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AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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OPINION REVIEW

Access to novel anti-diabetic agents in resource limited settings: A brief commentary

Poobalan Naidoo, Kiolan Naidoo, Sumanth Karamchand, Rory F Leisegang

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Abstract

The prevalence of diabetes mellitus is increasing in resource limited settings. Simultaneously, there has been an increase in the number of novel therapies for the management of diabetes mellitus. However, use of novel antidiabetic therapies is limited because of major market access challenges in resource limited settings. Niching products to those patients with the highest absolute risk for major adverse cardiovascular outcomes, and thus most likely to benefit from the therapy, are less likely to have negative budget impact for funders. To improve access, and reduce morbidity and mortality, requires alignment amongst key stakeholders including patient advocacy groups, health care professional councils, national departments of health, the pharmaceutical industry, treasury and finance departments.

Key Words: Type 2 diabetes mellitus; Novel anti-diabetic agents; Resource limited settings; Access

Core Tip: The manuscript addresses the problem of access to novel anti-diabetic agents in resource limited settings. Niching therapies for use in those with highest major adverse cardiovascular risk, may limit budget impact for funders. To improve access, and reduce morbidity and mortality, requires alignment amongst key stakeholders including patient advocacy groups, health care professional councils, national departments of health, the pharmaceutical industry, treasury and finance departments.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is increasing rapidly in resource limited settings[1]. This is likely to be multifactorial in aetiology including urbanisation, sedentary lifestyle and an increase of screening[2]. The diabetes epidemic has been paralleled by a rapid increase in the number of new therapies to manage type 2 diabetes[3]. These therapies include sodium - glucose transporter 2 inhibitors (SGLT2i), dipeptidyl peptidase-4 inhibitors and glucagon-like-peptide-1 receptor agonists (GLP-1 RAs). The American Diabetes Association and European Association for the Study of Diabetes 2022 consensus report of the management of hyperglycaemia in T2DM recommend an SGLT2i or GLP-1 RA with demonstrated cardiovascular benefit as initial therapy for individuals with T2DM with or at high risk for atherosclerotic cardiovascular disease, heart failure, and/or chronic kidney disease[4].

Unfortunately, in resource limited settings, treating clinicians and patients living with T2DM, have limited access to these therapies due to cost and access constraints. The situation was compounded by the coronavirus disease 2019 pandemic that consumed financial and human resources, that would otherwise have been used for non-communicable diseases such as diabetes.

The irony is that resource limited settings partake in clinical trials programs that test the safety, efficacy and tolerability of novel therapies. Although these countries partaking in the clinical trial programs, patients in these resource limited settings have constrained access to these interventions regardless of regulatory approval. Post-trial access and care are virtually non-existent in these settings[5]. In the absence of a robust post-trial access program, this places a substantial burden on the patient who contributes to the scientific body of evidence supporting a drug's approval but is unable to obtain treatment benefit beyond a predefined, finite period[6].

A major challenge is how to make novel therapies available to patients in resource limited settings. From a clinical perspective, a viable argument is for relevant authorities to facilitate product access for patients at the highest risk and most likely to benefit from therapies. This will niche these novel agents and thus minimise the number of patients on these therapies. For example, SGLT2is can be used in patients with congestive cardiac failure and with diabetes mellitus thus optimising glycaemic control while also reducing hospitalising for heart failure and subsequently reducing healthcare resource utilisation. This would be more cost effect than rolling out these therapies to all patients with diabetes, which is not financially sustainable in developing countries.

In our experience, requests for controlled access to novel drugs, with real world data collection to inform future clinical decisions, have not been successful. The prevailing perspective of focusing on short term drug costing of SGLT2is and not the future healthcare resource utilisation savings through reduced hospitalisations for heart failure, delayed progression of chronic kidney disease and reduction in mortality, requires a paradigm shift and political willingness to address medium and long-term costs and not just short-term expenditure.

An innovative approach is needed to ensure equity of access to novel treatments within a resource limited setting. As patient advocates, we feel that clinicians are best equipped to lead the process to enable access. Merely submitting drug access applications *via* existing systems without engagement on the core challenges at hand is frustrating and often futile. How do we as busy clinicians advocate for access? Perhaps the first step is a collective approach. We suggest engaging with relevant stakeholders to define the current challenges and outline potential solutions. This can be done at a national workshop during a diabetes congress. Alignment amongst key stakeholders including patient advocacy groups, health care professional councils, national departments of health, patient advocacy groups, the pharmaceutical industry, treasury and finance departments is needed in order to improve treatment access with the ultimate intention of improving patient outcomes.

CONCLUSION

In times of economic challenges, it may be necessary to invest funds in urgent related treatment. Furthermore, sourcing drugs from markets that are cost conscious may be an option.

Ultimately, after wide consultation and workshops, laws, acts and regulations will be required to protect the interests of patients and ensure access to novel antidiabetic therapies.

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REVIEW

Detection, management, and prevention of diabetes-related foot disease in the Australian context

Scott McNeil, Kate Waller, Yves S Poy Lorenzo, Olimpia C Mateevici, Stacey Telianidis, Sara Qi, Irina Churilov, Richard J MacIsaac, Anna Galligan

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Abstract

Diabetes-related foot disease (DFD) is a widely feared complication among people who live with diabetes. In Australia and globally, rates of disability, cardiovascular disease, lower extremity amputation, and mortality are significantly increased in patients with DFD. In order to understand and prevent these outcomes, we analyse the common pathogenetic processes of neuropathy, arterial disease, and infection. The review then summarises important management

considerations through the interdisciplinary lens. Using Australian and international guidelines, we offer a stepwise, evidence-based practical approach to the care of patients with DFD.

Key Words: Diabetes-related foot disease; Foot ulceration; Lower extremity amputation; Neuropathy; Peripheral arterial disease; Infection

Core Tip: In Australia, the interdisciplinary service is recognised as a critical component of providing care to people with diabetes-related foot disease (DFD). We give our perspective on the management of DFD based on 6 categories: (1) Assessment and education in high-risk patients; (2) Wound preparation, debridement, and dressing; (3) Offloading and footwear; (4) Diagnosis and management of infection; (5) Interventions including revascularisation, pharmacotherapy, and novel wound therapies; and (6) Integrated interdisciplinary care and patient information.

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INTRODUCTION

Diabetes affects an estimated 463 million people worldwide, a number expected to increase to over 700 million by 2045 [1]. The increasing prevalence of diabetes in Australia is driven largely by obesity and an ageing population. People with diabetes have an increased rate of vascular complications even when adjusting for other established risk factors. The combination of obesity and diabetes is associated with higher rates of hospital admission and mortality due to diabetes complications[2]. Foot ulceration and lower extremity amputation (LEA) are catastrophic but preventable complications of diabetes. We review the epidemiology, risk factors, and classification system of diabetes-related foot disease (DFD). Furthermore, we offer a clinical perspective from a National Association of Diabetes Centres accredited High Risk Foot Service Centre of Excellence on the management of DFD based on 6 categories: (1) Assessment and education in high-risk patients; (2) Debridement, wound preparation, and dressings; (3) Offloading; (4) Management of infection; (5) Interventions including revascularisation, pharmacotherapy, and novel wound therapies; and (6) Integrated interdisciplinary care and patient information which summarise and synthesise the major treatment principles in recent guidelines [3,4].

METHODS

We performed a literature review of PubMed, Medline, and Embase for articles published in English using the following key words: Diabetes-related foot disease; Foot ulceration; Lower extremity amputation; Neuropathy; Peripheral arterial disease; Infection. Recent international guidelines, Australian position statements, systematic reviews, and meta-analyses were preferred to provide an overview of Australian and International prevalence, best practices, and emerging strategies for the management of DFD. We have focused on the International Working Group on the Diabetic Foot (IWGDF) and Diabetes Foot Australia (DFA) guidelines to formulate our narrative review and recommendations.

EPIDEMIOLOGY OF DIABETES-RELATED FOOT DISEASE

DFD is defined as ulceration or infection of the foot associated with the key risk factors of peripheral neuropathy and/or peripheral arterial disease (PAD) in people with diabetes[3,5]. There is a huge global disability burden from DFD, which affected 131 million people worldwide in 2016, ranking 11th in the global disease burden[6].

At least 1 in 5 people with diabetes develop DFD in their lifetime, with an annual incidence of around 2%[7,8]. Aboriginal and Torres Strait Islander Australians have a 3-6 fold increased likelihood of experiencing diabetes-related foot complications compared to non-Indigenous Australians[9]. Ulcer recurrence is common, occurring at a rate of 40% within the 1st year after the ulcer has healed, almost 60% within 3 years, and 65% within 5 years[7,8,10].

DFD can be a surrogate marker of systemic disease. Armstrong *et al*[11] reported the 5-year mortality rates for DFD-related ulceration, and minor and major LEA were 30.5%, 46.2%, and 56.6% respectively, compared with a 5-year pooled mortality for all reported cancer of 31.0%[11]. The 10-year mortality outcomes in a large multi-centre meta-analysis were demonstrated to be 71% in those with DFD, compared to 5% in those without, with a median survival of 7.72 years compared to 12.6 years[12]. Patients with DFD have a 2-fold increased risk of all-cause mortality at 10 years compared to

people with diabetes who do not have DFD when controlled for age, sex, education, smoking, and waist circumference [13,14]. Established diabetic nephropathy has been shown to increase mortality in patients with DFD [Odds ratio (OR) 1.47], as has duration of diabetes (OR 1.31) and history of previous amputation (minor OR 1.85, major OR 2.96)[12]. The main causes of death are cardiovascular events (54.7%), respiratory causes (18.9%), and multi-organ failure (12.5%)[15].

Patients with DFD have increased frailty scores and demonstrate significant physical disability in activities of daily living[16]. Frailty is associated with a 5-fold increased risk of re-hospitalisation in patients with non-healing DFD[17]. Sarcopenia, a disorder of muscle mass and function that is highly prevalent in cases of frailty, has increased prevalence in people with diabetes and DFD, particularly in patients with underlying peripheral neuropathy[18]. A recent Australian study found a high likelihood of sarcopenia in 51% of patients with DFD which was associated with significantly lower quality of life based on a validated quality of life questionnaire[19].

DFD is a leading cause of hospitalisation and LEA in people with diabetes. While the association with mortality is clear, there is variability in reported rates of LEA over time[20]. A recent data linkage study in Australia found the overall rates of LEA were as high as 29.8 per 10000 patients (95%CI: 27.7-31.9)[21]. Trends in admission for LEA appear to be decreasing over time in patients with type 2 diabetes with an average annual percentage change of -4.9% between 2004 and 2016[21]. Conversely, no change in rates of LEA over time was observed in patients with type 1 diabetes (age-adjusted annual percentage change 1.4 (95%CI: -0.5-3.3)[21]. In the United States, Geiss *et al*[22] demonstrated an initial significant reduction in non-traumatic LEA in people with diabetes between 2000 and 2009 of 43%[22]. Subsequently, there was a rebound between 2009 and 2015 to just below the rates seen in 2000, with a particular increase in young and middle-aged adults[22]. Concerningly, increased trends of LEAs in young patients were seen in multiple Australian studies[23,24].

RISK CATEGORIES AND ULCER CHARACTERISTICS

Key risk factors for DFD are loss of protective sensation (LOPS), PAD, foot deformity, ulcer history, and previous amputation[8]. The IWGDF Risk Stratification System describes the risk of foot ulceration in people with diabetes and provides recommendations for screening for frequency for these key risk factors (Table 1).

Peripheral neuropathy can lead to changes in gait, foot deformity and soft tissue which can elevate mechanical stress[7,25,26]. The combination of mechanical stress and a LOPS from peripheral neuropathy leads to tissue damage, callus formation and subcutaneous haemorrhage, precipitating ulceration in the neuropathic foot (Figure 1)[7,25].

PAD is associated with an increased risk of non-healing ulcers, infection and LEA[27-29]. The Wound, Ischaemia, and foot Infection (WIfI) classification system was developed to provide risk stratification and predict the risk of amputation and requirement for revascularisation at 1 year[30]. A higher WIfI correlates with an increasing risk of infection, stenosis events and poor wound healing[31].

In patients with diabetic foot infection, other independent risk factors for LEA are osteomyelitis (OR 1.94), retinopathy (OR 1.32), history of amputation (OR 1.47), and history of osteomyelitis (OR 1.94). Male sex and smoking were also associated with increased risk of LEA (OR 1.31 and 1.38, respectively); these risk factors are also associated with the development of PAD caused by atherosclerotic plaque formation[32].

Assessment of risk factors and their management are the basic principles to prevent the development of DFD and the risk of subsequent LEA. Evaluation for LOPS and the presence of PAD, together with specific assessment and management of DFD form the basis of the management recommendations to follow. Optimising glucose control is an established approach to prevent the development and progression of diabetic peripheral neuropathy and LOPS. Attention to strict cardiovascular risk factor modification is the basic management strategy for the prevention of the development and progression of PAD. Furthermore, specific medications may reduce the risk of LEA and/or the development of cardiovascular disease[33,34].

MANAGEMENT PRINCIPLES

Based on IWGDF and DFA guidelines, DFD treatment can be summarised in 6 main categories[3,4]: (1) Assessment and education in high-risk patients; (2) Debridement, wound bed preparation and dressings; (3) Offloading; (4) Management of infection; (5) Interventions including revascularisation, pharmacotherapy and novel wound therapies; and (6) Integrated interdisciplinary care and patient information.

Cheng and Lazzarini performed Markov modelling to predict the efficacy of the first 4 interventions within a multidisciplinary team against long-term outcomes[35]. Comparing "optimal care" to the current standard of care, the model resulted in an overall cost saving to the health network of Australian dollar $2.7 billion over 5 years and improved the quality of life of participants compared to usual care. This theoretical outcome measure justifies the standardisation of DFD interventions for improved patient care and long-term health economics[35].

Management category 1: Assessment and patient education

The IWGDF and the DFA guidelines recommend an annual foot assessment for signs or symptoms of LOPS and PAD for people with diabetes at very low risk of foot ulceration (IWGDF risk 0)[3,36]. LOPS can be assessed using a 10-g Semmes-Weinstein monofilament, 128-Hz tuning fork or the Ipswich Touch Test[8]. At a minimum, in people with diabetes, taking a relevant history and palpating foot pulses should be done to assess for the presence of PAD. People with DFD

Table 1 International Working Group on the Diabetic Foot risk categories[8]

Risk category	Ulcer risk	Characteristics	Frequency
0	Very low	No LOPS and no PAD	Once a year
1	Low	LOPS or PAD	Once every 6-12 mo
2	Moderate	LOPS and PAD, or LOPS and foot deformity, or PAD + foot deformity	Once every 3-6 mo
3	High	LOPS or PAD and one of the following: History of foot ulcer, a previous LEA, end-stage renal disease	Once every 1-3 mo

LEA: Lower extremity amputation; LOPS: Loss of protective sensation; PAD: Peripheral arterial disease.

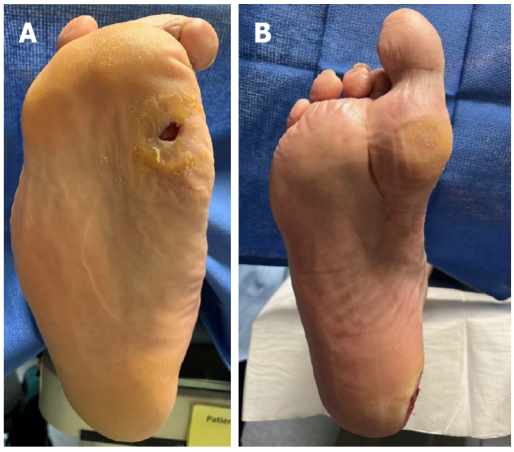

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Figure 1 The combination of mechanical stress and a loss of protective sensation from peripheral neuropathy leads to tissue damage, callus formation, and subcutaneous haemorrhage, precipitating ulceration in the neuropathic foot. A: Foot deformity with ulceration at the plantar forefoot; B: Foot deformity with healed ulceration at the plantar forefoot, a high-risk for ulcer recurrence.

will require further non-invasive vascular tests and/or imaging[37]. People with diabetes at higher risk of foot ulceration (IWGDF risk 1-3), including those with a history of foot ulceration or LEA, end-stage renal disease, presence or progression of foot deformity, limited joint mobility, abundant callus, or pre-ulcerative signs should have more frequent screening (*i.e.* 6-12 mo for IWGDF risk 1, 3-6 mo for IWGDF risk 2, 1-3 mo for IWGDF risk 3)[3,38]. People with diabetes and risk of foot ulceration should be instructed to protect their feet with appropriate footwear, perform daily inspections and be educated on the best foot care[36].

Management category 2: Wound preparation, debridement and dressings

A number of factors should be considered in the approach to wound bed preparation and dressing selection in DFD, including the underlying aetiology/s of the wound and factors impacting healing, the goals of management, patient-centred concerns including pain and access to resources and skilled clinicians[39,40].

Armstrong *et al*'s[41] mantra "it's not what you put on, but what you take off" for debriding and offloading techniques for diabetes-related foot ulcer guides the importance of addressing plantar pressure and devitalised tissue that will impact ulcer healing. In a wound with adequate arterial perfusion to heal, active sharp debridement to remove devitalised tissue such as callus, slough and necrosis will promote a moist wound environment to reduce infection risk encourage granulation and conditions conducive to healing[39].

An assessment of the ulcer exudate levels and other local wound conditions guides dressing choice and frequency of dressing change[40]. A dressing may either donate moisture to the wound bed, absorb exudate or maintain the current moisture levels. Moist wound healing is well established to improve outcomes with reduced healing time, pain management and infection rates in wounds with adequate arterial perfusion to heal[42]. In a dry and ischaemic ulcer that is not expected to heal, and where the goal of management is to prevent further deterioration, a dry dressing regime offers the best protection from infection and wound deterioration[39,43].

Management category 3: Offloading and footwear

An evidence-based and practical approach to offloading the neuropathic foot ulcer for best healing outcomes is outlined in the DFA and IWGDF guidelines[3,44]. A non-removable knee-high offloading device such as a total contact cast (Figure 2) or a removable cast walker rendered irremovable are the gold standard to promote healing in people with a plantar neuropathic/diabetes-related foot ulcer. Patient factors such as high wound exudate or infection, ischaemia or a risk of falling may preclude the use of an irremovable device. Knee-high removable devices (such as a Controlled Ankle

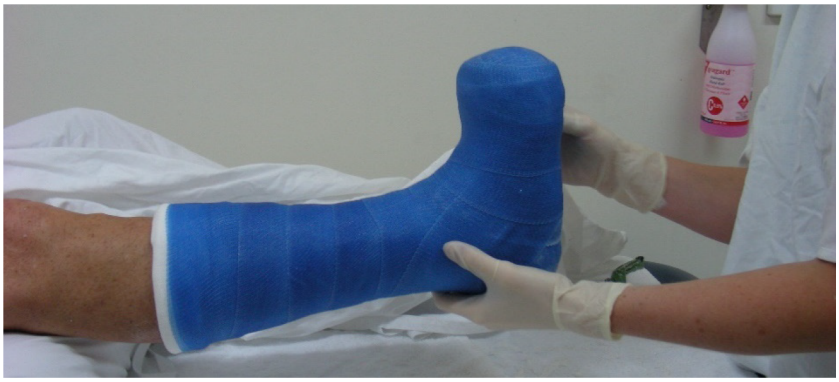

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Figure 2 Total contact cast. A total contact cast is an irremovable knee-high device that is suitable for some people for offloading treatment of diabetes-related foot disease. It is applied by a skilled clinician and changed every 3-14 d, depending on the foot and ulcer conditions.

Motion walker) may be a suitable compromise to irremovable devices, and if these are contraindicated or not well tolerated, an ankle-high offloading device should be worn during all weight-bearing activities. Felted foam in combination with an offloading device or custom-made shoe can assist healing when implemented by skilled clinicians. Where other ankle-high and knee-high devices are contraindicated or not tolerated, medical-grade footwear should be used rather than other standard shoes or no footwear.

Management category 4: Diagnosis and management of infection

Most wounds will become colonised with pathogenic organisms from commensal skin flora or the environment. The presence of bacteria in a wound can alter the local pH and prevent wound healing but does not necessarily represent infection[45].

Within a colonised wound there is a perpetual change in the microbiome composition, with certain bacteria (*e.g.*, anaerobes) favouring the development of invasive infection.

The development of infection in diabetic foot wounds is the result of a dynamic interaction between the local host immune response and bacteria colonising the wound. Following colonization, tissue invasion by these microorganisms is facilitated by a combination of diabetes-related factors including hyperglycaemic state, ischaemia-induced tissue injury, neuropathic trophic changes of the skin, impaired local immunity and reduced phagocytosis of bacteria by the macrophages[46,47].

Wound infection in DFD may present with the classical clinical signs of erythema, warmth, pain or tenderness overlying the region[48]. The depth of the wound may be enough to establish the presence of underlying bone infection (osteomyelitis) if clinical signs are present. In a diabetes-related foot ulcer that probes to the bone, a diagnosis of osteomyelitis is very likely[49]. The presence of systemic toxicity or systemic inflammatory response and/or metabolic instability are the clinical hallmarks of a severe infection and can help differentiate between moderate and severe diabetic foot infection[50]. Distinguishing between the two is important as a predictive factor for major amputation and prolonged hospital stay[51].

Once a clinical diagnosis of infection in DFD is made, this may then prompt the use of further radiological investigations depending on the depth and chronicity of the ulcer[47]. The choice of antibiotic, route of administration and duration of treatment relies on a combination of clinical assessment, the severity of presentation, imaging and microbiology. These management issues are discussed in detail below.

Imaging: Imaging can aid with the diagnosis of osteomyelitis when in doubt and inform management decisions and interventions. A plain X-ray can demonstrate cortical erosion to suggest established osteomyelitis, but early changes may not be perceptible. Magnetic resonance imaging (MRI) can detect more acute changes like marrow oedema as well as deep-seated collections. For the diagnosis of osteomyelitis in DFD, MRI has a very high sensitivity (90%) and specificity (79%)[52]. An active Charcot neuroarthropathy is difficult to differentiate from mid-foot septic arthritis with MRI imaging alone and should be further interpreted in a clinical context[53]. A standard three-phase bone scan has long been available for the evaluation of a foot ulcer for osteomyelitis, with a sensitivity of 80%-90% and specificity of 30%-45%. Leucocyte scans with radio-labelled white blood cells can increase specificity to 75%-80% and are now considered the most superior form of nuclear medicine scan for DFD. However, the IWGDF guidelines suggest that MRI is the more useful imaging test for this indication[54]. Fluorodeoxyglucose F18 positron emission tomography or combined 99m technetium white blood cell-labelled single-photon emission and computed tomography (CT) offer additional functional imaging assessment but are costly and are generally not required[54,55].

Microbiology and empirical antibiotics: An acute, superficial wound with clinical signs of spreading cellulitis is most often caused by Gram-positive skin bacteria and can generally be managed without the need for a formal microbiological diagnosis[56]. Similarly, microbiological investigations are often unhelpful for cellulitis without an open wound[57]. The clinician should commence empirical antibiotics with activity against key Gram-positive bacteria such as methicillin-susceptible *Staphylococcus aureus* (MSSA) and *Streptococcus* species. The empirical coverage should provide activity

against methicillin-resistant *Staphylococcus aureus* (MRSA) in patients with a known history of colonisation. Narrow-spectrum antibiotics are appropriate unless the patient has recent antibiotic exposure or presents with a water-immersed wound, in which case additional Gram-negative cover may be recommended (Tables 2 and 3). The ulcer should be monitored closely, and antibiotics ceased once infection signs have resolved[58].

In a chronic wound, erythema and oedema can suggest the development of a deep-seated infection. In these circumstances, pain often develops even in the presence of peripheral neuropathy. In order to obtain a microbiological diagnosis, a tissue specimen (curettage or biopsy) from the ulcer should be taken after the wound has been cleaned of debris and surface exudate with non-viable tissue debrided. If a tissue sample is not possible, a wound swab can be considered after appropriate cleaning and debridement. Gram-positive and Gram-negative organisms may be present on microbiology specimens, though one pathogen may be predominant. Anaerobic organisms can be difficult to culture but should be suspected if the wound is malodorous or gangrenous in appearance. If the patient with a chronically infected wound is systemically well, delaying antibiotic treatment until microbiology results are available can be considered. If treatment delay poses clinical concern or if microbiology investigations are not available, an empirical treatment with broad-spectrum oral antibiotics is indicated (Tables 2-4). Similar to acute infections, empirical antibiotic regimen should include MRSA coverage if colonisation is established. There is general agreement that empirical coverage for *Pseudomonas aeruginosa (P. aeruginosa)* is rarely necessary for mild- and most moderate- severity diabetic foot infections, especially in temperate climate regions where prevalence is low[59,60]. This is supported by indirect evidence from randomised controlled trials where outcomes were similar between groups treated with antibiotics with antipseudomonal activity *vs* those without[61-63]. Furthermore, if *P. aeruginosa* is identified in microbiological cultures, escalation of treatment may not be necessary in patients improving on antibiotics ineffective against *P. aeruginosa*[50,64]. Empirical *P. aeruginosa* coverage in mild- to moderate-severity foot infections may be more important in regions with tropical/sub-tropical climates or in wounds exposed to environmental water.

In the presence of severe deep-seated infection, gangrene or sepsis, the patient requires hospitalisation and empirical treatment with an intravenous broad-spectrum antibiotic regimen with antipseudomonal activity. Additional coverage for MRSA is recommended for those with risk factors (Table 2) but should cease after 48-72 h if microbiological investigations show no evidence of MRSA involvement. In a patient with sepsis, blood cultures should be taken before antibiotics are administered if this does not significantly delay the commencement of treatment. Surgical debridement with or without minor amputation may be required to control the infection. In the case of overwhelming sepsis with ascending infection or very poor distal blood supply, the surgeon may be required to perform a major amputation below or above the knee. The choice of antibiotics used should be reviewed alongside available microbiological investigations within 48-72 h of commencing.

After surgical debridement or distal amputation, taking proximal bone chips from the healthy-appearing bone edge for microbiological culture is useful in determining the choice and duration of antibiotics. When the entirety of the infected bone and soft tissue has been removed (*e.g.* negative proximal bone chips cultures), antibiotics can be promptly ceased[47,50,65]. If a pathogenic organism is isolated in residual bone, a longer course of 2-4 wk may be required pending clinical progress. Consultation with a medical microbiologist may be required to interpret reported microbiological culture results and/or obtain further antibiotic susceptibilities.

A CT-guided bone biopsy is traditionally considered the gold standard for the diagnosis of chronic osteomyelitis but is only required if an organism has not been isolated in appropriately collected wound swabs or soft tissue biopsy and/or the infection is not responding to empirical treatment[66]. To avoid contamination, the sample must be taken through intact skin, not through the ulcer or sinus[67].

Other investigations: Australian and international guidelines suggest a review of biochemical markers including white cell count, erythrocyte sedimentation rate, C-reactive protein, and/or procalcitonin. These markers are not specific for osteomyelitis in the presence of moderate to severe skin and soft tissue infection or sepsis and need to be considered an adjunct to other investigations[58].

Histopathology from a punch or deep tissue biopsy is indicated if the wound looks atypical in appearance, which may help to diagnose mycobacterial infection, papillomavirus, malignancy, or vasculitis.

Other antibiotic considerations: Beyond wound culture and susceptibilities, the clinician needs to consider several other factors when choosing an antibiotic. Pill burden and dosing interval frequency can be a major factor in patient compliance. For example, broad-spectrum regimens such as amoxicillin with clavulanic acid or trimethoprim with sulfamethoxazole require the patient to take one tablet, twice daily while clindamycin with ciprofloxacin involves up to 12 capsules per day (Table 3). Interactions with other medications, toxicity and tolerance profile, ease of access and out-of-pocket expense are other important considerations which may require consultation with an infectious disease physician and/or pharmacist. Furthermore, many antibiotics used for a long duration require monitoring of a variety of biochemical markers, which may pose an additional burden to the patient and/or health system. If the patient requires intravenous antibiotics beyond the acute presentation, usually for the treatment of *P. aeruginosa* or multi-drug resistant bacteria, consideration of community-based parenteral antimicrobial therapy for the remaining duration will reduce hospital length of stay and allow the patient to recover in their own home[68].

Management category 5: Therapeutic interventions including revascularisation, pharmacotherapy and novel wound therapies

Revascularisation strategies: PAD is present in up to 50% of people with DFD[38]. In a person with DFD, non –invasive bedside tests including palpation of pedal pulses and evaluation of pedal Doppler arterial waveforms in combination

Table 2 Common bacteria involved in diabetic foot infections according to infection grade, adapted from the International Working Group on the Diabetic Foot infection guidelines[47]

IWGDF classification	Recommended empirical cover				
	Gram-positive (MSSA, *Streptococcus spp.*)	Gram-negative (enteric, non-pseudomonal)	Obligate anaerobes	MRSA	Pseudomonal
Mild (grade 2) – no recent antibiotics	Yes	No	No	If at risk[1]	No
Mild (grade 2) – recent antibiotics or water-immersed wound	Yes	Yes	Consider if chronic	If at risk[1]	No
Moderate (grade 3)	Yes	Yes	Consider	If at risk[1]	Tropical climates or recently cultured
Severe (grade 4)	Yes	Yes	Yes	If at risk[1]	Yes

[1]Risk factors for methicillin-resistant *Staphylococcus aureus* (MRSA) include previous colonisation with MRSA, residence in a community, aged-care facility, or correctional facility with high MRSA prevalence, and frequent or prolonged stay in a hospital with a high prevalence of MRSA.
IWGDF: International Working Group on the Diabetic Foot; MSSA: Methicillin-susceptible *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*.

with ankle systolic pressure and systolic ankle brachial index or toe systolic pressure and toe brachial index can be used to assist in the diagnosis of PAD[37]. In people with diabetes, ankle-brachial index can be abnormally high due to non-compressibility of the tibial arteries secondary to calcific changes[69]. This process usually spares the digital arteries, making toe systolic pressures a useful predictor for likelihood of diabetic foot ulcer healing[70]. Vascular imaging in the form of colour duplex ultrasound or CT angiography can be completed for patients with DFD as this will illustrate the level at which the vascular disease is present and assist in surgical planning. Patients with diabetes and PAD typically present with multi-level, long-segment atherosclerotic disease below the knee[71,72]. The underlying pathophysiology is thought to be secondary to the upregulation of vasoconstrictors, abnormal platelet activity, activation of the coagulation cascade and a tendency towards plaque rupture[71]. Optimization of blood supply may be done by revascularisation either by open bypass operation or endovascular intervention (EVI). The revascularisation technique should be decided according to the morphological distribution of PAD, availability of autogenous vein, patient co-morbidities and local expertise[38]. Revascularisation should aim to establish direct blood flow into at least one pedal artery[73]. The preferred vascular target is angiosome-directed, meaning, targeting the vessel that supplies the region of tissue loss directly[72]. Studies have shown that the presence of a complete pedal arch following endovascular intervention is associated with increased wound healing, greater amputation free survival and increased survival at 1 year compared to those without a complete pedal arch[74]. A recent systematic review reported wound healing in patients with diabetes-related foot ulcer at 1 y following EVI was 75% while the healing rates following open revascularisation were reported to be lower, at 52% [72]. Limb salvage rates however, seemed to be greater amongst those undergoing open revascularisation with 85% at 1 year and 87% at 2 years and similar rates of 30 d perioperative mortality[72,75]. Despite these numbers, redo-revascularisation is required in up to 40% of patients undergoing EVI and 31% in those undergoing open surgery[76]. When revascularisation is unsuccessful, amputation may be necessary at a level with adequate perfusion for sufficient wound healing.

Hindfoot ulceration has the highest risk of primary major amputation and secondary major amputation with the risk being reduced in midfoot and forefoot ulcers respectively[77]. Another study has shown that smoking, diabetes duration, hypertension and number of debridements after surgery were significant risk factors for re-amputation[78].

Risk-factor modification and pharmacotherapy: Before the introduction of more recent medications which reduce cardiovascular outcomes independent of glucose lowering effect, early risk factor modification in patients with diabetes has been shown to reduce incident cardiovascular events. The STENO-2 study undertaken in the 1990s, and with over 20 years of mean follow-up time, has continued to demonstrate a reduction in cardiovascular disease and mortality in patients with diabetes who receive early and intensive targeted treatment of blood pressure, cholesterol, microalbuminuria and glycated hemoglobin (HbA1c) as well as smoking cessation[79,80].

All patients with PAD are generally treated with HMG-CoA reductase inhibition using statin therapy[81]. When used as part of optimal medical therapy (renin-angiotensin-aldosterone system inhibitors, beta-blockers and anti-thrombotic agents), they have been shown to reduce major adverse cardiovascular events and all-cause mortality[82]. Treatment with a statin has been shown to reduce LEA by up to 25%[83].

Anticoagulant and antiplatelet medications have long been established therapies for cardiovascular risk reduction in patients with PAD. In patients with stable atherosclerotic disease, rivaroxaban plus aspirin combination has been shown to reduce cardiovascular death, stroke, and myocardial infarction [Hazard ratio (HR) 0.76] compared to either medication alone with no significant increase in intracranial or fatal bleeding[84]. Furthermore, low dose rivaroxaban (2.5 mg) plus aspirin was associated with a significantly reduced incidence of acute limb ischaemia, LEA, myocardial infarction, ischaemic stroke, or death from cardiovascular cause compared to aspirin alone in patients post revascularisation for PAD[85]. In the CAPRIE trial (clopidogrel *vs* aspirin in patients at risk of ischaemic events), clopidogrel was superior to

Table 3 Spectrum of select antibiotics against common bacteria involved in diabetic foot infections, adapted from the International Working Group on the Diabetic Foot infection guidelines[47]

Antibiotic	Antibiotic spectrum					Oral dose frequency	Pill burden (per day)
	Gram-positive (MSSA, *Streptococcus spp.*)	Gram-negative (enteric, non-pseudomonal)	Obligate anaerobes	MRSA	Pseudomonal		
Penicillins, anti-staphylococcal[1]	Yes	No	No	No	No	4	4-8
Cefalexin	Yes	Some	No	No	No	4	4-8
Amoxicillin-clavulanate	Yes	Yes	Yes	No	No	2	2
Trimethoprim-sulfamethoxazole	Yes	Yes	No	Some[2]	No	2	2
Doxycycline	Yes	Some	No	Some[2]	No	2	2
Clindamycin	Yes	No	Yes	Some[2]	No	3-4	9-16
Metronidazole[3]	No	No	Yes	No	No	2-3	2-3
Cefazolin	Yes	Some	No	No	No		
Ceftriaxone	Yes	Yes	No	No	No		
Piperacillin-tazobactam	Yes	Yes	Yes	No	Yes		
Cefepime	Yes	Yes	No	No	Yes		
Meropenem	Yes	Yes	Yes	No	Yes		
Vancomycin	Yes	No	No	Yes	No		
Moxifloxacin	Yes	Yes	Yes	Some[2]	No	1	1
Ciprofloxacin[4]	No	Yes	No	No	Yes	2	2

[1]Includes flucloxacillin, dicloxacillin, and nafcillin.
[2]Some methicillin-resistant *Staphylococcus aureus* (MRSA) strains are resistant, however, in mild cases these oral MRSA-active antibiotics may be considered empirically for patients with MRSA risk.
[3]Metronidazole should not be used as a single agent, it is often combined with other antibiotics to add anaerobic activity to the regimen.
[4]Ciprofloxacin should not be used as a single agent empirically given the lack of Gram-positive cover, it is often combined with other antibiotics such as clindamycin or vancomycin.
MSSA: Methicillin susceptible *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*

aspirin in lowering the risk of ischaemic stroke, myocardial infarction, or vascular death[86,87].

Fenofibrate, a lipid-lowering therapy which works through peroxisome proliferator-activated receptor alpha, was shown to prevent microvascular complications of diabetes, particularly diabetic retinopathy through the Fenofibrate Intervention and Event Lowering in Diabetics (FIELD) study[88]. Post-hoc intention to treat analysis of this data demonstrated a reduction in first amputation (HR 0.64) and minor amputation (HR 0.53) in patients on fenofibrate 200 mg per day. This study did not establish statistical significance for a reduction in major amputations (HR 0.93, 95%CI: 0.53–1.62; $P = 0.79$)[89].

In recent years, two classes of anti-hyperglycaemic agents have shown significant cardiovascular risk reduction independent of their glucose lowering effect. Sodium-glucose cotransporter-2 (SGLT2) inhibitors are an oral medication which inhibits the receptor responsible for re-absorption of 90% of glucose filtered in the nephron. SGLT2 inhibitors induce glucosuria, osmotic diuresis and modest weight loss. In a meta-analysis of 27 cardiovascular outcome studies related to SGLT2 inhibitors, there was an early and sustained reduction in the composite primary outcome of cardiovascular death, non-fatal myocardial infarction or nonfatal stroke primarily driven by a major reduction in heart failure. This effect is independent of HbA1c reduction[90].

Both SGLT2 inhibitors available in Australia have been shown to reduce progression of chronic kidney disease and are generally considered safe to use in patients with renal impairment[91,92]. The main adverse effects of concern include genitourinary infections, euglycaemic diabetic ketoacidosis (in the fasting or unwell patient) and volume depletion[93]. In 2017 the Canagliflozin Cardiovascular Assessment Study, reported an increased risk of LEA in patients treated with canagliflozin (HR 1.97), particularly toe or metatarsal. The overall rates were low (6.3 *vs* 3.4 participants per 1000 patient years) however the finding caused significant pause in clinicians prescribing this class of medications to patients at risk of DFD[94]. Since then, multiple cardiovascular outcome studies have been published without any signal for increased LEA rates in patients treated with SGLT2 inhibitors. Several high-quality systematic reviews and meta-analyses were

Table 4 Empirical antibiotic choices in diabetes-related foot disease; adapted from the International Working Group on the Diabetic Foot infection guidelines[47]

IWGDF classification	Example of Empirical Antibiotic	If MRSA Risk[1]
Mild (grade 2) – no recent antibiotics	Flucloxacillin PO, or cefalexin PO	As a single agent clindamycin PO, or trimethoprim-sulfamethoxazole PO, or doxycycline PO
Mild (grade 2) – recent antibiotics or water-immersed wound	Amoxicillin-clavulanate PO	Add one of the agents above, OR as a single agent moxifloxacin PO
Moderate (grade 3)	Amoxicillin-clavulanate PO/IV Or cefazolin IV plus metronidazole PO/IV Or if *Pseudomonas* risk[2], piperacillin-tazobactam IV	Add one of the agents above, or if IV required, add vancomycin IV
Severe (grade 4)	Piperacillin-tazobactam IV	Add vancomycin IV

[1]Risk factors for methicillin-resistant *S. aureus* (MRSA) include previous colonisation with MRSA, residence in a community, aged-care facility, or correctional facility with high MRSA prevalence, and frequent or prolonged stay in a hospital with a high prevalence of MRSA.
[2]Risk factors for *Pseudomonas* infection include positive *Pseudomonas* microbiology in previous few weeks or located in a tropical/sub-tropical climate area.
IV: Intravenous; IWGDF: International Working Group on the Diabetic Foot; MRSA: Methicillin-resistant *Staphylococcus aureus*; PO: Oral.

undertaken to explore this association. One study demonstrated an increased risk of LEA for canagliflozin (Risk Ratio 1.59), but not for dapagliflozin or empagliflozin, whilst another demonstrated no increased risk of amputation across the entire class including canagliflozin[95,96]. It therefore appears that there is little contemporary data linking SGLT2 inhibitor use with increased LEA rates. Even if there was some risk of LEA associated with SGLT2 inhibitor use, this would need to be balanced against the substantial risk reduction in renal disease progression and protection from cardiovascular disease that this class of medications has shown in major clinical trials. Therefore, in our opinion, the benefits of SGLT2 inhibitor use in patients with DFD who are known to be at high risk for cardio-renal disease far outweighs any possible risk associated with LEA.

Glucagon-like peptide-1 receptor agonist (GLP1-RA) are injectable medications which reduce gastric emptying, stimulate endogenous insulin production and reduce glucagon secretion. Indirect effects of modulation in gut hormone signalling include appetite suppression, weight loss, improved peripheral insulin sensitivity and a more robust reduction in glucose levels. GLP-1RA have demonstrated a statistically significant reduction in major adverse cardiovascular events of 14% (HR 0.86, 95%CI: 0.79–0.95; P = 0.006), with the risk of cardiovascular death reducing by 13% (P = 0.016) and the risk of all-cause mortality reducing by 12% (P = 0.012)[97]. While SGLT2 inhibitor-related cardiovascular risk reduction is largely driven by improved heart failure outcomes, GLP-1 analogues appear to reduce atherosclerotic related cardiovascular events[97].

The GLP-1R analogues available in Australia that have been associated with cardiovascular risk reduction include liraglutide (a daily injection), dulaglutide, and semaglutide (both once weekly injections). Further studies are required to determine whether the reductions in coronary and cerebrovascular events will extrapolate to reduced complications of lower limb arterial disease. Liraglutide has been shown to reduce LEA (HR 0.65) but not other ulcer outcomes[98]. Dulaglutide and semaglutide have been shown to improve metabolic and inflammatory markers commonly associated with peripheral vascular disease but to date have not been shown to reduce DFD or amputation rates[99]. Gastrointestinal adverse effects are the main limitation to the use of GPL-1 analogues in the Australian setting. Utilisation of these two newer classes of glucose lowering agents which are weight-negative and infer cardiovascular protection is a modern part of the multidisciplinary management of patients with DFD.

Good glycaemic control with a goal HbA1c of less than 53 mmol/mol (7%) has been shown to reduce the incidence of diabetic foot ulcers and the risk of amputation. It has also been associated with improved sensory nerve function compared with less intensive glycaemic control[100]. Whilst many studies have suggested that good glycaemic control improves surrogate markers associated with wound healing, there is currently no randomised trial evidence that clearly shows that good glycaemic control improves the rate of diabetic foot ulcer wound healing[101,102]. However, in clinical practice our opinion is that patients with active ulcers should still aim to maintain good glycaemic control as it is likely that this approach will aid in wound healing and also help to prevent the onset and progression of other diabetes-related microvascular complications[103].

Newer therapies: Future directions in interventions to enhance wound healing include topical oxygen therapy (TOT), sucrose octasulfate-impregnated dressings, topical fibrin and leucocyte platelet patches as well as placenta-derived products[104]. Whilst these therapies rely on small studies, they are demonstrating a positive trend for the future direction of DFD and may be incorporated into future guidelines pending further study.

TOT is based on the idea that oxygen is a necessary factor for wound healing by its action on several oxygen-dependent enzymes, eventually increasing cell metabolism, bacterial defence, angiogenesis and vasodilation, and collagen deposition and crosslinking[105]. The method of delivery for TOT has different levels of effectiveness. Continuous delivery oxygen (CDO) system is a low continuous flow of oxygen (3–15 mL/h) through a sealed, disposable

dressing that is changed weekly. Multiple randomised sham control trials have demonstrated a statistically significant improvement in wound healing compared to standard of care, with 32.4%-54% of patients in the CDO group achieving healing, compared to 16.7%-49% in the sham therapy group ($P < 0.05$)[106,107].

Cyclically pressurised topical wound healing applies high flow oxygen with pressures from 7.5–37.5 mmHg, with the optional addition of humidification. It works by encapsulating the affected limb in an extremity chamber to enable local oxygen and pressure delivery. Cyclically pressurised TOT has been shown in two independent randomised controlled trials to not only improve ulcer healing (41.7% *vs* 13.5%, $P = 0.007$), but also to reduce amputations and hospitalisations at 1 year (54.1% *vs* 41.4%, $P < 0.001$)[108,109].

Hyperbaric oxygen therapy (HBOT) is a controversial healing modality currently being used at select centres. It is believed to improve wound healing through improving tissue hypoxia, improving perfusion and angiogenesis, as well as downregulating the inflammatory response. Treatment requires the patient to commit to an average of 60 h over many weeks with significant associated costs to the health care system. Although much of the existing evidence lacks the quality to support HBOT, multiple studies have demonstrated a positive trend towards healing rates. Patients most likely to benefit from treatment have indolent lower limb ulcers that have not healed after at least 1 mo of active treatment. Thus, where available, HBOT could be considered as an adjunct to appropriate wound care[110].

Sucrose octasulfate-impregnated dressings have been shown to reduce the action of matrix metalloproteinases and have a statistically significant benefit compared to placebo in wound closure[111]. Newer agents include topical fibrin and leucocyte platelet patches, thought to improve wound closure through the promotion of cytokines and growth factors involved in tissue repair[104]. These platelet-rich patches have been shown to increase wound healing (34% *vs* 22%; $P = 0.0235$), however, there are significant cost and organisational issues required to create these products, leading to a cautious recommendation for their use from the IWGDF[112].

A recent multicentre, randomized, double-blind vehicle-controlled study exploring the safety of topical esmolol hydrochloride showed minimal systemic concentration of the drug in plasma and a favourable safety profile in patients who received topical treatment to foot ulceration. Preliminary data suggests a trend to improved ulcer healing. The drugs general availability makes it a potential avenue for treatment of non-healing diabetes-related foot ulcers but larger phase III clinical trials are required to establish a statistically significant improvement in ulcer healing[113].

Finally, placenta-derived products are an area of growing research due to the combination of collagen-rich extracellular matrix and cells, growth factors and various stem cells thought to improve wound healing in human placental membranes. Cryopreserved amniotic membrane allograft has demonstrated increased ulcer closure (62% *vs* 21.3%; $P = 0.001$) with reduced median time to healing (42 d *vs* 69.5 d; $P = 0.019$), and umbilical cord product has also demonstrated a significant improvement in ulcer healing at 12 wk (70% *vs* 48%; $P = 0.0089$)[114,115]. Despite these outcomes, the cost is a major aspect ongoing for placenta-derived products, and these have yet to become utilised in everyday practice[104].

Management category 5: Integrated, interdisciplinary care

In order to provide the best care, a combination of guideline-based practice and clinical expertise is required. Nuanced decision-making is key, particularly in the presence of infection, vascular compromise and challenging patient factors. In Australia, The National Association of Diabetes Centres has implemented the Foot Forward diabetes education program aiming to detect foot problems early and ultimately prevent amputations[116]. A detailed and integrated Diabetes Foot Care pathway includes risk stratification and triage based on risk factors (Supplementary Materials)[116]. In a nationwide effort to standardise care, the program defines the interdisciplinary approach as the first core service indicator for a high-risk foot centre to achieve accreditation[117]. The standards outline that a High-Risk Foot Service must have access to the necessary core members of the multidisciplinary team, regular ongoing education for staff and patients, required administration and intake criteria, as well as resources to enact on supportive research. Core disciplines represented in the multidisciplinary team may include but are not limited to podiatry, prosthetics and orthotics, nursing (acute and in the home), physiotherapy, endocrinology, infectious diseases, vascular surgery and rehabilitation medicine.

Clinician-to-clinician discussion between disciplines, all within the immediate vicinity of the patient and each other, enables dialogue between specialists to reach the most appropriate outcome for each individual patient. Through case conferences, outpatient clinics or team ward rounds the interdisciplinary team can coordinate the offloading, dressings, infection management, revascularisation strategy, diabetes management and cardiovascular risk modification according to evidence-based guidelines (Supplementary Materials)[116].

Our High-Risk Foot Service is an interdisciplinary service based at a tertiary Australian hospital. Our integrated service includes endocrinologists, podiatrists, rehabilitation physicians, infectious diseases physicians, vascular surgeons, pharmacists, orthotists, diabetes nurse educators and nurses. We are supported by administration assistants and allied health assistants and have on-referral access to other medical, surgical and allied health clinicians. Our services operate multiple interdisciplinary outpatient clinics, an inpatient ward round, case conference and hospital in the home service. We have strong links with regional health services with increasing utilisation of telehealth.

In patients who have received acute hospital care, particularly surgical intervention, timely assessment of rehabilitation goals and options for continuation of hospital-based care in the home reduces readmission and utilization of long-term care facilities with improved patient satisfaction, without an increase in mortality[118].

CONCLUSION

DFD is a complex condition, acting as a marker of overall systemic disease, increasing morbidity and mortality, and causing a significant burden to health systems. Our review highlights the complexity of DFD and the improved patient

outcomes that are associated with an interdisciplinary management approach involving wound bed preparation and offloading, infection management, maintenance of vascular supply, glycaemic control, and cardiovascular risk modification.

We have discussed the above in the framework of our experiences in a multi-disciplinary high-risk foot service located in an Australian tertiary referral centre and university teaching hospital. To our knowledge this is the first review to describe the approach to the management of DFD with an Australian focus in the setting of the recently published national diabetes foot care pathway "Foot Forward for Diabetes" (Supplementary Materials) and to compare this approach to international guidelines. The strength of our recommendations for the care of patients with DFD are in the context of the limitations of a narrative review.

It is our opinion that a guideline-driven strategy with regular and efficient communication between medical, surgical, and allied health specialties within a High-Risk Foot Unit is paramount to the nuanced decision-making required to optimise patient outcomes in the most effective and efficient fashion. We await the further development of novel therapies for DFD. These include better tools and technologies for detecting high-risk feet and improved topical and systemic methods to promote ulcer healing. Unfortunately, apart from promoting good glycaemic control, little progress has been made in preventing the development and progression of diabetic neuropathy. In contrast, promising areas of interest for the management of PAD include the development of novel methods of perfusion assessment and revascularisation. We anticipate that rates of PAD may decrease with trends in improvement of risk factors and wider use of novel pharmacotherapies that infer vascular protection. In the interim, the early detection of high-risk feet and the initiation of strategies to prevent ulcer development within the multidisciplinary team remain essential elements for the care of people with diabetes and in the avoidance of the devastating consequences associated with DFD.

FOOTNOTES

Author contributions: McNeil S and Waller K contributed equally to this work; all authors contributed to the writing and editing of the manuscript according to their areas of specialty; McNeil S, Waller K and Poy Lorenzo YS created and formatted the tables and figures; MacIsaac RJ and Galligan A reviewed the final draft as senior authors.

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REVIEW

Novel insights regarding the role of noncoding RNAs in diabetes

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Abstract

Diabetes mellitus (DM) is a group of metabolic disorders defined by hyperglycemia induced by insulin resistance, inadequate insulin secretion, or excessive glucagon secretion. In 2021, the global prevalence of diabetes is anticipated to be 10.7% (537 million people). Noncoding RNAs (ncRNAs) appear to have an important role in the initiation and progression of DM, according to a growing body of research. The two major groups of ncRNAs implicated in diabetic disorders are miRNAs and long noncoding RNAs. miRNAs are single-stranded, short (17–25 nucleotides), ncRNAs that influence gene expression at the post-transcriptional level. Because DM has reached epidemic proportions worldwide, it appears that novel diagnostic and therapeutic strategies are required to identify and treat complications associated with these diseases efficiently. miRNAs are gaining attention as biomarkers for DM diagnosis and potential treatment due to their function in maintaining physiological homeostasis *via* gene expression regulation. In this review, we address the issue of the gradually expanding global prevalence of DM by presenting a complete and up-to-date synopsis of various regulatory miRNAs involved in these disorders. We hope this review will spark discussion about ncRNAs as prognostic biomarkers and therapeutic tools for DM. We examine and synthesize recent research that used novel, high-throughput technologies to uncover ncRNAs involved in DM, necessitating a systematic approach to examining and summarizing their roles and possible diagnostic and therapeutic uses.

Key Words: Noncoding RNA; miRNA; Diabetes; Circulating miRNA biomarkers; Therapeutic target; CRISPR/Cas9 system

Core tip: Diabetes mellitus is a chronic endocrinopathy characterized by disrupted glucose, lipid, and amino acid metabolism and has reached pandemic proportions. A vast body of evidence demonstrates that miRNAs play a key role in diabetic pathophysiology. Here, we explore numerous regulatory miRNAs involved in DM and discuss their potential diagnostic and therapeutic applications.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic endocrinopathy caused by genetic and environmental factors that lead to disruption of carbohydrate, lipid and amino acid metabolism. If DM is left out of control, the delayed effects of such a metabolic derangement promote systemic, mainly vascular consequences[1-3]. The prevalence of DM is increasing rapidly and has reached pandemic proportions[4,5]. According to studies and International Diabetes Federation (IDF), the global prevalence of type 2 DM (T2DM) was 9.3% in 2019, expected to rise to 10.9% by 2045, and affects ~629 million people[6,7].

The pathophysiology of DM is based on hyperglycemia induced by either insulin resistance (IR), insulin deficiency, or both[4]. Ineffective glucose utilization favors the activation of alternative glucose metabolic pathways (*i.e.* polyol, protein kinase C, and hexosamine), contributing to mitochondrial dysfunction, reactive oxygen species (ROS) generation, and the development of cellular and tissue hypoxia[8]. DM can cause endothelial cell destruction and low-grade systemic inflammation, leading to vascular problems, including diabetic foot ulcers, peripheral neuropathy, nephropathy, maculopathy, and retinopathy[9-11]. In DM patients, vascular issues contribute to a two to four-fold increase in myocardial infarction, stroke, and overall mortality[12-14].

There are at least five types of DM, with type 1 DM (T1DM) and T2DM being the most prevalent in clinical settings[2,15]. T1DM represents 10% of all cases and is one of the most frequent chronic diseases of childhood, with a worldwide incidence that is increasing by 3% annually[16-18]. Multiple mechanisms, including autoimmunity, genetic susceptibility, and epigenetic modulation, have been implicated in T1DM pathogenesis[19]. The presence of autoantibodies against β-cell antigens, such as insulin, decarboxylase, tyrosine phosphatases-2 and -2b, and glutamic acid, have been detected in T1DM patients[19,20]. More than 50 mutations that describe disease susceptibility were discovered at 50 different genetic loci, with HLA class II mutations being the most common. In addition, environmental factors such as nutritional habits, viruses, and other epigenetic factors directly affect the expression of insulin genes and genes responsible for autoimmune responses[21].

T2DM is more common in older or obese patients and is distinguished by cells and tissues that are resistant to high insulin levels and even high blood sugar levels. T2DM accounts for most (90%–95%) cases of DM[2,7]. While genetic predisposition and obesity are the main risk factors for the development of T2DM, autoimmunity plays a pivotal role in the development of T1DM[1,22].

Basic scientific research continuously aims to identify improved biochemical markers of early or advanced endothelial injury. Besides C-reactive protein, homocysteine, nitric oxide, and inflammatory cytokines, miRNAs are promising tools for assessing vascular complications risk in DM patients. Due to accumulating evidence regarding their role in the onset and progression of DM, miRNAs are gaining substantial attention as novel diagnostic biomarkers for DM and potential therapeutic agents.

In this review, we address the problem of the global prevalence of DM by providing a systematic and up-to-date summary of various miRNAs involved in the pathogenesis and pathophysiology of DM. We also summarize the recent findings of numerous studies that used a novel high-throughput methodology to identify miRNAs involved in the pathogenesis of DM and their potential diagnostic and therapeutic applications.

ROLES OF miRNAs IN DM

The rapid advancement of high-throughput sequencing technologies, such as microarray, deep RNA sequencing, and next-generation sequencing, revealed novel insights into the structure and function of the human genome and transcriptome. An unexpected finding of such studies is that only ~2% of the human genome is transcribed into protein-coding mRNA[23,24], while the remainder of the genome is transcribed into noncoding RNAs (ncRNAs), including structural RNAs (rRNAs and tRNAs), and regulatory RNAs such as miRNAs and long noncoding RNAs (lncRNAs)[25]. miRNAs are small (17–25 nucleotides) single-stranded ncRNA molecules that regulate gene expression post-transcriptionally primarily by binding to complementary cis-elements in the 3'-untranslated region (UTR) of target mRNAs[26]. They may, however, bind anywhere along the mRNA transcript to inhibit translation and regulate mRNA stability and degradation[27,28]. miRNA interactions with coding areas and the 5′-UTR are thought to mute gene expression[29,30], whereas miRNA interactions with promoter regions are thought to activate transcription[31].

miRNAs are transcribed by RNA polymerase II as long precursor molecules, which are cleaved by the nuclear RNase III-type endoribonuclease DROSHA to approximately 70-nucleotide precursor miRNAs (pre-miRNAs)[32], before being transported into the cytoplasm and further processed by another RNase III enzyme, DICER, to generate mature double-stranded miRNAs[33]. The guide strand of mature miRNA associates with Argonaute (AGO) proteins and is incorporated into the minimal miRNA-induced silencing complex (miRISC) that interacts with complementary sites within the target mRNAs[33] (Figure 1). It has been estimated that around 60% of human transcripts contain potential miRNA-binding sites within their 3′-UTRs[34] and that a single miRNA can potentially bind to more than 100 target mRNAs, where several miRNAs may act synergistically to finely tune the expression of the same transcript[35-37].

miRNAs as glucose homeostasis regulators

miRNAs regulate glucose homeostasis by controlling insulin production, secretion, and cell proliferation. Fine-tuned insulin secretion from pancreatic β-cells is required for blood glucose homeostasis; perturbations in this process can result in hyperglycemia and DM. The tissue-specific expression of miRNAs is an important aspect of their role in the pathophysiology of DM. For instance, miR-9, miR-375, miR-376 and miR-7 are highly expressed in the human pancreas, where they exhibit an important role in pancreatic islet function[38-40], participating in pancreas development and the regulation of islet mass, as well as β-cell proliferation and insulin secretion. Expanding knowledge of tissue-specific miRNA expression in animal models and human subjects is illustrated by several studies that provide valuable insights into connections between miRNAs and DM. For example, in streptozotocin-induced T1D mice, miRNA-microarray profiling revealed 64 upregulated and 72 downregulated pancreatic miRNAs, and subsequent qRT-PCR analysis validated the decreased expression of let-7, miR-148b-3p, miR-27a-3p, miR-7a-5p, miR-7b-5p, miR-26a-5p, and miR-26b-5p in diabetic mice[41]. Another study confirmed the downregulation of miR-26a-5p in pancreatic mouse tissues[42]. Regarding T2DM animal models, increased levels of miRNAs belonging to the miR-199 and miR-200 families, as well as miR-34a, miR-132, miR-146, let-7b, and miR-21, were observed in pancreatic islets of diabetic mice[43,44] whereas miR-30d, miR-184, miR-203, miR-210, miR-338–3p and miR-383 had significantly decreased levels[44-46]. miR-199a-5p and miR-184 were consistently dysregulated in several mouse models of obesity and/or IR. For instance, increased expression of miR-199a-5p was also reported in islets of diet-induced obese (DIO) mice[44,47], whereas decreased expression of miR-184 was confirmed in an independent study using the islets of mice on a high-fat diet[48]. In the nonobese spontaneous Goto-Kakizaki rat T2DM rat model, global evaluation of miRNA expression pattern in pancreatic islets identified 30 dysregulated miRNAs[49]. A study by Karolina *et al*[50] performed on pancreatic T2DM rat tissue showed a significant increase of miR-144, miR-150, miR-29a, miR-192, and miR-320a observed, while miR-146a, miR-30d, and miR-182 were highly downregulated[50].

Human studies show a cluster of highly expressed miRNAs expressed explicitly in human β-cells, such as miR-655, miR-656, miR-127, miR-136, miR-543, miR-369, miR-411, miR-432, miR-487, miR-495, miR-589, is significantly downregulated in islets from T2DM patients[51]. Other studies also found increased miR-124a and miR-187 expression in the islet tissue of T2DM patients[52,53]. However, miR-7a, and miR-184, implicated in the regulation of pancreatic β-cell function, showed a significantly decreased expression in human T2DM islets[47,48]. In T1DM patients, significant upregulation of miR-125a-5p compared to healthy controls was observed[54].

miR-375, which has an established role in regulating insulin secretion (Figure 2)[40], was the most highly expressed miRNA in human pancreatic islets[55]. The target of miR-375 is myotrophin (Mtpn) which is involved in the cytoskeletal remodeling by depolymerizing actin filaments[40] and mediating exocytosis by enabling the fusion of insulin vesicles on membranes of β-cells[40,56] (Figure 3). In addition, Mtpn was shown to upregulate the nuclear factor (NF)-κB), thus inducing the expression of proteins responsible for targeting insulin vesicles to the membrane[57,58].

Lovis *et al*[59] found that miR-124a and miR-96 could also operate as transcriptional regulators of proteins involved in insulin exocytosis and secretion (Figure 3)[59]. miR-124a increases the levels of Rab3A, SNAP25, and synapsin-1A while decreasing Noc2 and Rab27A levels (Figure 3). Rab27A is a GTPase that allows vesicles to be transported to the cell membrane. The direct binding of miR-124a to the 3'-UTR of Rab27A reduces Rab27A expression (Figure 3). miR-124a overexpression causes excessive insulin release at rest and decreases glucose-induced insulin secretion[59]. miR-96 has been shown to reduce glucose-induced insulin release by upregulating granuphilin, a negative regulator of insulin exocytosis, while suppressing Noc2 expression[59], a protein that is essential for the normal regulation of endocrine cell exocytosis[60]. miR-7 has also been found to inhibit glucose-induced insulin release in β-cells. It acts by directly regulating the expression of genes involved in the late stages of insulin granules fusion with the plasma membrane, including the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex, which mediates membrane fusion of vesicles with their target cellular compartments[47].

Several miRNAs are implicated in the regulation of insulin production. miR-375 has been shown to inhibit 3'-phosphoinositide-dependent protein kinase (PDK)1, a crucial component of the PI3K cascade (Figure 3)[61]. Decreased PDK1 levels lead to the downregulation of insulin gene expression in response to glucose stimulation[61]. miR-204 has also been found as a negative regulator of insulin production, whose expression is influenced by thioredoxin-interacting protein (TXNIP), a redox state regulator in β-cells. TXNIP levels are increased in DM, resulting in elevated miR-204 expression that mediates increased degradation of the insulin transcription factor MAFA[62]. Another target of miR-204 is the glucagon-like peptide 1 receptor 3'-UTR, and this interaction also downregulates glucose-induced insulin secretion [63].

miR-375 and several other pancreatic miRNAs, including miR-21, miR-34, and miR-200, have been found to influence β-cell proliferation, survival, and apoptosis (Figure 2). For instance, decreased proliferation and viability are associated with a miR-375 knockdown in mice, resulting in a severely diabetic state[56]. MiR-21, whose expression is regulated by the NF-κB proinflammatory cytokine pathway, has been reported to regulate β-cell number[64,65]. miR-21 overexpression *in vitro* was associated with decreased β-cell number[64]. miR-34a knockdown increased β-cell quantity and mass, which

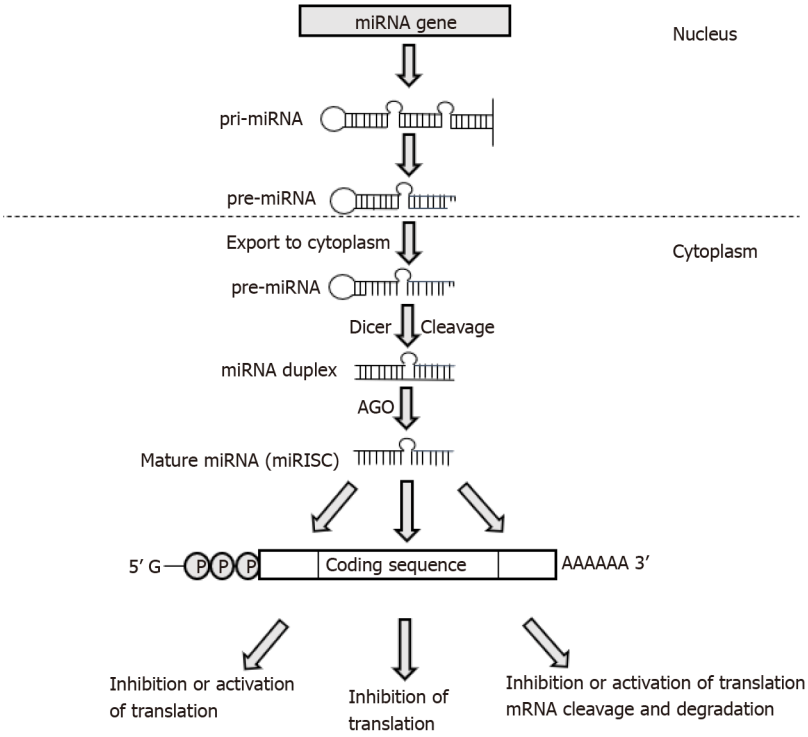

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Figure 1 Mechanism of action of miRNAs. AGO: Argonaute family of protein; miRNA: microRNA; miRISC: miRNA-induced silencing complex; pre-RNA: Precursor RNA; pri-miRNA: Primary miRNA; pre-miRNA: Precursor miRNA; 3'UTR: 3' untranslated region; 5' UTR: 5' untranslated region.

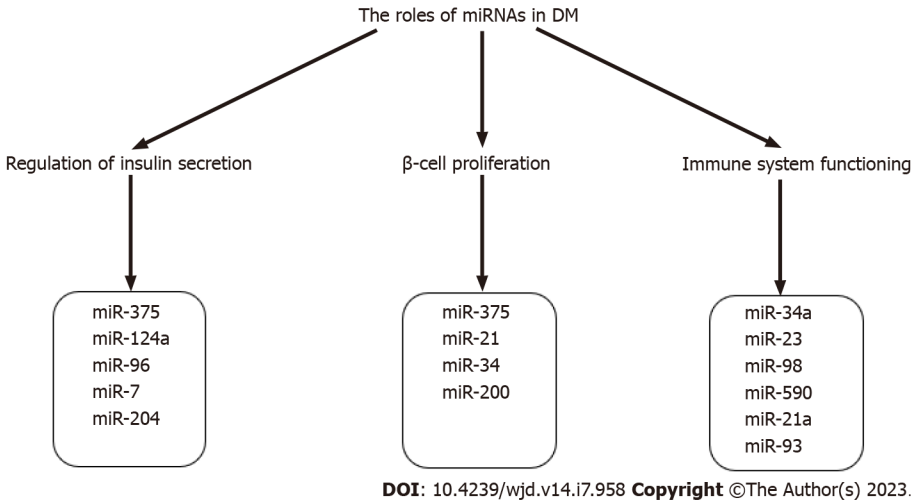

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Figure 2 The role of specific miRNAs in DM. miRNAs: micro RNAs; DM: Diabetes mellitus.

could be explained by the role of miR-34 in targeting sirtuin (SIRT)1 and, as a result, causing p53-mediated apoptosis[64]. miR-200 also induces expression of proapoptotic genes in both T1DM and T2DM by suppressing Xiap, a caspase inhibitor, and Dnajc3, a β-cell heat shock protein, while activating the tumor suppressor protein Trp53[66].

miRNAs as mediators of immune system equilibrium in DM

The significance of miRNAs as global cellular regulators of gene expression is evident in their function in immune system balance and regulating immune cell differentiation, maturation, and activation, which is especially relevant in the context of T1DM pathogenesis (Figure 2). Various immune cells, such as $CD4^+$, $CD8^+$ T cells, natural killer (NK) cells, type B lymphocytes, dendritic cells, and chemokines and cytokines, are characteristic of T1DM-associated β-cell damage[67]. B lymphocytes play a protective and defensive role for β-cells preventing T1DM development. Mone *et al*[68] reported that miR-34a overexpression in diabetic mice leads to reduced activity of B lymphocytes *via* a negative expression of the *Foxp1* gene[68] involved in B lymphopoiesis, resulting in a reduced ability of pancreatic islets to defend themselves against damage[68,69]. miRNA expression also regulates the production of specific T cells at the other end of the homeostatic

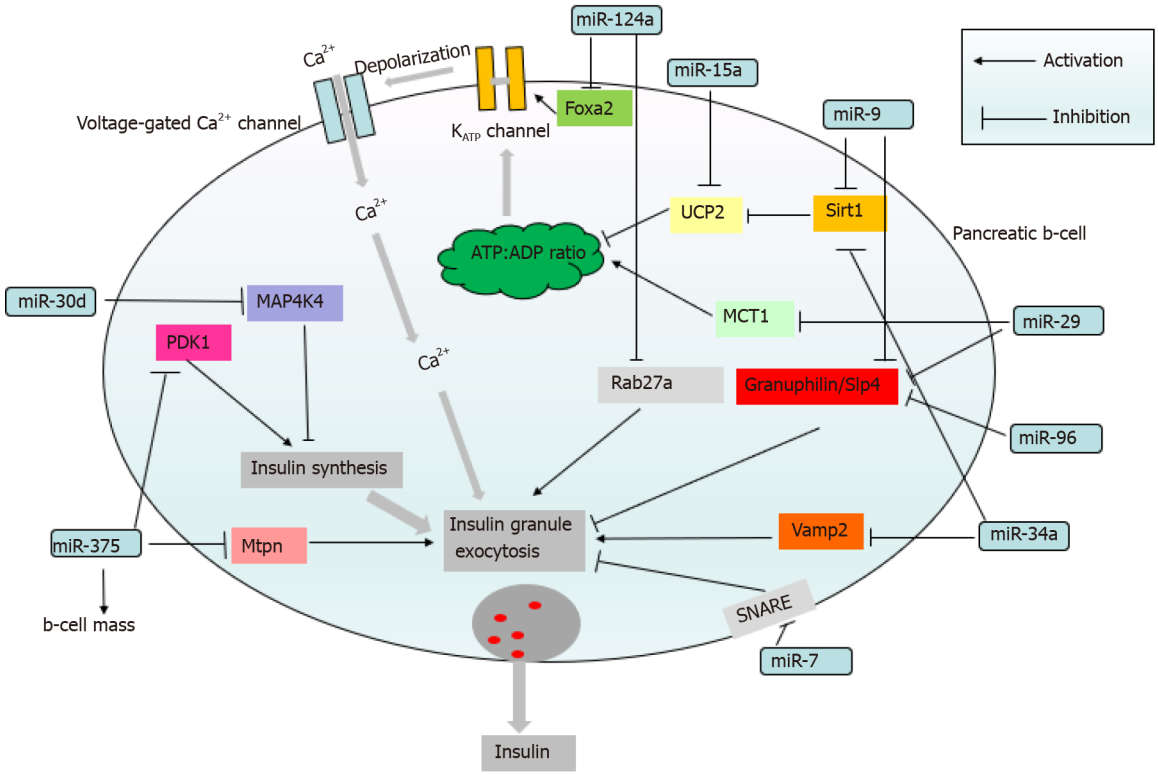

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Figure 3 miRNAs mediating glucose metabolism and insulin secretion in pancreatic β-cells. Foxa2: Forkhead box A2; KATP channel: ATP-sensitive potassium channel; MAP4K4: Mitogen-activated protein kinase kinase kinase kinase 4; MCT1: Monocarboxylate transporter 1; Mtpn: Myotrophin; PDK1: Phosphoinositide-dependent protein kinase 1; Rab27a: Member RAS oncogene family; Sirt1: Sirtuin (silent mating type information regulation 2 homologs) 1; SNARE: Soluble N-ethylmaleimide-sensitive factor activating protein receptor; Vamp2: Vesicle-associated membrane protein 2; UCP2: Uncoupling protein 2.

chain. miR-590, miR-98, and miR-23 overexpression has also been demonstrated to influence the production of $CD8^+$ T lymphocytes that target pancreatic islet antigens. The mechanisms of action operate by suppressing the expression of TRAIL and FAS ligands, implying that miRNA-mediated gene silencing may promote autoimmunity and the development of T1DM[70]. The three primary antibodies in T1DM occurring long before disease onset are autoantibodies against specific antigens of pancreatic islet cells IA, IA2B, and GAD (glutamic acid decarboxylase) and a cluster of 32 miRNAs participate in biosynthetic pathways that modify the expression of T1DM autoantibody sequences[71]. miRNA-21a and miR-93 are involved in the processes of inflammation and pathways leading to cell death in peripheral blood mononuclear cells (PBMCs) of T1DM patients[72], whereas miR-326, which targets significant immune system modulators such as the vitamin D receptor, was found to be overexpressed in T1DM patients' PBMC[73].

ROLE OF lncRNAs IN DM

lncRNAs are common long (> 200 nucleotides) linear transcripts that regulate gene expression at the transcriptional and post-transcriptional levels, influencing mRNA stability, pre-mRNA splicing, and translation[74-77]. Mechanistically, lncRNAs can act as miRNA sponges, scaffolds for protein complexes, and decoys for regulatory factors[76]. The interaction of lncRNAs with transcription factors results in transcription regulation[75,78]. It has also been observed that some lncRNAs may interact with pre-mRNAs to influence splicing[79]. lncRNAs can potentially block protein interactions with target mRNAs or change protein catalytic activity, acting as decoys[74,76]. It was also discovered that lncRNAs binding to translating mRNAs change the target mRNA's stability and translation[74].

lncRNAs regulate critical physiological processes such as cell proliferation, growth, differentiation, senescence, aging, and secretion[80,81]. They are also implicated in the pathogenesis of several diseases, including cardiovascular disease and DM. In humans, lncRNAs are primarily produced by RNA polymerase II or III[82] and are characterized as sense- or antisense-overlap, bidirectional, intronic, or intergenic lncRNAs based on their proximity to the next protein-coding gene [83]. The functions of lncRNAs are governed by their cellular location, with nuclear lncRNAs modulating transcription and splicing and cytoplasmic lncRNAs regulating post-transcriptional events such as mRNA stability, protein synthesis, and posttranscriptional alterations[76].

Various lncRNAs are expressed in a cell-type specific manner in pancreatic β-cells, such as *GAS5* (growth arrest-specific transcript 5), *PLUTO* (PDX1 locus upstream transcript), *TUG1* (taurine upregulated gene 1), *MEG3* (maternally expressed gene 3), and *βLINC* (β-cell long intergenic ncRNAs). *GAS5* is a lncRNA that regulates cell development and proliferation. *GAS5* levels in diabetic patients' serum are considerably lower than in healthy controls[84], and db/db mice

[85], and *GAS5* silencing *in vitro* is related to cell cycle arrest and decreased insulin production and secretion. *PLUTO* is an antisense transcript lncRNA upstream of the gene that codes for PDX1, a transcription factor involved in β-cell differentiation and pancreatic development. Both *PLUTO* and *PDX1* are significantly downregulated in T2DM patients[86]. Reduced *PLUTO* expression is related to chromatin changes that limit the interaction of the PDX1 promoter with its enhancer, resulting in lower PDX1 expression[86], implying that *PLUTO* plays a role in the control of β-cell function. *TUG1* and *MEG3* are extensively expressed in the pancreas and are controlled by glucose levels[87,88]. *TUG1* and *MEG3* knockdown reduces insulin synthesis and secretion and promotes β-cell death[88], supporting their roles in β-cell development and insulin production control. *βLINC1* is a highly conserved lncRNA linked to increased glucose intolerance and aberrant insulin secretion[89]. *βLINC2* and *βLINC3* are more abundant in pancreatic islets than other organs. *βLINC2* expression levels correlate favorably with body weight, glycemia, and insulinemia, but *βLINC3* expression correlates negatively with body mass index (BMI) and is considerably lower in T2DM patients compared to healthy controls[90].

FUNCTIONS OF CIRCULAR RNAs IN DM

Circular RNAs (circRNAs) are abundant, conserved tissue-specific covalently closed loop circular RNAs[91-95] produced by the direct ligation of 5′ and 3′ ends of linear RNAs as intermediates in RNA processing or generated by backsplicing where a downstream 5' splice donor attacks an upstream 3' splice acceptor site of pre-mRNA forming a covalently closed circRNA lacking the 5' and 3' ends[96,97]. circRNAs regulate gene expression by acting as miRNA sponges, modulating protein–protein interactions, binding to ribosomes, and interfering with translation or modifying transcription[98]. circRNAs are thought to play a role in the etiology of many diseases, including DM[99]. circRNA *Cdr1as*, for example, regulates insulin production and secretion by acting as a sponge for miR-7, reducing insulin secretion. *Cdr1as* contains about 60 miR-7 binding sites[93], and *Cdr1as* upregulation increases insulin secretion by inhibiting miR-7 activity[47]. miR-7 directly targets and inhibits the expression of paired box (Pax)6 and the myosin VIIA and Rab interacting protein (Myrip). Pax6 is a transcription factor that interacts with the promoters of the *ins1* and *ins2* genes to stimulate insulin production and secretion, whereas Myrip is involved in secretory granule transport and release. *Cdr1as* expression is downregulated in db/db mouse islets[100], but *Cdr1as* overexpression increases Pax 6 and Myrip expression, enhancing insulin transcription and secretion in pancreatic islets[101].

CircHIPK3 is abundantly expressed in pancreatic β-cells, and decreased *circHIPK3* levels are associated with reduced proliferation of β-cells[100]. *CircHIPK3* silencing decreases insulin mRNA levels and perturbs glucose-stimulated insulin secretion[100].

CircAFF1[101], another highly expressed circRNA in pancreatic islets, causes β-cell death *in vitro*, implying its role in β-cell growth and function[100].

FUNCTION OF miRNAs AS ENDOCRINE SIGNALING MOLECULES IN THE REGULATION OF INSULIN PRODUCTION AND FAT METABOLISM

Numerous investigations have shown that miRNAs act as endocrine signaling molecules, regulating insulin production and fat metabolism. Specific miRNAs directly regulated several insulin-signaling components, including insulin receptors (INSR) and several transcription factors. For example, miR-424-5p was found to target the 3′-UTR sequence of INSR mRNA, and its overexpression in human hepatocytes HepG2 cell line leads to decreased INSR levels and lipid accumulation[102]. Treatment of HepG2 cells with saturated fatty acids leads to increased miR-424-5 and a reduced expression of INSR[102]. Similarly, miR-15b[103], miR-195[104], and miR-96[105] bind to human INSR mRNA, and in the liver of mice on a high-fat diet or in HepG2 cells treated with saturated fatty acids, elevated expression of miR-96 and miR-195 was observed, accompanied by decreased INSR[104,105]. MiR-122, miR-144, and miR-146a were reported to indirectly modulate INSR by controlling the expression of protein tyrosine phosphatases that remove phosphate groups from tyrosine residues of the cytoplasmic domain of INSR and negatively affect insulin signaling[50,106,107]. In addition, INSR compartmentalization and signal transduction depends on the presence of caveolae, specialized microdomains in plasma membranes composed of caveolin proteins. It has been discovered that miR-107 and miR-103 bind to the 3′-UTR of caveolin-1 mRNA[108] and play a role in IR by increasing liver glucose production. AntagomiR-mediated silencing of miR-107 and miR-103 in adipocytes of DIO mice normalized glucose status[108].

miRNAs also target IR substrates (IRSs), altering insulin signaling and cholesterol and fatty acid metabolism. miR-96, miR-126, and miR-145 regulate IRS1 expression[109-111], whereas IRS2 is targeted by miR33a/b[112,113]. However, it has been reported that different miRNAs regulate IRS in various target tissues. IRS-1 is regulated in skeletal muscle by miR-29a, miR-29c, and miR-128a[104,114,115] and IRS2 by miR-135a[116]. IRS-1 mRNA contains a binding site for miR-29, which has been shown to activate lipid metabolism genes such as the peroxisome proliferator-activated receptor-coactivator-1 and 3-hydroxy-3-methylglutaryl-CoA synthase 2[117].

miR-26, which is significantly downregulated in the livers of overweight people and obese leptin-deficient mice, is another miRNA implicated in regulating insulin sensitivity, glucose and fat metabolism[118]. miR-26a levels correlate positively with BMI and are negatively related to homeostatic model assessment for IR (HOMA-IR). Furthermore, miR-26a overexpression prevented metabolic activity changes associated with obesity[118].

miRNAs regulate insulin-like growth factor (IGF)-1 and its receptor (IGF-1R) expression and secretion, as demonstrated by Ling *et al*[119], who revealed that miR-320 modulates the insulin signaling pathways by influencing IGF-1 expression and insulin sensitivity in adipocytes[119]. This finding implies that specific miRNAs may bind to multiple targets to regulate glucose metabolism, which is supported by the discovery that miR-1 regulates IGF-1 and IGF-1R expression in cardiac and skeletal muscles[120], whereas let-7 may bind to the 3'-UTR regions of INSR, IRS-2, and IGF-1R [121]. The discovery that miR-143-3p, which regulates IGF-2R receptor expression, is significantly up-regulated in the serum of T2DM patients and multiple tissues of obese mice (including pancreas, skeletal muscle, and heart), contributing to IR associated with metabolic syndrome, lends support to the role of IGF signaling in metabolic diseases[122].

SPECIFIC miRNA DYSREGULATION CAN CONTRIBUTE TO METABOLIC DISEASES

As previously mentioned, pancreatic β-cells are characterized by a high abundance of miR-375[40]. However, studies of miRNA distribution in other organs and tissues, such as liver and adipose tissue, suggest that miRNA expression profiles may serve as signatures of cell identity[123]. High-throughput omics studies have found a link between miRNA expression in different tissues, such as the pancreas, liver, and adipose tissue, and conditions like metabolic disease and obesity[124-126]. For instance, 221 out of 1736 genomic loci associated with obesity correspond to miRNAs[124]. The role of miRNAs in the pathophysiology of T2DM and associated metabolic disorders was investigated. Specific miRNAs such as miR-27a and miR-222 were upregulated in adipose tissue, whereas mir-122, miR-103, and miR-195 were enriched in the liver[127], with miR-122 accounting for nearly 70% of the total miRNA expressed in this tissue[128-130]. In addition, treating adipocytes with glucose results in increased expression of miR-27a, miR-29a, and miR-222 and downregulation of miR-10b in skeletal muscle. Other studies reported that muscle cells are enriched in miR-133a, miR-133b, miR-1, miR-486, miR-206, miR-208a, miR-208b, and miR-499[131-133].

Several studies show that specific miRNAs are differentially expressed in obese subjects' white adipose tissue compared to nonobese controls[109-112], and visceral adipose tissue miRNAs are more important in metabolic dysregulation than subcutaneous tissue miRNAs[134,135]. A correlation has been found between miRNA expression in adipose tissue and metabolic parameters such as BMI, glycemia, leptinemia, and adipogenesis[136,137]. For instance, elevated miR-21 expression was observed in the white adipose tissue of obese humans, and it positively correlated with BMI[138]. Treatment with a miR-21 inhibitor [locked nucleic acid (LNA)-miR-21] resulted in decreased adipocyte size, significant weight loss, and inhibition of expression of transforming growth factor β-receptor 2 (TGFBR2) and phosphatase and tensin homolog (PTEN)[139].

Circulating miRNAs exert an additional level of the regulation of metabolic homeostasis, which can mediate communications between various types of cells. Extracellular miRNAs may be utilized for evaluating an individual's metabolic condition because dysregulation of their expression is linked to various metabolic diseases, including T2DM, obesity, and cardiovascular diseases[140], and correlates with individual lifestyle characteristics such as exercise[141-144], dietary intake[145], and the composition of gut microbiota[146]. Adipocytes and adipose tissue macrophages (ATMs) can alter insulin-sensitive organs like the liver and muscles by releasing exosomal vesicles carrying miRNAs [147]. miRNA profiling of ATM exosomes revealed enhanced expression of miR-155, which targets the *PPARG* gene that encodes for peroxisome proliferator-activated receptor , which regulates glucose metabolism and fatty acid storage[148]. It has been established that adipose tissue is a major source of exosomal miRNAs that regulate gene expression in distant organs[149]. Several studies confirmed the link between adipose tissue and miRNA profiles, demonstrating that weight loss was associated with significant changes in circulating miRNA levels[150,151]. Thomou *et al*[149] observed that the liver could take up exosomal miR-99b from adipose tissue, resulting in a decreased expression of hepatic fibroblast growth factor 21 and, as a result, glucose intolerance[149].

Extracellular vesicles (EVs), specifically exosomes, play a vital role in interorgan communication by carrying lncRNAs and miRNAs that modulate metabolic pathways. EVs are tiny vesicles enclosed by a membrane, originate from endosomes, and are released by cells into the extracellular fluids depending on their cargo[152]. According to the Minimal Information for Studies of EVs 2018 recommendations, EVs are a component of the total secretome released by the cell, and no specific marker can distinguish EV subtypes and their subcellular origin[153]. Exosomes and microvesicles, two forms of EVs released by cells, are distinguished by their manner of synthesis rather than size. Cells undergo EV biogenesis, which includes inward invagination of the plasma membrane within the cytosol, forming early and late endosomes (LEs). These LEs join together to create multivesicular bodies, which invaginate to form intraluminal vesicles (ILVs)[154]. Exocytosis occurs when these ILVs fuse with the plasma membrane and release exosomes into the extracellular environment[155]. Exosomes are found in various bodily fluids and are released by various cell types, including lymphocytes and pancreatic islets[155]. The transfer of nucleic acids by exosomes is enhanced in inflammatory conditions, and miRNAs represent one of the main cargos transported by exosomes[156].

In DM, these molecules target specific tissues regulating their activity. Therefore, to understand the pathogenesis of T1DM and T2DM, it is crucial to investigate the communication between affected organs in response to elevated blood glucose levels. Exosomes act as messengers, linking the immune response to pancreatic damage and adipocyte stimulation, leading to IR in the liver and muscles. Exosomes containing lncRNAs and miRNAs also contribute to cellular communication by altering metabolic and insulin signals, impacting inflammatory processes in pancreatic cells. Exosome-carried miRNAs, particularly, hold great promise as biomarkers or in developing innovative therapeutics for diabetes and its consequences[157].

Islet insulitis is connected with the transfer of a specific group of miRNAs from lymphocytes to cells *via* exosomes, including miR-142-3p, miR-142-5p, and miR-155, leading to the selective death of insulin-secreting cells. Inactivation of

these miRNAs protected cells against apoptosis caused by T cell exosomes *in vitro* and reduced T1DM development in NOD mice *in vivo*. As a result, it has been postulated that miRNA transfer mediated by exosomes released by lymphocytes causes β-cell death and may be one of the mechanisms contributing to the development of T1DM[158].

Katayama *et al*[159] used microarrays to evaluate the expression profile of exosomal miRNAs in healthy people and those with T2DM[159]. They discovered that miR-20b-5p in exosomes was abundant in people with T2DM. Further *in vitro* studies revealed that this miRNA targets the AKT interacting protein, which modifies AKT protein activity and decreases glycogen buildup in muscles and IR[159].

Downregulation of exosomal lncRNA-p3134 in diabetic mice reduced glucose-stimulated insulin production by lowering key regulators (Pdx-1, MafA, GLUT2 and Tcf7 L2) in β-cells. This shows that lncRNA-p3134 regulates insulin secretion and that its downregulation leads to diabetes pathogenesis[160].

Exosomal miRNAs (miR-20b-5p, miR-155, miR-450b-3p, miR-151-3p and miR-29b-3p) were elevated in diabetic mice and targeted skeletal muscles *via* insulin signaling regulatory proteins (PPAR, AKT, GLUT4 and FOXO). This contributes to DM pathogenesis by influencing insulin signaling and glucose absorption in skeletal muscles[161]. Other exosomal miRNAs (miR-122, 192, 27a/b, 155 and 29b-3p) were upregulated in diabetic models and targeted adipocytes *via* PPAR proteins. This impairs lipid metabolism and contributes to diabetes development[162].

Exosomal miRNAs (miR-142-3p and miR-142-5p) were also found to be enhanced in diabetic mice and target pancreatic cells *via* cytokines that are elevated. This contributes to DM pathogenesis by encouraging immune cell recruitment and β-cell death during autoimmune attacks[158]. Other exosomal miRNAs that target organs such as astrocytes, retinal tissue, and renal cells (miR-106, miR-146a, miR-222 and miR-486) have potential therapeutic roles in protecting pancreatic cells or treating diabetic complications[163].

Exosomal miRNAs and lncRNAs influence DM development in various ways, including regulating pancreatic inflammation and metabolic and insulin signaling in target organs. Despite mounting evidence, research on the involvement of exosomes harboring ncRNAs in diabetes is still in its early stages, but they have promise and significant roles in pathogenesis, diagnosis and treatment of DM[164,165].

Finally, chronic inflammation is a feature of metabolic disorders such as DM and obesity[166], and several miRNAs are associated with regulating the expression of inflammatory markers[167-169]. The overexpression of miR-132 in human adipose-derived stem cells results in increased production of interleukin (IL)-8 and monocyte chemoattractant protein (MCP)1[170], which was also found to be regulated by miR-126 and miR-193b[171]. Other studies have found a link between low miR-221 expression and high tumor necrosis factor (TNF)-α levels in human adipose tissue-derived mesenchymal stem cells from obese women[172]. miR-145 was found to boost TNF-α expression in adipocytes by activating the NF-κB signaling pathway[173]. The investigation of miRNA expression in leptin-deficient ob/ob mouse adipocytes and TNF-α-treated adipocytes *in vitro* revealed a similar expression pattern[174]. In adipocytes isolated from mice, TNF-α increased the expression of a set of miRNAs, including miR-130, miR-150, miR-146a, miR-146b, miR-221 and miR-222, while decreasing miR-143 and miR-103 levels[174-177]. In human preadipocytes, both TNF-α and leptin induce decreased expression of miR-221 and upregulation of miR-335[177,178].

CIRCULATING miRNAs AS T1DM AND T2DM BIOMARKERS

Increasing evidence supports using extracellular circulatory miRNAs as biomarkers for various pathophysiological conditions[123,179]. Highly stable extracellular miRNAs that are protected from degradation due to their enclosure in exosomal vesicles[180] or association with AGO2 proteins[181,182] and high-density lipoproteins[183], are present in various biological fluids, such as serum[184], plasma[181], cerebrospinal fluid[185], saliva[180], urine and tears[186]. Although the concentration of miRNAs in body fluids is in the femtomolar range[187], highly sensitive assays such as qRT-PCR, microarrays, and RNA sequencing (RNA-seq) can detect them in small samples even after prolonged storage [186].

Several studies investigated the expression of miRNAs in the serum/plasma of diabetic patients, and several circulating miRNAs are consistently dysregulated in T1DM patients compared to controls. In T1DM, 11 circulating miRNAs implicated in immune system function, cell survival and proliferation, and insulin production were dysregulated[188]. This set comprised miR-100-5p, miR-21-5p, miR-150-5p, miR-24-3p, miR-146a-5p, miR-148a-3p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-1275 and miR-375. In a study by Nielsen *et al*[189], global profiling of serum miRNAs expression in new-onset T1DM in children and age-matched healthy controls revealed a set of 12 upregulated miRNAs in T1DM patients, comprising miR-24, miR-152, miR-30a-5p, miR-200, miR-148a, miR-181a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b and miR-25; some of which are implicated in β-cell function and glycemic control[189]. Erener *et al*[190] studied serum miRNA expression and signaling pathways involved in T1DM development[190]. They found six miRNA (miR-222-3p, miR-24-3p, miR-454-3p, miR-144-5p, miR-345-5p and miR-140-5p) that were specifically dysregulated in new-onset T1DM but not at later stages of DM, and were involved in Wnt, MAPK, and PI3K/Akt signaling pathways [190]. The plasma levels of miR-30d, miR29a, miR-21, miR-24, miR-34a, miR-148a, miR-126 and miR-146 were significantly upregulated in adult T1DM patients[191], and higher levels of miR-210, miR-21 and miR-181a in T1DM were also confirmed in other studies[192,193]. More than one independent study found upregulation of miR-21, miR-148a, miR-24, miR-210 and miR-181a-5p in T1DM, indicating their potential use as circulating biomarkers of T1DM.

Global profiling of circulating miRNAs in blood samples of T2DM patients revealed approximately 70 upregulated miRNAs and around 100 downregulated miRNAs in T2DM patients[50]. A subsequent meta-analysis suggested that plasma levels of miR-103, miR-29a, miR-107, miR34a, miR-142–3p, miR-132, miR-375 and miR-144 may potentially serve as biomarkers for T2DM[194]. miR-126a was the most significantly downregulated circulating miRNA in T2DM patients

[195-199]. Comparison of plasma miR-126 levels with healthy controls, T2DM-susceptible, and T2DM patients demonstrated significant miRNA-126 downregulation in both T2DM-susceptible individuals and T2DM patients, suggesting a close association of miR-126 with the T2DM manifestation, thus making this miRNA a potential biomarker for the early identification of susceptibility to T2DM[199]. In addition, numerous studies reported an association of other serum miRNAs with T2DM[197,200-208]. For example, miRNA profiles comparing T2DM patients to a control group with normal glucose tolerance revealed significantly lower expression levels of miR-486, miR-96, miR-23a, miR-191, miR-186, miR192 and let-7[202] whereas increased levels of miR-9, miR34a, miR-27a, miR-15b, miR-29a, miR-124a, miR-30d, miR-192, miR-150, miR-375, miR-146b, miR-320a, miR-571, miR-486, miR-661, miR-1303 miR-770 and miR-892b were observed[203-206]. Whole blood evaluations demonstrated upregulation of miR-320, miR-144, miR-29a, miR-192 and miR-150, downregulation of miR-30d, miR-15a and miR-182[50,207], and decreased miR-103b expression in platelets of T2DM patients[208].

Regarding the utility of miRNAs as potential biomarkers of metabolic diseases, circulatory miRNAs miR-17-5p, miR-15a-5p, miR-221 and let-7g were reported as reliable predictive biomarkers of metabolic syndrome (MetS)[209]. These miRNAs have a well-documented role in regulating IR, β-cell apoptosis, and central obesity[210,211]. Circulating miR-192 and miR-194 have been suggested as prospective DM risk biomarkers[212], whereas plasma levels of miR-29a, miR-9, miR-28-3p, miR-30a-5p, miR-103, and miR-150 are reliable predictive biomarkers that distinguish between incident-T2DM and non-T2DM patients[145]. It should be emphasized that this group of miRNAs is linked to cell proliferation, insulin sensitivity, and secretion and that alterations in their levels can occur up to three years before T2DM development[123]. However, more controlled studies on large patient cohorts are required to fully establish the potential of many proposed miRNA-based biomarkers for T1DM, T2DM, and other metabolic diseases.

miRNAs AND NANOTECHNOLOGY

In recent years, bio-nanomedicine has turned its attention to EVs as a novel disease treatment approach. One of the most promising applications is the delivery of tolerogenic nanoparticles (TNPs) to combat autoimmune diseases like T1DM. EVs and nanoparticles (NPs), as opposed to traditional medicines, provide advantages such as tailored delivery, lower toxicity, and enhanced stability. TNPs can induce immunological tolerance in T1DM patients by regulating the immune response *via* various mechanisms[213]. In contrast, EVs can deliver cargo such as cytokines, growth factors, and miRNAs to recipient cells, influencing immune responses *via* a paracrine impact and during the development of the immunological synapse[214].

A recent study has focused on developing EVs to contain TNPs to treat T1DM. For example, immunomodulatory NPs containing antisense oligonucleotides to CD40, CD80 and CD86 have been utilized to prevent T1DM in mice by increasing $Foxp3^+$ Treg cells[215]. Another study found that the coculture of islets and bone marrow stem cells enhanced islet-cell survival and functionality in mice, mediated by exosomes *via* a paracrine action[216]. Clinical studies[217] have shown that exosomes derived from mesenchymal stem cells can suppress immune targeting in allogeneic grafts. These findings imply that EVs have regenerative, antiapoptotic, immunomodulatory, and angiogenic activities, making them a prospective tool for restoring islet-cell function and treating autoimmune disorders[218].

Nanotechnology has gained prominence in diabetes research by leveraging nanomaterials, nanostructures, and NP design to obtain more exact information on DM diagnosis. NPs can be used to deliver RNA and proteins to identify and monitor illness progression[219]. A recent study aimed to identify critical miRNAs that are dysregulated in pancreatic islets during T1DM progression and to create a theranostic strategy to modulate their expression using an MRI-based nanodrug. Iron oxide NPs combined with miRNA-targeting oligonucleotides were used to treat a mouse model of T1DM [157,220].

miRNAs AS POTENTIAL THERAPEUTIC TARGETS IN DIABETES TREATMENT

RNA-based therapeutic approaches have several important advantages compared to other drugs. Theoretically, miRNAs can simultaneously target several mRNAs, and fine-tuning miRNAs expression may restore physiological homeostasis [221]. The mechanism of action of RNA molecules may be elucidated from various available bioinformatics tools, such as *in silico* analysis and RNA structure prediction. ncRNAs are not associated with the development of drug resistance[222]. Thus, miRNAs are intriguing pharmacological targets that can potentially treat various complex diseases such as DM, cardiovascular disease, and cancer at the molecular level[222-225]. In miRNA-based therapies, the ultimate goal is to restore specific miRNA functions to normal levels, achieved by either restoring the expression of downregulated miRNAs using miRNA mimics or inhibiting the activity of upregulated miRNAs with a miRNA inhibitor.

miRNA mimics are double-stranded RNAs with the same sequence as a specific endogenous miRNAs[226]. miRNA mimics have been used *in vitro* to stimulate the regeneration of insulin-producing cells from induced pluripotent stem cells[227-230]. The approach is based on discovering that different miRNA clusters control human iPSC reprogramming [231,232]. However, this approach is not used *in vivo* since it may be associated with unwanted side effects[233].

Antisense oligonucleotides (ASOs) containing a complementary sequence to the target miRNA are used as miRNA inhibitors. Since most miRNAs repress target gene expression, binding a miRNA inhibitor to the mature target miRNA typically activates target gene expression. Locked nucleic acid (LNA) anti-miRs and antagomiRs are *in vivo* efficient modified miRNA inhibitors[234,235]. Anti-miRs are ASOs fully or partially complementary to an endogenous miRNA and act by preventing its interaction with target genes. AntagomiRs are cholesterol-conjugated anti-miRs with improved

intracellular delivery[236]. At present, anti-miRs are the most commonly used miRNA-based therapeutic tool[237,238]. LNA anti-miR-122 treatment has been tested in mice and nonhuman primates, showing reduced plasma cholesterol, improved liver steatosis, and no indication of hepatic toxicity[239,240]. Several other miRNA-targeting therapeutics are in different phases of preclinical and clinical studies. Regarding the potential treatment of T2DM and IR, ASOs were used to inhibit miR-103 and miR-107 in the liver and adipose tissue of obese mice resulting in improved insulin sensitivity and glucose homeostasis[108]. Modified GalNAc-conjugated oligonucleotides RG-125 (AZD4076) developed by Regulus Therapeutics, which targets miR-103/mir-107, was tested in phase I and IIa clinical trials to assess their effect on insulin sensitivity and liver fat content in patients with T2DM and nonalcoholic steatohepatitis[241]. Another anti-miR developed by Regulus Therapeutics for treating metabolic illnesses is 2'-fluoro/methoxyethyl modified, phosphorothioate backbone modified anti-miR-33, which has been proven to reduce atherosclerotic plaque in T2DM patients[242,243]. An LNA-modified ASO targeting miR-208A (MGN-9103) was developed by Viridian Therapeutics, and it demonstrated the potential to ameliorate insulin sensitivity and systemic glucose tolerance in MetS[244]. Several candidate miRNA molecules, such as mir-21 and miR-181a, were suggested as potential therapeutic targets for metabolic disease. miR-21 suppression with LNA-modified anti-miR-21 in adipose tissue of db/db mice led to a significant decrease in body weight [139], whereas oligonucleotide-mediated downregulation of miR-181 in DIO mice increased levels of SIRT1 and ameliorated insulin sensitivity and glucose homeostasis[245].

It is also worth noting that dietary substances like conjugated linoleic acid (CLA), polyphenols, and long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) indirectly influence miRNA expression[246-249]. For instance, CLA treatment altered the expression of miRNAs related to adipocyte differentiation, lipid metabolism, and obesity, such as miR-143, miR-107, miR-222, miR-103 and miR-221[246]. Dietary polyphenols inhibited the increased expression of miR-103 and miR-107, which is associated with a high-fat diet[247], whereas n-3 PUFA consumption alters the expression of miRNAs involved in lipid metabolism and inflammation[249].

Gene editing based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 (CRISPR/Cas9) has recently emerged as a technique with broad applications in the research and treatment of various diseases. The CRISPR/Cas9 RNA-guided editing technology could be used for genome editing. It is composed of a Cas9 nuclease that binds to a conserved three-nucleotide sequence known as the proto-adjacent motif (PAM) and creates a double-stranded DNA break. CRISPR RNA (crRNA) is a guide for Cas9 and an adapter trans-activating RNA (tracrRNA). The crRNA and tracrRNA can be combined to create a single-guide RNA capable of directing Cas9 to any target within the PAM sequence[250-252]. The CRISPR/Cas9 platform has been used to target the expression of miRNAs implied in various pathophysiological conditions[253,254]. An innovative example of CRISPR/Cas9 use in the context of the development of antidiabetic therapeutics is a recent development of a therapeutic candidate VCTX210. In 2021, CRISPR Therapeutics and ViaCyt companies jointly developed CRISPR-edited stem cell therapy candidate VCTX210 for potentially treating T1DM and T2DM, which has been approved for a clinical trial in Canada. VCTX210 is a stem cell-derived therapy edited with CRISPR-Cas9 intended to replace the β-cells lost in diabetes. Nonetheless, the use of gene editing for therapeutic purposes in the future will necessitate precise delivery of CRISPR to specific target tissues as well as strict control of potential off-target effects[255].

CONCLUSION

Although most current animal and human studies focus on the detection rather than the functional analysis of various miRNAs associated with cell physiology and pathology, many highly specific miRNAs have recently been identified as potential prognostic markers and therapeutic tools in diabetes patients. MiRNAs are involved in the regulation of gene expression in glucose homeostasis, lipid metabolism, and immune system balance. Increasing evidence shows that miRNAs can be a biomarker to predict type 1 and type 2 diabetes. Future studies must extensively investigate the roles of identified miRNAs to find those with the most reliable prognostic and therapeutic potential. Developing highly precise miRNA-based therapies designed for clinical usage will improve predicting vascular risk and end-organ vascular damage. With further advancements in high-throughput methodologies, such as whole genome and transcriptome profiling, and the associated proteomic and metabolomic analyses, a more profound link between various miRNAs and the physiology and pathophysiology of glucose homeostasis and fat metabolism will be more firmly established.

FOOTNOTES

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REVIEW

Implications of receptor for advanced glycation end products for progression from obesity to diabetes and from diabetes to cancer

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Abstract

Obesity and type 2 diabetes mellitus (T2DM) are chronic pathologies with a high incidence worldwide. They share some pathological mechanisms, including hyperinsulinemia, the production and release of hormones, and hyperglycemia. The above, over time, affects other systems of the human body by causing tissue hypoxia, low-grade inflammation, and oxidative stress, which lay the pathophysiological groundwork for cancer. The leading causes of death globally are T2DM and cancer. Other main alterations of this pathological triad include the accumulation of advanced glycation end products and the release of endogenous alarmins due to cell death (*i.e.*, damage-associated molecular patterns) such as the intracellular proteins high-mobility group box protein 1 and protein S100 that bind to the receptor for advanced glycation products (RAGE) - a multiligand receptor involved in inflammatory and metabolic and neoplastic processes. This review analyzes the latest advanced reports on the role of RAGE in the development of obesity, T2DM, and cancer, with an aim to understand the intracellular signaling mechanisms linked with cancer initiation. This review also explores inflammation, oxidative stress, hypoxia, cellular senescence, RAGE ligands, tumor microenvironment changes, and the "cancer hallmarks" of the leading tumors associated with T2DM. The assimilation of this information could aid in the development of diagnostic and therapeutic approaches to lower the morbidity and mortality associated with these diseases.

Key Words: Type 2 diabetes; Cancer; Obesity; Advanced glycation end product receptor; Receptor for advanced glycation end products; Glycation end products, advanced

Core Tip: The receptor for advanced glycation products (RAGE) is involved in every stage of the pathophysiological pathways that lead to the progression of obesity, type 2 diabetes, and cancer. This article provides a focused discussion on the stages of obesity leading to the development of metabolic diseases and provides a broad overview of the contribution of RAGE to the development of diabetes and cancer.

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INTRODUCTION

Obesity, diabetes, and cancer are chronic diseases, the prevalences of which have all increased in parallel, and are leading causes of death worldwide[1]. However, the forecasts for these health problems are not encouraging. For example, the prevalence of diabetes is estimated to increase by 2045, specifically in middle-income countries to 21.1%, in high-income countries to 12.2%, and in low-income countries to 11.9%. Meanwhile, the incidence of malignant neoplasms in people under 50 years of age is also rising[2,3].

Although esophageal adenocarcinoma has a direct link to obesity, and pancreatic cancer can debut with type 2 diabetes mellitus (T2DM), there is an evident connection between the three disorders. Moreover, there is confusion about their shared lifestyle risk factors, including sedentariness and consumption of highly processed foods[4-6]. Regarding the common pathological mechanisms of obesity, T2DM, and cancer, expansion of adipose tissue (AT) results in the production of excess estrogen, adipokines, and inflammatory molecules that can lead to systemic or localized low-grade inflammation. In addition, omental and visceral adiposity is related to hyperinsulinemia and increased levels of insulin-like growth factor-1 (IGF-1)[7]. The metabolic abnormalities and lipo-glucotoxicity associated with insulin resistance and T2DM also cause an increase in inflammatory cytokines and oxidative stress. As a result, neoplastic processes can be triggered by T2DM and, likewise, obesity[8].

The pathogenic mechanisms that link obesity, T2DM, and cancer are complex and multifactorial. Because there is a notion of progression from obesity to T2DM towards cancer, our motivation for this review was to provide a detailed and up-to-date discussion on these mechanisms in the context of a single molecule known as the receptor for advanced glycation end products (RAGE). As such, this narrative review incorporates the conceptual framework and reports on findings extracted from two literature databases, the *Reference Citation Analysis* (https://www.reference-citationanalysis.com/) and PubMed, to provide a reflective discussion of RAGE's implications for the progression of obesity to T2DM and from T2DM to cancer.

RAGE is an immunoglobulin superfamily member and a type I pattern-recognition receptor. It is also a sensitive environmental sensor with several endogenous and external ligands. Furthermore, it is a widely expressed modulator of inflammatory and oxidative stress pathways with vast metabolic implications[9]. RAGE isoforms include soluble forms (sRAGE) that act as decoy receptors, sequester circulating ligands, and attenuate membrane RAGE signaling[10]. Soluble forms derived from membrane-localized RAGE are released into the circulation by proteolytic cleavage (cRAGE), and endogenously secreted RAGE (esRAGE) is formed by alternative splicing. In addition to the sRAGE isoforms and the full-length membrane receptor (flRAGE) - the only isoform that participates in signal transduction, there are also the dominant-negative isoforms lacking the cytoplasmic tail and the truncated isoform lacking the V-type immunoglobulin domain[11] (Figure 1A).

OBESITY AND T2DM

Initially, the function of RAGE was established in the context of chronic disease, specifically T2DM and its complications, in which persistent hyperglycemia triggers inflammation, oxidative stress, and endothelial damage[12,13]. However, there is more evidence that an increase in RAGE ligands is present in the early stages of metabolic dysfunction in obesity [14,15].

RAGE ligands

The most recognized ligands of RAGE are the advanced glycosylation end products (AGEs) and lipid oxidation adducts (ALEs). These are taken in from diet or produced by endogenous metabolism through non-enzymatic and spontaneous

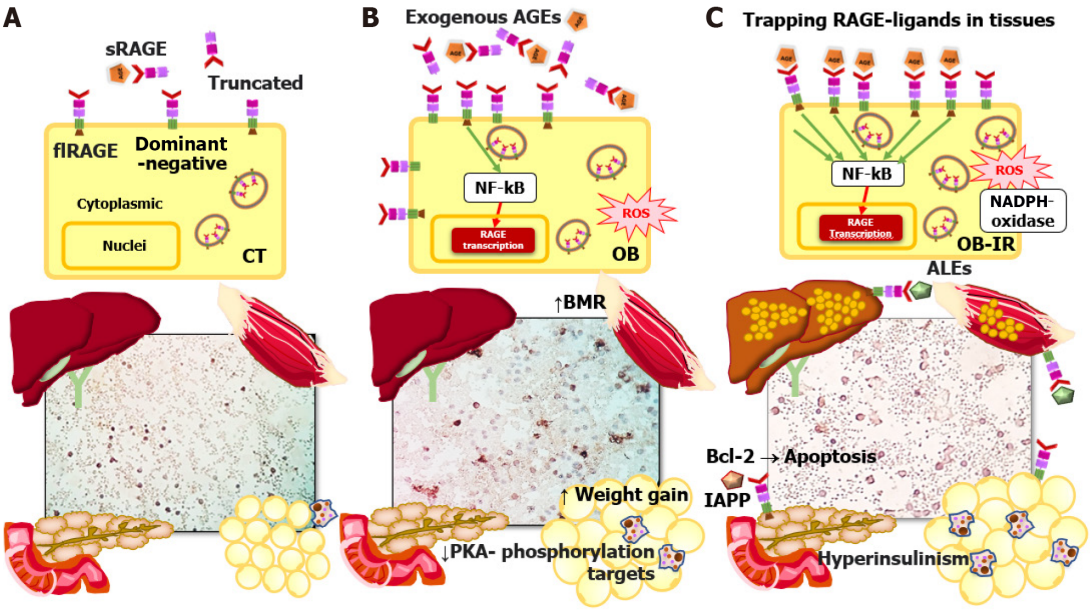

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Figure 1 Receptor for advanced glycation products signaling and molecular mechanisms involved in progression from obesity to type 2 diabetes mellitus. Receptor for advanced glycation products (RAGE)-ligand signaling in healthy control subjects, obese individuals (OB), and OB with insulin resistance is illustrated. A: Full-length, total soluble, dominant-negative (intracytoplasmic, lacking domain), and truncated (lacking a V-terminal) RAGE isoforms; B: Basal metabolic rate increase in muscle, decreased phosphorylation targets of protein kinase A, and weight gain (adipose tissue) are findings in obesity related to increased RAGE isoforms and ligands; C: The mechanism trapping RAGE-ligand in tissues involves translocation of cytoplasmic RAGE to the membrane, inflammation (nuclear factor-kappa B), and oxidative stress (NADPH-oxidase) in peripheral mononuclear blood cells, liver, muscle, pancreas, and adipose tissue. The B cell lymphoma-2 proto-oncogene mediates RAGE apoptosis signaling in pancreatic beta cells and leads to type 2 diabetes mellitus. Advanced glycosylation end products, advanced lipoperoxidation end products, and islet amyloid polypeptide (also known as amyloid) are RAGE ligands. RAGE: Receptor for advanced glycation products; CT: Control subjects; OB: Obese individuals; OB-IR: Obese individuals with insulin resistance; flRAGE: Full-length receptor for advanced glycation products; sRAGE: Soluble receptor for advanced glycation products; BMR: Basal metabolic rate; PKA: Protein kinase A; NF-kB: Nuclear factor-kappa B; PBMCs: Peripheral mononuclear blood cells; Bcl-2: B cell lymphoma-2; AGEs: Advanced glycosylation end products; ALEs: Advanced lipoperoxidation end products; IAPP: Islet amyloid polypeptide.

Maillard-type reactions in which proteins and nucleic acids react with carbohydrates, lipids, or their intermediate metabolites[16,17].

Foods cooked by roasting, grilling, frying, drying, heating, or adding artificial colorants, salt, oil, or sugar are often present in ultra-processed foods to make them suitable to store[6]. In addition to those above, an increase in the diet's caloric, fat, and glycemic indices leads to a significant rise in the levels of circulating AGEs. Some exogenous-derived food AGEs are Nδ-(5-hydro-5-methil-4-imidazolon-2-il)-ornithina (MG-H1), Nε-carboxyethyl lysine (CEL), and Nε-carboxy-methyl lysine (CML), in addition to the precursor methylglyoxal[18-20].

The problem gets worse when an individual also consumes other substances like alcohol and tobacco. Cigarettes are a source of AGEs, and smoking them causes RAGE expression to rise, which is linked to airway inflammation in chronic obstructive pulmonary disease and causes sRAGE to decrease in smoke-induced cardiovascular disease[21,22]. The increase in mitochondrial-derived reactive oxygen species (ROS) caused by the RAGE pathway in smoke-exposed skeletal muscle is one of the hypothesized mechanisms in this regard[23]. *In vitro*, oral squamous cell carcinoma treated with cigarette smoke extract showed an increase in RAGE with a link to a rise in invasive ability[24]. Additionally, RAGE is elevated in alcoholic liver disease, affecting blood triglycerides, low-density lipoprotein cholesterol, and alanine transaminase levels. RAGE also contributes to the accumulation of lipid droplets in the liver and modifies the expression of SREBP1, a transcription factor involved in lipid homeostasis[25].

Serum AGE accumulation from the diet can lead to cross-link formation that irreversibly changes endogenous proteins independent of glycemic control. Birukov *et al*[26] found that in people with prediabetes and T2DM, there were significant variations in the levels of AGEs in the skin. Additionally, AGE measurements in that study were related to factors such as waist circumference, glycated hemoglobin (commonly known as hemoglobin A1c) levels, C-reactive protein levels, and vascular stiffness. Further research is required to determine the sensitivity and accuracy of testing AGE accumulation and its relationship to disease status.

In addition to the above, other natural substances such as catechols, myeloperoxidase systems, and the polyol pathway are implicated in producing endogenous AGEs in obesity and states of insulin resistance[27,28]. Likewise, the link between AGEs in obesity and T2DM is the accumulation of lipids and their oxidized products. Thus, the accumulation of free fatty acids and subsequent ALE production aids in the progression of obesity to T2DM[29,30]. Oxidative stress promotes the lipoperoxidation of membranes and the production of metabolites such as 4-hydroxyl-trans2-nonenal, acrolein, aldehydes such as malondialdehyde (MDA), and ketoaldehydes such as 4-oxo-trans-2-nonenal. These may start with obesity and insulin resistance and can result in the creation of endogenous ALEs like MDA-Lys[17,31,32]. Further

studies are required on the mechanism by which the progression from obesity to T2DM is affected by the ALEs-RAGE interaction and their aldehyde precursors produced by lipid peroxidation.

In this regard, in obese subjects, RAGE induces migration of macrophages because of the rise in lipid peroxidation and the accumulation of ALEs in renal tissue that leads to kidney injury[33]. Patients with T2DM have high levels of ALE (MDA-Lys), which induces the activation and adherence of monocytes to endothelial cells by increasing the expression of monocyte chemotactic protein-1 (MCP-1) and activating the nuclear factor-kappa B (NF-kB) pathway causing inflammation[34]. Recent comprehensive reviews have addressed endogenous and exogenous AGE and ALE formation in obesity[17], T2DM, and cancer[28,35,36].

There is consistent evidence regarding how ultra-processed foods, ALEs, and AGEs disrupt the microbiota causing dysbiosis, the subsequent translocation of lipopolysaccharide (LPS), and endotoxemia[37,38]. Likewise, dysbiosis is related to obesity, low-grade inflammation, and the progression of insulin resistance and T2DM[39]. However, few publications implicate RAGE as an LPS ligand to mediate inflammatory processes in obesity[40]. This issue needs further investigation, and an exciting future research opportunity may focus on T2DM prevention with respect to the relationship between AGEs/ALEs, RAGE, and dysbiosis.

According to the most recent definitions, chronic low-grade inflammation begins when molecules and metabolites, resulting from altered cell function and structure and foods, stimulate receptors and activate their signaling cascades with dysregulated energy homeostasis. To this end, RAGE mediates danger signals to the body and metabolic stress characteristic of innate immune systems, since RAGE detects ligands from microbes *via* exogenous pathogen-associated molecular patterns such as LPS. Furthermore, damage-associated molecular pattern (DAMP) ligands are derived from endogenous sources such as high-mobility group box protein 1 (HMGB1), S100/calgranulins, amyloid deposits like β-amyloid peptide, and macrophage-1 antigen[41].

AGE and ALE metabolites can be considered DAMPs that are not derived from exogenous sources such as the diet, and the term "metabolism-associated molecular pattern" is proposed for these specific ligands. It is essential to differentiate between them and demonstrate that both endogenous and external components are involved in these responses [42]. An opportunity for experts in the field is to reach a consensus with respect to the classification of all exogenous and endogenous ligands for pattern-recognition receptors.

RAGE-trapping ligands

Several investigations in human subjects have found an association between obesity and low circulating AGE levels[43]. Complex detoxification and clearance kinetics of AGEs could lead to inconsistent study results. The concept of entrapment of AGE in tissues proposes that AGEs are no longer circulating because they are trapped in tissues as metabolic risks increase in individuals[44-46] (Figure 1C).

For instance, high RAGE expression in AT is implicated in its dysfunction and is evidence of a link between RAGE signaling and the progression of obesity to associated metabolic disorder. A high level of RAGE expression in human epicardial AT is related to its thickening, low glucose transporter type 4 expression, and high HMGB1 expression[47]. In this context, visceral omental AT and fetal membrane samples from women with gestational diabetes revealed higher levels of RAGE and the HMGB1 ligand, respectively[48]. RAGE signaling pathway proteins were also found to be expressed differently in omental and subcutaneous biopsies from obese people with healthy phenotypes. Subcutaneous AT showed a higher correlation between the RAGE signaling axis, inflammatory markers, and the homeostatic model assessment of insulin resistance (HOMA-IR)[49]. A study with a murine RAGE (-/-) model demonstrated protection against inflammation and oxidative stress and protection against insulin resistance. Interestingly, this model showed that the most beneficial characteristics of RAGE knockout were found in female mice[50]. Additionally, RAGE is related to the adaptive thermogenesis function of brown AT through the decline in energy expenditure caused by a high-fat diet, possibly mediated *via* the accumulation of AGEs[51,52] (Figure 1B).

In addition to dysregulation in AT discussed above, chronic inflammation also plays a pivotal role in obesity-related insulin resistance that leads to metabolic dysfunction in the liver and muscle. Insulin resistance is characterized by alterations in insulin signaling in sensitive tissues, hyperinsulinemia with defects in glucose uptake in muscle and AT, impaired suppression of hepatic glucose production, and ectopic accumulation of fat in the muscle and liver through re-esterification of fatty acids from AT[53,54] (Figure 1C). To this end, an increase in AGE accumulation in liver biopsies has been linked to RAGE expression, lipid accumulation, and the degree of liver damage without association with the measurements of sRAGE and circulating serum AGEs[55,56]. These studies demonstrate how RAGE affects hepatic conditions caused by the accumulation of AGEs in tissue in non-alcoholic liver disease.

RAGE expression and the accumulation of AGEs are linked to weight gain, inflammation, and oxidative stress markers in human muscle tissue[57]. For instance, one study demonstrated that RAGE expression and the accumulation of AGEs in skeletal muscle in a fructose-supplemented murine model were related to alterations in the oral glucose tolerance test curve, increased triglycerides, inflammatory response, increased basal metabolic rate, and resting metabolic rate[58]. Moreover, chronic AGE exposure is linked to sarcopenia[59]. However, the implications of obesity- and T2DM-induced RAGE expression in muscle tissue are less well explored in humans[60].

Along with the mechanism of trapping excess RAGE ligands in tissues, it is known that the sRAGE form eliminates dangerous circulating ligands and functions as a competitive inhibitor of ligands that might bind to cellular RAGE, supported by studies in which sRAGE levels were found to be low[61-64]. The role of sRAGE in metabolic diseases is debatable because it depends on the degree of disease development and the levels of cell and tissue damage[65]. The cRAGE levels are initially high in acute conditions, triggered by cleavage of flRAGE, which increases its AGE-binding activity. The main variations of sRAGE are attributed to the production of cRAGE shedding by metalloproteinases[66] to compensate for the increase in AGEs in the early stages of low-grade inflammation[67-69]. As the concentration of sRAGE decreases, sequestration and competitive inhibition of ligands decrease and as such they can reach cellular flRAGE,

leading to an inflammatory response and subsequent tissue damage[68-70] (Figure 1C).

In prediabetes, plasma levels of sRAGE and esRAGE are all negatively correlated with the HOMA-IR index of insulin resistance and MDA. This correlation matches their reduction as insulin resistance develops in an oxidative environment [67]. Another study with similar results comparing healthy people to those with prediabetes and T2DM found low levels of esRAGE and an inverse linkage with S100A12[71]. Miranda *et al*[62] showed that all RAGE isoforms were lower when grouped by pancreatic dysfunction (*i.e.*, healthy controls, individuals with glucose intolerance, and those with T2DM). Thus, according to the above, the negative correlation of sRAGE with RAGE ligands or increase of the AGE/esRAGE index seems to be more related to individuals with obesity-related insulin resistance and early T2DM[72], and low cRAGE concentrations are a marker of aging[72,73]. Even the elevated AGE/esRAGE index could distinguish between those with non-alcoholic fatty liver disease without T2DM and healthy individuals[74]. Further studies are needed to determine the precise interactions between sRAGE, esRAGE, cRAGE, and their ligands in these disease states.

Since sRAGE and resting energy expenditure are related, one of the most recent discoveries regarding the expression of soluble variants is sRAGE's contribution to adaptive negative energy balance. In an investigation of the influence of sRAGE on the change in energy expenditure that occurs during weight loss, it was found that, under caloric restriction, adaptive changes arise that slow down energy expenditure. Specifically, after a 3-mo intervention for weight loss due to caloric restriction, energy expenditure increased by 52.6 kcal/d for each 100 pg/mL increase in basal sRAGE levels. Increases in esRAGE and cRAGE similarly translated to concomitant rises in energy expenditure, by 181.6 kcal/d and 56.1 kcal, respectively. This finding illustrates the potential impact of a RAGE feedback mechanism, in which a reduction in sRAGE could slow energy expenditure during weight loss[75]. Furthermore, one mechanism by which RAGE controls energy expenditure is through the suppression of adaptative thermogenesis in white and brown AT *via* the decline of β-adrenergic signaling in adipocytes blocking protein kinase A (PKA) phosphorylation targets[76].

Still more, the subcellular localization of RAGE can change, a process related to oligomerization in the membrane after RAGE interaction with ligands[77]. A previous study demonstrated increased localization of RAGE in the cell membrane, rather than the cytoplasm, in peripheral blood mononuclear cells of obese individuals with insulin resistance compared with healthy individuals. As such, sRAGE correlates negatively with the HOMA-IR index and tissue damage markers[78] (Figure 1A-C). Peripheral blood mononuclear cells may provide an accessible platform to study the relationship between ligands and cellular RAGE, detect systemic inflammation, and relate these to tissue damage. The preceding argument needs to be tested by additional research.

In T2DM, the pancreas loses its ability to secrete enough insulin in response to meals. One of the mechanisms of pancreas failure is low-grade systemic inflammation. The activating signaling of RAGE in response to ligand binding results in RAGE autoregulation through the increase of its synthesis, which is mediated by NF-kB[79]. *In vivo* and *in vitro* models have shown that oxidative stress and inflammation are induced by AGE stimuli through NF-kB activation and the formation of ROS, respectively[80]. These events are evidenced by the increase in the inflammatory serum marker C-reactive protein, particularly in obesity[81]. Some antioxidants and drugs can modulate the AGEs-RAGE axis and the activation of NF-kB, leading to the reduction of lipid peroxidation products in obesity models[82-84].

RAGE expression in the pancreas may be an essential mechanism for the development of T2DM in humans, based on evidence from both *in vitro* and *in vivo* glycolipotoxicity studies[85-87]. In a rodent model of diet-induced hyperglycemia, endogenous AGE products are produced, and RAGE expression is observed in pancreatic islets[87]. RAGE inhibition prevented the increase of its expression, and decreased B cell lymphoma-2 (Bcl-2) expression and apoptosis of beta cells treated with glycation serum. However, RAGE inhibition did not restore the ability of the beta cells to secrete insulin in response to glucose[85]. RAGE endocytosis regulated by Rab31 ligand can inhibit apoptosis mediated by the pAkt/Bcl-2 pathway in beta cells treated with glycation serum[88]. In another study, the pancreas of db/db transgenic mice that lack the leptin receptor but express RAGE (+/+) have less beta cell mass and less apoptosis, is glucose intolerant, and has decreased insulin secretion. Likewise, when the MIN6 pancreatic beta cell line was treated with palmitate or oleate and leptin antagonists to induce RAGE expression, pancreatic damage occurred[86]. Another mouse model of diabetes induced by streptozotocin and a high-cholesterol diet treated with the water-soluble carotenoid crocin showed attenuated atrophic effects in pancreatic tissue and decreased blood glucose levels through decreases in the expression of RAGE and LOX-1[89].

DAMP/RAGE reports such as the activation of S100b/RAGE and the subsequent loss of beta cells by apoptosis *via* NADPH oxidase and the protection of sRAGE against amyloid deposition, beta cell loss, and glucose intolerance demonstrate that they interact[90,91]. All of these findings suggest that RAGE can lead to pancreatic failure and the progression of T2DM.

T2DM AND CANCER

Several studies have shown that the incidence of various malignancies increases in patients with T2DM. However, more rigorous statistical analyses of observational studies demonstrate a more significant association of T2DM with colorectal, pancreatic, hepatocellular, breast, and endometrial carcinomas. Even so, there are biases in these studies that make it challenging to study the confounding variables of T2DM leading to cancer[92]. A more recent study included statistical analysis of the "Mendelian randomization" studies to analyze genetic data from large-scale international consortia. Ultimately, it allowed to link a possible causal relationship between genetically predicted T2DM and endometrial and pancreatic cancer risks, and between the variable fasting insulin levels and breast cancer risk. In addition, numerous studies have demonstrated the impact of glycemic traits on the emergence of different malignancies, establishing a relationship between T2DM and cancer[93].

Metabolic and hormonal factors found in patients with obesity, insulin resistance, and T2DM, such as hyperinsulinism, hyperglycemia, IGF-1, adipokines, and estrogens, all of which are closely related to inflammation and oxidative stress, function in the long-term as risk factors that support transformation to neoplastic cells in diabetic patients[94].

Estrogens

The increase in estrogen levels in obese patients is due to the positive regulation of the aromatase enzyme, encoded by CYP19A1 and secreted by cells of the tumor stromal microenvironment. The activation mechanisms are triggered in response to hypoxia, with activation of hypoxia-inducible factor-1 alpha (HIF-1α), fat tissue hormones (*e.g.*, adipokine leptin, which increases aromatase expression by phosphorylating serine at position 485 of AMPK and inhibiting the aromatase suppressor), and inflammation processes[95]. Estrogen receptors are transcriptional factors of DNA reprogramming that transduce extranuclear signals, resulting in the regulation of ion channels or kinase cascades such as PKC/PKA/PI3-K/MAPK. The metabolic effects of estrogen on both tumor and normal cells are survival, cell proliferation, and immunomodulation[96]. Estrogens are the most relevant risk factor for endometrial and breast cancers, especially in postmenopausal women. Recently, studies have shown that the microbiota is a source of estrogen-like compounds or estrogen mimics that could be involved in cancer progression[97].

Hyperinsulinism and IGF-1

The insulin receptor (IR) and insulin receptor substrate (IRS) are phosphorylated at Ser/Thr residues by inflammatory cytokines and oxidative stress, resulting in insulin resistance and compensatory hyperinsulinemia[98]. Insulin induces proliferation in tissues not involved in metabolism. Binding to its receptor (*i.e.*, IR) activates the RAS/RAF/MAPK kinase-dependent/ERK signaling pathways and increases cell survival and migration[99]. Another mechanism is mediated by IGF-1, a hormone structurally and functionally similar to insulin that binds to IR and its receptor (*i.e.*, IGFR). This receptor, like IR, activates pathways that increase cell proliferation, and insulin enhances the liver's production of IGF-1, elevating the mitogenic activity of cancer cells expressing the IGFR[7,100]. The nuclear protein HMGA1 contributes to the potentiation of insulin action. In addition, the HMGA1 protein overexpressed in triple-negative breast cancer cells functions in chromatin remodeling and gene expression regulation, indirectly promoting enhanced IR expression through the inhibitory effect on p53 expression, which usually keeps IR expression turned off.

Hyperglycemia

Although hyperglycemia is the primary cause of T2DM pathophysiological abnormalities, it also contributes to the development of cancer through several processes that either directly or indirectly harm DNA, RNA, lipids, and proteins. The production of ROS, accumulation of mutations and inhibition of their repair, alteration of the immune system, alteration of metabolism, and activation of oncogenes and inactivation tumor suppressor genes are some of the carcinogenic effects that result from the formation of AGEs through non-enzymatic reactions and the subsequent activation of RAGE[101]. Endogenous AGEs are categorized according to their precursor as follows: Glyoxal (GO)-derived compounds including glyoxal lysine dimer, N7-(carboxymethyl)arginine, and CML; methylglyoxal-derived, including MG-H1, methylglyoxal lysine, argpyrimidine, and CEL; 3-deoxyglucosone-derived, including pyrraline, pentosidine, and deoxyglucosone lysine dimer; and derivatives of glucose, fructose, and glyceraldehyde that form DNA adducts or cross-link with lysine or arginine altering protein structure and function[102]. These non-enzymatic protein modifications elevate oxidative stress and inflammation by binding with cell surface receptors such as RAGE. Exogenous AGEs play a role in the progression of cancer in addition to endogenous AGEs[29,103,104]. The metabolism and pathogenic effects of endogenous and exogenous AGEs have recently been the subject of extensive reviews[105].

RAGE AND CANCER

RAGE, inflammation, and oxidative stress

Interactions between RAGE and its ligands in T2DM result in various cellular responses, including activation of signaling pathways that cause oxidative stress and inflammation, which in turn cause various pathophysiological effects such as apoptosis, autophagy[106], senescence, and osteogenic differentiation[107], remodeling processes of the extracellular matrix, and activation of fibroblasts significant in vascular, neuronal[108], and musculoskeletal processes[109]. AGEs in T2DM accumulate in the extracellular matrix, forming cross-links with type I collagen and allowing long-lasting activation of RAGE. This also initiates a complex signaling network that allows the formation of ROS, activates the signaling pathway through ERK1/2 which then phosphorylates and activates NF-kB, and directly induces inflammation. Another alternative signaling pathway to the AGE-RAGE/ERK1-2/PKC pathway involves Rap-1, which induces inflammation, remodeling of the extracellular matrix, and oxidative stress[110].

RAGE and hypoxia

Hypoxia is frequent in solid malignant neoplasms due to the high proliferation of neoplastic cells, which does not allow rapid vascularization of neoplastic tissue so that the oxygen demand exceeds the supply. Another factor is the formation of new blood vessels that do not have the integrity of their vascular wall; a continuous outflow of blood results in tissue oxygenation deficiency[111]. Under these conditions, a series of genes regulated by HIF-1α are activated, allowing survival through the expression of genes that promote angiogenesis, metabolic reprogramming, lipid accumulation[112], inhibition of apoptosis, invasion, and metastasis. HIF-1α also promotes inflammation *via* NF-kB signaling in hypoxic

environments. In this tumor niche with inflammation, hypoxia, and cell death, DAMPs activate the NF-kB pathway mediated by RAGE, thereby amplifying HIF-1α activity[113]. In this hypoxic setting, stromal cells are also affected by RAGE; this is the case in adipocytes, where the AGE/RAGE/NF-kB pathway is activated and prolongs the inflammatory and hypoxic processes. Other effects of hypoxia include the stimulation of cell adhesion mediated by MCP-1, chemotaxis, and the polarization of macrophages towards a proinflammatory phenotype, specifically through the RAGE/NF-kB pathway in tryptophan hydroxylase 1 monocytes[114].

RAGE, survival, and programmed cell death

Cell death is a physiological process that keeps tissues healthy by systematically removing damaged cells to prevent an immune response. Although necrosis is a kind of cell death, it is pathological and only happens when there has been a significant tissue injury coupled with an immune response. Non-pathological cell death can take many forms, including apoptosis, necroptosis, and autophagy[115]. RAGE is involved in all three death pathways and can be activated by AGEs, HMGB1, and S100. In normal tissues, both the intrinsic and extrinsic apoptosis pathways are activated[116], and ROS, NF-kB, and MAPK mediate the stimulation. High levels of ROS induce the apoptosis pathway, but if they are low, autophagy is activated. Reduced HMGB1 activates Beclin-1-mediated autophagy pathways, but if oxidized, it activates apoptosis[117].

RAGE promotes cancer cell autophagy, which eventually permits survival by utilizing nutrients through the catabolism of their cellular components in a blood-free environment with no access to external nutrients and hypoxia. RAGE-dependent signaling pathways that promote autophagy involve PI3K, NF-kB/Beclin-1, PKC, and/or RAF/p38-MAPK/ERK[118]. Likewise, in cancer cells, apoptosis is inhibited, which indirectly allows cell perpetuation and survival. The pathways that inhibit apoptosis start with the binding of HMBG1/RAGE, which induces the formation of ROS and activation of NF-kB; another pathway involves Akt and matrix metalloproteinase-9[119].

RAGE and senescent cells

Cell senescence is present in T2DM and cancer. Frequently occurring in tissues undergoing metabolic shock, chronic inflammation, or oxidative stress, cell senescence is a physiological response that aims to prevent genomic instability and the consequent DNA damage that leads to metabolic reprogramming. In addition, senescence relates to decreasing immune surveillance, thus facilitating cancer initiation and progression[109,119-121]. The same markers found in the carcinogenesis process discussed above, such as IGF, HIF-1α, AGEs, and RAGE, were discovered in a proteomics study looking for plasma proteins that indicate a senescence-related decline in health[122]. In a model of endothelial senescence induced by protein products of advanced oxidation, the presence of modified p53 at amino acid K386 by SUMOylation was associated with evasion of apoptosis[123].

RAGE ligands

RAGE aids in the removal of endotoxins and debris from apoptotic bodies during the processes of oxidative stress, hypoxia, and inflammation. Cellular damage occurs that causes the release of intracellular molecules that, outside the cell, behave as alarmins, specifically the S100 and HMBG1 proteins, also known as DAMPs, which act as endogenous RAGE ligands[124]. These proteins are also known as "moonlighting proteins" since they have various functions depending on their location. For example, when the HMGB1 protein locates inside the nucleus, it organizes chromatin[125]. In contrast, S100 is a protein that functions as a Ca^{2+} sensor[126], and like HMGB1, when located extracellularly, it functions as an alarmin. Tumor initiation and progression, as well as tissue damage, are significantly influenced by endogenous DAMP/RAGE ligand signaling. Numerous malignancies, including colorectal[127], hepatocarcinoma[128], pancreatic[129], breast, and endometrial cancers, overexpress HMGB1 and S100[35].

The primary ligands that bind to RAGE in cancer cells, such as AGEs, HMGB1, and S100, activate several signaling pathways such as PI3K/Akt, ERK 1/2, JAK/STAT, Ras/MAPK, Rac/cdc42, p14/p42, p38, and SAP/JNK/MAPK, and transcription factors such as NF-kB, STAT3, HIF-1α, AP-1, and CREB[118,130], and thus activate a series of genes whose functions are essential in the initiation, promotion, and extension of various malignant neoplasms. These functions are known as "cancer hallmarks" and include cell proliferation, inhibition of apoptosis, inhibition of tumor suppressor genes, evasion of immunity, increased survival, invasion, metastasis, angiogenesis, genomic instability due to failure to repair mutations, and metabolic dysregulation[35,124] (Table 1).

RAGE and tumor microenvironment

Tumorigenesis is the process by which healthy cells develop the capacity to become cancerous cells, which implies, in addition to genetic and epigenetic alterations in DNA, the formation of the tumor microenvironment. The tumor microenvironment is determined by the interaction between resident immune cells, mesenchymal stromal cells, and tumor cells, the paracrine signaling between them, and the anatomical niche built-up by the extracellular matrix and blood vessels. In addition to cancer-affected fibroblasts, the tumor microenvironment contains infiltrating tumor-associated macrophages that promote tumor survival[131,132]. The tumor microenvironment includes the extracellular matrix, blood vascular structures, and paracrine signaling between stromal cells and tumor cells (Figure 2).

Recent studies have revealed that the involved cells and specialized three-dimensional structures are unique to each tumor by tissue[133-135]. Table 1 outlines the traits of the tumor microenvironment in hepatocarcinoma, colorectal, breast, and pancreatic cancers with RAGE implications. These findings demonstrate that RAGE promotes different adaptive phenomena for the survival, initiation, and progression of malignant tumors. Nevertheless, it is necessary to mention that RAGE overexpression varies in cancer related to T2DM because of the cellular heterogeneity of the neoplastic process. The Human Protein Atlas database shows RAGE detection rates in malignant cells by immunohisto-

Table 1 Studies published between 2018 and 2022 on receptor for advanced glycation products-ligands, related activated pathways, and cancer hallmarks in the most frequent neoplasms found in diabetic patients

Neoplasia	Ligands and signaling pathway	Molecule expressed	Cancer hallmarks	TS/AM/CL	Ref.
Breast cancer	AGE/RAGE, ERK1/2; Akt, c-fos	IL-8/CXCR1/2	Migration and invasion	CL; CAFs, TNBC (MDA-MB-231 cells)	Santolla *et al*[137]
	HMGA1		Cell proliferation, metastasis, and EMT	CL; TNBC (MDA-MB-231 and Hs578)	Shah *et al*[138]
	HMGB1/RAGE		Motility, migration, invasion, and dysregulation of metabolism	TS; human breast cancer, AM; NOD/SCID mice, CL; human breast cancer cells (MCF-7, T-470, BT474, MDA-MB-231, ZR-75-30, BT549) and human fibroblast cells HFL1	Chen *et al*[139]
	HMGB1/PI3K/Akt	PD-L1	Cell proliferation, migration, invasion, and T-cell apoptosis	CL; human breast cancer cells (MDA-MB-231 P, MDA-MB-231 BM)	Amornsupak *et al* [140]
	HMGB1, PI3K/Akt, mTOR	HIF-1α, VEGF	Migration and angiogenesis	TS; human breast cancer CL; human breast cancer cells MCF-7	He *et al*[141]
	HMGB1/RAGE	Downregulation of miR-205	Cell growth, invasion, and EMT	TS; human breast cancer CL; TNBC (MDA-MB-231, MDA-MB-453, MDA-MB-468) and NTNBC (MCF-7, MCF-10F)	Wang *et al*[142]
	HMGB1/RAGE, ERK 1/2, CREB		Bone metastasis and neurite outgrowth of nervous system cells	AM; 4T1 mice CL; mouse breast cancer 4T1, primary rat nervous system cells DRG, rat DRG/mouse neuroblastoma hybrid cells F11, immortalized rat DRG neuronal cells 50B11	Okui *et al*[143]
	S100A14/RAGE, NF-kB	CCL2/CXCL5	Migration, invasion, and lung metastasis	TS; human breast cancer and paired adjacent breast normal, metastatic lymph node, and non-metastatic lymph node AM; BALB/c, BALB/c, SCID beige, C57BL/6J, CMV-CreC57BL/6J, S200-/- and S100A14-/- PyMT mice CL; human breast cancer cells MCF7, MCF10A, T47D, SKBR3, BT549, MDA-MB-231, MCF10AT, MCFCA1h, MCFCA1α and mouse breast cancer cells 4T1	Li *et al*[144]
	S100A7/RAGE, PI3K/Akt, ERK1/2, STAT3	IGF-1	Angiogenesis	CL; human breast cancer cells MCF-7, T47D, and HUVECs cells	Muoio *et al*[145]
	S100A7/RAGE, cPLA	PGE2, CD163+	Immunosuppression, M2-macrophages, CD4+, CD8+, and T cells	AM; NOD SCID gamma mice CL; human breast cancer cells MDA-MB-231, MDA-MB-468 and mouse mammary cancer cells MVT-1	Mishra *et al*[146]
	S100A8/A9-RAGE, FAK, Akt, Hippo-YAK	FLNA, CTGF, Cyr61	Cell proliferation and migration	CL; HEK293T and TNBC (MDA-MB-23 and BT-549)	Rigiracciolo *et al* [147]
	LPS/S100A7/TLR4/RAGE		Migration and invasion	AM; orthotopic breast cancer C57BL/6 mice model CL; murine mammary cancer cells EO771, MTV-1, murine metastatic mammary cells EO.2, human breast carcinoma cells SUM 159,	Wilkie *et al*[148]

				MDA-MB-231 and MDA-MB-468	
	acHMGB1/RAGE, S100A4/RAGE, Gas6/AXL	CXCR4, CXCL12, CCL2, CD151 and α3 β1-integrin	Cell proliferation, invasion, intravasation, and EMT	AM; murine orthotopic mammary cancer CL; human MSCs, geminin overexpressing breast tumors Gem197, Gem240, Gem256, Gem257 and Gem270 cells, CAFs, and M0- TAMs and M2-TAMs	Ryan *et al*[149]
Colorectal cancer	S100A16		Cancer prognostic marker	TS; human colorectal cancer	Sun *et al*[150]
	HMGB1/RAGE	PD-1	Cancer prognostic marker	TS; human colorectal cancer CL; human colorectal cancer cells SW480, and SW620	Huang *et al*[151]
	S100B/RAGE, NF-kB	VEGF-A	Proliferation, migration, and angiogenesis	CL; human colon cancer cells HCT116	Zheng *et al*[152]
	IGF1R-Ras/RAGE-HMGB1,		Oncogenesis	TS; Human colorectal from diabetic patients	Niu *et al*[153]
	AGEs/RAGE, KLF5	MDM upregulation and RB and p53 downregulation	Cancer initiation and development	AM; diabetic mouse model and CL; human colon cancer cells HCT116	Wang *et al*[154]
	TCTP, HMGB1/RAGE, NF-kB		Invasion and metastasis	TS; human colorectal AM; tumor xenografts BALB/c nude mice CL; human colon adenocarcinoma cells LoVo	Huang *et al*[155]
	S100A9/RAGE/TLR4	Arg-1, iNOS, IL-10 and ROS	Immune suppression and MDSC chemotaxis	TS; human colorectal cancer and normal colon CL; Human colorectal cells LoVo, and MDSCs	Huang *et al*[156]
	HMGB1/RAGE, Kras/Yap1		Cell proliferation	CL; human colorectal cancer cells HCT116 and SW480	Qian *et al*[157]
	S100B/RAGE, p38/pAkt/mTOR	VEGF-R2, iNOS, VEGF	Cell proliferation, migration, invasion, and angiogenesis	CL; human colon adenocarcinoma cells CaCo	Seguella *et al*[158]
	HMGB1/RAGE, pERK1/2, pDRP1		Cell viability, autophagy, and chemoresistance	TS; human colorectal AM; athymic nude BALBC/c mice CL; human colorectal cells SW480, SW620, and LoVo	Huang *et al*[159]
Hepatocellular carcinoma	S100A9-TLR4/RAGE-ROS,	NET	Cell proliferation, invasion, and metastasis	TS; HBV+ and HBV- hepatocellular carcinoma AM; BALB/c mice and C57BL/6 mice CL; human liver cells QSG-7701, human hepatocellular carcinoma cells HepG2.2.15, mouse hepatocellular carcinoma cells H22 and HUVEC cells	Zhan *et al*[160]
	HMGB1/RAGE		Cell proliferation and tumor differentiation	TS; primary hepatocellular carcinoma	Ando *et al*[161]
	HMGB1/RAGE, ATG7		Cell proliferation, fibrosis, and autophagy	TS; mouse hepatocellular carcinoma AM; Atg7, RAGE, HMGB1 transgenic C57BL/6Jmouse	Khambu *et al*[128]
	HMGB1/RAGE, JNK, OCT4/TGFb1	miR-21, CD44	Migration and invasion	TS; human hepatocellular carcinoma AM; BALB/c nu/nu mice CL; human hepatocellular carcinoma cells HepG2, HCCLM3, Huh7, SMMC7721 and MHCC97H	Li *et al*[162]

	S100A4/RAGE, b-catenin	OCT4, SOX2, CD44 and Nanog (stem cell-associated genes)	Fibrosis and carcinogenesis	TS; human hepatocellular carcinoma AM; S100a4-EGFP, S100A4$^{+/+GFP}$, S100A4$^{-/-}$ transgenic mice. CL; human hepatocellular carcinoma cells Huh7 and murine liver cancer cells Hep1-6	Li *et al*[163]
	HMGB1/RAGE, ERK1/2	CXCL2, IL-8, TNF, IL-6, IL-10, IL-23-p19	Macrophage activation and inflammation	AM; primary murine hepatocytes from male C57BI/6J mice, and primary murine splenocytes from male C57BI/CJ CC; murine hepatoma cells Hepa1-6 and Hep-56.1D, human hepatoma cells HepG2, RAW 264.7 macrophages and monocytic cells THP1	Bachmann *et al*[164]
	HMGB1/RAGE, NF-kB	circRNA 101368, miR-200a	Cell migration	TS; human hepatocellular carcinoma CL; human hepatocellular carcinoma cells HCCLM3, MHCC97L, SMMC7721, Hep3B, HepG2 cells, and normal hepatocyte cells THLE-3	Li *et al*[165]
Pancreatic cancer	RAGE	NET	Neutrophil autophagy	TS; human pancreatic carcinoma AM; Wild type C57BL6 mice and RAGE$^{-/-}$ C57BL6 mice, orthotopic pancreatic cancer model, CL; murine pancreatic cancer cells Panc02, MDSCs cells	Boone *et al*[166]
	RAGE, PI3K/AKT/mTOR		Cell viability	CL; human pancreatic cancer cells MIA Paca-2, BxPC-3, AsPC-1, HPAC, PANC-1, MIA Paca^{GEMR}	Lan *et al*[167]
	RAGE, ERK1/2/Akt	Alpha 2 and alpha 1 integrin downregulation	Cell proliferation, invasion, and migration	CL; human pancreatic cancer cells Panc-1	Swami *et al*[129]
	AGE/RAGE, TGFb1	a-SMA, collagen 1, IL-6	Fibrosis and EMT	TS; human pancreatic ductal adenocarcinoma AM; WT-C57BL/6 and RG-C57BL/6 mice CL; primary PSC, human pancreatic ductal adenocarcinoma cells BxPC-3 and AsPC-1	Uchida *et al*[168]
	HMGB1/RAGE, PI3K/Akt	Atg5, Beclin-1, LC3-II	Autophagia and apoptosis inhibition	CL; human pancreatic cancer cells MIA Paca-2 and MIA Paca^{GEMR}	Chen *et al*[169]

AGE: Advanced glycation end products; Akt: Protein kinase B; AM: Animal model; Arg-1: Arginase-1; ATG7: Autophagy related 7; CAFs: Cancer-associated fibroblasts; CCL2: CC-chemokine ligand 2; circRNA: Circular RNA; CL: Cell line; cPLA: Cytosolic phospholipase A2; CREB: cAMP response element-binding protein; CTGF: Connective tissue growth factor; CXCL: CXC motif chemokine ligand; CXCR: C-X-C Chemokine receptor; Cyr61: Cysteine-rich angiogenic 61; DRG: Dorsal rat ganglion; pDRP1: Phosphorylated dynamin-related protein 1; EMT: Epithelial-mesenchymal transition; ERK 1/2: Extracellular signal regulated kinase 1/2; FAK: Focal adhesion kinase; FLNA: Filamin A alpha; HIF-1α: Hypoxia-inducible factor-1 alpha; HBV: Hepatitis B virus; Gas6: Growth arrest-specific gene 6; HMG: High mobility group; acHMGB1: Acetylated high mobility group B1; HUVECs: Human umbilical vein endothelial cells; IGF-1: Insulin-like growth factor-1; JNK: Jun N-terminal kinase; KLF5: Kruppel-like factor 5; LPS: Lipopolysaccharide; MDA-MB-231 P: MDA-MB-231 Parental cells; MDA-MB-231 BM: MDA-MB-231 bone marrow; MDM: Mouse double minute 2 homolog; MDSCs: Myeloid-derived suppressor cells; MIA Paca^{GEMR}: MIA Paca gemcitabine resistant; MSCs: Mesenchymal stem cells; mTOR: Mammalian target of rapamycin; NET: Neutrophil extracellular traps; NF-kB: Nuclear factor-kappa B; iNOS: Inducible nitric oxide synthase; NTNBC: Non-triple-negative breast cancer; OCT-4: Octamer-binding transcription factor 4; PI3K: Phosphoinositide 3-kinase; PD-L1: Programmed death ligand 1; PGE2: Prostaglandin E2; PSC: Pancreatic stellate cell; Ras: Rat sarcoma virus; RB: Retinoblastoma; RAGE: Receptor for advanced glycation end products; S100: Soluble 100% protein; a-SMA: Alpha-smooth muscle actin; SOX2: SRY (sex determining region Y)-box 2; STAT3: Signal transducer and activator of transcription 3; TAMs: Tumor-associated macrophages; TCTP: Translationally controlled tumor protein; TGFb1: Tumor growth factor beta 1; THP1: Human monocytic cell line derived from acute monocytic leukemia; TLR: Toll-like receptor; TNBC: Triple-negative breast cancer; TNF: Tumor necrosis factor; TS: Tissue sample; VEGF: Vascular endothelial growth factor; Yap1: Yes associated protein 1.

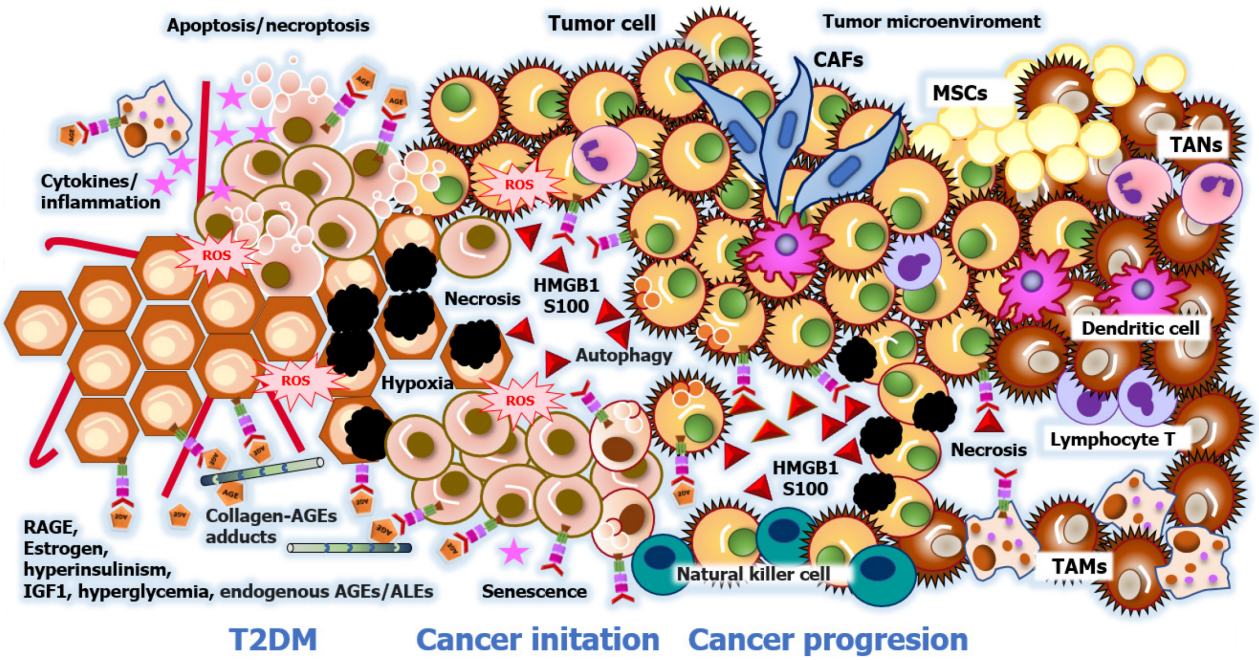

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Figure 2 Tumor microenvironment in type 2 diabetes mellitus. In type 2 diabetes mellitus patients, elevated estrogen levels, hyperinsulinemia, insulin-like growth factor-1 levels, hyperglycemia, endogenous advanced glycosylation end products (AGEs), and advanced lipoperoxidation end products (ALEs) promote cancer initiation and progression in the tumor microenvironment (TME). Receptor for advanced glycation products (RAGE) plays an essential role in the TME by promoting inflammation, oxidative stress, endotoxin clearance, senescence, and programmed cell death by binding to endogenous AGE/ALE ligands and damage-associated molecular patterns, primarily the high mobility group box 1 proteins and S100 proteins. To overcome a hypoxic and acidic microenvironment, tumor cells coordinate a metabolic program (Warburg effect), cell survival (senescence and cell death program), angiogenesis, extracellular matrix remodeling, proliferation, invasion, and metastasis. Tumor cells interact with resident immune cells and recruit mesenchymal stromal cells, cancer-associated fibroblasts, tumor-associated macrophages, tumor-associated neutrophils. RAGE: Receptor for advanced glycation products; ROS: Reactive oxygen species; T2DM: Type 2 diabetes mellitus patients; IGF-1: Insulin-like growth factor-1; AGEs: Advanced glycosylation end products; ALEs: Advanced lipoperoxidation end products; HMGB1: High mobility group box 1 proteins; MSCs: Mesenchymal stromal cells; CAFs: Cancer-associated fibroblasts; TAMs: Tumor-associated macrophages; TANs: Tumor-associated neutrophils.

chemistry as follows: Hepatocarcinoma at 50%; pancreatic cancer at 33.3%; breast cancer at 25%; endometrial cancer at 16.6%; and colorectal cancer at 8.3%[136].

CONCLUSION

RAGE is an environmental sensor with complex and multiple functions involved in every stage along the patho-physiological pathways that lead to the progression of obesity, T2DM, and cancer. Therefore, it is crucial to analyze each of the processes that RAGE is involved in, as the assimilation of this information could help in developing more accurate diagnostic and treatment approaches. For instance, this review has highlighted how RAGE acts from the earliest stages of the initiation and development of obesity, T2DM, and cancer. Recognizing all participating RAGE isoforms in their tissue and cellular locations could predict the progression points and provide diagnostic markers. In this manner, we would also be able to distinguish between a patient who is obese, has a low grade of inflammation, and is on the frontline of developing T2DM or most likely to respond to nutritional intervention.

On the other hand, RAGE participates in the initiation of neoplastic processes. Since its presence indicates cellular senescence and the presence of cancer cells with more aggressive activity, it is not surprising related to a poor prognosis and has potential as a cancer biomarker to help predict patient outcomes. Since RAGE participates even in the first stages, it has potential as a preventive and immunomodulator for therapeutic purposes to reduce morbidity and mortality associated with the development of obesity, T2DM, and cancer. Inhibitors of RAGE may be helpful in the treatment of obesity and diabetes mellitus. Studies have shown that RAGE is overexpressed in AT. Obesity is well known to contribute to inflammation and insulin resistance, which are hallmarks of obesity and diabetes. RAGE inhibitors could reduce inflammation and improve insulin sensitivity in obesity and T2DM; however, the majority of RAGE inhibitor studies have focused on cancer treatment. Some RAGE inhibitors under study are cromolyn, RAP, RAGE peptide antagonist, and gefitinib. While there are currently no RAGE-specific therapies approved for use in humans, there are pre-clinical studies investigating the potential of RAGE inhibitors as a treatment for various diseases. We review herein the topically relevant literature, delimiting by process, organ, and tissue to provide a progressive and systemic overview. It should be read and generalized with caution, as there are still many gaps in the knowledge about RAGE since most studies are experimental-based (in mice) and cross-sectional studies (in humans).

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REVIEW

Advanced glycation end product signaling and metabolic complications: Dietary approach

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Abstract

Advanced glycation end products (AGEs) are a heterogeneous collection of compounds formed during industrial processing and home cooking through a sequence of nonenzymatic glycation reactions. The modern western diet is full of heat-treated foods that contribute to AGE intake. Foods high in AGEs in the contemporary diet include processed cereal products. Due to industrialization and marketing strategies, restaurant meals are modified rather than being traditionally or conventionally cooked. Fried, grilled, baked, and boiled foods have the greatest AGE levels. Higher AGE-content foods include dry nuts, roasted walnuts, sunflower seeds, fried chicken, bacon, and beef. Animal proteins and processed plant foods contain furosine, acrylamide, heterocyclic amines, and 5-hydroxymethylfurfural. Furosine (2-furoil-methyl-lysine) is an amino acid found in cooked meat products and other processed foods. High concentrations of carboxymethyl-lysine, carboxyethyl-lysine, and methylglyoxal-O are found in heat-treated nonvegetarian foods, peanut butter, and cereal items. Increased plasma levels of AGEs, which are harmful chemicals that lead to age-related diseases and physiological aging, diabetes, and autoimmune/inflammatory rheumatic diseases such as systemic lupus erythematosus and rheumatoid arthritis. AGEs in the pathophysiology of metabolic diseases have been linked to

individuals with diabetes mellitus who have peripheral nerves with high amounts of AGEs and diabetes has been linked to increased myelin glycation. Insulin resistance and hyperglycemia can impact numerous human tissues and organs, leading to long-term difficulties in a number of systems and organs, including the cardiovascular system. Plasma AGE levels are linked to all-cause mortality in individuals with diabetes who have fatal or nonfatal coronary artery disease, such as ventricular dysfunction. High levels of tissue AGEs are independently associated with cardiac systolic dysfunction in diabetic patients with heart failure compared with diabetic patients without heart failure. It is widely recognized that AGEs and oxidative stress play a key role in the cardiovascular complications of diabetes because they both influence and are impacted by oxidative stress. All chronic illnesses involve protein, lipid, or nucleic acid modifications including crosslinked and nondegradable aggregates known as AGEs. Endogenous AGE formation or dietary AGE uptake can result in additional protein modifications and stimulation of several inflammatory signaling pathways. Many of these systems, however, require additional explanation because they are not entirely obvious. This review summarizes the current evidence regarding dietary sources of AGEs and metabolism-related complications associated with AGEs.

Key Words: Advanced glycation end products; Receptor for advanced glycation end products; Heat-treated diets; Food safety; Maillard reaction products; Metabolic disorder; Diabetes; Cardiac complication

Core Tip: All chronic illnesses involve protein, lipid, or nucleic acid modifications, including crosslinked and nondegradable aggregates known as advanced-glycation end products (AGEs). Endogenous AGE formation or dietary AGE uptake can result in additional protein modifications and stimulation of several inflammatory signaling pathways. Many of these systems, however, require additional explanation because they are not entirely obvious. This review summarizes the current evidence regarding dietary sources of AGEs and metabolism related complications associated with AGEs.

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INTRODUCTION

Advanced-glycation end products (AGEs) are heterogeneous compounds formed when glucose or other saccharides posttranslationally alter macromolecules such as proteins, lipids, and nucleic acids without the use of enzymes (fructose and pentose). Age-related illnesses and physiological aging are associated with higher plasma amounts of AGEs, which are toxic chemicals[1,2], causing diabetes mellitus (DM)[3], and autoimmune/inflammatory rheumatic diseases including systemic lupus erythematosus[4], rheumatoid arthritis[5], systemic sclerosis[6], and psoriasis[7]. More than 20 different AGEs have been discovered in dietary items, human blood, and tissues. These AGEs can be arbitrarily classified as fluorescent or nonfluorescent[8]. The three nonfluorescent substances that are most significant are pyrraline, carboxymethyl-lysine (CML), and carboxyethyl-lysine (CEL)[9]. The two fluorescent AGEs of most significance are pentosidine and methylglyoxal-lysine dimer (MOLD)[10]. The presence of lysine residue in the molecules serves as the primary distinguishing property of AGEs. The AGEs are discharged from the kidneys after being catabolized in renal proximal tubular cells on a metabolic level[11]. AGE formation after binding with AGE receptor (RAGE) can result in metabolic burdens such as hyperglycemia, hyperlipidemia, oxidative stress, inflammatory responses, and endothelial dysfunction [12]. AGE formation may be accelerated by a number of environmental factors such as sedentary lifestyles, high-carbohydrate and high-calorie diets, food cooked at high temperatures, and cigarette smoke[13]. Dietary AGE concentrations in a variety of commercial cow-based, goat-based, and soy-based infant formulas were measured using ultra-performance liquid chromatography-mass spectrometry, the degree of protein glycation in infant formulas is determined by the protein source, protein composition, and the number and type of carbohydrates. The soy-based formula studied contained significantly more arginine and arginine-derived dietary AGEs (dAGEs) than the cow- and goat-based formulas. The concentrations of dAGEs in infant formula with hydrolyzed proteins were higher than those in infant formula containing intact proteins, and lactose-containing formula was more susceptible to glycation than sucrose- and maltodextrin-containing formula[14]. Bakery products, with respect to their formation during baking, generate AGE content and have health effects. Phenolic components added to the formulation in bakery products greatly decrease the formation of AGEs; among these, ferulic acid showed the most significant lowering effect on AGEs. Dihydromyricetin outperformed the flavanones evaluated in the model cookie system in terms of AGE reduction. Furthermore, the addition of components that reduce water activity, such as dietary fiber, and the high temperature used in baking both enhance the formation of AGEs and the addition of fat, sugar, and protein-rich ingredients to bakery product formulations usually increases the AGE content. As a result, the food industry should concentrate on optimizing food production to reduce

AGE formation while maintaining bakery product safety and organoleptic properties[15]. In light of this, AGEs may establish a clear connection between modern nutrition and health[16].

Among the various AGEs receptors presently identified, RAGE is a critical receptor for AGEs to exert the main mechanism of cells and new pattern recognition receptor RAGE is a one of the members of the immunoglobulin superfamily. Numerous cells, including macrophages, mesangial cells, and endothelial cells, have RAGE receptors expressed on their surfaces[17], which can join forces with AGEs to create the AGE-RAGE axis, which activates intracellular signaling pathways and starts a chain of intracellular events.

The AGE-RAGE interaction has been demonstrated in experimental investigations to alter cell signaling, stimulate gene expression, generate oxidative stress, and cause the release of proinflammatory chemicals[18]. RAGE expression levels are extremely low in healthy individuals, but when the body's cells are stimulated or under stress, RAGE expression levels in damaged cells are markedly elevated. In light of this, RAGE is crucial for understanding how numerous diseases progress, including diabetes, Alzheimer's disease, vascular damage, and tumors. RAGE can also identify a variety of ligands, including some endogenous ligands like S100/calgranulins and high mobility group box-1[19], which interact with RAGE after being released by injured cells, activating some signaling pathways to enhance tissue damage and inflammation[20]. Nuclear factor-light-chain enhancer of activated B cells, also known as NF-κB, is translocated into the nucleus as a result of RAGE activation, which upregulates RAGE expression in a hyperglycemic environment[21].

MAILLARD REACTION AND AGE FORMATION

To create AGEs, the Maillard reaction (MR) proceeds through a series of processes. The primary regulators of AGEs formation including glycation of cellular and tissue proteins, are the rate of protein turnover, degree of hyperglycemia, and degree of oxidative stress[22]. The next sections explain the three stages of AGEs development *in vivo* (Figure 1).

Initial phase

The carbonyl group of reducing sugars such as glucose, fructose, or ribose reacts with the amino groups of proteins, primarily lysine and arginine residues, to create a Schiff base, which can also be formed *via* the polyol route. This unstable Schiff base is further modified to produce more stable ketoamines known as amadori products (APs), which can create free radicals and irreversible crosslinks with proteins and peptides. However, APs are still reversible, dependent on the minimal substrate concentration and time[23].

Proliferation phase

Glyoxal (GO), methylglyoxal (MG), and 3-deoxyglucosone (3-DG) are examples of AP that undergo additional chemical rearrangements in the presence of transitional metal ions to form active carbonyl intermediate groups known as dicarbonyls, which are precursors for the production of AGEs at an advanced stage. The previous phase's glucose, fructose, and Schiff base can also be transformed and stored into dicarbonyls, which are known as "carbonyl stress," and which have a propensity to react with amino and sulfhydryl groups of proteins to cause browning and crosslinking[24, 25].

Advanced phase

Dicarbonyls are eventually directly rearranged with AP and proteins as a result of multiple chemical modifications such as oxidation, nonoxidation, hydration, dehydration, glycation, glycosylation, fructosylation, and acid hydrolysis to create stable, irreversible AGEs such as DOLD, GOLD, MG-derived imidazolium crosslinking, and 3-deoxygluco. Table 1 depicts the key characteristics, sources, modes of production, and pathophysiology of the various forms of AGEs.

LITERATURE REVIEW

Strategy of article selection

The present narrative review of the literature was performed based on the data search from PubMed, Google Scholar, Scopus, The National Library of Medicine database, and Web of Science, at the beginning of 2023 focusing on keywords on AGEs, AGE generation, pathways, foods containing AGEs, and food sources for AGEs. The entire articles were screened for duplicate information and removed sequentially.

Search terms, keywords, and data extraction

Research retrieved information from various reputed biomedical reports/articles published until 2023. The information from prestigious journals using keywords such as AGEs, AGE production through sequential pathways, food items containing AGEs, and country data on AGEs was systematically compiled into tables and presented as narrative review. Based on the scientific search engine, the articles were screened for relevant information available in AGEs research and review articles, which were compiled into tables and figures and presented in the current review article.

Table 1 Advanced glycation end products content in carbohydrates rich food ready to eat food products and country origin

Type of AGEs	Country	Food products	AGE level	Ref.
Acrylamide	Poland	French fries	63-2175 μg/kg	[29]
		Potato chips	113-3647	
		Crispbread	65-1271 μg/kg	
		Crackers	566-2017 μg/kg	
		Daily Bread	35-110 μg/kg	
	United States	Biscuits	5-1796 μg/kg	[30]
	India	Potato chips	1456.5 μg/kg	[31]
		Biscuits bakery	126-665 μg/kg	[32]
		French fries	825.96-1143.15	
	Saudi Arabia	Biscuits	90-182 μg/kg	[33]
		Chocolate pies	439 μg/kg	
Furan	Brazil	Biscuits	38.1-105.3 μg/kg	[34]
	Belgium	Jarred baby food	61.7 μg/kg	[35]
	Spain	Vegetable-based baby food	10.9 to 143.0 μg/kg	[36]
		Fruit-based baby food	7.7 to 32.1	
	Germany	Ready to drink coffee	2-108 μg/kg	[37]
	Denmark	Instant coffee powder	39-1330 μg/kg	[38]
		Dried fruits	387 μg/kg	
HMF	Malaysia	Stored honey	118.47-1139.95 mg/kg	[39]
	Bangaldesh	Stored honey	3-703 mg/kg	[40]
	Turkey	Traditionally coffee	213-239 mg/kg	[41]
	Brazil	Corn syrup	406-2121 mg/kg	[42]
		Cane syrup	109-893 mg/kg	
	Polish Market	Roasted coffee	348 mg/kg	[43]
		Instant coffee	3351 mg/kg	
		Fruit juices	1-110 mg/L	
		Cola-carbonated drinks	2-40 mg/L	
	Syria	Instant coffee	526-1800 mg/kg	[44]
GO	Italy	Sugar cookies	362 mg/kg	[45]
	Spain	Commercial cookies	4.8-26.0 mg/kg	[46]
	Netherlands	Apple molasses	0.01-37.00 mg/kg	[47]
MGO	Italy	Sugar cookies	293.0 mg/kg	[45]
	Turkey	Dried apricots	20-41 mg/kg	[48]
	Netherlands	Dutch spiced cake, rusk, apple molasses	0.04-736.00 mg/kg	[47]
	Spain	Commercial cookies	3.7-81.4 mg/kg	[46]
Furosine	Denmark	Standard infant formula	1700-2800 mg/kg P	[49]
	Netherland	Standard infant formula	4719-6394 mg/kg P	[50]
	China	Charcoal-flavored milk	593.2 mg/100 g protein	[51]
		Branded fermented milk	25.40-1661.05 mg/100 g P	[52]
		Posturized milk	12.58-61.80 mg/100 g P	[53]
		Raw milk	8.85 mg/100 g P	

CML	United States	Fried beef	20.03 mg/100 g P	[54]
		Baked beef	14.31 mg/100 g P	
		Fried chicken breast	17.17 mg/100 g P	
		Baked chicken breast	13.58 mg/100 g P	
	China	Ground beef	3.00-19.96 mg/100 g P	[55]
		Fish	0.66-2.00 mg/100 g P	[56]
		Sea food (dry)	44.8-439.0 mg/100 g P	[57]
		Canned saury fishes	250-1608 mg/100 g P	[58]
CEL	China	Fish	3.08 mg/100 g P	[56]
		Canned saury fishes	721-3653 mg/100 g P	[58]

AGEs: Advanced glycation end products; CEL: N'-(1-carboxyethyl)lysine; CML: Carboxymethyl-lysine; GO: Glyoxal; HMF: 5-Hydroxymethylfurfural; MGO: Methyl glyoxal; MOLD: Methylglyoxal-lysine dimer.

DIETARY AGES IN DAILY FOOD PRODUCTS

People are modifying restaurant meals rather than traditional/conventionally cooked due to industrialization and marketing methods (Table 1). Nonvegetarian food contains more dietary AGEs than vegetarian food. Age level is directly influenced by cooking temperature and time[26]. The foods with the highest AGEs are those that are fried, barbecued, baked, or boiled[27]. Dry-heat processed foods like crackers, chips, and cookies have the highest AGEs level per gram of food in this group. This is most likely due to the addition of components such as butter, oil, cheese, eggs, and nuts, which significantly enhance AGE formation during dry-heat processing[28] (Table 1)[29-58].

Acrylamide

Worldwide, potatoes are a sustainable dietary alternative and source of energy from carbohydrates for all age groups. This root food is readily available all year, and boiling potatoes makes MR products more likely to occur[59]. Nonenzymatically, the reducing sugars glucose and fructose react with asparagine to make N-glucoside, which then produces melanoidin and the end product of the Schiff base reaction, which is decarboxylated to form acrylamide (ACR) [60]. Bread, coffee, fried potatoes, baked goods, and bread are the main sources of ACR[61], and browning increases its concentration[62]. The highest ACR production in diverse foods occurs at 120 °C[63]. Products made from cereal, coffee, and cocoa beans include 3-aminopropionamide[64] subsequently transformed into ACR in an aqueous MR[65]. Due to the fact that the MR occurs at the bread's surface, the ACR concentration is higher in the crust and lower in the crumb. Similar to this, fried chips with a double layer of chips create a large amount of ACR[66]. Fried potatoes can expose you to an estimated 272-570 g/kg ACR, as can bread goods (75-1044 g/kg) and breakfast cereals (149 g/kg)[67].

Furan

Furan has a planar enol-carbonyl structure, a cyclic dicarbonyl structure, and a caramel-like scent due to the MR[68]. It is created through a number of processes, including thermal deterioration, oxidation of polyunsaturated fatty acids, and the MR, which is the thermal rearrangement of carbohydrates in the presence of amino acids[69]. Acetaldehyde and glycolaldehyde are produced through the breakdown of serine and cysteine amino acids, and the addition of an aldol group allows for the production of furans[70]. Numerous chemical processes, such as the Strecker reaction and the oxidation of polyunsaturated fatty acids, take place during the heat processing of food[71]. High concentrations of furan are directly correlated with higher cooking temperatures (150-200 °C), yet some furan is vaporized when cooking in an open pan[72]. Reports from the Fromberg *et al*[73] study in open vessel cooking, furan is reduced by 50%, and chocolate has a low concentration. Furans provide meals with a variety of flavors and aromas including sweet, fruity, nutty, meaty, and burnt. In the course of manufacturing infant foods, cereal, coffee, preserved foods, meat, and fish, furan, and its derivatives are created[74]. Studies have shown that coffee is one of the most widely consumed nonalcoholic beverages, with little negative effect[75]. The processing of coffee and its products is thought to contribute the largest furan concentration, followed by baked cookies, bread, and chips. Furan levels are also high in packaged and bottled meals[76]. Due to variation in macronutrient ratios and processing methods, furan concentration in foods for infants varies. Infant meals with a meat foundation as opposed to ones with mixed fruits contain higher levels of furan[74].

Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is produced by the 1,2-enolation reaction in a mild alkaline medium, and HMF (6-carbon hetrocyclic aldehyde) is the main intermediate product of the Amadori rearrangement[77]. HMF is created *via* a variety of processes, including the thermal breakdown of sugars and interactions with other intermediates[78]. Under acidic conditions, disaccharide (sucrose) mostly degrades to glucose and fructose, which are then enolized and dried out to produce fructofuranosyl. Furthermore, at high temperatures, this cation changes to HMF[79]. Alternatively, the carbonyl group of reducing sugars such as maltose or glucose can join with lysine or another amino acid as a precursor. As a result,

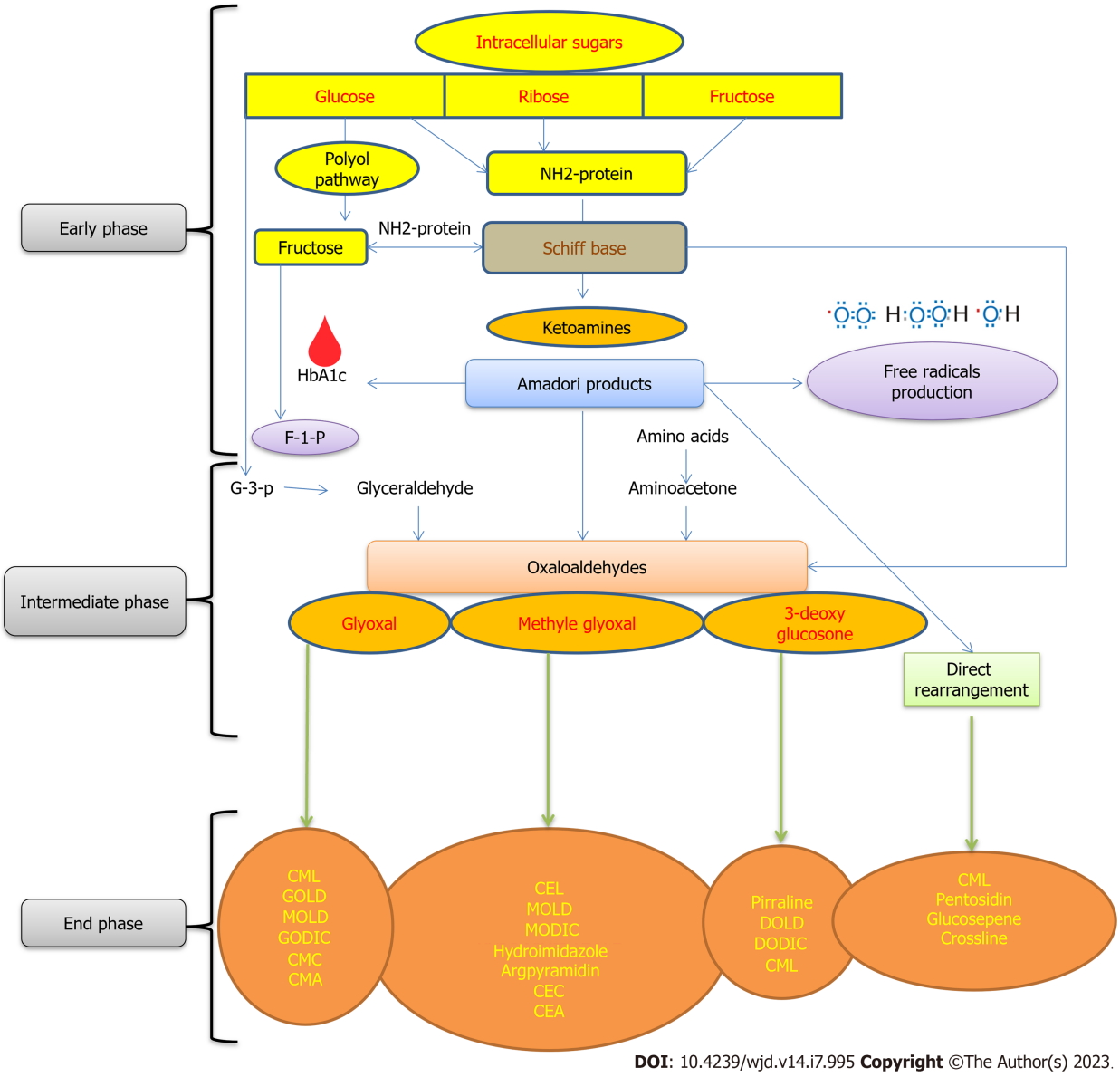

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Figure 1 Factors involved in accelerating advanced glycation end product formation and accumulation in the body. CEA: N7–(1-carboxyethyl)arginine; CEC: Carboxyethyl cysteine; CEL: N'-(1-carboxyethyl)lysine; CML: Carboxymethyl-lysine; MODIC: 2-ammonio-6-({2-[4-ammonio-5-oxido-5-oxopently)amino]4-methyl-4,5-dihydro-1H imidazol-5 ylidene}amino)hexanoate; MOLD: Methylglyoxal-lysine dimer; DODIC: N6-{2-{[(4S)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-[(2S,3R)-2,3,4-trihydroxybutyl]- 3,5-dihydro-4H-imidazol-4-ylidene}-L-lysinate; DOLD: 1,3-, di(N'-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium.

under controlled heat conditions, a sugar pyrolysis reaction occurs, forming browning and HMF[80]. Within a pH-controlled environment, the polymerization of HMF with a food product containing nitrogen results in melanoidins, which give the surface a brown color[81]. The presence of HMF in honey is utilized as a quality and freshness index indication. In honey and fruit juices, the level of thermal breakdown of sugar that results in the production of hazardous metabolites may also be clearly seen. Samples of honey from 29 different nations were shown to be directly correlated with storage temperature, time, and HMF content levels. According to country-specific honey samples, Malaysian honey had a value of 1132 mg/kg after more than 2 years of storage at 30 °C, Turkey's honey had a value of 0.0-11.5 mg/kg, and India's honey had a value of 0.15-1.70 mg/kg[82]. Other parameters that affect HMF levels, such as pH, water activity, kind of sugar, mineral content, and its origin, were explored by Kamboj *et al*[83]. Fructose-rich high fructose corn syrup (HFCS) is used in a variety of beverages and drinks. According to a study, fresh HFCS syrup has modest levels of HMF, which rise with temperature and storage time[84]. Similar findings have revealed that HMF level increases eight times in highly acidic media heated to 110 °C for 40 min[85] HMF levels in apple juice range from 0.06 mg/L to 18.12 mg/L as a result of heat exposure[86]. Date Syrup: Fresh 1000-2675 mg/kg and Industrial 12-456 mg/kg[87], Malaysian tropical fruit juices range 0.08-91.50 mg/L[88]. Towards the end of the baking process, volatile chemicals are formed. Longer periods of higher temperatures can lead to increased HMF production[89] and in biscuits and cookies, many aromas such as "bready," "almond," "pungent," and "sweet" form[90]. A recent study found that breakfast cereal has an HMF content of 13.3 mg/kg, but ultra-processed cereal has a content of 32.1 mg/kg. The HMF level of all cereals ranges from 0.3 mg/kg to 159.6 mg/kg. Due to the addition of sugar, refined wheat flakes have an elevated level of 159.6 mg/kg[91].

GO and methyl-GO formation in food products

While ketose (fructose) creates an equivalent Heyns compound, simple sugars such as glucose form an amadori intermediate (1-amino-1-deoxi-2-ketose) by losing a water molecule. Amadouri or Heyns compound breakdown then produces dicarbonyl intermediates[92]. Reactive dicarbonyl structures are created as intermediates during the MR as a result of a series of chemical events including isomerization, dehydration, fragmentation, and redox reactions. These compounds have an affinity to react with the side chains of the amino groups' lysine and arginine, producing stable protein adducts. Recent molecular structure investigations have shown that the amino acids arginine and lysine react with the molecules GO, methyl-GO (MGO), and 3-DG to form a number of crosslinkages[93]. Dehydration of hexose sugar produces 3-DG, and fragmentation of intermediate MR products produces 2,3-butanedione, GO, and MGO. On the other hand, these chemicals have also developed as a byproduct of the breakdown of lipids[94]. Carbonyl synthesis by lipid oxidation is supported by the advanced lipoxidation end products (ALE) process[95]. Group I chemicals as a result of lipid peroxidation include the following: acrolein, 4-hydroxy-2-nonenal, 4-hydroxy-hexenal, and 4-hydroxy-nonenal are examples of unsaturated aldehydes. Group (1): di-aldehydes include malondialdehyde and GO compounds. Group (3): cheto-aldehydes include MGO, 4-oxo-nonenal, and levuglandins[92]. By using a lipidomic technique, 35 aldehydes and ketones have so far been isolated from various fatty acid-rich sources. The researcher also highlighted how the oxidation of oleic acid and eicosapentaenoic acid helps to produce GO[96]. Depending on the manner of cooking and the type of processing used, these MR products alter the texture and flavor of food[97]. The production of MR intermediates is affected by caramelization and heat processing. Dicarbonyl concentration rises during baking in foods high in sugar and low in moisture. Cookies have been shown to have varying concentrations of 3-DG, GO, and MGO[98]. Early on, coffee roasting rises[99]. Due to nonenzymatic browning and fermentation, GO is primarily found in soybean paste, soy sauce, alcoholic beverages, and fermented coffee[100]. However, in both vegetarian and nonvegetarian food preparation, MGO production occurs during glycolysis[101].

Furosine

Early-stage MR products such as furosine bind to proteins that contain N-substituted 1-amino-1-deoxy-2-ketose, including fructose-lysine, lactulose-lysine, and maltose-lysine[102]. The N-substituted 1-amino-1-deoxy-2-ketose found in proteins such as fructose-lysine, lactulose-lysine, and maltose-lysine is bound by the early-stage MR product furosine. Degradation of the Amodori product results in formation of the dicarbonyl molecule, which either interacts with free amino acids to produce Strecker aldehydes or with amino groups of amino acids, peptides, and proteins to rearrange and produce AGEs. An amine group on a protein or peptide combines with a reducing sugar to produce various aromatic compounds and melanoidins, which are crosslinked proteins. This process is known as the dehydroalanine route[103]. Lactulosyl-lysine, a protein-bound AP, is the first stable chemical created during milk's MR process, and furosine is created following acid digestion. The primary causes of lysine blockage are temperature, time, and length of storage. Ten percent in ultra-high temperature (UHT) milk, 15% in sterilized container milk, and 25%-30% in newborn formula make up the percentage of lysine that is unavailable[104]. Dairy products' nutritional value is evaluated by their low furosin content. In their analysis of the furosine content of several heat-treated milk samples, Shi *et al*[51] found that charcoal-flavored fermented milk had the highest concentration, followed by flavored fermented milk, and low temperature (LT) pasteurized fresh milk had the lowest concentration of furosine. In hydrolyzed dairy samples, Montilla *et al*[105] estimated that furosine level ranged from 235-820 mg/100 g protein and increased by up to 90% after 4 mo of storage at 20 °C. Boitz and Mayer[106] calculated the amount of furosine in whipping cream for retailed pasteurized, extended shelf life, and UHT cream samples were 47.8 mg ± 14.0 mg, 72.2 mg ± 36.6 mg, and 172.5 mg ± 17.7 mg in $100g^{-1}$ protein. The amounts of furosine in soy and whey hydrolyzed protein-based infant formula were 379 mg/100 g and 1459 mg/100 g, respectively. Similar to the subsequent formula and other partially hydrolyzed milk formulas, casein makes up 945 mg/100 g of the protein in the latter[107]. Due to its higher lactose content than other dairy foods, infant food is more likely to include furosin. According to Lund *et al*[107], whey protein concentrate (WPC) underwent a number of alterations to the protein as a result of thermal treatment. The time of storage also enhances the quantity of furosin in both types of (DI-IF and IN-IF) processing, and recently, the role of WPC has created more furosine than other whey protein ingredients[107]. Other authors have noted that various newborn formulas contain furosin concentrations ranging from 471.9 mg/100 g to 639.5 mg/100 g[108]. Another investigation examined the impact of drying heat on various pasta samples. Artisanal pasta had the lowest furosine level, ranging from 107 to 186 mg/100 g protein, as a result of the LT drying method[109]. Due to the usage of durum wheat flour and other chemical components, whole grain pasta has a furosine concentration ranging from 229 to 836 mg/100 g protein[110]. Gluten-free spaghetti contains lower furosin 19-134 mg/100 g protein in another study by Gasparre *et al*[111] than durum wheat pasta.

CML and CEL

Animal proteins and processed plant foods contain furosine, ACR, heterocyclic amines (HCAs), and HMF[112]. Specifically cooked beef products and other processed foods include the amino acid furosine (2-furoil methyl lysine)[113]. High concentrations of CML, CEL, and MG-O are found in heat-treated nonvegetarian foods, peanut butter, and cereal items[114]. Infant milk formula contains CML as well because of the milk proteins in it. Lysines and other amino acids are released more freely after hydrolysis[115]. According to the AGE database, processed canned meats and nuts have the highest AGE levels, whereas fruits, vegetables, and butter have the lowest levels[116] (Figure 2).

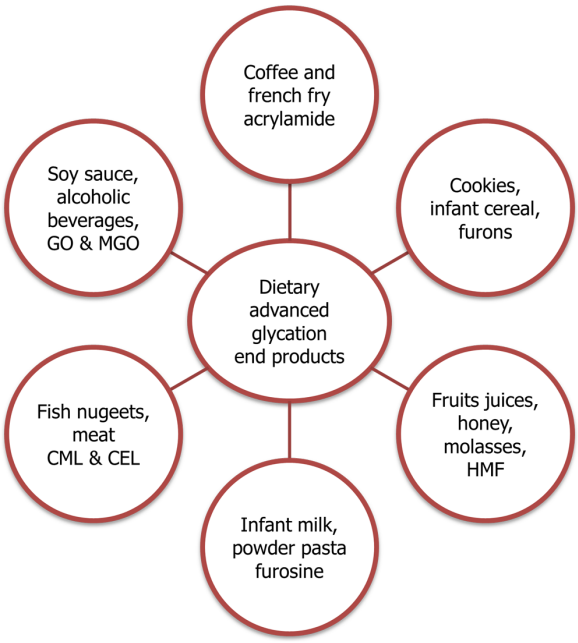

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Figure 2 Sources of dietary advanced glycation end products. CEL: Carboxyethyl-lysine; CML: Carboxymethyl-lysine; GO: Glyoxal; HMF: Hydroxymethylfurfural; MGO: Methylglyoxal.

FACTORS INVOLVED IN AGE$_s$ FORMATION

Several endogenous factors may hasten the generation of AGE in the body.

Hyperglycemia

AGE production and the stimulation of oxidative damage (OX) are two of hyperglycemia's main side effects [117]. Obese but healthy individuals could avoid the formation of AGEs and OX during metabolic stress by increasing the fractional excretion of AGEs *via* renal clearance. In particular, hyperglycemia induces excessive reactive oxygen species (ROS) production and OS, which in turn promotes the formation of AGEs, events eventually resulting in the development of insulin resistance, impaired insulin secretion, and endothelial dysfunction[118].

Aging, oxidative stress, and aging-related inflammatory disease

It is still unclear whether AGEs cause the aging process or aging process speeds up the buildup of AGEs[119]. In the natural progression of the aging process, some researchers have hypothesized that AGE production plays a critical role [117]. AGEs generate oxidative stress, and as a result, inflammatory and thrombogenic reactions *via* contact with RAGE, as well as metabolic changes[119]. Son *et al*[120] concluded that circulating glycotoxins are undoubtedly linked to oxidative stress and an inflammatory response that cause cell malfunction. They concluded that visceral fat was involved in the pathogenesis of inflammatory problems in the elderly. Biological aging, neuron related inflammatory illnesses, DM and its complications, bone-degenerative diseases, and renal disorders are all examples of AGE-related diseases[121]. The authors came to the conclusion that the common contributing factors to the inflammatory state in these noncommunicable chronic inflammatory disorders were AGE-RAGE signaling abnormalities.

Obesity

Obesity is typically linked to a higher risk of metabolic syndrome, which includes insulin-resistant type 2 DM, hypertension, fatty liver, and vascular problems due to the unnecessary production of adipokines by fat cells. Gaens *et al* [122] reported that obesity was associated with higher plasma and tissue levels of MGO, AGEs, and ALE surrogated by CML. Brix *et al*[123] showed that in patients with MO, soluble-form RAGE (sRAGE) levels were significantly lower than those in the nonobese group. But following bariatric surgery to lose weight, which stopped the AGE-mediated inflammatory process, sRAGE levels rose. Similarly, Sanchez *et al*[124] with an AGE reader, and skin autofluorescence (SAF) in the forearm to measure AGE buildup. It was found that SAF levels were higher in metabolic syndrome-affected MO patients than in nonobese people. SAF remained high following bariatric surgery until glycemic memory failed. Deo *et al* [125] examined how weight loss in overweight participants without diabetes affected their CML levels. After losing weight, CML readings dropped by 17%, but this was less beneficial in people with diabetes or prediabetes who were not overweight. These findings might imply that AGE formation and tissue accumulation in the body are influenced by both obesity and hyperglycemia.

Chronic renal insufficiency

Patients with uremia, whether or not they had diabetes, had significantly higher amounts of AGEs in their plasma[126]. Miyata *et al*[127] investigated the destiny of AGEs by administering pentosidine, a synthetic AGE, intravenously to rats. Pentosidine was found to be eliminated in urine after being filtered by the renal glomeruli, reabsorbed in the proximal renal tubules, and subjected to catabolic or metabolic changes. Later, Asano *et al*[128] studied the metabolism of protein-linked pentosidine using three cell lines: proximal tubular, distal tubular, and nonrenal, in contrast to the distal tubular and nonrenal cell lines, they showed that pentosidine was quickly found in the cytoplasm of the proximal renal tubular cell line. They came to the conclusion that renal proximal tubular cells were crucial for the elimination of plasma pentosidine. Adriamycin-induced chronic nephropathy in nondiabetic rats was directly associated with renal pentosidine buildup[129]. Chronic heart failure, cardiovascular illnesses, diabetes, neurological diseases, osteoarthritis, and nondiabetic atherosclerosis all developed together with AGE accumulation in chronic kidney disease[130]. A high-AGE diet may also increase the chance of developing chronic illnesses, including chronic kidney disease[131]. According to Inagi, this alleged "glycation stress" was discovered to be directly related to kidney aging[132].

Glyoxalase I deficiency

Reactive carbonyl compounds, which are pentosidine's precursors, are detoxicated by glyoxalase in a hemodialysis patient with uremia. By chance, the authors discovered that this patient's renal blood vessels (RBVs) had far higher plasma levels of pentosidine and CML than those of hemodialysis patients. Further analysis revealed that this patient's RBVs had very low glyoxalase activity. They came to the conclusion that high AGE levels in uremia patients were largely caused by glyoxalase I deficiency (GLO-I), which was unable to detoxify AGEs[133]. In addition, Shinohara *et al*[134] reported that the bovine endothelial cells that overexpress GLO-I reduce intracellular AGE production and stop hyperglycemia from causing an increase in macromolecular endocytosis in the circulation. Similarly, Brouwers *et al*[135] revealed that in mesangial cells taken from diabetic rats and mice, overexpression of GLO-I decreased hyperglycemia-induced AGE formation and oxidative stress. Furthermore, Kurz *et al*[136] demonstrated that glycation stress may be prevented from causing cell damage by reducing the hazardous levels of MGO, GO, and other AGEs. Xue *et al*[137] explored the molecular underpinnings of erythroid 2-related factor 2's transcriptional regulation of GLO-I. The team identified a defense mechanism against stress caused by decarbonyl glycation (MGO) in high glucose concentration, inflammation, cell aging, and senescence as a result. Recently, Garrido *et al*[138] reported that MGO-derived AGE buildup might be prevented by fatty acid production working with GLO-I to protect against glycation damage.

AGES AND METABOLIC DISORDERS

The fast rise in the consumption of foods and beverages with added sugar during the past three decades, in both industrialized and developing nations, has been linked to an increase in metabolic illnesses. The function of advanced glycation end products in the pathophysiology of metabolic illnesses associated with modern nutrition is a new area of research (AGEs) (Figure 3)[139].

Diabetes and related complications

In vivo AGE formation is dependent on particular intracellular and extracellular circumstances. The rate at which proteins are turned over, oxidative stress in the intra- or extracellular environment, and the degree of hyperglycemia are some of the elements that have been explored as contributing to the creation of AGEs[140]. After 1 wk of hyperglycemia, endothelial cells have been shown to produce considerably more intracellular AGEs. Additionally, the type of reducing sugar has an impact on how quickly AGEs form when combined with intracellular proteins, with glucose having the slowest reaction when compared to fructose, glyceraldehyde-3-phosphate, and glucose-6-phosphate[140]. Healthy aging individuals have been shown to accumulate AGEs in their blood and tissues, and this buildup is greater when their blood glucose levels are high. In addition, In situations of metabolic and vascular illnesses such DM, atherosclerosis, and renal disease, AGEs have been observed to be raised in human tissues, plasma, and urine[141]. Semba *et al*[142] showed that higher circulating AGEs were a reliable indicator of renal function in an older group. Study found that after 3 years and 6 years of follow-up, the estimated glomerular filtration rate (a measure of kidney function) at baseline and chronic kidney disease were independently related with a higher plasma content of CML[143] and results indicate that the overall population of older community-dwelling persons may be affected by the potential negative effects of AGEs on the kidney [143]. In a different investigation, 51.6% of the 548 women from the Women's Health and Aging Study I in Baltimore had worse glomerular filtration rates, which were linked to higher serum levels of CML and sRAGE (the soluble form of RAGE)[143]. Normal renal function involves the kidneys clearing circulating AGEs, although elevated AGE levels have been seen in individuals with uremia and diabetic nephropathy, likely due to insufficient renal clearance[144].

Additionally, individuals with DM have peripheral nerves with high amounts of AGEs[145]. Ahmed conducted a recent study and discovered that diabetes has been linked to increased myelin glycation in *in vitro* investigations. By phagocytosing the glycated myelin, macrophages could explain the nerve demyelination found in diabetic neuropathy. When AGEs are injected into peripheral nerves in animal tests, blood flow, nerve action potentials, and sensory motor conduction velocities all decrease[146].

In terms of developmental diseases, AGE accumulation and obesity interact with health risk factors, as a result, the development of glucose levels is influenced and said that AGEs, glycated hemoglobin, and obesity are all linked to glucose levels, and obesity may be one of the health risk factors pathophysiological mechanisms leading to increased glucose level due to AGE accumulation; thus, obesity could be health risk factors leading to increased glucose level in

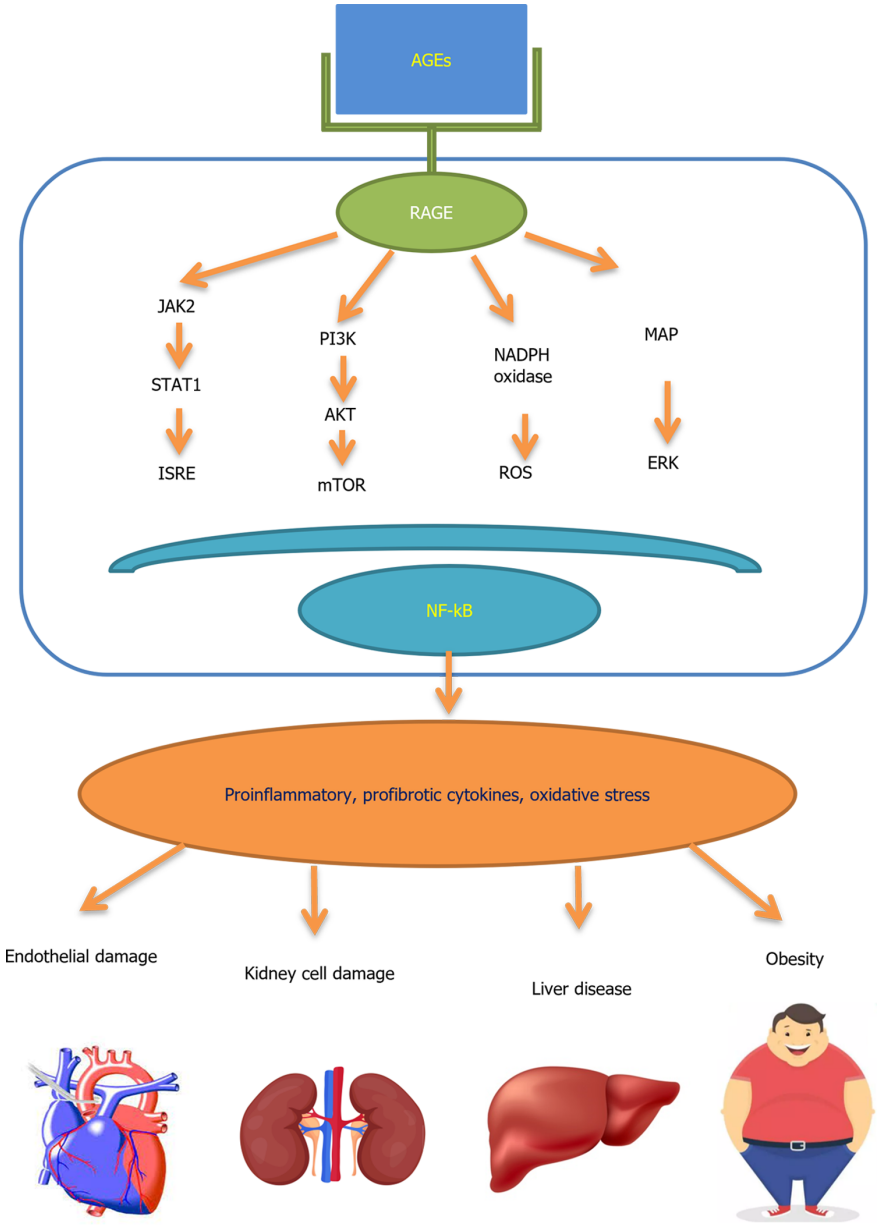

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Figure 3 Advanced glycation end products, advanced glycation end products receptor mediated pathways, production of cytokines, oxidative stress and organ involvement. AGEs: Advanced glycation end products; ERK: Extracellular signal regulated kinase; ISRE: Interferon-sensitive response element; JAK: Janus kinase; PI3K: Phosphoinositide 3-kinase; mTOR: Mammalian target of rapamycin; RAGE: Advanced glycation end products receptor; ROS: Reactive oxygen species.

AGE accumulation[147]. Local inflammatory response is linked to elevated systemic inflammatory cytokines, which are responsible for impaired glucose regulation[148]. It is now well established that external AGEs considerably contribute to the body's AGE pool[149]. The increased inflammation further triggers the activation of additional mediators which increases inflammation, as well as induces insulin resistance in muscles[150]. Under identical settings of hyperglycemia and AGE accumulation, the interaction of RAGE-induced cellular dysfunction, protein kinases, and inflammation leads to a reduction in insulin sensitivity in target cells[151]. Hofmann and colleagues demonstrated in a RAGE knock-out mouse model that both AGEs and RAGE are implicated in aortic leaflet calcification and consequent aortic stenosis[152]. Reduced sRAGE and endogenous secretory receptor for RAGE (esRAGE), both of which are assumed to be protective against AGEs, have been identified as an early indicator of first target organ damage in moderate hypertensives[153] or diabetics have negative coronary artery remodeling[154]. In DM patients, AGEs and RAGE build within stenotic aortic valves, and the extent of this accumulation is related to the severity of the aortic stenosis and plasma AGE and sRAGE levels were linked to aortic valve area, they may be regarded novel biomarkers of the aortic stenosis course in patients with type 2 diabetes[155], dual character of RAGE, combined with increased AGE consumption by sRAGE in people with poor glucose metabolism, may disrupt direct correlations between RAGE and markers reflecting the degree of aortic stenosis[156].

Cardiovascular complications

Insulin resistance and hyperglycemia can impact numerous human tissues and organs, leading to long-term difficulties in a number of systems and organs, including the cardiovascular system[157,158]. Left ventricular concentric hypertrophy, perivascular fibrosis, and interstitial fibrosis are signs of pathological remodeling of the heart, which results in diastolic dysfunctions[159]. Cardiovascular illness affects the former more severely and extensively than the latter, has a worse prognosis, and manifests earlier in the former. Heart failure is 2-4 times more likely to occur in people with type 2 diabetes than in people without the disease[160]. About 70%-80% of diabetics pass away from cardiovascular issues at the end[161]. Additionally, almost 3/4 of people with type 2 diabetes also have a number of cardiovascular risk factors, including obesity, dyslipidemia, and hypertension. The accumulation of these risk factors may directly encourage the development of diabetic cardiovascular problems[162]. The primary fundamental mechanism thought to be responsible for diabetic cardiovascular disorders is increased oxidative stress[163]. In diabetic cardiovascular problems, hyperglycemia causes NADPH oxidase to become active[164], oxidative stress causes myocardial fibrosis, endothelial dysfunction, hypertrophy and apoptosis of cardiomyocytes, inflammation, endothelial dysfunction, decreased left ventricular compliance, diastolic dysfunction, and ultimately heart failure, arrhythmia, and/or sudden cardiac death [165]. Nin *et al*[166] confirmed that plasma AGE levels in fatal or nonfatal coronary artery disease are related to all-cause mortality. Steine *et al*[167] found that Plasma AGE levels are related to left ventricular dysfunction in people with type 1 diabetes. Jia *et al*[159] also found that the plasma AGE levels are related to left ventricular dysfunction in people with type 1 diabetes. Type IV collagen and laminin, two extracellular matrix proteins of endothelial cells, can be directly modified by AGEs[168]. This mechanism accelerates cardiac fibrosis and damages the natural structure and function of blood vessels[169]. In addition to harming endothelium cells, AGEs also cause endothelial progenitor cells to die and become dysfunctional[170]. Atherosclerosis can be accelerated by circulating AGEs, which can increase lipid oxidation and deposition in atherosclerotic plaques and encourage macrophage infiltration, T cell migration, and proliferation[171]. Additionally, recent research has demonstrated that AGE binding to the platelet membrane receptor cluster of differentiation 36 results in the production of thrombi, which may be a key mechanism by which AGEs encourage myocardial ischemia episodes in diabetes patients[172]. As a result of the AGE-RAGE interaction, numerous signal transduction cascades and downstream pathways are activated, including mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, p38, and nuclear factor kappa B. This causes oxidative stress to increase, ROS to be produced, and the development of cardiovascular problems in diabetes[173]. Additionally, it was discovered that AGEs increased endothelial cells' NADPH oxidase production and activity, which is a significant source of oxidative stress in diabetic cardiovascular problems[174,175]. Currently, it is accepted that diabetic patients who take metformin regularly can lower their chance of developing cardiovascular disease[176]. Its antioxidant qualities that lower OX activity and lipid peroxidation in type 2 diabetic patients are responsible for its cardiovascular protective benefit [177]. Metformin treatment reduced AGE plasma levels in diabetic rats, decreasing AGE-induced heart remodeling and oxidative stress [178].

Interestingly, sustained high dietary AGEs have been shown to cause increased arterial stiffness, which leads to an increase in systolic blood pressure and inflammatory activation, leading to vascular issues in type 2 diabetes[179]. Regardless of aortic diameter, elevated circulating sRAGE levels have been connected to the presence of bicuspid aortic valves and linked aortopathies[180]. The AGEs/sRAGE ratio has been recommended as a more effective biomarker of organ damage than either AGEs or sRAGE variants alone[181] Furthermore, differing prediction abilities of esRAGE and cRAGE as cardiovascular risk factor markers have recently been demonstrated[182]. AGEs can also glycate and crosslink basement membrane protein, changing cell-matrix interactions and reducing endothelial cell adhesion leading microvascular and macrovascular problems[183]. AGEs cause oxidative stress, as well as inflammatory and fibrotic reactions, all of which contribute to the development and progression of life-threatening cardiovascular illnesses[184]. AGEs mainly induce arterial damage and exacerbate the development of atherosclerotic plaques by triggering cell receptor-dependent signal resulting in arterial wall injury and plaque formation[185].

CONCLUSION

The worldwide increase in consumption of highly processed, calorie-dense food is fueling an obesity, diabetic, kidney, and cardiometabolic disease crisis. Focusing on the effects of dietary AGEs has been shown to increase circulating AGEs, accumulate in tissues, to affect endothelial function, increase pro-inflammatory cytokines and oxidation markers, and to act as a ligand for the advanced glycation end products receptor (RAGE). AGEs intake was higher in participants with obesity, diabetes, cardiovascular disease complications when compared with those without complications. AGEs have been found in dietary items, human blood, and tissues such as pyrraline, CML, CEL, pentosidine, and MOLD. In both industrialized and developing countries over the past three decades, consumption of AGE-containing foods and beverages has been associated with an increase in metabolic diseases. Cardiovascular disorders are made worse by diabetes, and patients with diabetic cardiovascular problems have worse clinical outcomes. Since AGEs not only influence oxidative stress but also are impacted by it, it is well known that AGEs and oxidative stress play a central role in the cardiovascular problems associated with diabetes. However, many of these mechanisms are still unclear and require more explanation. Beyond blood glucose control in this population, it has been discovered that glucose-lowering medications have a protective effect on the cardiovascular system.

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FOOTNOTES

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REVIEW

Tight junction disruption and the pathogenesis of the chronic complications of diabetes mellitus: A narrative review

Ma Ludivina Robles-Osorio, Ernesto Sabath

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Abstract

The chronic complications of diabetes mellitus constitute a major public health problem. For example, diabetic eye diseases are the most important cause of blindness, and diabetic nephropathy is the most frequent cause of chronic kidney disease worldwide. The cellular and molecular mechanisms of these chronic complications are still poorly understood, preventing the development of effective treatment strategies. Tight junctions (TJs) are epithelial intercellular junctions located at the most apical region of cell-cell contacts, and their main function is to restrict the passage of molecules through the paracellular space. The TJs consist of over 40 proteins, and the most important are occludin, claudins and the zonula occludens. Accumulating evidence suggests that TJ disruption in different organs, such as the brain, nerves, retina and kidneys, plays a fundamental pathophysiological role in the development of chronic complications. Increased permeability of the blood-brain barrier and the blood-retinal barrier has been demonstrated in diabetic neuropathy, brain injury and diabetic retinopathy. The consequences of TJ disruption on kidney function or progression of kidney disease are currently unknown. In the present review, we highlighted the molecular events that lead to barrier dysfunction in diabetes. Further investigation of the mechanisms underlying TJ disruption is expected to provide new insights into therapeutic approaches to ameliorate the chronic complications of diabetes mellitus.

Key Words: Tight junctions; Blood-brain barrier; Diabetic neuropathy; Blood-retinal barrier; Diabetic retinopathy; Diabetic nephropathy

Core Tip: Chronic complications of diabetes mellitus constitute a major public health problem. Tight junctions are epithelial intercellular junctions, and their main function is to restrict the passage of molecules through the paracellular space. TJ disruption plays a fundamental pathophysiological role in the development of diabetic chronic complications. Increased permeability of the blood-brain barrier and the blood-retinal barrier are related to development of diabetic neuropathy and diabetic retinopathy.

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INTRODUCTION

Tight junctions (TJs) are epithelial intercellular junctions located at the most apical region of cell-cell contacts. TJs serve two main functions: (1) Gate function, which restricts the passage of molecules through the paracellular space; and (2) Fence function, which confers cell polarity by preventing the movement of solutes and proteins between the apical and basolateral plasma membrane. Additional functions in cell-signaling processes, cell proliferation and gene expression have been identified[1].

At a molecular level, the TJs consist of over 40 proteins including members of the four-pass trans-membrane proteins that are part of the occludin and claudin families. TJs are also composed of cytoplasmic proteins, such as members of the zonula occludens (ZO-1,-2,-3) family, which connect TJs to the cytoskeleton by binding to actin filaments[2] (Figure 1).

Claudins are 21-28 kDa proteins and consist of four transmembrane domains, two extracellular loops, amino- and carboxyl-terminal cytoplasmic domains, and a short cytoplasmic turn. Claudins interact with the ZO-family of scaffolding proteins *via* their cytoplasmic region and are an essential component of the TJs regulating assembly and permeability[2].

Occludin is a 65 kDa protein that interacts with other TJ proteins such as membrane-associated guanylate kinase-scaffolding proteins. Occludin is expressed in endothelial and epithelial tissues, and its expression is regulated by different tyrosine and threonine kinases such as the non-receptor tyrosine kinase c-Yes and the protein kinase C (PKC). Madin-Darby Canine Kidney (MDCK) cells that express terminally truncated occludin have an increase in the paracellular permeability but preserve the formation of TJ strands[3]. However, occludin null mice did exhibit defects in certain organs, and histological abnormalities were found in several tissues including hyperplasia of the gastric epithelium, brain calcifications and testicular atrophy, suggesting an unknown role of occludin in the homeostasis of these organs[4].

The ZO proteins (members of the membrane-associated guanylate kinase family) are scaffolding proteins that bind and regulate the expression of cytoplasmic (cytoskeleton) and transmembrane components of the TJs. ZO proteins regulate gene transcription, cell proliferation and claudin polymerization. Phosphorylation of these proteins by the PKC and tyrosine kinases regulates TJ permeability and assembly. ZO-1 depletion in MDCK and endothelial cells lead to TJ disruption, delayed formation of TJs and reorganization of the actin and myosin cytoskeleton[5]. Maintenance of the cellular barriers and regulation of the transepithelial permeability to prevent diffusion of small molecules and bacteria to specific organs such as brain and retina is essential to keep the homeostasis at these organs.

Type 2 diabetes mellitus (DM) is a chronic disease that has reached epidemic proportions. Chronic hyperglycemia (CH) combined with defects on insulin secretion and action impair the microvasculature and activate intracellular signaling pathways, eventually leading to diabetic nephropathy (DN), retinopathy and neuropathy with significant negative effects on the quality of life and life expectancy[6].

Many studies have demonstrated that TJ disruption and increased leakage of water, solutes and proteins is associated with development of diabetic chronic complications [diabetic eye disease (DED), diabetic neuropathy and DN][7,8]. Therefore, this review aimed: (1) To summarize the normal structure of the TJs at the different barrier structures (brain, nerves, retina and kidney); (2) To describe the pathophysiological changes caused by DM leading to TJ disruption and increase in paracellular permeability that are associated with the chronic complications; and (3) To summarize these findings with the clinical consequences and pharmacological treatments used in the management of these complications.

SEARCH STRATEGY

This systematic review was conducted according to the 2021 guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses[9]. Both authors (MLRO and ES) systematically searched PubMed, Google Scholar and the *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) databases to identify published articles from 1978 to December 2022 describing the role of TJs and the chronic complications of DM. Seminal references from selected articles were also searched and included. Both authors independently reviewed the database search results, assessed the

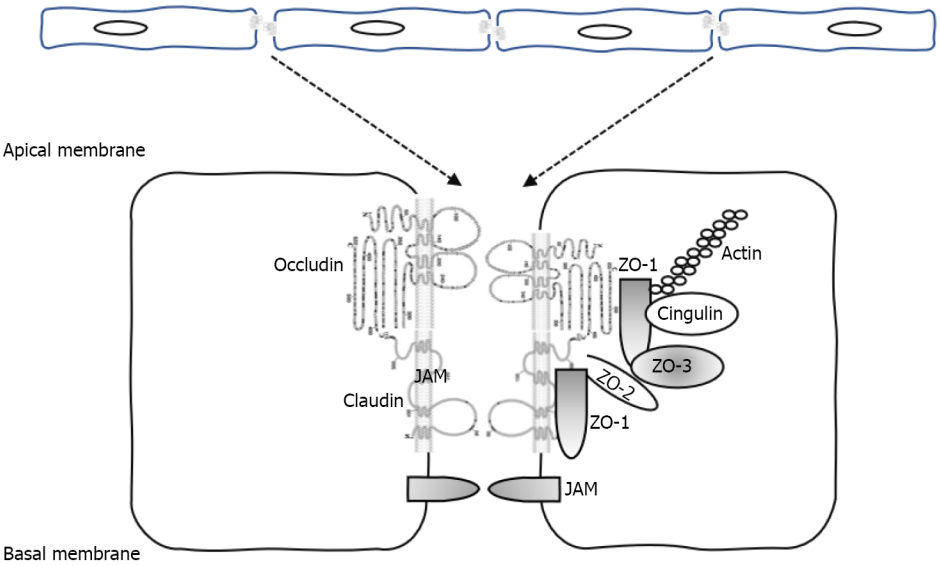

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Figure 1 Molecular organization of the tight junctions. Claudins, occludin and junctional adhesion molecules are the major integral membrane proteins of tight junctions (TJs). Claudins form TJ strands, corresponding to membrane kissing points. TJ-associated membrane proteins are localized at apical cell-cell junctions by interacting with the zonula occludens family of scaffolding proteins, serving as links between TJs and the actin cytoskeleton. JAM: Junctional adhesion molecules; ZO: Zonula occludens.

titles, evaluated the abstracts and considered the study for full review. The search was performed combining the texts "tight junction" OR "occludin" OR "ZO (zonula occludens) proteins" OR "claudin" OR "blood retinal barrier" OR "brain-blood-barrier" OR "glomeruli" OR "renal tight junctions" with "diabetes mellitus" OR "diabetic retinopathy" OR "diabetic neuropathy" OR "diabetic nephropathy." Only articles written in English were included (Figure 2). For the final analysis we evaluated 109 research papers.

DM AND TJ DISRUPTION

DM, TJs and nervous system disease

The blood-brain-barrier (BBB) and the blood-nerve-barrier (BNB) are highly selective semipermeable barriers that regulate the exchange of water and solutes between the blood and the nerve tissue. Both the BBB and the BNB play important roles in maintaining the integrity of the nervous system, and many recent reports suggest that their breakdown drives a cascade of pathogenic events leading to many nervous system diseases[10].

DM and increased permeability of the BBB: Numerous epidemiological studies have shown that DM is an important risk factor for central nervous system (CNS) disorders such as stroke[11], mild cognitive impairment and dementia[12]. The underlying causes related to these complications are multifactorial and are not well understood, although it is now evident that BBB damage adversely affects CNS homeostasis and function[10] (Figure 3).

The BBB consists of a confluent layer of non-fenestrated endothelial cells to tightly regulate the movement of molecules between the blood and the nervous system. Its basic structure is formed by the TJs located between the endothelial cells. The brain capillaries are shielded by pericytes and the foot processes of the astrocytes. These cells are important for the secretion of proteins that forms the basement membrane. The BBB is permeable to small molecules and lipid-soluble proteins, but receptor-mediated transcytosis is required by large molecules to enter the nervous system[13].

The endothelial TJs of the BBB are formed by the transcellular proteins claudins, occludin and junctional adhesion molecules. The loss of claudins increases barrier permeability, suggesting that this family of proteins are particularly important for barrier function. Claudins -1, -3, -5 and -12 take part in the formation of TJs between the endothelial cells [14]; claudin-5 is the most abundant claudin at the BBB and is a critical regulator of brain endothelial cell permeability. In claudin-5 knockout mice the blood vessels of the brain showed normal development and morphology, but the size-selectivity of the BBB was impaired allowing diffusion of small molecules[15].

Occludin is highly expressed at the BBB but does not appear to be essential to barrier function, as occludin-deficient mice have normal BBB permeability. ZO-1, ZO-2 and ZO-3 cross-link the claudins and other TJ proteins to the endothelial cytoskeleton[16]. Increased permeability of the BBB has been demonstrated in both type 1 DM[17] and type 2 DM[18,19], and significant efforts have been made to identify the molecular mechanisms related to BBB breakdown in DM.

Huber *et al*[20] demonstrated a progressive increase in the BBB permeability to small molecules in mice with streptozotocin-induced DM; the midbrain was particularly susceptible to DM-induced microvascular damage. Insulin administration attenuated BBB disruption during the first few weeks of treatment. However, as DM progressed the microvascular damage occurred even if hyperglycemia was controlled.

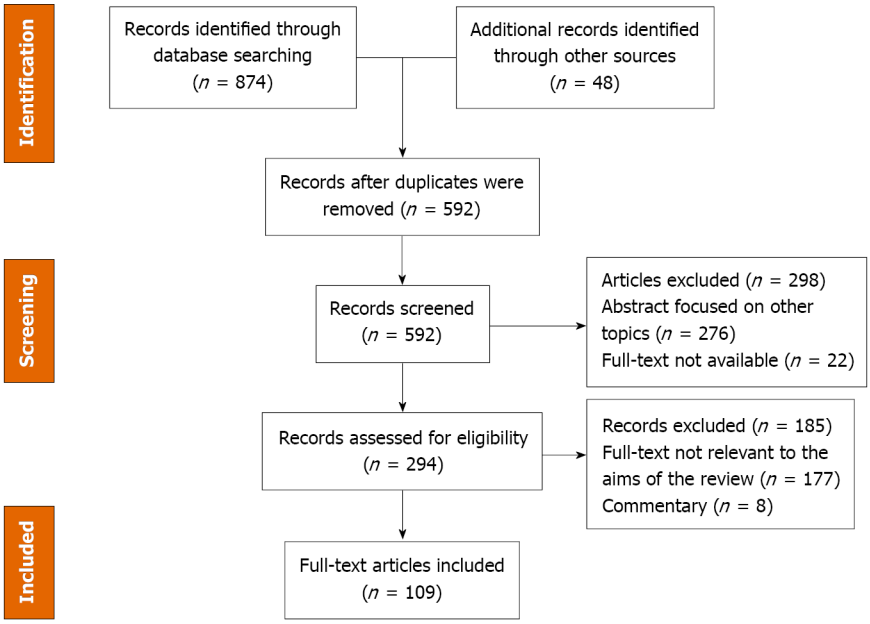

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Figure 2 Study flowchart according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.

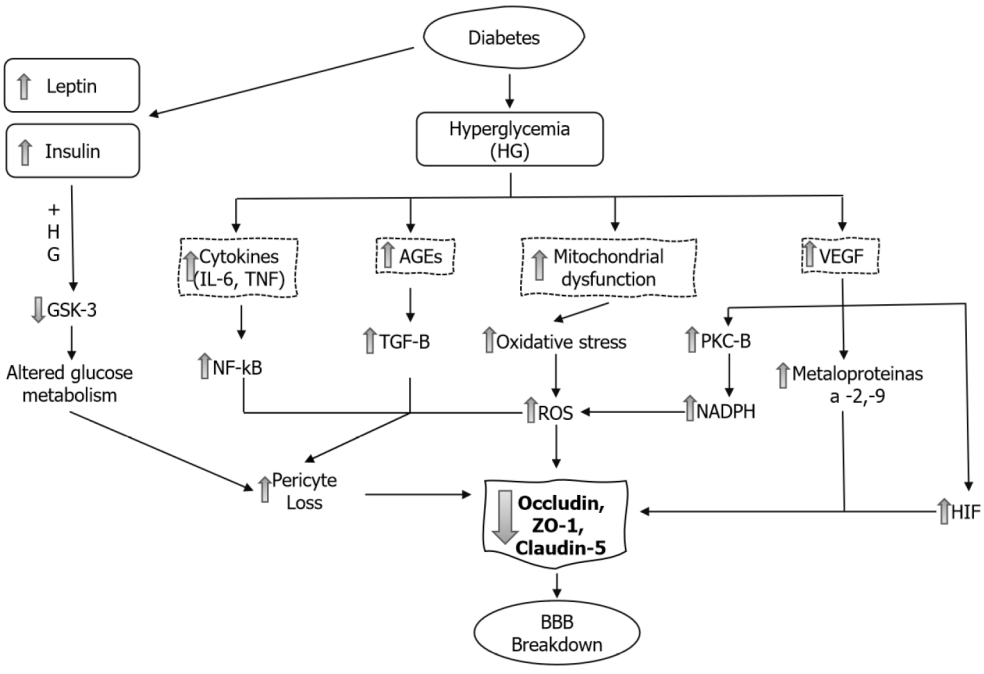

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Figure 3 Mechanisms of blood-brain-barrier dysfunction in diabetes mellitus. AGE: Advanced glycation end-product; BBB: Blood-brain-barrier; GSK-3: Glycogen synthase kinase 3; HG: Hyperglycemia; HIF: Hypoxia-inducible factor; IL-6: Interleukin 6; NADPH: Nicotinamide adenine dinucleotide phosphate; NF-κB: Nuclear factor-kappa B; PKC-B: Protein kinase C; ROS: Reactive oxygen species; TGF-β: Transforming growth factor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor; ZO-1: Zonula occludens.

There are many proposed mechanisms by which DM leads to pericyte loss and BBB breakdown. Hyperglycemia causes mitochondrial dysfunction and synthesis of reactive oxygen species (ROS) and increases oxidative stress, activation of nuclear factor-kappa B (NF-κB) and the synthesis of inflammatory cytokines[21]. In pericytes and endothelial cells, both hyperglycemia and formation of advanced glycation end-products (AGEs) downregulate the TJ proteins claudin-5, ZO-1 and occludin. There is also a significant increase in the amount of occludin and claudin-5 on the membrane-bound extracellular vesicles[22]. This allows greater influx of blood components into the perivascular space.

Hyperglycemia also stimulates the synthesis of vascular endothelial growth factor (VEGF), increasing both angiogenesis and vascular permeability. Downstream, VEGF activates PKC-β, causing an increase in nicotinamide

adenine dinucleotide phosphate-oxidase and an increase in ROS formation. VEGF increases the activation of different matrix metalloproteinases (MMP-2 and MMP-9). These mechanisms increase brain barrier permeability through the decrease in occludin expression and phosphorylation[23].

The hypoxia-inducible factor-1 (HIF-1) is a transcriptional factor that activates cellular adaptation to hypoxia. High glucose upregulates the transcriptional activity and protein level of HIF-1α in brain endothelial cells. In addition, it increased the paracellular permeability and diminished the expression of the TJ proteins occludin and ZO-1[24].

The development of cognitive impairment in diabetic rats was associated with an increase in the BBB permeability. These rats showed an increase in brain levels of interleukin (IL)-6 and a decrease in occludin and claudin-5 expression [25].

Recent studies suggested that many factors other than hyperglycemia, like insulin and leptin, have a pathophysiological role increasing BBB permeability[26]. Insulin crosses the BBB using a saturable transporter, affecting brain functions through mechanisms largely independent of glucose utilization. Insulin transport across the BBB is highly regulated and altered in obesity, starvation and DM[27].

Insulin receptor signaling regulates the integrity of the BBB *via* inactivation of glycogen synthase kinase 3, a key enzyme in many cellular functions, specifically regulating glycogen synthesis and blood glucose levels. Administration of insulin alone increases BBB resistance, but the combined administration of high glucose/high insulin synergistically impairs TJ integrity[28].

Some drugs have been shown to have effects on BBB structure and function. Statins are known to improve endothelial cell function, and simvastatin treatment improved the barrier function in cerebral tissue of diabetic rats[29]. Administration of valsartan (AT1R antagonist) to db/db mice ameliorated BBB leakage. This finding suggested that neurovascular protection can be obtained blocking the AT1-receptor mediated signaling pathways[30]. Exogenous administration of exendin-4, a glucagon-like peptide 1 agonist that crosses the BBB, reverses the functional changes and restores levels of TJ proteins[31].

In many CNS disorders the BBB integrity is compromised, and treatment with glucocorticoids improves the tightness of the BBB[32]. However, there are no reports about its effects on diabetic animal models.

Diabetic neuropathy and increase permeability of the BNB: Diabetic polyneuropathy (DPN) is the most common chronic complication, with a prevalence of 30%-50%. The duration of DM and HbA_{1c} levels are major predictors of DPN. Other risk factors consistently associated with DPN are hypertriglyceridemia, hypertension, abdominal obesity, low high-density lipoprotein levels, smoking and alcohol ingestion[33].

The BNB is localized in the microvessels of the endoneurium or perineurium, and consists of endothelial cells, pericytes and the basement membrane (Figure 4). TJs are an essential component of the BNB cellular architecture to restrict the paracellular flow into the endoneurial milieu and are constituted by occludin, ZO-1 and claudins. Cells of the perineurium express claudin-1, -3 and -19, whereas the endoneurial vessels express claudin-5[34].

There are many mechanisms involved in the axonopathy associated with DPN. Hyperglycemia increases sorbitol pathway activity, reduces myo-inositol nerve content, induces mitochondrial dysfunction with an increase in the synthesis of free radical species and activates metalloproteinases. The formation of AGEs increases protein glycosylation and Schwann cell injury[35].

Initial studies on the effects of hyperglycemia on BNB structure and permeability were controversial as some initial studies conducted in streptozotocin-diabetic rat models did not show increased permeability to large molecules, even in experiments performed with exposition to severe hyperglycemia[36,37]. Other studies showed severe impairment and increased permeability of the BNB[38]. More recent studies showed that the BNB was leaky for small but not for large molecules. Even though no gross changes in TJ proteins were observed, there was a downregulation in the expression of claudin-1[39]. In human subjects with type 1 DM an increase in the extravasation of albumin and immunoglobulin G through the BNB has been demonstrated[40].

Pathological BNB breakdown leads to an increase in the paracellular leakage of potentially harmful molecules into the nerve tissue and the upregulation of adhesion molecules on the vessel walls to permit the transcellular entry of inflammatory cells to the endoneurium initiating a local inflammatory cascade. Inflammation, endoneurial hypoxia and pericyte degeneration are some of the mechanisms associated with BNB disruption[13]. AGE exposition induces basement membrane hypertrophy and disrupts the BNB by increasing autocrine VEGF and transforming growth factor-β signaling. Claudin-5 synthesis was also significantly reduced[41].

The consequences of the breakdown of the BNB are the access of hematogenous cells and inflammatory molecules to the endoneurium. These phenomenon take part in the local inflammatory cascade generating neuropathic pain[42]. Unfortunately, there are no effective treatments for this complication. Current analgesics have limited beneficial impact alleviating neuropathic pain, and other than glucose and metabolic control there are no disease-modifying therapies[35].

TJ disruption in the physiopathology of DED

DED is the most common microvascular complication of DM and manifests as vascular disease with vessel proliferation [diabetic retinopathy (DR)] and vascular leakage (diabetic macular edema). The latest prevalence data from a pooled analysis estimated a prevalence of 35%, and this prevalence increased with DM duration. The most important risk factors associated with DED are CH, age, cholesterol levels and high blood pressure[43].

The retina is the innermost, light-sensitive layer of tissue of the eye that turns light energy from photons into three-dimensional images. The blood–retina barrier (BRB) separates the retina from the systemic circulation to regulate the flow of water, electrolytes, nutrients and metabolic waste products. The BRB is composed of both an inner barrier (iBRB) and an outer barrier (oBRB)[44].

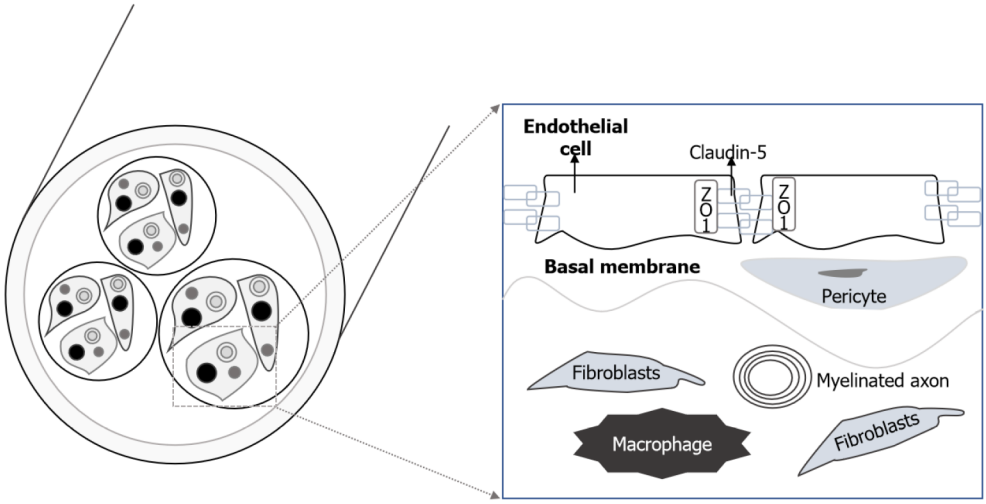

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Figure 4 Blood-nerve-barrier and cellular structure of the endothelial cells and tight junctions. ZO: Zonula occludens.

The iBRB is composed of retinal vascular endothelial cells (REC) that line the retinal vasculature, which originates from the central retinal artery and supplies the inner retinal layers. The iBRB has some transport properties because substances from the blood can cross it by transcellular (caveolae-mediated transport) and paracellular transport (dependent on TJs). Intercellular TJs are crucial for the formation of endothelial barriers, as they regulate paracellular diffusion[44].

Claudins are the main determinants to regulate TJ properties. Claudin-5 is the most abundant claudin isoform in the BRB and is essential for the maintenance of the iBRB integrity[45,46]. Claudin-5 interacts with the PDZ domains of ZO-1 to cross-link the transmembrane proteins to the cytoskeleton. ZO-1 has an important role to maintain the iBRB permeability as loss of ZO-1 disrupts TJs and increases the barrier permeability[47]. Claudin-1 is also expressed in TJs on REC and is an important component of these structures to keep the barrier function[48].

The oBRB consists of the choroid, Bruch's membrane and the retinal pigment epithelial cells. The retinal pigment epithelial cells are a group of epithelial cells divided into apical and basolateral sides. The apical surface is in direct contact with the photoreceptors, and the basolateral side acts as a barrier that interacts with the capillaries of the choroid layer. The TJs of the RPE are located at the apical surface and are mainly responsible for maintaining oBRB integrity. The oBRB is essential for the survival of the photoreceptors by supporting the absorption of out of focus light, the retinal adhesion and the transport of retinoids and other nutrients[49].

Effects of DM on iBRB: Clinical studies strongly suggest that diabetic macular edema is the result of abnormal fluid accumulation as a consequence of the breakdown and vascular leakage of the iBRB. The predominant molecular mechanisms leading to iBRB breakdown include hypoxia and the direct effects of glucose on the endothelium, activation of VEGF and other intracellular signaling transduction pathways (such as PKC) and the triggering of inflammatory factors like tumor necrosis factor alpha, prostaglandins and toll-like receptor 4 (TLR-4)[50] (Figure 5). Hypoxia activates PKC and directly affects the TJs redistributing occludin and ZO-1[51]; hypoxia is also a key factor to induce the synthesis of VEGF.

VEGF has an important role in the homeostasis of endothelial cells as is an important regulator of vascular permeability, migration and cell proliferation. CH and oxidative stress upregulate VEGF-α and VEGF-β, which induces retinal neovascularization and vascular leakage. In the retina, VEGF is mainly expressed in Müller cells, endothelial cells, astrocytes, RPE cells and ganglion cells. However, recent studies suggest that Müller cell-derived VEGF induces retinal neovascularization, vascular leakage and inflammation playing a major causative role in DR[52].

The process whereby VEGF induces paracellular permeability involves binding to its receptor VEGFR-2 and activation of both the Src family cytoplasmic tyrosine kinases and PKC-β. Tyrosine kinases of the Src family are critically involved in TJ regulation through occludin and ZO-1 tyrosine phosphorylation[53,54]. VEGF also decreases occludin expression[55] and induces occludin serine-threonine phosphorylation through a mechanism mediated by activation of PKC- β. PKC-β is the most crucial PKC isoform that regulates the retinal microvascular permeability[56], and administration of PKC inhibitors prevented this increase in permeability[57]. Endothelial cells with the phosphorylation-resistant Ser490 to Ala form of occludin have preserved TJ organization and reduced VEGF-induced permeability[58].

Hyperglycemia increases the permeability of the REC through decreasing the levels of both ZO-1 and occludin[49]. The expression of claudin-1 and -5 is also decreased[59]. The formation of AGEs also decreases the expression of occludin, ZO-1 and ZO-2 in REC increasing permeability. Interestingly, the administration of silver-nanoparticles inhibited AGE-induced permeability by increasing the expression of the TJ proteins[60].

The increase of intracellular glucose leads to an increase in the synthesis of diacylglycerol, the main endogenous activator of PKC[61]. PKC regulates the function of TJ proteins through the phos-phorylation of serine and threonine amino acids. The pathologic effects of PKC activation are mediated through increased vascular permeability, disruption of nitric oxide regulation, increased leukocyte adhesion to vessel walls, changes in blood flow, overexpression of VEGF and increased oxidative stress[62].

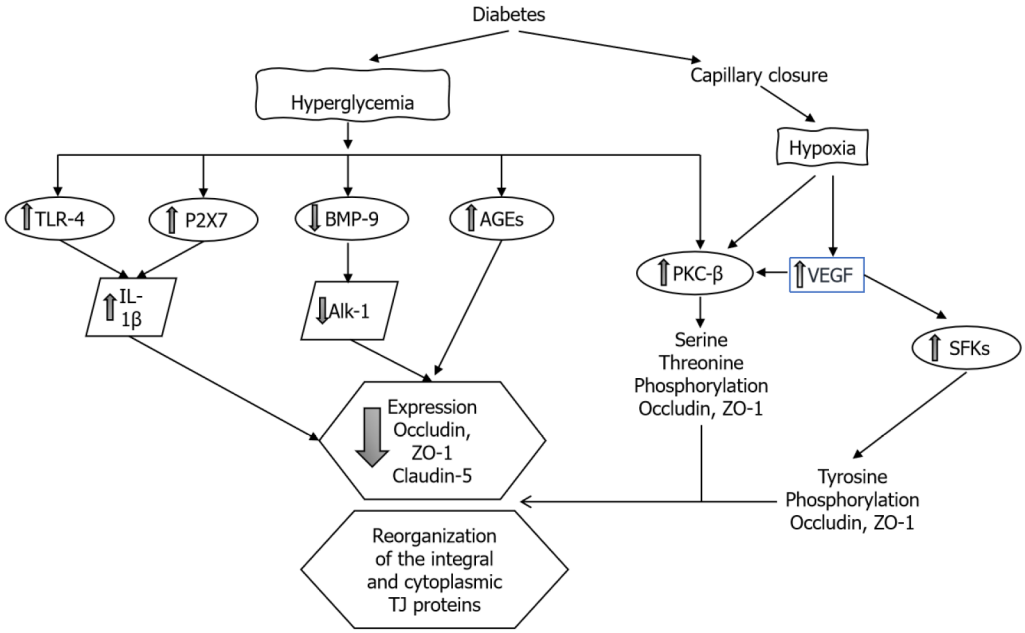

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Figure 5 Mechanisms of blood-retina-barrier dysfunction in diabetes mellitus. AGEs: Advanced glycation end-products; Alk-1: Activin-like kinase receptor type I; BMP-9: Bone morphogenetic protein 9; IL-1: Interleukin 1 beta; P2X7: Purinergic receptor; PKC-: Protein kinase C beta; SFK: Src family of cytoplasmic proteins; TJ: Tight junction; TLR-4: Toll-like receptor 4; VEGF: Vascular endothelial growth factor; ZO-1: Zonula occludens 1.

High glucose impairs other signaling cascades in retinal endothelial cells. The bone morphogenetic protein 9/activin receptor-like kinase 1 signaling cascade is necessary to maintain the endothelium integrity; this system is impaired in endothelial cells exposed to hyperglycemic conditions. A decrease in bone morphogenetic protein 9 and alterations in the activin receptor-like kinase 1 cascade contributes to increasing vascular permeability through the disruption of the occludin junctions[63].

Many cell components of the retina including the REC and the RPE express the purinergic receptor (P2X7R). It has been shown that activation of the P2X7R by hyperglycemia has a role in the breakdown of the BRB. Activation of the P2X7R induces the release of IL-1β. IL-1β reduces the transendothelial electrical resistance by decreasing the expression of claudin-5 and ZO-1. These effects were inhibited with the exogenous administration of an P2X7R antagonist[64].

β-adrenergic receptors regulate TLR-4 signaling in the retina, and inhibition of TLR-4 significantly reduces retinal barrier permeability. The exogenous administration of forskolin (a PKA agonist) or compound 49b (β-adrenergic receptor agonist) to retinal endothelial cells restored the high glucose-associated decrease in ZO-1 and occludin through the inhibition of the TLR-4 inflammatory cascade[65]. Histamine increases paracellular permeability and reduces the expression of the TJ protein ZO-1 in cultures of retinal endothelial cells[66].

Angiopoietin 1, derived from pericytes, is known to be an antipermeability factor in the vascular system. Angiopoietin 1 has also been proven to have a protective effect on BRB *via* inhibiting VEGF-induced retinal vascular leakage[67].

Hydrocortisone increases barrier properties of the retinal endothelial cells. Hydrocortisone increases the occludin content, decreases occludin phosphorylation and promotes the TJ assembly. These changes decrease water and solute endothelial permeability[68].

Effects of DM on oBRB: The role of the oBRB in the pathophysiology of the macular edema has gained importance in recent years. Recent evidence suggests that the TJs of RPE cells are also compromised in DR and may contribute to macular edema. Leaky TJs would dissipate the chloride gradient that RPE uses to pump fluid out of the retina[69]. Treatment of RPE cells with tumor necrosis factor alpha or IL-1 decreased transendothelial electrical resistance, increased permeability and altered the expression or content of TJ molecules[70].

Villarroel *et al*[71] studied the effects of high glucose concentration in ARPE-19 cells; there was a reduction of permeability with overexpression of claudin-1 and no changes in ZO-1 or occludin. These findings suggested that hyperglycemia per se is not the only factor accounting for the impairment of the oBRB in DR but requires the release of cytokines and ROS to induced damage and increase permeability[72]. At higher glucose exposure, the ARPE-19 cells increased miR-132 expression and decreased the expression of occludin and increased cell permeability[73].

High glucose induces a loss of Na-K-ATPase function impairing the transport of water from the subretinal space contributing to the development of macular edema[74]. Erythropoietin (EPO) is upregulated in DR. EPO overexpression has been found in both the RPE and neuroretina of diabetic eyes. EPO maintains the oBRB integrity through downregulation of HIF-1α and JNK signaling, thus upregulating ZO-1 and occludin expression in RPE cells[75]. Although VEGF has an important role in the pathogenesis of this disease, the RPE has mechanisms for maintaining low concentrations of VEGF in the retinal space. Peng *et al*[76] showed that VEGF and anti-VEGF drugs (bevacizumab, ranibizumab) have no effects on the TJs of RPE cells.

TJ and diabetic kidney disease

Diabetic kidney disease or DN is the leading cause of end-stage kidney disease[77]. CH leads to structural, metabolic and hemodynamic changes in the renal glomeruli and tubules, but the pathophysiology of the DN is complex and still poorly understood. CH activates the renin-angiotensin system and increases the activity of PKC, ROS formation and many cytokines and transcription factors that result in structural and functional abnormalities in the kidney[78]. However, the effects of hyperglycemia on the renal TJs have received little interest, except for the important focus on the podocyte slit diaphragms (SD).

TJs are necessary for the proper function of glomeruli and tubules and are the most important structures involved in the paracellular transport of water and solutes. The transepithelial electrical resistance and the complexity of the TJ increases from the proximal to the collecting tubule as does the expression of ZO-1, ZO-2 and occludin[79]. The distribution of claudins through the glomerular endothelium and tubules form selective pores and barriers for water and electrolytes such as sodium, potassium, magnesium, calcium and chloride[80].

The distribution and localization of claudins varies along the nephron. In the glomerular endothelium, claudin-5 forms a barrier for high molecular weight proteins. In the proximal tubule (leaky epithelium), claudin-2 forms a pore for sodium and potassium ions. In the thick ascending limb, claudin-14, -16 and -19 regulate the paracellular reabsorption of calcium and magnesium. In the renal collecting duct (tight epithelium), claudin-4 is expressed (together with claudin-3, -7 and -8) and is the major modulator of the paracellular chloride pathway[81] .

Aldosterone is the main hormonal stimulus of sodium reabsorption in the distal segments of the nephron by increasing the expression and activity of the epithelial sodium channel. Recent evidence has shown that aldosterone also has a role regulating the paracellular flow of sodium. Aldosterone phosphorylates claudin-4 and increases claudin-8 expression. These mechanism in the distal nephron are aimed to prevent the luminal back-flux of reabsorbed sodium as well to reinforce the paracellular chloride reabsorption pathway[81,82].

Effects of DM on TJs of the glomerulus: The glomerulus is a highly specialized structure that functions as an efficient filtration barrier that restricts passage of large molecules but remains highly permeable to water and small molecules. The glomerulus is composed by a network of capillaries, mesangial cells, podocytes and the Bowman's capsule. The blood is filtered across the fenestra of the glomerular endothelial cells (GEC) and the other components of the glomerular filtration barrier yielding a fluid composed of water plus soluble substances that accumulates at the Bowman's capsule to enter the renal tubules[83].

The GECs form the first cellular barrier, and the TJs between cells are important for maintaining capillary permeability. Injury to the GECs with disruption of the TJs increases its permeability and induces inflammatory cell infiltration, podocyte damage, albuminuria and progression of kidney disease[84]. High glucose decreases the expression of occludin and translocates ZO-1 to the cytoplasm by activation of RhoA (a member of the family of small GTPases)/ROCK1 system. Simvastatin inhibits the RhoA/ROCK1 signaling, increases occludin expression and restores ZO-1 localization. In db/db mice simvastatin decreases albuminuria by suppressing the RhoA/ROCK1 system[85]. AGEs significantly increase the permeability of GEC monolayers through activation of MMP-2 and MMP-9, which downregulate the expression of occludin and claudin-5[86].

Glomerular podocytes (Figure 6) are highly differentiated cells that cover the glomerular capillaries and have a characteristic morphology with numerous foot processes. The formation of SD between the foot processes serves as a final filtration barrier and is composed by many transmembrane proteins such as nephrin, podocin, Neph1 and Fat1. Podocyte damage causes disruption of the filtration barrier, proteinuria and glomerulosclerosis[87].

During the early stages of embryonic development the TJs connect immature podocytes, but in mature stages they disappear along with the widening of the intercellular spaces and the appearance of SD[88]. TJ proteins such as occludin, claudin-5 and ZO-1, but not claudin-1, have also been found in the SD of the mature podocytes. Their expression and localization are altered in glomerular diseases[89].

DN and other diseases with nephrotic proteinuria are characterized by the loss of the filtration slit, appearance of TJ-like structures and the presence of multiple membrane "fusion" points between the foot processes. This finding has been called the SD to TJ transition and is mediated by the upregulation of claudin-1 in podocytes[90-92].

In normal conditions, claudin-1 is usually absent from podocytes but present in the glomerular parietal cells. In DN, claudin-1 is upregulated in parietal cells and extended ectopically to podocytes[90]. The presence of claudin-1 led to podocyte effacement and albuminuria, presumably through the activation of the β-catenin/Snail signaling system and pathological interactions with nephrin and podocin, which disrupts the SD[92].

Sirtuin-1 (Sirt1) is an NAD(+)-regulated deacetylase with numerous known positive effects on cellular functions, and accumulating evidence shows that Sirt1 plays a crucial role in the pathogenesis of DN[92]. Hasegawa *et al*[93] found reduced expression of Sirt1 in the proximal tubules and higher expression of claudin-1 in glomeruli in streptozotocin-induced diabetic mice, which led to morphological changes on podocytes and albuminuria. Overexpression of Sirt1 in these mice inhibited the rise of claudin-1 and morphological changes. In kidney biopsy samples from subjects with DN, lower expression of Sirt1 and higher expression of claudin-1 were correlated with higher levels of albuminuria. Altogether, these data indicate a protective role of Sirt1 in glomerular and tubular injury.

Claudin-5 has been classified as a cation barrier and is expressed throughout the plasma membrane of podocytes. Molina-Jijón *et al*[82] reported that early DN decreases the expression of claudin-5 in glomeruli. This finding was attributed to an increase in oxidative stress and was associated with changes in the localization of ZO-1. Administration of all-trans retinoic acid ameliorated these changes[94]. Spironolactone prevented depletion of claudin-5 in glomeruli, suggesting a role of aldosterone in the regulation of claudin-5 expression and function[82].

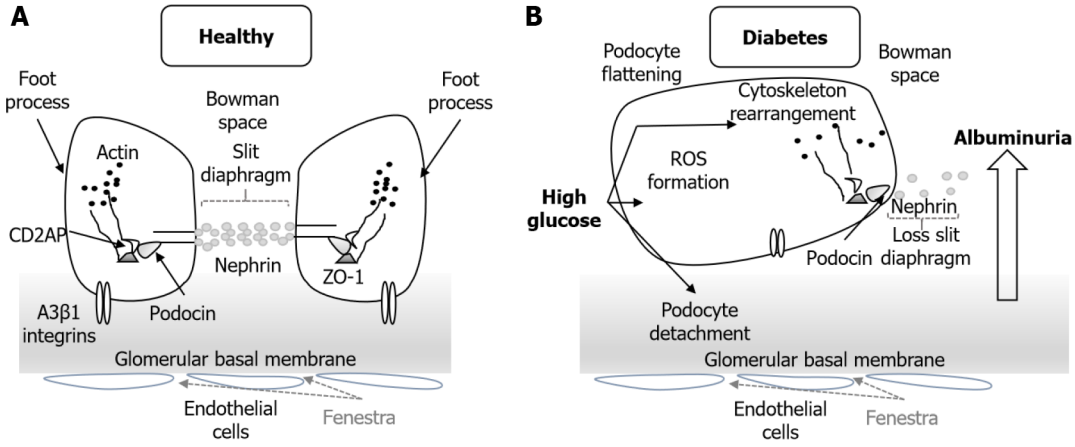

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Figure 6 Podocyte structure. A: Normal structure of the podocyte with the morphology of the foot process and the slit diaphragm; B: In diabetic nephropathy the podocyte structure and slit diaphragm are injured with slit diaphragm disruption, podocyte detachment and cytoskeleton rearrangement. These changes lead to albuminuria and progressive kidney disease. ROS: Reactive oxygen species; ZO: Zonula occludens.

Sun *et al*[95] showed that claudin-5 deletion reduced ZO-1 expression and nuclear translocation of ZO-1-associated nucleic acid-binding protein, followed by activation of the WNT signaling pathway that led to podocyte injury and dysfunction. ZO-1-associated nucleic acid-binding protein is a member of a family of DNA-binding proteins that regulate the expression of genes involved in proliferation and other nuclear signaling processes[96].

As previously stated, the scaffolding protein ZO-1 helps to maintain the permselective properties of the glomerular capillary wall. Experimental proteinuria is associated with cellular redistribution of this protein in the glomeruli, and administration of lisinopril [angiotensin converting enzyme (ACE) inhibitor] prevented these changes[97]. In glomeruli exposed to high glucose ZO-1 expression decreased, was redistributed from the podocyte membrane to the cytoplasm and inhibited serine and tyrosine phosphorylation. Administration of angiotensin II type 1 receptor blockers attenuated these changes[98]. An increase of bradykinin levels associated with the use of ACE-inhibitors also prevented ZO-1 changes[99]. These findings explain some of the beneficial effects of drugs acting on inhibition of the renin-angiotensin system.

Modulation of claudins and other TJ-SD proteins remains a key area of research from a clinical and therapeutic point of view. Many current drugs such as ACE inhibitors and simvastatin have a positive effect on these proteins limiting the glomerular injury and progressive kidney disease. Other potential drugs are shown in Table 1. Further research is necessary to develop specific drugs that target these proteins to evaluate their effect on glomerular cells.

Effects of DM on TJs of the renal tubules and tubular transport: The renal tubules and specifically the proximal tubule are uniquely susceptible to a variety of metabolic and hemodynamic factors associated with DM. The development of tubule-interstitial injury is an important risk factor associated with progressive diabetic kidney disease . In early stages of DN, tubular hypertrophy with thickening of the basal membrane is observed, but in advanced stages tubular atrophy with interstitial fibrosis is more prominent[100]. Studies on the effects of DM on tubular TJs are scarce.

The exposition of MDCK II cells to high glucose induced a decrease in the TJ content of claudins-1 and -3, a significant increase in claudin-2 and a decrease in the expression of occludin and ZO-1 junctional content. These changes decreased transendothelial electrical resistance and increased TJ permeability[101]. Claudin-2 expression in the proximal tubule decreased in streptozotocin-induced diabetic rat models[102,103]. The administration of spironolactone and all-trans retinoic acid prevented the decrease in claudin-2 and occludin in proximal tubules by decreasing oxidative stress[82,94].

The consequences of these tubular cell TJ changes on kidney function or progression of kidney disease are currently unknown.

IMPLICATIONS

TJs have an important role in maintaining organ homeostasis and are highly selective structures that regulate the paracellular exchange of water and solutes. Altered TJs have an important role in the pathogenesis of the chronic complications of DM. Identification of the mechanisms that lead to TJ disruption will provide better tools for prevention and treatment of these complications in people with DM.

An area of particular interest is the measurement of TJ proteins on plasma and its correlations with clinical outcomes. Halbgebauer *et al*[104] found significantly increased levels of plasma claudin-5 in trauma patients with hemorrhagic shock that were positively correlated with lactate levels and blood transfusions. These findings indicate that a breakdown of TJ barriers can be related with clinical outcomes in this group of patients. In other diseases, such as bipolar disorders [105] and chronic migraine[106], claudin-5 plasma levels have been found to be significantly higher than in healthy subjects. There are no studies about plasma levels of TJ proteins and clinical outcomes in diabetic patients. This is an area

Table 1 Drugs used to decrease proteinuria and progressive kidney disease and their effects on slit diaphragms /tight junction proteins

Drug	Type	Mechanism of action
Spironolactone[82]	Mineralocorticoid inhibitor	Decrease oxidative stress
		Prevent decrease of claudin-5 in glomeruli
		Prevent decrease of claudin-2 and occludin in PT
Simvastatin[85]	Inhibits HMG-CoA reductase	Inhibit RhoA/ROCK1 signaling
		Increase occludin expression
		Restore ZO-1 localization
atRA[94]	Retinoid	Decrease oxidative stress
		Prevent decrease of claudin-5 in glomeruli
		Prevent decrease of claudin-2 and occludin in PT
Lisinopril[97]	ACE inhibitor	Preserve glomerular ZO-1 distribution
Irbesartan[107]	Antagonist	Avoid nephrin depletion on SD
	Ang II receptor	
Sitagliptin[108]	Inhibits DPP-4	Decrease levels of mitochondrial ROS, ameliorate reduction of claudin-5 in GEC
Sinomenine[109]	Alkaloid isolated from the root of *Sinomenium acutum*	Attenuate ROS level, tight junction dysfunction and RhoA/ROCK activation

ACE: Angiotensin converting enzyme; Ang: Angiotensin; atRA: All-trans retinoic acid; DPP-4: Dipeptidyl peptidase 4; GEC: Glomerular endothelial cells; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; PT: Proximal tubule; ROS: Reactive oxygen species; SD: Slit diaphragms; ZO: Zonula occludens.

of opportunity for early detection of chronic complications in diabetic subjects.

New findings about the pathophysiology of TJs on the retina, nervous system and kidney may advance the development of delivery systems of insulin and other drugs by targeting these structures.

CONCLUSION

TJs are essential to the integrity and function of the epithelial and endothelial barriers in the retina, nervous system and kidney. Disruption of these structures contributes to the pathophysiology of the chronic complications in DM. There are many mechanisms of TJ disruption in DM, and hyperglycemia triggers many of the mechanisms that induce TJ disruption. Activation of PKC phosphorylates ZO-1, occludin and claudin increasing the permeability of the TJ; an increase in the oxidative stress, activation of metalloproteinases, synthesis of AGEs and hypoxia induces changes on TJ proteins increasing permeability in these barriers. Claudin-5 is an essential component of the BBB and BRB. A better understanding of the functions of these protein may allow better diagnosis and treatment to prevent injury at these organs.

In the kidney, hyperglycemia induces podocyte detachment and changes in the morphology and function of the SD that leads to albuminuria and progressive kidney disease. More research is required to identify the role of TJ disruption with clinical outcomes in diabetic subjects. Future studies should be directed to develop drugs that target TJ proteins to prevent disruption of these barriers and to improve drug delivery to these organs.

The main limitation of this review was the lack of clinical studies conducted on humans, as most of studies were carried out in animal and cellular models. This increases the difficulty for translating whether the molecular changes and severity of the TJ disruption are associated with worse clinical outcomes.

FOOTNOTES

Author contributions: Robles-Osorio ML and Sabath E designed the research study, performed the review of the literature and the article selection; Sabath E wrote the manuscript; Both authors read and approved the final manuscript.

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MINIREVIEWS

Klotho: A new therapeutic target in diabetic retinopathy?

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Abstract

Klotho (Kl) is considered an antiaging gene, mainly for the inhibition of the insulin-like growth factor-1 signaling. Kl exists as full-length transmembrane, which acts as co-receptor for fibroblast growth factor receptor, and in soluble forms (sKl). The sKl may exert pleiotropic effects on organs and tissues by regulating several pathways involved in the pathogenesis of diseases associated with oxidative and inflammatory state. In diabetic Patients, serum levels of Kl are significantly decreased compared to healthy subjects, and are related to duration of diabetes. In diabetic retinopathy (DR), one of the most common microvascular complications of type 2 diabetes, serum Kl levels are negatively correlated with progression of the disease. A lot of evidences showed that Kl regulates several mechanisms involved in maintaining homeostasis and functions of retinal cells, including phagocytosis, calcium signaling, secretion of vascular endothelial growth factor A (VEGF-A), maintenance of redox status, and melanin biosynthesis. Experimental data have been shown that Kl exerts positive effects on several mechanisms involved in onset and progression of DR. In particular, treatment with Kl: (1) Prevents apoptosis induced by oxidative stress in human retinal endothelial cells and in retinal pigment epithelium (RPE) cells; (2) reduces secretion of VEGF-A by RPE cells; and (3) decreases subretinal fibrosis and preserves autophagic activity. Therefore, Kl may become a novel biomarker and a good candidate for the treatment of DR.

Key Words: Klotho; Diabetic retinopathy; Retinal pigment epithelium; Vascular endothelial growth factor A; Epithelial to mesenchimal transition; Ocular neovascularization

Core Tip: In diabetic Patients, serum levels of Klotho (Kl) are significantly decreased compared to healthy subjects. Moreover, serum Kl levels are negatively correlated with worsening of diabetic retinopathy (DR). Several evidence suggests that retina homeostasis may be affected by altered expression of membrane Kl, as well by reduced levels of soluble Kl. In this review we focused on the role of Kl in DR, highlighting the importance of Kl in maintaining retinal homeostasis and its positive effects on several mechanisms involved in DR onset and progression. Therefore, Kl could be a novel biomarker and a good candidate for the treatment of DR.

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INTRODUCTION

Klotho

The name Klotho (Kl) derives from that of the youngest of the Three Fates who spins the thread of human life[1]. Indeed, it is considered an antiaging gene, since phenotypes of mice with mutation in this gene are similar to those of patients with premature-ageing syndromes. Kl shares sequence similarity with members of the glycosidase family 1 and it has been reported to function as a novel β-glucuronidase[2,3]. It encodes for 3 proteins: α-Kl, β-Kl and Kl-related protein (Klrp)[4]. β-Kl is mainly expressed in liver and adipose tissue and is involved in metabolic processes[4]; whereas Klrp is a cytosolic β-glucocerebrosidase[5]. α-Kl, generally simply referred as Kl, is a type I single-pass trans-membrane glycoprotein mainly expressed in the kidneys, liver, brain, and at lower level in the pituitary, skeletal muscle, urinary bladder, pancreas, testis, ovary, colon, thyroid gland, placenta and vascular tissue[1]. Both the intracellular and the transmembrane domains of α-Kl are very short, whereas the extracellular domain is longer and contains two repeated sequences (KL1 and KL2)[4,6]. After association with fibroblast growth factor receptors (FGFRs), the full-length transmembrane Kl (mKl) acts as coreceptor for the bone-derived phosphaturic hormone FGF23, thus taking part to phosphate excretion and calcium homeostasis by regulating the expression and activity of the calcium channel transient receptor potential vanilloid 5 (TRPV5)[7]. Besides mKl, there are 2 isoforms of α-Kl: A shed soluble form (sKl), which derives from the cleavage of the extracellular domain of Kl from the cell surface by the metalloproteinases ADAM10 and ADAM17, and a secreted form that is produced by alternative splicing of Kl mRNA[4]. The shed soluble form of Kl seems to be dominant on both the secreted and the membrane forms in humans[8]. It has been proposed that the soluble forms of Kl function as a hormone[9]. Moreover, since circulating levels of sKl increase following exercise training, it has been also hypothesized that Kl may be related to the antiaging effects of physical activity[10]. The sKl has pleiotropic effects on a lot of organs and tissues, thus regulating several pathways[8]. Indeed, after the release in blood, urine and cerebrospinal fluid, sKl exerts biological effects involved in preservation of endothelial integrity and permeability, and affect intracellular signaling pathways including those related to insulin, insulin-like growth factor-1 (IGF-1), PI3K, NF-kB, p53/p21, cAMP, protein kinase C and Wnt[8,11-13]. In particular, a lot of evidence demonstrated that the anti-ageing effects of sKl have been associated with the inhibition of IGF-1 signaling and its downstream actions especially by enhancing resistance to oxidative stress[14,15]. Indeed, inhibition of the IGF-1 signaling by sKl results in increased production of antioxidant enzymes[16]. Therefore, activity of sKl may regulate several pathways involved in the pathogenesis of diseases associated with oxidative and inflammatory state.

It is not yet clear whether intracellular signaling of circulating Kl is mediated by a membrane receptor. Recent hypothesis suggests that sKL may act as a circulating co-receptor for membrane-bound FGFRs, thus allowing the interaction with FGF23 and regulating FGFR-mediated signaling also in cells lacking the full length form of Kl[17]. Moreover, it has been demonstrated that sKl is able to bind membrane lipid rafts, alter their organization, and affect caveolae-mediated TRPV5 endocytosis[18], suggesting that the intracellular signaling of sKl may occur at the level of caveoale.

KI AND DIABETES

In diabetic patients, serum levels of Kl have been found significantly decreased compared with those of healthy subjects [19]. In addition, the amount of sKl is related to duration of diabetes and is negatively correlated to HbA1c. Kidneys are considered the main source of sKl[17], and are also the principal organ involved in the clearance of sKl from the circulation into the urine, thus playing a dual role in the homeostasis of Kl[9]. Therefore, altered kidney function may affect the systemic effects of Kl. Consequently, the anti-aging effects of Kl have been extensively investigated in kidneys, reporting that increased levels of Kl inhibit the progression of various kidney diseases[20,21]. In animal models of diabetes, Kl counteracts podocytic and glomerular albumin permeability induced by hyperglycemia[22], and prevents epithelial-mesenchymal transition (EMT) in diabetic kidneys[21]. Interestingly, expression of Kl has been found decreased in the renal cortices of mice with diabetes[22]. Moreover, Typiak *et al*[23] showed that decreased levels of membrane-bound Kl are associated to increased shedding of Kl, to higher levels in serum of diabetic rats and a to reduced urinary

excretion[23]. In diabetic patients, the amount of soluble Kl is reduced in the early stage of chronic kidney disease (CKD), but increased with disease progression and the decrease of glomerular filtration rate[24]. A recent meta-analysis of data on sKl amount in patients with diabetic nephropathy (DN) confirms that levels of sKl are further lowered in the early stage of DN[25], suggesting that Kl might be considered as an early biomarker of DN[23,26]. However, although levels of sKl still remain lower in patients with DN, they seem to increase during the worsening of diabetic CKD probably linked to the decline in glomerular filtration rate that leads to reduced urinary excretion of Kl[23,27].

Expression of Kl has been detected also in mouse pancreatic islets and in beta-cell line[28,29]. It has been showed that Kl is involved in regulation of glucose-induced insulin secretion, probably, through regulation of TRPV2 expression[28,29]. Indeed, overexpression of Kl increases both insulin secretion and plasma membrane levels of TRPV2; whereas silencing of Kl negatively affects plasma membrane levels of TRPV2, glucose-induced calcium entry and insulin secretion [28]. Moreover, treatment with α- or β-Kl protects human beta-cells by cytokine-induced apoptosis and improved insulin secretion[30,31].

DIABETIC RETINOPATHY

Diabetic retinopathy (DR) is a common microvascular complications of type 2 diabetes and represents the primary cause of blindness in working age adults[32]. Actually, retinal neurodegenerative lesions may occur earlier than microvascular ones, therefore DR has been defined as a highly tissue-specific neurovascular complication of diabetes by the American Diabetes Association[33]. The early manifestations of DR involves damages to both microcirculation and retinal neurons and are associated with oxidative stress[34]. The resulting sustained proinflammatory environment, in turns, increases oxidative stress, due to the reduced levels of antioxidant enzymes in the retina. Photoreceptors and the retinal pigment epithelium (RPE) cells are highly susceptible to oxidative stress in the early stage of DR and their dysfunction lead to progression of retinal degeneration[34]. Furthermore, chronic inflammation causes vasoregression and alters vascular permeability, leading to formation of microaneurysms and exudates. Then, hypoxia and the release of proangiogenic factors, such as vascular endothelial growth factor A (VEGF-A), may promote pathological ocular neovascularization[34]. In the retina, VEGF-A is mainly produced by RPE cells, a monolayer of highly specialized cells located between the choroid and photoreceptors that forms the outer blood-retinal barrier[35]. Due to their localization, RPE cells may affect retinal homeostasis by altering the function and maintenance of both the photoreceptors and capillary endothelium[36]. Indeed, under normal condition, VEGF-A is released at low concentrations from the basal side of the RPE to maintain endothelial cell function[37]. However, under pathological condition, such as chronic hyperglycemia, secretion of VEGF-A increases leading to activation of endothelial cells and altered permeability of the choroidal vessels[37,38]. It is well known that dysfunction of RPE cells contributes to onset and progression of DR. Therefore, maintaining the function of RPE and controlling the levels of VEGF-A are of great importance in preventing worsening of DR to the proliferative state.

KI AND RETINAL HOMEOSTASIS

It has been found that Kl is expressed in the human retina, optic nerve, and lens[39,40]. Several evidence showed that Kl regulates a lot of mechanisms involved in maintaining homeostasis and functions of retinal cells[39,41,42]. Firstly, Kl knockout mice display several morphological changes as compared to wild type mice: Decreased pigmentation of the RPE layer, large choroidal vessels, thinner and deformed basal membrane, and signs of degeneration in the outer segment of photoreceptors (POS)[41]. Proteomics analysis reveals that proteins involved in eye development, visual perception and mitochondrial function are downregulated in Kl knockout mice[42]. Accordingly, Kl knockout mice have reduced retinal function, with functional deficit comparable to those observed in IGF-1 knockout mice[39]. Considering that Kl knockout mice are hypoglycemic, it can be hypothesized that the effects observed in the retina may be attributable to increased sensitive to the insulin and IGF-1 signaling.

Kokkinaki *et al*[41] demonstrated that Kl is expressed in primary cultures of RPE cells, mainly in the cell membrane, and that its depletion compromises several important function of RPE cells[41]. Moreover, they demonstrated that treatment with recombinant Kl protein has protective effects on RPE function, including phagocytosis, VEGF-A secretion, oxidative stress response, and melanogenesis.

Phagocytosis of POS is of particular importance in maintaining visual function and the visual cycle. It has been shown that transfection of RPE cells with Kl siRNA significantly reduced phagocytosis[41], suggesting that Kl is involved in the regulation of this important function. Evidences that treatment of RPE cells with Kl significantly increased phagocytosis in RPE cells confirm this hypothesis[41]. POS phagocytosis is regulated by several factors, among them, the Ca2+ signaling and the expression of Mer Tyrosine Kinase (MerTK) seem to play an important role[43]. Rise in intracellular Calcium is required for maintaining POS phagocytosis rate[44-46]. It has been reported that secreted Kl may regulate calcium homeostasis by affecting activity of calcium channels, including TRPVs and the Ca2+ release-activated Ca2+ channel (CRAC)[28,47,48]. Interestingly, human RPE expresses both TRPV5 and CRAC, which regulate calcium entry in this cells[49,50]. However, Kokkinaki *et al*[41] showed that treatment of RPE cells with Kl did not increase intracellular Calcium concentration[41], suggesting that Kl increases phagocytosis through a mechanism independent to calcium. Internalization of POS requires the engagement of MerTK, a cell surface receptor member of the tyro/Axl/Mer family of receptor tyrosine kinase, therefore MerTK expression is critical for POS phagocytosis[43]. Interestingly, it has been demonstrated that Kl regulates phagocytosis by upregulating MerTK expression, indeed treatment of RPE cells with Kl

Table 1 Main effects of Klotho on retinal cells

Functions	Effects of Klotho depletion	Effects of treatment with Klotho	Type of cell	Ref.
Phagocytosis	Reduced	Improved	RPE cells	[41,43]
		Increased expression of Mertk		
VEGF-A		Decreased secretion	RPE cells	[41]
		Reduced signaling mediated by VEGFR2- and IGF-1R		
Redox balance	Increased oxidative stress	Restored	RPE cells	[41,53]
		Prevention of ROS production		
		Increased NRF2 expression and nuclear translocation		
	Reduced expression of SOD2	Restored expression of SOD2 and CAT		
Pigmentation	Reduced		RPE cells	[41]
	Decreased melanin granules			
Mitochondrial function	Reduced biogenesis of mitochondria	Preserved	RPE cells	[53]
Autophagy		Improved	Retina	[42]
	Decreased activation of AMPK			
	Reduced expression of SIRT1			
EMT		Decreased expression of mesenchymal cell markers	RPE cells	[66]
Apoptosis		Reduced	RPE and retinal endothelial cells	[42,53,54]
		Increased expression of Bcl-2		
		Decreased expression of Bax		
		Decreased activity of Caspase-3		

VEGF-A: Vascular endothelial growth factor A; IGF-1R: Insulin-like growth factor-1; RPE: Retinal pigment epithelium; ROS: Reactive oxygen species; AMPK: 5' adenosine monophosphate-activated protein kinase; SIRT1: Silent information regulator 1; EMT: Epithelial-mesenchymal transition; NRF2: Nuclear factor E2-related factor 2; SOD2: Superoxide dismutase 2; CAT: Catalase.

induces intracellular signaling that leads to increased expression of MerTK and, consequently, improves phagocytosis efficiency[41].

VEGF-A is one of the main important pro-angiogenic factor and its excessive secretion is implicated in promoting the pathological neovascularization of the choroidal vasculature[51,52]. RPE cells are the major responsible of VEGF-A production in the retina. Treatment of the RPE cell line ARPE-19 with Kl significantly decreases VEGF-A secretion from both the apical and the basal sides[41]. Moreover, the presence of Kl inhibits the phosphorylation of VEGFR2 induced by VEGF-A, thus affecting intracellular signaling activated by VEGF-A.

Due to its extremely active metabolism, the retina is one of the organ with major request of oxygen, therefore it may be susceptible to overproduction of reactive oxygen species (ROS). Under normal conditions, ROS take part to the retinal physiological signaling, however, when generation of ROS exceeds the natural antioxidants defenses, oxidative stress may contribute to the pathogenesis of several retinal diseases, including DR. Experimental data demonstrate that Kl contributes to maintain the redox balance in the retina. Indeed, mRNA levels of Kl have been found significantly decreased in ARPE-19 cells treated with hydrogen peroxide (H_2O_2)[53]. Moreover, Kokkinaki *et al*[41] demonstrated that down-regulation of Kl expression leads to reduced expression of the anti-oxidant Superoxide dismutase 2 (SOD2) in RPE cells[41]. On the contrary, pretreatment with sKl prevented rise in ROS induced by H_2O_2 enhancing the antioxidant activities of ARPE-19[53], and decreased apoptosis induced by oxidative stress in human retinal endothelial cells[54].

Eye pigmentation is essential to maintain visual function. The RPE contribute to absorption of scattered light and to reduce retinal damage from ultraviolet light by forming a dark-brown pigmented wall[35,55]. Studies on models in which Kl expression has been down-regulated revealed that Kl is involved in regulation of genes encoding for melanin biosynthesis[41]. Indeed, pigmentation of eyes from Kl k/o mice was reduced and their RPE cells contained fewer melanin granules than normal RPE cells[41].

All these findings suggest that retina homeostasis may be affected by altered expression of Kl, as well altered levels of soluble Kl (Table 1).

Kl AND DR

Levels of sKl has been found reduced in ocular pathologies characterized by inflammatory state[56-59], suggesting that the reduced levels of sKl may be a common feature in several ocular diseases. In particular, decreased levels of Kl may be associated with increased risk of onset and worsening of DR. Indeed, circulating levels of Kl are lower in diabetic subject with DR than in those without this complication[54,60]. Moreover, serum Kl levels are negatively correlated with progression of DR[54,60]. Following the onset of DR in diabetic patients reveals that patients with progression of retinopathy had lower levels of serum Kl as compared to those without[60]. In addition, Ji *et al*[54] found that levels of sKl are gradually reduced among patient with diabetes without DR, non-proliferative DR (PDR) and PDR, independently of DN[54]. Corcillo *et al*[60] hypothesize that a halving of circulating Kl levels may increase the risk of retinopathy progression by 44%[60]. On the other hand, the incidence of the functional "KL-VS" variant of the Kl gene, which is associated with higher longevity in humans, is lower in people with DR and is associated with reduced serum levels of inflammatory markers and pro-angiogenic factors, suggesting that this genotype may be protective against retinopathy incidence[61].

As reported in the previous section, several experimental models demonstrated that depletion of Kl negatively affects important function of retinal cells, including oxidative stress response, VEGF-A secretion, and phagocytosis, leading to activation of mechanisms that may contribute to onset and progression of DR. On the other hand, there are also several evidence that treatment with recombinant sKl or overexpression of Kl ameliorate retinal function.

Oxidative stress and inflammation have been causative associated with DR[62,63]. It has been reported that Kl exerts protective effects against oxidative stress in retinal cells[13,41,42,53]. Firstly, it has been observed that pretreatment with sKl prevents increment of ROS production in ARPE-19 cells exposed to H_2O_2[41,53]. In particular, Wen *et al*[53] demonstrated that sKl improves redox balance in H_2O_2-treated ARPE-19 cells by increasing expression and nuclear translocation of nuclear factor E2-related factor 2 (Nrf2), thus restoring glutathione peroxidase, SOD2 and catalase to the levels of untreated cells[53]. In addition, pretreatment with sKl prevents H_2O_2-induced apoptosis of ARPE-19 cells[42,53], by increasing expression of Bcl-2 and decreasing the activation of caspase-3[53].

It is well established that VEGF-A plays an important role in driving pathological neovascularization of the retina during DR, and that neovascularization due to severe hypoxia is a hallmark of PDR[34]. The expression of VEGF-A is regulated by hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor involved in cellular response to hypoxia and hyperglycemia[64,65]. Interestingly, Kl levels have been found decreased in ARPE-19 cells exposed to hypoxia and in laser-induced CNV lesions in mice[66]. Xie *et al*[66] demonstrated that HIF-1α, besides directly increase VEGF-A transcription, may be responsible of down-regulation of Kl expression during hypoxia[66]. Indeed, HIF-1α activates p53, which, in turns, leads to the increased levels of miRNA34, that targets Kl thus reducing its expression[66]. Given that Kl is expressed in ocular tissues, it is possible that part of the sKl that acts in the eye derives by local shedding of mKl, therefore its contribution may be lost when expression of Kl is down-regulated. It has been reported that treatment with Kl reduces VEGF-A secretion from ARPE-19 cells[41]. In particular, Kl was able to decrease VEGF-A secretion by reducing phosphorylation of both IGF-1 receptor (IGF-1R) and VEGR2. The pathogenic role of IGF-1 in the development of PDR is still debated, several studies indicate that increased activation of IGF-1 signaling may contribute to retinal neovascularization, however a strong relationship between IGF-1 and the development of proliferative retinopathy has not been still clearly demonstrated[67-69]. Several studies reported that IGF-1R signaling is regulated by lipid raft integrity and interaction with caveolin-1[70-74]. In particular, down-regulation of caveolin-1 expression in RPE cells significantly reduces both basal and IGF-1-stimulated VEGF-A secretion[72]. These data together with the ability of Kl to modify the lipid organization within lipid rafts/caveolae[18] suggest that Kl may reduce the phosphorylation of IGF-1R by altering these microdomains. Hyperglycemia increases production and secretion of VEGF-A by Muller cells in the retina. In particular, Yu *et al*[75] demonstrated that hyperglycemia increases the production of VEGF-A in Muller glial cells through the activation of FGFR1[75]. It is well known that sKl acts as a co-receptor for FGFs at non-renal sites and activates protective pathways in several cell types[76,77]. Interestingly, screening the potential pathogenic genes associated with DR revealed that hyperglycemia increases the expression of FGF23[78], and of its membrane receptor FGFR1 on Muller glial cells[75,79]. Considering that absence of Kl may allow Kl-independent activation of FGFRs resulting in pathological cellular changes[17,76,77], and that Kl-independent action of FGF23 has been reported to contribute to endothelial dysfunction[17], these findings suggest that lower levels of Kl together with increased production of FGF23 may contribute to the onset of DR and to progression to PDR by increasing VEGF-A production.

Autophagy is a highly conserved lysosomal pathway for the turnover of cytoplasmic organelles and long-lived proteins that acts as an adaptive response to cellular stresses and regulates homeostasis, differentiation, development and survival in several cell types[80]. In retinal cells, autophagy plays an important role by participating to POS degradation, visual pigment recycling, and lipofuscin degradation[81-83]. Altered activation of autophagy has been found in experimental models of DR and in the retina of diabetic patients[84,85]. For instance, RPE cells exposed to high glucose concentration increase formation of autophagosome, suggesting that induction of autophagy is a cytoprotective response against high glucose (HG)[84,85]. However, the excessive activation of this mechanism may lead to its impairment as occur in retinal Muller cells, where the process of degradation cannot be completed due to the lysosomal dysfunction[85]. It has been reported that autophagic activity is reduced in DM mice and human renal proximal tubule cells exposed to HG[86]. Recent studies showed that Kl may act as a regulator of autophagy even in diabetic condition[87]. Specific expression of Kl significantly improves autophagy in both pancreatic beta cells and in renal tubule cells exposed to HG[29,86]. Moreover, Zou *et al*[21] showed that activation of 5' adenosine monophosphate-activated protein kinase (AMPK), a positive regulator of autophagy, is significantly decreased in the retina of Kl deficient mice as compared to that of WT mice[42]. Although there is no direct evidence, these finding suggest that Kl may affect autophagy also in retinal cells. A decreased activation of AMPK has been observed also in arterial endothelial cells of Kl deficient mice[88], confirming that

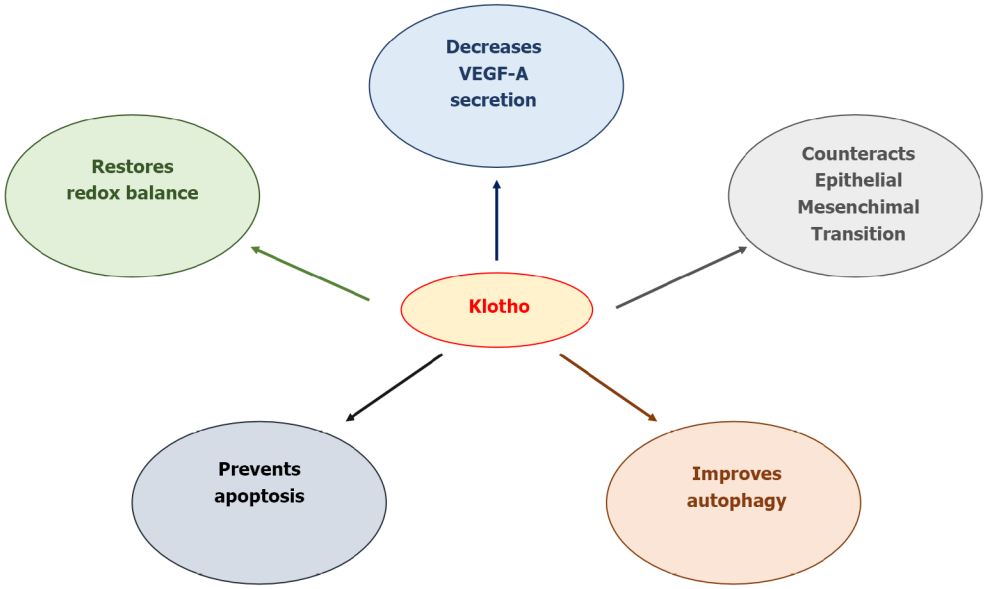

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Figure 1 Positive effects of Klotho in diabetic retinopathy. VEGF-A: Vascular endothelial growth factor A.

AMPK is a crucial mediator of protective effects of Kl. Moreover, Kl deficient mice have also reduced activity of silent information regulator (SIRT) 1[88], another important player in autophagy[89]. Interestingly, the expression of SIRT1 is reduced in DR and intravitreal administration of SIRT1 reverses DR in a mouse model of type 2 diabetes[90]. These results suggest that regulation of SIRT1 may be another mechanism through which Kl improve DR.

PDR is also characterized by formation of fibrous proliferative anterior membrane[91]. Subretinal fibrosis is mediated by EMT, a process that leads RPE cells to the acquisition of a mesenchymal phenotype[92]. Several evidence demonstrated that HG induce EMT in RPE[93,94]. It has been shown that Kl expression is down-regulated in models of induced fibrosis, suggesting a protective role of Kl[22,95,96]. In particular, the protective effects of Kl have been related to inhibition of the Wnt/β-catenin and the Egr-mediated signaling pathways. Recently, it has been reported that overexpression of Kl decreased the expression of mesenchymal cell markers induced by hypoxia in ARPE-19 cells[66]. Moreover, overexpression of Kl was able to reduce subretinal fibrosis in a mouse laser-induced CNV model[66]. Here, under hypoxic conditions, Kl was able to block the axis that through HIF-1α leads to the activation of p53 and promotes EMT in RPE cells, confirming that Kl may be useful in preventing EMT also in RPE cells.

Besides hyperglycemia, dyslipidemia is another important actor in the progression of DR[97,98]. Palmitic acid (PA) is involved in the onset of DR and may induce endothelial cell damage[98]. It has been demonstrated that Kl pretreatment significantly reduces apoptosis induced by PA in human retinal endothelial cells[54]. This effect implies the activation of the PI3K and subsequent phosphorylation of AKT[54]. Moreover, Kl affects expression of proteins involved in apoptosis leading to increased expression of the anti-apoptotic Bcl-2 and down-regulation of the pro-apoptotic Bax[54]. Consistent with these data, pretreatment with Kl reduced the apoptosis rate in ARPE-19 cells exposed to H_2O_2 by up-regulating Bcl-2 expression and decreasing levels of Bax[53]. In addition, Kl was able to prevent the decrease of mitochondrial membrane potential and the activation of Caspase-3 induced by H_2O_2[53].

CONCLUSION

DR is a common complication of diabetes. The International Diabetes Federation estimated the global population with diabetes mellitus to be 463 million in 2019 and 700 million in 2045[99]. These data require the development of strategies able to prevents the onset and the progression of DR. To date, the first line treatment for PDR is intravitreal anti-VEGF therapy. However, it is not so successful for routine treatment of non-PDR[32,100]. Therefore, new molecules in development have been designed to target other pathways involved in pathogenesis of DR[101,102]. It has been demonstrated that Kl has protective effects in DN and that pathological mechanisms between DR and DN share similarities[19,29], suggesting that Kl may be a good candidate in counteracting DR. Experimental models targeting Kl have been shown to have positive effects on several mechanisms involved in DR onset and progression (Figure 1). Therefore, Kl may become a novel biomarker and a good candidate for the treatment of DR[60].

FOOTNOTES

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Abstract

Diabetes is a complex condition, and the causes are still not fully understood. However, a growing body of evidence suggests that exposure to air pollution could be linked to an increased risk of diabetes. Specifically, exposure to certain pollutants, such as particulate Matter and Ozone, has been associated with higher rates of diabetes. At the same time, air pollution has also been linked to an increased risk of thyroid cancer. While there is less evidence linking air pollution to thyroid cancer than to diabetes, it is clear that air pollution could have severe implications for thyroid health. Air pollution could increase the risk of diabetes and thyroid cancer through several mechanisms. For example, air pollution could increase inflammation in the body, which is linked to an increased risk of diabetes and thyroid cancer. Air pollution could also increase oxidative stress, which is linked to an increased risk of diabetes and thyroid cancer. Additionally, air pollution could increase the risk of diabetes and thyroid cancer by affecting the endocrine system. This review explores the link between diabetes and air

pollution on thyroid cancer. We will discuss the evidence for an association between air pollution exposure and diabetes and thyroid cancer, as well as the potential implications of air pollution for thyroid health. Given the connections between diabetes, air pollution, and thyroid cancer, it is essential to take preventive measures to reduce the risk of developing the condition.

Key Words: Air pollution; Diabetes mellitus; Health risk; Thyroid cancer; Thyroid disorders

Core Tip: Although the direct link between diabetes and air pollution on thyroid cancer is not yet established, recent research has suggested a strong correlation between air pollution exposure and the risk of endocrinopathies and developing certain types of cancer, including thyroid cancer. This suggests that people with diabetes may be at an increased risk of developing thyroid cancer if exposed to high levels of air pollution. It is essential for people with diabetes to be aware of the potential health risks associated with air pollution and to take steps to reduce their exposure to air pollution and to control their blood glucose levels as well as eat healthy food.

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INTRODUCTION

Diabetes mellitus (DM) and thyroid dysfunction are the most common endocrinopathies[1]. There is accumulating evidence indicating a contribution of thyroid hormone dysfunction to type 2 DM (T2DM) and vice versa[1,2]. Thyroid hormones have a direct effect on insulin production and clearance. Fluctuations in thyroid hormones raise the risk of developing T2DM and can worsen diabetic symptoms and complications[1,3]. In 2017, patients with DM reached 476 million affected people worldwide, with an expected projection of 570.9 and 783.2 million in 2025 and 2045, respectively [4,5]. Patients with DM are at higher risk of vascular disease and poor lung function, rendering them vulnerable to declining air quality[6]. A growing body of evidence suggests that exposure to air pollution could be linked to an increased risk of diabetes[7]. Specifically, exposure to certain pollutants, such as particulate matter (PM) – the primary carbon-based component of air pollution – and ozone, has been associated with higher rates of diabetes[7]. At the same time, air pollution has also been linked to an increased risk of thyroid disorders, including thyroid cancer (TC)[8]. The latter is an endocrine tumor with the highest occurrence, and its incidence has increased in recent decades[9]. By 2030, this type of cancer is anticipated to rank as the fourth-most frequent cancer in the United States[10]. While there is less evidence linking air pollution to TC than to diabetes, it is clear that air pollution could have severe implications for thyroid health[11].

This narrative review aims to explore the link between diabetes and air pollution on thyroid cancer. The evidence for an association between air pollution exposure and both diabetes and thyroid cancer, as well as the potential mechanisms underlying this type of synergism, will be discussed.

LITERATURE SEARCH

Literature was screened *via* several electronic databases such as PubMed, Google Scholar, and Web of Science. The compiled literature included peer-reviewed articles published from 1991 to 2022 written in English. Authors utilized the phrases "Diabetes mellitus, type 1 diabetes, type 2 diabetes, particulate matter, air pollution, hyperthyroidism, hypothyroidism, thyroid carcinoma, insulin resistance" in the screening process. Organizational reports, literature reviews, cross-sectional studies, cohort studies, clinical studies, animal studies, and time series categories of literature were retained, and letters of opinion were excluded. Literature deemed acceptable was screened with a focus on: (1) The prevalence and incidence of DM and thyroid pathology and their respective etiologies; (2) Air pollution and particulate matter trends globally stemming from anthropogenic PM production; and (3) Non-duplicate studies, in which examples of comparative literature were decided upon by more recent publication. Additionally, data mining in the publicly available "comparative toxicogenomic database; CTD" (http://ctdbase.org/) (last accessed 25 March, 2023) was done to unravel how environmental exposures to the specified pollutant of the current review could impact human health[12].

PATHOGENESIS

An overview of the problem

Many factors play significant roles in the development of DM and thyroid diseases, such as genetic liability, environmental factors, lifestyle, family history, and comorbidities[13-15]. Exposures to specific environmental toxicants, such as air pollution, have been reported to have a negative impact on the thyroid gland and pancreas[7]. Global populations are growing annually, and an expanding populace comes with an increased demand for industrialization[16]. The World Health Organization (WHO) has identified industrial development as a significant driver of air pollution, with fossil fuel consumption, large-scale agriculture, and the accelerating need to meet comfortable lifestyle parameters as significant contributors[17]. The WHO defines air pollution as "contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere"[17]. The air pollutants with the most significant negative impact on public health are sulfur dioxide, carbon monoxide, nitrogen dioxide (NO_2), ozone, and fine PM[18] (Tables 1-5), respectively. According to the International Agency for Research on Cancer Working Group, air pollution was categorized as carcinogenic in 2013[19]. The damaging effect of these pollutants substantially depends on the pollutants' type, the dose and time of exposure, and the body's accumulation of pollutants over time[20]. PM, also known as atmospheric aerosol, comprises the deleterious component of air pollution established to be harmful to human health[21] and has been associated with numerous cancers, endocrine disorders, cardiovascular diseases, and other forms of significant inflammation[22]. Patients with high-risk pulmonary conditions such as asthma, chronic obstructive pulmonary disease, lung cancer, and so forth are of frequent consideration with rising PM levels globally, yet impacts on the endocrine system are substantial[23]. Increasing DM cases globally pose a point of concern, as complications of the disease may manifest in acute and chronic settings, with consequences including declining patient quality of life, healthcare costs, and economic burden[5]. Coronary artery disease, stroke, peripheral vascular disease, end-stage renal disease, neuropathy, and lower-extremity amputation comprise the most burdensome complications. Notably, excluding confounding factors such as environmental conditions, physical activity, family history of TC, genetic sustainability, dietary habits, and history of radiation exposure should be done to link air pollution to DM and thyroid diseases [24].

Diabetes is multifactorial in origin, with T2DM being more so reliant on lifestyle and environmental risk factors[25], as opposed to its more genetic-reliant counterpart type 1 DM (T1DM) (still influenced by environment and lifestyle, although a lesser degree). Recently, T2DM was also occurring increasingly frequently in children[26]. A recent meta-analysis from Yang *et al*[27] has highlighted the substantial role PM exposure plays in the development of T2DM, with proposed mechanisms predominantly pertaining to increased systemic inflammation, mitochondrial dysfunction, and cardiovascular stress, with the contribution of some epigenetic changes. When controlling for genetic risk factors, air pollution was still found to impact T2DM development significantly[23]. While the weight of these findings alone is undoubtedly essential, with air pollution rates rising globally and a curbing solution yet to be implemented, it is of utmost importance to examine the intricate web of PM's impact on the endocrine system and alternate routes of exacerbation in the diabetes crisis. Diabetes may be the most common endocrine disease, but thyroid disease follows closely as one of the most prevalent endocrine organ diseases[28].

Patients diagnosed with DM, interestingly, exhibit a higher rate of hyperthyroidism than the non-diabetic remainder [29]. About 4.4% of T2DM patients over eighteen exhibit overt hyperthyroidism, and 2%-4% exhibit subclinical hyperthyroidism[30]. Glycemic control deteriorates in hyperthyroid diabetic individuals. Excess TH in the blood is linked to hyperglycemia, low circulating insulin levels, and poor glycemic control in hyperthyroidism. Nearly 2%-3% of patients having hyperthyroidism progress into developing overt diabetes[31]. In Grave's disease, a hyperthyroid condition of autoimmune origin, modest glucose intolerance is seen in over 50% of patients[31]. Thyrotoxicosis has been found to lead to endothelial dysfunction[32] and diabetic ketoacidosis[33], among other consequences. As a result, cardiovascular comorbidities are at a higher rate due to endothelial dysfunction, potentially contributing to the worsening of vascular integrity in patients diagnosed with existing T2DM or progression toward it. With accumulating data establishing connections between the two endocrine disease groups, it is crucial to assess possible physiologic links further to bolster clinical intervention methods, identify prevention strategies, and, in time, mitigate risk of T2DM development.

Air pollution role in thyroid disease and type 2 diabetes

Air pollution is a significant issue that affects human health on a global scale, mainly in crowded industrial cities where the daily emission of PM and other pollutants continuously exceeds permitted levels[34]. More people are affected by PM than by any other pollution[35]. Sulfate, nitrates, ammonia, sodium chloride, black carbon, mineral particles, and water are the main components of PM, which comprises a complex mixture of solid and liquid particles of organic and inorganic materials suspended in the air. The Environmental Protection Agency classified PM based on aerodynamic diameter into ($PM_{2.5}$: ≤ 2.5 mm) and (PM_{10}: ≤ 10 mm)[36]. $PM_{2.5}$ comprises "secondary" particles formed in the atmosphere by the chemical reactions of gaseous emissions, whereas PM_{10} is composed of coarse or "primary" particles, such as dust and carbon dioxide combustion[36]. These particles can be inhaled and enter the bloodstream[37].

According to the WHO, $PM_{2.5}$ is frequently used to indicate air pollution, and the upper limit concentration of $PM_{2.5}$ is set at 10 mg/m^3[38]. Globally, PM pollution in the atmosphere is increasing. $PM_{2.5}$ levels in India and China increased by 69.8% and 52.7%, respectively. These raise alarming signs in areas where the health burden of air pollution is high[39]. However, a few studies have evaluated the impact of $PM_{2.5}$ on human health[39]. High levels of $PM_{2.5}$ are linked with negative impacts on cardiovascular diseases, cognitive deterioration, and mortality, among others[40] (Table 5). Even though there have been a few studies regarding the relationship between air pollution and TC, it has been suggested that air pollution is a potential risk factor for rising TC risks[24]. Remarkably, In the Chinese population, industrial waste gas air pollution was significantly linked to an increased risk of TC[9,41]. A recent study reported that the incidence of

Table 1 Summary of the impact of sulfur dioxide on human health

Type of interaction	Ref. (PMID)
Sulfur Dioxide results in increased interleukin-6 production	20056584
Sulfur Dioxide affects the glucose metabolic process	26166095
[Air Pollutants results in increased abundance of Sulfur Dioxide] which affects the regulation of heart rate	28129768
[Air Pollutants results in increased abundance of Sulfur Dioxide] which affects the regulation of systemic arterial blood pressure	27015811
[Air Pollutants results in increased abundance of Sulfur Dioxide] which results in increased response to oxidative stress	27015811
Sulfur Dioxide results in decreased leukocyte homeostasis	30826618
Sulfur Dioxide decreases the respiratory system process	32000783
Sulfur Dioxide affects cytokine production involved in the immune response	32000783
[[*TNF* gene SNP affects the susceptibility to [[Air Pollutants results in increased abundance of Fuel Oils] which results in increased abundance of Sulfur Dioxide]] which results in increased tumor necrosis factor production] which results in increased secretion of TNF protein	24056475

Data source: The comparative toxicogenomic database (http://ctdbase.org/)[12].

Table 2 Summary of the impact of carbon monoxide on human health

Type of interaction	Ref. (PMID)
Carbon Monoxide inhibits the reaction [Rotenone results in increased apoptotic process]	23593279
Carbon Monoxide results in the decreased xenobiotic catabolic process	7908050
Carbon Monoxide inhibits the reaction [NADP results in increased oxidative demethylation]	8498088
[*IL6* gene SNP results in increased susceptibility to Carbon Monoxide] which results in increased positive regulation of interleukin-6 production	19750100
[Air Pollutants results in increased abundance of Carbon Monoxide] which results in decreased response to bronchodilator	26187234
Carbon Monoxide results in an increased inflammatory response	23717615
[Air Pollutants result in an increased abundance of Carbon Monoxide] which affects the regulation of blood pressure	28732501
[Air Pollutants results in increased abundance of Carbon Monoxide] which affects the regulation of heart rate	28129768
Carbon Monoxide inhibits the reaction [HMOX1 protein affects the reaction [Ammonium Chloride inhibits the reaction [[TNF protein co-treated with Cycloheximide] results in decreased cell growth]]]	27867098
Carbon Monoxide inhibits the reaction [[TNF protein co-treated with Cycloheximide] results in decreased cell growth]	27867098
Carbon Monoxide results in decreased leukocyte homeostasis	30826618
Carbon Monoxide results in the decreased respiratory system process	31861594 | 32000783
Carbon Monoxide affects cytokine production involved in the immune response	32000783
[Air Pollutants results in increased abundance of Carbon Monoxide] which affects T cell homeostasis	33603036
[Air Pollutants result in an increased abundance of Carbon Monoxide] which affects the regulation of blood pressure	33603036
[[[Vehicle Emissions results in increased abundance of Air Pollutants] which results in increased abundance of Carbon Monoxide] which results in increased membrane lipid catabolic process] which results in increased abundance of 8-epi-prostaglandin F2alpha	34417545

Data source: The comparative toxicogenomic database (http://ctdbase.org/)[12].

papillary thyroid carcinoma with 2 and 3 years of $PM_{2.5}$ exposure is directly linked to the dose and duration of exposure to $PM_{2.5}$[42]. Although Yanagi *et al*[43] stated that the statistical correlation between overall exposure to urban PM_{10} and TC incidence was high and significant, Park *et al*[24] reported a negative correlation between PM_{10} and TC.

A retrospective population-based study conducted in Shanghai, China, by Cong *et al*[41] recruited 550000 new cancer patients for assessment, and the investigators found that TC incidence was positively correlated with ambient air pollution from waste gas emissions, linking thyroid pathology and PM. Air pollution and its insidious hazards garnered attention in the American public's concerns following the aftermath of 9/11, in which first responders and other persons exposed to the explosion's remains began reporting alarmingly high rates of TC[44]. The Solan *et al*[45] study of 9/11 first responders, including 20984 participants, found that those assisting on-site exhibited an increased TC standardized

Table 3 Summary of the impact of nitrogen dioxide on human health

Type of interaction	Ref. (PMID)
Regulation of inflammatory response	18560490
Regulation of gene expression	22306530
Glucose metabolic process	26166095
[Air Pollutants result in an increased abundance of NO_2] which affects the regulation of blood pressure	27219456
[Nitrogen Dioxide results in decreased mitochondrial DNA metabolic process] which affects the expression of ND1 mRNA	26317635
[Air Pollutants results in increased abundance of Nitrogen Dioxide] which affects DNA methylation on cytosine within a CG sequence	27448387
[Air Pollutants results in increased abundance of NO_2] which results in decreased hemoglobin biosynthesis	28153527
[[Vehicle Emissions results in increased abundance of Air Pollutants] which results in increased abundance of Nitrogen Dioxide] which results in increased positive regulation of interleukin-6/10/13/ tumor necrosis factor (TNF) production	28669936
[Air Pollutants results in increased abundance of NO_2] which results in increased response to oxidative stress	27015811
Nitrogen Dioxide affects musculoskeletal movement	29364820
[Air Pollutants results in increased abundance of NO_2] which results in decreased cognition	28921105
[Air Pollutants results in increased abundance of NO_2] which results in decreased motor behavior	28921105
Decreased leukocyte homeostasis	30826618
cytokine-mediated signaling pathway	29114965
[Air Pollutants results in increased abundance of NO_2] which results in increased negative regulation of telomere maintenance	31393792
Cytokine production is involved in the immune response	32000783
[[Air Pollutants results in increased abundance of NO_2] which affects glucose homeostasis] which affects the abundance of Blood Glucose	32552747
[[Air Pollutants results in increased abundance of NO_2] which affects the regulation of cholesterol metabolic process] which affects the abundance of Cholesterol	31622905
[Air Pollutants results in increased abundance of NO_2] which affects T cell homeostasis	33603036
[Air Pollutants results in increased abundance of NO_2] which affects B cell homeostasis	33603036
[[[Vehicle Emissions results in increased abundance of Air Pollutants] which results in increased abundance of NO_2] which results in increased negative regulation of cholesterol metabolic process] which results in decreased abundance of cholesterol, HDL, and membrane lipid catabolic process	34417545

Data source: The comparative toxicogenomic database (http://ctdbase.org/)[12].

incidence rate of 2.39, seven years post-exposure. While it is not incorrect to assert that TC rates have increased globally due in part to enhanced detection capability, data from the Solan *et al*[45] study suggests a robust correlative effect. Should the higher incidence be a product of screening opportunity, one would expect increased detection of small, localized, early-stage cancer; yet, 40% of patients exposed to Ground Zero diagnosed with TC presented with more advanced disease, including lymph node metastasis[44], suggesting PM exposure to be of significance in thyroid disease etiology and progression. Ghassabian *et al*[46] reported that only high exposure to $PM_{2.5}$ was linked to hypothyroxinemia. It is firmly established that hyperthyroidism is associated with a high incidence of TC[47]; however, hyperthyroidism may be the pathological link between PM exposure and TC development and progression, and further investigation is necessary to confirm or deny the actual mechanism.

NO_2 is a reactive compound and a potential endocrine-disrupting chemical in polluted air with several health impacts [24] (Table 3). A significant association between chronic exposure to NO_2 and TC (1.33, 95%CI: 1.24-1.43, $P < 0.001$) has been documented[24]. Zaccarelli-Marino *et al*[48] found that a raised NO_2 concentration in air pollutants revealed a strong correlation with elevated odds of primary hypothyroidism (spearman correlation coefficients; adolescent female = 0.94, adolescent male = 0.94). Exposure to NO_2 was linked to TC in a study conducted in cohort data of 4632 patients with TC from 2002 to 2015[24]. Additionally, exposure to ambient NO_2 was significantly associated with reduced free thyroxine (FT4) concentration and a rise in thyroid-stimulating hormone (TSH)[49]. Interestingly, the increased circulating TSH level due to NO_2 exposure was followed by increased TSH receptor signaling and, consequently, a rise in thyroid cancer [24,50].

Furthermore, Zeng *et al*[51] performed a retrospective cross-sectional study and found that a 10 μg/m^3 increase in $PM_{2.5}$ was linked with a decrease in FT4 and an increase in FT3, and the FT4/FT3 ratio was inversely associated with $PM_{2.5}$ (coefficient: −0.06, $P < 0.01$). Dong *et al*[52] stated that $PM_{2.5}$ exposure could perturb TH homeostasis by affecting TH biosynthesis, biotransformation, and transport, affecting TH receptor levels, and inducing oxidative stress and inflammatory responses in female rats. $PM_{2.5}$ induced oxidative stress accompanied by pathologic changes in rat thyroid and

Table 4 Summary of the impact of ozone on human health

Type of interaction	Ref. (PMID)
Ozone results in increased gene expression	18332784
Ozone affects heart contraction	18091001
Ozone affects the regulation of inflammatory response	18560490
Ozone results in increased interleukin-6 production	20056584
[Vehicle Emissions co-treated with Ozone] affects neutrophil, lymphocyte, and monocyte homeostasis	27058360
[Air Pollutants results in increased abundance of Ozone] which results in increased DNA methylation	27219456
DNMT1 gene polymorphism affects the reaction [[Air Pollutants results in increased abundance of Ozone] which affects the regulation of blood pressure]	27219456
Ozone results in increased cholesterol metabolic process	27703007
[Cholesterol co-treated with Ozone] results in increased protein lipidation	27703007
Ozone results in increased mRNA and rRNA transcription	28652203
[Dust co-treated with Ozone] results in increased negative regulation of lymphoid progenitor cell differentiation	29767793
[Dust co-treated with Ozone] results in increased positive regulation of reactive oxygen species biosynthetic process	29767793
Ozone results in increased positive regulation of glycolytic process and cellular response to oxidative stress	29471466
Ozone results in increased positive regulation of proteolysis and amino acid metabolic process	29471466
Ozone affects the regulation of the membrane lipid metabolic process	29471466
Ozone results in increased tissue regeneration	29471466
[Air Pollutants results in increased abundance of Ozone] which affects the regulation of heart rate	28129768
Ozone results in increased positive regulation of ERK1, ERK2, and p38MAPK cascade	29925859
Ozone results in increased iron ion transport, homeostasis	24862973
Ozone results in increased viral entry into the host cell and the viral life cycle	22496898
Ozone results in increased chloride transmembrane transport	27886375
Ozone affects cytokine production involved in the immune response	32000783
[Air Pollutants results in increased abundance of Ozone] which results in increased positive regulation of heart rate	31349208
[Ozone results in increased oxidation of dimethylselenide] which results in increased ncRNA transcription	33656867
Ozone affects the aspartate/glutamate/ornithine/taurine metabolic process	33993003
[Oxygen co-treated with Ozone] results in the decreased cellular metabolic process	32992648
[Oxygen co-treated with Ozone] results in increased necrotic cell death	32992648
[Air Pollutants results in increased abundance of Ozone] which affects T cell homeostasis	33603036

Data source: The comparative toxicogenomic database (http://ctdbase.org/)[12].

liver characterized by increased follicular cavity size and decreased amounts of follicular epithelial cells and fat vacuoles [52]. Activation of the hypothalamic-pituitary-thyroid axis and altered hepatic transthyretin levels, therefore, play a crucial role in $PM_{2.5}$-induced thyroid dysfunction[52]. In addition, NO and PM with a diameter of fewer than 10 μm are the air pollutants most influential on diabetes[20].

CO exposure has been shown to have a negative impact on thyroid function and the pancreas, particularly in cigarette smoking[53,54]. A national cohort study from Taiwan confirmed that exposure to CO increases the risk of developing hypothyroidism[55]. A study of adult Koreans shows that a significantly high serum concentration of TSH and low FT4 could be attributed to CO exposure, especially in overweight or obese older people than younger adults[49].

Air pollution could play a role in genomic instability, driving the tumorigenesis process[34]. PM and NO_2 have been reported to be endocrine-disruptive compounds and carcinogenic in humans[24,42]. Exposure to $PM_{10,}$ $PM_{2.5}$, and NO_2 was closely associated with thyroid cancer occurrence[24,42]. At the cellular level, PM and NO_2 can have several impacts, including inflammation, DNA damage, and genomic instability[34,56]. NO_2 exposure mediates oxidative stress and inflammation pathways; thus, it has been classified as a carcinogen[56]. NO_2 induces oxidative stress, interacts with unsaturated fatty acids, and causes organic molecules to undergo autooxidation, which can start free radical processes

Table 5 Some examples of the impact of particulate matter on human health

Type of interaction	Ref. (PMID)
[Air Pollutants results in increased abundance of Particulate Matter] which affects glucose homeostasis	27219535
Affects the glucose metabolic process	29616776 | 31851346
[Particulate Matter results in increased lipid oxidation] which results in an increased abundance of 4-hydroxy-2-nonenal	30716388
Affects the thyroid hormone metabolic process	27623605
[Vehicle Emissions results in increased abundance of Particulate Matter] which results in increased positive regulation of superoxide anion generation	28013216
Results in increased cell death	26856867
Results in increased reactive oxygen species metabolic process	21384498
Affects the positive regulation of cellular response to oxidative stress	23542817
[Particulate Matter co-treated with Biological Products] affects positive regulation of the apoptotic process	23454527
Particulate Matter affects the positive regulation of interleukin-6/8 production and NF-kB transcription factor activity	23201440
Results in decreased cell population proliferation	23722391
Results in increased T-helper 2 cell chemotaxis	16890758
Results in increased cell population proliferation	16455839
Results in increased negative regulation of mitotic cell cycle	25336953
Results in increased lipid catabolic process	21233593
Results in increased positive regulation of p38MAPK cascade	23900936
Results in increased positive regulation of apoptotic DNA fragmentation	26507108
Affects the vascular process in the circulatory system	25233101
Affects inflammatory response	25233101
Affects the insulin metabolic process	25233101
Results in increased inflammatory response	25479755
Results in decreased cognition	27128166
Affects the cholesterol biosynthetic process	26967543
Affects the positive regulation of telomere maintenance *via* telomere lengthening	21169126
Results in increased positive regulation of autophagosome assembly	27125970
[Air Pollutants result in an increased abundance of Particulate Matter] which affects the regulation of endothelial cell differentiation	27311922
[Vehicle Emissions results in increased abundance of Particulate Matter] which results in increased respiratory burst after phagocytosis	28013216
Affects the electron transport chain, mitochondrial translation, and tricarboxylic acid cycle	28821289
Affects the regulation of mitochondrial membrane potential	26989813
Results in decreased superoxide dismutase activity	26989813
Results in increased positive regulation of endothelial cell activation	29244817
Affects histone modification	27918982
Affects gene expression	25564368 | 28821289 | 29114965 | 29342453
Affects T and B cell homeostasis	20678227
[Vehicle Emissions results in increased abundance of Particulate Matter] which results in increased cellular senescence	31551408
[[Vehicle Emissions results in increased abundance of Particulate Matter] which co-treated with Oleic Acid] results in increased triglyceride biosynthetic process	31340670
[Air Pollutants results in increased abundance of Particulate Matter] which affects negative regulation of DNA-	26298100

templated transcription	
Results in increased cell migration and cell chemotaxis	29913439
Results in decreased learning or memory	31881430
Results in increased activation of protein kinase B activity and p38MAPK cascade	32687961
Results in decreased endothelial cell-cell adhesion	33159583
[Air Pollutants result in an increased abundance of Particulate Matter] which affects ATP metabolic process	32487172

Data source: The comparative toxicogenomic database (http://ctdbase.org/)[12].

[57]. The induced systemic inflammation and the immune response to autoantigens resulting in the production of reactive oxygen species have been proposed as mechanisms of PM carcinogenesis in thyroid cancer patients[56]. Oziol *et al*[58] reported that ambient air in French urban areas had thyroid receptor alpha-1 agonistic effects without competitive effects concerning T3-dependent transcriptional activity. Similarly, Nováková *et al*[59] conducted an *in vitro* experiment and found that exposure to PM_{10} in ambient air significantly increased thyroid receptor-mediated activity.

Numerous air pollutants have also been linked to other diseases of systemic inflammation[60]. Air pollution modifies T-cell-dependent immunity, predisposing to autoimmune illnesses and inflammation[61]. It may also cause oxidative stress and lung formation of reactive oxygen species to harm the beta cells in the pancreas, which would limit insulin release and contribute to T2DM risk[62,63]. According to research by Chuang *et al*[64], exposure to PM_{10} alters blood pressure, blood lipids, and hemoglobin A1c. Chronic exposure to such particles increases the risk of lung cancer, as well as respiratory and cardiovascular problems, further fueling T2DM morbidity. In an Iranian study by Kelishadi *et al*[63], the investigators found that PM_{10} was positively correlated with insulin resistance in children. The risk of developing insulin resistance was later discovered to be positively correlated with residential proximity to high levels of automotive traffic – and subsequently a high degree of PM – among a German cohort of children[65]. Impaired glucose tolerance in pregnancy is also linked to exposure to traffic-related air pollution[66]. The possible inhibition of T suppressor cells is also one of the main links in the genetic predisposition for autoimmune TD. In this situation, T helper cells have a great deal to do, both in the activation of B lymphocytes, which create enhanced thyroid antibodies, and so also interferon[18]. High exposure to $PM_{2.5}$ and NO_2 in the first trimester of pregnancy is associated with mild thyroid dysfunction with positive thyroid peroxidase antibodies[46]. Figure 1 summarizes the synergetic impact of air pollution and diabetes on thyroid tumorigenesis risk.

Thyroid dysregulation as a diabetes risk factor

The lab of Brandt *et al*[30] found, in a Danish study conducted on a national level, that patients exhibiting hyperthyroidism – clinical or subclinical – had a greater risk of developing T2DM. TSH levels in patients with subclinical hyperthyroidism and pre-existing diabetes can be returned to normal function as diabetes control improves, indicating that T2DM therapies may help restore normal thyroid function prior to progression to overt hyperthyroidism for these patients[67]. However, a recent study found that hyperthyroidism patients who did not have diabetes had a higher chance of progressing to T2DM later in life than euthyroid cohorts. Thus, it is likely that thyroid dysfunction may occur before diabetogenic processes as a primary catalyst[68].

Insulin resistance in hyperthyroidism

Hyperthyroidism can often be detected clinically by characteristic symptoms, including palpitations, fatigue, tremor, weight loss, anxiety, and excessive sweating. However, subclinical hyperthyroidism may exist with few, if any, symptoms and is characterized by low TSH levels despite adequate TH levels. A study assessing individuals with either overt or subclinical hyperthyroidism who underwent a glucose tolerance test found that higher blood levels of both glucose and insulin may be found in either form[69]. Increased Cory cycle activity, which suggests that muscle tissue serves as a source of substrates for hepatic gluconeogenesis, supports higher rates of gluconeogenesis (lactate and certain amino acids such as alanine and glutamine). This process entails a dynamic glucose buffer that enables other tissues to utilize it as necessary when they have a glucose demand. Phosphoenolpyruvate carboxykinase is the rate-limiting step in gluconeogenesis, and it is known that TH – specifically triiodothyronine (T3) – increases its expression in the liver, indicating a direct involvement for THs in the control of endogenous glucose production[69]. High THs also increase gluconeogenesis through accelerated lipid mobilization as well[69]. Inducing Sterol response element-binding protein 2 expression and enhancing LDL receptor expression, TH lowers blood levels of TGs and cholesterol-containing lipoproteins. This potentiates hepatic cholesterol absorption. The mechanism is presumed to occur through increasing the expression of acetyl CoA carboxylase and carnitine palmitoyltransferase Iα, which will increase the hepatic uptake of fatty acids[70].

It has been demonstrated that hepatic insulin resistance in hyperthyroid patients increases gluconeogenesis and, subsequently, hepatic glucose production[71,72]. Studies mimicking hyperthyroidism in mice *via* exogenous T4 have shed light on insulin signaling concerning TH; despite fasting conditions, insulin target tissues demonstrate active insulin signaling, presumed to result from deregulated insulin production from the endocrine pancreas[73]. Compared to healthy people, hyperthyroid patients have higher basal hepatic glucose production and fasting insulin levels; however, when treated with methimazole (an antithyroid agent), these levels were dramatically minimized, reducing THs to the levels of

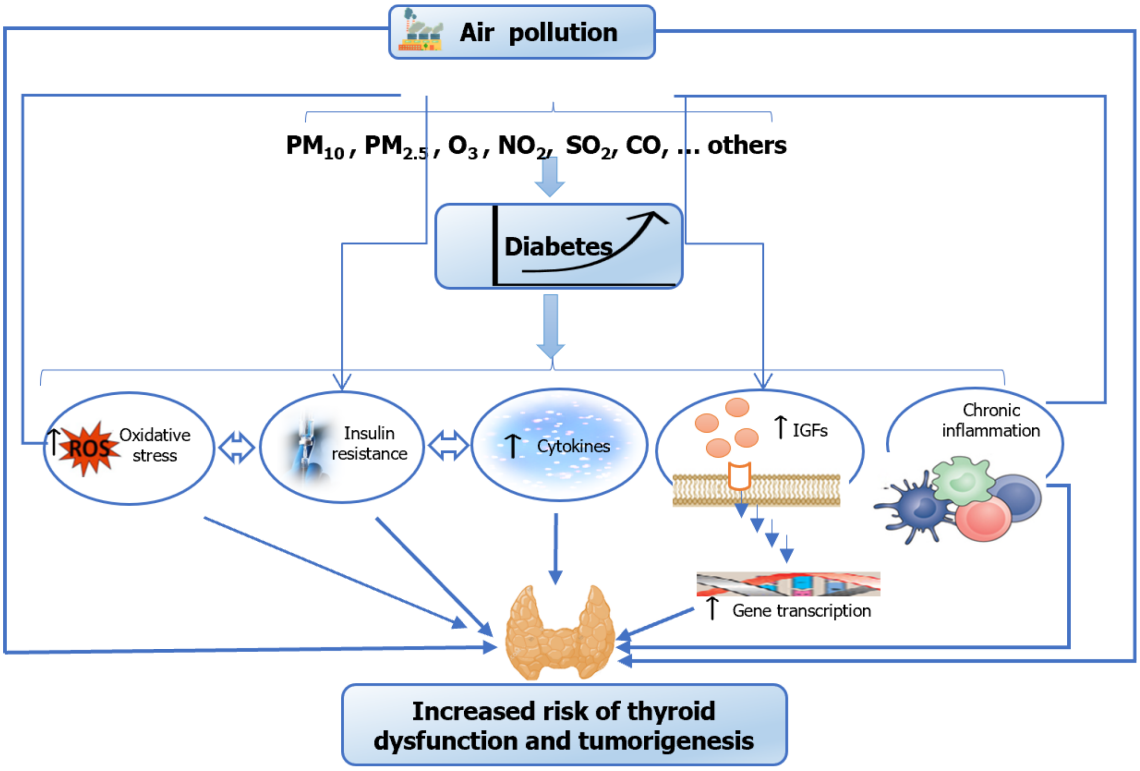

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Figure 1 Air pollution could increase the risk of diabetes and thyroid cancer through several mechanisms. For example, it could increase inflammation and oxidative stress in the body and disrupt the production of cytokines and several hormones, such as insulin and thyroid hormones, linked to increased risk of diabetes and thyroid cancer. IGFs: Insulin-like growth factors; CO: Carbon monoxide; NO_2: Nitrogen dioxide; O_3: Ozone; PM: Particle matter; ROS: Reactive oxygen species; SO_2: Sulfur dioxide.

the healthy control group[74].

Collectively, this review consolidates links between thyroid dysfunction and diabetes development, common pathways of synergy, and the catalytic role PM plays in the emergence of diabetes and thyroid cancer. However, while the connections between PM and thyroid cancer, and between hyperthyroidism and PM, have been established, further exploration is needed to support or reject the presumption that PM contributes to thyroid cancer with hyperthyroidism as the pathogenic liaison. Future focus areas should prioritize longitudinal assessment of thyroid pathology following significant PM exposure to identify possible cancer development courses and mechanisms.

CONCLUSION

Air pollution, specifically PM, contributes significantly to developing thyroid disease and T2DM, both independently and synergistically. Identifying these interconnections within the unique endocrine system is essential to mitigate the exacerbation of insulin resistance, reduce T2DM development and progression, and identify PM-exacerbated specific risk factors for diabetic patients in the face of ever-accumulating air pollution.

FOOTNOTES

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MINIREVIEWS

Liver or kidney: Who has the oar in the gluconeogenesis boat and when?

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Abstract

Gluconeogenesis is an endogenous process of glucose production from non-carbohydrate carbon substrates. Both the liver and kidneys express the key enzymes necessary for endogenous glucose production and its export into circulation. We would be remiss to add that more recently gluconeogenesis has been described in the small intestine, especially under high-protein, low-carbohydrate diets. The contribution of the liver glucose release, the net glucose flux, towards systemic glucose is already well known. The liver is, in most instances, the primary bulk contributor due to the sheer size of the organ (on average, over 1 kg). The contribution of the kidney (at just over 100 g each) to endogenous glucose production is often under-appreciated, especially on a weight basis. Glucose is released from the liver through the process of glycogenolysis and gluconeogenesis. Renal glucose release is almost exclusively due to gluconeogenesis, which occurs in only a fraction of the cells in that organ (proximal tubule cells). Thus, the efficiency of glucose production from other carbon sources may be superior in the kidney relative to the liver or at least on the level. In both these tissues, gluconeogenesis regulation is under tight hormonal control and depends on the availability of substrates. Liver and renal gluconeogenesis are differentially regulated under various pathological conditions. The impact of one source *vs* the other changes, based on post-prandial state, acid-base balance, hormonal status, and other less understood factors. Which organ has the oar (is more influential) in driving systemic glucose homeostasis is still inconclusive and likely changes with the daily rhythms of life. We reviewed the literature on the differences in gluconeogenesis regulation between the kidneys and the liver to gain an insight into who drives the systemic glucose levels under various physiological and pathological conditions.

Key Words: Gluconeogenesis in the kidney and liver; Diabetes; Hormonal regulation; Metabolic acidosis; Insulin resistance; Net glucose metabolism

Core Tip: The liver and kidneys have an essential role in regulating glucose homeostasis through gluconeogenesis. However, the two tissues prefer different substrates. The contribution of kidney *vs* liver gluconeogenesis may vary under certain physiological and pathological conditions. However, increased gluconeogenesis in the liver and kidneys contributes to hyperglycemia in the pathogenic stage of type 2 diabetes mellitus. While in the case of metabolic acidosis, which develops in response to diabetes, gluconeogenesis induction occurs exclusively in the kidneys. Nevertheless, the two organs often compensate for each other by inter-organ coordination to maintain glucose and energy homeostasis.

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INTRODUCTION

Glucose is the primary or even requisite source of energy for many tissues, including the brain, kidney medulla, and red blood cells. Blood glucose levels are maintained within a very narrow range between 3.9-7.1 mmol/L. In addition to dietary glucose, glucose produced through the process of glycogenolysis and gluconeogenesis results in the release of additional glucose into the circulation when blood levels drop. Glycogenolysis involves the breakdown of glycogen to glucose-6-phosphate and its subsequent hydrolysis by glucose-6-phosphatase (G6PC) to free glucose. Gluconeogenesis involves the formation of glucose-6-phosphate from non-carbohydrate carbon substrates such as lactate, glycerol, and amino acids with its subsequent hydrolysis by G6PC to free glucose. The process requires several enzymatic steps and counters the glycolytic breakdown of glucose. The key enzymes in the gluconeogenesis pathway are pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase, and G6PC[1]. There are three rate-limiting, unidirectional steps in gluconeogenesis, which all occur in the cytosol. The first is the phosphorylation of decarboxylated oxaloacetate to form phosphoenolpyruvic acid, which is catalyzed by PEPCK[2]. The phosphoenolpyruvic acid is converted into fructose 1,6-bisphosphate through a series of reactions, which is hydrolyzed to fructose 6-phosphate in the second rate-limiting step *via* the fructose 1,6-bisphosphatase enzyme. Glucose phosphate isomerase converts fructose 6-phosphate to glucose 6-phosphate. Finally, in the third rate-limiting step, glucose 6-phosphatase dephosphorylates glucose 6-phosphate to release glucose into the bloodstream (Figure 1).

GLUCONEOGENESIS IN THE LIVER AND KIDNEYS

The liver and kidneys are the primary organs that can synthesize glucose through the process of gluconeogenesis and can also export the synthesized glucose into the bloodstream.

WHO USES WHAT?

Lactate, glycerol, and certain glucogenic amino acids, *e.g.*, alanine and glutamine, are the primary substrates accounting for 90% of overall gluconeogenesis[3,4]. For liver gluconeogenesis, lactate, which is produced during anaerobic glycolysis, is the primary substrate. In the kidney, glutamine appears to be the major substrate. Although a few studies have suggested lactate as the main substrate, the renal conversion of lactate to glucose was found to be less than that of glutamine (50% *vs* 70% of its overall systemic gluconeogenesis)[3,5]. Moreover, in the post-absorptive phase, glutamine contributes 73% toward renal gluconeogenesis, while alanine contributes only 4%. It is the opposite for the liver, where alanine majorly contributes to gluconeogenesis. Moreover, hepatic gluconeogenesis from lactate and alanine is an endergonic process that consumes energy, while renal gluconeogenesis by utilizing glutamine is an exergonic process that produces four ATP/mole of synthesized glucose[6]. The transport systems for gluconeogenic amino acids also vary between the liver and kidneys. In renal tubular cells, glutamine transport depends on the A amino acid transport system, while in hepatocytes the transport depends on the N system. Nevertheless, the differences in glucogenic amino acid substrates would indicate differences in the regulatory mechanisms of glucose production in the two organs.

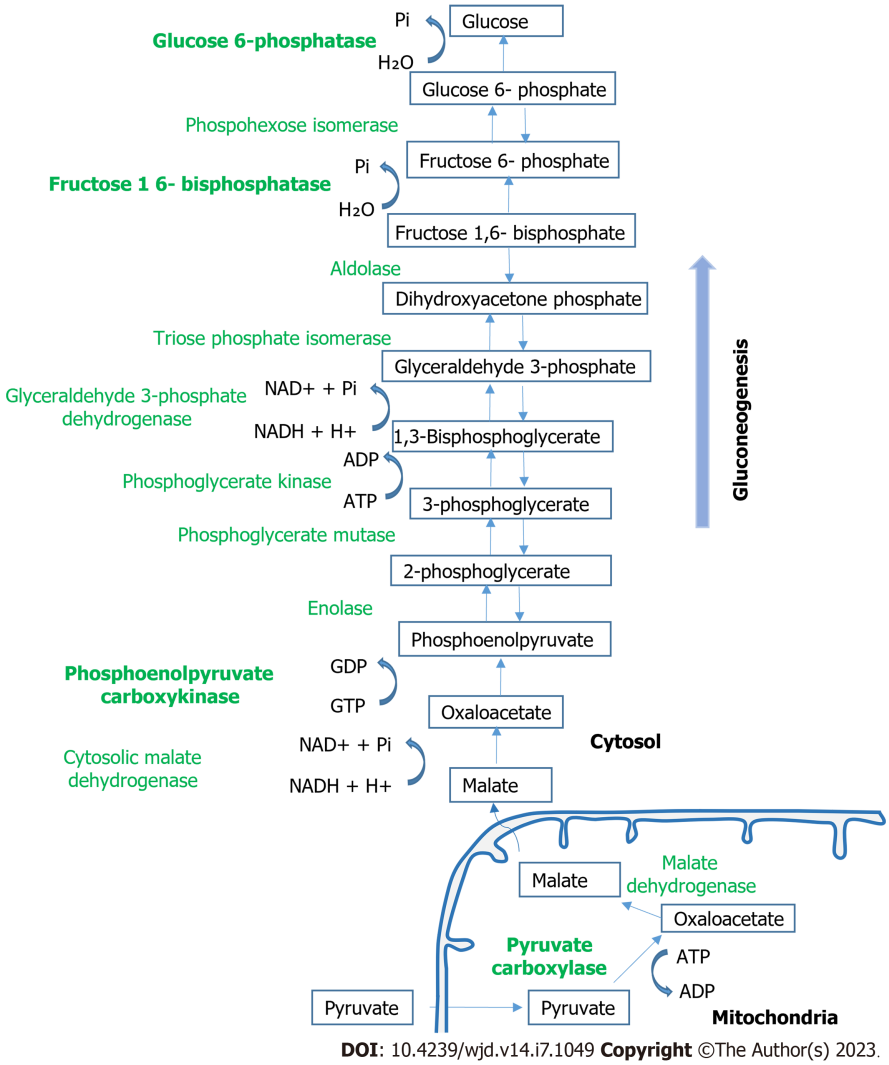

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Figure 1 Overview of gluconeogenesis metabolic pathways. NADH: Nicotinamide adenine dinucleotide.

WHO IS MORE SENSITIVE TO HORMONAL REGULATION?

Insulin, glucagon, and catecholamines regulate plasma glucose levels within minutes through their acute glucoregulatory actions on the liver and kidney gluconeogenesis. The effects of growth hormone, thyroid hormone, and cortisol take a long time either by altering the sensitivity of the liver towards the acute regulatory hormones or by affecting the glycogen stores regulating enzyme activity and gluconeogenic precursor availability[7]. Moreover, most of these studies have been conducted in animals and their effect on renal glucose release in humans is largely unknown.

Insulin is by far the most well-known negative regulator of gluconeogenesis in both the liver and kidneys. Insulin can act by directly activating or deactivating the rate-limiting enzymes for gluconeogenic substrate availability or by acting on gluconeogenic activators. The insulin-dependent transcriptional control of gluconeogenic gene expression involves the FOXO family of transcription factors, which act through the IRS1/Akt2/mTORC1/2 and IRS/PI3k/Akt/FOXO1 pathways[8-12]. Recent studies suggest that the kidney may be more sensitive than the liver to hormonal downregulation of gluconeogenesis[13]. Moreover, insulin receptor-specific signaling may be necessary for the downregulation of renal gluconeogenesis. Proximal tubule-targeted insulin receptor deletion in mice resulted in an elevation in fasting blood glucose and increased renal protein and mRNA expression of G6PC[14]. Also, in proximal tubule cell culture, knockdown of the insulin receptor, but not the insulin-like growth factor type 1 receptor abrogated the inhibitory effects of insulin on glucose production[15].

Unlike the liver, where glucagon increases gluconeogenesis[16], the regulation of gluconeogenesis in the kidneys by glucagon is still controversial. Upregulation in PEPCK, IRS2, and PGC1α expression and glucose production by human proximal tubule cells, independent of the action of insulin, was observed upon glucagon stimulation[17]. Similar gluconeogenic effects of elevated glucagon levels were also reported in type 2 diabetes mellitus (T2DM) subjects[18]. Catecholamines also affect glucose release by the two organs by increasing the availability of gluconeogenic substrates and by decreasing insulin secretion[19,20]. In addition, both glucagon and catecholamines may positively regulate hepatic gluconeogenesis through cyclic AMP-dependent activation of protein kinase A[21,22] and acutely by the phosphorylation of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 at Ser36[23].

WHO DRIVES SYSTEMIC GLUCOSE RELEASE DURING STARVATION?

After overnight fasting, endogenous glucose production is approximately 10-11 μmol/kg/min in humans[3]. The liver contributes to systemic glucose production through both glycogenolysis and gluconeogenesis, while the kidney produces glucose only through renal gluconeogenesis as it does not store glycogen in a healthy state. In the first hour of fasting, hepatic glycogen stores break down to glucose to meet the energy demand. Thus, in the liver, glycogenolysis is considered the primary (approximately 75%) source of glucose production in the early phase of the post-absorptive period while gluconeogenesis contributes approximately 25%[24]. It was suggested that only upon the depletion of glycogen stores, hepatic gluconeogenesis take over glucose production. However, other studies reported the contribution of gluconeogenesis at approximately 50% of hepatic glucose production, even in the early post-absorptive period when liver glycogen stores were maximal[25]. At the other extreme, Landau *et al*[26] reported a 54% contribution of gluconeogenesis after 14 h of fasting, and the percentage rose to 64% after 22 h of fast. Thus, the length of fasting gluconeogenesis and glycogenolysis in the liver were considered to contribute equally toward glucose production. These assumptions were made as the net organ balance studies suggested the liver as the primary site for glucose production, as kidneys showed little or no net glucose production in healthy humans during starvation[27-29].

A breakthrough in determining the role of kidney gluconeogenesis in whole-body glucose homeostasis came from the studies of Mutel *et al*[30]. They showed using liver-specific deletion of the *G6pc* gene (L-G6pc-/-mice) that the absence of hepatic glucose release had no major effect on the control of fasting plasma glucose concentration. The authors also suggested that in early fasting an induction of gluconeogenesis in the kidneys sustained endogenous glucose production and maintained euglycemia. Re-evaluation of the renal contribution to glucose release during starvation using net renal glucose balance together with a deuterated glucose dilution method suggested that renal glucose production handled approximately 20% of whole-body glucose release[24]. In prolonged fasting, renal gluconeogenesis increased and accounted for about 40% of the total systemic gluconeogenesis. New methodologies demonstrated that the kidneys not only produce glucose in the cortex but also utilize glucose for energy in the medullary region, thus the net organ balance of glucose may not truly reflect renal glucose production. This paradigm-shifting set of studies brought into effect new thinking that de novo systemic glucose production is likely provided equally by glycogenolysis (in the liver) and gluconeogenesis (approximately 30% by the liver and 20% by the kidney) during periods of extended fasting.

Overall, it has been realized that the contribution of the kidneys and liver towards endogenous glucose production changes under various nutritional situations, including long-term fasting. This repartition seems necessary for the body to maintain constant plasma glucose and simultaneously preserve the energetic status of the body for anabolic purposes. However, the predominant mechanism for glucose release into the circulation by the two organs varies in the fed state. In the kidneys, two mechanisms are in operation for the net release of glucose: The high energy-consuming gluconeogenesis and a relatively lower energy-driven glucose reabsorption process[12]. Whereas in the liver, glucose release occurs solely through gluconeogenesis. In the fasting state, however, the inability to reabsorb sufficient glucose, together with inactivated insulin signaling, promotes ATP-consuming gluconeogenesis. The role of the insulin receptor in the fast-fed regulation of gluconeogenesis in the human proximal tubule with insulin receptor substrates as direct effectors has recently been described[17].

WHO DRIVES HYPERGLYCEMIA IN DIABETES?

Increased liver as well as renal glucose release have been reported in T2DM[31-33,34-36]. The liver was commonly believed to be the primary source for this increased release of glucose into the circulation in humans with T2DM. Although renal glucose release has only been assessed in a handful of studies in humans with T2DM, the absolute increase in renal glucose release seems to be comparable to the liver by the combined isotopic-net renal glucose balance technique[36-38]. Unlike the liver, where glycogenolysis also contributes to the release of new glucose into the circulation, the increased release of new glucose by the kidney into the circulation is exclusively a result of the rise in gluconeogenesis.

In humans with or without diabetes, renal glucose release into the circulation increased for 2-3 h after a 75-g oral glucose load, whereas hepatic glucose release was reduced throughout the entire postprandial period[39]. However, the average rate of postprandial glucose release was roughly twice as high in diabetic patients as it was in non-diabetic subjects, and renal glucose release accounted for nearly 49% of the overall glucose release. This was predominantly due to defective endogenous glucose release regulation and to a lesser extent, decreased initial ingested glucose splanchnic sequestration. This effect is expected in patients with diabetes having lower postprandial insulin release or insulin resistance[9].

"Carryover" of the elevated renal gluconeogenesis observed in the post-absorptive state may have also contributed to endogenous glucose release[36], in addition to the higher availability of free fatty acid[40] and gluconeogenic precursors observed in T2DM patients[41]. Nevertheless, increased gluconeogenesis (both liver and kidneys) contributes to hyperglycemia in T2DM. However, in the kidneys enhanced glucose reabsorption *via* sodium-glucose cotransporters (SGLT1 and SGLT2) may also sustain hyperglycemia in T2DM. Inhibiting SGLT2 lowers blood glucose levels in T2DM [42]. Two distinct mechanisms have been indicated to improve glycemic control and reduce the plasma glucose levels by SGLT2 inhibitors: (1) By increasing the removal of plasma glucose; and (2) By ameliorating glucotoxicity, which leads to improved insulin sensitivity in peripheral tissues and enhanced β cell function[43].

Paradoxically, SGLT2 inhibitors also increased the hepatic gluconeogenic response while decreasing plasma insulin and offset by approximately 50% the increase in urinary glucose excretion[43-45]. The increase in endogenous glucose

production by SGLT2 inhibitors corroborated well with the observed increase in plasma glucagon concentration[44]. Glucagon is a powerful stimulator of hepatic gluconeogenesis as already discussed in the previous section.

Glucosuria-induced glucagon secretion by SGLT2 inhibitors is beyond the scope of this review. However, glucosuria through neural reflex might activate the kidney-liver axis directly or through neuronal centers in the central nervous system[43]. Nevertheless, there are studies to suggest SGLT2 inhibitors might enhance gluconeogenesis predominantly in the kidney[44,46]. Moreover, the influence of diet intake control on the metabolic effects of SGLT2 inhibitors, including gluconeogenesis, has been observed[47].

The increase in gluconeogenesis in diabetes has been attributed to impaired insulin suppression of PEPCK and other gluconeogenic enzyme activities[31,48-50]. Elevated gluconeogenic gene expression in the kidneys was reported in proximal tubule-specific IRS1/2 double-knockout (KO) mice. These mice also exhibited attenuated phosphorylation of insulin signaling molecules including Akt and FOXO1[12]. Similarly, proximal tubule-specific insulin receptor KO increased fasting glucose concentration and renal *G6pc* mRNA in KO mice[14]. Moreover, studies conducted in a rat model of T2DM[51] and T2DM patients[52] also demonstrated the downregulation of insulin receptor subunit protein levels, the activation of glycogen synthase kinase 3 beta kinase, and increased gluconeogenic enzymes in proximal tubules.

Another mechanism by which insulin resistance can enhance gluconeogenesis is through impaired insulin-induced suppression of lipolysis. Accelerated lipolysis in insulin resistance or insulin deficiency releases free fatty acids and glycerol into the circulation, demonstrating a role for adipose tissue as another source of increased substrate supply for gluconeogenesis[3,53]. The rates of glycerol turnover and gluconeogenesis from glycerol increase in overnight fasted T2DM patients[54,55]. In renal tissues of human diabetes patients, an increase in plasma concentrations of alanine, glycerol, and lactate were detected demonstrating the role of increasing substrate availability enabling the possibility of enhanced gluconeogenesis[56,57]. In diabetic rats, the elevated renal Nicotinamide adenine dinucleotide phosphate oxidase activity and oxidative stress were suggested to upregulate PEPCK expression *via* CREB and the ERK1/2 pathway leading to accelerated renal gluconeogenesis[48,58].

Unlike diabetes where gluconeogenesis is regulated in both the liver and kidney, metabolic acidosis, such as what occurs in T2DM, regulation is primarily in the kidneys[59,60]. To counterbalance acidosis, the kidney generates ammonia, mainly from glutamine deamination, which forms α-ketoglutarate and $NH4^+$ *via* the ammonia genesis pathway[61]. The proximal tubule imports glutamine and catalyzes it into glutamate, freeing up $NH4^+$ to secrete into the lumen to eliminate acid equivalents and reabsorbs basolaterally bicarbonate to normalize blood pH. Glutamate in the proximal tubules is then converted to α-ketoglutarate, which is a substrate for gluconeogenesis[62]. It is the transcription of the *PEPCK-C* gene in the kidney cortex by metabolic acidosis that is unique to the kidney, whereas the transcription of *PEPCK-C* in the liver does not respond to changes in pH[63].

REPARTITIONING ENDOGENOUS GLUCOSE PRODUCTION AMONG ABLE ORGANS

Inter-organ coordination among the liver, kidneys, and potentially intestine may be expected if glucose and energy homeostasis is to be maintained[30]. A similar regulation may be expected during the anhepatic phase of liver transplantation in humans. In mice with liver-specific deletion of the *G6PC* gene, the absence of hepatic glucose production, glucagon was suggested to account for the basal induction of the renal *G6PC* gene[30]. Moreover, glucose production was suggested to counter-regulate insulin-induced hypoglycemia in humans during increased glucagon and cortisol secretions[64]. These studies highlight the important role of the kidney in endogenous glucose production. Similarly, the liver is also expected to compensate for hypoglycemia due to renal insufficiencies. However, it does not appear to always be the case as patients with renal failure are prone to hypoglycemia[65,66]. Underlying hepatic issues in such patients could be a possibility in individuals with reduced hepatic glycogen stores or less available gluconeogenic substrates[67]. Moreover, acidosis would limit the ability of the liver to compensate *via* hepatorenal reciprocity[68].

In this vein, renal gluconeogenesis diminution was shown to promote the repartition of endogenous glucose production in intestinal gluconeogenesis leading to the sparing of glycogen stores in the liver in mice lacking kidney-specific *G6pc*[69]. Thus intestine-liver crosstalk might take place in the situations of deficient renal glucose production, such as chronic kidney disease. However, studies are warranted to determine the contribution of intestinal gluconeogenesis to systemic glucose release and to confirm that the repartition of endogenous glucose production takes place and contributes to a glycemic reduction in chronic kidney disease with reduced renal gluconeogenesis. More studies are needed to understand the relative role of the liver vis-à-vis extrahepatic gluconeogenic organs in glucose homeostasis.

CONCLUSION

Gluconeogenesis in the liver as well as kidneys is now considered important in maintaining glucose homeostasis. The difference in the preference for gluconeogenic substrates by the liver and kidneys and the hormonal regulation of the process in the two organs would imply that the regulatory mechanisms of glucose production are not the same in the two organs. Moreover, the contribution of kidney *vs* liver gluconeogenesis may vary under certain physiological and pathological conditions. For example, in the early phase of fasting as the hepatic glycogen gets depleted, the systemic glucose production was considered equally by glycogenolysis (in the liver), and gluconeogenesis (approximately 30% by the liver and 20% by renal gluconeogenesis). In prolonged fasting, renal gluconeogenesis increases and accounts for about 40% of the total systemic gluconeogenesis. In the pathological state of T2DM, increased gluconeogenesis in both the liver

and kidneys contributes towards hyperglycemia. In metabolic acidosis in response to diabetes, gluconeogenesis induction exclusively occurs in the kidneys, and liver gluconeogenesis remains unaffected. Similarly, differential effects of SGLT2 inhibitors on renal and liver gluconeogenesis have been reported in the liver and kidneys. In addition, the two organs can compensate, at least partially, for the impaired glucose release due to renal or liver insufficiency suggesting an inter-organ coordination to maintain glucose and energy homeostasis. For translational implications, more studies in the area are needed to know the real driver of systemic glucose production under pathological states, such as in patients with liver or renal insufficiency.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Network-pharmacology-based research on protective effects and underlying mechanism of Shuxin decoction against myocardial ischemia/reperfusion injury with diabetes

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Abstract

BACKGROUND

Patients with diabetes mellitus are at higher risk of myocardial ischemia/reperfusion injury (MI/RI). Shuxin decoction (SXT) is a proven recipe modification from the classic herbal formula "Wu-tou-chi-shi-zhi-wan" according to the traditional Chinese medicine theory. It has been successfully used to alleviate secondary MI/RI in patients with diabetes mellitus in the clinical setting. However, the underlying mechanism is still unclear.

AIM

To further determine the mechanism of SXT in attenuating MI/RI associated with diabetes.

METHODS

This paper presents an ensemble model combining network pharmacology and biology. The Traditional Chinese Medicine System Pharmacology Database was accessed to select key components and potential targets of the SXT. In parallel, therapeutic targets associated with MI/RI in patients with diabetes were screened from various databases including Gene Expression Omnibus, DisGeNet, Genecards, Drugbank, OMIM, and PharmGKB. The potential targets of SXT and the therapeutic targets related to MI/RI in patients with diabetes were intersected and subjected to bioinformatics analysis using the Database for Annotation, Visualization and Integrated Discovery. The major results of bioinformatics analysis were subsequently validated by animal experiments.

RESULTS

According to the hypothesis derived from bioinformatics analysis, SXT could possibly ameliorate lipid metabolism disorders and exert anti-apoptotic effects in MI/RI associated with diabetes by reducing oxidized low density lipoprotein (LDL) and inhibiting the advanced glycation end products (AGE)-receptor for AGE (RAGE) signaling pathway. Subsequent animal experiments confirmed the hypothesis. The treatment with a dose of SXT (2.8 g/kg/d) resulted in a reduction in oxidized LDL, AGEs, and RAGE, and regulated the level of blood lipids. Besides, the expression of apoptosis-related proteins such as Bax and cleaved caspase 3 was down-regulated, whereas Bcl-2 expression was up-regulated. The findings indicated that SXT could inhibit myocardial apoptosis and improve cardiac function in MI/RI in diabetic rats.

CONCLUSION

This study indicated the active components and underlying molecular therapeutic mechanisms of SXT in MI/RI with diabetes. Moreover, animal experiments verified that SXT could regulate the level of blood lipids, alleviate cardiomyocyte apoptosis, and improve cardiac function through the AGE-RAGE signaling pathway.

Key Words: Chinese herbal drugs; Network-pharmacology; Diabetes; Myocardial reperfusion injury; Shuxin decoction

Core Tip: Patients with diabetes are susceptible to myocardial ischemia/reperfusion injury (MI/RI). The efficacy of implementing strict glycemic control to reduce cardiovascular mortality in patients with diabetes has not been established to yield significant benefits. Here, we evaluated a recipe [Shuxin decoction (SXT)], which was modified from the classic herbal formula "Wu-tou-chi-shi-zhi-wan" in traditional Chinese medicine. Animal experiments based on findings from network pharmacology indicated that SXT could regulate lipid metabolism, alleviate cardiomyocyte apoptosis, and attenuate MI/RI in diabetes through the advanced glycation end products (AGE)-receptor for AGE signaling pathway. These findings could potentially facilitate developing a novel complementary or alternative form of medicine for effectively managing MI/RI with diabetes.

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INTRODUCTION

According to the latest report of the International Diabetes Federation, diabetes is responsible for about 6.7 million deaths globally every year[1]. Most mortality in diabetic patients is associated with cardiovascular disease[2]. Increasing evidence has revealed that larger infarct size and worse cardiac function in diabetes follow with myocardial ischemia/reperfusion injury (MI/RI)[3-6]. Obesity, hyperglycemia, and hyperlipidemia are the most common metabolic diseases in diabetes mellitus, which are recognized as cardiovascular risk factors[7]. However, no significant benefits were obtained from strict glycemic control to decrease cardiovascular mortality in diabetes[8,9]. Thus, regulating lipid metabolism may be a novel strategy for alleviating MI/RI in diabetes.

Shuxin decoction (SXT) is a traditional Chinese medicine (TCM) compound based on modification of "Wu-tou-chi-shi-zhi-wan" recorded in the medical classic "Jin Gui Yao Lue" written by Zhongjing Zhang in the Eastern Han Dynasty. "Wu-tou-chi-shi-zhi-wan" was used to protect the cardiovascular system from various injuries in TCM. SXT was a modification of "Wu-tou-chi-shi-zhi-wan" into seven herbs: *Astragalus*, *Zanthoxylum*, *Rhizoma zingiberis*, *Cinnamon*, *Salvia miltiorrhiza*, *Panax notoginseng*, and *Ligusticum wallichii*. Recent studies have shown that *Astragalus* extract can reduce the

levels of triglyceride (TG), total cholesterol (TC), and low density lipoprotein (LDL)[10]. *Zanthoxylum* extract exerts anti-obesity and hypolipidemic effects by reducing liver oxidative stress[11]. *Rhizoma zingiberis* extract reduces heart structural abnormalities in diabetic rats by improving the levels of apolipoproteins, leptin, cathepsin G, and homocysteine in serum [12]. *Cinnamic acid* alleviates MI/RI by inhibiting NLRP3/Caspase-1/GSDMD signaling[13]. *Salvia miltiorrhiza* and *Panax notoginseng* saponins can reduce oxidative stress and apoptosis to ameliorate myocardial damage[14-17]. *Ligusticum wallichii* attenuates myocardial injury by activating PI3K/Akt signaling in the myocardium[18]. Our research suggested the effect of SXT in alleviating symptoms of cardiovascular injury in MI/RI in diabetes. However, the details of the SXT mechanism are still unclear due to the complexity of diabetes mellitus with MI/RI.

Network pharmacology is a commonly used tool in identifying multiple components and investigating the mechanisms of herbal medicine. In this study, based on network pharmacology, the main targets and pathways of SXT in the treatment of MI/RI in diabetes were predicted, analyzed, and verified, which will provide evidence for the development of drugs for MI/RI in diabetes.

MATERIALS AND METHODS

Screening of active compounds and potential targets of SXT

The Traditional Chinese Medicine System Pharmacology Database (TCMSP) database was used to predict the active compounds and potential targets of SXT with an oral bioavailability ≥ 30% and drug similarity (DL) ≥ 0.18 (http://Lsp.nwu.edu.cn/tcmsp.php)[19]. Then, we constructed the relationship network between the active compounds and potential target genes of SXT *via* the Cytoscape 3.9.0 software (http://cytoscape.org/)[20].

Identification of therapeutic targets for diabetes and MI/RI

The therapeutic targets were identified by searching the Gene Expression Omnibus (GEO), DisGeNet, Genecards, Drugbank, OMIM, and PharmGKB with "MI/RI", "myocardial ischemia/reperfusion injury", "diabetes mellitus", and "diabetes" as keywords. We merged the three diabetes related datasets (GSE118139, GSE161355, and GSE193626) and two MI/RI related datasets (GSE36875 and GSE210611) identified in the GEO database separately and then obtained differentially expressed genes (DEGs) *via* the R package "limma" for batch correction and screening $|\log 2 \text{ (fold change)}| > 1$ and $P < 0.05$). Then, we standardized the target names through the UniProt database (https://www.uniprot.org/)[21].

Identification of potential therapeutic targets of SXT for attenuating MI/RI in diabetes

The obtained DEGs from the GEO database were combined with diabetes related targets or MI/RI related targets from the DisGeNet, Genecards, Drugbank, OMIM, and PharmGKB databases separately. Targets that appeared at least twice were regarded as therapeutic targets for diabetes or MI/RI. Then, therapeutic targets for diabetes were intersected with those for MI/RI to obtain potential therapeutic targets for MI/RI in diabetes. Finally, potential targets of SXT obtained from the TCMSP database were intersected with therapeutic targets for MI/RI in diabetes to identify prospective SXT therapeutic targets for MI/RI in diabetes.

Network construction and enrichment analysis

We obtained the interactions among potential therapeutic targets of SXT *via* the STRING (https://string-db.org/)[22] database to construct a protein-protein interaction (PPI) network. Then, we imported the comprehensive data into Cytoscape 3.9.0 software and used its Molecular Complex Detection plugin to select the key subnetworks and therapeutic targets[20]. Default parameters (Degree Cutoff: 2; Node Score Cutoff: 0.2; K-core: 2; maximum depth: 100) were used. The key therapeutic targets were further selected according to the degree value *via* CytoNCA plugin. To investigate the probable molecular mechanisms of SXT for attenuating MI/RI in diabetes, the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) (https://david.ncifcrf.gov/home.jsp)[23] was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, and the results were visualized using the clusterProfiler package in R[24].

Chemicals and reagents

SXT was purchased from Sichuan Hongpu Pharmaceutical Co., Ltd. (Sichuan, China). Triphenyltetrazolium chloride (TTC), Evan's blue (EB), streptozotocin (STZ), and sodium citrate buffer (SSC, 0.1 mol/L, pH 4.5) were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). BCA protein analysis reagents were obtained from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China). Rat insulin (INS), troponin T (cTnT), TG, TC, free fatty acids (FFA), creatine kinase isoenzyme MB (CKMB), lactate dehydrogenase (LDH), oxidized LDL (ox-LDL), LDL cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and advanced glycation endproducts (AGEs) antibodies for ELISA were obtained from Jianglai Company (Shanghai, China). Bax antibody used for Western blot was purchased from Abcam (Shanghai, China), Bcl-2 and receptor for AGE (RAGE) antibodies were purchased from Affinity (Jiangsu, China), and cleaved caspase-3 antibody was purchased from PTGCN (Wuhan, China). Chemical standards (verisoflavone glucoside, tanshinone IIA, ginsenosides Rb1, ferulic acid, 6-gingerol, and cinnamaldehyde) with a purity higher than 98 % were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China).

Preparation of SXT and quality control

SXT is composed of *Astragalus* (Huang-Qi, 40 g), *Zanthoxylum* (Shu-Jiao, 6 g), *Rhizoma zingiberis* (Gan-Jiang, 12 g),

Cinnamon (Rou-Gui, 12 g), *Salvia miltiorrhiza* (Dan-Shen, 24 g), *Panax notoginseng* (San-Qi), and *Ligusticum wallichii* (Chuan-Xiong, 18 g). SXT extract was obtained after sterilization and filtration through a 0.22-μm filter. Mass spectrometry of SXT was performed for quality control by using an HPLC-VWD mass spectrometer (Figure 1A and B).

Animal experiments

The animal experimental protocol for this study was approved by the General Hospital of Western Theater Command (No. 2022EC2-ky004). We obtained 60 male Sprague-Dawley rats weighing 120-140 g from Chengdu Dashuo Laboratory Animal Co., Ltd. [Certificate number: SCXK (Chuan) 2020-030]. The animals were housed in an SPF-rated environment. After 1 wk of adaptation, the rats were randomly divided into six groups (n = 10): Normal control group (C), diabetic rats with sham operation group (DS), MI/RI in diabetes group (DMR), MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group (SXTL), MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group (SXTM), and MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group (SXTH). Except group C, other groups were given a high-fat diet (60.65% fat, 18.14% protein, 21.22% carbohydrate; Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd., China). The intraperitoneal glucose tolerance test (IPGTT) and the intraperitoneal insulin tolerance test (IPITT) were performed on each group of rats. After 4 wk of high-fat diet feeding, rats in all groups except group C were intraperitoneally injected with a single dose of STZ (35 mg/kg, dissolved in 0.1 mol/L citrate buffer, pH 4.5; Solarbio, China). Rats in group C were injected with an equal volume of citrate buffer. After 1 wk, fasting blood glucose (Roche, Germany) level in blood collected from the tail vein was measured, and rats with a blood glucose level ≥ 11.1 mmol/L were considered diabetic[25]. Four weeks after diabetes induction, rats in the SXTL, SXTM, and SXTH groups started to receive SXT gavage treatment. The C, DS, and DMR groups received pure water gavage.

After 8 wk of treatment, the second IPGTT and IPITT experiments were performed. After an overnight fast, an MI/RI model[26] was created by ligation of the left anterior descending artery in the DMR, SXTL, SXTM, and SXTH groups. Briefly, rats were anesthetized with pentobarbital sodium (60 mg/kg) *via* intraperitoneal injection, and artificial respiration was established using a ventilator (Anhui Zhende Medical Company, Anhui, China) with a respiratory rate of 75 breaths/min, respiratory ratio of 1:1, and tidal volume of 20 mL. After disinfection of the skin, the chest was opened through the left third intercostal space, and a slipknot was made with an 8-0 surgical silk suture to ligate the left anterior descending coronary artery. Coronary artery occlusion was confirmed by ST-segment elevation on electrocardiogram. After 30 min of ligation, the slipknot was released to allow reperfusion for 2 h. The rats in the DS group underwent the same surgical procedure except for ligation of the heart. Cardiac function was assessed by echocardiography 2 h after reperfusion using an M-mode Vevo3100LT high-resolution *in vivo* imaging system (Visualsonic, Toronto, Canada). The rats were anesthetized with 2.5% isopentyl ether inhalation, and their body temperature was maintained at about 37 °C. We measured the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS).

At the end of reperfusion, three rats from each group were randomly selected for TTC and EB staining. The coronary arteries were ligated, and 1% EB was injected into the left ventricular cavity. The heart was rapidly excised. After freezing at -20 °C, sections were stained with 1% TTC at 37 °C for 10 min[27]. The stained area was analyzed with Image J software. Areas at risk (AARs) were indicated by TTC staining in red (infarct border area) and white (infarct area), and normal myocardium was stained dark blue by EB. The AAR was calculated as a percentage of the total area.

Finally, serum and plasma were collected and stored at -20 °C for later experiments. We selected three hearts from each group to fix in 10% formalin for 3 d and then embed in paraffin for hematoxylin and eosin (HE) and immunofluorescence staining. The hearts from the remaining rats were stored at -80 °C.

Enzyme-linked immunosorbent assay

An ELISA kit was used to detect the levels of INS, ox-LDL, HDL-C, LDL-C, cTnT, CKMB, and LDH in serum and AGEs in plasma. We followed the instructions in the ELISA kit and calculated the concentration of the sample according to the standard concentration and optical density.

Western blot analysis

Cells in each group were immediately lysed and homogenized with lysis buffer. Total protein samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, anti-rat RAGE (Affinity, AF5309), cleaved caspase-3 (Affinity, AF7022), Bax (Abcam, ab32503), Bcl-2 (PTGCN, 60178-1-lg), and DAPDH (Affinity, AF7021) antiboides were applied. The membranes were incubated overnight at 4 °C. After three washes, the membranes were incubated with secondary antibody (Bioss, 0295G) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (Millipore, WBKLS0100). The integrated optical density of each band was measured with Image J software.

TUNEL fluorescent staining

The paraffin sections were dewaxed to water and repaired with proteinase K. After the membranes were ruptured, the buffer was incubated at room temperature for 10 min. According to the number of slices and tissue size, TDT enzyme, dUTP, and buffer from the TUNEL kit at a ratio of 1:5:50 were mixed at a temperature of 37 °C and incubated for 2 h. The nuclei were then counterstained with DAPI and finally mounted with anti-fluorescence quenching mounting medium. Sections were observed under a fluorescence microscope. Nuclei are blue under UV excitation, and positive apoptotic nuclei are green.

Immunofluorescence

The paraffin sections were dewaxed to water and then antigen repair was performed. After blocking, the sections were

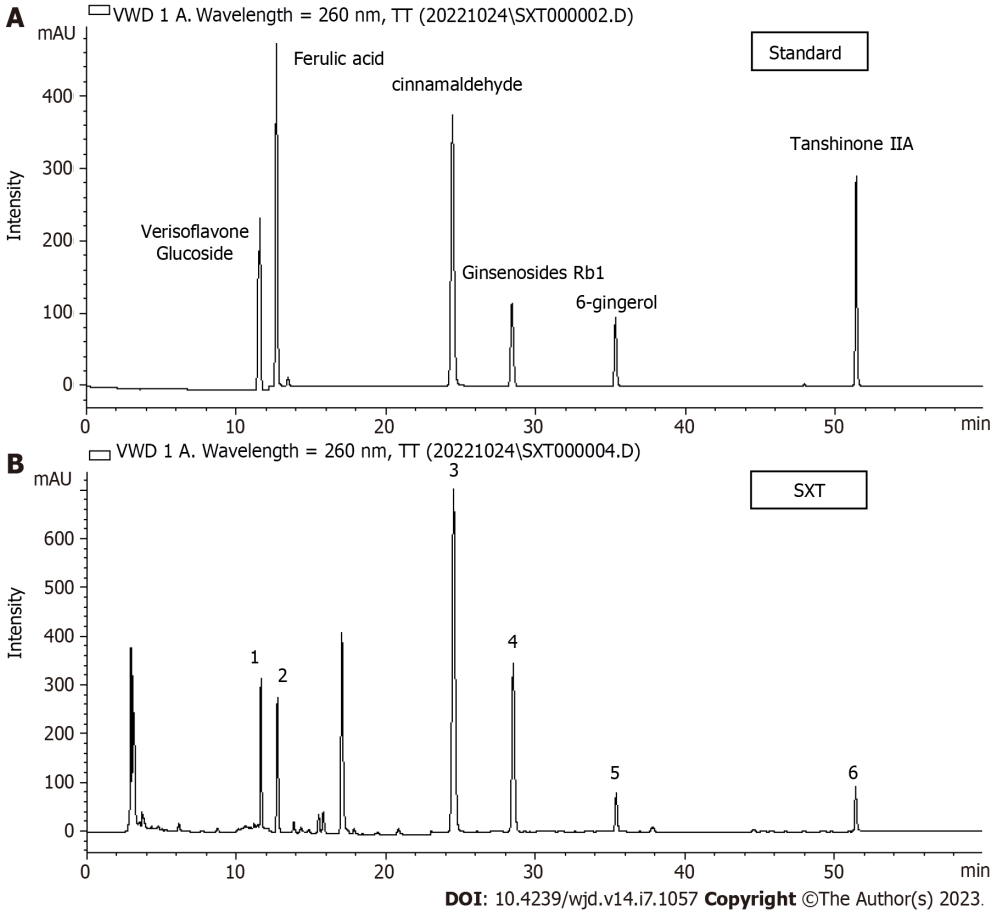

Figure 1 HPLC-VWD mass spectrometry. A: Key components of Shuxin decoction (SXT) identified by HPLC-VWD mass spectrometry; B: HPLC-VWD mass spectrometry of SXT. 1-6 represent verisoflavone glucoside, ferulic acid, cinnamaldehyde, ginsenosides Rb1, 6-gingerol, and tanshinone IIA, respectively. SXT: Shuxin decoction.

incubated with anti-rat RAGE (Affinity, AF5309) at 4 °C overnight. Then, we added a secondary antibody and incubated the sections at room temperature for 50 min in the dark. Nuclei were counterstained with DAPI, autofluorescence quencher was added for 5 min, and the sections were washed with running water for 10 min. After drying, the sections were mounted using anti-fluorescence quenching mounting medium. Sections were observed under a fluorescence microscope. Nuclei are blue under UV excitation, and RAGE is stained red.

Statistical analysis

The data were evaluated by one-way ANOVA, and *t*-test was used if the variances were not uniform. A $P < 0.05$ was considered statistically significant. SPSS version 16.0 (SPSS Inc., Chicago, IL) was used to analyze the data.

RESULTS

Active ingredients and targets of SXT

As shown in Figure 2, the pharmacological network of SXT was constructed to indicate the relationships among all the herbs, compounds, and corresponding targets. Finally, 92 active compounds and 237 targets of SXT were identified as the predicted targets for further research (Supplementary Table 1). The top three pharmaceutical compounds of SXT based on degree of value were quercetin, beta-sitosterol, and kaempferol.

Therapeutic targets for diabetes and MI/RI

We utilized the R package "limma" to detect 2404 DEGs linked to diabetes and 174 DEGs linked to MI/RI. In Figure 3A and B, the red dots on the right represent up-regulated genes in diabetes or MI/RI patients, while the blue dots on the left represent down-regulated genes in diabetes or MI/RI patients. Figure 3C and D shows the expression of the top 40 DEGs that were ranked high and low in patients *vs* healthy individuals, respectively. Next, we found 2359, 11539, 119, 6012, and 8 therapeutic targets related to diabetes and 300, 962, 70, 39, and 237 therapeutic targets related to MI/RI in the DisGeNet, Genecards, Drugbank, OMIM, and PharmGKB datasets, respectively. Finally, targets that appeared at least twice were regarded as therapeutic targets for diabetes or MI/RI, and this resulted in 4380 potential therapeutic targets for diabetes and 276 potential therapeutic targets for MI/RI (Figure 4A and B).

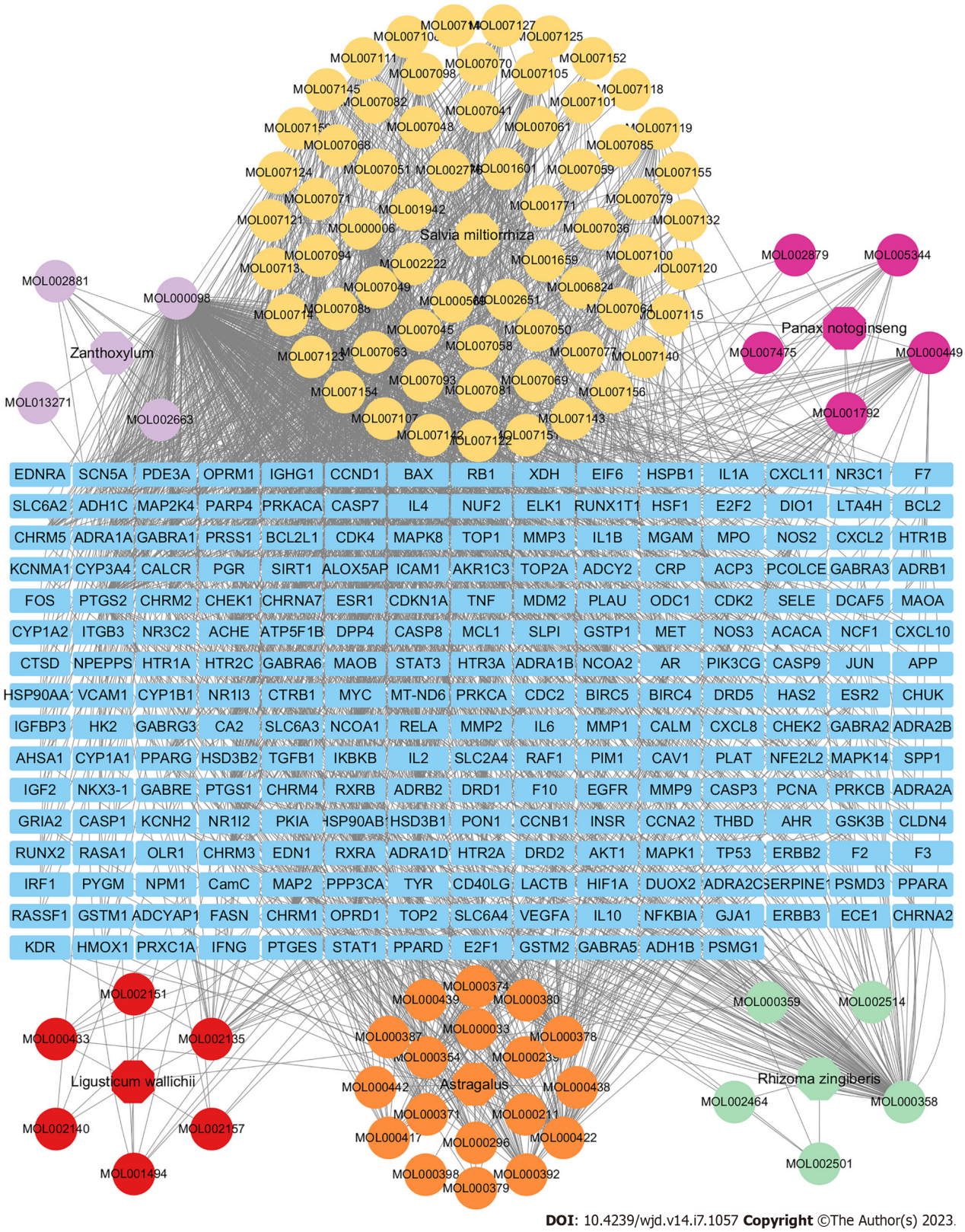

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Figure 2 Relationship network among herbs, active compounds, and targets of Shuxin decoction. In the network, blue rectangle indicates targets. The colored ellipses represent respectively the main components of six herbs: *Astragalus* (orange), *Zanthoxylum* (light purple), *Rhizoma zingiberis* (green), *Salvia miltiorrhiza* (yellow), *Panax notoginseng* (deep purple), and *Ligusticum wallichii* (red). Grey lines indicate the interrelationships among the herbs, active compounds, and targets.

Potential therapeutic targets of SXT for attenuating MI/RI in diabetes

After the therapeutic targets for diabetes and MI/RI were intersected, we obtained 220 potential therapeutic targets for MI/RI in diabetes (Figure 4C). Then, 220 potential therapeutic targets were intersected with 237 targets of SXT to identify 58 potential SXT therapeutic targets for MI/RI in diabetes (Figure 4D).

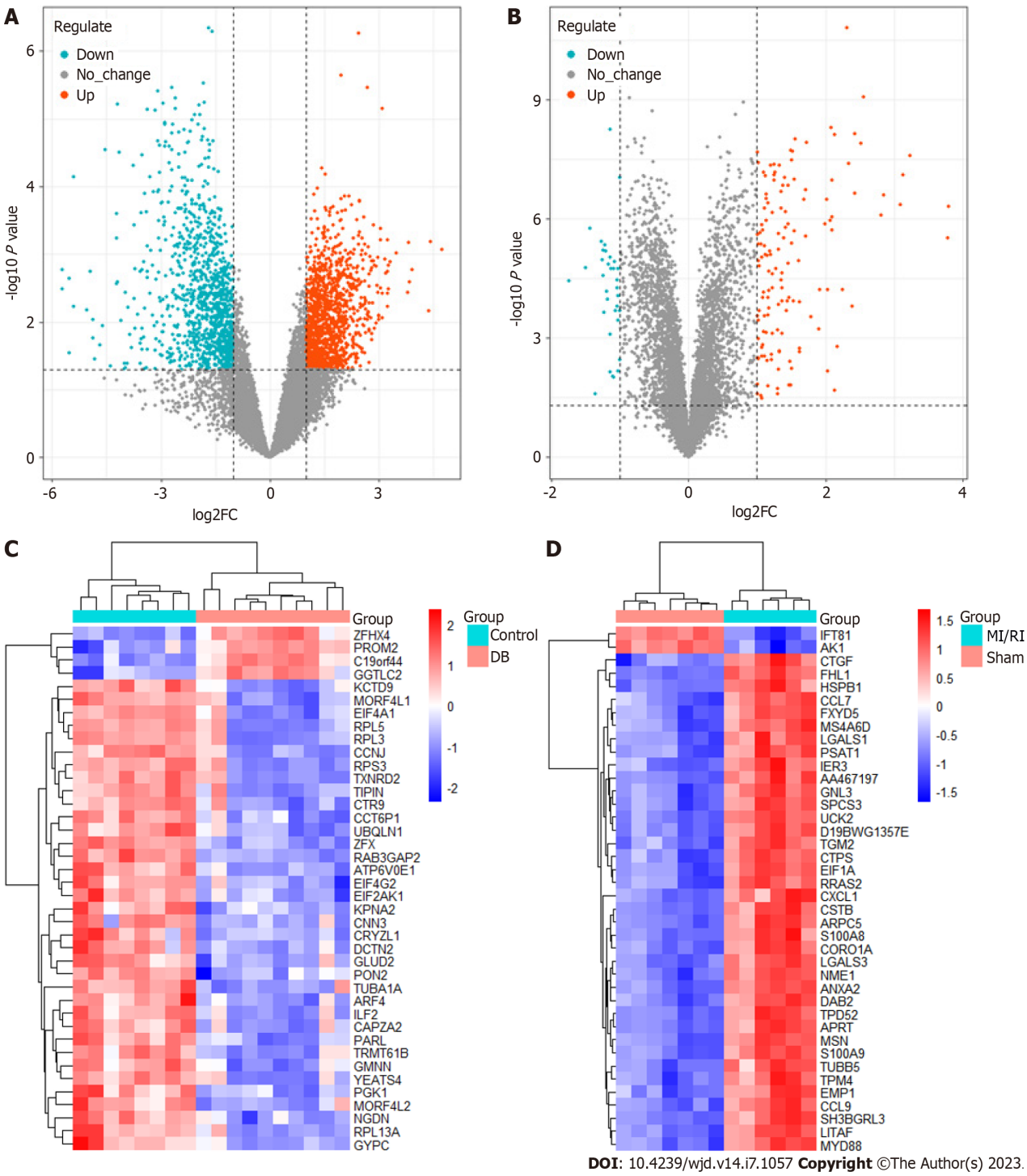

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Figure 3 Differentially expressed genes related to diabetes or myocardial ischemia/reperfusion injury in Gene Expression Omnibus datasets. A: Volcano map of differentially expressed genes (DEGs) related to diabetes (GSE118139, GSE161355, and GSE193626); B: Volcano map of DEGs related to myocardial ischemia/reperfusion injury (MI/RI) (GSE36875 and GSE210611); C: Heat map of DEGs related to diabetes (GSE118139, GSE161355, and GSE193626); D: Heat map of DEGs related to MI/RI (GSE36875 and GSE210611). MI/RI: Myocardial ischemia/reperfusion injury.

Network construction and results of GO and KEGG analyses

To obtain a PPI network, 58 potential SXT therapeutic targets for MI/RI in diabetes were uploaded to the STRING database (Figure 5A). Then, we imported the comprehensive data into Cytoscape to obtain 41 key therapeutic targets by MCODE plugin (Table 1). A total of 41 key therapeutic target nodes were connected by 680 edges, with an average node degree of 32.8 and clustering coefficient of 0.799 (Figure 5B). According to the DAVID database, a total of 489 GO items were obtained, including 395 biological processes (BPs), 26 cellular components, and 68 molecular functions. The first 10 items were selected in terms of the *P* value for visual analysis (Figure 6A-C). The results showed that the treatment of MI/RI in diabetes with SXT mainly involves BPs such as angiogenesis, cellular response to hypoxia, apoptotic process, and inflammatory response. These targets have enzyme binding, protein binding, cytokine activity, transcription factor

Table 1 Forty-one Shuxin decoction key therapeutic targets for myocardial ischemia/reperfusion injury in diabetes

ID	Target	Protein name	Degree	Betweenness	Closeness
1	IL-6	Interleukin-6	54	0.047667394	0.95
2	IL-1β	Interleukin-1beta	53	0.029250817	0.93442623
3	TNF	Tumor Necrosis Factor	53	0.029250817	0.93442623
4	VEGFA	Vascular Endothelial Growth Factor A	51	0.022294667	0.904761905
5	MMP9	Matrix Metallopeptidase 9	49	0.016493589	0.876923077
6	CXCL8	C-X-C Motif Chemokine Ligand 8	48	0.017645476	0.863636364
7	STAT3	Signal Transducer and Activator of Transcription 3	48	0.014004747	0.863636364
8	PTGS2	Prostaglandin-Endoperoxide Synthase 2	48	0.012914899	0.863636364
9	CASP3	Caspase 3	48	0.017524977	0.863636364
10	TP53	Tumor Protein P53	47	0.013024421	0.850746269
11	JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	47	0.010160499	0.850746269
12	PPARG	Peroxisome Proliferator Activated Receptor Gamma	45	0.017202087	0.826086957
13	HIF1A	Hypoxia Inducible Factor 1 Subunit Alpha	45	0.01117217	0.826086957
14	IL-10	Interleukin-10	45	0.010195923	0.826086957
15	ICAM1	Intercellular Adhesion Molecule 1	43	0.009172336	0.802816901
16	NOS3	Nitric Oxide Synthase 3	42	0.021291387	0.791666667
17	HMOX1	Heme Oxygenase 1	41	0.005343813	0.780821918
18	FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	40	0.017888649	0.77027027
19	MYC	MYC Proto-Oncogene, BHLH Transcription Factor	39	0.007362402	0.76
20	IFNγ	Interferon Gamma	38	0.003891145	0.75
21	EDN1	Endothelin 1	37	0.007764542	0.74025974
22	CASP8	Caspase 8	37	0.007408849	0.74025974
23	MAPK8	Mitogen-Activated Protein Kinase 8	37	0.009018794	0.74025974
24	VCAM1	Vascular Cell Adhesion Molecule 1	37	0.00621228	0.74025974
25	CCND1	Cyclin D1	36	0.005854834	0.730769231
26	SERPINE1	Serpin Family E Member 1	36	0.004739025	0.730769231
27	MAPK14	Mitogen-Activated Protein Kinase 14	35	0.0033994	0.721518987
28	STAT1	Signal Transducer and Activator of Transcription 1	35	0.002891632	0.721518987
29	ESR1	Estrogen Receptor 1	34	0.005648692	0.7125
30	MPO	Myeloperoxidase	33	0.007272867	0.703703704
31	NOS2	Nitric Oxide Synthase 2	33	0.002943975	0.703703704
32	CASP1	Caspase 1	32	0.003275919	0.695121951
33	SPP1	Secreted Phosphoprotein 1	32	0.002473838	0.695121951
34	IL1A	Interleukin 1 Alpha	31	0.001235526	0.686746988
35	SELE	Selectin E	31	0.003528291	0.686746988
36	NFE2L2	Nuclear Factor, Erythroid 2 Like 2	30	0.003874065	0.678571429
37	CASP9	Caspase 9	30	0.003883128	0.678571429
38	PPARA	Peroxisome Proliferator Activated Receptor Alpha	30	0.001620936	0.678571429
39	KDR	Kinase Insert Domain Receptor	28	0.001361612	0.662790698
40	CXCL10	C-X-C Motif Chemokine ligand 8	27	0.000671	0.655172414
41	CD40LG	CD40 ligand	27	0.003513977	0.655172414

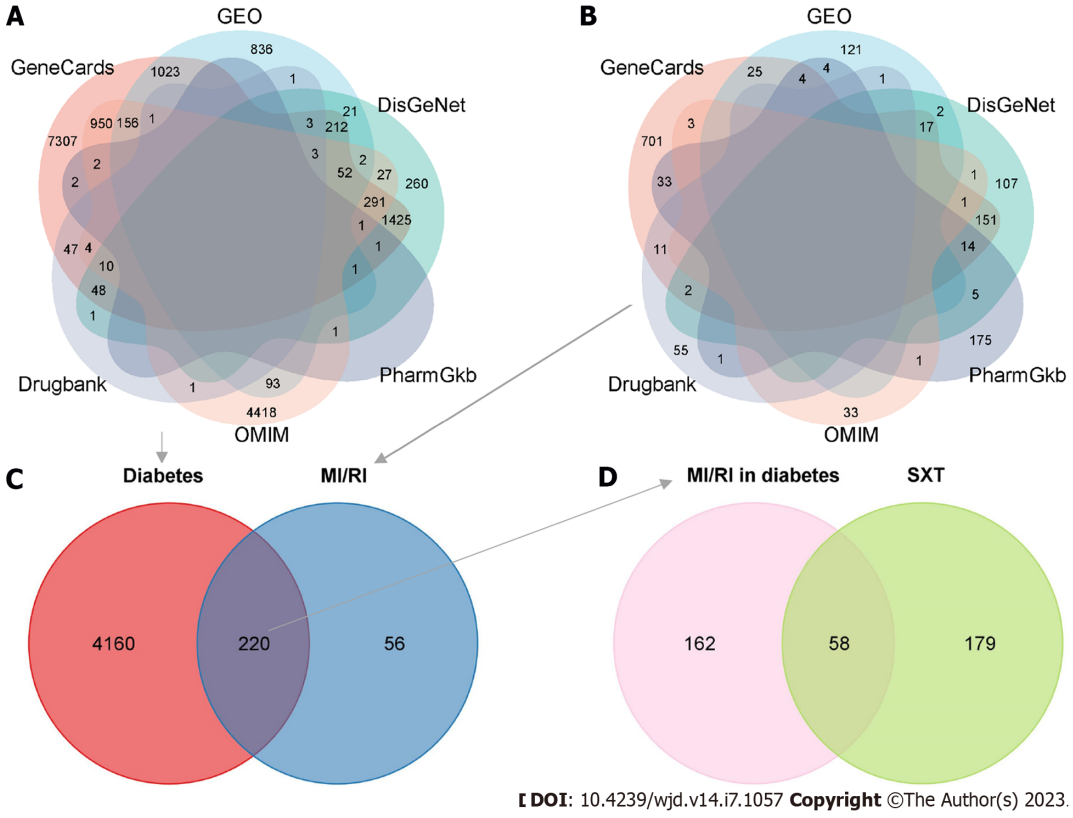

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Figure 4 Targets related to Shuxin decoction for attenuating myocardial ischemia/reperfusion injury in diabetes. A: Venn diagram of diabetes therapeutic targets in six disease databases; B: Venn diagram of myocardial ischemia/reperfusion injury (MI/RI) therapeutic targets in six disease databases; C: Venn diagram of diabetes related targets and MI/RI related targets; D: Venn diagram of the targets in at least two databases in C and the therapeutic targets of Shuxin decoction. MI/RI: Myocardial ischemia/reperfusion injury; SXT: Shuxin decoction; GEO: Gene Expression Omnibus.

binding, cysteine-type endopeptidase activity, and other functions, and they play a role in the extracellular space, macromolecular complex, membrane raft, nucleoplasm, external side of plasma membrane, and the nucleus. KEGG enrichment analysis showed that these targets were mainly enriched in the AGE-RAGE signaling pathway in diabetic complications and the lipids and atherosclerosis signaling pathway (Figure 6D).

Based on the above results of network pharmacology analysis, we observed that the AGE-RAGE signaling pathway in diabetic complications is a downstream pathway of the lipids and atherosclerosis signaling pathway. As shown in Figure 7, among 41 key therapeutic targets, the AGE-RAGE signaling pathway in diabetic complications, lipids and atherosclerosis signaling pathway, and apoptosis were mainly enriched. Interestingly, these two signaling pathways largely participate in lipid metabolism and apoptotic processes. LDL, AGEs, and RAGE are key proteins of the lipids and atherosclerosis signaling pathway, along with the AGE-RAGE signaling pathway in diabetic complications. LDL is subject to oxidative modifications to become ox-LDL and promotes the binding of AGEs to their receptor RAGE[28]. Studies have shown that AGE level in diabetic patients is much higher than that in non-diabetic patients, and its level is positively correlated with the risk of cardiovascular diseases[29]. AGE-RAGE subsequently activates the expression of nicotinamide adenine dinucleotide phosphate to produce many reactive oxygen species, which further promotes the generation of AGEs and forms a positive cycle, constantly aggravating the occurrence of oxidative stress in the body, and further promoting apoptosis[30-33]. The above results provided great support for clarifying the anti-lipid metabolism disorders and anti-apoptotic mechanisms of SXT on MI/RI in diabetic rats. These indicated that SXT may inhibit the AGE-RAGE signaling pathway *via* reducing ox-LDL to ameliorate lipid metabolism disorders and anti-apoptotic effects in MI/RI in diabetes. However, further experimental validation is required to confirm the predicted results of network pharmacology.

Effect of SXT on blood glucose and blood lipids in diabetic rats with MI/RI

At baseline, there were no significant differences in IPGTT or IPITT between each group of rats, and no insulin resistance or increase in blood glucose was observed (Figure 8A-D). At the end of the experiment, the rats in the DS, DMR, SXTL, SXTM, and SXTH groups exhibited impaired glucose tolerance and significantly increased blood glucose levels at all time points compared with group C ($P < 0.001$) (Figure 8E and F). The average areas under the curves of the DS, DMR, SXTL, SXTM, and SXTH groups during IPGTT and IPITT were all increased (Figure 8G and H), and the international sensitivity index was significantly decreased compared with group C ($P < 0.001$) (Figure 8I). However, there were no differences among the DS, DMR, SXTL, SXTM, and SXTH groups. This indicated that SXT could not reduce blood glucose levels in MI/RI in diabetic rats, nor could it relieve the impaired insulin sensitivity and insulin resistance.

Table 2 Results of serum lipid metabolism indexes in rats

Group	TC (mmol/L)	TG (mmol/L)	FFA (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
C	1.10 ± 0.20	0.60 ± 0.10	0.41 ± 0.02	0.74 ± 0.08	1.66 ± 0.11
DS	2.50 ± 0.20[c]	1.54 ± 0.07[c]	0.78 ± 0.02[c]	1.53 ± 0.06[c]	0.81 ± 0.07[c]
DMR	2.63 ± 0.15[c]	1.54 ± 0.08[c]	0.77 ± 0.03[c]	1.51 ± 0.03[c]	0.81 ± 0.06[c]
SXTL	2.17 ± 0.21[c]	1.46 ± 0.09[c]	0.69 ± 0.03[c,d]	1.47 ± 0.03[c]	0.97 ± 0.06[c]
SXTM	1.90 ± 0.20[b,e]	1.30 ± 0.08[c,d]	0.66 ± 0.04[c,e]	1.32 ± 0.04[c,e]	1.05 ± 0.08[c]
SXTH	1.85 ± 0.15[b,e]	1.30 ± 0.02[c,d]	0.61 ± 0.02[c,f]	1.34 ± 0.05[c,d]	1.08 ± 0.14[c,d]

[b]$P < 0.01$.
[c]$P < 0.001$ *vs* group C.
[d]$P < 0.05$.
[e]$P < 0.01$.
[f]$P < 0.001$ *vs* group DMR ($n = 3$ rats per group).
Results are expressed as the mean ± SD. C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group; TC: Total cholesterol; TG: Total triglycerides; FFA: free fatty acids; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol.

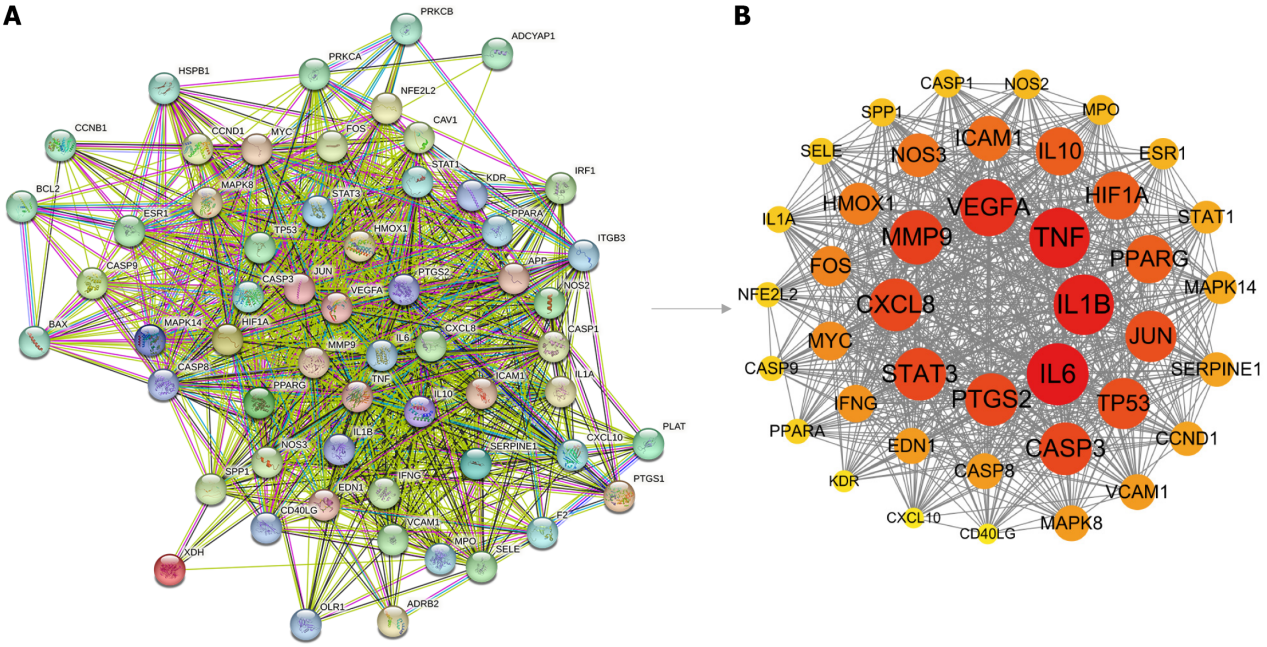

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Figure 5 Protein-protein interaction network of targets related to Shuxin decoction for attenuating myocardial ischemia/reperfusion injury in diabetes. A: Protein-protein interaction (PPI) network of 58 targets generated by STRING 11.5; B: PPI network of 41 key therapeutic targets constructed *via* Cytoscape 3.9.0 software. In accordance with the degree value, the targets are organized in a descending order, ranging from the highest degree to the lowest degree.

The ELISA results given in Table 2 show that compared with group C, TC, TG, FFA, and LDL-C values in each group were significantly increased, and HDL-C was significantly decreased ($P < 0.001$). Compared with the DMR group, TC, TG, FFA, and LDL-C values were significantly decreased in the SXTH group, and HDL-C was significantly increased ($P < 0.05$).

SXT improves cardiac dysfunction in diabetic rats with MI/RI

To verify the cardioprotective effects of SXT on MI/RI in diabetic rats, we initially assessed left ventricular function, cardiac damage markers, and histopathologic changes. As shown in Figure 9A-C, echocardiography showed that LVEF and LVFS values were remarkably reduced in the DMR group compared with the C and DS groups ($P < 0.001$). In the SXTH group, LVEF and LVFS values were significantly increased compared with the DMR group ($P < 0.05$). Figure 9D-F shows that the cardiac damage markers CKMB, cTnT, and LDH levels in serum were significantly higher in the DMR

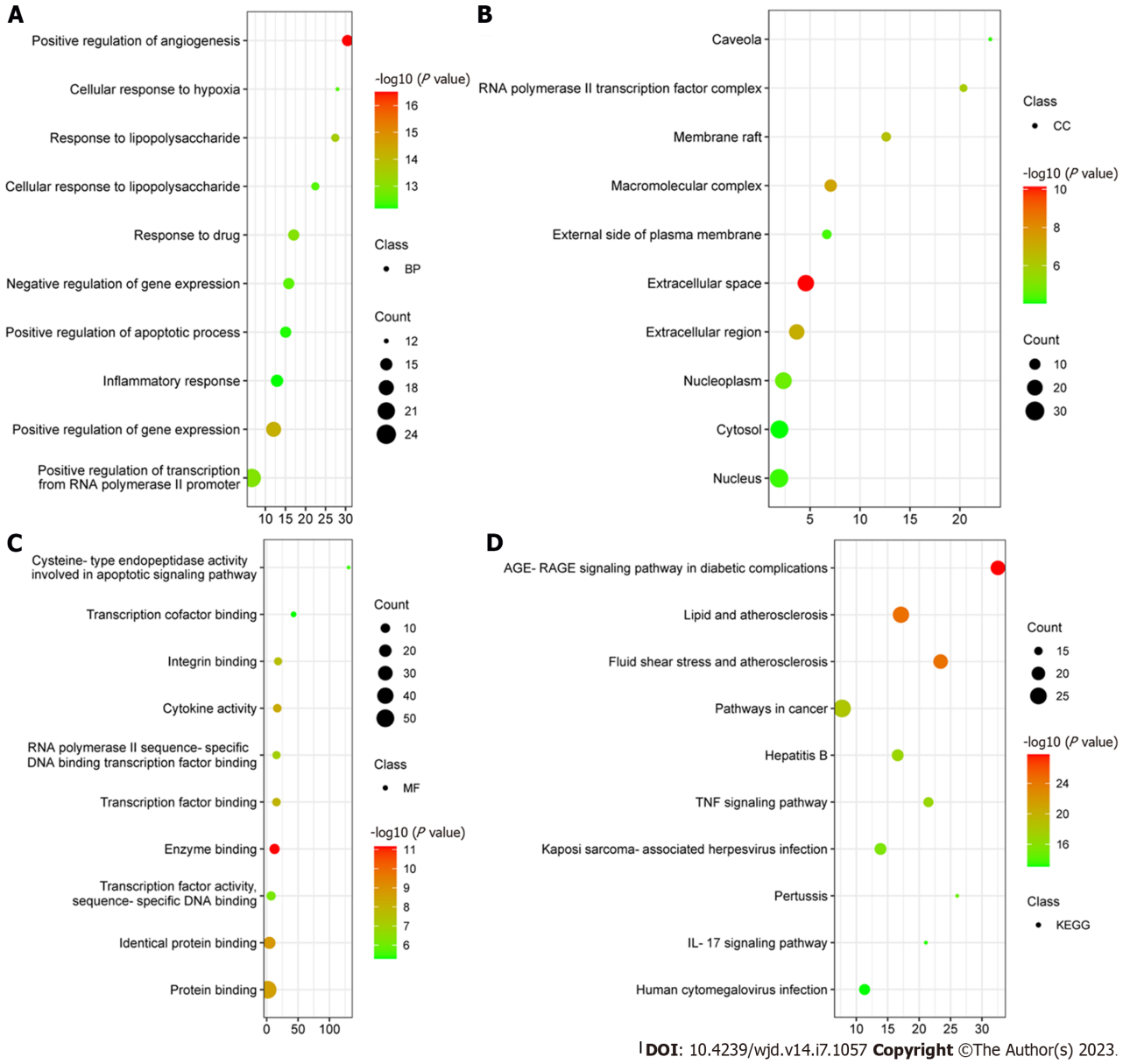

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Figure 6 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis. A: Top ten biological process terms according to the degree value; B: Top ten cellular component terms according to the degree value; C: Top ten molecular function terms according to the degree value; D: Top ten Kyoto Encyclopedia of Genes and Genomes terms according to the degree value. BP: Biological process; CC: Cellular component; MF: Molecular function; KEGG: Kyoto Encyclopedia of Genes and Genomes; IL: Interleukin; TNF: Tumor necrosis factor.

group than in the C and DS groups ($P < 0.001$). Conversely, a high dose of SXT markedly attenuated these changes ($P < 0.05$). Furthermore, HE staining showed that the DMR group showed regional necrosis, interstitial edema, inflammatory cell infiltration, disordered and swollen muscle fibers, rupture of myocardial fibers, and dark staining. However, in the group that received different doses of SXT, these histopathologic changes were replaced by well-arranged myocardial cells (Figure 10A). The percentage of AAR to total area was calculated *via* the EB-TTC double-staining method. As shown in Figure 10B and C, compared with the C group, the proportion of AAR in the DMR group was significantly higher ($P < 0.001$). However, in the SXTM and SXTH groups , the proportion of AAR was significantly reduced compared with the DMR group ($P < 0.01$). These results demonstrate that SXT could improve the cardiac dysfunction of diabetic rats with MI/RI.

SXT attenuates myocardial apoptosis in MI/RI in diabetic rats

TUNEL assay was used to detect myocardial apoptosis. As shown in Figure 11A and B, the DMR group showed a significant increase in the number of apoptotic myocytes compared with the C and DS groups ($P < 0.001$). Compared with the DMR group, SXT significantly decreased the number of apoptotic myocytes ($P < 0.001$). Moreover, the expression of apoptosis-related proteins was evaluated by Western blot analysis. As shown in Figure 11C-F, compared with the C group, the DMR group had significantly decreased anti-apoptotic protein Bcl-2 expression and increased pro-apoptotic proteins Bax and cleaved caspase-3 expression ($P < 0.05$). The SXTH group had increased Bcl-2 expression and decreased

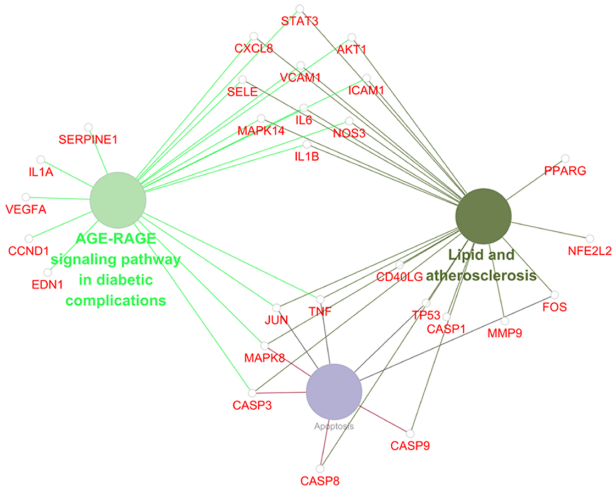

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Figure 7 Forty-one key therapeutic targets enriched in advanced glycation end products-receptor for advanced glycation end products signaling pathway in diabetic complications, lipids and atherosclerosis signaling pathway, and apoptosis.

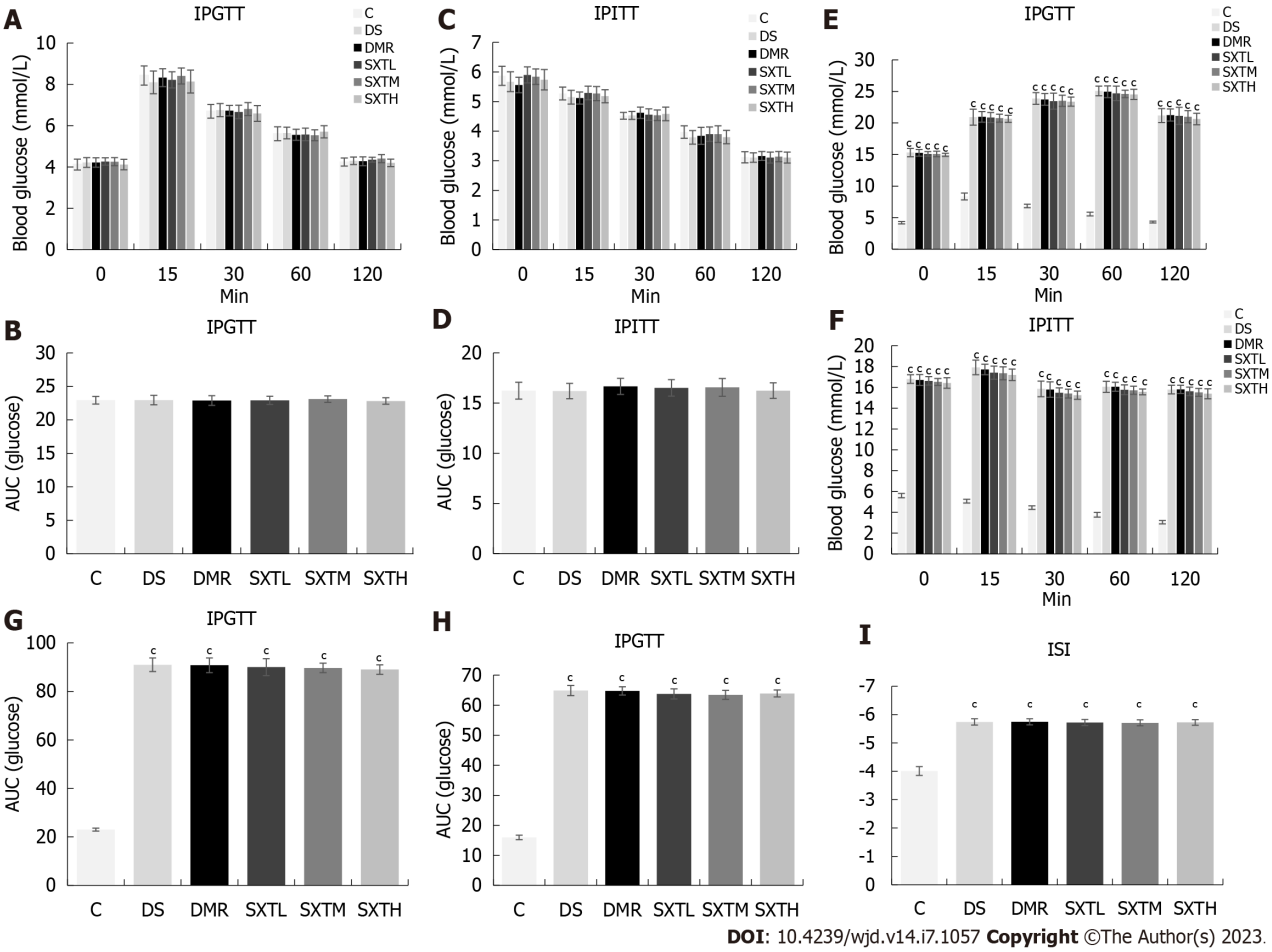

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Figure 8 Intraperitoneal glucose tolerance test, intraperitoneal insulin tolerance test, and international sensitivity index of rats. A: Intraperitoneal glucose tolerance test (IPGTT) at baseline; B: Average area under the curve (AUC) of IPGTT at baseline; C: Intraperitoneal insulin tolerance test (IPITT) at baseline; D: Average AUC of IPITT at baseline; E: IPGTT at the end of the experiment; F: IPITT at the end of the experiment; G: Average AUC of IPGTT at the end of the experiment; H: Average AUC of IPITT at the end of the experiment; I: International sensitivity index (ISI) at the end of the experiment. ISI = 1(/Log FPG × Log FINS). $^{c}P < 0.001$ *vs* group C (n = 8-10 rats per group). IPGTT: Intraperitoneal glucose tolerance test; IPITT: Intraperitoneal insulin tolerance test; C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group; AUC: Area under the curve.

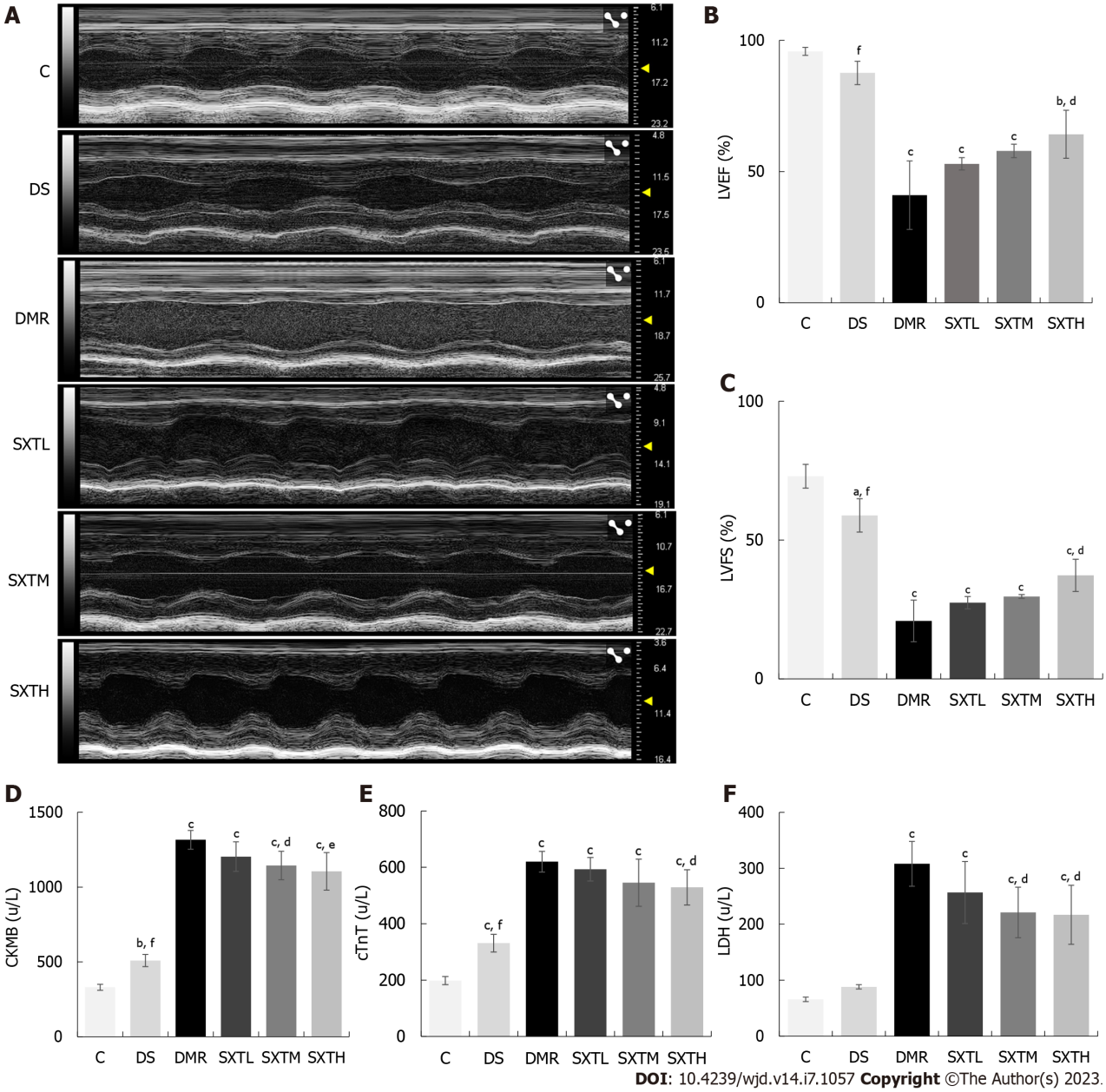

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Figure 9 Echocardiography and cardiac damage markers. A: Echocardiography after 2 h reperfusion; B: Left ventricular ejection fraction after 2 h reperfusion; C: Left ventricular fractional shortening after 2 h reperfusion; D: Creatine kinase isoenzyme MB at the end of the experiment; E: Troponin T at the end of the experiment; F: Lactate dehydrogenase at the end of the experiment. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* group C. $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* myocardial ischemia/reperfusion injury in diabetes group (n = 3 rats per group). C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening; CKMB: Creatine kinase isoenzyme MB; cTnT: Troponin T; LDH: Lactate dehydrogenase.

Bax and cleaved caspase-3 expression ($P < 0.05$). These results indicated that a high dose of SXT attenuated MI/RI in diabetic rats by inhibiting apoptosis.

SXT attenuates blood lipids and myocardial apoptosis in diabetic rats with MI/RI by reducing ox-LDL and activating AGE-RAGE signaling pathway

To explore the mechanism of SXT regulating lipid metabolism and attenuating myocardial apoptosis in diabetic rats with MI/RI, we measured the ox-LDL, AGE, and RAGE protein expression based on the results of network predictive analysis. As shown in Figure 12A and B, ELISA revealed that, compared with the C group, the levels of ox-LDL and AGEs in the DMR group were significantly increased ($P < 0.001$). In the SXTM and SXTH groups, the levels of ox-LDL and AGEs were significantly decreased compared with those in the DMR group($P < 0.05$). The results of immunofluorescence (Figure 12C and D) revealed that the average density of RAGE in the DMR group was significantly higher than that of the C group ($P < 0.001$). The average density of RAGE was significantly lower in the SXTL, SXTM, and SXTH groups compared with the DMR group ($P < 0.001$). As shown in Figure 12E and F, the expression of RAGE was significantly up-regulated compared

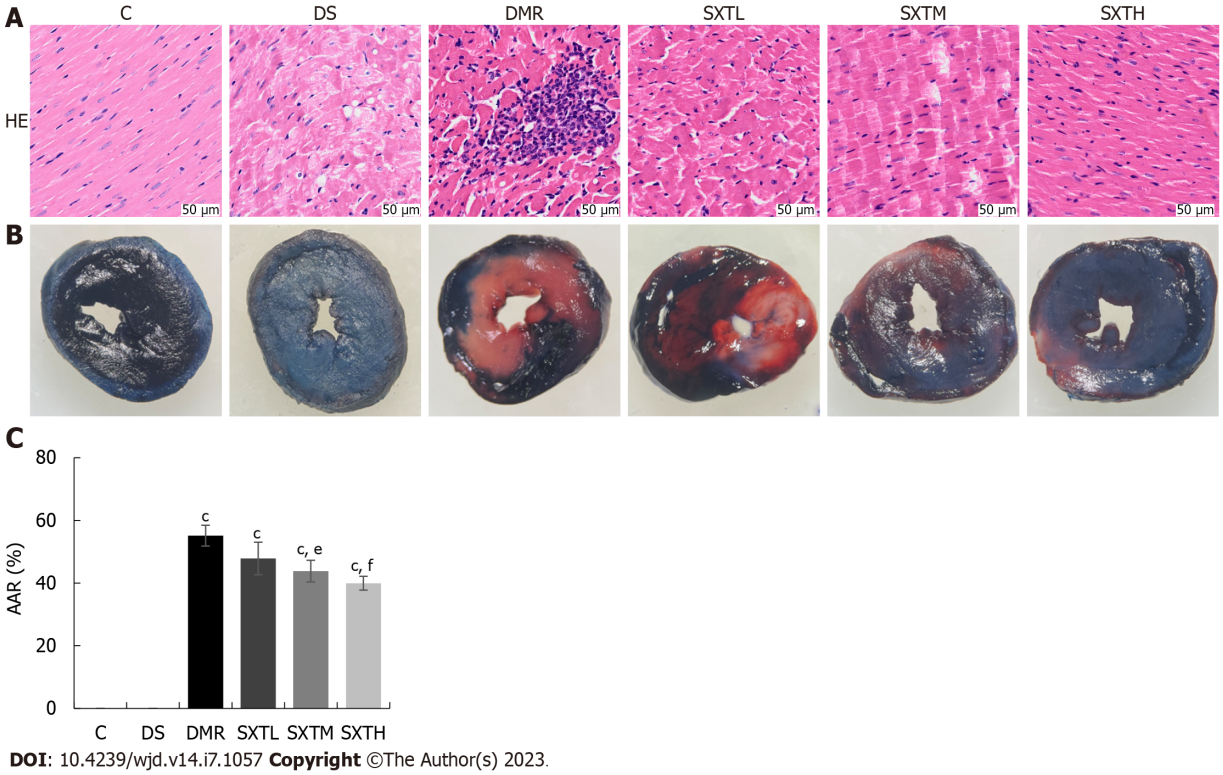

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Figure 10 Pathological staining. A: Hematoxylin and eosin staining; B: Evan's blue-triphenyltetrazolium chloride double-staining; C: Proportion of areas at risk. [c] $P < 0.001$ *vs* group C. [e]$P < 0.01$, [f]$P < 0.001$ *vs* myocardial ischemia/reperfusion injury in diabetes group ($n = 3$ rats per group). C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group; AAR: Areas at risk; HE: Hematoxylin and eosin.

with the C group ($P < 0.001$), while SXTM and SXTH down-regulated the expression of RAGE compared with the DMR group ($P < 0.05$). These results suggested that the anti-apoptosis mechanism of SXT in MI/RI of diabetic rats might be related to a reduction in ox-LDL and the inhibition of the AGE-RAGE signaling pathway.

DISCUSSION

In this study, we discovered that SXT could significantly reduce the level of blood lipids, and alleviate cardiomyocyte apoptosis and myocardial injury without glycemic control. SXT targets the pathogenesis of MI/RI in diabetes by reinforcing Qi and promoting blood circulation, regulating the level of blood lipids, alleviating cardiomyocyte apoptosis, and improving cardiac function. It is a problem for TCM formulations to be examined at the molecular level in terms of their multi-component and multi-target features. However, with the rapid development of network pharmacology, systematic research of TCM formulations has been in progress. Therefore, we explored and verified the molecular mechanisms of SXT in the treatment of MI/RI in diabetes *via* network pharmacology and experimentation.

Based on network pharmacology, quercetin, beta-sitosterol, and kaempferol were found to be the key components of SXT in reducing MI/RI in diabetes according to the degree of value. Quercetin and kaempferol ameliorated lipid metabolism disorders by activating AMPK[34,35], while quercetin could work against mitochondrial apoptosis by regulating ERK1/2/DRP1 signaling[36]. Beta-sitosterol, a plant sterol that has antioxidant activity, has been suggested to increase resistance to oxidative stress and lipid peroxidation[37]. A total of 41 key SXT therapeutic targets for MI/RI in diabetes were identified through network pharmacology analysis, and they were mainly related to the AGE-RAGE signaling pathway in diabetic complications together with the lipids and atherosclerosis signaling pathway. Coincidentally, the AGE-RAGE signaling pathway in diabetic complications is a downstream pathway of the lipids and atherosclerosis signaling pathway, which is closely related to lipid metabolism and apoptosis[38-40]. Therefore, we selected key proteins in these two pathways for validation and predicted that SXT may inhibit the AGE-RAGE signaling pathway *via* reducing ox-LDL to ameliorate lipid metabolism disorders and exerting anti-apoptotic effects in MI/RI in diabetes. Finally, this study confirmed that a dose of SXT (2.8 g/kg/d) could inhibit the expression of ox-LDL and blood lipids, suppress the expression of AGEs, RAGE, cleaved caspase 3, and BAX proteins, and increase the expression of Bcl-2 protein, thereby reducing MI/RI in diabetes.

Previous studies have found that about half of all patients with type 2 diabetes have complications in the form of dyslipidemia, which is one of the important causes of cardiovascular disease in patients with diabetes[41]. In this study, SXT was not effective in reducing blood sugar and insulin resistance, while it could reduce blood lipids in diabetic rats.

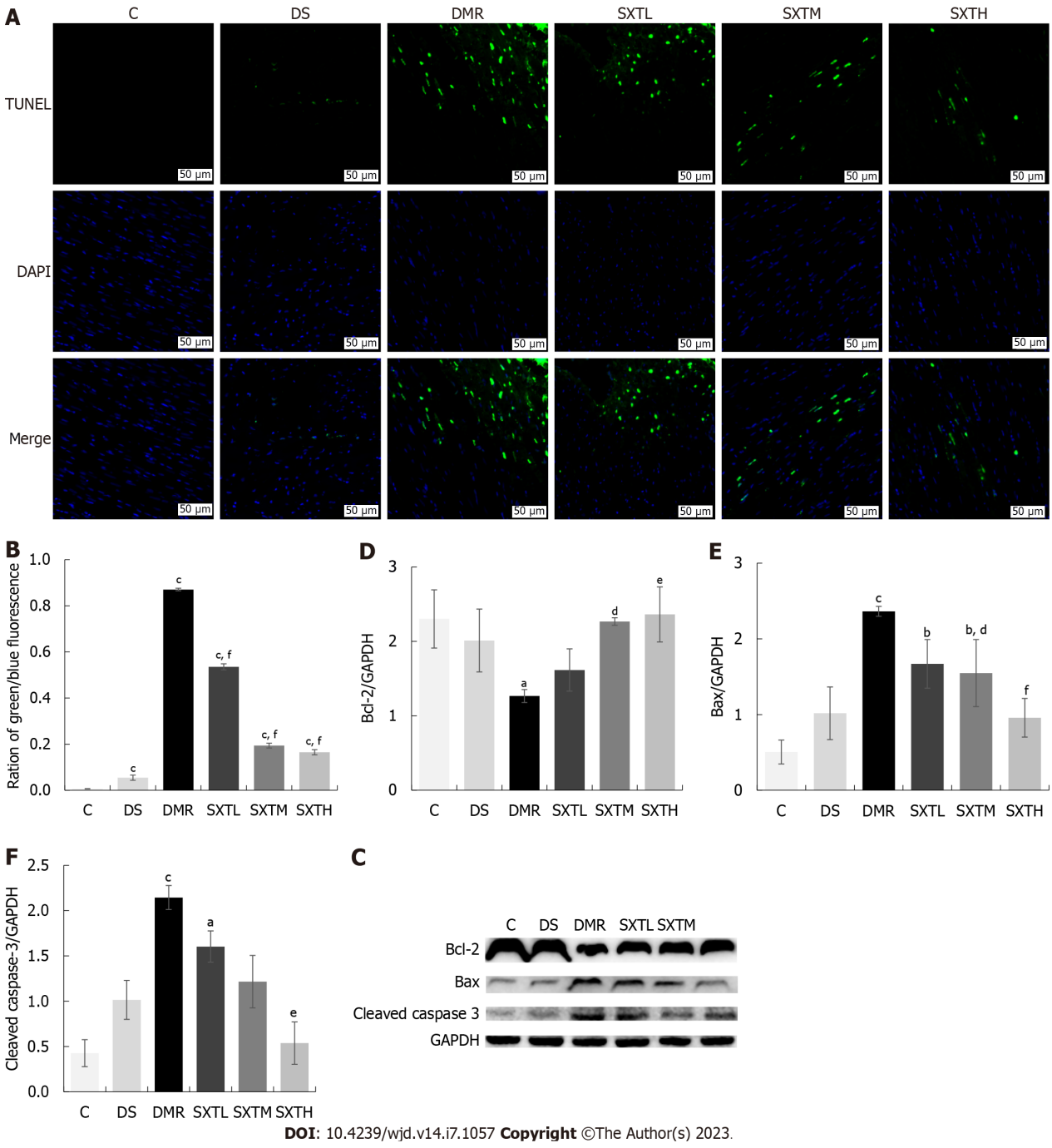

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Figure 11 Shuxin decoction attenuates myocardial apoptosis in diabetic rats with myocardial ischemia/reperfusion injury. A: TUNEL staining. TUNEL-positive nuclei are stained green, while nuclei of cardiomyocytes are blue; B: Percentage of positive apoptosis cardiomyocyte (green/blue fluorescence, magnification × 20, scale bars, 50 μM); C: Bcl-2, Bax, and cleaved caspase-3 protein levels detected by Western blot; D: Statistics of gray value of Bcl-2/GAPDH based on Western blot; E: Statistics of gray value of Bax/GAPDH based on Western blot; F: Statistics of gray value of cleaved caspase-3/GAPDH based on Western blot. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* group C. $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* myocardial ischemia/reperfusion injury in diabetes group (n = 3 rats per group). C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group.

This indicates that SXT regulates dyslipidemia, but not due to its hypoglycemic effect. The liver is the main site of lipid metabolism, and ox-LDL plays an important role in lipid metabolism and cardiovascular diseases[42,43]. VLDL is produced in the liver and released into the plasma, where it is metabolized to LDL *via* intermediate-density lipoproteins [44]. LDL is subjected to oxidization modifications to activate the AGE-RAGE signaling pathway, aggravating oxidative stress and myocardial cell apoptosis[28]. A recent study in the journal of *Science* suggested a new perspective that liver-heart cross-talk mediated by coagulation factor XI protects attenuated heart failure, which coincides with TCM theory [45]. According to the five elements theory of TCM, the liver pertains to wood, representing the mother organ, while the heart pertains to fire, representing the child organ. Pathologically, disorders of the mother organ involve the child organ, which means that liver disease will lead to heart disease. Our study verified that SXT reduced blood lipids, inhibited the

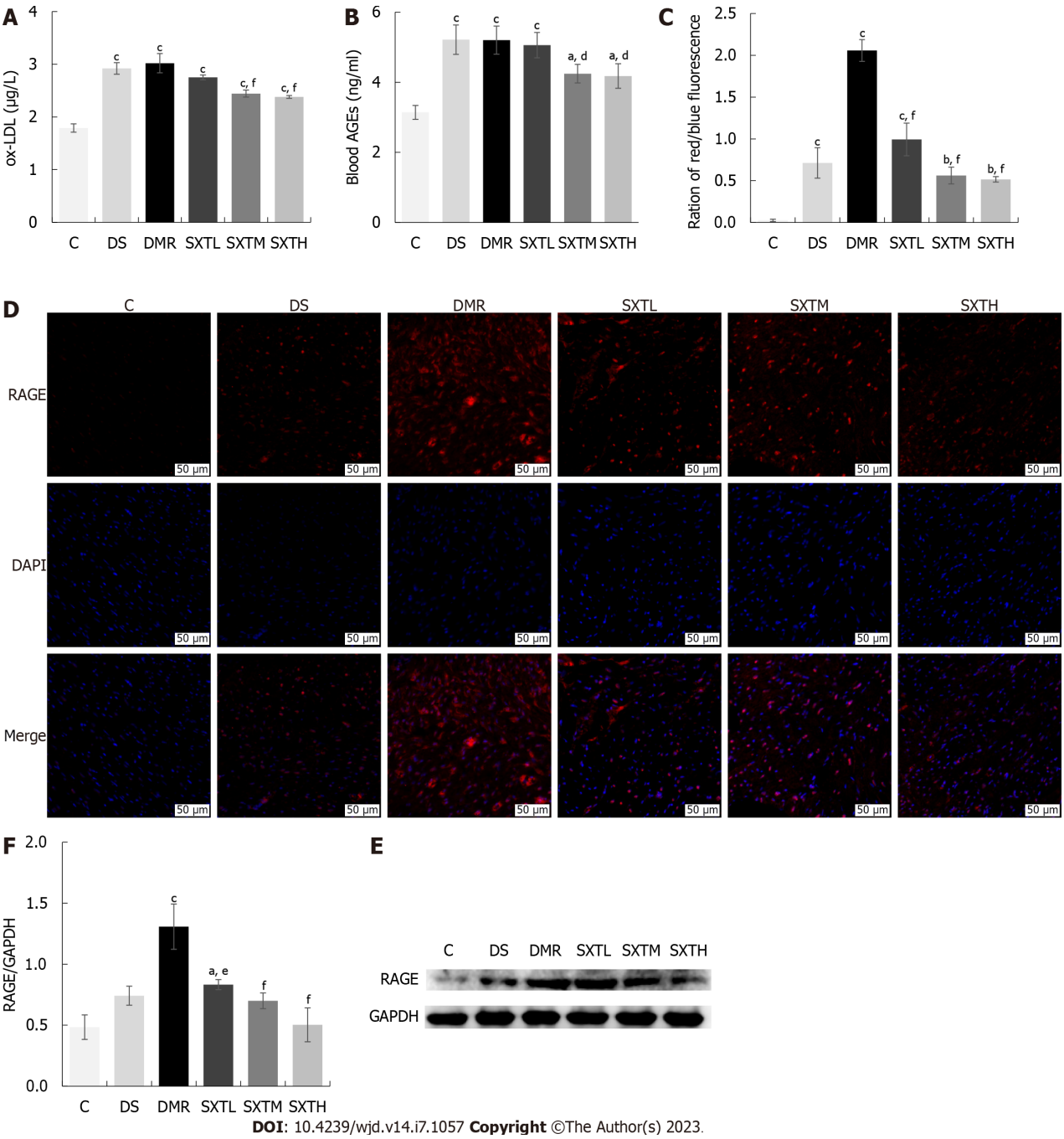

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Figure 12 Shuxin decoction reduces oxidized low density lipoprotein and activates advanced glycation end products-receptor for advanced glycation end products signaling pathway. A: Oxidized low density lipoprotein levels at the end of the experiment; B: Blood advanced glycation end products (AGEs) at the end of the experiment; C: Percentage of positive receptor for AGE (RAGE) (red/blue fluorescence, magnification × 20, scale bars, 50 μM); D: Immunofluorescence of RAGE. The RAGE-positive cells are stained red, while nuclei of cardiomyocytes are blue; E: RAGE protein levels detected by Western blot; F: Statistics of gray value of RAGE/GAPDH based on Western blot. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* group C. $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* myocardial ischemia/reperfusion injury in diabetes group (n = 3 rats per group). C: Normal control group; DS: Diabetic rats with sham operation group; MI/RI: Myocardial ischemia/reperfusion injury; DMR: MI/RI in diabetes group; SXTL: MI/RI in diabetic rats receiving SXT 0.7 g/kg/d group; SXTM: MI/RI in diabetic rats receiving SXT 1.4 g/kg/d group; SXTH: MI/RI in diabetic rats receiving SXT 2.8 g/kg/d group; ox-LDL: Oxidized low density lipoprotein.

expression of ox-LDL, suppressed the AGE-RAGE signaling pathway, and ultimately alleviated MI/RI in diabetes, which also hinted at the theory of liver-heart crosstalk. However, the specific mechanism of how liver-heart crosstalk mediated by lipid metabolism attenuated MI/RI in diabetes needs further study.

CONCLUSION

Considering all these results, we uncovered the targets and molecular mechanisms of SXT for attenuating MI/RI in

diabetes and confirmed that SXT exerted anti-apoptotic effects *in vivo* through regulating the AGE-RAGE signaling pathway. Quercetin, beta-sitosterol, and kaempferol are the key components of SXT in reducing MI/RI in diabetes and need further verification.

ARTICLE HIGHLIGHTS

Research background

The occurrence of myocardial ischemia/reperfusion injury (MI/RI) in diabetic individuals is often accompanied by larger infarct sizes and diminished cardiac function, which can have significant implications for patient prognosis. However, the effectiveness of strict glycemic control for the purpose of reducing cardiovascular mortality in diabetes was found to be insignificant. Notablely, Shuxin decoction (SXT) has been successfully used to alleviate secondary MI/RI in patients with diabetes mellitus in the clinical setting.

Research motivation

There is an urgent need to identify and facilitate developing novel complementary or alternative forms of medicine for effectively managing MI/RI with diabetes.

Research objectives

To investigate the protective effects and underlying mechanism of SXT against MI/RI with diabetes.

Research methods

The Traditional Chinese Medicine System Pharmacology Database was employed to identify critical components and potential targets of SXT. Additionally, various databases such as Gene Expression Omnibus, DisGeNet, Genecards, Drugbank, OMIM, and PharmGKB were searched to identify potential therapeutic targets associated with MI/RI in diabetic patients. The intersection of the potential targets of SXT and the therapeutic targets related to MI/RI in diabetic patients were analyzed through bioinformatics techniques using the Database for Annotation, Visualization and Integrated Discovery. Subsequently, the major results of the bioinformatics analysis were validated through animal experiments.

Research results

Through animal experiments, it was demonstrated that the hypothesis generated by network pharmacology pertaining to the potential of the SXT to ameliorate MI/RI in diabetes through the reduction of oxidized low density lipoprotein (ox-LDL) and inhibition of the advanced glycation end products (AGE)-receptor for AGE (RAGE) signaling pathway was valid. The administration of a dose of SXT (2.8 g/kg/day) led to a decline in ox-LDL, AGEs, and RAGE, along with modulation of blood lipid levels. Furthermore, the treatment resulted in a decrease in the expression of apoptosis-related proteins such as Bax and cleaved caspase 3, while increasing the expression of Bcl-2.

Research conclusions

SXT could regulate the level of blood lipids, alleviate cardiomyocyte apoptosis, and improve cardiac function through the AGE-RAGE signaling pathway.

Research perspectives

The potential utilization of SXT as a complementary or alternative medicinal intervention could represent a valuable strategy for effectively managing MI/RI in diabetes.

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FOOTNOTES

Author contributions: Yang L designed the study, performed the network pharmacological analysis, and wrote the manuscript; Jian Y, Zhang ZY, Qi BW, Jiang CQ, and Yang Y performed the production and identification of SXT; Li YB, Huang S, Huang J, and Ma J performed the animal experiments; Long P performed the statistical analysis; Wang X, Zhou LF, and Hu YH designed the study; Xiao WJ revised the manuscript and approved the final proof as the corresponding author; all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Analysis of N6-methyladenosine-modified mRNAs in diabetic cataract

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Provenance and peer review: Unsolicited article; Externally peer reviewed.

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Peer-review report's scientific quality classification

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Abstract

BACKGROUND

Cataracts remain a prime reason for visual disturbance and blindness all over the world, despite the capacity for successful surgical replacement with artificial lenses. Diabetic cataract (DC), a metabolic complication, usually occurs at an earlier age and progresses faster than age-related cataracts. Evidence has linked N6-methyladenosine (m6A) to DC progression. However, there exists a lack of understanding regarding RNA m6A modifications and the role of m6A in DC pathogenesis.

AIM

To elucidate the role played by altered m6A and differentially expressed mRNAs (DEmRNAs) in DC.

METHODS

Anterior lens capsules were collected from the control subjects and patients with

DC. M6A epitranscriptomic microarray was performed to investigate the altered m6A modifications and determine the DEmRNAs. Through Gene Ontology and pathway enrichment (Kyoto Encyclopedia of Genes and Genomes) analyses, the potential role played by dysregulated m6A modification was predicted. Real-time polymerase chain reaction was further carried out to identify the dysregulated expression of RNA methyltransferases, demethylases, and readers.

RESULTS

Increased m6A abundance levels were found in the total mRNA of DC samples. Bioinformatics analysis predicted that ferroptosis pathways could be associated with m6A-modified mRNAs. The levels of five methylation-related genes-*RBM15*, *WTAP*, *ALKBH5*, *FTO*, and *YTHDF1*-were upregulated in DC samples. Upregulation of *RBM15* expression was verified in SRA01/04 cells with high-glucose medium and in samples from DC patients.

CONCLUSION

M6a mRNA modifications may be involved in DC progression *via* the ferroptosis pathway, rendering novel insights into therapeutic strategies for DC.

Key Words: N6-methyladenosine; Diabetic cataract; RNA; Ferroptosis; Epitranscriptomic microarray

Core Tip: Diabetic cataracts (DCs) are associated with elevated blood sugar levels and usually occur at an earlier age with more rapid progression than age-related cataracts. However, the specific molecular mechanisms underlying DC progression remain to be elucidated. As environmental factors are essential in the pathogenesis of diabetes mellitus, epigenetic changes may be particularly important. Recently, N6-methyladenosine (m6A) has been suggested to play a part in DC progression. The present study elucidated the m6A landscape in DC and simultaneously analyzed the methylation and expression of related mRNA. These analyses indicate that m6A mRNA modifications in lens epithelial cells might be involved in DC progression.

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INTRODUCTION

Over the last several decades, the prevalence of diabetes mellitus (DM) in adults has increased globally. There were approximately 110 million DM cases in China in 2015, and the number is estimated to be 150 million by 2040, as indicated by the International Diabetes Federation[1]. DM, a systemic condition, affects various organs and thus can induce several complications, including cataracts[2]. Despite the increasing maturity of modern cataract surgery technology, cataracts remain a prime reason for vision loss and blindness globally[3,4]. Diabetic cataracts (DCs) usually develop at an earlier age and pro-gresses more rapidly than age-related cataracts do[5]. Evidence has linked DC to polyol pathway, nonenzymatic glycation, and oxidative stress (OS)[4]. Yet, the molecular mechanism underlying DC progression remains largely unknown.

As environmental factors play critical roles in the pathogenesis of DM, epigenetic changes may be particularly important[6]. N6-methyladenosine (m6A), one of the most prevalent epigenetic modifications in mammals[7], is increasingly shown to be crucial in several pathological processes (*e.g.*, tumorigenesis, angiogenesis, tissue degeneration, and inflammatory responses)[8,9]. A study on DC patho-genesis based on m6A-RNA immunoprecipitation (MeRIP)-sequencing reported that the level of the methyltransferase protein complex, methyltransferase-like 3 (METTL3), is upregulated in high glucose-induced human lens epithelial cells (LECs) and that METTL3 mediates a higher methylation level[10]. However, the RNA m6A modification landscape in DC and the role of m6A in DC pathogenesis are still largely undetermined.

Herein, we performed an m6A epitranscriptomic microarray analysis to identify differentially methylated mRNAs and determined their potential roles using bioinformatics analyses, rendering novel insights into the pathogenic mechanisms of DC as well as clues for future biological interventions.

MATERIALS AND METHODS

Participants and specimen collection

The anterior lens capsule (ALC) tissue of three DC patients had been living with diabetes for more than 5 years was collected, and the cataract severity was graded using the Lens Opacities Classification System III[11]. In addition, ALCs collected from age-matched transparent crystals of cadaveric eyes were used as normal controls (NC). Patients with other eye diseases, such as high myopia, trauma, uveitis, or glaucoma were excluded from the study. Patients' information is presented in Table 1. The workflow of sample collection and processing is shown in Figure 1. This study has obtained approval from the Ethics Committee of the Eye and ENT Hospital of Fudan University and written informed consent from all participants, and the principles of the Declaration of Helsinki were strictly follows throughout the research period. This study was registered with ClinicalTrials.gov, number NCT05682001.

Cell culture

The human LEC line SRA01/04, obtained from Genechem, was immersed in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, United States) where 5.5 mmol/L glucose, 10% fetal bovine serum (Invitrogen, Carlsbad, CA, United States), 100 IU/mL penicillin (Thermo Fisher Scientific), and 100 mg/mL streptomycin (Thermo Fisher Scientific) were added, for cultivation in a 5% CO_2 humidified atmosphere with the temperature maintained at 37 °C. Confluent cells (75%-80%) were then randomly grouped as a normal- (NG group; 5.5 mmol/L glucose-supplemented medium) and a high-glucose group (HG group; 25.0 mmol/L glucose-supplemented medium), and cultured for 24 h for subsequent examinations.

Total RNA extraction and m6A immunoprecipitation

Using TRIzol (Invitrogen) and following kit recommendations, total RNA was isolated from the LECs of the included DC patients and controls, as well as SRA01/04 cells, followed by RNA quantification and purity evaluation with a NanoDrop ND-1000 spectrophotometer purchased from Thermo Fisher Scientific. This was followed by immunoprecipitation (IP) of the extracted total RNA from the NC (n = 3) and DC samples (n = 3) with an anti-m6A antibody by referring to the manufacturer's recommendations. In brief, we placed 2 μg total RNA and m6A spike-in control mixture into a 300 μL IP buffer supplemented with 2 μg anti-m6A rabbit polyclonal antibody (Synaptic Systems, Goettingen, Germany), and let the reaction mixture rotate head-over-tail for 2 h at 4 °C. A DynabeadsTM M-280 sheep anti-rabbit immunoglobulin G (IgG) suspension (20 μL) was blocked with freshly prepared 0.5% bovine serum albumin at 4 °C for 2 h, followed by three rinses with IP buffer (300 μL) and resuspension in the total RNA-antibody mixture prepared. The RNA was then allowed to bind to the m6A-antibody beads for 2 h at 4 °C *via* head-over-tail rotation. After washing the beads thrice with 500 μL 1 × IP buffer and twice with 500 μL wash buffer, and incubation with 200 μL elution buffer (50 °C, 1 h), the enriched RNA was eluted and extracted using acid phenol-chloroform for ethanol precipitation.

Two-color RNA labeling and hybridization

The immunoprecipitated m6A-enriched RNAs were eluted from the magnetic beads as "IP", while the unmodified RNAs were collected from the supernatant as "Sup", which were then labeled with Cy5 and Cy3 (cRNAs), respectively, using an Arraystar Super RNA Labeling Kit (Arraystar, AL-SE-005). Purification of the synthesized cRNAs employed a RNeasy Mini Kit (QIAGEN, 74105), and the determination of concentrations and specific activities used the NanoDrop ND-1000. Following Arraystar's standard protocol, microarray hybridization was performed. We combined and hybridized Cy3 and Cy5 Labeled cRNAs to an Arraystar Human mRNA Epitranscriptomic Microarray (4 × 44 K, Arraystar, China), after which the slices were washed for array scanning using an Agilent Scanner G2505C (Agilent, Beijing, China).

Data analysis

Analyses of the acquired array images were carried out using Agilent's Feature Extraction software *v*11.0.1.1. Cy5-labeled IP and Cy3-labeled Sup raw intensities were normalized to the mean of log_2-scaled spike-in RNA intensities. The m6A methylation level was counted as a percentage of modified RNA (% modified) from total RNA, based on IP and Sup normalized intensities. The m6A quantity of each transcript was calculated according to normalized IP (Cy5-labeled) intensities. RNA expression was determined from the total IP and Sup normalized RNA intensities.

Gene ontology and pathway analysis

The online gene ontology (GO) (URL: http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (URL: http://www.genome.jp/kegg) were utilized for determining the enriched GO terms and pathways in the mRNAs with significantly different m6A expression levels.

MeRIP coupled with real-time quantitative polymerase chain reaction

To validate microarray data quality, MeRIP-quantitative polymerase chain reaction (qPCR) was performed on four randomly selected mRNAs. In brief, the IP RNAs from ALC tissues of patients with DC and NCs were analyzed for microarray data validation, with primers used presented in Supplementary Table 1.

Reverse transcription-qPCR

Reverse transcription of total RNA to cDNA was performed as per the instruction of the PrimeScript RT Reagent Kit (Takara, Dalian, Liaoning province, China). Reverse transcription-qPCR (qRT-PCR) primers, designed with the use of

Table 1 Features of diabetic cataract patients included in microarray analysis

No.	Gender	Age (yr)	AL (mm)	Lens opacity grading	Duration of DM (year)	FBG (mmol/L)	HbA1c (%)
1	Male	64	22.09	C4N3P3	10	7.89	7.50
2	Female	68	21.77	C3N4P4	7	8.30	7.80
3	Female	63	23.43	C4N4P5	7	8.30	7.90

AL: Axial length; FBG: Fasting blooding glucose; HbA1c: Hemoglobin; DM: Diabetes mellitus.

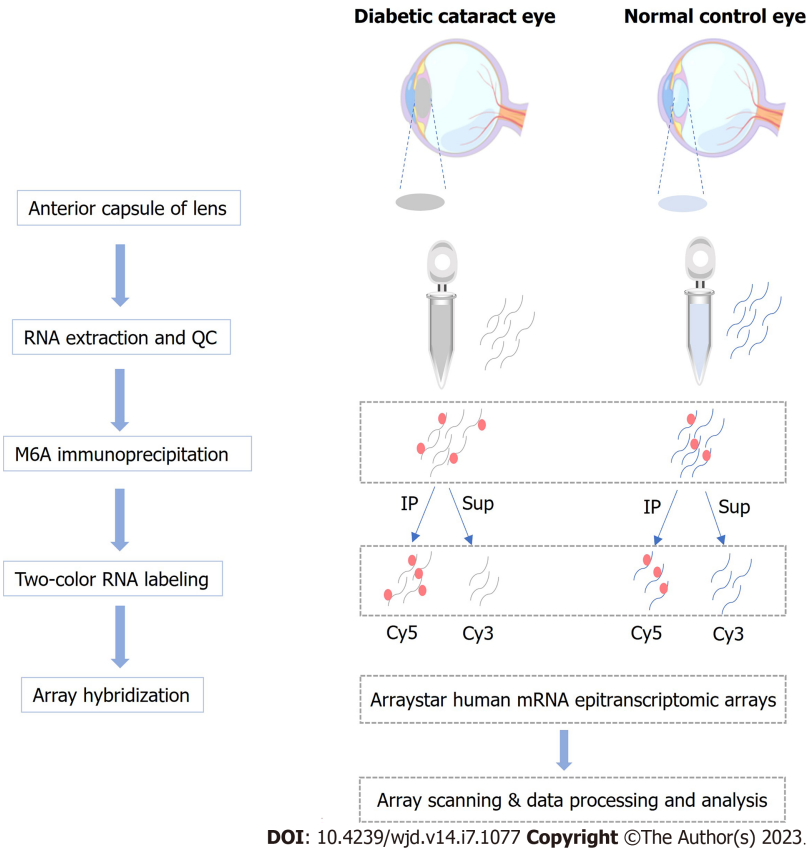

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Figure 1 Workflow of the experimental design. m6A: N6-methyladenosine; QC: Quality control; IP: Immunoprecipitation.

Primer 5.0, were blasted for specificity in NCBI (Supplementary Table 1). An Applied Biosystems ViiA 7 Real-Time thermal cycler (Thermo Fisher Scientific) and SYBR Green PCR Master Mix (Arraystar) were then utilized to perform the qRT-PCR. The expression of target mRNAs were normalized against Actin, and fold changes were determined by the comparative CT ($2^{-\Delta\Delta CT}$) method.

Statistical analysis

The significance threshold was $P < 0.05$ in this study. For the microarray analysis, statistical significance in methylation levels between DC cases and NCs was identified using an unpaired two-sided t-test. For GO and KEGG analyses, GO terms and KEGG pathway identifiers with significant differences were identified using the Fisher's exact test p-value and -log10(p) transformed as the enrichment score. While the relative genes' expression in MeRIP-qPCR and qRT-PCR was worked out by $2^{-\Delta\Delta CT}$.

RESULTS

Epitranscriptomic microarray analysis reveals the differential m6A modification of mRNAs in DC samples

Microarray analyses of the mRNAs extracted from the lens anterior capsule tissues of the DC and NC samples showed differential m6A-methylated mRNAs, as identified by the "m6A-mRNA quantity and m6A-mRNA methylation level". The results have been presented as heatmaps (Figure 2A and C) and volcano plots (Figure 2B and D). According to the

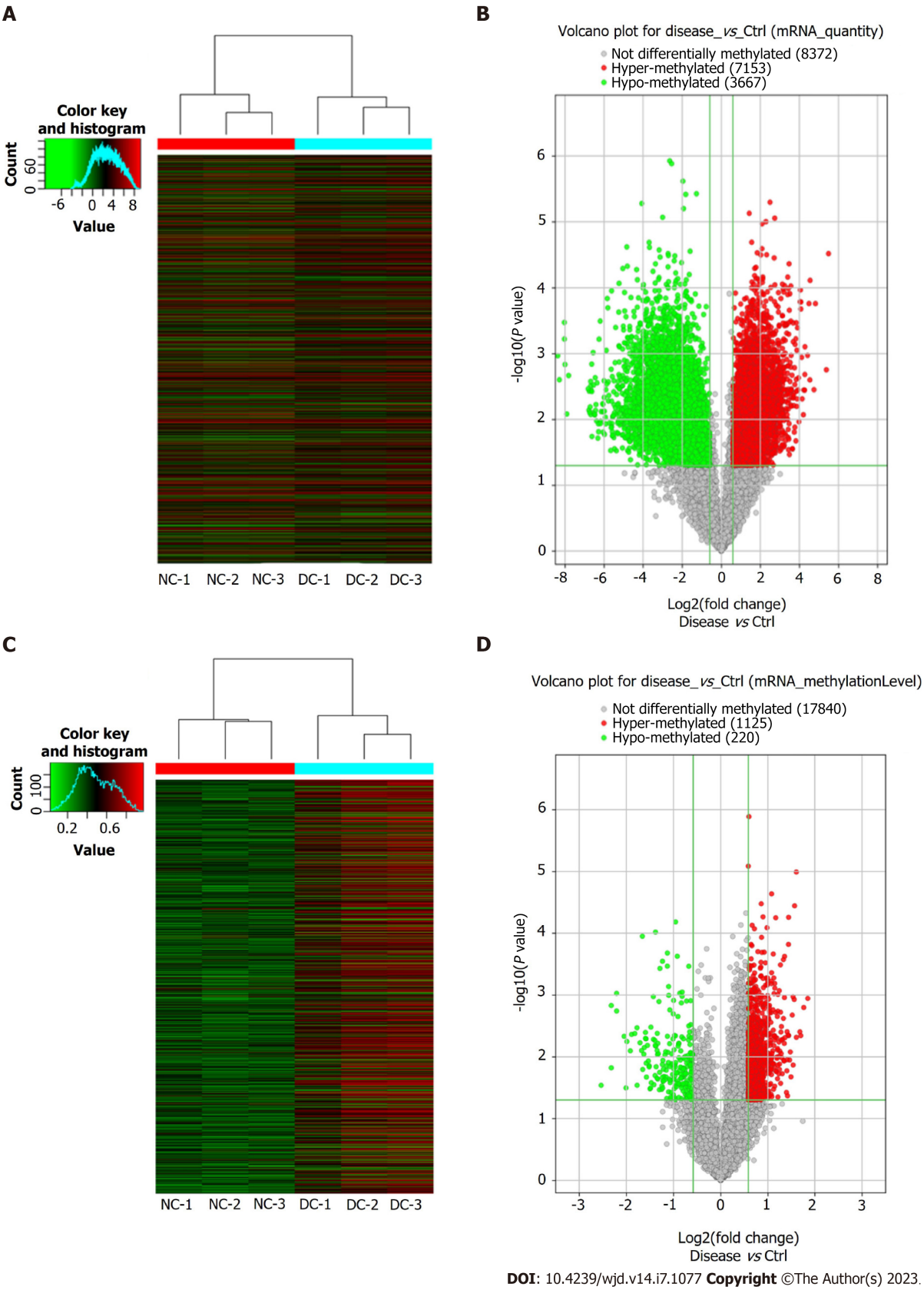

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Figure 2 Microarray data analysis showing expression profile of methylated mRNAs. A: Visualization of differential N6-methyladenosine (m6A) quantity profiles of mRNAs between the diabetic cataract (DC) and normal control (NC) groups through heat map and hierarchical clustering, where red and green colors indicate up- and down-regulated mRNAs, respectively; B: Volcano plot showing significant dysregulation of 10820 (7153 upregulated and 3667 downregulated) mRNAs in DC cases compared to NCs; C: Visualization of differential m6A mRNA methylation level profiles between DC cases and NCs through heat map and hierarchical clustering, where red and green colors indicate up- and down-regulated mRNAs, respectively; D: Volcano plot showing significant dysregulation of 1345 (1125 upregulated and 220 downregulated) methylated mRNAs in DC cases versus NCs.

m6A quantity results, there were 7153 hypermethylated mRNAs and 3667 hypomethylated mRNAs. As per the m6A methylation level results, 1125 mRNAs had higher m6A methylation levels, whereas 220 mRNAs had lower levels. See Table 2 for the top 20 mRNAs with the most significant hyper- and hypomethylation levels between DCs and NCs.

GO and KEGG pathway analyses reveal the biological function of differentially methylated mRNAs in DC

The enriched GO annotations can be fall into biological process (BP), cellular component (CC), or molecular function (MF). For hypermethylated mRNAs, 580 BPs, 110 CCs, and 100 MFs were enriched. The quantity of differentially methylated mRNAs related to the listed GO ID was recorded; of them, the top 10 most significantly enriched terms are presented as pie charts (Figure 3A-C, G). In addition, the top four terms with the highest enrichment score are shown in Figure 3G. For the hypomethylated mRNAs, 288 BPs, 47 CCs, and 67 MFs were enriched. See Figures 3D-F and H for the top 10 most significantly enriched terms and the top 4 terms with the highest enrichment scores.

Based on KEGG pathway analysis, the mRNAs differentially methylated by m6A participated in 27 pathways (Figure 4A and B). Most of the hypermethylated mRNAs were primarily enriched in "ferroptosis", "PPAR axis", and "alpha-linolenic acid metabolism". The ferroptosis pathway map is illustrated in Figure 4C.

Functional analysis of differentially expressed mRNAs in DC specimens

Besides m6A modification levels, the m6A microarray analyses provided data for mRNA expression (Figure 5A and B). A total of 12015 mRNAs in the DC and NC groups showed significantly different expression [$P \leq 0.05$, fold change (FC) ≥ 1.5], 7698 of which were upregulated, whereas 4317 were downregulated. The functions of the top 20 differentially expressed mRNAs (DEmRNAs) (Supplementary Table 2) were analyzed using GO and KEGG pathway analyses. Among the enriched GO terms, 780 BPs, 137 CCs, and 101 MFs were associated with downregulated mRNA expression, with the top 10 displayed in Figure 5C. Moreover, 1199 BPs, 119 CCs, and 190 MFs were identified to be linked to upregulated mRNA expression, with the top 10 presented in Figure 5D. Among the upregulated mRNAs of the BP category, "cellular component organization" had the highest GO term enrichment score, whereas for the downregulated mRNAs, the highest score belonged to "positive regulation of immune effector process". For the CC category, "intracellular" and "plasma membrane" were the most prominent GO terms for up- and down-regulated mRNA expression, respectively. In the MF category, "protein binding" was the most significant term for both up- and downregulated mRNAs.

According to KEGG pathway analysis, DEmRNAs participated in 55 pathways, most of which were primarily enriched in the "MAPK axis", "Type II DM", and "cAMP axis" (Figure 5E and F).

Combined analysis of m6A methylation and mRNA expression in DC samples

Using the thresholds FC ≥ 1.5 and $P \leq 0.05$, the combined analysis revealed significantly altered m6A methylation and mRNA expression levels in 1,320 mRNAs. Conjoint analysis of these 1320 mRNAs resulted in the formation of four mRNA groups: Group I, 958 hypermethylated and upregulated mRNAs; Group II, 105 hypermethylated and downregulated mRNAs; Group III, 207 hypomethylated and downregulated mRNAs; Group IV, 50 hypomethylated and upregulated mRNAs (Figure 6A). Several key genes of ferroptosis (*PRNP*, *SLC39A8*, *VDAC2*, *P53*, *CYBB*, *ATG7*, and *SLC3A2*) were found in Group I.

Hypermethylated-upregulated (hyper-up) and -downregulated (hypo-down) mRNAs were further identified using GO and KEGG pathway analyses. For Group I mRNAs, the most enriched GO terms in BP, CC, and MF categories were found to be "protein membrane anchor", "early phagosome", and "sodium ion binding", respectively. For Group III mRNAs, the terms were "lens fiber cell development", "cohesin complex", and "translation release factor activity binding" (Figure 6B). KEGG pathway analysis showed that DEmRNAs participated in 26 pathways. Most mRNAs in Group I were mainly enriched in "alpha-linolenic acid metabolism," "ferroptosis", and "apoptosis", whereas Group III mRNAs were primarily enriched in "calcium axis", "cGMP-PKG axis", and "tight junction" (Figure 6C and D).

Validation of the diverse methylated mRNA and RNA methyltransferase expression patterns in vivo and in vitro

We randomly selected four mRNAs (BECN2, METTL21A, NFE2, and TIPRL) for MeRIP-qPCR to validate the microarray data quality; specifically, we screened the differentially methylated mRNAs under the criteria of P value ≤ 0.05 and FC ≥ 1.5, and then we selected genes with multiple expression folds for verification with the primers can be designed for those mRNAs. Finally, we selected 4 methylated mRNAs with different fold changes for verification to ensure the reliability of the results to a certain extent. Details of the selected genes are listed in Supplementary Table 3, and the results accorded with the microarray data (Figure 7A). Furthermore, to explore the possible genes participating in m6A modification, we compared the expression of the DEmRNAs in our epitranscriptomic micro-array with that of 24 known methylation-related genes (METTL3, METTL14, WTAP, VIRMA, KIAA1429, RNA binding motif protein 15 (RBM15), RBM15B, ALKBH5, FTO, AlkB-H9, HNRNPA2, HNRNPB1, HNRNPC1, HNRNPC2, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, EIF3A, EIF3B, IGF2BPs, DGCR8, and ELAVL1). The expression of five genes (RBM15, WTAP, ALKBH5, FTO, and YTHDF1) was found to be upregulated in the microarray results (Figure 7B; the FC values of these genes are shown in Figure 7C); and of these, RBM15 exhibited the highest change in expression level. Additionally, the upregulation of RBM15 in SRA01/04 cells cultured in HG medium verified its expression *in vitro*, supporting the qRT-PCR results in DC specimens (Figure 7D).

Table 2 The top 20 most hyper- and hypomethylated mRNAs

Gene symbol	*P* value	Fold change	Regulation	Chromosome
AQP2	0.001135123	3.60	Hyper	chr12
RPL10	0.001594064	3.40	Hyper	chrX
ACP5	0.004544885	3.25	Hyper	chr19
ACAP1	0.003944032	3.21	Hyper	chr17
CALML6	0.00484061	3.05	Hyper	chr1
DDX31	1.02006E-05	3.04	Hyper	chr9
KRTAP29-1	3.59728E-05	2.97	Hyper	chr17
TRIM39-RPP21	0.001179594	2.95	Hyper	chr6
DGCR8	0.013600714	2.90	Hyper	chr22
LRAT	0.001067196	2.90	Hyper	chr4
NR1H3	0.028959935	5.78	Hypo	chr11
PNPT1	0.001479448	4.99	Hypo	chr2
TSEN2	0.015094744	4.99	Hypo	chr3
C3orf80	0.000939962	4.61	Hypo	chr3
TBCD	0.001813039	4.61	Hypo	chr17
RPS19	0.004668423	4.12	Hypo	chr19
TEAD2	0.031922173	4.02	Hypo	chr19
RAB3IP	0.005656491	3.96	Hypo	chr12
HN1L	0.008004647	3.77	Hypo	chr16
TFEB	0.004349132	3.70	Hypo	chr6

DISCUSSION

The present study elucidated the m6A landscape in DC using an epitranscriptomic microarray, which simultaneously analyzed the methylation and expression of related mRNA. According to the microarray results, a total of 1345 mRNAs exhibiting significantly different m6A modification levels between DC cases and NCs were identified. Most of these mRNAs (1125/1345) had higher m6A methylation levels in the DC samples. First identified in the 1970s, abundant m6A modifications in polyadenylated RNA were accidentally discovered by some research groups when they were characterizing the 5′ structures of mRNA in mammalian cells[12]. In multiple human pathophysiological processes, m6A extensively modifies RNA transcription and protein generation[13]. Modification by m6A modulates gene expression by affecting mRNA splicing, localisation, stability, and translation. Over the past few years, the development of techniques such as MeRIP-sequencing and epitranscriptomic microarrays has made the high-throughput measurement of m6A modification sites possible[14-16]. These approaches allow simultaneous screening of modified transcript types and modification changes under different con-ditions, as well as detection of modification proportions per transcript. The development of microarray method has allowed for a more subtle mapping of the m6A modification, providing better insights into its importance in gene regulation.

In this study, RBM15 was found most upregulated in the DC group, which was verified in DC samples and HG-cultured LECs. Methylation through m6A is a reversible process, dynamically regulated by three different types of protein complexes: methyltransferases, demethylases, and readers[17]. RBM15 and its paralog, RBM15B, are additional components of the methyltransferase complex[18]. RBM15, a split-end protein family member, modulates m6A methylation for RNA modification[19]. As part of the methyltransferase complex, it participates in hematopoietic cell homeostasis and alternative mRNA splicing[20]. The main role of RBM15 in m6A methylation catalysis is recruiting the m6A methyltransferase complex to U-rich regions adjacent to m6A sites[18,21]. Pollreisz *et al*[22] reported markedly increased global mRNA m6A methylation level and RBM15 expression in laryngeal squa-mous cell cancer patients; however, inhibiting RBM15 led to a notable reduction in the m6A methylation level. But the potential roles played by RBM15 in DC pathogenicity need further research. It could be suggested that RBM15-mediated m6A modification of LECs may promote DC progression. Further studies are warranted to clarify the mechanisms underlying m6A modification in DC.

Further, based on the KEGG pathway analysis, ferroptosis was identified as one of the most enriched pathways in the m6A-hypermethylated and upregulated mRNAs in DC samples (Figure 4C). In human lens development, LECs play a key role in transport, metabolism, and detoxification[23]. The integrity and survival of LECs are critical for lens transparency[24]. LEC death due to apoptosis and autophagy plays pathophysiological roles in DC progression[25].

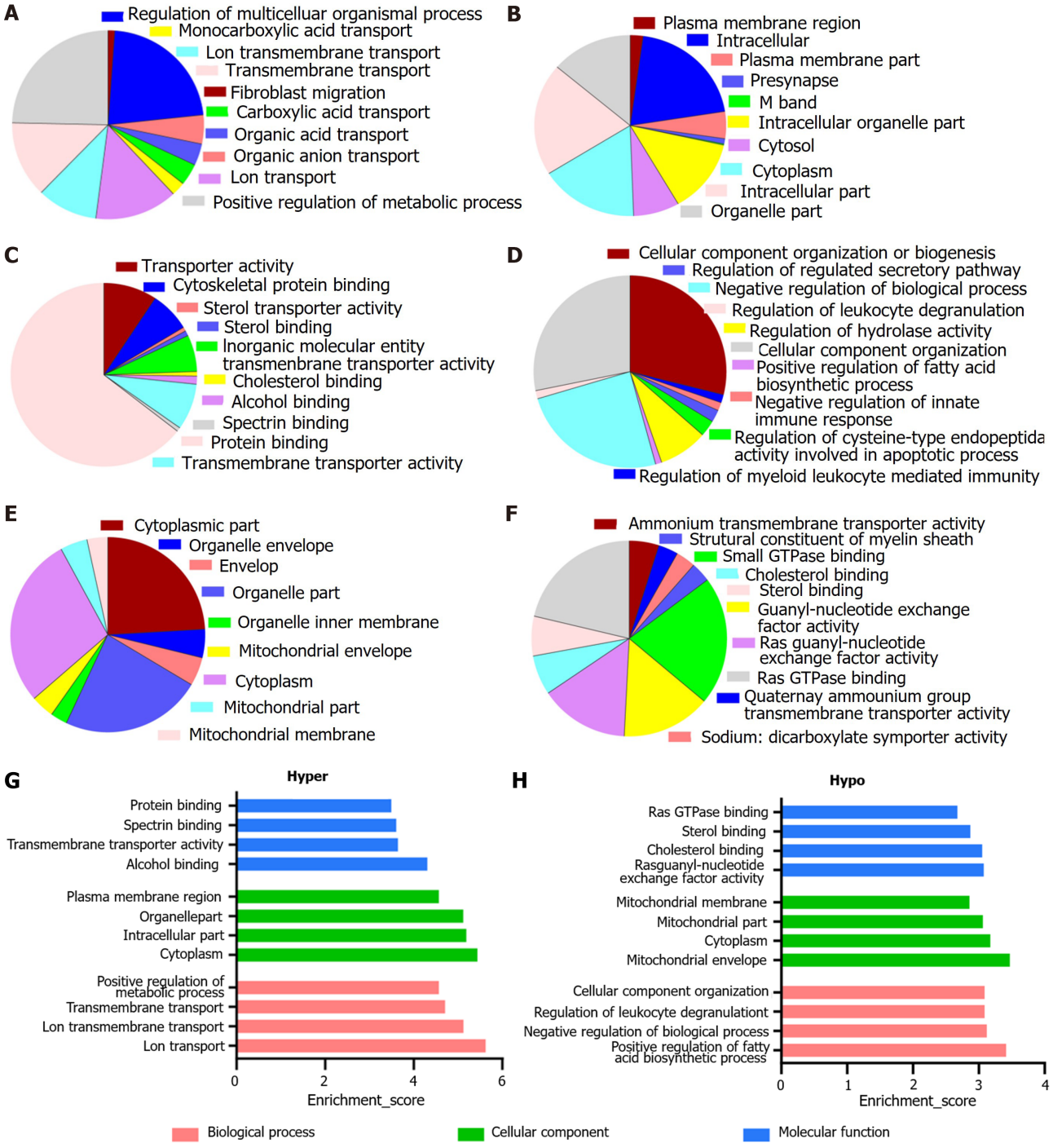

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Figure 3 Overall distribution of Gene Ontology analysis. A-C: Classification of hypermethylated mRNAs in the biological process (BP), cellular component (CC), and molecular function (MF) categories. Among the enriched Gene Ontology (GO) terms, 580 BPs, 110 CCs, and 100 MFs had higher mRNA methylation levels; D-F: Classification of hypomethylated mRNAs in the BP, CC, and MF categories. For the hypomethylated mRNAs, 288 BPs, 47 CCs, and 67 MFs were identified; G: The top four most enriched GO terms of the hypermethylated mRNAs; H: The top four most enriched GO terms of the hypomethylated mRNAs.

Ferroptosis is a newly defined programmed death mode that is implicated in various reactive oxygen species (ROS)-related pathophysiological states, such as age-related macular degeneration and cardiovascular diseases[26,27]. OS is vital in DC pathogenesis[28]. The process of ferroptosis is characterized by glutathione (GSH) depletion, lipid peroxidation, and intracellular ROS accumulation with iron overload as well as accelerated cell death[29-31]. GSH levels are markedly lower in DC patients than in non-diabetic senile cataract patients and non-diabetic type 2 DM patients as well as in healthy individuals[32].

The subsequent combined analysis of m6A methylation and mRNA expression levels showed several ferroptosis-associated key genes (PRNP, SLC39A8, VDAC2, P53, CYBB, ATG7, and SLC3A2) to be hypermethylated and upregulated in the DC group, suggesting enhanced ferroptosis in LECs of patients with DC. P53 can potentiate ferroptosis by inhibiting the transcription of system xc-subunit SLC7A11[33]. Reportedly, its expression was upregulated in the LECs of

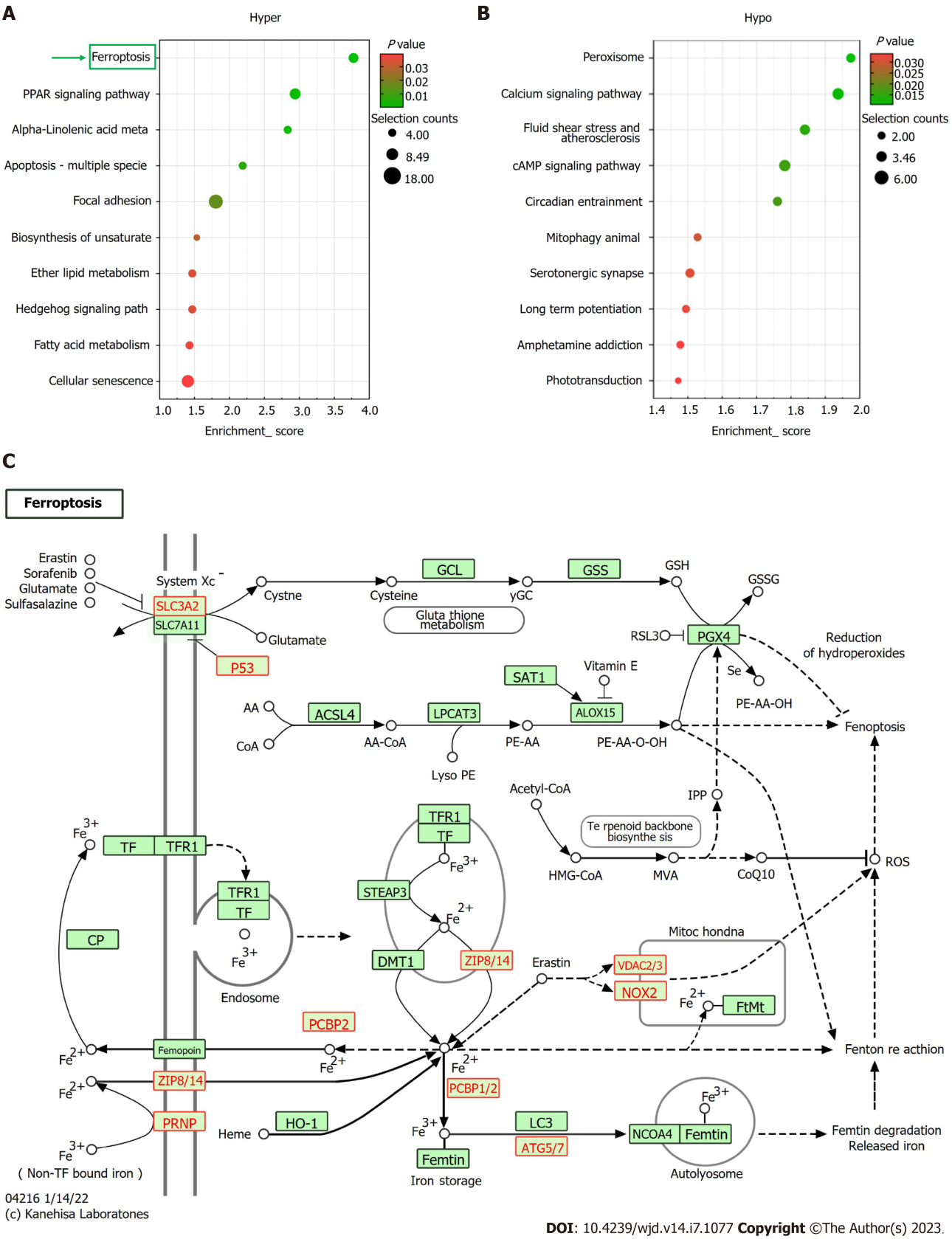

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Figure 4 Kyoto encyclopedia of Genes and Genomes pathway analysis. A: The top 10 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of hypermethylated mRNAs; ferroptosis is the most enriched axis; B: The top 10 KEGG pathways of hypomethylated mRNAs; C: The KEGG map of ferroptosis pathway.

patients with DC[34]. Therefore, we speculate that m6A mRNA modifications of LECs are involved in DC progression *via* the ferroptosis pathway. In future, more comprehensive research is warranted to elucidate ferroptosis-associated mechanisms in DC pathogenesis.

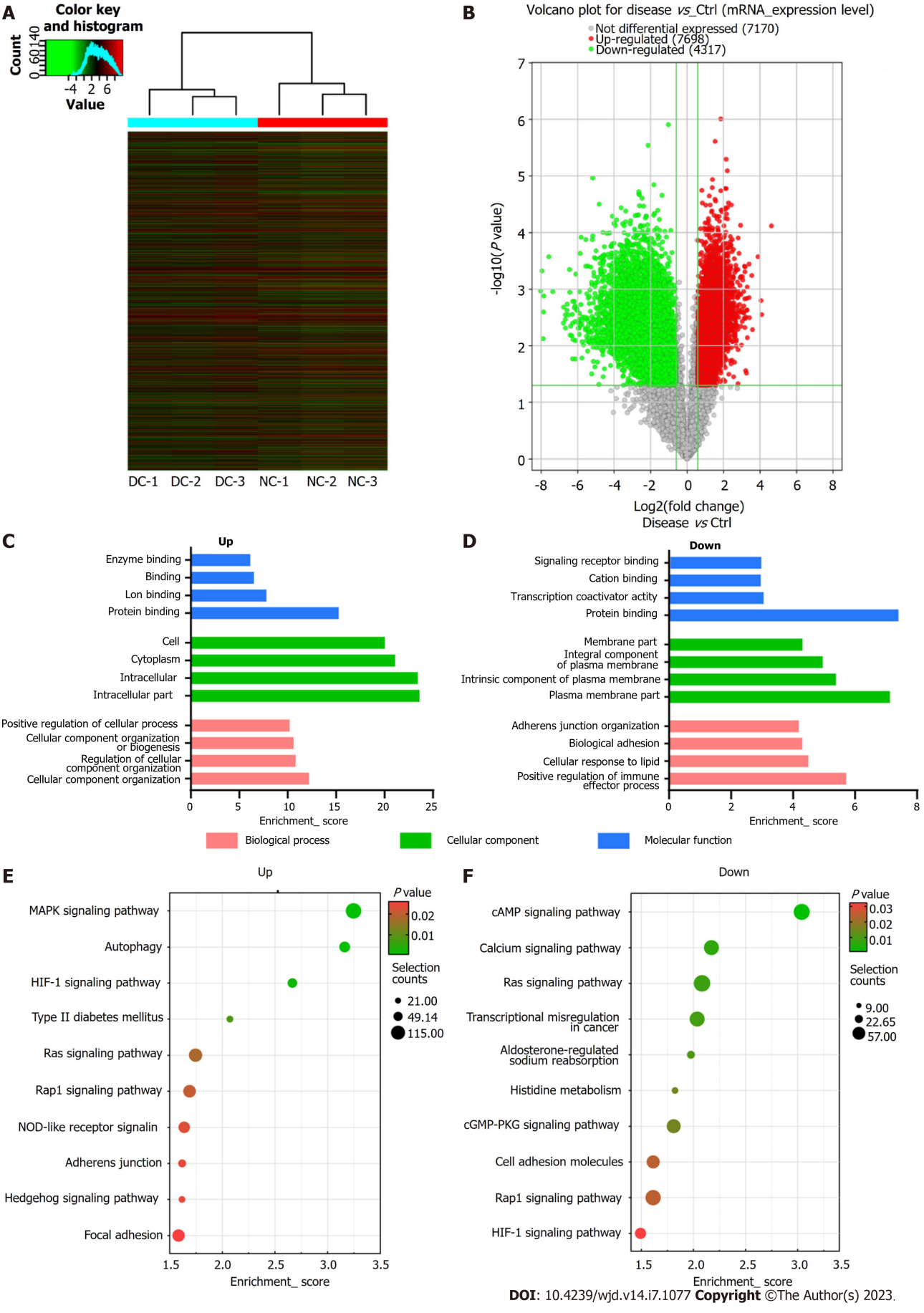

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Figure 5 Microarray data showing the profiles of differentially expressed mRNAs. A: Visualization of differentially expressed mRNAs (DEmRNAs)

profiles between diabetic cataract (DC) cases and normal controls (NCs) through heat map and hierarchical clustering, where red and green colors indicate up- and down-regulated mRNAs, respectively; B: Volcano plot showing significant dysregulation of 12015 mRNAs in DC cases than in NCs; C and D: The top four most enriched Gene Ontology terms of down- (C) and up-regulated mRNAs (D); E and F: The top 10 Kyoto Encyclopedia of Genes and Genomes pathways of down (E) and up-regulated mRNAs (F).

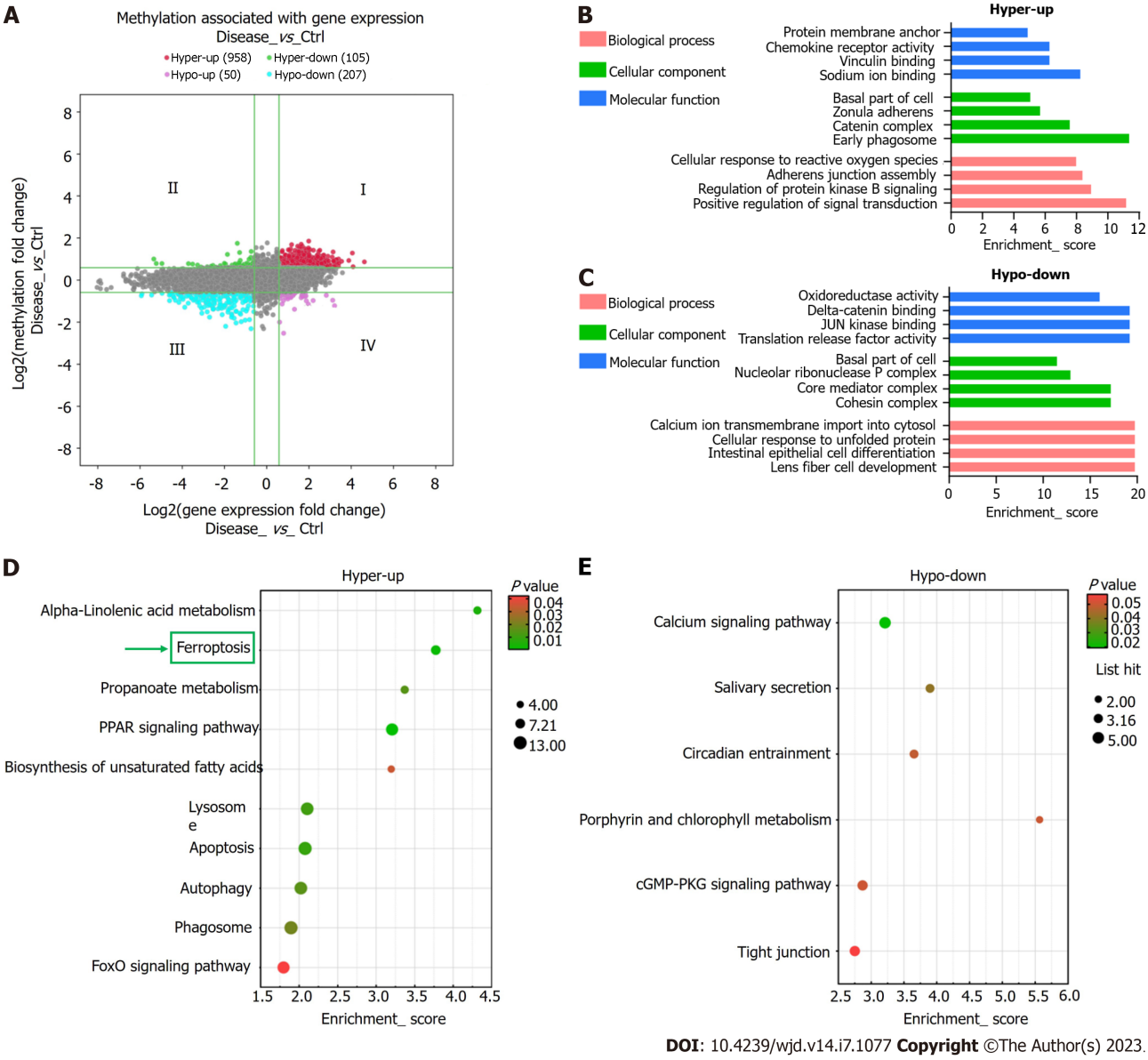

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Figure 6 Combined analysis of N6-methyladenosine methylation and mRNA expression levels. A: Visualization of the positive correlation of differential N6-methyladenosine methylation with differential mRNA expression *via* a four-quadrant graph; B and C: The top four Gene Ontology terms significantly enriched for the hypermethylated-upregulated (hyper-up) genes (B) and the hypomethylated-downregulated (hypo-down) genes (C); D and E: The top 10 Kyoto Encyclopedia of Genes and Genomes pathways significantly enriched for the hyper-up (D) and hypo-down genes (E).

CONCLUSION

Collectively, the m6A abundance level in total mRNA increased in patients with DC. Conjoint analysis indicated that m6A mRNA modifications of LECs might be involved in DC progression *via* the ferroptosis pathway. The expression level of RBM15 increased, which provided a better understanding of the mechanisms underlying upregulated m6A demethylation levels.

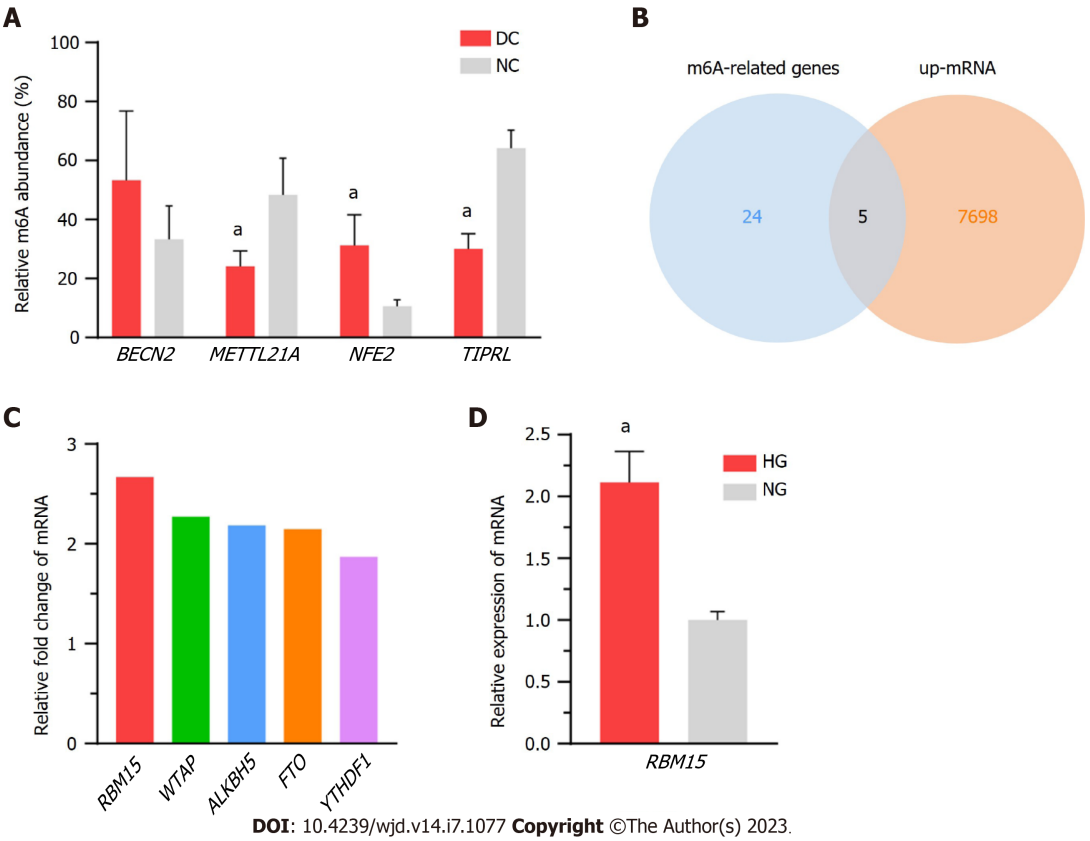

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Figure 7 Validation of the diverse expression levels of methylated mRNA and RNA methyltransferase, using *in vivo* and *in vitro* models. A: Methylation levels of *BECN2*, *METTL21A*, *NFE2*, and *TIPRL* are consistent with the microarray data for the diabetic cataract and normal control groups; B: Intersection results of upregulated mRNAs and N6-methyladenosine-related genes; C: Fold change values of five genes (*RBM15*, *WTAP*, *ALKBH5*, *FTO*, and *YTHDF1*) in microarray results; D: The mRNA levels of *RBM15* are significantly higher in high-glucose cultured SRA01/04 cells than in normal-glucose cultured ones. DC: Diabetic cataract; NC: Normal control; HG: High-glucose; NG: Normal-glucose. $^{a}P < 0.05$.

ARTICLE HIGHLIGHTS

Research background

Cataract remains a prime reason for visual disturbance and blindness all over the world, despite successful surgical replacement with artificial lenses. Diabetic cataract (DC) usually occurs at an earlier age with more rapid progression than age-related cataracts. The polyol pathway, oxidative stress, and nonenzymatic glycation have been shown to be linked to the pathogenesis of DC. But the exact molecular mechanisms underlying DC progression remains largely unknown. As environmental factors play critical roles in the pathogenesis of diabetes mellitus, epigenetic changes may be particularly important.

Research motivation

Despite successful surgical replacement with artificial lenses, cataract remains a prime reason for visual disturbance and blindness globally. It has been recently suggested that N6-methyladenosine (m6A) plays a role in DC progression. However, there exists a lack of understanding regarding RNA m6A modifications and the role of m6A in DC pathogenesis.

Research objectives

To investigate the roles played by altered m6A and differentially expressed mRNAs (DEmRNAs) in DC.

Research methods

M6A epitranscriptomic microarray was used to investigate altered m6A modifications and determine DEmRNAs. The possible roles played by dysregulated m6A modification was predicted through Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. Real-time polymerase chain reaction was carried out to identify dysregulated expression patterns of RNA methyltransferases, demethylases, and readers.

Research results

Increased m6A abundance levels were found in the total mRNA of DC samples. Bioinformatics analysis predicted that ferroptosis pathways could be associated with m6A-modified mRNAs. The levels of five methylation-related genes-

RBM15, WTAP, ALKBH5, FTO, and YTHDF1-were upregulated in DC samples. Upregulation of RBM15 expression was verified in SRA01/04 cells with high-glucose medium and in samples from patients with DC.

Research conclusions

M6A abundance level in total mRNA increased in patients with DC. Ferroptosis pathways could be associated with m6A-modified mRNAs.

Research perspectives

M6A mRNA modifications may be involved in DC progression *via* the ferroptosis pathway.

FOOTNOTES

Author contributions: Cai L and Han XY contributed equally to this work; Lu Y and Yang J contributed equally to this work; Cai L performed the experiments, analyzed the data, and wrote the original draft; Han XY collected the samples, performed the experiments, and also wrote the original draft; Li D designed the experiments; Ma DM and Shi YM performed the experiments; Lu Y and Yang J designed the experiments and revised the draft; and all authors read and approved the final manuscript.

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Informed consent statement: Written informed consent was obtained from all patients.

Conflict-of-interest statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement: The data for this study can be obtained from the corresponding author upon request.

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

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Long-term quality-of-care score for predicting the occurrence of acute myocardial infarction in patients with type 2 diabetes mellitus

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Abstract

BACKGROUND

Cardiovascular disease (CVD) is the leading cause of death globally, and diabetes mellitus (DM) is a well-established risk factor. Among the risk factors for CVD, DM is a major modifiable factor. In the fatal CVD outcomes, acute myocardial infarction (AMI) is the most common cause of death.

AIM

To develop a long-term quality-of-care score for predicting the occurrence of AMI among patients with type 2 DM on the basis of the hypothesis that good quality of care can reduce the risk of AMI in patients with DM.

METHODS

Using Taiwan's Longitudinal Cohort of Diabetes Patient Database and the medical charts of a medical center, we identified incident patients diagnosed with type 2 DM from 1999 to 2003 and followed them until 2011. We constructed a summary quality-of-care score (with values ranging from 0 to 8) with process indicators (frequencies of HbA_{1c} and lipid profile testing and urine, foot and retinal examinations), intermediate outcome indicators (low-density lipoprotein, blood pressure and HbA_{1c}), and co-morbidity of hypertension. The associations between the score and the incidence of AMI were evaluated using Cox regression models.

RESULTS
A total of 7351 patients who had sufficient information to calculate the score were enrolled. In comparison with participants who had scores ≤ 1, those with scores between 2 and 4 had a lower risk of developing AMI [adjusted hazard ratio (AHR) = 0.71; 95% confidence interval (95%CI): 0.55-0.90], and those with scores ≥ 5 had an even lower risk (AHR = 0.37; 95%CI: 0.21-0.66).

CONCLUSION
Good quality of care can reduce the risk of AMI in patients with type 2 DM. The quality-of-care score developed in this study had a significant association with the risk of AMI and thus can be applied to guiding the care for these patients.

Key Words: Acute myocardial infarction; Cardiovascular disease; Diabetes mellitus; Quality-of-care; Score

Core Tip: Cardiovascular disease is the leading cause of death globally, and diabetes mellitus (DM) is a major modifiable factor. Hypothesizing that good quality of care can reduce the risk of acute myocardial infarction (AMI) in patients with DM, we developed a long-term quality-of-care score for predicting the occurrence of AMI in patients with type 2 DM. In 7351 patients, we observed a good association between the score and the risk of AMI. Therefore, good quality of care can reduce the risk of AMI in patients with DM, and the score can be applied to guiding the care for these patients.

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INTRODUCTION

Diabetes mellitus (DM) is prevalent worldwide, and it was approximated that there were 422 million individuals suffering from it in 2014[1]. It was projected that this number will reach 592 million by 2035[2]. In Taiwan, it was estimated that around 1.6 million people (7% of the total population) had DM in 2012, and 90% of them had type 2 DM. For over 30 years, this has been one of the most frequent causes of mortality, resulting approximately 11.5% of overall health care costs in recent times[3]. In addition, DM is associated with a two- to three-fold increased risk of heart attacks and strokes[4], and cardiovascular disease (CVD) is the leading cause of death and disability for those with type 2 DM[5, 6].

Results from randomized controlled trials have demonstrated conclusively that strict glycemic control reduces microvascular complications (retinopathy, nephropathy and neuropathy) in patients with type 1[7,8] and type 2 DM[9-11]. However, there is a lack of firm evidence of the beneficial effects of intensive glycemic control on great vessel disease, especially CVD, from large, long-term randomized controlled trials[12,13]. According to the United Kingdom Prospective Diabetes Study, intensive control (median HbA_{1c} < 7.0%) could reduce the overall microvascular complication rate by 25%, but had only a slight benefit for the prevention of CVD (16% decrease; $P = 0.052$)[11,13,14].

The argument that strict control of blood sugar control has no benefit in terms of reducing mortality is largely driven by the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, which did not observe a positive effect[15,16]. However, other studies such as the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive)[17], have suggested that controlling blood sugar can lead to improvements. Interventions that simultaneously control common comorbidities of DM, such as hypertension and hyperlipidemia, have been shown to be more effective in reducing deaths related to CVD than solely focusing on regulating blood sugar levels[18]. Adherence to frequent blood testing for blood sugar and lipid profile has been linked to fewer hospital visits for people with DM, including those for vascular and renal complications[19]. Multifactorial risk factor reduction (controlling blood sugar levels, stopping smoking, keeping blood pressure (BP) in check, treating cholesterol issues, and daily use of aspirin for secondary prevention) appears to be the most effective preventive approach for the macrovascular complications of type 2 DM. Nonetheless, studies found that screening tests, including those for HbA_{1c} and lipid, as well as urine and retinal examinations, generally fell well below the frequencies recommended by the American Diabetes Association[19].

Many initiatives have been focused on the evaluation and enhancement of healthcare for people suffering from DM[20-24]. The Diabetes Quality Improvement Program (DQIP), one of the most important such initiatives, has proposed a uniform set of process and intermediate outcome indicators for quality of care, selected under the hypothesis that as a whole they can predict macrovascular complications of type 2 DM[25]. Only a small number of studies have combined process (*e.g.*, the frequency of HbA_{1c} testing) and intermediate outcome (*e.g.*, HbA_{1c} < 8.0%) indicators to predict the occurrence of specific complications of DM, and the combination of DQIP process indicators and intermediate outcome indicators was found to be associated with CVD events and mortality[26,27]. While DQIP chose HbA_{1c} as an intermediate

outcome indicator of blood sugar control and applied 9.5% (80 mmol/mol) as the cut-off[25], some recent studies used 8% (64 mmol/mol) based on American Diabetes Association recommendations under the hypothesis that stricter blood sugar control leads to a lower risk of macrovascular complications[25-28]. Nonetheless, the choice of process indicators remained a problem[26,27]. According to the American Diabetes Association, blood sugar should be tested at least twice yearly as an indicator of effective healthcare management. However, studies conducted on an Italian insurance database suggest that less frequent testing may result in better diabetic control[26,27]. Despite the studies having a 28-mo[26,27] average follow-up period, it may not be enough time to get an accurate assessment of the long-term effects like macrovascular complications.

We took into account past research while combining process indicators, intermediate outcome indicators, and the presence of hypertension to construct a score that allowed us to analyze its relationship with AMI. In this research, we obtained information from both hospital medical charts and national health insurance claims. We followed the American Diabetes Association's advice concerning the frequency of testing to measure the quality of healthcare and kept tabs on the progress over an extended period. Among intermediate outcome indicators, we adopted the American Diabetes Association recommended cut-off of 100 mg/dL for low-density lipoprotein (LDL) instead of 130 mg/dL, which was adopted by DQIP and some other previous studies[28].

MATERIALS AND METHODS

Study population

Patients who had type 2 DM and were covered by the National Health Insurance system in Taiwan were enrolled from a medical facility located in the southern of Taiwan. The insurance program was launched in March 1995 and had reached a coverage rate of 99% in 2014. For research purpose, the National Health Research Institute of Taiwan constructed and maintains a Longitudinal Cohort of Diabetes Patient Database (LHDB), which contains claim data on 120000 individuals who are randomly selected annually since 1999 from incident patients of DM, identified using the International Classification of Diseases, Clinical Modification (ICD-9-CM) codes 250, A181, and 648.0. The inclusion criteria are having at least: (1) One hospitalization for DM or receiving a prescription for DM medication during hospitalization; (2) Two outpatient visits for DM within one year; or (3) One outpatient visit for DM and receiving at least one prescription for DM medication within one year. The incident year was defined as the year when the first claim for DM was filed, and all the patients included were traced back to January 1, 1997 for their claim records.

Data collection

In the current study, participants were identified from the LHDB in 2013. We identified incident patients of DM who were diagnosed between January 1, 1999 and December 31, 2003, with a two-year washout period from January 1, 1997 to December 31, 1998, and followed them till December 31, 2011. In 2011, the Taiwanese health authority initiated a quality control campaign of diabetes care, in which the care indicators of each hospital are compared with the whole country. Because the frequency of care indicators is an important component of the quality of care in our study, this campaign will interfere the study results. Therefore, we used the data before 2011 for this study. Candidates who were diagnosed with Type 1 DM or gestational diabetes were excluded. We also excluded those who had myocardial infarction events before the diagnosis of DM, who were under 20 years of age, who had no information on sex, and who were followed up for less than 3 years (Figure 1).

The LHDB does not have information about lab tests, so we figured out which patients got care at the medical center by pairing their outpatient visit times, ICD-9-CM codes, and date of birth in the LHDB, and then gathering the information from the patient's medical charts. We extracted information from the medical charts of each participant until the end of follow-up (Figure 1). The medical facility eliminated any identifying details from the medical charts prior to making them public, in order to protect the confidentiality of the information. The study protocol was reviewed and approved by the Ethics Committees of the Chi Mei Medical Center.

Quality of care summary score

On the basis of the scoring systems used in previous studies[26,27], we constructed a quality-of-care score (Table 1). The score includes items of process indicators (frequencies of tests), intermediate outcome indicators (values of test results), and co-morbidity of hypertension for which clear associations with CVD complications have been documented and effective preventive measures are available. The intermediate outcome indicators included LDL < 100 mg/dL, BP < 130/80 mmHg, and HbA_{1c} < 8.0%. The process indicators encompassed how often HbA1c and lipid profiles were examined, along with the regularity of urine, foot, and retinal examinations. Data on the process indicators and co-morbidity of hypertension were extracted from the LHDB, and data on intermediate outcome indicators were extracted from the medical charts retrieved from the medical center.

We modified cut-offs values of the intermediate outcome indicators according to the most recent American Diabetes Association guidelines, and so they were not exactly the same as those used in the previous studies: 130 mg/dL instead of 100 mg/dL for LDL, 130 mmHg instead of 140 mmHg for systolic BP, and 80 mmHg instead of 90 mmHg for diastolic BP. Similarly, cut-offs for the process indicators were also modified: ≥ 2/year instead of < 1/year for tests of HbA_{1c}, ≥ 1/year instead of < 1/year for tests of lipid profile, and ≥ 1/year instead of < 1/year for urine examination. In addition, we included frequencies of foot examination and retinal examinations (both with 1/year as the cut-off) as process indicators.

Table 1 Quality-of-care scoring system

Item	Score
HbA1c	
HbA1c measurement < 2/yr & HbA1c ≥ 8% (64 mmol/mol)	0
HbA1c measurement < 2/yr & HbA1c < 8% (64 mmol/mol)	1
HbA1c measurement ≥ 2/yr & HbA1c ≥ 8% (64 mmol/mol)	1
HbA1c measurement ≥ 2/yr & HbA1c < 8% (64 mmol/mol)	2
Blood pressure	
Co-morbidity of hypertension, never used anti- hypertension agents	0
SBP ≥ 130 mmHg or DBP ≥ 80 mmHg, never used anti-hypertension agents	0
No blood pressure data, ever used anti-hypertension agents	0
SBP ≥ 130 mmHg or DBP ≥ 80 mmHg, ever used anti-hypertension agents	0
No blood pressure data, no co-morbidity of hypertension, and never used anti-hypertension agents	1
SBP < 130 mmHg and DBP < 80 mmHg	1
Lipid profile	
Lipid profile measurement < 1/yr & LDL cholesterol ≥ 100	0
Lipid profile measurement < 1/yr & LDL cholesterol < 100	1
Lipid profile measurement ≥ 1/yr & LDL cholesterol ≥ 100	1
Lipid profile measurement ≥ 1/yr & LDL cholesterol < 100	2
Eye exam	
Eye measurement < 1/yr	0
Eye measurement ≥ 1/yr	1
Foot exam	
Foot exam < 1/yr	0
Foot exam ≥ 1/yr	1
Urine exam	
Urine exam < 1/yr	0
Urine exam ≥ 1/yr	1

HbA1c: Hemoglobin; SBP: Systolic blood pressure; DBP: Diastolic bold pressure; LDL: Low density lipoprotein.

We assigned the scores according to the data during the 3-year period before the censor date. In scoring the control of lipid and blood sugar, we assigned the value 2 when both the process and the intermediate outcome indicators met the targets, the value 1 when only one of the indicators met the target, and 0 when none of the indicators met the target. For the frequency of examinations of urine, foot and retinal, the value 1 was assigned when the target was met, and 0 otherwise. For BP, the values were assigned to the status during the study period: 1 to cases with good BP control and cases with no co-morbidity of hypertension. When the information on a specific indicator was missing, a value of 0 was assigned. Consequently, the quality-of-care score has a range between 0 and 8, and a higher score indicates better quality of care.

Event date and censoring date

We identified AMI events using ICD-9-CM diagnostic codes[29]. On the basis of prior research[30,31], the event date was determined to be the day when an applicable ICD-9-CM diagnostic code appeared on claims for outpatient visits for a second time or on claims for inpatient care for its initial time. For those who have survived till end of the study period without any AMI events, a censoring date of December 31, 2011 was assigned.

Statistical analysis

To evaluate the differences in continuous variables among groups, we used one-way ANOVA. For categorical variables, we used χ^2 tests to evaluate differences among groups. We used the Kaplan-Meier method to calculate the probability of AMI in each group defined by the score and the Breslow test to evaluate differences in the AMI disease event-free

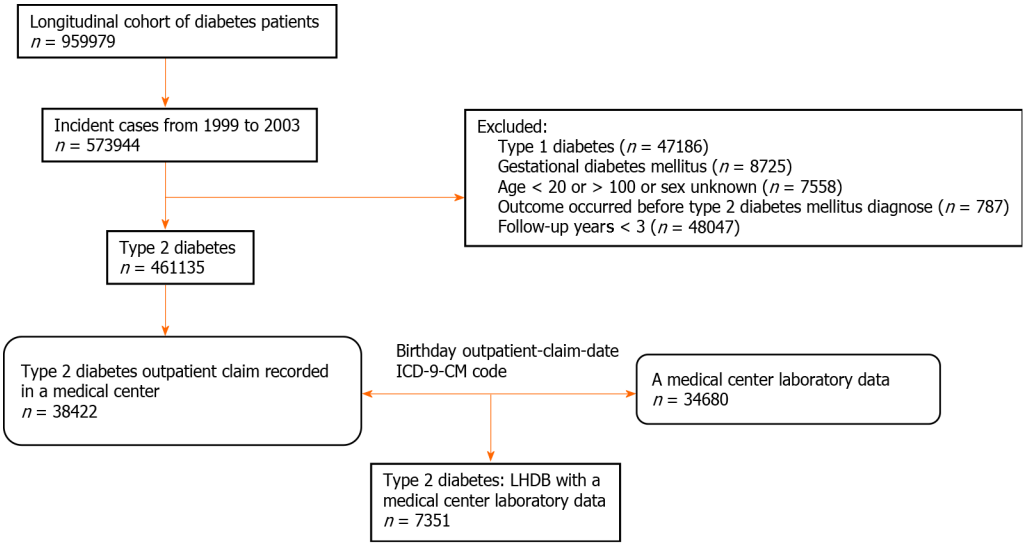

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Figure 1 Study flow diagram. LHDB: Longitudinal cohort of diabetes patients database; ICD-9-CM: International classification of diseases, clinical modification.

probabilities among groups. To evaluate the association between the score and AMI, we used Cox proportional hazards regressions. We looked into age, sex, types of medication, compliance with treatment, the Diabetes Complications Severity Index (DCSI)[32], and BP or lipid disorder history in Cox proportional hazards analyses to account for and adjust for possible distorting effects. The DCSI was constructed in a previous study using automated diagnostic, pharmacy, and laboratory data, and a score from 0 to 13 can be assigned accordingly.

Taking into account the stability of estimates, we defined "high" quality of care as having a score higher than half of the maximum value (≥ 5). Accordingly, we divided the participants into three groups: With scores ≤ 1 (the reference group), with scores between 2 and 4, and with scores ≥ 5 (the high-quality group).

Because a portion of the participants did not have information on all the variables evaluated, we conducted a sensitivity analysis by including participants with complete data only. There are two possible reasons why the information is missing. One is that the test/examination was not ordered or administered on the patient. The other is that the patient received the test/examination at other facilities, not the medical center, which rarely happens. Besides, due to the potential for large fluctuations in BP and the lack of routine foot examinations in Taiwan, these two items were excluded from the quality-of-care score in the sensitivity analysis. In other words, participants included in the sensitivity analysis were those who had a complete set of data, except for data on BP or foot examinations. All the statistical analyses were performed using SAS software, Version 9.2 (SAS, Cary, NC).

RESULTS

A total of 7351 participants with type 2 DM were enrolled in this study, including 3963 (53.9%) men and 3388 (46.1%) women (Table 2). The mean age at diagnosis was 56.0 years old, and 66.5% of them were between 40 and 65 years old. Most of the participants (64.3%) took oral antidiabetic drugs (OAD) only, followed by those who received insulin injections only, and then those who received both OAD and insulin treatment. Using records of pharmacy refill, we defined a ratio between 90% and 110% as good adherence[33], which was found in 23.1% of the participants. According to the DCSI, we divided the participants into six groups, from 0 to ≥ 5, as in a previous study[32] and found 66.7% of them were categorized in the first group while only 0.4% were categorized in the last group. During the one-year period before diagnosis, 25.3% of the participants had hypertension, 3.6% had dyslipidemia, and 6.0% had both.

While 52% of the participants had a quality-of-care score of ≤ 1, only 9% had high quality of care (score ≥ 5). In comparison with those in the other two groups, participants in the lowest score group were older, predominantly male, and more likely to be prescribed with insulin only. This group also had the worst adherence to treatment and the shortest history of DM (Table 2).

We followed up the participants for a mean period of 9.95 years, and more than 97% of them were followed for more than 5 years. During the follow-up period, 308 (4.2%) participants had AMI, and the incidence rate correlated with the quality-of-care score: 5.1 per 1000 person-years in those having a score of ≤ 1, 3.6 per 1000 person-years in those having a score between 2 and 4, and 1.87 per 1000 person-years in those having a score of ≥ 5. Kaplan-Meier curves also show that a score of ≥ 5 was associated with a lower likelihood of developing AMI.

After adjusting for age, sex, type of DM medicine, adherence to medication, DCSI, and past history of hypertension or dyslipidemia, we found that participants with a score of ≥ 5 had a lower risk of developing AMI [adjusted hazard ratio (AHR) = 0.37; 95% confidence interval (95%CI): 0.21-0.66] in comparison with those with a score of ≤ 1 (Table 3). Female participants had a lower risk of developing AMI (AHR = 0.53; 95%CI: 0.42-0.67) in comparison with male participants (Table 3). Other independent predictors identified in this study included age 40 years to 65 years (AHR = 1.90; 95%CI:

Table 2 Characteristics of quality-of-care score of patients with type 2 diabetes mellitus

	n (%)				*P* value
	Total, *n* = 7351	Score ≤ 1, *n* = 3858	1 < score < 5, *n* = 2819	Score ≥ 5, *n* = 674	
Age (mean ± SD)	55.96 ± 11.94	57.08 ± 12.70	54.99 ± 10.94	53.62 ± 10.70	< 0.0001
≤ 40	643 (8.8)	330 (8.6)	245 (8.7)	68 (10.1)	< 0.0001
40 < age ≤ 65	4886 (66.5)	2369 (61.4)	2022 (71.7)	495 (73.4)	
> 65	1822 (24.8)	1159 (30.0)	552 (19.6)	111 (16.5)	
Sex					
Male	3963 (53.9)	2146 (55.6)	1477 (52.4)	340 (50.5)	< 0.01
Female	3388 (46.1)	1712 (44.4)	1342 (47.6)	334 (49.6)	
Duration of diabetes mellitus (mean ± SD)	9.95 ± 1.94	9.76 ± 2.06	10.14 ± 1.81	10.32 ± 1.60	< 0.0001
≤ 5 yr	216 (2.9)	161 (4.2)	52 (1.8)	3 (0.5)	< 0.0001
> 5 yr	7135 (97.1)	3697 (95.8)	2767 (98.2)	671 (99.6)	
Anti-diabetic drugs					
Oral only	4729 (64.3)	2483 (64.4)	1818 (64.5)	428 (63.5)	< 0.0001
Insulin only	1607 (21.9)	919 (23.8)	608 (21.6)	80 (11.9)	
Oral + insulin	1015 (13.8)	456 (11.8)	393 (13.9)	166 (24.6)	
Adherence to medication (%)					
< 90	5340 (72.6)	3087 (80.0)	1872 (66.4)	381 (56.5)	< 0.0001
90 ≤ adherence < 110	1698 (23.1)	637 (16.5)	786 (27.9)	275 (40.8)	
≥ 110	313 (4.3)	134 (3.5)	161 (5.7)	18 (2.7)	
Comorbidity (DCSI)					
0	4904 (66.7)	2444 (63.4)	1951 (69.2)	509 (75.5)	< 0.0001
1	1160 (15.8)	634 (16.4)	436 (15.5)	90 (13.4)	
2	911 (12.4)	546 (14.2)	313 (11.1)	52 (7.7)	
3	227 (3.1)	131 (3.4)	82 (2.9)	14 (2.1)	
4	122 (1.7)	80 (2.1)	34 (1.2)	8 (1.2)	
≥ 5	27 (0.4)	23 (0.6)	3 (0.1)	1 (0.2)	
Hypertension/dyslipidemia					
None	4782 (65.1)	2458 (63.7)	1845 (65.5)	479 (71.1)	< 0.0001
Hypertension only	1861 (25.3)	1054 (27.3)	669 (23.7)	138 (20.5)	
Dyslipidemia only	265 (3.6)	124 (3.2)	118 (4.2)	23 (3.4)	
Both	443 (6.0)	222 (5.8)	187 (6.6)	34 (5.0)	
Acute myocardial infarction event					
No	7043 (95.8)	3666 (95.0)	2716 (96.4)	661 (98.1)	< 0.001
Yes	308 (4.2)	192 (5.0)	103 (3.7)	13 (1.9)	
Incidence rate (per 1000 person-year)	4.21	5.1	3.6	1.87	

DCSI: Diabetes complications severity index.

1.10-3.28 in comparison with those ≤ 40 years old), age older than 65 years (AHR = 2.48; 95%CI: 1.39-4.40 in comparison with those ≤ 40 years old), and a history of both hypertension and dyslipidemia (AHR = 1.82; 95%CI: 1.20-2.75 in comparison with those who had no history of hypertension nor dyslipidemia).

To compare the scoring system developed in this study with a well-established system[26,27], we used 5-point increments to assign the scores. When applying that scoring system to the data in this study, we did not observe an association between the score and the risk of developing AMI (Table 4). There was a U-shaped relationship between the

Table 3 Crude and adjusted hazard ratio associated with each predictor

Item	Crude HR (95%CI)	Adjusted HR (95%CI)
Quality-of-care score		
Score ≤ 1	1	1
1 < Score < 5	0.69 (0.55-0.88)	0.71 (0.55-0.90)
Score ≥ 5	0.36 (0.20-0.63)	0.37 (0.21-0.66)
Age		
≤ 40	1	1
40 < Age ≤ 65	1.93 (1.12-3.32)	1.90 (1.10-3.28)
> 65	2.64 (1.51-4.62)	2.48 (1.39-4.40)
Sex		
Male	1	1
Female	0.57 (0.45-0.73)	0.53 (0.42-0.67)
Anti-diabetic drugs		
Oral only	1	1
Insulin only	0.80 (0.60-1.07)	0.78 (0.59-1.05)
Oral + insulin	0.70 (0.49-1.01)	0.77 (0.54-1.11)
Adherence to medication (%)		
< 90	0.89 (0.69-1.15)	0.79 (0.61-1.02)
90 ≤ adherence < 110	1	1
≥ 110	0.71 (0.37-1.37)	0.66 (0.34-1.28)
Comorbidity (DCSI)		
0	1	1
1	1.13 (0.83-1.54)	0.95 (0.69-1.31)
2	1.31 (0.94-1.81)	1.02 (0.72-1.43)
3	1.27 (0.69-2.33)	0.96 (0.52-1.80)
4	1.56 (0.73-3.31)	1.07 (0.49-2.31)
≥ 5	1.04 (0.15-7.43)	0.66 (0.09-4.77)
Hypertension/dyslipidemia		
None	1	1
Hypertension only	1.38 (1.07-1.78)	1.30 (0.99-1.71)
Dyslipidemia only	1.19 (0.64-2.18)	1.17 (0.64-2.17)
Both	1.89 (1.27-2.79)	1.82 (1.20-2.75)

HR: Hazard ratio; 95%CI: 95% confidence interval; DCSI: Diabetes complications severity index.

score and the risk of AMI. Initially, the risk went down as the score increased, reaching its lowest at 25. After that, the risk increased again, peaking when the score was between 35 and 40. When evaluating the system developed in this research, we observed similar risks for scores ranging from 0 to 10. Afterwards, there was a reduction in the risk as scores decreased, up until scores between 35 and 40. However, the number of individuals included in this group was small (only 103 people), so the risk assessment might not be accurate.

The sensitivity analysis, based on data from people who had all the indicators present, yielded a similar dose-response relationship as seen in the main investigation; however, the risk decreased (> 50%) even at a score of 3 (Table 4).

Table 4 Comparisons of the number and percentage in each score of study subjects group defined by old and new score systems

Score	Present study (*n* = 7351)			Score	Previous study[1] (*n* = 7351)		Sensitivity analysis[2] (*n* = 3433)	
	n (%)	Crude HR	Adjusted HR		*n* (%)	Adjusted HR	*n* (%)	Adjusted HR
0	2107 (28.7)	1	1	0	6 (0.1)	1	969 (28.2)	1
1	1751 (23.8)	1.21 (0.91-1.61)	1.22 (0.92-1.63)	5	720 (9.8)	0.19 (0.03-1.46)	828 (24.1)	1.17 (0.77-1.76)
2	1355 (18.4)	0.97 (0.70-1.33)	0.98 (0.71-1.35)	10	1333 (18.1)	0.23 (0.03-1.69)	654 (19.1)	1.00 (0.63-1.58)
3	925(12.6)	0.56 (0.36-0.86)	0.57 (0.37-0.89)	15	1148 (15.6)	0.24 (0.03-1.78)	420 (12.2)	0.49 (0.25-0.98)
4	539 (7.3)	0.60 (0.36-1.02)	0.63 (0.37-1.07)	20	2932 (39.9)	0.27 (0.04-1.98)	249 (7.3)	0.62 (0.28-1.38)
5	343 (4.7)	0.59 (0.31-1.14)	0.63 (0.33-1.26)	25	857 (11.7)	0.18 (0.02-1.35)	173 (5.0)	0.49 (0.17-1.36)
6	228 (3.1)	0.27 (0.08-0.84)	0.26 (0.08-0.83)	30	344 (4.7)	0.20 (0.03-1.54)	97 (2.8)	0.00 (0.00-NA)
7-8	103 (1.4)	0.00 (0.00-4.23E266)	0.00 (0.00-1.57E265)	35-40	11 (0.2)	0.77 (0.05-12.47)	43 (1.3)	0.00 (0.00-NA)

[1]De Berardis *et al*[27], 2008; Rossi *et al*[26], 2011.

[2]In order to compare the performance of our scoring system with that in the previous study, we used 5-point increments to assign the scores.

HR: Hazard ratio.

DISCUSSION

It is well known that DM has a close association with major CVD[34], including ischemic heart disease, heart failure, stroke, and peripheral artery disease, which may affect as many as 50% of the patients[35]. Despite the advances in our understanding of the pathophysiology underlying its relationship with CVD, the effects of DM still remain not fully understood. DM, in particularly type 2, is often fraught with additional risk factors contributing to the risk for developing CVD[36]. The additional risk factors include, but are not limited to, dyslipidemia, hypertension, poor blood sugar control, hypercoagulability, smoking, obesity, and lack of physical activity[37].

The relative risk of myocardial infarction is 50% greater in diabetic males and 150% greater in diabetic females[38], and the prevalence of AMI is 3 to 5 times higher in patients with DM in population studies in the United States[39,40]. Women with DM had a lower risk for myocardial infarction than men with DM to experience whichever myocardial infarction events[41]. In our study, the risk of developing AMI was 47% lower in female patients (AHR = 0.53; 95%CI: 0.42-0.67) in comparison with male patients, which is compatible with findings in the United States.

Diabetic patients are at increased risk of developing coronary artery disease (CAD)[42] and experience worse clinical outcomes following AMI[43]. Due to the high prevalence of AMI in diabetic patients, the quality-adjusted life years associated with diabetes lost was 32.8 years[44]. DM is an independent risk factor for the development of CAD[34] and clinical outcomes following the various manifestations of CAD. Despite a clear improvement in the treatment and survival rate of myocardial infarction, the mortality and morbidity of myocardial infarction remain high in diabetic patients[45,46].

DM is a complex chronic progressive metabolic disorder which requires continuous medical care as well as multifactorial risk-reduction strategies extending beyond blood sugar control. Research has proven that managing hypertension and cholesterol levels properly can lead to remarkable declines in CVD[47-49]. For this reason, it is important for those with diabetes to control these factors in combination for reducing the chance of CVD[50,51]. All six components of our quality care score, which are HbA1c, BP, LDL, urine examination, foot examination, and retinal examination, are also included in the conditions established by the Taiwanese government's pay for performance (P4P) program for diabetes[52,53]. The program incentivizes healthcare providers to register patients who have diabetes, with the intention of increasing the quality of care. Those who join the P4P program are more likely to obtain tests associated with diabetes, and an extended investigation assessing the sustained impacts of the program found it to be economical [52]. Our study confirmed the finding and supports that good quality of care can greatly reduce the risk of developing AMI, and even other CVD, in patients with DM. Therefore, the quality-of-care score developed in this study can be used for prediction and surveillance.

DM poses huge financial burdens to many countries, but data on the clinical care for DM have varied substantially across countries[54]. In Italy, the Quality of Care and Outcomes in Type 2 Diabetes Study combined HbA_{1c}, BP, LDL, and microalbuminuria to construct a quality-of-care score for DM ranging from 0 to 40 and found a close relationship between the score and long-term CVD outcomes[27]. The Quality Assessment Score and Cardiovascular Outcomes in Italian Diabetes Patients study confirmed the finding[26]. However, a large variation in the quality-of-care score among participating centers was observed[26]. Our investigation collected all the information from the same healthcare facility and used factual details to calculate the scores directly. Our scoring system follows the guidelines laid out by the American Diabetes Association in order to properly care for those with diabetes. In addition to using the scoring system developed in this study, we adopted the scoring system used by previous studies[26,27] and found that the other scoring system had a poor correlation with the risk of AMI. Results of this comparison showed that the same scoring system may not work well in prediction of CVD in different countries. It seems that the quality of care may differ from one nation to another, and the indicator used to measure it could have different effects in different health care systems. In Italy,

frequent testing may be regarded as a sign of poor care quality[26,27], while in Taiwan it signifies good quality of care, which is in agreement with the American Diabetes Association's guidelines.

While our study has the strength coming with a large study population and a long follow-up period, it still suffers from some limitations. First of all, lifestyle characteristics such as diet, smoking, and exercise are also predictors for AMI, but was not included in our scoring system because the LHDB does not have the information. Nonetheless, these predictors were not included in the scoring system used by previous studies. Furthermore, although we could not adjust for the effect of smoking, due to the low prevalence of smoking in female Taiwanese (*e.g.*, 2.3% in adults above 18 years of age in 2017[55]), it has been roughly adjusted indirectly when we adjusted for the effects of sex. Secondly, some of the data required for the calculation of the quality-of-care score were missing on a portion of the participants. Nonetheless, in the sensitivity analysis that included only patients with complete data, we observed findings similar to those in the main analysis. It should also be noted that our study was conducted in Taiwan, where there is a health insurance program with an almost complete coverage rate and a high density of medical care facilities. Subsequently, research must be conducted to determine if the observed results are also true in regions where healthcare is limited or costly.

CONCLUSION

The new quality-of-care score developed in this study had a good correlation with the risk of AMI. Thus, the score can be utilized to recognize those receiving substandard treatment, as well as the components of care that should be advanced. In fact, the scoring systems have been demonstrated as having good correlations with other long-term complications. A previous study revealed that the likelihood of developing chronic kidney illness dropped as the score rose, so strategies focusing on each indicator should be adjusted to reduce the development of diabetes-induced nephropathy[56]. Another study showed that a reduction in macrovascular complication events was associated with a score of 5 or higher[57], similar to findings in this study. Therefore, in order to reduce the risk of AMI in patients with DM, multifactorial interventions should be taken. Checking laboratory tests and combining treatments directed at high blood sugar, high BP, and unhealthy cholesterol levels are among the steps that can be taken. The score we developed is easy to calculate. It can also be applied to comparison of performance across health care facilities and evaluation of the efficacy of quality improvement programs. Nonetheless, it should be kept in mind that various healthcare systems may modify the scoring system to make it more useful.

ARTICLE HIGHLIGHTS

Research background

Cardiovascular disease (CVD) is the leading cause of death globally and diabetes mellitus (DM) is a well-established risk factor. Of the fatal outcomes of CVD, acute myocardial infarction (AMI) is the most common.

Research motivation

DM is a major modifiable factor for CVD, and good quality of care can reduce the risk of AMI in patients with DM. Therefore, a long-term quality-of-care score for DM may predict the occurrence of AMI among patients with type 2 DM and thus guide the care.

Research objectives

To develop a long-term quality-of-care score for predicting the occurrence of AMI among patients with type 2 DM.

Research methods

Using Taiwan's Longitudinal Cohort of Diabetes Patients Database and the medical charts of a medical center, we identified incident patients diagnosed with type 2 DM. We constructed a summary quality-of-care score consists of process indicators, intermediate outcome indicators, and a hallmark co-morbidity. The associations between the score and the incidence of AMI were evaluated using Cox regression models.

Research results

A total of 7351 patients were enrolled. In comparison with participants who had scores ≤ 1, those with scores between 2 and 4 had a lower risk of developing AMI [adjusted hazard ratio (AHR) = 0.71; 95% confidence interval (95%CI): 0.55-0.90], and those with scores ≥ 5 had an even lower risk (AHR = 0.37; 95%CI: 0.21-0.66). The performance of this score in predicting the risk of AMI is better than that of a widely used scoring system.

Research conclusions

Good quality of care can reduce the risk of AMI in patients with type 2 DM. The quality-of-care score developed in this study had a significant association with the risk of AMI and thus can be applied to guiding the care for these patients.

Research perspectives

The quality-of-care score developed in this study can be applied to guiding the care for these patients, but different

healthcare systems may make modifications to the scoring system for better application.

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FOOTNOTES

Author contributions: Li PI and Guo HR conceived and designed the study; Li PI obtained and analyzed the data, and drafted the manuscript; Guo HR supervised the data analysis and helped interpretation of the results; Guo HR edited and revised the manuscript; both authors critically reviewed the manuscript and have approved the final version.

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ORIGINAL ARTICLE

Retrospective Study

Correlation between glycated hemoglobin A1c, urinary microalbumin, urinary creatinine, β2 microglobulin, retinol binding protein and diabetic retinopathy

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Abstract

BACKGROUND

Retinopathy is the most common microvascular disease of type 2 diabetes, and seriously threatens the life, health and quality of life of patients. It is worth noting that the development of diabetic retinopathy (DR) can be hidden, with few symptoms. Therefore, the preliminary screening of diabetic patients should identify DR as soon as possible, delay disease progression, and play a vital role in its diagnosis and treatment.

AIM

To investigate the correlation between glycated hemoglobin A1c (HbA1c), urinary microalbumin (U-mALB), urinary creatinine (U-CR), mALB/U-CR ratio, β2 microglobulin (β2MG), retinol binding protein (RBP) and DR.

METHODS

A total of 180 patients with type 2 diabetes mellitus attending the Second People's Hospital of Hefei from January 2022 to August 2022 were retrospectively enrolled by ophthalmologists. Based on whether they had combined retinopathy and its degree, 68 patients with diabetes mellitus without retinopathy (NDR) were assigned to the NDR group, 54 patients with non-proliferative DR (NPDR) to the NPDR group, and 58 patients with proliferative DR to the PDR group. General data, and HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR results were collected from the patients and compared among the groups. Pearson's correlation method was used to analyze the correlation between HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR indices, and multiple linear regression was applied to identify the risk factors for DR. Receiver operator characteristic (ROC) curves

were also drawn.

RESULTS

The differences in age, gender, systolic and diastolic blood pressure between the groups were not statistically significantly ($P > 0.05$), but the difference in disease duration was statistically significant ($P < 0.05$). The differences in fasting blood glucose, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total cholesterol, and triglyceride between the groups were not statistically significant ($P > 0.05$). HbA1c in the PDR group was higher than that in the NPDR and NDR groups ($P < 0.05$). The levels of mALB, β2MG, RBP, mALB/U-CR and U-CR in the PDR group were higher than those in the NPDR and NDR groups ($P < 0.05$). Multiple linear regression analysis showed that disease duration, HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR were risk factors for the development of DR. The ROC curve showed that the area under the curve (AUC) for the combination of indices (HbA1c + mALB + mALB/U-CR + U-CR + β2MG + RBP) was 0.958, with a sensitivity of 94.83% and specificity of 96.72%, which was higher than the AUC for single index prediction ($P < 0.05$).

CONCLUSION

HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP can reflect the development of DR and are risk factors affecting PDR, and the combination of these six indices has predictive value for PDR.

Key Words: Diabetic retinopathy; β2 microglobulin; Retinol-binding protein; Urinary microalbumin; Urinary creatinine

Core Tip: Diabetes retinopathy (DR) is a common complication of diabetes, which can eventually lead to blindness in diabetic patients and seriously affect the quality of life of patients. The identification of risk factors for DR is significant for early intervention. Here we retrospectively analyzed 180 patients with type 2 diabetes mellitus to examine the correlation between glycated hemoglobin A1c, microalbumin (mALB), mALB/urinary creatinine (U-CR), U-CR, ββ2 microglobulin, retinol binding protein and DR in diabetic patients in order to provide a scientific basis and guidance for clinical application.

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INTRODUCTION

Diabetic retinopathy (DR) is an irreversible blindness-causing disease[1]. The prevalence of diabetes in China accounts for 26.2% of the global diabetic population, and the prevalence of DR is approximately 35%-50%[2]. The prevalence of DR in Singapore and the United States is 20.1% and 25.7%, respectively[3]. The disease progresses rapidly and if not diagnosed and treated early, it will seriously affect the visual field and vision. In severe cases, patients may even lose their sight, which causes many inconveniences to their life and work and hinders their normal life. Therefore, early clinical diagnosis is important for the subsequent treatment of DR patients[3]. Currently, the clinical diagnosis of this disease is mainly based on fundus photography and fluorescein angiography, but the application process is complicated and may cause adverse reactions in diabetic patients. In addition, there is a lack of convenient and intuitive biochemical markers providing guidance for the diagnosis of DR[4]. Therefore, it is important to identify relevant biochemical markers to predict DR. Urinary β2 microglobulin (β2MG) has been found to be closely associated with microvascular complications such as diabetic nephropathy. It is known that DR is a microvascular complication, so it is assumed that the pathogenesis of the two diseases is similar and β2MG may be a useful marker for predicting DR[5]. Retinol-binding protein (RBP), a lipid-derived cytokine, has been shown to be closely associated with the development of diabetes mellitus and diabetic vasculopathy[6]. Urinary microalbumin (U-mALB), urinary creatinine (U-CR) and the mALB/U-CR ratio are predictors of diabetic vasculopathy and are risk factors for endothelial cell function and microvascular function[7]. In this study, we aimed to examine the correlation between glycated hemoglobin A1c (HbA1c), β2MG, RBP, mALB, U-CR, mALB/U-CR and DR lesions in patients with DR. The innovation of this study is determination of the predictive value of the combined detection of HbA1c, mALB, mALB/U-CR, U-CR, β2MG, and RBP in DR using real clinical data. The clinical significance is to provide a scientific basis and guidance for the clinical use of the combined detection of HbA1c, mALB, mALB/U-CR, U-CR, β2MG, and RBP to evaluate the risk of DR.

MATERIALS AND METHODS

General data

A total of 180 type 2 diabetic patients attending the Second People's Hospital of Hefei from January 2022 to August 2022 were enrolled retrospectively, including 68 patients with diabetes without retinopathy (NDR group), 54 patients with non-proliferative diabetic retinopathy (NPDR group), and 58 with proliferative diabetic retinopathy (PDR group).

Inclusion criteria

(1) The study subjects met the diagnostic criteria for type 2 diabetes mellitus[8]; (2) The diagnosis of DR was based on the International Clinical Classification Criteria for Diabetic Retinopathy[9]. NPDR: microaneurysm alone was observed or 4 quadrants with intraretinal hemorrhage and microangioma; or moderate retinal mesangiopathy occurring in more than 2 or more quadrants; PDR: If the retina had new abnormal blood vessels, this was considered PDR. The diagnosis was confirmed by satisfying one or more of the following: neovascularization, vitreous hematopoiesis or anterior retinal hemorrhage; and (3) None of the study subjects had a history of trauma or ocular surgery.

Exclusion criteria

(1) Those with combined non-fundus pathology, *e.g.*, cataract, glaucoma; (2) Those with poorly graded fundus visual field images due to blurring of large blood vessels adjacent to the optic disc, and whose diagnosis was more difficult to further confirm on fundus examination; (3) Those with organ disease, such as coronary artery disease, heart failure, diabetic nephropathy, *etc.*; (4) Combined with diabetic complications, such as diabetic gangrene, stroke, or atherosclerosis; and(5) Difficult to cooperate in the completion of the study.

Methods

General information of the patients was collected, including age, gender, duration of disease, systolic and diastolic blood pressure. Blood was collected in the morning after a 12-h fast to measure HbA1c, fasting blood glucose (FPG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride (TG) using a glycated hemoglobin analyzer and supporting reagents.

Urinary mALB and U-CR concentrations were measured using a special protein analyzer and the urinary mALB/U-CR ratio was calculated three times. β2MG was measured by the immunoturbidimetric method and RBP was measured using an automatic biochemical analyzer.

Observation indicators

General information: age, gender, duration of disease, systolic and diastolic blood pressure. Clinical indicators: FPG, HDL-C, LDL-C, TC, TG, and HbA1c. Combined indicators: mALB, mALB/U-CR, U-CR, β2MG, and RBP levels.

Statistical analysis

GraphPad Prism 9 was used to analyze the study data and for image export. The measurement data were expressed as mean ± SD, and compared by one-way ANOVA for multiple groups of data or for two groups of data. The count data were expressed by n (%), and compared using the χ^2 test. Correlation analysis and risk factor identification were performed using Pearson's correlation method and multiple linear regression, respectively. A receiver operator characteristic (ROC) curve was plotted to predict the value of PDR. $P < 0.05$ was considered statistically significant.

RESULTS

General information in each group

The differences in age, gender, systolic and diastolic blood pressure between the three groups were not significant ($P > 0.05$), but the differences in disease duration were significant ($P < 0.05$, Table 1).

Clinical indicators among the groups

No significant differences in FPG, HDL-C, LDL-C, TC and TG were observed among the groups ($P > 0.05$); HbA1c in the PDR group was higher than that in the NPDR and NDR groups ($P < 0.05$, Table 2).

Comparison of mALB, mALB/U-CR, U-CR, β2MG and RBP levels among the groups

The levels of mALB, β2MG, RBP, mALB/U-CR, and U-CR in the PDR group were higher than those in the NPDR and NDR groups ($P < 0.05$, Table 3).

Correlation analysis

By Pearson's correlation analysis, mALB, mALB/U-CR, U-CR, β2MG, and RBP were positively correlated with disease duration and HbA1c, ($P < 0.05$, Figure 1).

Risk factors for the development of PDR

With PDR as the dependent variable (yes = 1, no = 0) and the above meaningful results as independent variables all

Table 1 General information of the three groups (mean ± SD)

Group	Age (yr)	Sex (M/F)	Duration of illness (yr)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
NDR (n = 68)	57.71 ± 7.18	37/31	4.21 ± 0.81	117.47 ± 19.38	76.05 ± 9.48
NPDR (n = 54)	58.00 ± 8.93	29/25	6.22 ± 1.26	118.32 ± 16.02	75.34 ± 11.91
PDR (n = 58)	56.59 ± 7.12	31/37	8.12 ± 1.47	111.33 ± 18.09	75.69 ± 7.96
F/χ^2 value	0.534	0.013	169.133	2.606	0.178
P value	0.587	0.994	< 0.001	0.078	0.836

NDR: Diabetes mellitus without retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy.

Table 2 Clinical indicators among the groups (mean ± SD)

Group	HbA1c (%)	FPG (mmol/L)	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
NDR (n = 68)	8.01 ± 1.86	8.60 ± 1.96	4.86 ± 0.98	1.68 ± 0.21	2.61 ± 0.42	1.15 ± 0.22
NPDR (n = 54)	9.14 ± 2.12	8.55 ± 1.94	4.42 ± 0.75	1.69 ± 0.27	2.62 ± 0.41	1.24 ± 0.20
PDR (n = 58)	10.28 ± 2.66	8.92 ± 2.16	4.55 ± 0.84	1.77 ± 0.29	2.74 ± 0.54	1.22 ± 0.27
F value	15.385	0.572	0.319	2.216	1.476	1.073
P value	< 0.001	0.565	0.726	0.112	0.231	0.344

NDR: Diabetes mellitus without retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; HbA1c: Glycated hemoglobin A1c; FBG: Fasting blood glucose; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglyceride.

Table 3 Comparison of microalbumin, microalbumin/urinary creatinine, urinary creatinine, β2 microglobulin and retinol binding protein levels in each group (mean ± SD)

Group	mALB (mg/L)	mALB/U-CR (mg/mmoL)	U-CR (μmol/L)	β2MG (mg/L)	RBP (μg/L)
NDR (n = 68)	15.04 ± 1.94	2.19 ± 0.86	6.86 ± 1.67	2.28 ± 0.66	12.29 ± 2.82
NPDR (n = 54)	65.69 ± 7.30	3.29 ± 1.26	19.97 ± 5.81	3.13 ± 0.84	21.58 ± 4.83
PDR (n = 58)	170.29 ± 11.63	5.09 ± 1.02	33.35 ± 11.45	4.53 ± 0.97	36.78 ± 7.84
F value	147.103	121.668	206.027	117.619	69.460
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

NDR: Diabetes mellitus without retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; U-mALB: Urinary microalbumin; U-CR: Urinary creatinine; β2MG: β2 microglobulin; RBP: Retinol binding protein.

included as original values, multiple linear regression analysis was performed and the results revealed that disease duration, HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR were all risk factors for the development of PDR (Table 4).

ROC curve analysis of HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP for predicting PDR

As shown in Table 5 and Figure 2, the ROC curve indicated that the combined diagnostic area under the curve of the indicators was 0.904, with a sensitivity of 92.53% and specificity of 90.65%, which was higher than the prediction of HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP alone (P < 0.05).

DISCUSSION

DR is a diabetes-induced retinal vascular complication and causes irreversible visual impairment and vision loss[10]. Currently, irreversible visual impairment due to DR accounts for approximately 1.9% worldwide, while visual loss accounts for approximately 2.6% worldwide. However, there are significant reported differences in the prevalence of DR in China and abroad[11]. Some scholars have reported that the prevalence of DR in diabetes is about 34.6% globally, and

Table 4 Multiple linear regression analysis of risk factors associated with the development of proliferative diabetic retinopathy

Independent variable	B value	SE	β value	*t* value	*P* value
Course of disease	1.203	0.293	0.220	4.106	< 0.001
HbA1c	0.942	0.192	0.755	4.906	< 0.001
mALB	0.874	0.128	0.256	6.828	< 0.001
mALB/U-CR	0.743	0.284	0.525	6.959	< 0.001
U-CR	0.842	0.121	0.254	6.959	< 0.001
β2MG	1.048	0.123	0.157	8.520	< 0.001
RBP	1.262	0.184	0.215	3.271	< 0.001

HbA1c: Glycated hemoglobin A1c; U-mALB: Urinary microalbumin; U-CR: Urinary creatinine; β2MG: β2 microglobulin; RBP: Retinol binding protein.

Table 5 Receiver operator characteristic curve analysis of glycated hemoglobin A1c, microalbumin, microalbumin /urinary creatinine, urinary creatinine, β2 microglobulin, retinol binding protein for predicting proliferative diabetic retinopathy

Item	Cut-off	Standard error	AUC	95%CI	Sensitivity (%)	Specificity (%)
mALB	56.84 mg/L	0.040	0.641	0.530-0.688	68. 82	71.24
mALB/U-CR	2.45 mg/mmoL	0.046	0.726	0.728-0.876	70.38	73.85
U-CR	25.96 μmol/L	0.004	0.757	0.508-0.722	72.49	75.58
β2MG	3.18 mg/L	0.027	0.748	0.637-0.882	76.84	79.84
RBP	26.58 μg/L	0.036	0.807	0.637-0.882	82.48	79.38
HbA1c	9.05%	0.043	0.710	0.638-0.775	72.41	63.11
Combination	-	0.017	0.958	0.917-0.982	94.83	96.72

AUC: Area under the curve; U-mALB: Urinary microalbumin; U-CR: Urinary creatinine; β2MG: β2 microglobulin; RBP: Retinol binding protein; HbA1c: Glycated hemoglobin A1c; Combination: Glycated hemoglobin A1c + microalbumin + microalbumin/urinary creatinine + urinary creatinine + β2 microglobulin + retinol binding protein.

is 16.4% and 25.9% in the UK and Australia, respectively. The incidence of PDR is approximately 7.0%. In China, the results of the six provinces of the Guangdong Provincial Flow Survey showed that the prevalence of DR in 13473 diabetic patients ranged from 33.28% to 34.88%[12,13]. The above studies suggest that DR is a common and highly prevalent chronic microangiopathy, which endangers public health safety. Therefore, early diagnosis of DR in diabetic patients is essential in clinical settings.

In recent years, studies have found that persistent poor glycemic control was a risk factor for the development and progression of DR, disrupting polyol metabolic pathways, contributing to the release of protein kinase C in large amounts and stimulating the onset of oxidative stress, inflammatory cell infiltration and other metabolic imbalances[14]. The above cascade of reactions further affects endothelial cells and microcirculatory function, leading to abnormal retinal microvascular biology and hemodynamics, and the development of DR. It has been found that persistent poor glycemic control is associated with alterations in mALB and U-CR, which are stimulated by oxidative stress and inflammation, and persistent high expression of mALB and U-CR[15]. The mALB/U-CR ratio is a novel index that is more accurate and reliable than traditional 24 h urine protein quantification, and is a valid marker for qualitative or quantitative prediction of proteinuric changes in the clinic[16]. DR severity has been reported to be positively correlated with decreased renal function and is independent of renal pathology[17]. An 8-year follow-up study reported that patients with DR with upregulated expression of mALB/U-CR had a progressively reduced glomerular filtration rate[18]. In the current results, mALB, mALB/U-CR, U-CR, β2MG, and RBP levels were found to be consistently increased as DR progressed from NDR, NPDR, to the PDR stage. It is hypothesized that mALB, mALB/U-CR, U-CR, β2MG, and RBP upregulated expression in DR patients is closely associated with progressive loss of renal function in diabetic patients.

Urinary β2MG was also expressed at high levels with the progressive of DR, which is a recognized early predictor of diabetic nephropathy in the clinic with high sensitivity and specificity[19]. This is consistent with previous studies by Cheng *et al*[20] and others, although altered β2MG levels have been associated with systemic lupus erythematous nephritis and globular nephropathy. However, the present study combined urinary mALB, mALB/U-CR, U-CR, and RBP to positively verify the association between DR occurrence and altered renal function. RBP is a low molecular mass vitamin A transporter protein, synthesized by the liver, expressed in large amounts in urine, blood, and cerebrospinal fluid, and reaches the blood *via* retinol in the liver[21]. It has been found that free RBP can normally be filtered by the

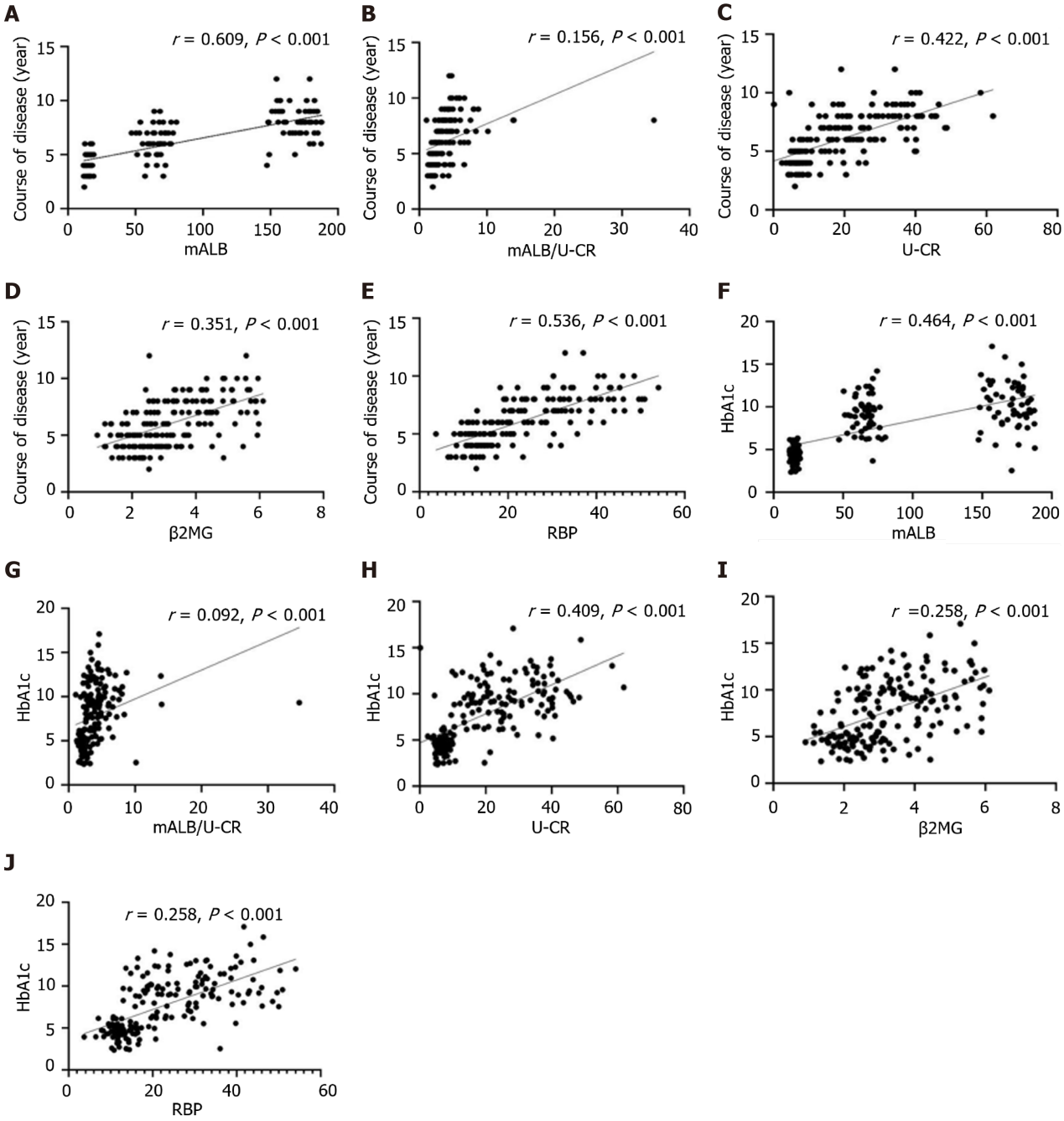

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Figure 1 Correlation analysis. A-E: The relationship between microalbumin (mALB), mALB/urinary creatinine (U-CR), U-CR, β2 microglobulin (β2MG), retinol binding protein (RBP) and course of disease; F-J: The relationship between mALB, mALB/U-CR, U-CR, β2MG, RBP and glycated hemoglobin A1c. U-mALB: Urinary microalbumin; U-CR: Urinary creatinine; β2MG: β2 microglobulin; RBP: Retinol binding protein; HbA1c: Glycated hemoglobin A1c.

glomerulus in healthy populations[22]. Lu *et al*[23] reported that urinary RBP correlated significantly with changes in renal function as the disease progressed in patients with diabetic nephropathy, elevating the rate of thylakoid cell proliferation, basement membrane synthesis and impaired glomerular filtration in patients with diabetic nephropathy, with subsequent upregulation of urinary RBP. Our study showed that mALB was involved in the regulation of renal function.

In addition, the results showed that mALB, mALB/U-CR, U-CR, β2MG and RBP were related to disease duration and HbA1c ($P < 0.05$); and disease duration, HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR were risk factors for the development of PDR. This indicates that the progression of diabetic microangiopathy is related to duration of the disease and the degree of abnormal glucose metabolism. It was found that persistent elevation of HbA1c accelerates damage to structural proteins in the glomerular basement membrane, causing disruption of polyol pathways, oxidative stress onset, and inflammatory infiltration involved in microvascular injury[24]. With the onset and progression of DR, disease duration and HbA1c levels increased abnormally, suggesting that persistent disease duration and abnormal HbA1c expression are involved in the development of diabetic microangiopathy, consistent with the findings of Casadei *et al*[25] and others. mALB, mALB/U-CR, U-CR, β2MG, RBP, disease duration and HbA1c were positively correlated in DR patients suggesting a synergistic role in promoting disease progression. The physiological characteristics of the

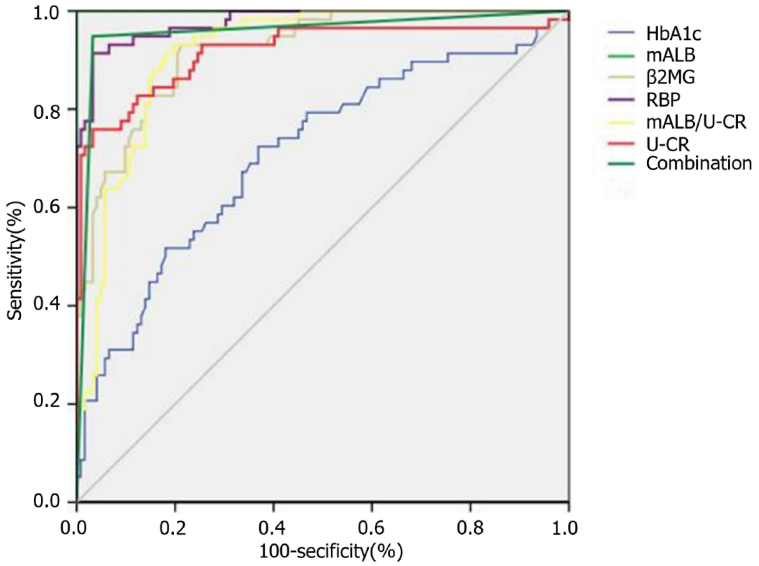

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Figure 2 Receiver operator characteristic curve analysis of glycated hemoglobin A1c, microalbumin, microalbumin/urinary creatinine, urinary creatinine, β2 microglobulin, and retinol binding protein for predicting proliferative diabetic retinopathy. U-mALB: Urinary microalbumin; U-CR: Urinary creatinine; β2MG: β2 microglobulin; RBP: Retinol binding protein; HbA1c: Glycated hemoglobin A1c.

glomerular and retinal vasculature, both of which are microcirculatory systems, suggest that persistent disease progression and elevated HbA1c levels induce disruption of the body's metabolic homeostasis and activation of oxidative stress, leading to damage to the vascular endothelium and the release of large amounts of inflammatory cytokines, inducing damage to the blood-retinal barrier and the glomerular filtration membrane barrier. In a state of persistently high glucose levels, oxides in vascular endothelial cells cannot be excreted, activating multiple signaling pathways and accelerating the impairment of vascular endothelial function, which may manifest as diabetic nephropathy if the abnormality is only in the kidney, or as DR if it occurs in the retina. Therefore, further studies found that the combination of HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP levels is predictive of the occurrence of PDR and can be used as a biochemical marker of DR. However, this study is a single center small sample study, and the results require further verification by follow-up multicenter and large sample studies.

CONCLUSION

HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP levels were up-regulated in DR patients, and their levels were closely related to disease duration, HbA1c and severity, all of which are risk factors for the development of PDR and can be used as markers to screen for DR progression. In the future, multi-center or propensity matching methods will be adopted to exclude the interference of multiple factors and provide new directions for clinical targeted therapy.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is a common complication of diabetes, which can eventually lead to blindness and seriously affect the quality of life of diabetic patients. Therefore, identification of the risk factors of DR is significant for early intervention.

Research motivation

This study explored the risk factors for DR and their predictive effect on retinopathy.

Research objectives

This study aimed to investigate the correlation between glycated hemoglobin A1c (HbA1c), urinary microalbumin (U-mALB), urinary creatinine (U-CR), mALB/U-CR ratio, β2 microglobulin (β2MG), retinol binding protein (RBP) and DR.

Research methods

Based on real population data, a retrospective study was carried out.

Research results

Duration of disease, HbA1c, mALB, β2MG, RBP, mALB/U-CR and U-CR were found to be risk factors for the development of DR. The area under the curve of the combined indices (HbA1c + mALB + mALB/U-CR + U-CR + β2MG + RBP) was 0.958.

Research conclusions

The combination of HbA1c, mALB, mALB/U-CR, U-CR, β2MG and RBP has predictive value for proliferative DR.

Research perspectives

Large multicenter studies are needed to further verify these results.

FOOTNOTES

Author contributions: Song JJ contributed to the conceptualization, funding acquisition, resources, supervision, methodology, software, investigation, formal analysis, writing - original draft, visualization, writing, review and editing of the manuscript; Han XF contributed to the data curation, writing and original draft of the manuscript; Chen JF contributed to the visualization, investigation, resources, supervision of the study; Liu KM contributed to the software and validation of data.

Institutional review board statement: This study was approved by the Medical Ethics Committee of the Second People's Hospital of Hefei (No. 2023014).

Informed consent statement: This study only used anonymous data in the system, and did not require informed consent according to institutional policy.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Data sharing statement: According to institutional policy, the third party has no access to obtain the data.

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ORIGINAL ARTICLE

Observational Study

Glucose metabolism profile recorded by flash glucose monitoring system in patients with hypopituitarism during prednisone replacement

Min-Min Han, Jia-Xin Zhang, Zi-Ang Liu, Lin-Xin Xu, Tao Bai, Chen-Yu Xiang, Jin Zhang, Dong-Qing Lv, Yan-Fang Liu, Yan-Hong Wei, Bao-Feng Wu, Yi Zhang, Yun-Feng Liu

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Abstract

BACKGROUND

Commonly used glucocorticoids replacement regimens in patients with hypopituitarism have difficulty mimicking physiological cortisol rhythms and are usually accompanied by risks of over-treatment, with adverse effects on glucose metabolism. Disorders associated with glucose metabolism are established risk factors of cardiovascular events, one of the life-threatening ramifications.

AIM

To investigate the glycometabolism profile in patients with hypopituitarism receiving prednisone (Pred) replacement, and to clarify the impacts of different Pred doses on glycometabolism and consequent adverse cardiovascular outcomes.

METHODS

Twenty patients with hypopituitarism receiving Pred replacement [patient group (PG)] and 20 normal controls (NCs) were recruited. A flash glucose monitoring system was used to record continuous glucose levels during the day, which

provided information on glucose-target-rate, glucose variability (GV), period glucose level, and hypoglycemia occurrence at certain periods. Islet β-cell function was also assessed. Based on the administered Pred dose per day, the PG was then regrouped into Pred > 5 mg/d and Pred ≤ 5 mg/d subgroups. Comparative analysis was carried out between the PG and NCs.

RESULTS

Significantly altered glucose metabolism profiles were identified in the PG. This includes significant reductions in glucose-target-rate and nocturnal glucose level, along with elevations in GV, hypoglycemia occurrence and postprandial glucose level, when compared with those in NCs. Subgroup analysis indicated more significant glucose metabolism impairment in the Pred > 5 mg/d group, including significantly decreased glucose-target-rate and nocturnal glucose level, along with increased GV, hypoglycemia occurrence, and postprandial glucose level. With regard to islet β-cell function, PG showed significant difference in homeostasis model assessment (HOMA)-β compared with that of NCs; a notable difference in HOMA-β was identified in Pred > 5 mg/d group when compared with those of NCs; as for Pred ≤ 5 mg/d group, significant differences were found in HOMA-β, and fasting glucose/insulin ratio when compared with NCs.

CONCLUSION

Our results demonstrated that Pred replacement disrupted glycometabolic homeostasis in patients with hypopituitarism. A Pred dose of > 5 mg/d seemed to cause more adverse effects on glycometabolism than a dose of ≤ 5 mg/d. Comprehensive and accurate evaluation is necessary to consider a suitable Pred replacement regimen, wherein, flash glucose monitoring system is a kind of promising and reliable assessment device. The present data allows us to thoroughly examine our modern treatment standards, especially in difficult cases such as hormonal replacement mimicking delicate natural cycles, in conditions such as diabetes mellitus that are rapidly growing in worldwide prevalence.

Key Words: Hypopituitarism; Prednisone; Flash glucose monitoring system; Glucose-target-rate; Glucose variability; Period glucose level

Core Tip: Glucocorticoids (GCs) replacement regimens for patients with hypopituitarism are hard to mimic physiological cortisol rhythms and carry risks of over-treatment, which can have adverse effects on glucose metabolism. We assessed the glucose metabolism profile of patients with hypopituitarism receiving prednisone (Pred) replacement, using a flash glucose monitoring system, to clarify impacts of different GCs preparations and prescriptions on glycometabolism, along with the resultant risks of consequent cardiovascular events. The study showed that Pred replacement disturbed glycometabolic homeostasis in patients with hypopituitarism. A dose of > 5 mg/d Pred caused more adverse effects on glycometabolism than ≤ 5 mg/d, contributing to the higher risks of cardiovascular events.

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INTRODUCTION

As the growing amount of information consolidated in the field of glucocorticoids' (GCs) hyperglycemia effect[1,2], whether GCs replacement therapy disturbs glycometabolism homeostasis in patients with hypopituitarism has garnered considerable interest. Timely and adequate GCs replacement has been commonly recognized as a lifesaving prescription for those patients with hypopituitarism, which aims to restore hormone deficiency and improve well-being. Hydrocortisone (HC) is the default choice for GCs replacement because of its similarity to endogenously-generated cortisol. Prednisone (Pred), cortisone, and dexamethasone represent other viable alternatives[3].

Choosing an optimum GCs replacement regimen for patients with hypopituitarism continues to be a challenging problem as the physiological cortisol rhythm is difficult to replicate. The most commonly used GCs replacement regimen is usually accompanied by a risk of insufficient trough levels or subtly excessive post-dose peaks[4,5]. An inability to mimic physiological cortisol rhythms or over-treatment may make those patients receiving GCs replacement susceptible to metabolic disturbances and subsequent cardiovascular events[6,7]. To date, the majority of evidence collected suggests that the occurrence of cardiovascular events is reportedly higher in patients with hypopituitarism who undergo GCs replacement than that in healthy controls[8,9]. New evidence has also emerged revealing that GCs replacement increases

the prevalence of glycometabolism disorders[10], which are established risk factors for cardiovascular disease.

As the prevalence of adverse events increases in patients with hypopituitarism receiving GCs replacement, greater emphasis has been placed on choosing a suitable replacement regimen with as little influence on glycometabolism as possible. Therefore, this study was designed to assess the glucose metabolism profile recorded by a flash glucose monitoring system (FGMS) in patients with hypopituitarism, illuminating the impact of GCs preparation and prescription doses on glucose metabolism. In doing so, we hope to add novel insights into the existing body of evidence and provide references to guide the treatment choices for those patients with hypopituitarism, in order to reduce the incidence of cardiovascular events.

MATERIALS AND METHODS

This study was conducted at the Department of Endocrinology, First Hospital of Shanxi Medical University from December, 2018 to August, 2022. The study protocol was approved by Ethic Committee in First Hospital of Shanxi Medical University. Written informed consent was obtained from all the subjects after explanation of study design and purpose.

Subjects

Patients diagnosed with hypopituitarism and receiving Pred replacement were recruited in this study as patient group (PG). The hypopituitarism was diagnosed by the following criteria[3]: Medical history (including postpartum hemorrhage, hypophysectomy, and pituitary crisis); clinical manifestations (including hyponatremia, hypotension, and hypoglycemia); pituitary magnetic resonance imaging (including empty sella, pituitary hypoplasia, and pituitary stalk interruption); hormone assay (lower levels of hormones relevant to pituitary-adrenal/thyroid/gonad function); corticotropin stimulation test (stimulated cortisol < 500 nmol/L). Those who were under interventions known to influence cortisol metabolism and glucose metabolism were excluded. Age- and sex-matched normal controls (NCs) without known hypopituitary dysfunction or glycometabolic disorders were enrolled. At baseline, all the recruited patients underwent hypopituitary-adrenal/thyroid function assessment, along with electrolyte and glucose metabolism evaluation, including plasma sodium, glycosylated hemoglobin, fasting blood glucose, and fasting insulin. The NCs received laboratory tests similar to those of PG.

Subgroup analysis

Relevant studies have corroborated that > 20 mg/d of HC correlated with unfavorable metabolic profile and cardiovascular events[11-13], however, whether an equivalent dose of Pred (> 5 mg/d) contributes to similar consequences is a relatively unexplored field. Due to the limited availability of HC in China, most patients with hypopituitarism received a Pred replacement regimen. In this study, we enrolled patients with hypopituitarism treated with Pred as PG, and divided PG into Pred > 5 mg/d and Pred ≤ 5 mg/d groups, based on the recommended Pred dose per day.

FGMS

FGMS (FreeStyle Libre, Abbott Diabetes Care, Witney, United Kingdom) was used to record glucose profiles of those in PG and NCs. Due to unexpected dropping of the sensor or other unpredictable interferences, we failed to obtain a complete two-week monitoring data in each person. The data from the first-day of monitoring were removed due to poor accuracy. In the end, a total 222 d of monitoring data were collected from patients in PG (134 d for the Pred > 5 mg/d group, and 88 d for the Pred ≤ 5 mg/d group), while NCs provided 184 d of glucose data. The FGMS required a real-time scanned value within 8-h in order to ensure complete glucose data during the surveillance. Some glucose values at certain time points were therefore missing, owing either to the subjects' poor adherence or other reasons, such as sleep time exceeding 8 h. These missing values were filled in with the mean of values before and after the missing values.

Islet function assessment

β-cell function and insulin resistance (IR) were assessed by calculating the homeostasis model assessment (HOMA)-β along with HOMA-IR, fasting glucose/insulin ratio (G/I), and quantitative insulin sensitivity check index (QUICKI).

Statistical analysis

The statistical methods used in this study were reviewed by Chunni Zhao from Shanxi Medical University. The data of laboratory tests and FGMS in PG, subgroups, and NCs were allocated in a Microsoft Excel datasheet. Data analysis and graph plotting were performed using SPSS 21.0 and Sigmaplot 12.5. All data are shown as mean ± standard error (SE) unless otherwise stated. Some data are represented as median (first quartile, third quartile) due to their wide distribution. A compared two-group Student's t-test (for normally distributed data) or Mann-Whitney U test (for skewed data) was conducted between PG, each subgroup, and NCs, respectively. Statistical significance was set as $P < 0.05$.

FGMS data were analyzed according to methods used in previous publications[14,15]. Specifically, glucose-target-rate, glucose variability (GV), and period glucose level were analyzed. A sensor glucose value within 3.9-7.8 mmol/L was set as the normal range. Accordingly, parameters representing glucose-target-rate were analyzed, including percentile time 1 (PT1, the percentage of sensor glucose values less than 3.9 mmol/L during the day), PT2 (the percentage of sensor glucose values within the range of 3.9-7.8 mmol/L during the day), PT3 (the percentage of sensor glucose values above 7.8 mmol/L during the day), time in range (TIR, the time of sensor glucose values within 3.9-7.8 mmol/L during the day),

and time out of range (the time of sensor glucose values less than 3.9 mmol/L or above 7.8 mmol/L during the day). GV was analyzed from the following perspectives: General GV including 24-h mean glucose and coefficient of variance (CV); within-day GV consisting of SD and mean amplitude of glycemic excursion; mean of daily difference (MODD) and area of interquartile range (IQR) reflecting day-to-day GV.

The endogenous cortisol secretion rhythm begins with a rise at around 3 am towards a peak after awaking, and then falls throughout the day until culminates in a nadir around the midnight[16]. Accordingly, nocturnal and fasting periods were merged and readjusted to periods of 0-3 am and 3-8 am. Glucose levels and area under the curve of glucose level at 0-3 am, 3-8 am, and postprandial periods were analyzed. In addition, hypoglycemia occurrence (glucose value less than 3.9 mmol/L) was analyzed during the 0-3 am and 3-8 am periods[17].

Formulas for calculating β-cell function and IR from a previous publication were used[14].

RESULTS

Twenty patients diagnosed with hypopituitarism, including nine with Sheehan's syndrome, four with empty sella, six with hypophysectomy, and one with pituitary hypoplasia, and receiving Pred replacement were enrolled in this study. Of these patients, 16 had suffered acute hypopituitarism, presenting symptoms of hyponatremia, hypotension, hypoglycemia, *etc.*, and four of them were diagnosed by corticotropin stimulation test (stimulated cortisol < 500 nmol/L). Significantly reduced levels of 24-h urinary free cortisol were detected in all of the patients during the course of the disease. Twelve patients were treated with doses of > 5 mg/d Pred and eight patients were treated with doses of ≤ 5 mg/d. There were also 12 patients undergoing concurrent thyroid hormone replacement therapy. The general characteristics and laboratory results of PG, subgroups, and NCs, including age, sex, disease duration (duration since hypopituitarism had been definitely diagnosed), blood pressure, plasma sodium, and endocrine hormone levels, are listed in the Table 1. Blood pressure, plasma sodium, and hormone levels were within the normal range in PG under the recommended replacement regimen.

Glucose-target-rate

Significantly increased PT1 (P = 0.018) and PT3 (P = 0.002) along with decreased TIR (P < 0.001) were identified in PG when compared with that of NCs (Figure 1A).

Remarkable elevations in PT1 (P = 0.02) and PT3 (P < 0.001) along with reduction in TIR (P < 0.001) were identified in Pred > 5 mg/d group when compared with those of NCs (Figure 1B). Comparable PT1, PT3, and TIR were found between Pred ≤ 5 mg/d group and NCs (Figure 1B).

GV

In PG, parameters of general GV were identified significance in CV (P = 0.003) compared with that of NCs. With regard to within-day GV, a notable elevation was found in SD (P = 0.003) when compared to those of NCs. There were no significant differences found in indices of day-to-day GV (Figure 2A).

In Pred > 5 mg/d group (Figure 2B), remarkable elevations were identified in parameters of general GV (CV, P < 0.001), within-day GV (SD, P < 0.001) and day-to-day GV (MODD, P = 0.019; area of IQR, P = 0.002), compared to that of NCs. However, no significant difference was observed in GV parameters between Pred ≤ 5 mg/d group and NCs (Figure 2B).

Period glucose level

For PG, period glucose level analysis indicated that glucose level was significantly lower at period of 3-8 am (P = 0.004) than that of NCs (Figure 3). Consistent results were found in the analysis of hypoglycemia occurrence with a remarkable elevation during this period (P = 0.012, Figure 4A). In addition, significantly increased glucose levels were identified at postprandial phase of PG (after lunch, P = 0.028; after dinner, P < 0.001) when compared to that of NCs (Figure 3).

In Pred > 5 mg/d group, notable alterations were found during the 3-8 am period with decreased glucose level (P = 0.025, Figure 5A) and increased hypoglycemia occurrence (P = 0.008, Figure 4B) in comparison with those in NCs. In addition, a remarkable elevation in glucose level was observed at postprandial phase (after lunch, P = 0.015; after dinner, P < 0.001) when compared with that of NCs (Figure 6A).

In Pred ≤ 5 mg/d group, a significant reduction of glucose level at 3-8 am period (P = 0.021) were found in comparison with NCs (Figure 5B), whereas, hypoglycemia occurrence was comparable to that of NCs (Figure 4B). Comparable postprandial glucose levels were also identified at postprandial periods between Pred ≤ 5 mg/d group and NCs (Figure 6B).

Islet function assessment

In PG, glucose metabolism indicators showed a significant difference in HOMA-β (P = 0.003) compared with that of NCs (Table 2).

Glucose metabolism indicators showed a notable difference in HOMA-β (P = 0.021) in Pred > 5 mg/d group when compared with those of NCs (Table 2). As for Pred ≤ 5 mg/d group, significant difference was found in HOMA-β (P < 0.001), and G/I (P = 0.018) in comparison with that in NCs (Table 2).

Table 1 General characteristics and laboratory results of patient groups and normal controls

Parameters	PG	Pred (> 5 mg/d)	Pred (≤ 5 mg/d)	NCs	Reference range
Age (year-old)	52.85 ± 3.49	51.5 ± 4.78	49.88 ± 5.33	50.75 ± 3.05	-
Male:famle	5:15	3:9	2:6	5:15	-
BMI (kg/m^2)	19.46 ± 1.39	20.02 ± 1.44	18.61 ± 0.81	20.5 ± 1.58	18.5-23.9
Disease duration (day)	730 (88.75, 1095)	730 (50, 1642.5)	730 (638.75, 1095)	-	-
Blood pressure (mmHg)	(108.65 ± 1.15)/(74.85 ± 0.89)	(108.25 ± 1.62)/(75.08 ± 1.36)	(109.25 ± 1.65)/(74.5 ± 0.98)	(111.95 ± 1.17)/(73.5 ± 0.7)	
Pred dose (mg/d)	6.19 ± 1.54	7.29 ± 0.48	4.53 ± 0.93	-	-
ACTH (pmol/L)	3.5 ± 0.33	3.58 ± 0.51	3.37 ± 0.33	4.40 ± 0.41	1.6-13.9
Cortisol at 8 am (nmol/L)	228.01 ± 4.42	230.24 ± 6.84	224.66 ± 4.44	372.90 ± 16.69	171-536
Cortisol at 4 pm (nmol/L)	153 ± 11.99	165.93 ± 17.48	133.6 ± 12.87	159.10 ± 14.62	64-327
Cortisol at 0 am (nmol/L)	111.55 ± 8.85	113.31 ± 12.01	108.91 ± 13.76	-	-
UFC (nmol/24h)	141.33 ± 11.34	131.80 ± 10.41	155.61 ± 23.86	-	100-279
FT3 (pmol/L)	4.25 ± 0.13	4.24 ± 0.15	4.27 ± 0.25	5.20 ± 0.14	3.1-6.8
FT4 (pmol/L)	14.11 ± 0.55	13.45 ± 0.50	15.09 ± 1.10	12.98 ± 0.92	10-23
TSH (μIU/ml)	1.076 ± 0.16	1.1 ± 0.19	1.04 ± 0.28	2.30 ± 0.19	0.27-4.2
Plasma sodium (mmol/L)	141.95 ± 0.86	140.3 ± 0.65	142.63 ± 1.07	140.6 ± 0.55	137-147

Pred: Prednisone; NCs: Normal controls; BMI: Body mass index; ACTH: Adrenocorticotropic-hormone; UFC: Urine free cortisol; FT3: Free triiodothyronine; FT4: Free thyroxine; TSH: Thyroid stimulation hormone. The laboratory tests were conducted in patient group during prednisone treatment.

Table 2 Glucose metabolism indictors in patient groups and normal controls

Parameters	PG	Pred (> 5 mg/d)	Pred (≤ 5 mg/d)	NCs	Reference range
HbA1c (%)	5.61 ± 0.101	5.71 ± 0.15	5.47 ± 0.11	5.46 ± 0.06	4.8-5.9
FBG (mmol/L)	4.71 ± 0.12	4.81 ± 0.19	4.56 ± 0.11	5.01 ± 0.13	3.9-6.1
FINS (μU/mL)	7.10 ± 0.64	6.82 ± 0.98	7.52 ± 0.72	5.76 ± 0.62	2.6-24.9
HOMA-β	130.22 ± 13.43[a]	113.511 ± 16.18[a]	155.28 ± 21.37[a]	81.02 ± 6.83	-
HOMA-IR	1.52 ± 0.17	1.52 ± 0.27	1.54 ± 0.17	1.32 ± 0.16	-
G/I	0.79 ± 0.09	0.898 ± 0.14	0.64 ± 0.048[a]	1.02 ± 0.082	
QUICKI	0.69 ± 0.03	0.71 ± 0.04	0.66 ± 0.02	0.73 ± 0.03	

[a]$P < 0.05$.

Pred: Prednisone; NCs: Normal controls; HbA1c: Glycosylated hemoglobin; FBG: Fasting blood glucose; FINS: Fasting insulin; HOMA-IR: Homeostasis-insulin resistance; G/I: Fasting glucose/fasting insulin ratio; QUICKI: Quantitative insulin check index.

DISCUSSION

In this study, we investigated the glucose metabolism profile in patients with hypopituitarism receiving GCs treatment. Significantly decreased glucose-target-rate and glucose level at nocturnal period, along with increased GV, hypoglycemia occurrence, and glucose level at postprandial phase were identified in PG when compared with those of NCs. These results demonstrated that glucose metabolism homeostasis was perturbed in patients with hypopituitarism receiving Pred replacement, despite careful administration. This disturbance may carry a risk of leading to cardiovascular diseases.

A dose of > 5 mg/d Pred was associated with a notable reduction in glucose-target-rate and glucose level at nocturnal period, along with elevation in GV, hypoglycemia occurrence, and glucose level at postprandial phase. However, only glucose level at 3-8 am period was changed significantly in Pred ≤ 5 mg/d group. Accordingly, we concluded that a dose

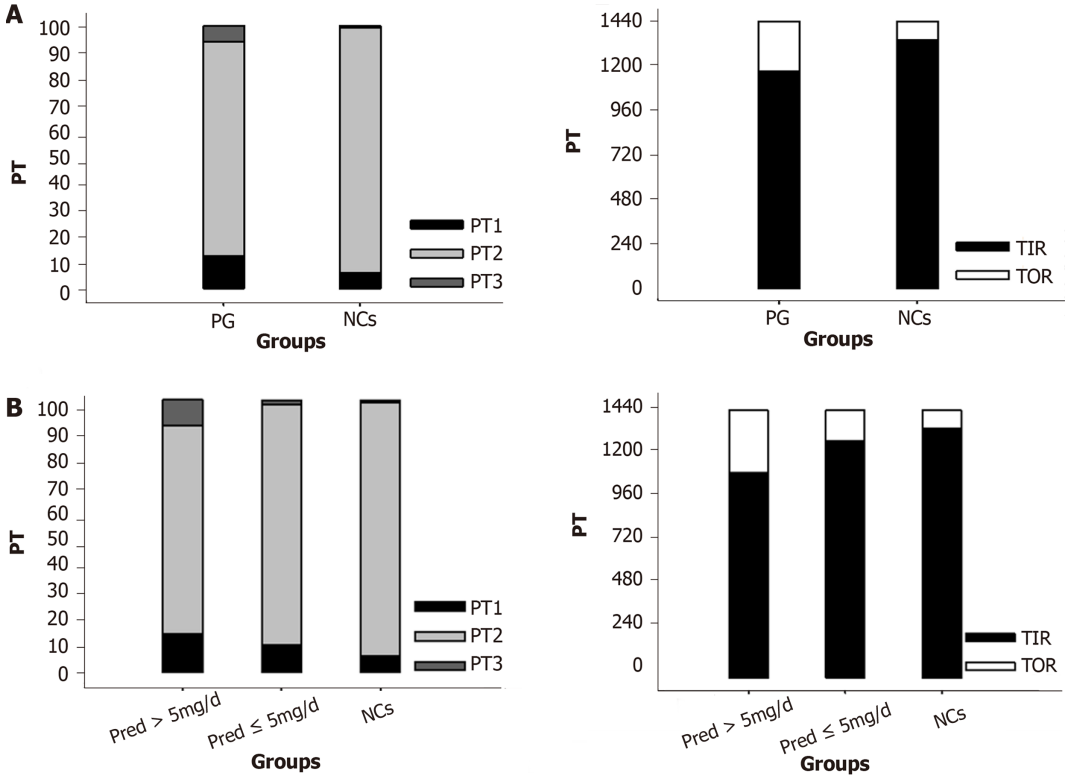

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Figure 1 Glucose-target-rate profile. A: Comparison of patient group to normal controls (NCs); B: Comparison of prednisone (Pred) > 5 mg/d group and Pred ≤ 5 mg/d group to NCs. Data are denoted as means. PG: Patient group; NCs: Normal controls; Pred: Prednisone; PT: Percentile time; TIR: Time in range; TOR: Time out of range.

of > 5 mg/d Pred may have a more adverse impact on glucose metabolism.

Given the essential role of GCs in maintaining normal life, authoritative guidelines strongly endorse the paramount importance of exogenously GCs replacement in those patients with endogenous insufficiency[3,18]. In this context, GCs replacement has been recognized as a fundamental therapeutic paradigm for patients with hypopituitarism. The diurnal rhythm of physiological cortisol secretion has been recognized for many years[19]. It is challenging for a GCs replacement regimen to accurately mimic this endogenously rhythmic pattern[20,21], usually leading to nonphysiological and subtly excess cortisol levels.

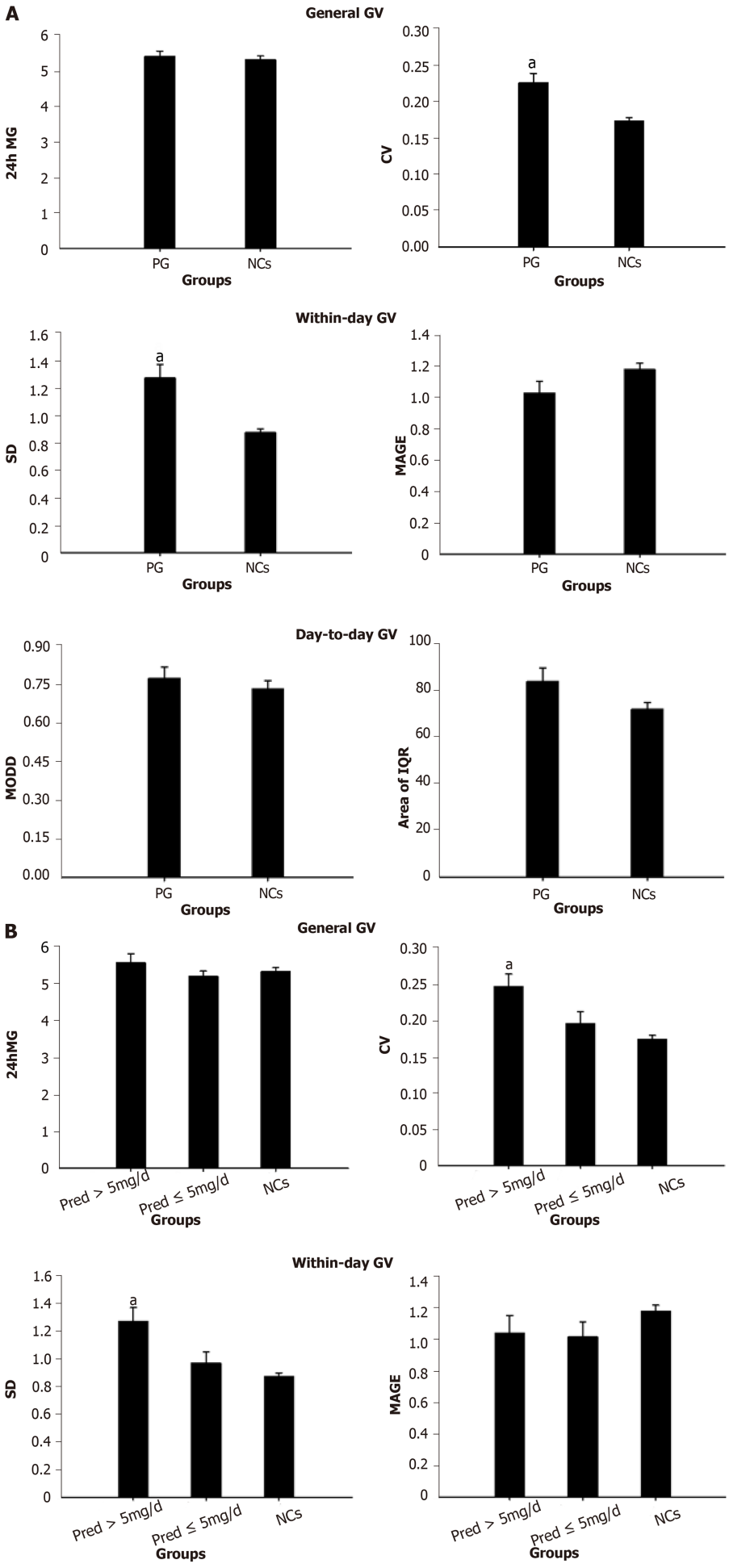
There is a growing awareness of highly dynamic synchronization in cortisol secretion into the blood circulation and its binding to GCs receptor (GR) in peripheral tissues[22]. Non-physiological GCs replacement fails to achieve a circadian rhythmic pattern, and further disturbs the tissue response mode, ensuing compromised hormone action, such as impaired glycometabolism and water-electrolyte metabolism. Pred is a kind of synthetic steroids, endowed with a great and enduring stimulatory effect on GR[23], and by continuously acting on the target tissues of glycometabolism, it can lead to metabolic disturbance.

In this study, significantly altered PT and TIR were identified in PG when compared with that of NCs. The significantly increased prevalence of hyperglycemia and hypoglycemia led to poor TIR. As an emerging indicator for blood glucose control in diabetic patients, TIR has been demonstrated to be inversely correlated with the risk of cardiovascular events [24,25]. The statistically decreased TIR identified in Pred group may herald a higher risk of cardiovascular events in these patients.

The results of significantly increased GV than that of NCs suggested that Pred replacement brought about an adverse impact on glycometabolism. GV is known to be positively associated with incidence of cardiovascular events in patients with diabetes[26,27]. Accordingly, we hypothesized that this notable elevation of GV found in PG implied that these patients would be prone to developing cardiovascular diseases during the long-term replacement therapy regimen.

The average glucose level throughout the whole day in PG was within the normal range. Nonetheless, a notable reduction was indicated at period of 3-8 am. Increased hypoglycemia occurrence was also identified at this period but not at 0-3 am period. Taken together, one could postulate that there existed relatively insufficient cortisol level at 3-8 am period, which was responsible for the elevated occurrence of hypoglycemia.

It is crucial to reiterate the basic fact that cortisol secretion follows a circadian rhythm in normal subjects, which commences with a rise at approximately 3 am, reaches a peak at around 8-9 am, and then progressively decreases towards a nadir at around midnight[16]. The cortisol trough level seemed sufficient in PG according to the comparable glucose level and hypoglycemia occurrence observed at 0-3 am period, when compared to those of NCs, which allowed us to hypothesize that Pred produced long-term steroids effects due to its delayed disassociation from GR. The applied Pred regimen was enough to maintain sufficient a trough level, although it failed to adequately and synchronously


A
General GV
24h MG
CV
a
PG
NCs
Groups
Within-day GV
SD
MAGE
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MODD
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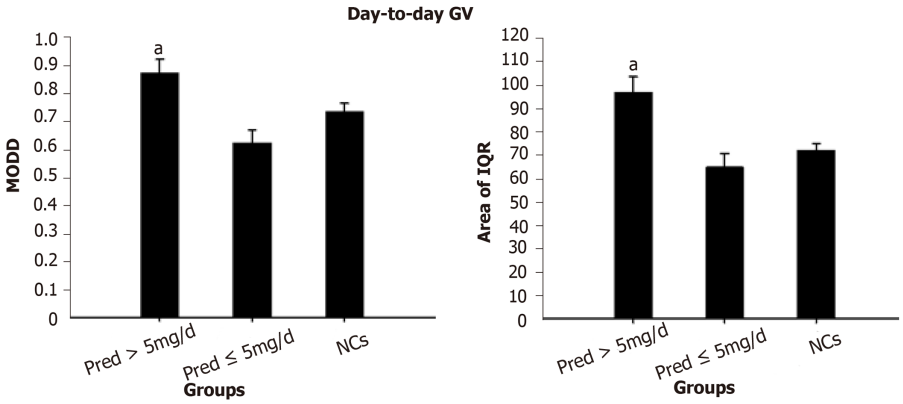

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Figure 2 Glucose variability profile. A: Comparison of patient group to normal controls (NCs); B: Comparison of prednisone (Pred) > 5 mg/d group and Pred ≤ 5 mg/d group to NCs. Data are denoted as mean ± SE. $^{a}P < 0.05$. PG: Patient group; NCs: Normal controls; Pred: Prednisone; GV: Glucose variability; MG: Mean glucose; CV: Coefficient of variance; MODD: Mean of daily difference; IQR: Interquartile range.

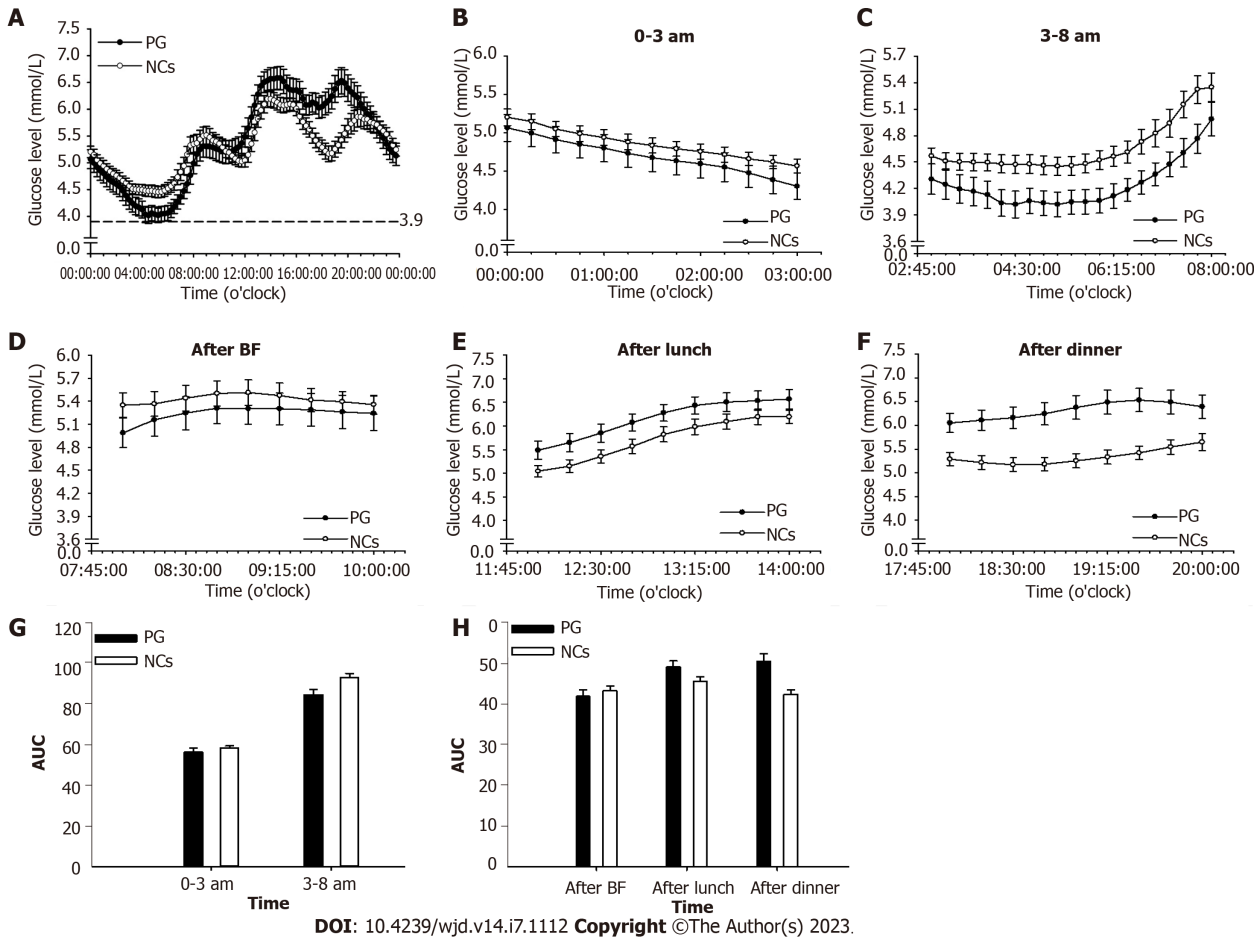

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Figure 3 Comparison of glucose level and area under the curve between patient group and normal controls. A: Glucose level during whole day; B: Glucose level at 0-3 am period; C: Glucose level at 3-8 am period; D: Glucose level after breakfast; E: Glucose level after lunch; F: Glucose level after dinner; G: Area under the curve (AUC) at 0-3 am period and 3-8 am period; H: AUC at postprandial periods. Data are denoted as mean ± SE. $^{a}P < 0.05$. AUC: Area under the curve; PG: Patient group; NCs: Normal controls; BF: Breakfast.

maintain cortisol elevation from 3 am to 8 am compared to the normal cases in NCs. Consequently, there may have existed a pre-dose cortisol insufficiency at period of 3-8 am when the steroids effect of the last administration had been washed out, leading to decreased glucose level and increased hypoglycemia occurrence.

A significantly increased postprandial glucose level was found in PG compared with that of NCs, seemingly indicating a subtly excess cortisol level during the daytime. As an allegedly long-acting GCs preparation, Pred possesses a great affinity for GR, occupying and stimulating the GR over a lengthy period until it is finally degraded[23]. Prolonged

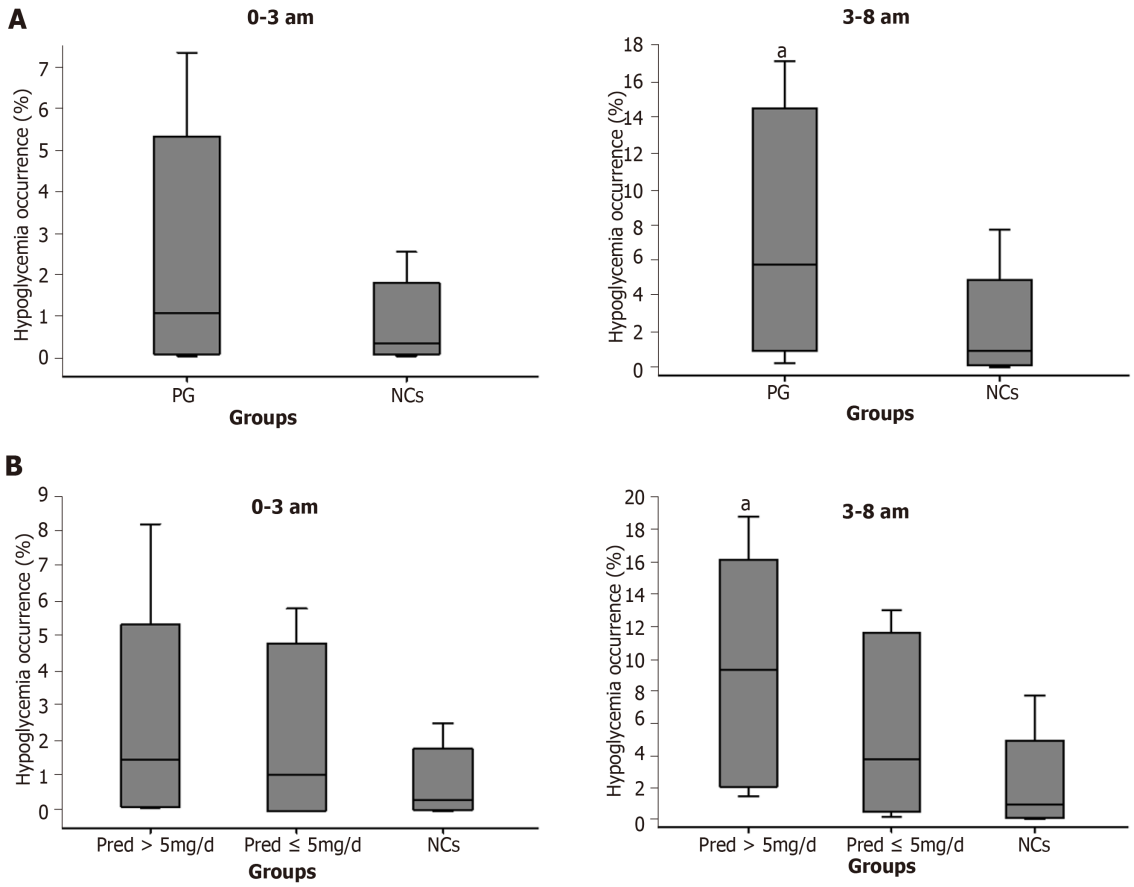

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Figure 4 Hypoglycemia occurrence. A: Comparison of patient group to normal controls (NCs); B: Comparison of prednisone (Pred) > 5 mg/d group and Pred ≤ 5 mg/d group to NCs. Data are showed as median, interquartile range, and interdecile range. [a]*P* < 0.05. PG: Patient group; NCs: Normal controls; Pred: Prednisone; IQR: Interquartile range.

exposure to steroids allows continuous accesses to target tissues, eliciting unfavorable side effects such as increased postprandial glucose level, as discovered in this study.

An important consideration when interpreting the influence of GCs replacement on glucose metabolism is whether the daily dose matches with the measured cortisol secretion rate over the course of an entire day in a normal subject. The physiological cortisol production rate is lower than the traditionally recommended GCs replacement dose[28,29]. Clinical evidence suggests that a dose of > 20 mg/d HC correlates with increased incidence of adverse events[11,12,30]. A dose of 5 mg Pred is equivalent to 20 mg HC. Assuming that patients under a replacement regimen of > 5 mg/d Pred were associated with a higher risk of adverse metabolic profile, PG was distributed into two subgroups: Pred > 5 mg/d and Pred ≤ 5 mg/d.

Significantly altered PT and TIR were found in Pred > 5 mg/d group, revealing that poor TIR was attributed to increased hyperglycemia and hypoglycemia occurrence. However, all indicators of glucose-target-rate showed no significant difference between Pred ≤ 5 mg/d group and NCs. According to TIR results, Pred > 5 mg/d group may have a predisposition towards developing cardiovascular diseases.

Significantly increased GV was detected in Pred > 5 mg/d group, while no statistical difference was identified in these parameters when Pred ≤ 5 mg/d group and NCs were compared. It is tempting, therefore, to speculate that patients in Pred > 5 mg/d group experienced more aggressive glycometabolism impairment and a great tendency to experience adverse cardiovascular events.

The average daily glucose level was normal in both Pred > 5 mg/d group and Pred ≤ 5 mg/d group. In Pred > 5 mg/d group, a notable reduction of glucose level was documented at 3-8 am period, highlighting the possibility of insufficient nocturnal hormone level. Moreover, a remarkable elevation of hypoglycemia occurrence was identified at this period, adding credence to the speculation of insufficient nocturnal hormone level. The Pred ≤ 5 mg/d group exhibited a significantly decreased glucose level at 3-8 am period, however, hypoglycemia occurrence at this period was comparable to that in NCs, prompting the assumption that insufficient nocturnal hormone level was relatively mitigated in comparison with the situation in Pred > 5 mg/d group. In addition, the glucose level and hypoglycemia occurrence at 0-3 am period were comparable to those of NCs in Pred > 5 mg/d group and Pred ≤ 5 mg/d group, demonstrating that a sufficient trough level may have been achieved in these two group.

A significantly increased glucose level at postprandial phase was identified in Pred > 5 mg/d group, but not in Pred ≤ 5 mg/d group. The underlying mechanism was assumed to be continuous hormone accessing to the target tissue due to

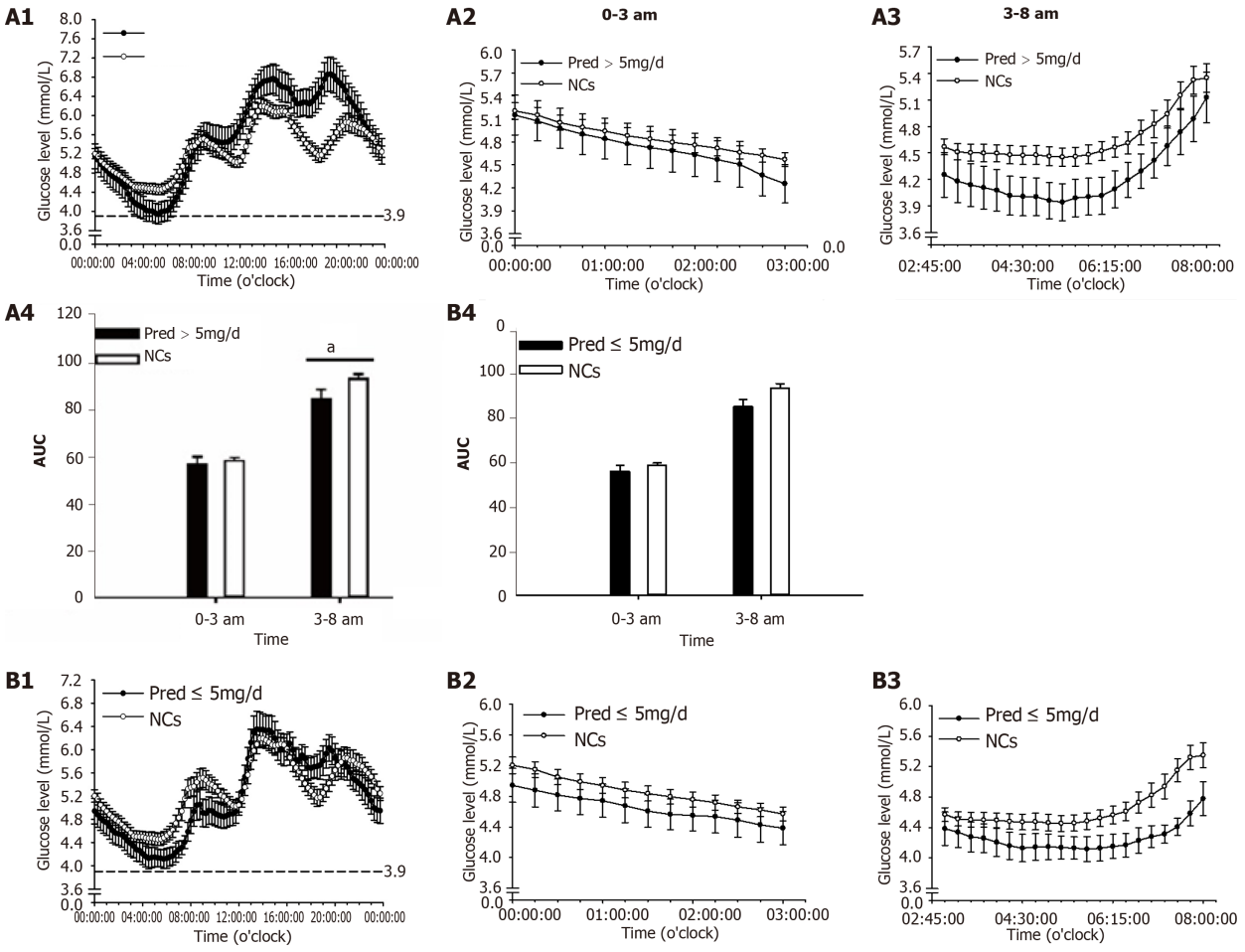

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Figure 5 Glucose level and area under the curve during the day, at 0-3 am, period and at 3-8 am period. Comparison of prednisone (Pred) > 5mg/d group to normal controls (NCs): A1, during the day; A2, at 0-3am; A3, at 3-8am; A4, AUC. Comparison of prednisone (Pred) ≤ 5mg/d group to normal controls (NCs): B1, during the day; B2, at 0-3am; B3, at 3-8am; B4, AUC. Data are denoted as mean ± SE. $^{a}P < 0.05$. AUC: Area under the curve; NCs: Normal controls; Pred: Prednisone.

the long-acting property of Pred during the daytime. This continuous stimulation may have disturbed glucose regulation in the relevant organs, leading to reduced glucose disposal and elevated glucose production. The present data allowed us to hypothesize that a Pred dose of > 5 mg/d posed a more profound effect on glucose regulation during the efficacy period than a dose of ≤ 5 mg/d.

Impaired β-cell function was indicated by relevant parameters in PG when compared to that of NCs. Summarizing the yet published literature, there are no consistent data supporting the presence of impaired β-cell function in patients with hypopituitarism undergoing GCs treatment[31-33]. Results generated in this study allowed us to hypothesize that the physiological Pred replacement regimen may exert adverse effects on glucose metabolism, leading to compromised β-dell function.

Of special note is that adrenocorticotropic-hormone (ACTH) levels measured in all the groups were within the normal range, probably indicating partial ACTH deficiency. The normal ACTH levels were suggestive of a reasonable Pred treatment because an excess dose of Pred might suppress the ACTH secretion. However, the usage of FGMS identified impaired glucose metabolism, which is relevant to higher risks of cardiovascular diseases. In this light, FGMS attests its importance in providing reliable information for evaluating a suitable Pred replacement regimen.

CONCLUSION

Pred replacement in patients with hypopituitarism impaired glucose metabolism, leading to an increased risk of cardiovascular events. A dose of > 5 mg/d Pred had a more significant influence on glucose metabolism than a dose of ≤ 5 mg/d. A suitable Pred replacement regimen necessitates comprehensive and accurate evaluation, for which FGMS is a kind of promising and reliable assessment device. Altogether, the integration of results in this study adds weight to the existing knowledge, and further provides new reference and guidance for future clinical work to effectively avoid the risk of cardiovascular events and improve well-being in patients with hypopituitarism.

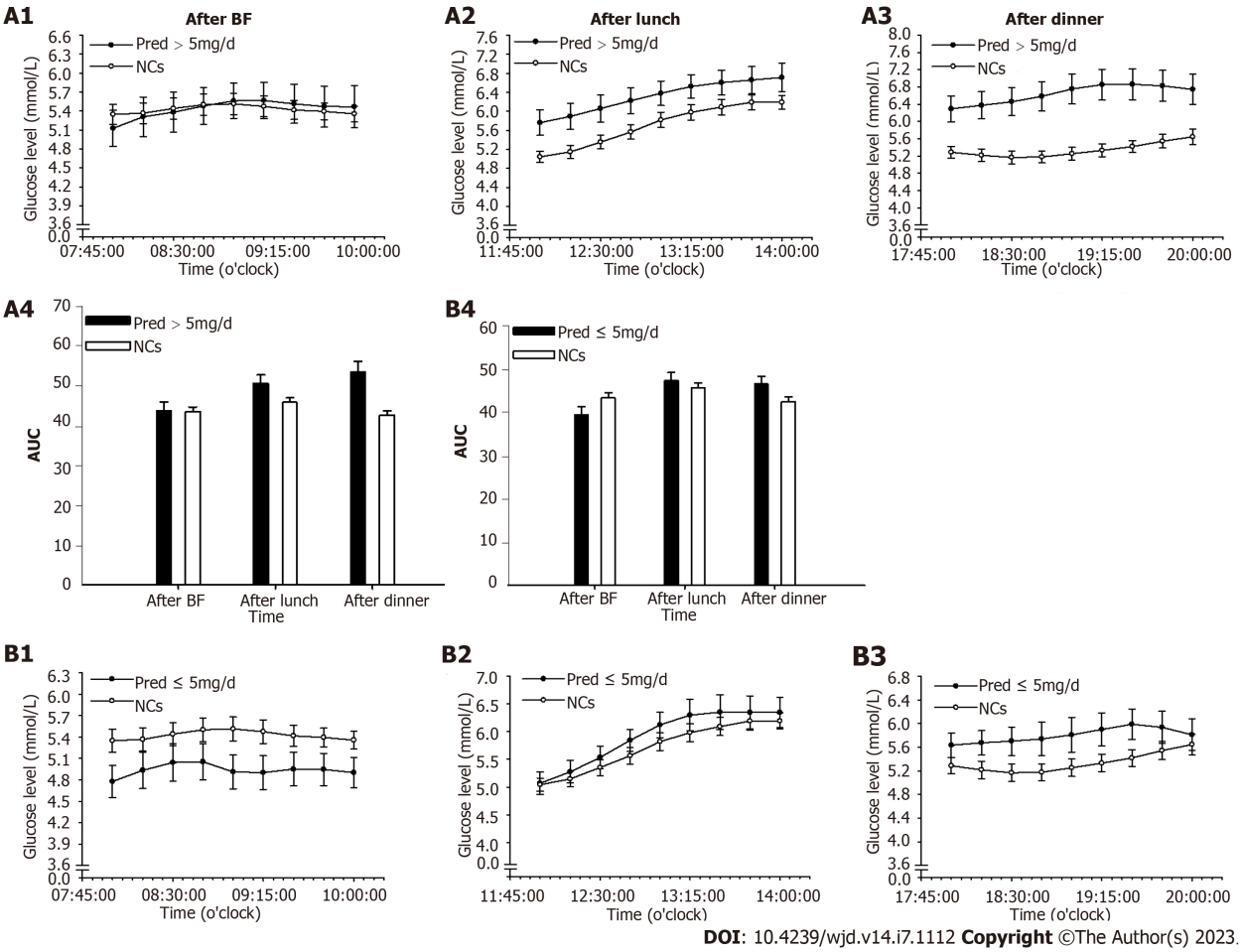

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Figure 6 Glucose level and area under the curve at postprandial phase. Comparison of prednisone (Pred)>5mg/d group to normal controls (NCs): A1, afetr BF; A2, after lunch; A3, after dinner; A4, AUC. Comparison of prednisone (Pred) ≤ 5mg/d group to normal controls (NCs): B1, after BF; B2, after lunch; B3, after dinner; B4, AUC. Data are denoted as mean ± SE. $^{a}P < 0.05$. AUC: Area under the curve; NCs: Normal controls; Pred: Prednisone; BF: Breakfast.

ARTICLE HIGHLIGHTS

Research background

As the growing amount of information consolidated in the field of glucocorticoids' (GCs) hyperglycemia effect, whether GCs replacement therapy disturbs glycometabolism homeostasis in patients with hypopituitarism has garnered considerable interest. Timely and adequate GCs replacement has been commonly recognized as a lifesaving prescription for those patients with hypopituitarism, which aims to restore hormone deficiency and improve well-being. Choosing an optimum GCs replacement regimen for patients with hypopituitarism continues to be challenging problem as the physiological cortisol rhythm is difficult to replicate. An inability to mimic physiological cortisol rhythms or over-treatment may make those patients receiving GCs replacement susceptible to metabolic disturbances and subsequent cardiovascular events.

Research motivation

Commonly used glucocorticoids replacement regimens in hypopituitarism patients have difficulty mimicking physiological cortisol rhythms and are usually accompanied with risks of over-treatment, which will pose adverse effects on glucose metabolism. Disorders associated with glucose metabolism are established risk factors of cardiovascular events, one of the life-threatening ramifications. As the increasing prevalence of adverse events occurs in hypopituitarism patients under GCs replacement, greater emphasis has been placed on choosing a suitable replacement regimen with as little influence on glycometabolism as possible.

Research objectives

This study was designed to assess the glucose metabolism profile recorded by a flash glucose monitoring system in patients with hypopituitarism, illuminating the impact of GCs preparation (Pred) and prescription doses on glucose metabolism. In doing so, we hope to add novel insights into the existing body of evidence and provide references to guide the treatment choices for those patients with hypopituitarism, in order to reduce the incidence of cardiovascular events.

Research methods

In this study, patients with hypopituitarism treated with Pred were enrolled as patient group (PG), and regrouped into Pred > 5 mg/d group and Pred ≤ 5 mg/d group based on the recommended Pred dose per day. Age- and sex-matched normal controls (NCs) without known hypopituitary dysfunction or glycometabolic disorders were enrolled. At baseline, all the recruited patients underwent hypopituitary-adrenal/thyroid function assessment, along with electrolyte and glucose metabolism evaluation, including plasma sodium, glycosylated hemoglobin, fasting blood glucose, and fasting insulin. The NCs received laboratory tests similar to those of PG. Flash glucose monitoring system (FGMS) was used to record glucose profile of both PG and the NCs. Parameters of glucose-target-rate, glucose variability (GV), and period glucose level were analyzed. β-cell function and insulin resistance (IR) were assessed by calculating the homeostasis model assessment (HOMA)-β along with HOMA-IR, fasting glucose/insulin ratio, and quantitative insulin sensitivity check index.

Research results

Twenty patients diagnosed with hypopituitarism receiving Pred replacement were enrolled in this study. Of these, twelve patients were treated with doses of > 5 mg/d Pred and eight patients were treated with doses of ≤ 5 mg/d. Significantly decreased glucose-target-rate and glucose level at nocturnal period, along with increased GV, hypoglycemia occurrence, and glucose level at postprandial phase were identified in PG when compared with those of NCs. These results demonstrated that glucose metabolism homeostasis was perturbed in patients with hypopituitarism receiving Pred replacement, despite careful administration. This disturbance may carry a risk of leading to cardiovascular diseases. A dose of > 5 mg/d Pred was associated with a notable reduction in glucose-target-rate and glucose level at nocturnal period, along with elevation in GV, hypoglycemia occurrence, and glucose level at postprandial phase. However, only glucose level at 3-8 am period was changed significantly in Pred ≤ 5 mg/d group. Accordingly, we concluded that a dose of > 5 mg/d Pred may have a more adverse impact on glucose metabolism. Impaired β-cell function was indicated by relevant parameters in PG when compared to that of NCs.

Research conclusions

Pred replacement in patients with hypopituitarism impaired glucose metabolism, leading to an increased risk of cardiovascular events. A dose of > 5 mg/d Pred had a more significant influence on glucose metabolism than a dose of ≤ 5 mg/d. A suitable Pred replacement regimen necessitates comprehensive and accurate evaluation, for which FGMS is a kind of promising and reliable assessment device. Altogether, the integration of results in this study adds weight to the existing knowledge, and further provides new reference and guidance for future clinical work to effectively avoid the risk of cardiovascular events and improve well-being in patients with hypopituitarism.

Research perspectives

The integration of results in this study adds weight to the existing knowledge, and further provides new reference and guidance for future clinical work to effectively avoid the risk of cardiovascular events and improve well-being in patients with hypopituitarism.

FOOTNOTES

Author contributions: Liu YF was the guarantor and designed the study; Liu ZA, Xu LX, Bai T, Xiang CY, Zhang J, Lv DQ, Liu YF, Wei YH, and Wu BF participated in the acquisition of the data; Han MM, Zhang JX, and Liu ZA analyzed and interpreted the data; Han MM and Zhang JX drafted the initial manuscript; Liu YF, Zhang Y, and Han MM revised the article critically for important intellectual content.

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Informed consent statement: Written informed consent was obtained from all the subjects after explanation of study design and purpose.

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ORIGINAL ARTICLE

Observational Study

Association between cardiorespiratory fitness level and insulin resistance in adolescents with various obesity categories

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Abstract

BACKGROUND

An association between cardiorespiratory fitness (CRF) and insulin resistance in obese adolescents, especially in those with various obesity categories, has not been systematically studied. There is a lack of knowledge about the effects of CRF on insulin resistance in severely obese adolescents, despite their continuous rise.

AIM

To investigate the association between CRF and insulin resistance in obese adolescents, with special emphasis on severely obese adolescents.

METHODS

We performed a prospective, cross-sectional study that included 200 pubertal adolescents, 10 years to 18 years of age, who were referred to a tertiary care center due to obesity. According to body mass index (BMI), adolescents were classified as mildly obese (BMI 100% to 120% of the 95th percentile for age and sex) or severely obese (BMI ≥ 120% of the 95th percentile for age and sex or ≥ 35 kg/m^2, whichever was lower). Participant body composition was assessed by bioelectrical impedance analysis. A homeostatic model assessment of insulin resistance (HOMA-IR) was calculated. Maximal oxygen uptake (VO_2max) was determined from submaximal treadmill exercise test. CRF was expressed as VO_2max scaled by total body weight (TBW) (mL/min/kg TBW) or by fat free mass (FFM) (mL/min/kg FFM), and then categorized as poor, intermediate, or good, according to VO_2max terciles. Data were analyzed by statistical software package SPSS (IBM SPSS Statistics for Windows, Version 24.0). $P < 0.05$ was considered

statistically significant.

RESULTS

A weak negative correlation between CRF and HOMA-IR was found [Spearman's rank correlation coefficient (r_s) = -0.28, P < 0.01 for CRF_{TBW}; (r_s) = -0.21, P < 0.01 for CRF_{FFM}]. One-way analysis of variance (ANOVA) revealed a significant main effect of CRF on HOMA-IR [$F_{(2200)}$ = 6.840, P = 0.001 for CRF_{TBW}; $F_{(2200)}$ = 3.883, P = 0.022 for CRF_{FFM}]. Subsequent analyses showed that obese adolescents with poor CRF had higher HOMA-IR than obese adolescents with good CRF (P = 0.001 for CRF_{TBW}; P = 0.018 for CRF_{FFM}). Two-way ANOVA with Bonferroni correction confirmed significant effect of interaction of CRF level and obesity category on HOMA-IR [$F_{(2200)}$ = 3.292, P = 0.039 for CRF_{TBW}]. Severely obese adolescents had higher HOMA-IR than those who were mildly obese, with either good or poor CRF. However, HOMA-IR did not differ between severely obese adolescents with good and mildly obese adolescents with poor CRF.

CONCLUSION

CRF is an important determinant of insulin resistance in obese adolescents, regardless of obesity category. Therefore, CRF assessment should be a part of diagnostic procedure, and its improvement should be a therapeutic goal.

Key Words: Cardiorespiratory fitness; Insulin resistance; Obese adolescents; Severe obesity; Obesity category

Core Tip: The association between obesity and insulin resistance is well established. However, data concerning the relationship between cardiorespiratory fitness (CRF) and insulin resistance in obese adolescents, especially in those with varying obesity categories, are quite limited. The results of present study show that obese adolescents with good CRF have lower homeostatic model assessment of insulin resistance (HOMA-IR) than obese adolescents with poor CRF. Moreover, there is no difference in HOMA-IR between severely obese adolescents with good CRF and mildly obese adolescents with poor CRF. Thus, the improvement of CRF in obese adolescents, including those with severe obesity, should be a therapeutic goal.

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INTRODUCTION

The global obesity epidemic is accompanied by rapid increase in the prevalence of cardiometabolic disorders. The association between obesity and insulin resistance is well established, along with the fact that insulin resistance represents a pivotal step in the progression towards prediabetes and type 2 diabetes[1,2]. The phenomenon of pubertal insulin resistance has been confirmed in cross-sectional and longitudinal studies[3]. Therefore, obese adolescents who are in puberty should be regarded as a particularly vulnerable group for glucose metabolism dysregulation. Growing evidence supports the notion that young-onset type 2 diabetes has a more aggressive disease phenotype, leading to early development of complications, adversely affecting quality of life and long-term outcomes. As more than half of the world's population is expected to be overweight or obese within the next 12 years, expanding the options to manage adolescent obesity is essential to treat the epidemic.

Cardiorespiratory fitness (CRF) refers to ability of the circulatory and respiratory systems to supply oxygen to skeletal muscle mitochondria for energy production during sustained physical activity. In adults, poor CRF is associated with the risk of insulin resistance, irrespective of body weight[4]. Moreover, health benefits are most apparent at the low end of the CRF continuum, providing the evidence that interventions aimed at CRF improvement of the least fit individuals should be encouraged[5]. In children and adolescents, CRF is an important marker of health which shows an inverse relationship with obesity, insulin resistance and cardiometabolic risk[6-8]. Available data confirm that association between obesity and cardiometabolic risk scores could be partially decreased with improvements in fitness levels[9]. It seems that early intervention and prevention strategies targeting youth CRF may be associated with reduced risk for obesity and cardiometabolic disease later in life[10].

However, the relationship between obesity, CRF, and insulin resistance in the adolescent population is still insufficiently explored. According to a recently published study, high CRF was associated with lower total and regional fat and higher insulin sensitivity in overweight and obese adolescents[11]. Also, obese adolescents with low CRF had higher insulin resistance indices and insulin secretion response than adolescents with normal CRF, irrespective of body mass index (BMI) *z*-score[12]. According to another study which included children aged 8 years to 11 years, as BMI categories

rose, CRF attenuated the metabolic risk score, with the biggest differences observed in the most obese children, although the attenuation was significant only in mild obesity[13].

The aim of our study was to investigate the association between CRF and insulin resistance in obese adolescents, with special emphasis on those with severe obesity, for whom the data about this topic are scarce.

MATERIALS AND METHODS

Study population

Two hundred adolescents who had been referred to the Department of Pediatric Endocrinology and Diabetology at the University Hospital Center "Sestre milosrdnice" due to obesity from February 2019 to July 2022 participated in this cross-sectional study. Prior to enrolment, all the participants and their parents provided written informed consent. The study was approved by the University Hospital Ethics Committee and complied with the Declaration of Helsinki.

The inclusion criteria were: 10 years to 18 years of age, presence of puberty, and BMI ≥ of the 95th percentile for age and sex according to the Centers for Disease Control and Prevention BMI-for-age growth charts[14]. The exclusion criteria were: Chronic diseases which prevent CRF assessment or affect either body mass or body composition (hypothyroidism, hypercortisolism, and syndromes associated with obesity), history of disorders of glucose metabolism, and the use of drugs affecting glucose metabolism or body composition.

According to obesity category, adolescents were classified into groups with mild (class I obesity, BMI 100% to 120% of the 95th percentile for age and sex) or severe obesity (class II obesity, BMI 120% to 140% of the 95th percentile for age and sex or ≥ 35 kg/m^2, whichever was lower; class III obesity, BMI more than 140% of the 95th percentile for age and sex or BMI ≥ 40 kg/m^2, whichever was lower), and according to the terciles of CRF into groups with poor, intermediate, or good CRF.

Anthropometric measurements

During anthropometric measurements, adolescents were wearing minimal clothing and no shoes. Height was measured using a wall stadiometer (Holtain Ltd., Harpenden, United Kingdom) with a precision of 0.1 cm. Weight was determined using an electronic scale (Seca 704; BIS, Hamburg, Germany) with a precision of 0.1 kg. BMI (kg/m^2) was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured in a standing position, with a flexible, non-elastic measuring tape, midway between the most inferior rib and the top of the iliac crest, with a precision of 0.1 cm. Body composition was assessed by bioelectrical impedance analysis (BIA) (MC-780 analyzer; Tanita, Nagano, Japan). The pubertal stages were determined using Tanner's criteria, based on breast size and contour in girls and testicular volume in boys.

Laboratory tests

Plasma glucose in mmol/L (Abbott Architect c8000; Abbott Laboratories, Chicago, IL, United States) and insulin concentrations in mU/L (ECLIA, Cobas e601; Roche Diagnostics, Basel, Switzerland) were measured after a 10 h-12 h overnight fast. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5.

CRF assessment

CRF was assessed using a submaximal treadmill walking test, according to a validated protocol for overweight and obese adolescents[15]. After a 4-min warm-up at a self-selected comfortable walking speed (treadmill incline 0%), the participants were asked to maintain this speed for 4 min while the treadmill incline increased to 5%. Heart rate was recorded at rest (HR 0') and at the end of the 4 minutes on a 5% incline (HR 4'), as well as the self-selected speed. Based on these two variables, maximal oxygen uptake (VO_2max) was estimated from the equation that also included sex (female-F, male-M), weight and height.

VO_2max (mL/min) = -1772.81 + 318.64 × sex (F = 0, M = 1) + 18.34 × weight (kg) + 24.45 × height (cm) - 8.74 × HR 4'-0.15 × weight (kg) × (HR 4' - HR 0') + 4.41 × speed (km/h) × 0.6213711922 × (HR 4'-HR 0').

To facilitate comparison among adolescents of different sizes, VO_2max was scaled by total body weight (TBW) (mL/min/kg TBW) and by fat free mass (FFM) (mL/min/kg FFM).

Statistical analysis

All statistical analyses were conducted using SPSS version 24.0 (IBM Corp., Armonk, NY, United States). Descriptive statistics were employed to summarize the demographic characteristics of the study population, and the variables being investigated. The normality of data distribution was tested with Shapiro-Wilk test. For data deviating from normal distribution, Levene's test of homogeneity of variances was used. For comparisons, a *t*-test for independent samples was employed, and if Levene's test was statistically significant, the corrected value of the t-test and the associated *P* were used. Spearman's correlation coefficient was calculated as a measure of association between continuous variables. A chi-square test was employed for categorical variables. A one-way analysis of variance (ANOVA) was used to test the influence of CRF on HOMA-IR, while the differences between the groups of adolescents with mild and severe obesity were analyzed using two-way ANOVA with Bonferroni correction. All analyses were adjusted for age and sex. *P* values < 0.05 were considered statistically significant.

RESULTS

Characteristics of the study population

Two hundred obese, pubertal adolescents (average age of 14.54 years ± 1.90 years) were included in the study. There were more girls (60.5%) than boys. The majority of adolescents were in advanced puberty (72.5% Tanner stages IV and V), and were severely obese (64.5% class II and class III obesity) (Figure 1). More adolescent girls were in advanced puberty, while more adolescent boys were severely obese (Table 1).

Differences in anthropometric, CRF and insulin resistance parameters among groups with various obesity categories

Groups of adolescents with various obesity categories differed according to majority of the measured variables (Table 2). All 3 groups differed with respect to BMI, BMI z-score, and CRF_{TBW} (expressed as VO_2max in ml/min/kg TBW) in a way that BMI and BMI *z*-score increased and CRF_{TBW} decreased from class I to class III obesity group ($P < 0.001$). Subjects with class I and class II obesity had lower fasting insulin and HOMA-IR than subjects with class III obesity ($P < 0.001$ for fasting insulin, $P = 0.001$ for HOMA-IR). Finally, the class I obesity group had lower waist circumference and waist to height ratio, and higher CRF_{FFM} (expressed as VO_2max in mL/min/kg FFM) than class III obesity group ($P = 0.017$ for waist circumference, $P = 0.019$ for waist to height ratio, $P = 0.003$ for CRF_{FFM}).

Correlation between CRF and HOMA-IR in obese adolescents

In obese adolescents, a weak negative Spearman's correlation between CRF_{TBW} and HOMA-IR (r_s =-0.28, $P < 0.01$), and CRF_{FFM} and HOMA-IR (r_s =-0,21, $P < 0.01$) was found.

Association between CRF level and HOMA-IR in obese adolescents

A statistically significant main effect of CRF_{TBW} on HOMA-IR was detected [$F_{(2200)} = 6.840$, $P = 0.001$]. Subsequent comparisons revealed that HOMA-IR was higher in the group of adolescents with poor than in the groups of adolescents with intermediate ($P = 0.021$) or good CRF_{TBW} ($P = 0.001$) (Figure 2A).

Furthermore, a statistically significant main effect of CRF_{FFM} on HOMA-IR was determined [$F_{(2200)} = 3.883$, $P = 0.022$]. Subsequent comparisons revealed that HOMA-IR was higher in the group of adolescents with poor compared to the group with good CRF_{FFM} ($P = 0.018$) (Figure 2A).

Association of CRF level and obesity category with HOMA-IR

Separate main effects of CRF_{TBW} level and obesity category (class I-mild obesity, class II and III-severe obesity) on HOMA-IR were not statistically significant, but their interaction was [$F_{(2200)} = 3.292$, $P = 0.039$] (Table 3).

Adolescents with mild obesity had lower HOMA-IR than severely obese adolescents, regardless of their poor or good CRF_{TBW}, while adolescents with intermediate CRF_{TBW} did not differ significantly with regard to HOMA-IR (Figure 2B). In severely obese adolescents, HOMA-IR was the highest in subjects with poor CRF_{TBW}. HOMA-IR of mildly obese adolescents with poor CRF_{TBW} did not differ significantly from HOMA-IR of severely obese adolescents with good CRF_{TBW} (Figure 2B).

The separate main effect of CRF_{FFM} level on HOMA-IR was not statistically significant, while the influence of obesity category was of borderline statistical significance [$F_{(2200)} = 3.846$, $P = 0.051$] (Table 4). HOMA-IR of mildly obese adolescents with poor CRF_{FFM} was not significantly different from HOMA-IR of severely obese adolescents with good CRF_{FFM} (Figure 2C).

DISCUSSION

In people living with obesity, the current widely accepted management strategies are based on diet and lifestyle modifications. However, the therapeutic emphasis is most often on calorie restriction and weight reduction, while the importance of regular physical activity and CRF improvement is insufficiently stressed. Moreover, physical activity is perceived primarily as a means to create a negative energy balance. Such an approach overlooks the important health benefits of CRF improvement, independent of weight loss[16].

Rates of obesity among children and adolescents are high and the prevalence of severe obesity in pediatric population is increasing[17]. Obese young people tend to participate in less physical activity than youths of healthier weight[18].

In this study, among 200 adolescents, 129 (64.5%) met the criteria for severe obesity. This should not come as a surprise, given that adolescents were referred for obesity evaluation to a tertiary care center. The proportion of participants with severe obesity was higher in adolescent boys than in adolescent girls, which is in line with other published data[17,19].

The hyperinsulinemic-euglycemic clamp is the gold standard for insulin sensitivity assessment, but it is expensive and labor-intensive. Alternative tests, including the frequently sampled intravenous glucose tolerance test, insulin tolerance test, insulin sensitivity test, and continuous infusion of glucose with model assessment are also quite impractical for routine use. The oral glucose tolerance test is easier to perform, but still time consuming. Fasting methods for assessment of insulin resistance such as fasting insulin, glucose/insulin ratio, quantitative insulin sensitivity check index, and HOMA-IR are inexpensive and less difficult to apply in clinical practice, although each of them has its merits and deficiencies[20]. In this study, HOMA-IR was used as a surrogate marker of insulin resistance, due to its correlation with clamp techniques and wide employment in clinical research.

Table 1 Distribution of adolescents according to puberty stage and obesity category, *n* (%)

Variable	All	Females	Males	[1]*P* value
Tanner stage				< 0.001
II	33 (16.5)	7 (5.8)	26 (32.9)	
III	22 (11.0)	9 (7.4)	13 (16.5)	
IV	41 (20.5)	21 (17.4)	20 (25.3)	
V	104 (52.0)	84 (69.4)	20 (25.3)	
Obesity category				0.004
Class I	71 (35.5)	54 (44.6)	17 (21.5)	
Class II	79 (39.5)	42 (34.7)	37 (46.8)	
Class III	50 (25.0)	25 (20.7)	25 (31.6)	

[1]χ^2 test.

Table 2 Anthropometric characteristics, maximal oxygen uptake, fasting glucose, fasting insulin, and homeostatic model assessment of insulin resistance in adolescents with different obesity classes, mean ± SD

Variable	Class I obesity, *n* = 71 (35.5%)	Class II obesity, *n* = 79 (39.5%)	Class III obesity, *n* = 50 (25.0%)	[1]*P*
Height, cm	166.65 ± 9.49	168.11 ± 10.10	167.32 ± 8.64	0.646
Weight, kg	88.51 ± 15.29	99.38 ± 18.59	106.52 ± 16.64	< 0.001
WC, cm	102.42 ± 10.05	107.00 ± 16.82	110.39 ± 18.37	0.017
WC/height	0.61 ± 0.05	0.64 ± 0.10	0.66 ± 0.11	0.019
BMI, kg/m^2	30.65 ± 2.29	34.34 ± 2.54	41.02 ± 4.44	< 0.001
BMI *z*-score	1.97 ± 0.19	2.35 ± 0.15	2.68 ± 0.24	< 0.001
VO_2max, L/min	2.55 ± 0.44	2.66 ± 20.54	2.77 ± 0.53	0.064
VO_2max, mL/min/kg TBW	29.68 ± 3.53	27.69 ± 3.83	23.95 ± 3.60	< 0.001
VO_2max, mL/min/kg FFM	46.48 ± 4.46	45.38 ± 5.90	43.63 ± 6.71	0.003
Fasting glucose, mmol/L	4.99 ± 0.41	4.99 ± 0.47	5.05 ± 0.38	0.708
Fasting insulin, mU/L	25.00 ± 12.80	28.45 ± 16.22	37.31 ± 21.11	< 0.001
HOMA-IR	5.78 ± 3.09	6.31 ± 3.68	8.49 ± 5.23	0.001

[1]ANOVA/Kruskal-Wallis, Scheffe post hoc test.

BMI: Body mass index; FFM: Fat free mass; HOMA-IR: Homeostatic model assessment of insulin resistance; SD: Standard deviation; TBW: Total body weight; VO_2max: Maximal oxygen uptake; WC: Waist circumference.

Previous research revealed positive association between BMI and HOMA-IR in adults and in children[21,22]. In this study, adolescents with class I and class II obesity had lower HOMA-IR than adolescents with class III obesity, which is in line with already published data showing that HOMA-IR rose linearly throughout the whole spectrum of BMI from underweight to severely obese children[13].

Although obesity and increased proportion of body fat are strongly associated with cardiometabolic risk, some individuals with excess body fat have HOMA-IR in the normal range and no metabolic abnormalities[23]. Factors responsible for preserved insulin sensitivity are not clear, but could be related to their lifestyle and alterations in adipose tissue biology. The results from a meta-analysis with pooled data from 15 studies found that CRF, assessed as VO_2max, was higher in obese people without than in obese people with metabolic abnormalities[24].

Scaling of VO_2max by TBW leads to a considerable underestimation of CRF in obese individuals[25]. Some authors suggest lean body mass to be the strongest determinant of VO_2max, while fat mass does not significantly affect VO_2max after adjustment for lean mass[26]. To eliminate the confounding factor of adiposity, it is recommended to express CRF in relation to FFM[27]. However, body composition analysis is not routinely available nor performed in everyday practice. Therefore, in the present study, CRF was expressed in both ways, scaled by TBW and FFM.

Table 3 Association of cardiorespiratory fitness level scaled by total body weight and obesity category with homeostatic model assessment of insulin resistance

CRF level	Mildly obese			Severely obese			*F*	*P*
	Mean	SD	*n*	Mean	SD	*n*		
Poor CRF_{TBW}	6.12	2.97	8	8.51	5.36	58	Main effect CRF_{TBW}, F (2200) = 1.249	0.289
Intermediate CRF_{TBW}	7.18	3.67	26	5.82	3.04	42	Main effect obesity, F (2200) = 1.746	0.188
Good CRF_{TBW}	4.72	2.23	37	6.37	3.41	29	Interaction CRF_{TBW} and obesity, F (2200) = 3.292	0.039

CRF_{TBW}: Cardiorespiratory fitness scaled by total body weight; SD: Standard deviation.

Table 4 Association of cardiorespiratory fitness level scaled by fat free mass and obesity category with homeostatic model assessment of insulin resistance

CRF level	Mildly obese			Severely obese			*F*	*P*
	Mean	SD	*n*	Mean	SD	*n*		
Poor CRF_{FFM}	7.35	3.20	13	7.91	5.65	54	Main effect CRF_{FFM}, F (2200) = 2.027	0.135
Intermediate CRF_{FFM}	6.12	3.77	30	7.04	3.23	37	Main effect obesity, F (2200) = 3.846	0.051
Good CRF_{FFM}	4.68	1.58	28	6.19	3.33	38	Interaction CRF_{FFM} and obesity, F (2200) = 0.167	0.846

CRF_{FFM}: Cardiorespiratory fitness scaled by fat free mass; SD: Standard deviation.

When otherwise healthy obese children and adolescents and their peers with appropriate BMI were compared, despite being expressed in relation to lean mass, CRF was still significantly lower in the obese group[28]. According to our knowledge, there are no published data regarding the comparison of CRF between the groups of adolescents with different obesity categories. In this study adolescents from the class I obesity group had, in comparison with subjects from class III obesity group, significantly higher values of both CRF_{TBW} and CRF_{FFM}.

Also, in the entire study population, a weak negative correlation of HOMA-IR with CRF_{TBW} and CRF_{FFM} was found. Similar results were obtained in several studies. In a cross-sectional, multi-ethnic study, which included 1445 children aged 9 years to 10 years, a negative association between CRF and HOMA-IR was established. After adjustment for fat mass index the association still remained statistically significant. The adjustment for FFM index did not further reduce the negative association between CRF and HOMA-IR[29]. In 1710 children with an average age of 11.4 years ± 2.4 years, VO_2 max expressed in relation to lean and total body mass were correlated with HOMA-IR as follows: r = -0.076, P < 0.002; r = -0.264, P < 0.001[27]. However, somewhat different results were obtained from a study including 452 children aged 6 years to 8 years. CRF expressed in relation to TBW was negatively associated with HOMA-IR, while CRF appropriately controlled for body size and composition using lean mass was not related to HOMA-IR[30]. It is worth mentioning that participants included in our study were, in comparison with subjects from all the aforementioned studies, older and exclusively pubertal.

Although the association between CRF and insulin resistance is weak, it is not negligible. Prospective, longitudinal studies indicate a negative association of CRF in childhood with fasting insulin levels and HOMA-IR in adulthood, suggesting that CRF during adolescence is important for preserving insulin sensitivity in later life. A prospective study, which followed 317 adolescents from the age of 15 years for up to a maximum of 12 years, showed that CRF and isometric muscle strength in adolescence are negatively related to fasting insulin and HOMA-IR in young adulthood, regardless of obesity[31]. In a study with more than 2000 involved subjects, CRF and muscle fitness in children aged 7 years to 15 years were negatively associated with fasting insulin and HOMA-IR 20 years later. The association remained statistically significant after adjustment for childhood abdominal circumference[32].

To examine in more detail the association of CRF with HOMA-IR, participants were divided according to terciles of VO_2max into groups with poor, intermediate and good CRF_{TBW} and CRF_{FFM}.

The group of adolescents with poor CRF had a significantly higher HOMA-IR than the group of adolescents with good CRF, both for CRF_{TBW} and CRF_{FFM}. Also, HOMA-IR was higher in the group with poor, compared to the group with intermediate CRF_{TBW}. Other researchers came to similar results after the analysis of data collected for overweight or obese children (n = 115, average age 10.6 years ± 1.1 years, 54% girls), although they used a different protocol for CRF assessment. Namely, children with good CRF assessed by the beep test had lower HOMA-IR than children with poor CRF[33]. The results of our study are to the greatest extent comparable with the results of a large study which included 1710 children (mean age of 11.4 years ± 2.4 years; 920 normal-weight, 340 overweight and 450 obese). A progressive increase in HOMA-IR was found with decreasing CRF_{TBW}, while HOMA-IR scores remained similar between the groups with moderate and low CRF_{FFM}[27]. The stronger association between CRF_{TBW} and HOMA-IR is partially due to the

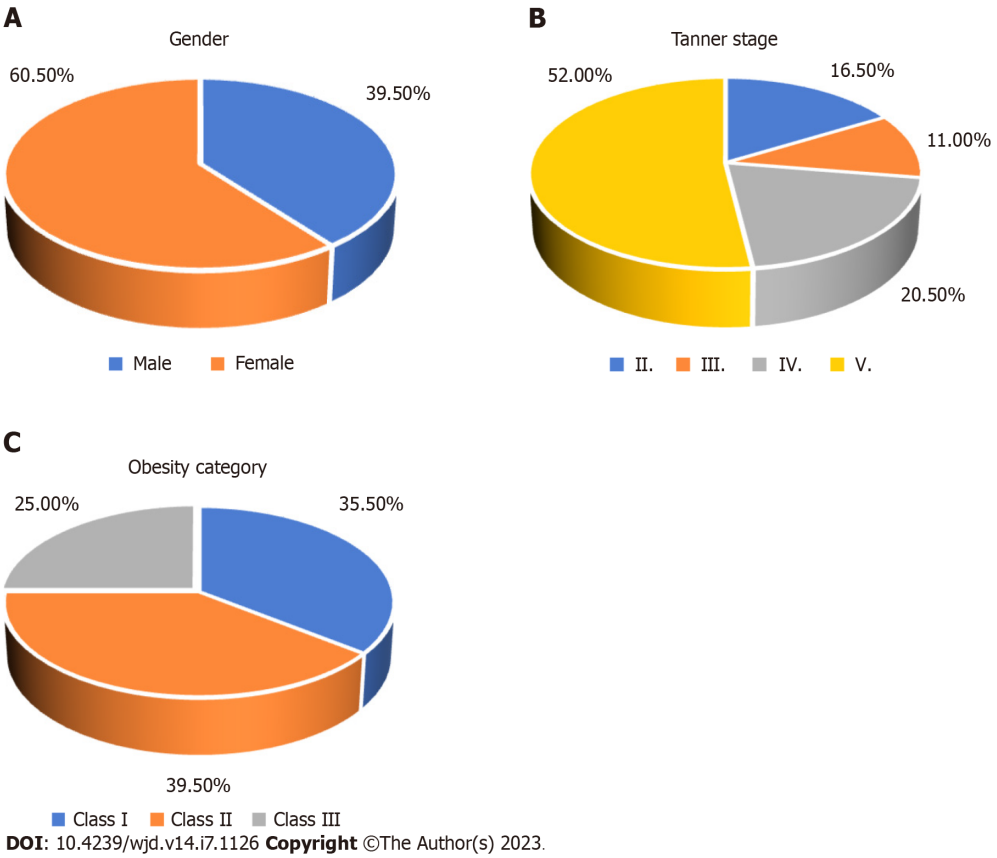

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Figure 1 Distribution of participants according to sex, stage of puberty, and obesity category. A: Sex; B: Stage of puberty; C: Obesity category.

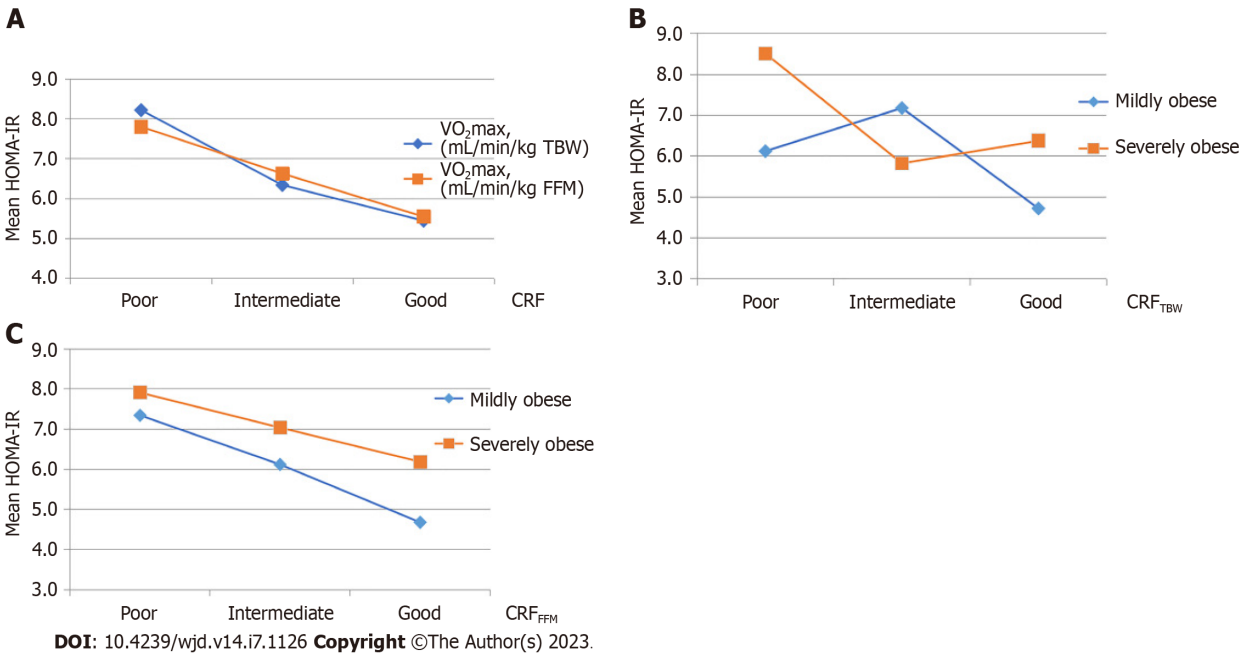

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Figure 2 Association of cardiorespiratory fitness level. A: Association of cardiorespiratory fitness (CRF) level with the homeostatic model assessment of insulin resistance in obese adolescents; B: Association of CRF scaled by total body weight (CRF_{TBW}) and obesity category with homeostatic model assessment of insulin resistance; C: Association of CRF scaled by fat free mass (CRF_{FFM}) and obesity category with homeostatic model assessment of insulin resistance (HOMA-IR).

significant association of obesity with HOMA-IR. The influence of obesity on HOMA-IR is however reduced if CRF is scaled by FFM, but still remains significant as shown in this study.

Although the number of severely obese youth continues to grow, studies that explore modifying factors for cardiometabolic risk and insulin resistance in such a group of adolescents are lacking. Therefore, the secondary goal of our study was to examine the association of CRF level with HOMA-IR in adolescents with different obesity categories, including those with severe obesity.

In this study, mildly obese participants had lower HOMA-IR than severely obese, both in the groups with good and poor CRF, regardless of scaling. HOMA-IR was the highest in severely obese adolescents with poor CRF. Interestingly, HOMA-IR of severely obese participants with good CRF did not differ significantly from HOMA-IR of mildly obese subjects with poor CRF. Therefore, it seems that CRF attenuates the adverse effects of obesity on insulin resistance. This is in line with findings of a pooled study suggesting that CRF may play an important role in lowering the risk of cardiometabolic diseases in obese children[13].

One of the main limitations of our study is its cross-sectional design, which makes it impossible to establish a causal relationship between CRF and insulin resistance. Also, body composition including FFM was assessed by BIA, but the hydration status could not be fully controlled for all the participants.

CONCLUSION

In obese adolescents, independent of obesity category, poor CRF is associated with the highest HOMA-IR. This highlights the need to include the assessment of CRF in routine diagnostic algorithm and to encourage lifestyle-based strategies, with special emphasis on CRF improvement in obese adolescents, including those with severe obesity. Further research is needed to determine which interventions should be implemented in obese youth with low CRF in order to achieve optimal cardiometabolic effects. Current recommendations include combined aerobic and resistance training, as well as high-intensity interval training.

ARTICLE HIGHLIGHTS

Research background

The global obesity epidemic, not sparing children and adolescents, is accompanied by rapid increase in the prevalence of cardiometabolic disorders. The association between obesity and insulin resistance is well established, along with the fact that insulin resistance represents a pivotal step in the progression towards prediabetes and type 2 diabetes. Obese adolescents who are in puberty should be regarded as a particularly vulnerable group for glucose metabolism dysregulation. Growing evidence supports the notion that young-onset type 2 diabetes has a more aggressive disease phenotype, leading to early development of complications, and adversely affecting quality of life and long-term outcomes. As more than half of the world's population is expected to be overweight or obese within the next decade, expanding the options to manage adolescent obesity is essential to treat the epidemic.

Research motivation

Cardiorespiratory fitness (CRF), referring to ability of the circulatory and respiratory systems to supply oxygen to skeletal muscle mitochondria for energy production during sustained physical activity has been associated with the insulin resistance, irrespective of body weight. In children and adolescents, CRF is an important marker of health which shows an inverse relationship with obesity, insulin resistance and cardiometabolic risk. Available data confirm that association between fatness and cardiometabolic risk scores could be partially decreased with improvements in fitness levels. It seems that early intervention and prevention strategies targeting youth CRF may be associated with reduced risk for obesity and cardiometabolic disease later in life.

Research objectives

To investigate the association between CRF and insulin resistance in obese adolescents, with special emphasis on severely obese adolescents.

Research methods

This was a prospective, cross-sectional study including 200 pubertal adolescents, 10 years to 18 years of age. According to body mass index (BMI), adolescents were classified as mildly obese (BMI 100% to 120% of the 95th percentile for age and sex) or severely obese (BMI ≥ 120% of the 95th percentile for age and sex or ≥ 35 kg/m^2, whichever was lower). Participant body composition was assessed by bioelectrical impedance analysis (BIA). A homeostatic model assessment of insulin resistance (HOMA-IR) was calculated. Maximal oxygen uptake (VO_2max) was determined from submaximal treadmill exercise test. CRF was expressed as VO_2max scaled by total body weight (mL/min/kg TBW) or by fat free mass (mL/min/kg FFM), and then categorized as poor, intermediate or good, according to VO_2max terciles. Data were analyzed by statistical software package SPSS (IBM SPSS Statistics for Windows, Version 24.0). P value < 0.05 was considered statistically significant.

Research results

We observed a weak negative correlation between CRF and HOMA-IR [Spearman's rank correlation coefficient (r_s) = -

0.28, $P < 0.01$ for CRF_{TBW}; (r_s) =-0.21, $P < 0.01$ for CRF_{FFM}]. A one-way analysis of variance (ANOVA) revealed a significant main effect of CRF on HOMA-IR [$F_{(2200)} = 6.840$, $P = 0.001$ for CRF_{TBW}; $F_{(2200)} = 3.883$, $P = 0.022$ for CRF_{FFM}]. Subsequent analyses showed that obese adolescents with poor CRF had higher HOMA-IR than obese adolescents with good CRF ($P = 0.001$ for CRF_{TBW}; $P = 0.018$ for CRF_{FFM}). Two-way ANOVA with Bonferroni correction confirmed significant effect of interaction of CRF level and obesity category on HOMA-IR [$F_{(2200)} = 3.292$, $P = 0.039$ for CRF_{TBW}]. Severely obese adolescents had higher HOMA-IR than mildly obese, with either good or poor CRF. However, HOMA-IR did not differ between severely obese adolescents with good and mildly obese adolescents with poor CRF.

Research conclusions

CRF is important determinant of insulin resistance in obese adolescents, regardless of obesity category. Therefore, CRF assessment should be a part of diagnostic procedure, and its' improvement should be a therapeutic goal.

Research perspectives

Large scale prospective studies are needed to expand the knowledge of CRF, IR, and cardiometabolic health. Also, determination of participants' body composition by using different methods (such as abdominal MR scans) would offer more precise insight into type and distribution of body fat.

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FOOTNOTES

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CASE REPORT

Maturity-onset diabetes of the young type 9 or latent autoimmune diabetes in adults: A case report and review of literature

Guang-Hong Zhou, Min Tao, Qing Wang, Xing-Yu Chen, Jing Liu, Li-Li Zhang

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Abstract

BACKGROUND

Maturity-onset diabetes of the young (MODY) is a monogenic genetic disease often clinically misdiagnosed as type 1 or type 2 diabetes. MODY type 9 (MODY9) is a rare subtype caused by mutations in the *PAX4* gene. Currently, there are limited reports on *PAX4*-MODY, and its clinical characteristics and treatments are still unclear. In this report, we described a Chinese patient with high autoimmune antibodies, hyperglycemia and a site mutation in the *PAX4* gene.

CASE SUMMARY

A 42-year-old obese woman suffered diabetes ketoacidosis after consuming substantial amounts of beverages. She had never had diabetes before, and no one in her family had it. However, her autoantibody tested positive, and she managed her blood glucose within the normal range for 6 mo through lifestyle interventions. Later, her blood glucose gradually increased. Next-generation sequencing and Sanger sequencing were performed on her family. The results revealed that she and her mother had a heterozygous mutation in the *PAX4* gene (c.314G>A, p.R105H), but her daughter did not. The patient is currently taking liraglutide (1.8 mg/d), and her blood glucose levels are under control. Previous cases were retrieved from PubMed to investigate the relationship between *PAX4* gene mutations and diabetes.

CONCLUSION

We reported the first case of a *PAX4* gene heterozygous mutation site (c.314G>A, p.R105H), which does not appear pathogenic to MODY9 but may facilitate the progression of latent autoimmune diabetes in adults.

Key Words: Maturity-onset diabetes of the young; *PAX4*; Latent autoimmune diabetes in adults; Type 1 diabetes; Case report

Core Tip: Maturity-onset diabetes of the young type 9 (MODY9), as a subtype of MODY caused by mutations in the *PAX4* gene, has been poorly reported, and its clinical features and treatments remain unclear. We reported a heterozygous mutation in the *PAX4* gene (c.314G>A, p.R105H) in a patient with latent autoimmune diabetes in adults (LADA). Based on the analysis of the cases indexed in PubMed, it is the first reported case of *PAX4* with LADA. The *PAX4* heterozygous mutation reported in the present case may not be considered for MODY9 and may be facilitated for the onset and progress of LADA.

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INTRODUCTION

Maturity-onset diabetes of the young (MODY) is a monogenic genetic disease inherited predominantly and is often associated with impaired pancreatic β cell function[1,2]. The prevalence in adults is estimated to be 1 in 10000 and in children to be 1 in 23000, accounting for 1%-3% of diabetes cases[3,4]. A definitive diagnosis of MODY relies on genetic testing. According to the Standard of Care for Diabetes proposed in 2022[5], children diagnosed with diabetes within 6 mo or children or young adults who do not have typical characteristics of type 1 or type 2 diabetes but have a family history of diabetes for several generations should have genetic testing for MODY. MODY is often misdiagnosed as type 1 or type 2 diabetes[6,7].

MODY is classified into subtypes based on genetic mutations; 14 gene mutations have been proven to cause MODY. The most common types are *HNF4A*, *GCK* and *HNF1A*[8]. MODY9 is a subtype caused by mutations in the *PAX4* gene. *PAX4* belongs to the paired cassette homology domain family primarily expressed in pancreatic islets and is a key factor in the normal differentiation of β cells and δ cells[9]. Inactivation of *PAX4* causes a lack of mature β and δ cells in the pancreas, resulting in the body's inability to produce sufficient insulin and growth inhibitory hormone[10]. Numerous studies have shown that *PAX4* can promote the differentiation of stem cells to β cells[11,12], promote β cell survival and proliferation[13,14], induce the conversion of mature α cells to β cells[15,16], regulate cell cycle proteins[17] and maintain endoplasmic reticulum integrity[18] and other pathways that play a crucial role in diabetes. Reports on the diagnosis of *PAX4* mutations are still controversial, and the clinical features and treatment of *PAX4*-related hyperglycemia have not been identified. Here, we reported a patient with high autoimmune antibodies and hyperglycemia with a novel site mutation in the *PAX4* gene.

CASE PRESENTATION

Chief complaints

A 42-year-old woman presented with xerostomia, polydipsia, polyuria and blurred vision for 4 d.

History of present illness

The patient experienced xerostomia, polydipsia and polyuria after consuming substantial amounts of beverages and fruits 4 d before admission to the local hospital. She also had blurred vision and fatigue. She went to the local hospital, where her lab results revealed that her fasting blood glucose (FBG) was 18.15 mmol/L, and her glycated hemoglobin (HbA1c) was 10.3%. She was then prescribed metformin and another oral drug (details unknown) to control her blood glucose. However, her symptoms were not relieved, and her FBG remained at 14.54 mmol/L at the time of admission.

History of past illness

The patient had a history of cesarean section 18 years prior to admission and had uterine fibroids for 12 years.

Personal and family history

The patient reported no knowledge of diabetes in her family.

Physical examination

The patient was sane, conscious and had dry lips. Her body mass index was 31.85 kg/m^2, and her blood pressure was 133/96 mmHg. She was generally in good condition, and no other obvious abnormality was detected at admission.

Laboratory examinations

At admission, the patient arterial pH was 7.29, PO_2 was 93 mmHg, bicarbonate was 14.6 mmol/L, FBG was 14.54 mmol/L, islet cell antibody was 45 times higher than normal, glutamic acid decarboxylase (GAD) was 200 times higher than normal, and insulin autoantibody was two times higher than normal. Her urine ketone was significantly positive. Her liver function was slightly abnormal, but her blood lipids, albumin/creatinine ratio and thyroid function were normal (Table 1).

Next-generation sequencing

The patient was tested with next-generation sequencing (DNBSEQ-T7) to detect 130 genes related to diabetes, which include 14 pathogenic genes associated with MODY (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, *APPL1*). The patient's mother and daughter also underwent Sanger validation. The findings revealed that she had a heterozygous mutation in the *PAX4* gene (c.314G>A, p.R105H), and subsequent Sanger validation revealed that her mother also suffered the same mutation. Her daughter was normal (Figure 1).

FINAL DIAGNOSIS

Diabetic ketoacidosis and type 1 diabetes mellitus (T1DM).

TREATMENT

The patient was given a fluid replacement and insulin treatment at admission until her arterial pH and urine ketone levels returned to normal. She was then administered a hypodermic injection of mixed protamine zinc recombinant human insulin injection (70/30), 8 IU before breakfast and 8 IU before dinner, and her FBG level was 6-7 mmol/L at discharge. She maintained lifestyle interventions (balanced diet and regular exercise 30 min/d). One month after discharge, the patient discontinued insulin therapy, and her blood glucose appeared to be normal with lifestyle interventions.

OUTCOME AND FOLLOW-UP

The patient visited our outpatient clinic regularly for check-ups. She also regularly tested capsular blood glucose at home, and the data showed her blood glucose was well controlled. About 3 mo after discharge, we administered an oral glucose tolerance test (OGTT) to evaluate her cell function. Her HbA1c was 6.2%, OGTT (fasting, 30 min, 1 h and 2 h) was 5.96 mmol/L, 12.44 mmol/L, 12.64 mmol/L and 8.33 mmol/L, respectively, oral glucose-insulin release test (fasting, 30 min, 1 h and 2 h) was 6.82 μU/mL, 35.97 μU/mL, 44.81 μU/mL and 56.74 μU/mL, respectively, and the autoantibodies of GAD were still higher than the upper limit. At the 9-mo follow-up, she informed us that her capsular blood glucose was always around 7 mmol/L or slightly higher; hence, we further scheduled an HbA1c and an OGTT test. Her HbA1c was 7.3%, OGTT (fasting, 30 min, 1 h and 2 h) was 8.88 mmol/L, 11.26 mmol/L, 15.72 mmol/L and 18.17 mmol/L, respectively, and oral glucose-insulin release test (fasting, 30 min, 1 h and 2 h) was 11.93 μU/mL, 18.26 μU/mL, 30.93 μU/mL and 33.13 μU/mL. Furthermore, her GAD was still higher than the upper limit (GAD ≥ 10.0 IU/mL). Considering her gradually increasing blood glucose and relatively remaining cell function, she was administered liraglutide 1.8 mg once a day. Her fasting blood glucose was 5-6 mmol/L, and her postprandial blood glucose was 6-8 mmol/L (Figure 2).

DISCUSSION

Here, we reported a rare case of diabetes with a heterozygous mutation in the *PAX4* gene (c.314G>A, p.R105H). The patient, a middle-aged obese woman, had no obvious diabetic syndrome until she consumed substantial amounts of beverages and fruits. Her HbA1c was 10.3%, indicating that her blood glucose was increased for at least 3 mo. Her high body mass index and insidious onset diabetes are characteristics of type 2 diabetes. However, the repeated high level of autoantibodies (GAD, islet cell antibody and insulin autoantibody) suggested the diagnosis of latent autoimmune diabetes in adults (LADA). Furthermore, this was further supported by her short remission time after lifestyle interventions (about 3-6 mo) and progressive declining cell function and increased blood glucose. We performed genetic testing to exclude other reasons for hyperglycemia. We found that the patient and her mother had a heterozygous mutation in the *PAX4* gene (c.314G>A, p.R105H), while her daughter did not. We then drew her family pedigree (Figure 3), which confirmed that the mutation was indeed heterozygous, and the mother carried the mutation but with normal blood glucose. Therefore, we concluded that the mutation might not be the primary cause of her hyperglycemia. So, we did not diagnose her with MODY. To the best of our knowledge, this is the first case of LADA combined with a heterozygous mutation in the *PAX4* gene.

MODY9 is the result of a *PAX4* mutation. However, few studies have reported MODY9 in detail. Here, we conducted a literature review of case reports of *PAX4* mutation. We searched the PubMed database with the terms "maturity-onset

Table 1 Clinical features and laboratory results of the patient

Parameter	Values
Age at onset (yr)	42
Weight (kg)	79.5
Height (cm)	158
BMI (kg/m^2)	31.85
FBG (mmol/L)	14.54
HbA1c (%)	10.3
pH	7.29
HCO_3^- (mmol/L)	14.6
ABE	16.6
SBE	16.7
ICA (COI)	45.20
GAD (IU/mL)	> 2000.00
IAA (COI)	2.10
KET (mmol/L)	+-
UA (μmol/L)	484.7
TG (mmol/L)	1.23
TC (mmol/L)	4.22
HDL (mmol/L)	1.01
LDL (mmol/L)	2.89
ALT (U/L)	44.8
AST (U/L)	40.4
ALP (U/L)	66.6
GGT (U/L)	34.0

ABE: Actual base excess; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; FBG: Fasting blood glucose; GAD: Glutamic acid decarboxylase; GGT: γ-glutamyl transpeptidase; HbA1c: Glycated hemoglobin; HCO_3^-: Bicarbonate; HDL: High-density lipoprotein cholesterol; IAA: Insulin autoantibodies; ICA: Islet cell autoantibodies; KET: Urinary ketones; LDL: Low-density lipoprotein cholesterol; SBE: Standard base excess; TC: Total cholesterol; TG: Triglyceride; UA: Uric acid.

diabetes of the young or MODY" and "paired cassette homology domain or *PAX4*" and selected the case reports, pedigree analyses, and cross-sectional studies. If the article was not related to MODY9 or *PAX4* gene mutations, or if the specifics of the patient were not described, it was excluded. Finally, nine articles with 17 cases were included[19-27] (Table 2).

Of these cases, 6 cases[19,22,24,25] with heterozygous *PAX4* mutation and 1 case[20] with homozygous *PAX4* mutation were diagnosed with MODY, indicating that both homozygous and heterozygous mutations were pathogenic. However, in our case, the patient's mother had normal blood glucose, possibly because the present site mutation had little pathogenic function, or the mother may progress to diabetes in the future and have longer follow-up needs. The above 6 cases with heterozygous mutations had a family history, while the patient with the homozygous mutation had no family history. Moreover, our case also had no family history. Therefore, it is difficult to determine whether diabetic family history is a characteristic of *PAX4* mutation.

Six cases[21,26] were diagnosed with ketosis-prone diabetes, two-thirds of them were homozygous mutation, all were male, and most of them had a family history. One Japanese case of homozygous mutation[23] was diagnosed with type 2 diabetes mellitus (T2DM), and three Japanese cases of homozygous mutation[27] were diagnosed with late-onset diabetes. All of these patients were lean and had no obvious sex and family history differences. Of the 17 cases, only 1 female case with the homozygous mutation had a slightly high level of positive insulin antibody but with a relatively low HbA1c. She was treated with an oral drug and no detailed follow-ups; that case was diagnosed with late-onset diabetes.

Although the c.314G>A mutation has been reported in the dbSNP database, there is no article reporting the specific clinical features of the patients with this mutation nor has it been reported that this mutation is related to LADA. Therefore, our case is significant since it is the first to be reported in China with a mutation site and a high level of autoimmune antibodies. It had a 1-year follow-up to assess the changes in cell function and the progression of the

Table 2 Articles describing the characteristics of clinical cases carrying the *PAX4* mutant gene

Ref.	Diagnosis	*PAX4* variant	Ethnicity	Family history	Diagnostic age (yr)	Sex	BMI (kg/m^2)	HbA1c, %	Insulin antibody, +/-	Treatment	HbA1c % at remission
Sujjitjoon *et al*[22]	MODY9	Heterozygous IVS7-1G>A	Thailand	Yes	44	Female	NA	NA	-	NA	NA
Chapla *et al* [25]	MODY	Heterozygous c.92G>T	Asian-Indian	Yes	14	Male	23	NA	-	Glimepiride and insulin	NA
Jo *et al*[19]	MODY	Heterozygous c.374-412 del 39	Japanese	Yes	15	Male	18.2	14.5	-	Insulin	7.4
Cho *et al* [20]	MODY	Homozygous c.575G>a	Korean	No	22	Male	25.3	13.8	NA	NA	NA
Abreu *et al* [24]	MODY	Heterozygous c.491G>A	Brazilian	Yes	32	Female	21.6	NA	-	Insulin	NA
			Brazilian	Yes	56	Female	29.48	11.3	-	Metformin and gliclazide	NA
			Brazilian	Yes	49	Female	23.61	6	-	Metformin	NA
Schmidt *et al*[21]	Ketosis-prone diabetes	Heterozygous c.109C>T	African	No	38	Male	28.4	> 14	-	Insulin	7.0
Mauvais-Jarvis *et al* [26]	Ketosis-prone diabetes	Homozygous R133W	West African	Yes	47	Male	29.1	13.8	-	Drugs	6.6
			West African	Yes	22	Male	18.5	12.2	-	Drugs	5.1
			West African	Yes	38	Male	28.3	14.1	-	Insulin	6.2
			West African	Yes	20	Male	26.5	12.5	-	Insulin	7.3
		Heterozygous R37W	West African	Yes	39	Male	30.4	11.6	-	Insulin	8.2
Kanatsuka *et al*[27]	Late-onset diabetic	Homozygous R121W	Japanese	Yes	37	Male	21.5	7.6	-	Insulin	NA
			Japanese	No	71	Male	22.8	7.1	-	Insulin	NA
			Japanese	Yes	71	Female	20.3	6.2	+	Drugs	NA
Shimajiri *et al*[23]	T2DM	Homozygous R121W	Japanese	No	29	Female	22.2	12.6	-	Insulin	7.3
Present case	T1DM	Heterozygous c.314G>A	Chinese	No	42	Female	31.85	10.3	+	Lifestyle control	7.3

BMI: Body mass index; MODY: Maturity-onset diabetes of the young; NA: Not available; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

disease.

The literature on the diagnosis of *PAX4* mutation with hyperglycemia was controversial. Of the above 17 cases, only 1 case was diagnosed with MODY9, 6 cases were diagnosed only as MODY, and the other cases were diagnosed with ketosis-prone diabetes, late-onset diabetes and T2DM. No case was diagnosed as LADA. While cross-sectional studies found *PAX4* gene mutations to be associated with T2DM or ketosis-prone diabetes[21,23,26], population-based studies from China[28], Finland, Hungary[29] and the United Kingdom[30] found no significant association between the *PAX4* gene and the risk of developing T1DM. After Biason-Lauber *et al*[31] proposed that the *PAX4* variant 1168C>A was associated with T1DM, Geng *et al*[32] rejected this point the same year. Mechanically, *PAX4* plays a crucial role in the normal differentiation of β cells[9], including promoting the differentiation of stem cells to β cells[11,12], converting mature α cells to β cells[15,16] and maintaining β cell survival and proliferation[13,14]. Therefore, in our case, we considered that the heterozygous mutation in the *PAX4* gene might facilitate cell function decline, which coupled with autoimmune antibody destruction accelerates the progression of diabetes. However, this hypothesis also depends on the outcome of her mother's follow-up.

According to the treatment, in cases with mutations in the *PAX4* gene, 9 patients were treated with insulin (52.9%) and 6 patients with oral medication (35.3%). Liraglutide, an incretin hormone that can increase glucose-stimulated insulin secretion, has also been demonstrated to promote β cell proliferation, reduce apoptosis[33,34] and improve β cell function in high-lipid environments by activating the PI3K/Akt pathway[35]. For obese T1DM patients, clinical trials have

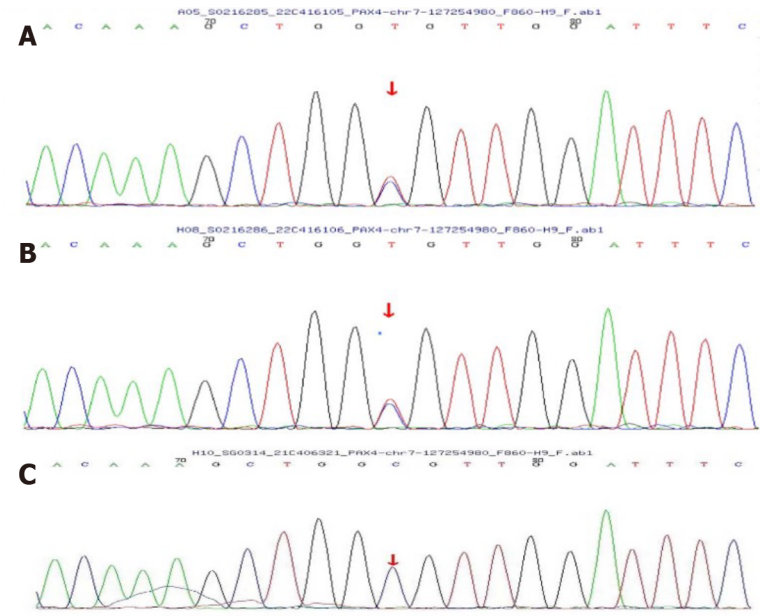

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Figure 1 Sequencing profile of exon 5 of *PAX4* in the mutation region (R105H). A: Sequencing result of the propositus; B: Sequencing result of the mother; C: Sequencing result of the daughter. The whole exome sequencing and Sanger sequencing verification showed the propositus and her mother had the heterozygous variant of *PAX4*, c.314G>A; p.R105H, and the daughter was normal.

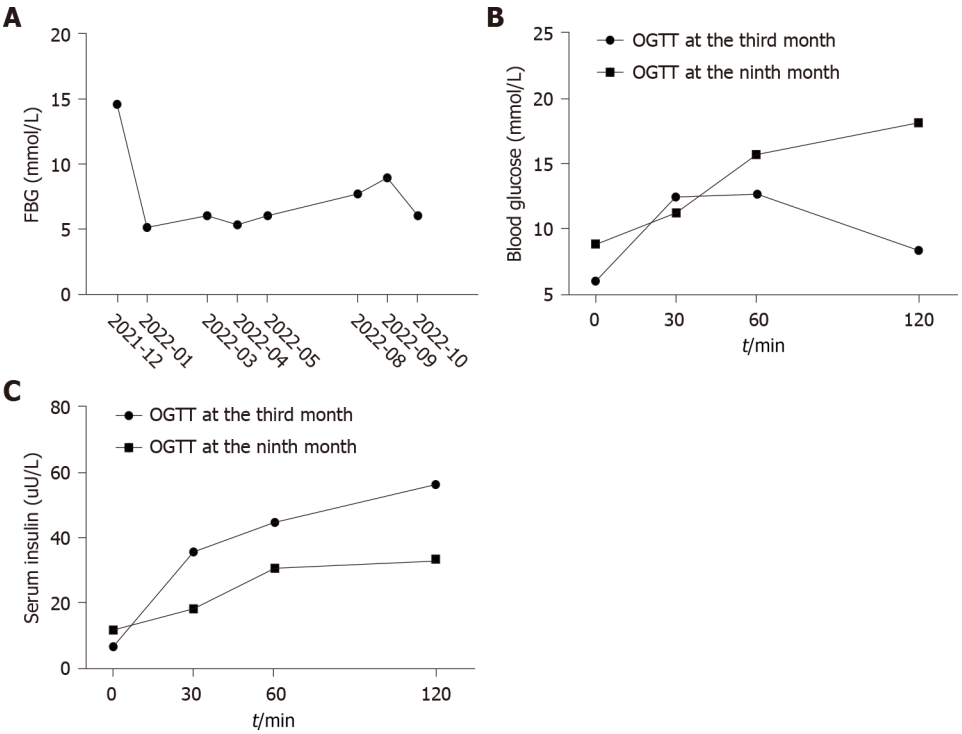

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Figure 2 Changes of blood glucose and serum insulin during the follow-up. A: Fasting blood glucose levels from onset to follow-up; B: Oral glucose tolerance test levels during follow-up; C: Oral glucose-insulin release test levels during follow-up. FBG: Fasting blood glucose; OGTT: Oral glucose tolerance test; OGIRT: Oral glucose-insulin release test.

demonstrated that liraglutide can improve blood glucose, stimulate lipid oxidation and increase thermogenesis while maintaining lean body mass[36]. In T1DM patients with residual islet function, adjuvant therapy with liraglutide has also been proven to reduce HbA1c levels, reduce insulin requirements and increase C-peptide levels[37-39]. We finally added liraglutide to control blood glucose levels and was effectively controlling the patient's glucose levels at the last follow-up.

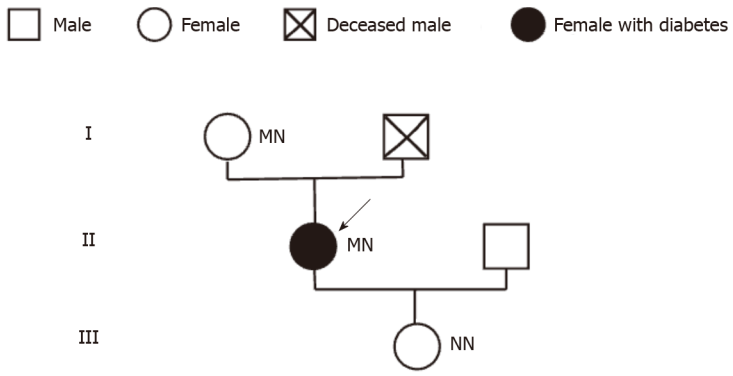

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Figure 3 Family pedigree of the patient. To the right of the symbol, it shows the genotype of *PAX4* c.314G>A mutation. M: Mutant allele; N: Normal allele.

CONCLUSION

In this report, we discovered a heterozygous mutation in *PAX4* (c.314G>A, p.R105H) that can coexist with LADA and does not appear pathogenic to MODY9 but may facilitate the progression of LADA. Further functional experiments are needed to confirm this in future.

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FOOTNOTES

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