to PRISMA recommendations[12]. The protocol is registered in PROSPERO (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022385007). PubMed, Scopus, the ClinicalTrials.gov registry, and Center for Drug Evaluation and Research were searched for eligible CVOT and associated *post-hoc* analyses (Figure 1). The study search was initially performed on October 19, 2023 and further updated on February 12, 2024. Studies included in analysis were those only conducted in adult patients with T2D receiving DPP-4I, GLP-1RA, or SGLT-2I (Table 1 and Supplementary Figure 1). CVOT of these antidiabetic medications while recruiting patients without T2D (which were determined at baseline) were excluded. The Cochrane Collaboration's Risk-of-Bias tool was applied for quality assessment (Supplementary Figure 2).

Data extraction and synthesis

Effect sizes [*i.e.*, hazard ratios (HR)] indicating treatment effects of these newer antidiabetic medications on primary and secondary cardiovascular outcomes, were extracted from the eligible CVOT (Supplementary Figures 3-7), and converted to statistics including mean logHR and their standard errors [calculated using HR and 95% confidence intervals (95%CI)] for network meta-regressions and meta-analyses[13]. Covariates including percentages of patients receiving the co-treatment with GLP-1RA or SGLT-2I and having baseline prior MI or HF in the placebo and treatment groups, were retrieved from the CVOT of GLP-1RA and SGLT-2I for network meta-regressions (Table 1).

Statistical analysis

A set of Bayesian network meta-analyses were initially performed to compare the cardiovascular outcomes among these antidiabetic medications (including DPP-4I). The between-study heterogeneities were assessed using the I^2 and τ^2 statistics (Supplementary Figures 3-7). Surface under the cumulative ranking curve (SUCRA) was also calculated for efficacy comparisons.

Furthermore, we conducted multiple Bayesian network meta-regressions to explore the effect modification of GLP-1RA on treatment efficacies of SGLT-2I and *vice versa*, which is equivalent to answering the main research question of whether the combination treatment of GLP-1RA and SGLT-2I can provide additional cardiovascular benefit. The network meta-regression model was constructed to establish a correlation between the covariate and effect size (*i.e.*, HR) observed in the CVOT. The correlation, namely, effect modification, can be numerically quantified as a coefficient (β)[14]. Given that this statistical model is linear[14], the percentages of patients ever receiving the co-treatment during the CVOT, namely, the postbaseline co-treatment, were incorporated as the covariate. This approach could yield results with more accuracy than those incorporating the baseline co-treatment. $HR_{0/GLP\text{-}1RA/SGLT\text{-}2I\ vs\ Placebo}$ and $HR_{1/Combination\ vs\ GLP\text{-}1RA/SGLT\text{-}2I}$ were thus estimated when assigning covariate = 0 or 1, assuming either 0% or 100% patients receiving the co-treatment in the CVOT, and compared with HR_{NA} from the parallel network meta-analyses (indicating the effect size observed from the CVOT with the actual percentages of patients receiving the co-treatment).

Likewise, cardiovascular outcomes of GLP-1RA or SGLT-2I were explored under effect modification of prior cardiovascular diseases. Percentages of patients having baseline MI or HF were incorporated as the covariates. $HR_{0/Disease-}$ and $HR_{1/Disease+}$, were estimated when assigning covariate = 0 or 1 with the assumption being that either 0% or 100% patients having MI or HF in the CVOT; and compared with HR_{NA} from meta-analyses (indicating the effect size observed from the CVOT with the actual percentages of patients having the co-morbidities).

In addition, I^2 or τ^2 in the network meta-regressions and run-in-parallel network meta-analyses (without covariate incorporation) was compared to determine the covariate effects on between-study heterogeneity. All the analyses were conducted with R version 4.2.3 using the GEMTC packages. We used four Markov chains with 150000 iterations after an initial burn-in of 20000 and a thinning of 1 for all the analyses (Supplementary material). As all the eligible CVOT for analysis were double-blind and randomized placebo-controlled trials, inconsistency was not assessed (Supplementary Figure 1).

Effect modification analysis

The credibility of all the proposed effect modifications was assessed using the instrument for the credibility of effect modification analyses (ICEMAN)[15]. For the credibility question 5, considering a Bayesian network meta-regression model applied in this study, 95%CI of the β (instead of *P* values) was included to indicate the results of the interaction test (Supplementary material).

RESULTS

GLP-1RA or SGLT-2I can improve primary and secondary cardiovascular outcomes in T2D

To determine the cardiovascular benefits of GLP-1RA and SGLT-2I, a total of 150423 participants in the CVOT were incorporated in the overall network meta-analysis to compare primary and secondary cardiovascular outcomes among DPP-4I, GLP-1RA, and SGLT-2I in T2D (Table 1, Supplementary Figure 1, and Figure 2). Based on our preliminary test results (not shown), network meta-analyses using relative risks would significantly underestimate the cardiovascular benefit of SGLT-2I, hence the results using survival (*i.e.*, HR) rather than count statistics have greater robustness. The

Table 1 Study-level characteristics of included major cardiovascular outcome trials and associated *post-hoc* analyses

Year	CVOT	Intervention	Median follow-up (year)	History of MI (yes, %)	History of HF (yes, %)	Post-baseline GLP-1RA/SGLT-2I (yes, %), intervention/placebo
2013	EXAMINE[25]	Alogliptin	1.5	N/A	N/A	N/A
2013	SAVOR-TIMI 53[26]	Saxagliptin	2.1	N/A	N/A	N/A
2015	TECOS[27]	Sitagliptin	3.0	N/A	N/A	N/A
2019	CARMELINA[28]	Linagliptin	2.2	N/A	N/A	N/A
2015	ELIXA[29]	Lixisenatide	2.1	22	22	N/A
2016	SUSTAIN-6[30]	Semaglutide	2.1	32	17[31]	1.5/1.2
2016	LEADER[32]	Liraglutide	3.8	31	18[33]	2.1/2.8[1]
2017	EXSCEL[34]	Exenatide	3.2	32[35]	16	4.4/5.8
2018	HARMONY OUTCOMES[36]	Albiglutide	1.5	47	20	9.7/10.8
2019	REWIND[37]	Dulaglutide	5.4	16[38]	9	5.2/7.3
2019	PIONEER-6[39]	Semaglutide	1.3	36[31]	12[31]	13.5/15.8
2021	AMPLITUDE-O[40]	Efpeglenatide	1.8	N/A	18	17.5/21.2
2015	EMPA-REG OUTCOME[41]	Empagliflozin	3.1	47	10	N/A
2017	CANVAS[42]	Canagliflozin	2.4	29[43]	14	6.2/7.7[2]
2019	DECLARE-TIMI 58 [44]	Dapagliflozin	4.2	21[45]	10	9.5/11.5[19]
2019	CREDENCE[46]	Canagliflozin	2.6	10[47]	15	6.5/6.9
2020	VERTIS CV[48]	Ertugliflozin	3.5	48	24	4.9/5.6

[1]Data kindly provided by Dr. Kajsa Kvist from Novo Nordisk.

[2]Data extracted from the drug approval package (application number: 204042Orig1s027) kindly provided by Dr. Frank Vercruysse from Janssen Pharmaceuticals.

Data for analysis were extracted from 17 primary investigations and 9 *post-hoc* analyses (including a drug approval package retrieved from Center for Drug Evaluation and Research). Covariates of percentages of patients having baseline prior myocardial infarction or heart failure; percentages of patients receiving postbaseline co-treatment with sodium-glucose co-transporter-2 inhibitors (SGLT-2I)/glucagon-like peptide-1 receptor agonists (GLP-1RA) in the placebo and GLP-1RA or SGLT-2I groups of the cardiovascular outcome trials (CVOT), were incorporated into the network meta-regression analyses. The numbers in square brackets denoting the CVOT and data correspond to the cited references. CVOT: Cardiovascular outcome trials; GLP-1RA: Glucagon-like peptide-1 receptor agonists; SGLT-2I: Sodium-glucose co-transporter-2 inhibitors; MI: Myocardial infarction; HF: Heart failure; N/A: Not applicable.

overall heterogeneities were low, with I^2 of 0% and 19% and τ^2 of 0.001 to 0.005 (Supplementary Figures 3-7). Compared with placebo, DPP-4I demonstrated no risk-reducing effects on any of the cardiovascular outcomes. Both GLP-1RA and SGLT-2I significantly reduced risks of MACE ($HR_{GLP\text{-}1RA\ vs\ Placebo}$ = 0.85, 95%CI: 0.79-0.90; $HR_{SGLT\text{-}2I\ vs\ Placebo}$ = 0.90, 95%CI: 0.83-0.96) and cardiovascular death ($HR_{GLP\text{-}1RA\ vs\ Placebo}$ = 0.87, 95%CI: 0.74-0.95; $HR_{SGLT\text{-}2I\ vs\ Placebo}$ = 0.84, 95%CI: 0.74-0.95). Moreover, GLP-1RA might have modest benefit over SGLT-2I in reducing risks of MACE ($HR_{GLP\text{-}1RA\ vs\ SGLT\text{-}2I}$ = 0.95, 95%CI: 0.86-1.04; $SUCRA_{GLP\text{-}1RA}$ = 0.95, $SUCRA_{SGLT\text{-}2I}$ = 0.70). Whereas for cardiovascular death, SGLT-2I might be associated with lower risk compared with GLP-1RA ($HR_{SGLT\text{-}2I\ vs\ GLP\text{-}1RA}$ = 0.97, 95%CI: 0.83-1.14; $SUCRA_{SGLT\text{-}2I}$ = 0.87, $SUCRA_{GLP\text{-}1RA}$ = 0.77). GLP-1RA also demonstrated significant risk-reducing effects on fatal and non-fatal MI ($HR_{GLP\text{-}1RA\ vs\ Placebo}$ = 0.90, 95%CI: 0.83-0.99), and fatal and non-fatal stroke ($HR_{GLP\text{-}1RA\ vs\ Placebo}$ = 0.84, 95%CI: 0.75-0.93). Compared with the other interventions, SGLT-2I achieved the most significant and superior benefit in reducing risk of hospitalization for HF (*e.g.*, $HR_{SGLT\text{-}2I\ vs\ GLP\text{-}1RA}$ = 0.74, 95%CI: 0.63-0.88; $SUCRA_{SGLT\text{-}2I}$ = 1; Figure 2).

The combination treatment of GLP-1RA and SGLT-2I may provide additional cardiovascular benefit in T2D

Estimation of the combination treatment for cardiovascular disease prevention in T2D was further conducted in 107049 participants only in the CVOT of GLP-1RA and SGLT-2I. Potential effect modification of co-treatment with SGLT-2I on cardiovascular outcomes of GLP-1RA (and *vice versa*) was analyzed using network meta-regressions. The overall negative β (-0.13 to -0.01) indicated that there might be a positive effect modification of the co-treatment on improvement of the primary and secondary cardiovascular outcomes, *i.e.*, the higher the percentages of patients receiving the combination treatment, the lower the HR (Supplementary Table 1), which is in consistent with comparisons among HR_0, HR_1, and HR_{NA} (*e.g.*, $HR_0 > HR_{NA} > HR_{1/Combination\ vs\ GLP\text{-}1RA/SGLT\text{-}2I}$; Table 2). In patients not receiving the co-treatment, GLP-1RA or SGLT-2I (alone) could improve cardiovascular outcomes (compared to placebo). The lack of statistical significance in

Table 2 Effect modification of co-treatment with sodium-glucose co-transporter-2 inhibitors on cardiovascular benefit of glucagon-like peptide-1 receptor agonists and *vice versa* in type 2 diabetes

Cardiovascular outcome	Covariate	Intervention	HR with 95%CI
MACE	NA	GLP-1RA *vs* Placebo	0.84 (0.77-0.90)
MACE	0	GLP-1RA *vs* Placebo	0.89 (0.77-0.99)
MACE	NA	SGLT-2I *vs* Placebo	0.90 (0.82-0.98)
MACE	0	SGLT-2I *vs* Placebo	0.95 (0.82-1.08)
MACE	1	Combination *vs* GLP-1RA	0.51 (0.16-1.65)
MACE	1	Combination *vs* SGLT-2I	0.48 (0.15-1.54)
Cardiovascular death	NA	GLP-1RA *vs* Placebo	0.85 (0.76-0.94)
Cardiovascular death	0	GLP-1RA *vs* Placebo	0.88 (0.73-1.07)
Cardiovascular death	NA	SGLT-2I *vs* Placebo	0.90 (0.79-1.02)
Cardiovascular death	0	SGLT-2I *vs* Placebo	0.93 (0.76-1.16)
Cardiovascular death	1	Combination *vs* GLP-1RA	0.58 (0.08-3.39)
Cardiovascular death	1	Combination *vs* SGLT-2I	0.55 (0.07-3.25)
Fatal and non-fatal MI	NA	GLP-1RA *vs* Placebo	0.89 (0.79-0.98)
Fatal and non-fatal MI	0	GLP-1RA *vs* Placebo	0.94 (0.79-1.09)
Fatal and non-fatal MI	NA	SGLT-2I *vs* Placebo	0.92 (0.81-1.05)
Fatal and non-fatal MI	0	SGLT-2I *vs* Placebo	0.98 (0.81-1.19)
Fatal and non-fatal MI	1	Combination *vs* GLP-1RA	0.45 (0.10-2.18)
Fatal and non-fatal MI	1	Combination *vs* SGLT-2I	0.44 (0.09-2.10)
Fatal and non-fatal stroke	NA	GLP-1RA *vs* Placebo	0.81 (0.72-0.91)
Fatal and non-fatal stroke	0	GLP-1RA *vs* Placebo	0.82 (0.67-1.00)
Fatal and non-fatal stroke	NA	SGLT-2I *vs* Placebo	0.94 (0.82-1.08)
Fatal and non-fatal stroke	0	SGLT-2I *vs* Placebo	0.95 (0.75-1.20)
Fatal and non-fatal stroke	1	Combination *vs* GLP-1RA	0.86 (0.12-6.23)
Fatal and non-fatal stroke	1	Combination *vs* SGLT-2I	0.74 (0.10-5.47)
Hospitalization for HF	NA	GLP-1RA *vs* Placebo	0.90 (0.79-1.02)
Hospitalization for HF	0	GLP-1RA *vs* Placebo	0.97 (0.80-1.19)
Hospitalization for HF	NA	SGLT-2I *vs* Placebo	0.68 (0.59-0.79)
Hospitalization for HF	0	SGLT-2I *vs* Placebo	0.75 (0.59-0.96)
Hospitalization for HF	1	Combination *vs* GLP-1RA	0.26 (0.03-1.88)
Hospitalization for HF	1	Combination *vs* SGLT-2I	0.33 (0.04-2.53)

The covariates of percentages of patients receiving postbaseline co-treatment with sodium-glucose co-transporter-2 inhibitors (SGLT-2I)/glucagon-like peptide-1 receptor agonists (GLP-1RA) were incorporated into the meta-regressions. Hazard ratios (HR)$_{0/\text{GLP-1RA/SGLT-2I}}$ *vs* $_{\text{Placebo}}$ and HR$_{1/\text{Combination}}$ *vs* $_{\text{GLP-1RA/SGLT-2I}}$ with 95% confidence intervals (95%CI) were estimated, assuming either 0% or 100% patients receiving the co-treatment. The network meta-analyses were run in parallel to calculate HR$_{NA}$ with 95%CI. The HR$_{NA}$ indicated effect sizes observed from the cardiovascular outcome trials with the actual percentages of patients receiving the co-treatment. HR: Hazard ratios; 95%CI: 95% confidence intervals; MACE: Major adverse cardiovascular events; CVOT: Cardiovascular outcome trials; GLP-1RA: Glucagon-like peptide-1 receptor agonists; SGLT-2I: Sodium-glucose co-transporter-2 inhibitors; MI: Myocardial infarction; HF: Heart failure; NA: Not available.

some of the results could stem from EMPA-REG OUTCOME being excluded from the network meta-regressions, as this trial did not report percentages of patients receiving the post co-treatment of GLP-1RA in the placebo and SGLT-2I groups (Table 1). In patients receiving the co-treatment, the combination treatment was estimated to be associated with additional cardiovascular benefit in preventing MACE compared to either GLP-1RA (HR$_1$ = 0.51, 95%CI: 0.16-1.65) or SGLT-2I (HR$_1$ = 0.48, 95%CI: 0.15-1.54) alone. Similar effect sizes were also assessed for cardiovascular death and fatal and non-fatal MI. Although to a lesser extent, the combination treatment might further lower the risk of fatal and non-fatal stroke compared with GLP-1RA (HR$_1$ = 0.86, 95%CI: 0.12-6.23) or SGLT-2I (HR$_1$ = 0.74, 95%CI: 0.10-5.47) alone. Moreover,

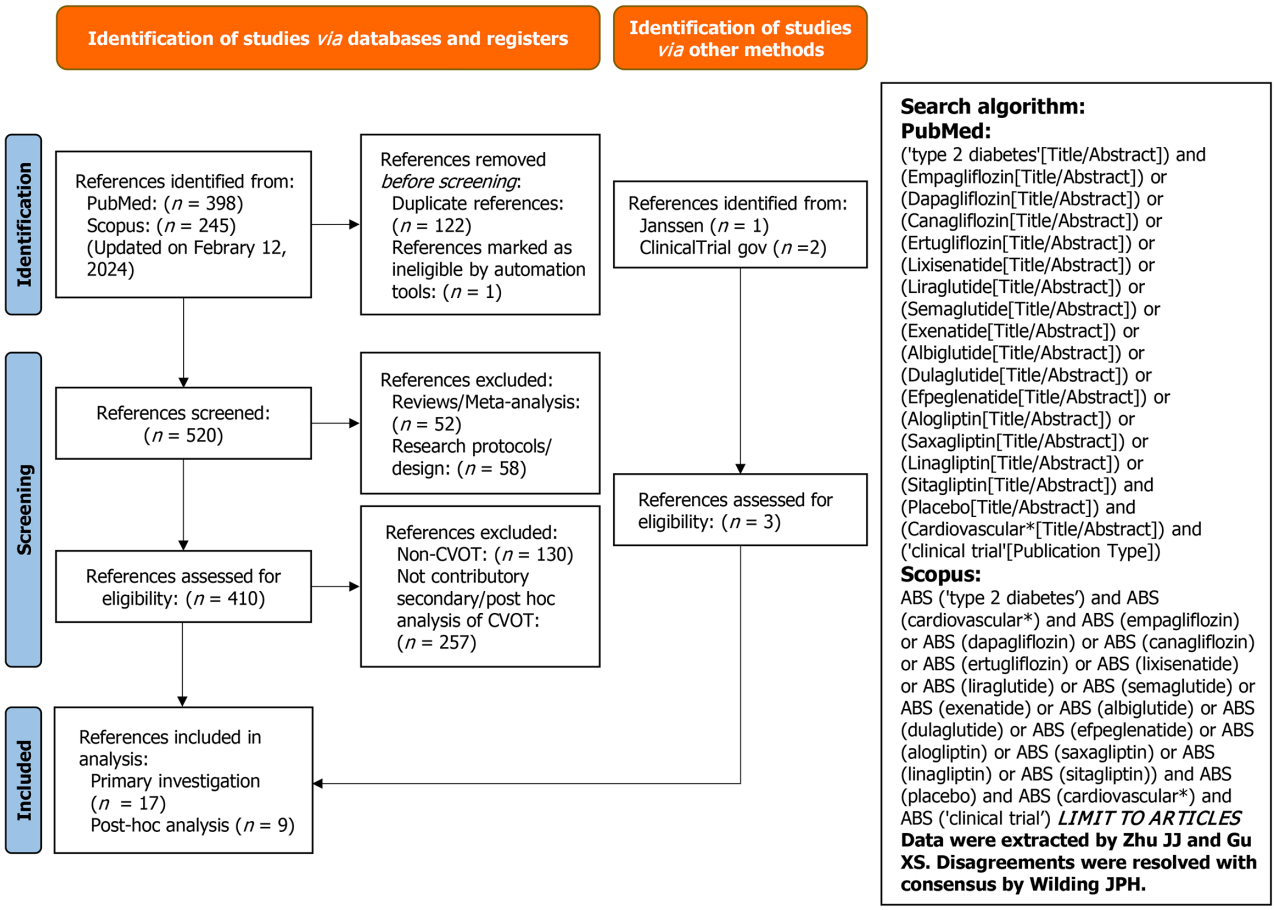

Figure 1 PRISMA flow diagram with search algorithm. CVOT: Cardiovascular outcome trials.

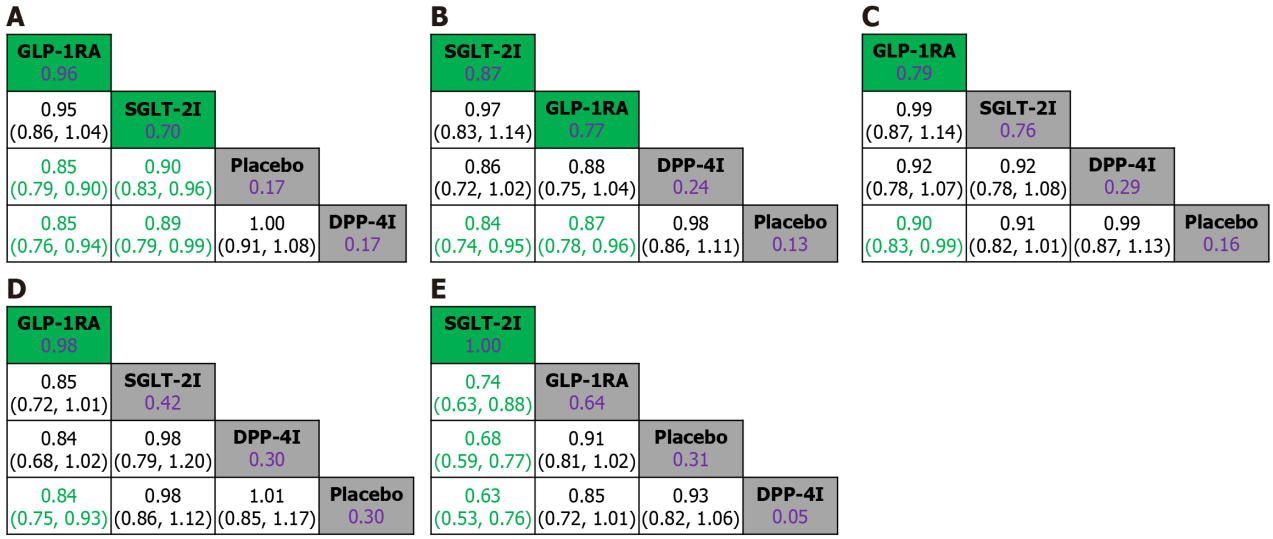

Figure 2 Comparisons of primary and secondary cardiovascular outcomes among newer glucose-lowering medications in type 2 diabetes. A: Major adverse cardiovascular events; B: Cardiovascular death; C: Fatal and non-fatal myocardial infarction; D: Fatal and non-fatal stroke; E: Hospitalization for heart failure. The treatments are reported in order of cardiovascular outcome ranking according to surface under the cumulative ranking curve (indicated in purple). Comparisons in the network meta-analyses should be read from left to right. The results, *i.e.*, hazard ratios (HR) with 95%CI, are located at the intersection of the column-defining treatment and the row-defining treatment (indicated in green and black). For the observed primary and secondary outcomes of the cardiovascular outcome trials, HR (< 1) favors the column-defining treatment. Significant results and treatments of significant cardiovascular benefit are indicated in green.

hospitalization for HF might be prevented to a greater extent in patients receiving the combination treatment rather than receiving GLP-1RA (HR_1 = 0.26, 95%CI: 0.03-1.88) or SGLT-2I (HR_1 = 0.33, 95%CI: 0.04-2.53; Table 2) alone. Taken together, the estimated effect sizes, *i.e.*, HR_1, were all numerically but not significantly favorable to the combination treatment, suggesting that the combination treatment may achieve greater benefit than the monotherapies in preventing cardiovascular diseases in patients with T2D.

Regarding the primary and secondary cardiovascular outcomes, low degrees of variations between I^2 or τ^2 in the meta-regressions and meta-analyses might eliminate the probability of the co-treatments being sources of between-study heterogeneity (Supplementary Table 1). However, for the effect modifications of the co-treatments, the overall credibility ratings ranged from low to moderate (Supplementary material).

Cardiovascular outcomes of GLP-1RA or SGLT-2I could be modified by cardiovascular co-morbidities in T2D

Effect modification of prior MI or HF on cardiovascular outcomes in patients receiving GLP-1RA or SGLT-2I were likewise explored in network meta-regressions. The negative β (-0.07 to -0.01) indicated that GLP-1RA and SGLT-2I might be more effective in prevention of cardiovascular death and hospitalization for HF in trial populations with higher rates of MI and HF, respectively (Supplementary Table 2). In patients without prior MI, GLP-1RA were estimated to be associated with a significant risk reduction in cardiovascular death (HR_0 = 0.88, 95%CI: 0.76-0.99), whereas the effect size might modestly increase in patients with prior MI (HR_1 = 0.74, 95%CI: 0.26-2.01). Similarly, in patients without prior HF, SGLT-2I could significantly reduce the risk of hospitalization for HF (HR_0 = 0.68, 95%CI: 0.60-0.76), and additional risk reduction was estimated in patients with prior HF (HR_1 = 0.62, 95%CI: 0.14-2.80; Table 3). However, the estimated cardiovascular benefit of GLP-1RA and SGLT-2I was numerically but not statistically conclusive in patients with these preexisting cardiovascular co-morbidities.

In contrast, the positive β (0.05-0.08) indicated that GLP-1RA and SGLT-2I might demonstrate reduced effectiveness in preventing cardiovascular death and recurrent MI as the prevalence of MI and HF within trial populations increased (Supplementary Table 2). In patients without prior HF, both GLP-1RA and SGLT-2I could significantly reduce the risk for cardiovascular death ($HR_{0/GLP\text{-}1RA}$ = 0.86, 95%CI: 0.76-0.97; $HR_{0/SGLT\text{-}2I}$ = 0.84, 95%CI: 0.73-0.96). However, these risk reduction effects were estimated to be neutral in patients with prior HF ($HR_{1/GLP\text{-}1RA}$ = 1.52, 95%CI: 0.30-10.07); $HR_{1/SGLT\text{-}2I}$ = 1.51, 95%CI: 0.29-10.38; Table 3).

Compared with fatal and non-fatal MI or hospitalization for HF, cardiovascular death demonstrated the greatest heterogeneities as I^2 = 19% indicated (Supplementary Figures 4, 5, and 7). Notably, the I^2 and τ^2 were reduced when incorporating covariates of prior HF or MI, suggesting that these co-morbidities could be also considered sources of the between-study heterogeneities (Supplementary Table 2). With respect to the effect modifications of these cardiovascular diseases in patients receiving the mono-antidiabetic treatment with GLP-1RA or SGLT-2I, the overall credibility ratings ranged from low to moderate (Supplementary material).

DISCUSSION

The initial network meta-analyses confirmed the cardiovascular benefit of GLP-1RA and SGLT-2I in T2D. GLP-1RA demonstrated remarkable risk reductions in various adverse cardiovascular outcomes. SGLT-2I had superior benefit in preventing cardiovascular death and hospitalization for HF. Compared with previous analyses[16], our study exhibited lower heterogeneities and generated results with higher robustness. These advantages can be attributed to analysis using survival rather than count statistics and incorporation of CVOT exclusively conducted in patients with T2D.

To date, there has not been any systematic review examining whether the combination treatment of GLP-1RA and SGLT-2I can prevent cardiovascular diseases in T2D. It should be noted that running separate subgroup analyses is not a correct method to investigate effect modification in network meta-analysis as it cannot guarantee same estimates of between-trial variation nor produce test of interaction to reject the null hypothesis of equal effects[17]. Therefore, our study used a robust network meta-regression model to explore the cardiovascular benefit of the combination treatment *via* estimating the effect modification of GLP-1RA on treatment efficacies of SGLT-2I (and *vice versa*). Moreover, from a methodological standpoint, covariate incorporation in meta-regression can avoid unbalanced hazards between intervention groups (which can be introduced *via* covariate stratification in sub-group analysis[18-21]), thereby estimating effect sizes with greater precision. Consistent with previous *post hoc* subgroup and propensity score matching analyses [17-21], our results suggest that the combination treatment may achieve additional cardiovascular benefit in T2D[17-21]. A recent published real-world data based study further confirmed that the combination treatment was associated with both lower cardiovascular and risks compared with the monotherapies[22]. Mechanistically, their complementary actions on glucose, blood pressure, and lipid regulation might have contributed to the greater cardiovascular benefits[23].

Cardiovascular co-morbidities have been recognized as risk factors capable of potentially modifying cardiovascular benefit of GLP-1RA and SGLT-2I[3,4]. Our results indicated that SGLT-2I could significantly lower hospitalization for HF but not cardiovascular death in patients with HF, which are consistent with observations from the CVOT conducted in HF with preserved ejection fraction (*e.g.*, EMPEROR-Preserved and DELIVER)[5,7]. In patients with prior MI, the EMPACT-MI trial showed that the SGLT-2I was not associated with improved cardiovascular outcomes[10], whereas our results indicated that the risk of cardiovascular death might be further reduced compared with those without prior MI, but the estimation remains statistically inconclusive as the 95%CI indicated. Similar effect modifications were also estimated in GLP-1RA. As GLP-1RA and SGLT-2I have become the most recommended second-line and, in some cases, first-line antidiabetic treatments, particularly for patients with "high risk" (*e.g.*, atherosclerotic cardiovascular disease) [24], these specific cardiovascular conditions may be considered "above high risk" at which patients should receive the

Table 3 Effect modification of prior cardiovascular diseases on cardiovascular outcomes of glucagon-like peptide-1 receptor agonists and sodium-glucose co-transporter-2 inhibitors in type 2 diabetes

Cardiovascular outcome	Covariate		Intervention	HR with 95%CI
Fatal and non-fatal MI	Prior history of MI	NA	GLP-1RA *vs* Placebo	0.91 (0.84-1.01)
Fatal and non-fatal MI	Prior history of MI	0	GLP-1RA *vs* Placebo	1.13 (0.85-1.51)
Fatal and non-fatal MI	Prior history of MI	1	GLP-1RA *vs* Placebo	0.57 (0.30-1.05)
Fatal and non-fatal MI	Prior history of MI	NA	SGLT-2I *vs* Placebo	0.91 (0.82-1.02)
Fatal and non-fatal MI	Prior history of MI	0	SGLT-2I *vs* Placebo	0.84 (0.66-1.07)
Fatal and non-fatal MI	Prior history of MI	1	SGLT-2I *vs* Placebo	1.09 (0.66-1.80)
Cardiovascular death	Prior history of MI	NA	GLP-1RA *vs* Placebo	0.88 (0.76-0.99)
Cardiovascular death	Prior history of MI	0	GLP-1RA *vs* Placebo	0.93 (0.59-1.48)
Cardiovascular death	Prior history of MI	1	GLP-1RA *vs* Placebo	0.74 (0.26-2.01)
Cardiovascular death	Prior history of MI	NA	SGLT-2I *vs* Placebo	0.84 (0.72-0.96)
Cardiovascular death	Prior history of MI	0	SGLT-2I *vs* Placebo	0.92 (0.62-1.32)
Cardiovascular death	Prior history of MI	1	SGLT-2I *vs* Placebo	0.68 (0.32-1.48)
Hospitalization for HF	Prior history of HF	NA	GLP-1RA *vs* Placebo	0.91 (0.82-1.02)
Hospitalization for HF	Prior history of HF	0	GLP-1RA *vs* Placebo	0.93 (0.61-1.42)
Hospitalization for HF	Prior history of HF	1	GLP-1RA *vs* Placebo	0.84 (0.20-3.67)
Hospitalization for HF	Prior history of HF	NA	SGLT-2I *vs* Placebo	0.68 (0.60-0.76)
Hospitalization for HF	Prior history of HF	0	SGLT-2I *vs* Placebo	0.69 (0.52-0.90)
Hospitalization for HF	Prior history of HF	1	SGLT-2I *vs* Placebo	0.62 (0.14-2.80)
Cardiovascular death	Prior history of HF	NA	GLP-1RA *vs* Placebo	0.86 (0.76-0.97)
Cardiovascular death	Prior history of HF	0	GLP-1RA *vs* Placebo	0.77 (0.51-1.08)
Cardiovascular death	Prior history of HF	1	GLP-1RA *vs* Placebo	1.52 (0.30-10.07)
Cardiovascular death	Prior history of HF	NA	SGLT-2I *vs* Placebo	0.84 (0.73-0.96)
Cardiovascular death	Prior history of HF	0	SGLT-2I *vs* Placebo	0.76 (0.52-1.04)
Cardiovascular death	Prior history of HF	1	SGLT-2I *vs* Placebo	1.51 (0.29-10.38)

The covariates of percentages of patients having baseline prior cardiovascular diseases including myocardial infarction and heart failure were incorporated in the network meta-regressions. Hazard ratios $(HR)_{0/Disease-}$ and $HR_{1/Disease+}$ with 95% confidence intervals (95%CI) were estimated with the assumption of either 0% or 100% patients having the co-morbidities in the cardiovascular outcome trials (CVOT). The network meta-analyses were run in parallel to calculate HR_{NA} with 95%CI. The HR_{NA} indicated effect sizes observed from the CVOT with the actual percentages of patients having the co-morbidities. HR: Hazard ratios; 95%CI: 95% confidence intervals; CVOT: Cardiovascular outcome trials; GLP-1RA: Glucagon-like peptide-1 receptor agonists; SGLT-2I: Sodium-glucose co-transporter-2 inhibitors; MI: Myocardial infarction; HF: Heart failure.

combination treatment of GLP-1RA and SGLT-2I to optimize the overall cardiovascular outcomes.

The overall credibility of these effect modifications was rated as low to moderate using ICEMAN. This is considered a major limitation of our study. Factors that underestimated the credibility include the over-specification of the network meta-regression model due to scarcity of the data points (*e.g.*, only 13 available trials/baselines were included for analysis)[14]. Consequently, the β values were generated with less statistical power, which also contributes to the generally low to moderate credibility and may explain the very wide 95%CI of some estimated HR. Multiple interaction models using individual patient data should be undertaken in the future, to investigate cardiovascular and renal benefits of the combination treatment under effect modification of these cardiovascular co-morbidities. Nevertheless, further definitive trials are still in need to be able to support a strong recommendation to this effect.

CONCLUSION

The combination treatment of GLP-1RA and SGLT-2I may achieve additional cardiovascular benefit in T2D. In patients with prior cardiovascular co-morbidities including MI and HF, GLP-1RA or SGLT-2I alone may not significantly improve the overall cardiovascular outcomes, hence the combination treatment can be prioritized in such clinical scenarios.

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FOOTNOTES

Author contributions: Wilding JPH and Gu XS contributed equally to this study as co-corresponding authors. Wilding JPH proposed to investigate cardiovascular benefit of the combination treatment of GLP-1RA and SGLT-2I; Zhu JJ and Gu XS conducted the systematic review; Zhu JJ performed all the statistics and took responsibility for the accuracy of the data analysis; Wilding JPH and Gu XS supervised the findings of this study; all the authors discussed the results, and contributed to and approved the final manuscript (including the registered protocol).

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LETTER TO THE EDITOR

Interleukin-35: A key player managing pre-diabetes and chronic inflammatory type 1 autoimmune diabetes

Ratul Chakraborty, Ashis Kumar Mukherjee, Asis Bala

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Abstract

Interleukin-35 (IL-35) is a novel protein comprising IL-12α and IL-27β chains. The IL12A and *EBI3* genes are responsible for its production. The study of IL-35 has experienced a substantial increase in interest in recent years, as demonstrated by many research papers. Recent clinical studies have shown that individuals who do not have a C-peptide have notably reduced amounts of IL-35 in their blood serum. This is accompanied by a drop in the percentage of IL-35$^+$ Treg cells, regulatory B cells, and CD8$^+$ FOXP3$^+$ cells that produce IL-35. This article emphasizes the potential significance of IL-35 expression in governing the immune response and its involvement in chronic inflammatory autoimmune diabetes in pancreatic inflammation. It demonstrates IL-35's ability to regulate cytokine proportions, modulate B cells, and protect against autoimmune diabetes. However, further investigation is necessary to ascertain the precise mechanism of IL-35, and meticulous planning is essential for clinical studies.

Key Words: Interleukin-35; Chronic inflammatory type diabetes; Autoimmune diabetes; Pancreatic inflammation; Gene disease association

Core Tip: Studies suggest interleukin (IL)-35 protects against prediabetes and autoimmune diabetes by regulating immune system function. Development of type 1 diabetes (T1D) can be influenced by various cytokines produced by immune and pancreatic cells. Some cytokines, such as IL-10, transforming growth factor beta (TGF-β), IL-5, IL-4, IL-2, IL-15, IL-33, and IL-35, can stimulate regulatory cells in the immune system, releasing anti-inflammatory cytokines. Regulatory dendritic cells release IL-7, important for maintaining Tregs. In T1D, Tregs express IL-7Rα. Inhibiting TGF-β and activating IFN-γ can increase TC, Th1, and Th17 cells, while TGF-β can stimulate Runx1 expression to convert Th1 cells into Th17 cells.

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TO THE EDITOR

The study by Ping *et al*[1], published in 2024 in the *World Journal of Diabetes*, elucidates the etiology of prediabetes and its corresponding treatment medications. Nevertheless, the role of interleukin (IL)-35 in regulating the progression of prediabetes has not been investigated, which deserves due attention.

IL-35 has garnered considerable interest in recent years as a potential pivotal controller of diabetes, namely in prediabetes and chronic inflammatory autoimmune diabetes, which are progressively impacting children and adolescents across various locations globally[2,3]. Two separate genes encode IL-35 called IL12A and Epstein–Barr virus-induced 3 (EBI3)[4,5]. Both the genes IL-12A and EBI3 are networked with various diseases, as represented in Figure 1. The data in the PubMed database indicated a direct correlation between IL-35 and EBI3 genes in many immune-inflammatory, autoimmune, cancer, and endocrine diseases[6-8].

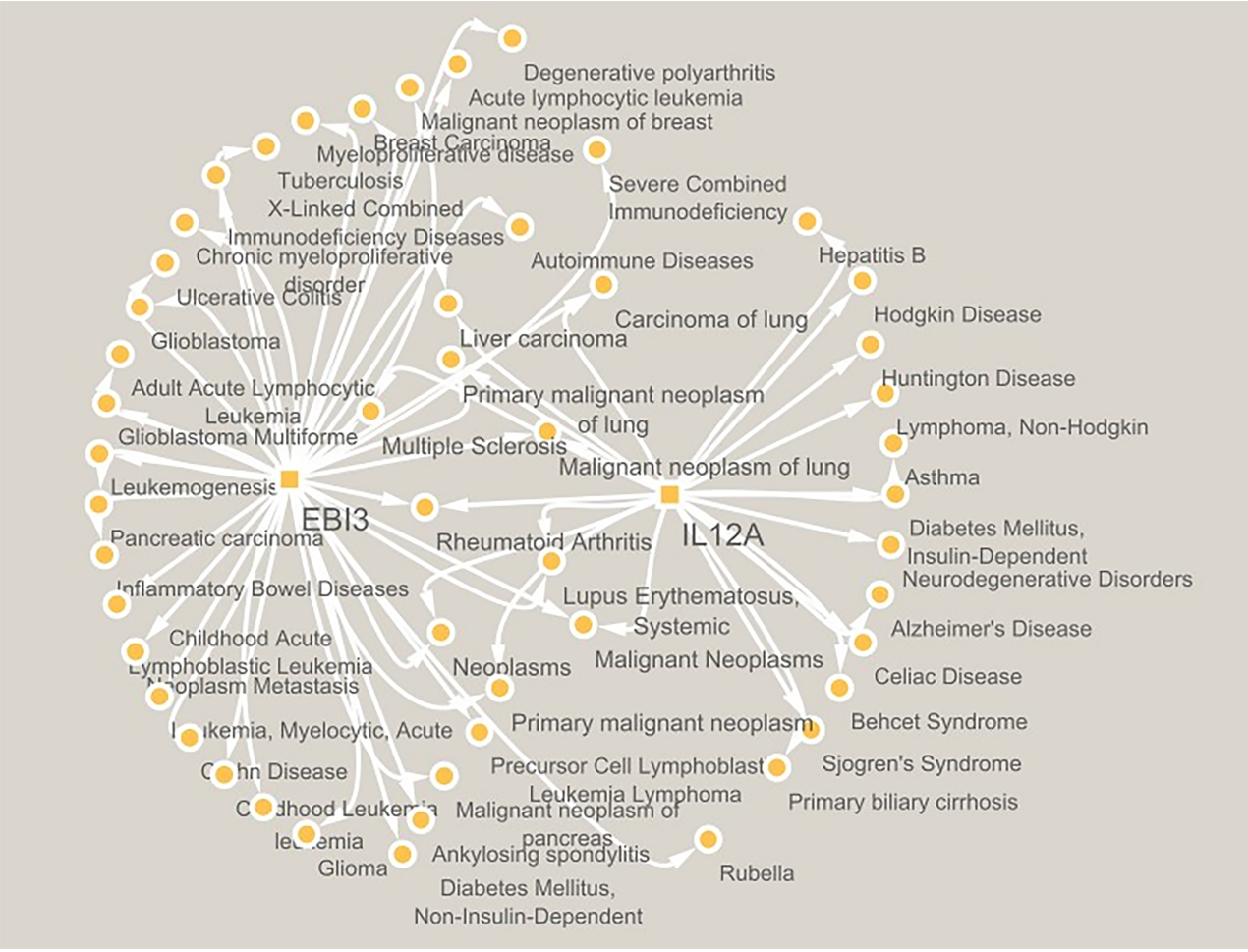

Figure 1 Schematic representation of interleukin-12A and Epstein–Barr virus-induced 3 individually and mutually networked with

different diseases by white solid lines. Data were collected from the PubMed database. The disease-gene association is searched in the DisGeNET database v 7.0 for gene-disease associations, whereas the "N_PMIDs (citation)" ≥ 3 were considered, and a gene-disease target network was created and analyzed using CYTOSCAPE version 3.10.0. Schematic representation of networking in Figure 1, in which interleukin-12A and Epstein–Barr virus-induced 3 are found to be individually and mutually networked by solid white lines.

Further, we selected 5 protein/enzyme markers clinically identified with IL12A and EBI3 from the PubMed database. We then searched for their UniProt ID and human gene names in the UniProt databases and looked for their disease associations in the DisGeNET database v7.0. Gene-disease associations with N_PMIDs (citation) greater than or equal to 10 were considered. Finally, we created a gene-disease target network using CYTOSCAPE version 3.10.0[9]. The schematic representation of networking of the total of five genes named ADIPOQ, CRP, IL18, IL1RN, and SERPINE1 encodes the protein Progestin and adipoQ receptor family member 3, C-reactive protein, IL-18, IL-1 receptor antagonist protein, and SERPINE1 mRNA-binding protein 1, respectively are shown in Figure 2.

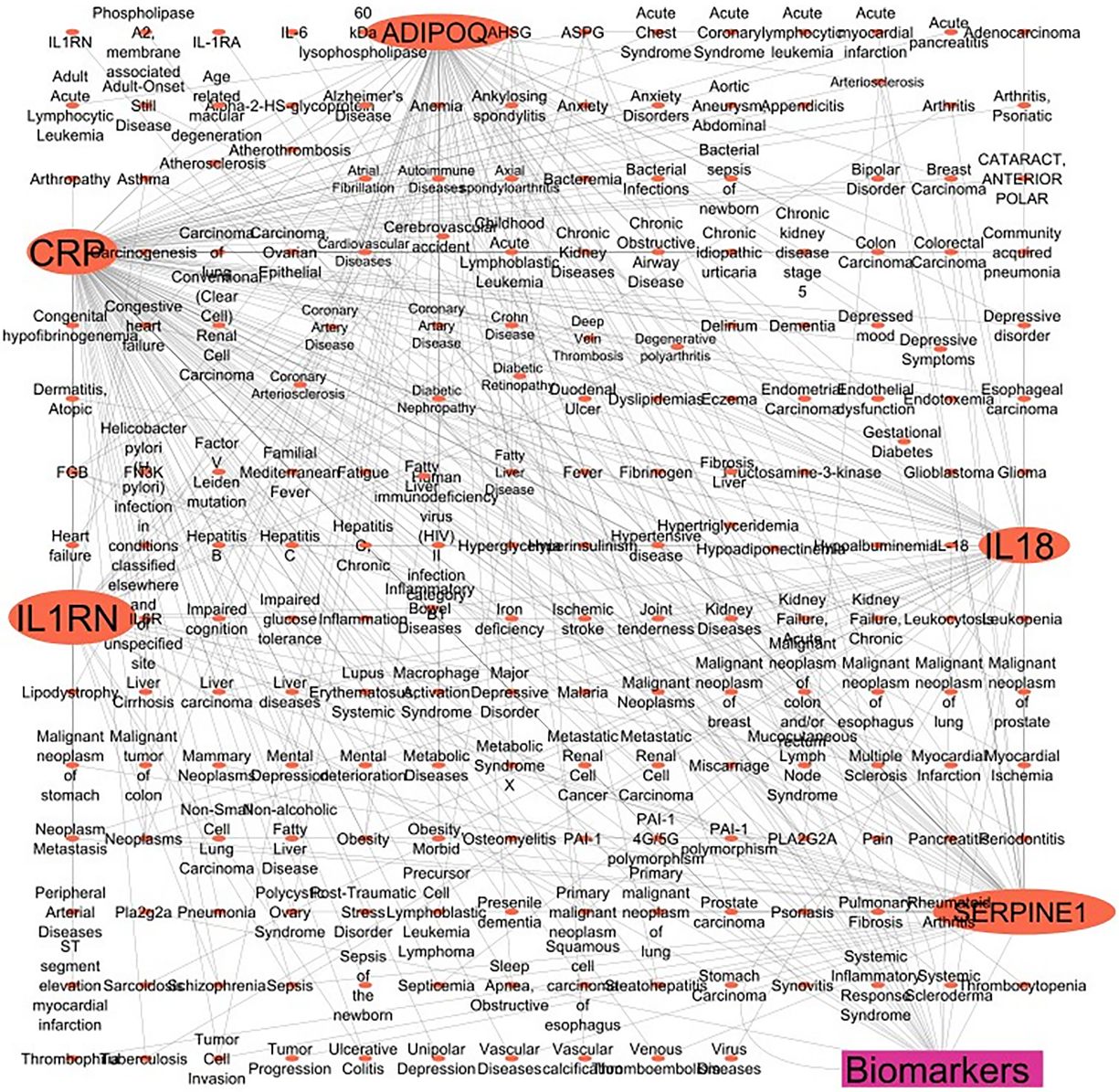

Figure 2 Schematic representation of networking of the 5 genes encodes 5 unique protein/enzymatic markers. A total of 5 clinically identified protein/enzyme markers were identified from the PubMed database. Next, their UniProt ID and human gene names were searched in the UniProt databases, and their disease associations were found in the DisGeNET database v7.0. A note on gene-disease associations with N_PMIDs (citation) greater than or equal to 10 was taken. Lastly, the gene-disease target network was constructed and analyzed using CYTOSCAPE version 3.10.0.

The network pharmacological analysis revealed that these five genes, highlighted in the figure, exhibited the most significant interaction with the disease. The PubMed database establishes a correlation between 5 genes and various disorders, encompassing immune-inflammatory, autoimmune, cancer, and endocrine diseases.

Based on several PubMed literature searches, there is a direct correlation between proinflammatory mediators such as CRP and IL-6R, which has been re-validated through networking. As a result, IL-35, an anti-inflammatory immune suppressant, may help counteract the proinflammatory signals that occur during prediabetes, diabetes, and its complications.

KEY POINTS

IL-35 is a protective factor against prediabetes and plays a significant role in macrophage polarization[10]. Treg and Th1 cells are crucial for this protection[11]. Studies on non-obese diabetic mice have revealed that IL-35 expression reduces conventional T cells, dendritic cells, and Treg cells against beta cells[12]. The administration of IL-35 also reduces the number of Th1 and Th17 cells and IFN-γ or IL-17A-expressing CD8+ T cells[13]. Thereby, IL-35 plays a critical regulatory role in T1D by decreasing the infiltration of mononuclear cells in the islets[14,15]. Clinical research has provided additional insights, indicating that C-peptide-negative patients exhibit markedly lower serum levels of IL-35[8-13]. This decrease is associated with a simultaneous reduction in the proportion of IL-35+ Treg cells, IL-35+ regulatory B cells, and IL-35-producing CD8+ FOXP3+ cells[15,16].

CONCLUSION

The results above emphasize the possible importance of IL-35 expression in regulating the immune response and its involvement in the autoimmune mechanisms underlying type 1 diabetes. Immunotherapy with IL-35 has demonstrated encouraging outcomes in combating the consequences of prediabetes and diabetes. Research indicates that IL-35 can alter the balance of cytokines, modulate the activity of B cells, and offer defense against autoimmune diabetes. Nevertheless, additional investigation is necessary to ascertain the precise mechanism of action, accompanied by meticulous design of clinical studies.

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FOOTNOTES

Author contributions: Bala A was responsible for planning and writing; Chakraborty R systematically formatted the manuscript; Mukherjee AK revised the manuscript with data analysis.

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World Journal of Diabetes

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LETTER TO THE EDITOR

Gut microbiota modulating therapy for diabetes mellitus should be individualized

Jin Wang, Hong-Juan Wei, Rui-Feng Mao, Xin Chang

Specialty type: Endocrinology and metabolism

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Abstract

In this editorial, we commented on two articles published online in August and September 2024 in the *World Journal of Diabetes*, which focused on modifying the gut microbiota (GM) to prevent or delay the progression of diabetes mellitus (DM) and DM-related complications. Numerous studies, many of which are animal studies, have indicated the potential role of GM in the pathogenesis of DM. However, the detailed causality and mechanisms between GM and DM have not been fully clarified. Although there have been some reports of a potential role of modifying the GM in treating DM, most lack long-term observations and are not mechanistic. Additionally, the GM and its role in DM might vary among individuals; therefore, GM-targeted interventions should be individualized to realize their therapeutic potential.

Key Words: Diabetes mellitus; Gut microbiota; Dysbiosis; Causality; Individualized interventions

Core Tip: This editorial focuses on the research progress regarding the gut microbiota (GM) in the development of diabetes mellitus (DM). Revealing and understanding the precise causality and mechanisms between the GM and DM may facilitate investigations focused on modifying the GM to ameliorate DM and its complications. Additionally, as a result of the considerable interindividual heterogeneity in the GM, more precise and personalized GM-targeting therapeutic interventions should be considered.

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TO THE EDITOR

As a common metabolic and endocrine disease, diabetes mellitus (DM) is characterized by glycometabolism disorders resulting from malfunctions in insulin secretion and/or its action. The high and steadily increasing global prevalence of DM poses an increasing burden on health care, particularly in developed countries[1]. DM has various subtypes, the most important being type 1 DM (T1DM) and type 2 DM (T2DM), and more than 90% of DM cases worldwide are T2DM[2]. As a chronic autoimmune disease, T1DM is characterized by insulin deficiency resulting from inflammation and the destruction of insulin-producing pancreatic β-cells mediated by autoantibodies. Although T1DM can occur at any age, it primarily affects children and adolescents[3]. Individuals with T1DM require a lifelong dependence on exogenous insulin as there is no cure[3]. In contrast, T2DM mainly occurs due to insulin resistance, or insulin deficiency resulting from β-cell dysfunction caused by mechanisms other than the autoimmune process similar to T1DM[4]. As a result of its progressive nature, T2DM is treated by lifestyle changes, oral antidiabetic drugs, or insulin injections depending on its stage[5]. After the diagnosis of T1DM or T2DM, glycemic control is the central goal of DM management, as hyperglycemia contributes greatly to DM itself and to its various complications[6]. However, it is not easy to achieve and maintain optimal glycemic control, and only 25% of T2DM patients receiving insulin therapy achieve good glycemic control[7]. Thus, there is a considerable need to further improve the management of DM, which requires a comprehensive consideration of various aspects.

The optimal management of DM depends on a full understanding of its associated risk factors and pathogenesis. Various potential risk factors for developing DM, such as a family history of DM (genetics), altered and/or unhealthy lifestyle behaviors, obesity, and environmental irritants, have been suggested[8]. Scientific research has shown that the balance of the gut microbiota (GM) is closely related to the state of health, and the diversity, activity and composition changes of the GM, as an important part of this huge ecosystem, can significantly affect the metabolic function, and the immune response. Furthermore, gastrointestinal microflora has been shown to play a key role in maintaining homeostasis in, and GM dysbiosis might contribute to various diseases, including DM[9]. Therefore, GM might be an important environmental factor associated with the pathogenesis of DM. In the 8th[10] and 9th[11] issues of the *World Journal of Diabetes* this year, there were reports on the modification of the GM in the treatment of DM and DM-related complications. Inspired by these two papers, we wrote this editorial to describe the progress in research on modifying the GM in the treatment of DM.

As a large reservoir of microorganisms occupying up to 95% of the entire human microbiota, the GM mainly contains six phyla, of which *Firmicutes* and *Bacteroidetes* are the most dominant. In fact, an altered GM composition is an important characteristic of DM patients. In addition, increasing evidence indicates an underlying association between GM and DM [12,13]. Compared with healthy children, those with either new-onset T1DM or autoantibody positivity show GM dysbiosis, characterized by reduced diversity, reduced vitality and a lower ratio of *Firmicutes*-to-*Bacteroidetes*. Additionally, various specific gut microbiome changes have been observed in T1DM patients. These microbial changes might induce a proinflammatory environment and increase paracellular permeability, ultimately stimulating autoimmunity[13,14]. The occurrence of GM dysbiosis might contribute to the development and progression of T2DM *via* various mechanisms, such as increasing intestinal permeability by decreasing short-chain fatty acid (SCFA)-producing bacteria and modifying glucose homeostasis by altering bile acid signaling[15]. Therefore, it is generally believed that profiling the gut microbial composition and function in diagnosed or potential DM patients and then rebalancing or modifying gut dysbacteriosis could reverse DM or delay its development.

Under normal circumstances, as a dynamically changing microbial community, the complex gut microbial ecosystem can be affected by several factors, such as genetics, age, place of residence, diet, use of probiotics and prebiotics, medications, and environmental factors (*i.e.*, air pollution)[16]. Various strategies aimed at preventing or treating DM by modifying the GM have shown encouraging results, among which dietary intervention may be among the most effective, as dietary changes can easily affect the composition of the GM[17]. For example, a Mediterranean diet, which is rich in monounsaturated fats and fiber, could induce changes in the GM to increase SCFA production, especially butyrate, to exhibit immunoregulatory activities, suggesting a potential role in preventing or delaying the progression of T1DM and T1DM-related complications[18]. Currently, various studies have revealed that different dietary components may contribute to primary prevention, secondary prevention, tertiary prevention, or complication prevention in patients with T1DM[19]. However, whether these dietary interventions function *via* GM-mediated processes is unclear; and indeed, the

protective effects of some dietary interventions on T1DM are not mediated by GM[20]. There are more studies on the associations between diet and GM with T2DM than with T1DM, and strong associations have been reported[21]. Similarly, a Mediterranean diet improved glycemic control in patients with T2DM, possibly by increasing GM diversity and richness. Therefore, GM richness has potential as a biomarker for analyzing the efficacy of dietary interventions in T2DM patients[22]. A high-fiber diet resulted in increased levels of *Lactobacillus*, *Bifidobacterium* and *Akkermansia* and decreased levels of *Desulfovibrio*, *Klebsiella* and other opportunistic pathogens, thus leading to better glycemic control[23]. However, it should be noted that alterations in the GM induced by dietary changes seem temporary; therefore, long-term studies of dietary interventions that evaluate the microbiome and the quality of DM control are needed[24]. Future research will focus on developing diet plans rich in specific prebiotics, dietary fiber or oligosaccharides that promote the growth of beneficial bacteria and inhibit that of harmful bacteria, thereby optimizing the composition of the GM and improving the metabolic status of diabetic patients. Additionally, nutrition recommendations personalized according to the characteristics of the GM, such as by adjusting the diet plan, would likely achieve the best results. In addition to dietary interventions, numerous medications, especially antibiotics, can significantly impact or regulate GM homeostasis and could be used to modify the GM composition in patients with DM. Indeed, antidiabetic medications, such as metformin, might also act by regulating the GM balance[25]. Additionally, probiotics and prebiotics have been reported to ameliorate DM by affecting the GM[17,26,27].

As described above, various studies have investigated the association between the GM and DM and the effect of modifying the GM on the progression of DM and DM-related complications. However, it should be noted that most studies of the causality of the relationship between GM and DM used mouse models, and of the few studies conducted in human subjects, most were observational. In addition, human studies have focused on patients with one type of DM. In studies of T2DM, newly diagnosed patients are most frequently used, whereas studies of T1DM typically involve children with an unformed or unstable GM[28]. The reproducibility of human research might be poor due to variations in the types of interventions, geographic locations, participants, and study designs and settings. Moreover, some human studies have reported contradictory observations[17]. In other words, research on the association between the GM and DM is at a very early stage, and it is difficult to clarify the detailed causality of the relationship between a specific gut bacterium and phenotypic exposure; thus, more research is still required. This ambiguous association is a major obstacle to preventing or delaying the progression of DM and DM-related complications by modifying the GM. Future studies evaluate how the GM directly or indirectly participate in the regulation of blood glucose and the maintenance of insulin sensitivity through the production of SCFAs, the regulation of bile acid metabolism, and the release of inflammatory factor. An in-depth analysis of these mechanisms will provide a solid theoretical basis for the treatment of DM by modulating the intestinal flora.

Interindividual heterogeneity is another important factor that should be considered. Being influenced by various factors, the composition and richness of the GM are highly variable among individuals. In the future, DM treatment is likely to be personalized. Precise analysis of the GM through high-throughput sequencing technology can identify members of the microbiota or metabolites associated with the diabetes risk, enabling the design of targeted dietary interventions or probiotic/prebiotic supplementation programs to achieve optimal treatment outcomes. Therefore, given that the altered GM of patients with DM may be individual specific, analysis of the GM composition by cutting-edge technologies, such as next-generation sequencing is needed. However, despite the popularity of next-generation sequencing, the cost and time constraints preclude regular monitoring of changes in the GM. As an alternative, a multiplex TaqMan qPCR assay targeting various gut microbial phyla could be used to monitor alterations in the GM[29]. On the basis of the potential role of an altered GM in DM, individualized therapeutic interventions targeting the GM, such as personalized nutrition and/or probiotics or prebiotic mixtures, should be designed and evaluated for their ability to prevent or delay the progression of DM and DM-related complications. Moreover, the long-term effects and sustainability of these individualized therapeutic interventions should be investigated.

The implementation of GM-based treatment strategies for DM requires close collaboration among endocrinology, nutrition, microbiology, genetics and bioinformatics. Interdisciplinary research can provide insight into the pathogenesis of DM, thereby accelerating the development and application of new treatments. As data accumulate, GM regulation therapy is expected to be included in the clinical treatment guidelines for DM alongside drug therapy and lifestyle intervention. Moreover, governments and health institutions should strengthen relevant policies, encourage scientific research and innovation, promote the clinical translation of scientific research, and improve DM prevention and control.

CONCLUSION

In conclusion, various genetic and environmental factors contribute to the risk of DM. Accumulating evidence suggests that GM dysbiosis should be considered a causative environmental factor of T1DM and T2DM. The discovery of the relationship between the GM and DM opens a new horizon for the management and treatment of DM and heralds the arrival of a new era of DM control based on the regulation of the human microecology. However, the precise causality and mechanisms of the relationship between the GM and DM have not been revealed and deserve further elucidation, which will facilitate modification of the GM to ameliorate DM and its complications. Moreover, the role of the GM in DM might show interindividual heterogeneity, highlighting the importance of more precise and personalized GM-targeting therapeutic interventions. As research continues and technologies advance, we believe that further breakthroughs in this field will lead to the development of safer and more effective treatment options for DM.

FOOTNOTES

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