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EDITORIAL

Considerations for management of patients with diabetes mellitus and acute COVID-19

Efterpi Mougakou, Maria Kyziroglou, Alexandra Tsankof, Evangelos Cholongitas, Konstantinos Tziomalos

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Abstract

Diabetes mellitus (DM) is an independent risk factor for admission to intensive care unit and death in patients with coronavirus disease 2019 (COVID-19). On the other hand, medications used in the management of COVID-19 are potentially associated with increases in blood glucose levels and a higher incidence of infections. Accordingly, care of patients with DM and acute COVID-19 requires careful consideration of both diseases. Hyperglycemia and hypoglycemia are associated with adverse outcomes and therefore frequent measurement of blood glucose levels and a basal-bolus insulin regimen are required in most patients. Regarding the management of COVID-19, dexamethasone increases blood glucose levels and might also increase the risk for infections. On the other hand, limited data suggest that antiviral and immunomodulatory agents used in COVID-19 are not strongly associated with higher incidence of infections in this population. As knowledge evolves in this field, optimization of the management of both DM and COVID-19 will hopefully improve the outcome of these patients.

Key Words: Diabetes mellitus; COVID-19; Insulin; Antidiabetic agents; Dexamethasone; Tocilizumab; Remdesivir

Core Tip: Diabetes mellitus is a frequent comorbidity in patients hospitalized with coronavirus disease 2019 and is associated with adverse outcomes. Strict glycemic control using insulin is necessary in most of these patients. Dexamethasone, antiviral agents and immunomodulation are also frequently administered and require vigilance and careful monitoring for adverse effects, particularly infections.

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INTRODUCTION

Several studies showed that diabetes mellitus (DM) is an independent risk factor for contracting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19)[1,2]. Moreover, DM is associated with longer hospitalization, increased risk for admission to an intensive care unit (ICU) and higher mortality in patients with COVID-19[1-3]. Elderly patients and those with poor glycemic control and comorbidities, including hypertension and established cardiovascular disease (CVD), are at higher risk for adverse outcomes[1-3]. In addition, patients with DM and COVID-19 appear to have higher risk for acute complications of DM, particularly diabetic ketoacidosis (DKA)[4]. On the other hand, SARS-CoV-2-induced insulin resistance and impaired insulin production, stress and dexamethasone, which is frequently used for the management of COVID-19, often cause substantial increases in blood glucose levels[5-7]. Furthermore, immunomodulatory agents, which are also part of the treatment of COVID-19, might increase the risk for infection, which is higher in patients with DM[8,9]. Therefore, the management of patients with both DM and COVID-19 requires special considerations, which are briefly summarized in the present commentary.

BLOOD GLUCOSE GOALS

In patients with DM and COVID-19, both hyperglycemia and hypoglycemia have been associated with worse outcome[10,11]. Therefore, maintaining a strict glycemic control in this population appears to be of critical importance. Blood glucose levels between 110 and 180 mg/dL have been recommended as targets in hospitalized diabetic patients with COVID-19, aiming at the higher end of range[12]. However, this target should be individualized, blood glucose levels up to 220 mg/dL are considered acceptable and glucose control should be less strict in patients at high risk for hypoglycemia, including the elderly, the underweight, and patients with severe COVID-19 and/or renal impairment[12].

MONITORING OF BLOOD GLUCOSE LEVELS

Glucose measurement should be performed at least 4 times per day, before meals and at bedtime, but in certain cases has to be done more frequently, particularly in patients who are not eating or are receiving parenteral nutrition[12]. Continuous blood glucose monitoring devices can also be used, particularly in ICU, and appear to be feasible, accurate and reduce the need for point of care glucose measurements [13]. Given the increased risk for DKA in patients with DM and COVID-19, blood ketone levels should ideally be measured in all diabetic patients at admission[12].

ANTIDIABETIC TREATMENT

Regarding antidiabetic treatment, insulin is the agent of choice in most patients. In those who are already receiving long-acting basal insulin, this should be continued[12]. If the patient is not on long-acting insulin and has ≥ 2 blood glucose measurements > 220 mg/dL within the previous day, basal insulin should be started at a total daily dose of 0.25 units/kg[12]. However, in elderly or frail patients and in those with impaired kidney function, the total daily dose of basal insulin should be lower (approximately 0.15 units/kg)[12]. In patients who are receiving glucocorticoids, the dose of basal insulin should be increased by 20%-40%, depending on blood glucose levels[8]. Basal insulin dose are then titrated once-daily according to blood glucose levels, the severity of COVID-19 and caloric intake [12]. Regarding rapid-acting insulin, corrective doses should be administered in patients with blood

glucose levels > 220 mg/dL and the dose should depend on glucose levels and either on total daily dose (in patients who were already using insulin) or on body weight (in patients naïve to insulin)[12]. In critically ill patients and in those who cannot eat, insulin should be administered intravenously[12]. Notably, sliding scale insulin and premixed insulin have been associated with higher risk for iatrogenic hypoglycemia and are not recommended[14]. Patients with type 1 DM can be treated with either subcutaneous or intravenous insulin, depending on their clinical condition. Insulin is administered intravenously at a rate between 1-5 units/h whereas in patients who cannot eat, glucose-dextrose solutions are preferred to avoid hypoglycemia[12]. Regarding patients on insulin pump therapy, this can be maintained provided that their clinical status is stable[12].

Regarding the use of oral antidiabetic agents, metformin should be stopped at admission but if and when the risk of lactic acidosis is considered low, it should be restarted since it appears to improve the outcome of COVID-19[12,15,16]. If used, the dose of metformin should be reduced in patients with estimated glomerular filtration rate (eGFR) between 30 and 45 mL/min and should be discontinued in patients with eGFR < 30 mL/min, liver failure, high risk for lactic acidosis and before iodine contrast imaging[17]. Sulfonylureas are not recommended because of reduced efficacy due to COVD-19-related impaired insulin production and increased insulin resistance and also due to the risk for hypoglycemia, particularly in elderly and in patients with renal impairment or poor oral intake[12]. However, emerging data suggest that these agents might also reduce mortality risk in diabetic patients with COVID-19[18]. Sodium-glucose cotransporter-2 inhibitors should also be discontinued in hospitalized patients, particularly in severely ill patients, due their association with euglycemic DKA[12]. Thiazolidinediones are also not recommended due to their association with edema and heart failure exacerbation, especially in patients with severe COVID-19 and hemodynamic instability[19]. In contrast, dipeptidyl peptidase-4 (DPP-4) inhibitors could be used alone or in combination with insulin in patients with mild hypoglycemia; however, they should be avoided in critically ill patients due to their association with increased risk for heart failure[12]. Notably, some studies suggested that continued use of DPP-4 inhibitors after hospitalization was associated with a decrease in mortality compared with discontinuation but others did not confirm this finding[18,20]. Finally, glucagon-like peptide-1 receptor agonists should also be stopped in hemodynamically unstable and severely ill patients due to risk of gastrointestinal side effects[12].

MANAGEMENT OF COVID-19

Regarding the management of COVID-19, in patients who require supplemental oxygen or ventilatory support, low-dose dexamethasone (6 mg daily for 10 d or until discharge) is recommended, according to data suggesting a clear benefit on all-cause 28-day mortality[21-23]. Indeed, in the controlled, open-label RECOVERY trial (n = 2104 patients assigned to receive dexamethasone and 4321 to receive usual care), the 28-day mortality was 36% lower in the dexamethasone group among patients on mechanical ventilation and 18% lower among those on supplemental oxygen[23]. Of note, 24% of the total study population had DM and no excess serious adverse events related to dexamethasone were recorded[23]. The incidence of death due to infections other than COVID-19 also did not differ between patients treated with dexamethasone and those assigned to usual care[23]. According to a meta-analysis by the World Health Organization Rapid Evidence Appraisal for COVID-19 Therapies Working Group, which included 7 trials in 1703 critically ill patients with COVID-19, administration of glucocorticoids was associated with 34% lower 28-d mortality with no suggestion of a higher risk of adverse effects compared with standard of care or placebo[22]. Despite these reassuring findings, patients with diabetes receiving glucocorticoids should be carefully monitored for bacterial or fungal infections, with prompt initiation of empirical antibiotic treatment if needed[8].

In patients with COVID-19 who require supplemental oxygen, but not in those on mechanical ventilation or extracorporeal membrane oxygenation, the antiviral agent remdesivir (200 mg intravenously on day 1 followed by 100 mg/d for 5 d) should be considered because it shortens recovery time and shows a trend for reduced need for mechanical ventilation and improved survival[21,24,25]. In a trial in 1062 patients hospitalized with COVID-19 pneumonia randomized to receive remdesivir or placebo (30.6% with DM), hyperglycemia was a common non-serious adverse effect, occurring in 6% of patients, but with a similar incidence in the remdesivir and the placebo group[24]. The rate of infections was also similar in the 2 groups[24]. Remdesivir can also be considered in hospitalized patients without requirement for supplemental oxygen. In a randomized, open-label trial (n = 584 patients with moderate COVID-19, defined as any radiographic evidence of pulmonary infiltrates and oxygen saturation > 94% on room air), clinical status at day 11 was better in patients randomized to a 5-d course of remdesivir compared with standard care whereas the incidence of adverse events was similar in the 2 groups[26]. Of note, 40% of patients enrolled in this trial had DM but it was not evaluated whether the benefits and risks of remdesivir differed between this subgroup and non-diabetic patients[26].

Immunomodulatory agents can also be considered in diabetic patients who are hospitalized due to COVID-19. Tocilizumab, an interleukin-6 inhibitor (8 mg/kg as a single intravenous dose), may be used

Table 1 Principles of the management of patients with diabetes mellitus and acute coronavirus disease 2019

	Principles of the management
Blood glucose goals	Between 110 and 180 mg/dL in most patients. Less strict goals in patients at high risk for hypoglycemia
Monitoring of blood glucose levels	At least 4 times daily. More frequently in selected patients (*e.g.*, in the intensive care unit)
Antidiabetic treatment	Insulin in most patients. Metformin and dipeptidyl peptidase-4 inhibitors might be considered. Other antidiabetic agents should be avoided
Management of COVID-19 in hospitalized patients	Similar to non-diabetic patients. Patients receiving glucocorticoids or immunomodulatory agents should be carefully monitored for infections
Management of COVID-19 in the outpatient setting	Patients with symptomatic COVID-19 are eligible for treatment with monoclonal antibodies, remdesivir, nirmatrelvir-ritonavir or molnupiravir

COVID-19: Coronavirus disease 2019.

in patients who require high-flow oxygen or mechanical ventilation and it may also be an option for selected patients on low-flow oxygen with significantly elevated inflammatory markers (C-reactive protein levels ≥ 75 mg/L) or with a rapid increase in oxygen requirements despite dexamethasone therapy, within 96 h of hospitalization[25]. In a meta-analysis of 10930 patients hospitalized for COVID-19, administration of tocilizumab was associated with a 17% lower all-cause 28-d mortality with no increased risk of infection compared with standard of care or placebo[27]. Sarilumab may be an alternative interleukin-6 inhibitor option if tocilizumab is not available, but with limited trial data[27]. Another treatment option is baricitinib (4 mg/day orally for 14 d), a Janus Kinase inhibitor with immunomodulatory properties, that may be used with the same indications as tocilizumab, with the exception of patients on mechanical ventilation due to limited trial data in this subgroup of patients[25]. In a randomized, placebo-controlled trial in 1525 patients (30% had DM), baricitinib reduced 28-d and 60-d mortality by 38% without an increased risk for infection or other adverse events[28]. Notably, it has not been evaluated whether these immunomodulatory agents have different safety or efficacy in patients with DM[27,28].

Notably, patients with acute COVID-19 are at higher risk for thrombosis than the general inpatient population and the presence of DM further increases this risk[29]. Accordingly, this population should be carefully monitored for the occurrence of thrombotic events and should receive prophylactic dose of heparin[21,25]. In patients who are already receiving antiplatelet agents for DM or for established CVD, these should be continued and low-dose heparin should be added[21,25].

Regarding the outpatient management of COVID-19, even in the absence of symptoms of severe disease, both patients with type 1 and 2 DM are considered at high risk for evolution to severe disease, especially if they are ≥ 65 years-old or have obesity, chronic kidney disease or established CVD[30]. Therefore, patients with DM and symptomatic COVID-19 are eligible for treatment with monoclonal antibodies, remdesivir, nirmatrelvir-ritonavir or molnupiravir, to reduce the risk of hospitalization[21,25]. The choice between these agents depends mainly on availability and should start as soon as possible after symptom onset[21,25]. There is no specific agent that is contraindicated in patients with DM, however nirmatrelvir-ritonavir cannot be used if eGFR is < 30 mL/min[21,25]. Moreover, before prescription of ritonavir-boosted nirmatrelvir, a careful review of concomitant medications is required, because it has significant drug-drug interactions with commonly prescribed medications in patients with DM and CVD, including rosuvastatin, clopidogrel and rivaroxaban[21,25].

Patients with DM also appear to be at higher risk for persistence of COVID-19-related symptoms (*i.e.*, long COVID)[31]. It has also been reported that an aggravation of insulin resistance persists for up to 2 mo after recovery from COVID-19[32]. Accordingly, patients with DM should be followed up closely after the resolution of COVID-19.

CONCLUSION

Diabetic patients with acute COVID-19 are a particularly vulnerable population at a high risk for complications. Close monitoring of blood glucose levels and careful administration of insulin with appropriate titration are needed to achieve glycemic control without complications. On the other hand, management of COVID-19 in these patients requires individualization and heightened attention for the occurrence of adverse events, particularly hyperglycemia and infections (Table 1). There are currently limited data regarding the safety and efficacy of both antidiabetic and antiviral treatments in diabetic patients with acute COVID-19. As knowledge evolves in this field, optimization of the management of both DM and COVID-19 will hopefully improve the outcome of these patients.

FOOTNOTES

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REVIEW

Growing importance of urogenital candidiasis in individuals with diabetes: A narrative review

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Abstract

Both diabetes and fungal infections contribute significantly to the global disease burden, with increasing trends seen in most developed and developing countries during recent decades. This is reflected in urogenital infections caused by *Candida* species that are becoming ever more pervasive in diabetic patients, particularly those that present with unsatisfactory glycemic control. In addition, a relatively new group of anti-hyperglycemic drugs, known as sodium glucose cotransporter 2 inhibitors, has been linked with an increased risk for colonization of the urogenital region with *Candida* spp., which can subsequently lead to an infectious process. In this review paper, we have highlighted notable virulence factors of *Candida* species (with an emphasis on *Candida albicans*) and shown how the interplay of many pathophysiological factors can give rise to vulvovaginal candidiasis, potentially complicated with recurrences and dire pregnancy outcomes. We have also addressed an increased risk of candiduria and urinary tract infections caused by species of *Candida* in females and males with diabetes, further highlighting possible complications such as emphysematous cystitis as well as the risk for the development of balanitis and balanoposthitis in (primarily uncircumcised) males. With a steadily increasing global burden of diabetes, urogenital mycotic infections will undoubtedly become more prevalent in the future; hence, there is a need for an evidence-based approach from both clinical and public health perspectives.

Key Words: Balanitis; Balanoposthitis; Candida; Candidiasis; Diabetes; Pregnancy; Urogenital infections; Vulvovaginitis

Core Tip: The global health burden of both diabetes and *Candida* spp. infections is on the rise, and these two clinical entities can have a compounding effect on the development of different urogenital diseases and syndromes. Pathophysiological changes observed in diabetes mellitus can predispose individuals to *Candida* colonization, increased virulence of this fungus, and subsequent infection. Diabetic females are more prone to recurrent vulvovaginal candidiasis that can endanger the pregnancy, while diabetic males have higher rates of balanitis/balanoposthitis. In both females and males, there is an increased risk of candiduria and urinary tract infections, with complications such as emphysematous cystitis.

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INTRODUCTION

Diabetes is a salient global health issue, with an enormous disease burden that has increased substantially in recent decades for the majority of developed and developing countries. The estimations from the International Diabetes Federation reveal that 537 million adults are living with diabetes around the world, with a projected growth to 693 million or more by 2045 without effective preventative methods [1,2]. On the other hand, the estimates from the Global Action Fund for Fungal Infections show that every year there are over 300 million individuals of all ages suffering from a fungal infection that can seriously impact their health[3], which also includes urogenital infections caused by yeasts belonging to the genus *Candida*.

Taking into account such considerable global prevalence of these two frequently coexistent clinical conditions, it is of no wonder that diabetic patients with genitourinary candidiasis are currently pervasive not only in primary practice but also in secondary and tertiary care facilities. In addition, a relatively new group of anti-hyperglycemic drugs known as sodium glucose cotransporter 2 (SGLT2) inhibitors made both females and males more prone to *Candida* colonization of the urogenital region as well as for subsequent infection[4-6]. All of this means urogenital *Candida* infections may become even more ubiquitous among diabetic patients in the future. Therefore, given the scarcity of recent and comprehensive sources that provide an integrative and critical overview of the available literature on this topic (Table 1), in this review we aimed to summarize microbiological, pathophysiological, and clinical facets of urogenital infections with *Candida* species in both females and males with diabetes.

DIABETES AND IMMUNE RESPONSE AGAINST INFECTIONS

According to the classification published by the American Diabetes Association, diabetes occurs in four basic forms, of which diabetes mellitus type 1 and diabetes mellitus type 2 are the most common forms of the disease[7]. In the quotidian clinical approach, fasting blood glucose levels up to 5.6 mmol/L are normal. When these values are above 7 mmol/L, this represents a key criterion for diagnosing diabetes mellitus, while values between 5.6 mmol/L and 6.9 mmol/L indicate prediabetes[8]. Therefore, it is always necessary to perform two glucose measurements: the first one on an empty stomach; and the second 1-2 h after a meal. Glucose values 2 h after a meal should fall below 7.8 mmol/L; if these values are still above 11 mmol/L, then we can diagnose diabetes mellitus with a substantial amount of certainty. If these values are between 7.8 to 11 mmol/L, we consider prediabetes or glucose intolerance [9]. The vital difference between prediabetes and diabetes is that prediabetes can be reversed. Of course, the most crucial factors are lifestyle changes, but there are also several viable pharmacological approaches.

Type 1 diabetes mellitus is caused by an absolute (or almost absolute) lack of insulin due to autoimmune destruction of pancreatic β-cells, which leads to insulin insufficiency and hyperglycemia [10]. Conversely, type 2 diabetes mellitus is characterized by insulin resistance with an inadequately compensatory increase in insulin secretion[11]. Gestational diabetes occurs in pregnancy, most often during the second trimester of pregnancy. Insulin resistance is potentiated by hormones produced by

Table 1 Keywords, database, and search time

Keywords	MeSH term	Database
Balanitis	Balanitis	PubMed, Scopus, RCA
Balanoposthitis	-	PubMed, Scopus, RCA
Vulvovaginitis	Vulvovaginitis	PubMed, Scopus, RCA
Urogenital infections	Urogenital system; infections; pathogenicity	PubMed, Scopus, RCA
Pregnancy	Pregnancy	PubMed, Scopus, RCA
Candida	Candida	PubMed, Scopus, RCA
Candidiasis	Candidiasis	PubMed, Scopus, RCA
Diabetes	Diabetes mellitus; diabetes insipidus	PubMed, Scopus, RCA

RCA: Reference Citation Analysis.

the placenta[12]; therefore, it occurs in females whose pancreatic function does not overcome pregnancy-related insulin resistance. The main consequences are increased risks of preeclampsia, macrosomia, as well as Cesarean delivery and their associated morbidities[13].

Diabetes mellitus is one of the most common endocrine disorders characterized by a disorder in insulin secretion and its action. Due to its frequency, it is currently a global health problem[14]. The prevalence of diabetes mellitus is constantly increasing in developed and developing countries alike. According to the data from 2017, its prevalence is around 8.8% worldwide[15]. In addition to a myriad of co-occurring problems characteristic of patients with diabetes mellitus, a particular issue is immune system dysfunction resulting from complex interactions between the endocrine and immune systems [16]. Immune dysfunction occurs due to elevated insulin levels (hyperglycemia) and leptin present in affected individuals, resulting in an increased risk of various organ damage[17].

Decreased immunity is manifested in decreased T lymphocyte count, reduced cytokine release, increased programmed leukocyte cell death, reduced neutrophil function, impaired ability to fight infectious agents, and increased susceptibility to infection[14]. The increased risk of opportunistic infections is a particular problem due to the weakened ability to fight invasive pathogens[18]. In patients with diabetes mellitus, the recovery time after infection is significantly prolonged compared to individuals without it[19]. One of the salient indicators that should raise a suspicion of underlying diabetes is a propensity for recurrent infections caused by opportunistic pathogenic fungal species belonging to the genus *Candida*[20]. The pathogenic abilities of *Candida* species and their colonization factors depend on host-related immune factors due to the intricate homeostatic relationship of fungi with the host's current immune status, a key determinant of commensalism or parasitism[21]. From a pathophysiological perspective, we find a suitable environment in diabetic patients for *Candida* multiplication and proliferation due to alteration of gut microbiota, dietary changes, reduced intestinal secretions and altered liver function, continued usage of antimicrobial agents (and other drugs), coexisting diseases, as well as the pervasive deficiency of key nutrients, as demonstrated in the literature [21].

CANDIDA AS A PARAMOUNT FUNGAL PATHOGEN

The profile of Candida albicans and non-albicans Candida species

Fungal infections caused by *Candida* species lead to a significant health burden, causing high mortality rates, hospitalizations, and increased treatment costs[22]. Lethal outcomes are most commonly seen as a result of sepsis and invasive systemic candidiasis[23].

Candida albicans was the most widespread fungal pathogen isolated during episodes of candidiasis for a long time. Still, recent literature reports reveal an increasingly important role of other non-*albicans* species such as *Candida glabrata* (*C. glabrata*), *Candida parapsilosis* (*C. parapsilosis*), *Candida krusei* (*C. krusei*), *Candida tropicalis* (*C. tropicalis*), and more recently *Candida auris* (*C. auris*)[24]. However, the most commonly isolated *Candida* spp. from clinical specimens are non-*albicans* species. These other non-*albicans Candida* species are becoming more noticeable due to the production of virulence factors that were once attributed exclusively to *C. albicans*; furthermore, they are also characterized by reduced sensitivity to the most commonly used antifungal drugs[25]. The prevalence and virulence of non-*albicans Candida* species show varied geographical distribution, but more importantly many non-*albicans Candida* species cause more frequent fungal infections in patients with diabetes. That is especially pertinent for patients with type 1 and 2 diabetes mellitus with foot ulcers and skin and nail lesions[6].

Considering all of the above, species-level identification of *Candida* spp. should be introduced into routine laboratory work-up[26].

But notwithstanding such global prominence of non-*albicans* candida, *C. albicans* is still the most common cause of candidiasis[27]. It can be a colonizer of skin and many mucosal surfaces and can thus easily act as an opportunistic pathogen in the genitourinary system[28]. Approximately 75% of females have at least one episode of vulvovaginal candidiasis during their lifetime, and the most common cause (*i.e.* in 90% of cases) the putative species is *C. albicans*[29]. According to available data, in females with diabetes mellitus who presented with a vulvovaginal infection caused by *Candida*, *C. albicans* is the most prevalent fungus in over 50% of cases, while different non-*albicans Candida* species are present in about 40% of cases[30].

Candida is a polymorphic fungus that, contingent on the environment in which it is located, can alter its morphology from yeast form (blastoconidia) to pseudohyphae and hyphae[31]. Indeed, this is one of the most important differences from other *Candida* species because it can create true hyphae *in vivo* when met with favorable conditions[32]. Two serotypes of *C. albicans* have been identified, namely type A and type B[33], and numerous factors contribute to the noticeable increase in invasive fungal infections, including hyperglycemia[19].

Major virulence factors in C. albicans

Virulence represents the ability of a microorganism to damage a host[34], and *C. albicans* possesses a panoply of virulence factors[35]. One of the most important factors is dimorphism (already mentioned), which represents the ability of *C. albicans* to change its shape from yeast to mold, with subsequent formation of true hyphae under favorable conditions. The latter trait significantly increases its invasiveness and proteolytic activity; however, in yeast form, it shows the propensity for greater dissemination[36]. Genes that are important for these activities are *ALS3*, *SAP4-6*, *HWP1*, *HYR1*, and *ECE1*, and their expression can be variable[37], while *SAP1* and *SAP3* and *SAP8* genes have been correlated with vaginal infections[38].

In the first phase of the infection, which is the adhesion phase, adhesins and invasins allow *C. albicans* cells to adhere to the substrate, forming a basal layer of cells[39]. Adhesins are glycoproteins that enable yeast to adhere to epithelial and endothelial cells[40]. Invasins are specialized proteins by which *C. albicans* stimulates host cells towards endocytosis by binding to host cell ligands[41]. The target ligands are E-cadherin on epithelial cells and N-cadherin on endothelial cells[42]. Numerous genes are involved in adhesion to epithelial cells, and the large cell surface area of the glycoprotein encodes eight genes belonging to the *C. albicans* agglutinin-like sequence family[43].

Biofilm production is recognized as a crucial virulence factor (Table 2)[44]. In the proliferation stage of *C. albicans* cells, filaments are formed, in which yeast cells begin to develop filamentous hyphae. That is the most critical step in which cells can change their morphology, facilitating in turn biofilm formation on the mucosal surfaces of the host[45,46]. The biofilm formation process is controlled by six genes (*EFG1, BCR1, BRG1, NDT80, TEC1,* and *ROB1*) that belong to the transcriptional regulatory network[47,48].

Alongside the aforementioned virulence factors, it is also becoming clear that *C. albicans* isolated from patients with diabetes mellitus has more pronounced pathogenic properties[49]. Namely, the hyperglycemic environment, rich in carbohydrates, serves as a source of energy indispensable for producing biofilms and matrices that protect fungal cells from external influences[6]. Most pathological conditions caused by *C. albicans* are associated with biofilm formation on abiotic surfaces or host surfaces[50]. Yeast cells dispersed from mature biofilm are more virulent and have a more remarkable ability to adhere to surfaces to form new biofilms than planktonic ones[51]. Biofilm production also complicates treatment and contributes to high morbidity and mortality rates[52].

C. albicans can produce the cytolytic enzyme known as candidalizine, and hyphae are responsible for its secretion[44]. This enzyme plays a vital role in developing vaginal mucosal infections[53]. More specifically, candidalizine has immunomodulatory properties critical in host cell damage[54] and plays a role in neutrophil recruitment during disseminated systemic fungal infections[55].

A direct contribution to the virulence of *C. albicans* is the secretion of hydrolytic enzymes aspartyl proteinase and phospholipase as well as hemolysin, which all enhance pathogenic effects such as binding to host tissue and rupture of the cell membrane. As a result of their activity, the invasion of the mucosal surface is facilitated, and they are also responsible for avoiding the host's immune response[46,56,57]. In *C. albicans*, at least ten members of the aspartyl proteinase gene family are present, while phospholipase has been reported in four families[58].

Finally, one of the essential contributors to *C. albicans* virulence is thigmotropism (contact sensing), which is regulated by extracellular calcium intake and aids significantly in spreading into host tissues and biofilm development[44].

Table 2 Biofilm production process

Phase	Phase name	Description
1	Adherence	In the first 3 h, individual *C. albicans* cells adhere to the substrate, which forms the basal layer of the biofilm
2	Intermediate phase	In 11-14 h, biofilm is shaped during this phase of cell proliferation and filamentation, in which the formation of hyphae occurs, marking the beginning of true biofilm formation
3	Maturation phase	In 20-48 h, there is a complete penetration of all layers of cells attached to the surface; extracellular polysaccharide matrix accumulates at this stage of maturation
4	Dispersion	After 24 h, the final phase involves separation of non-adherent cells from the biofilm, resulting in possible development of new biofilms and dissemination in the tissue

C. albicans: *Candida albicans*.

TYPES OF UROGENITAL CANDIDIASIS IN PATIENTS WITH DIABETES

Vulvovaginal candidiasis in females with diabetes

Several important pathophysiological mechanisms are involved in the occurrence of vulvovaginitis and vulvovaginal candidiasis (VVC) in individuals with uncontrolled hyperglycemia, leading to increased glucose levels in vaginal mucosa[6]. First of all, yeasts can utilize the glucose found in secretions as a viable nutrient, and additional influence of the overall change in pH and temperature can result in increased *Candida* spp. virulence[59]. Furthermore, the binding of *Candida* spp. to epithelial cells on the vaginal surface represents a pivotal initial step in colonization and ensuing infection with yeasts[60], with an indispensable role of intercellular adhesion molecule 1 expression for facilitating adhesion after the episodes of hyperglycemia[61]. Recurrent episodes of VVC are more frequent in diabetic patients due to immune suppression, altered leukocyte function, and a myriad of other factors[21].

Several different author groups appraised the association between VVC and diabetes mellitus. For example, Gunther *et al*[30] studied females with diabetes from Brazil and found that *Candida* species were more frequently isolated in them than in those without it (18.8% *vs* 11.8%); likewise, the development of VVC (both isolated and recurrent forms) has been more frequently observed in the diabetic group of patients, together with lower cure rates. In a study on postmenopausal females with diabetes and symptoms of VVC, *Candida* spp. were isolated in 15.6% of involved patients using culturing techniques and molecular confirmation with *C. albicans* leading the way in frequency (59.30%), followed by C. *glabrata* (24.41%) and *C. krusei* (16.27%)[62]. These studies also showed different antifungal susceptibilities of isolated species, which is why mycological culture is often endorsed, even though microscopy is often sufficient for visualizing recognizable fungal elements such as pseudohyphae of *C. albicans* (Figure 1).

However, non-*albicans Candida* species are increasingly implicated in VVC in cases of patients with diabetes. In a research endeavor by Ray *et al*[63], which explored cure rates of different treatment modalities, *C. glabrata* has been cultured in 61.3% and *C. albicans* in 28.8% of 111 female individuals with VVC and diabetes. A study by Goswami *et al*[64], conducted on diabetic females from India, showed a relatively high prevalence (46%) of VVC with a relative risk of 2.45 and a predominance of *C. glabrata* and *C. tropicalis*. Such dominance of *C. glabrata* in the same context was confirmed by another study from India[65], showing that all therapeutic considerations have to consider country- and region-specific pathogen distribution (Table 3).

The problem is further aggravated with the use of relatively novel hypoglycemic agents that are known to induce glycosuria, and this specifically pertains to SGLT2 inhibitors. More specifically, the colonization rate with *Candida* spp. (and subsequently the risk of VVC) can increase substantially with the use of these agents, reaching up to 37%[4,5]. Another important issue is selecting the optimal treatment approach in females with recurrent VVC and diabetes, especially since many author groups recommend routine prophylactic administration of antimicrobial drugs in preventing candidiasis when faced with uncontrolled diabetes[6,21]. The best approach is still a matter of debate, as even a recent and comprehensive Cochrane review on different pharmacological and non-pharmacological treatment modalities highlighted that more research is necessary to ascertain the optimal medication choices as well as dose and frequency for females with diabetes[66].

Balanitis/balanoposthitis due to Candida spp. in males with diabetes

The influence of diabetes on the development of balanitis/balanoposthitis caused by *Candida* spp. is well known due to the well-established association of uncontrolled blood glucose levels and the proliferation of *Candida* beneath the prepuce[67]. While *Candida* is a causative agent of less than 20% of all balanoposthitis cases, it is the most commonly observed pathogen in males with diabetes, habitually presenting as a pruritic rash with sores, erosions, or papules (with possible sub-preputial discharge)[68]. In addition, coinfection with other pathogens can worsen the clinical presentation in males with

Table 3 Studies of the prevalence of candidiasis in individuals with diabetics

Ref.	Yr	Study population	Study outcome
Goswami *et al* [64]	2000	*n* = 78 diabetics, *n* = 88 non-diabetics	A total of 46% of diabetic patients showed vaginal *Candida* sp. and 23% healthy subjects demonstrated *Candida* spp.
Goswami *et al* [65]	2006	*n* = 85 diabetics, *n* = 62 non-diabetics	A total of 67.1% of diabetic patients showed vaginal *Candida* spp. and 47.3% healthy subjects demonstrated *Candida* spp. following fluconazole treatment
Gunther *et al*[30]	2014	*n* = 48 diabetics; *n* = 669 non-diabetics	A total of 18.8% of diabetics showed vaginal *Candida* spp. and 11.8% healthy subjects demonstrated *Candida* spp.
Yokoyama *et al* [5]	2019	65 diabetic patients	A total of 36.9% of diabetic patients converted to a positive vaginal *Candida* spp.
Halteet *et al*[62]	2020	550 diabetic patients	A total of 15.6% of diabetics showed vaginal *Candida* spp.
Lisboa *et al*[71]	2010	*n* = 38 diabetics; *n* = 440 non-diabetics	A total of 26.2% of males had *Candida* spp. and 18% of males had balanitis; 13.8% of diabetic patients had balanitis
Kofteridis *et al* [84]	2009	*n* = 88 diabetics; *n* = 118 non-diabetics	A total of 12.7% of diabetic patients showed urinary tract *Candida* spp. and 1.7% healthy subjects demonstrated *Candida* spp.
Yismaw *et al*[90]	2013	422 diabetic patients; *n* = 387 with asymptomatic UTI; *n* = 35 with symptomatic UTI	A total of 17.1% of symptomatic diabetic patients showed significant candiduria and 7.5% of asymptomatic diabetic patients
Falahati *et al*[91]	2016	305 diabetic patients	A total of 12.5% of diabetic patients were positive for candiduria
Esmailzadeh *et al*[89]	2018	400 diabetic patients	A total of 10% of diabetic patients showed *Candida* spp. in the urinary tract
Gharanfoli *et al* [92]	2019	500 patients with UTI; *n* = 106 diabetics; *n* = 394 non-diabetics	A total of 21.1% of diabetic patients showed *Candida* sp. in urinary tract and 4.2% of UTI patients were positive for *Candida* spp.

UTI: Urinary tract infections.

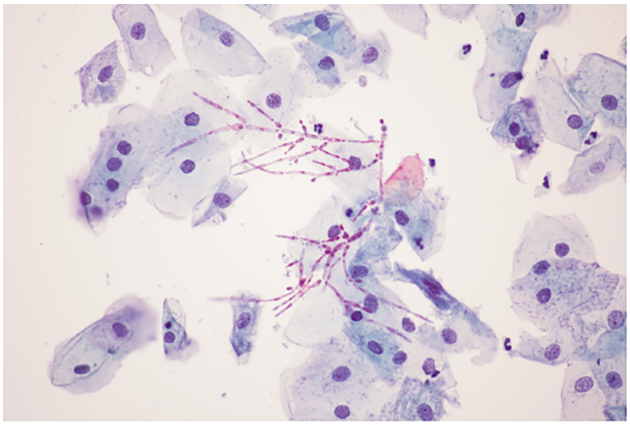

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Figure 1 Filamentous growth of *Candida albicans* in a vaginal specimen, with visible pseudohyphae and hyphae (magnification × 400).

diabetes (not only with common sexually transmitted infections but also pathogens such as *Streptococcus pyogenes*[69]).

The aforementioned connection between diabetes and penile infection is reflected in population studies as well; for example, the appraisal of all male patients with balanoposthitis from the Longitudinal Health Insurance Database in Taiwan revealed that the incidence of type 2 diabetes mellitus was higher in the balanoposthitis cohort than those without it, with a hazard ratio of 2.55 after age and comorbidity adjustments[70]. Furthermore, a large study from Portugal demonstrated that diabetes mellitus was significantly more prevalent in patients with clinically frank balanitis when compared to the asymptomatic group, and there was also higher colonization with *Candida* species[71]. In addition, an extensive survey of dermatology specialists from across India, with more than 60000 outpatients in their care, showed that up to 75% of individuals with *Candida* balanoposthitis were known cases of diabetes mellitus[72].

Even novel hypoglycemic agents that can induce glycosuria, most notably already mentioned SGLT2 inhibitors, can also increase the risk of genital candidiasis in males (Figure 2). For example, a recent

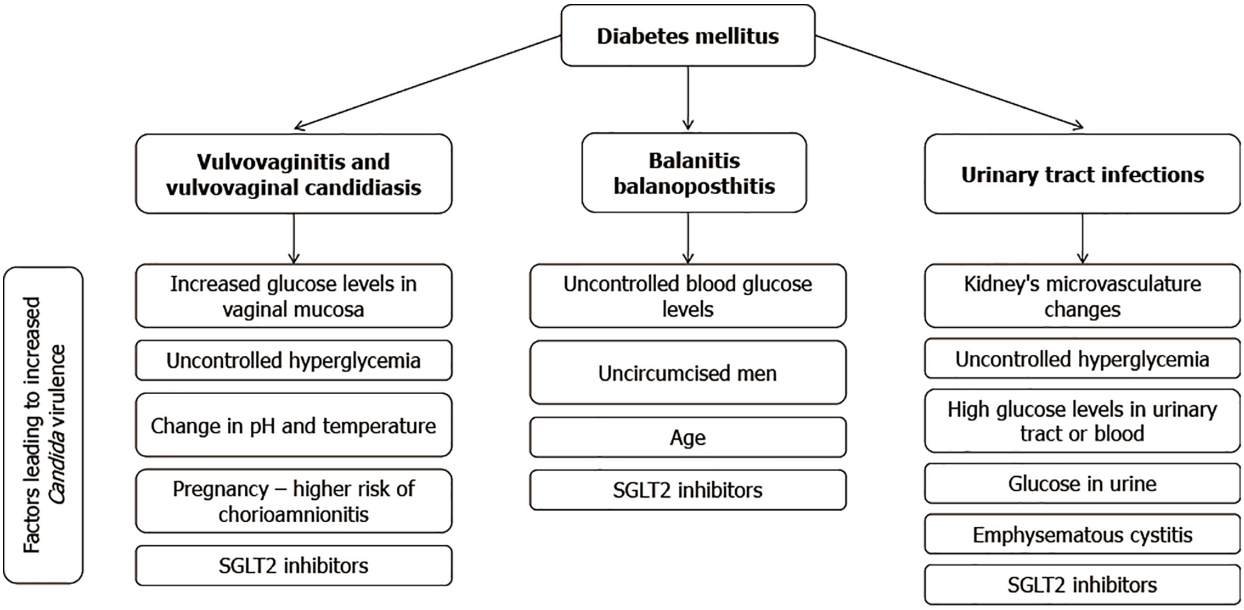

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Figure 2 A summary of factors leading to urogenital candidiasis in patients with diabetes mellitus. SGLT2: Sodium glucose cotransporter 2.

report by Bartolo *et al*[73] showed the development of balanitis due to *C. albicans* and subsequent candidemia but also the potential role of other species such as *C. glabrata*. A severe form of balanoposthitis caused by *C. albicans* after treatment with SGLT2 was also described in a 57-year-old with type 2 diabetes coupled with oral candidiasis[74]. Of note, balanitis is rarely seen in circumcised males, as the moist space beneath the foreskin represents an ideal environment for facilitated yeast proliferation[4].

The compounding effect of diabetes and genital candidiasis in pregnancy

The observed incidence of VVC during pregnancy is approximately 15%[75], but this percentage is even higher in pregnant females with either type 1/type 2 diabetes mellitus or gestational diabetes[76]. This means both pregnancy as a physiological process and diabetes as a pathological condition may have a compounding effect in the development of VVC (Figure 2). In a study on 251 pregnant females from Poland, Nowakowska *et al*[77] demonstrated a four times increased risk of developing vaginal mycosis in those with type 1 diabetes mellitus as well as a two times increased risk in those with gestational diabetes in comparison with healthy controls. A study on pregnant females from a Malaysian tertiary-care hospital showed that the first and second trimester of pregnancy and diabetes mellitus are significant risk factors for developing VVC[78]. A prospective study from China showed a significantly higher frequency of VVC in females with gestational diabetes (22.6% *vs* 9.7%)[76].

This is important due to the possible development of candida chorioamnionitis in diabetic pregnant females stemming from VVC, with potentially detrimental consequences for the unborn child. Although this clinical entity is relatively uncommon, it was repeatedly described in the medical literature. One of the gravest examples is a case reported by Obermair *et al*[79] on *Candida* chorioamnionitis that successively led to a late stillbirth in a pregnant woman with gestational diabetes mellitus. Unfortunately, there were no prior obstetrics procedures in this case, and infection with *C. albicans* triggered an inflammatory cascade that resulted in the occlusion of umbilical cord blood vessels, ultimately resulting in fetal death[79].

Recently, Shazniza Shaaya *et al*[80] reported 2 cases of *Candida* chorioamnionitis linked to gestational diabetes and originating from VVC where manifold red and yellowish spots were observed during pathohistological observation on the superficial area of the umbilical cord. Microscopically, these spots were microabscesses laden with yeasts and pseudohyphae, while peripheral funisitis was highlighted as a prominent feature of such *Candida* chorioamnionitis. Other reported cases of *Candida* chorioamnionitis associated with diabetes mellitus also led to adverse perinatal outcomes such as preterm birth, neonatal sepsis due to *C. tropicalis*, and the death of one twin as an unfortunate outcome of twin pregnancy[81-83]. The imputable role of diabetes mellitus in the development of *Candida* chorioamnionitis after VVC (with potentially serious sequelae for the fetus) cannot be overstated.

Candidiasis in the urinary tract of diabetic patients

Urinary tract infections (UTI) are much more common in individuals with diabetes, and this is also valid for potential complications such as emphysematous cystitis, pyelonephritis, and kidney abscesses[84, 85]. Furthermore, type 2 diabetes mellitus is a well-recognized risk factor for both community- and

healthcare-associated acquired UTIs, but UTIs are linked to catheterization and following renal transplantation. In all of these scenarios, different *Candida* species have a prominent role[6,86]. In addition, in patients with diabetes, the duration of disease and poor glycoregulation in the long run lead to changes in the kidney's microvasculature and frequent polyuria/glycosuria, which can predispose them to more frequent urinary tract infections[87].

Delineating candiduria from frank UTI is still a controversial topic, as there are no steadfast laboratory criteria. Furthermore, *Candida* is a recognized commensal of the urogenital tract. Therefore, its presence in the urine sample adds ambiguity to making a definitive diagnosis of *Candida* UTI[88]. A further issue is that candiduria by itself may be the sole indicator of invasive candidiasis, with potentially serious outcomes (particularly in immunocompromised patients)[88]. In any case, the prevalence of candiduria in individuals with type 2 diabetes mellitus ranges between 2.27% and 30.00% in studies conducted worldwide, with notably higher rates in females[89] (Table 3).

A study from Ethiopia found significant candiduria in 7.5% of asymptomatic and 17.1% of symptomatic patients presenting with diabetes, with *C. albicans*, *C. glabrata,* and *C. tropicalis* being the most commonly implicated species[90]. In one study by Falahati *et al*[91] from Iran, uncontrolled diabetes, increased fasting blood sugar levels, and glucose in urine were all significantly related with candiduria, with the most frequent species being *C. glabrata* and *C. albicans* in 50.0% and 31.6% of cases, respectively, followed by *C. krusei*, *C. tropicalis*, and *C. kefyr*. This was corroborated by another study from Iran, where the candiduria rate was also high in individuals with type 2 diabetes mellitus that presented with inadequate blood glucose control[89]. The most frequently isolated species in the latter study was *C. albicans* (47.5%), followed by *C. glabrata* (37.5%), *C. kefyr* (10.0%), and *C. krusei* (5.0%)[89]. A recent study from Iran on 1450 urine samples highlighted diabetes as the most frequent risk factor for the development of candiduria and the three most common species as *C. albicans*, *C. glabrata,* and *C. tropicalis*[92].

Emphysematous cystitis is a rare complication that is almost exclusively seen in diabetic patients, while fungal microorganisms are seldom implicated in its pathogenesis[6]. Still, uncontrolled diabetes is viewed as a major risk factor for an increasing role of *Candid*a species (especially non-*albicans* species) in this specific pathology. Wang *et al*[93] described the case of a 53-year-old man with diabetes from China who presented with two rare and concomitant complications of *C. tropicalis* infection in the urinary tract: a discrete mass known as a fungus ball and emphysematous cystitis. Another study from the United States presented a case of a 49-year-old male with diabetes and emphysematous pyelitis caused by *C. tropicalis*, where early diagnosis and treatment led to a favorable outcome[94].

Treatment of candidiasis in patients with diabetes

Antifungal therapy is often not justified, even in UTIs caused by different types of *Candida*[95]. The assumption is that people with predisposing factors (*e.g.*, diabetes) should first be treated, which may in turn resolve the infection[96]. For individuals who have symptomatic UTIs caused by *Candida* spp. and when it is assumed that predisposing factors have been eliminated or at least kept to a minimum, the use of fluconazole is recommended due to the possibility of achieving high concentrations in urine. It can be administered orally, 200-400 mg daily, in a single dose for 14 d. Exceptions are infections caused by *C. krusei* and *C. glabrata*, where amphotericin B deoxycholate is often used (due to inadequate urine concentrations of other azole antifungals and echinocandins)[95]. In instances of resistant *Candida* spp. or in high-risk patients, amphotericin B is given intravenously at a dose of 0.3 to 0.6 mg/kg per day in the case of cystitis and given intravenously in a dose of 0.5 -0.7 mg/kg in the case of pyelonephritis[97]. In the case of resistant pyelonephritis, 25 mg/kg of flucytosine is added orally four times a day. The standard treatment regimen is 2 wk. The patient's kidney function should be taken into account[98]. The use of flucytosine, although very effective in the eradication of *Candida* spp., requires extra caution due to the toxicity it possesses[98]. If used alone, resistance to it occurs very quickly, and therefore therapy is not carried out longer than 7-10 d. Also, the drug is administered every 6 h at a dose of 25 mg/kg[99]. It is important to note that the recurrences of infections caused by *Candida* spp. are very common[100].

CONCLUSION

In conclusion, there is an increasing body of evidence that shows how patients with diabetes (particularly those characterized by unsatisfactorily controlled glycemia) are vulnerable to urogenital mycotic infections with *C. albicans* and other non-*albicans Candida* species of increasing importance. We have highlighted virulence factors of *C. albicans* and shown how the interplay of many pathophysiological factors can give rise to VVC with increased risk of recurrent episodes and dire pregnancy outcomes. There is also an increased risk of candiduria and UTI development caused by species of *Candida* in females and males alike (with the possibility of further complications such as emphysematous cystitis) as well as balanitis and balanoposthitis in (primarily uncircumcised) males. With a steadily increasing global burden of diabetes, these clinical conditions will undoubtedly become more prevalent in the future. All of this underscores the importance of establishing and preserving euglycemia, alongside any introduced antifungal treatment approaches, if our end-goal is to successfully manage urogenital

candidiasis in affected individuals with diabetes. Moreover, in order to minimize this high burden of yeast infections in individuals with diabetes, it is pivotal to identify those at high risk for developing type 2 diabetes mellitus and forestall the rise of complications; consequently, many lifestyle interventions (such as dietary changes, exercise, and weight reduction) have a much better impact than pharmacologic treatment. If the condition arises and the patient is faced with urogenital *Candida* infections, an early and appropriate treatment regimen should be introduced, especially to avoid several complicated conditions, which we have described.

FOOTNOTES

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REVIEW

Everything real about unreal artificial intelligence in diabetic retinopathy and in ocular pathologies

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Abstract

Artificial Intelligence is a multidisciplinary field with the aim of building platforms that can make machines act, perceive, reason intelligently and whose goal is to automate activities that presently require human intelligence. From the cornea to the retina, artificial intelligence (AI) is expected to help ophthalmologists diagnose and treat ocular diseases. In ophthalmology, computerized analytics are being viewed as efficient and more objective ways to interpret the series of images and come to a conclusion. AI can be used to diagnose and grade diabetic retinopathy, glaucoma, age-related macular degeneration, cataracts, IOL power calculation, retinopathy of prematurity and keratoconus. This review article intends to discuss various aspects of artificial intelligence in ophthalmology.

Key Words: Artificial intelligence; Diabetic retinopathy; Deep learning; Machine learning; Ophthalmology

Core Tip: It is said that necessity is the mother of all inventions and converging global trends make multiplying eye care efficiency an increasingly urgent necessity. Artificial intelligence refers to an artificial creation of human-like intelligence of computer machines that can learn, reason, plan, perceive or process natural language.

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INTRODUCTION

Artificial intelligence (AI) refers to a machine's ability to mimic human cognitive functions, such as learning, reasoning, problem-solving, knowledge representation, social intelligence and general intelligence. It represents a significant advance in computer science and enables doing tasks using a computer with little human mind involvement following human training. AI was developed in the 1940s, but major advances ensued during the 1990s with significant improvements in machine learning, multi-agent planning, case-based reasoning, scheduling, data mining, natural language understanding and translation, vision, virtual reality, games, *etc*. Researchers have created an algorithm that can guess whether patients with cardiovascular diseases have lived or died based on their condition within a year. The algorithm could predict patient survival in 85% of cases based on data obtained by measuring the heart's electrical activity using electrocardiography. The rapid development in AI technology requires physicians and computer scientists to have a good mutual understanding of the technology and the medical practice to improve medical care. This review article presents the role of AI in various fields of ophthalmology.

METHODOLOGY

We searched highly cited articles in PubMed, Scopus database, Google Scholar, Web of Science, Cochrane library and Embase database on Artificial - Intelligence in Diabetic - Retinopathy, Age-related macular degeneration, Glaucoma, Keratoconus, Cataract, Dry Eye and other common ocular diseases published between the year 2000 to 2021. We also used Reference Citation Tool (RCA) for searching the keywords and articles were ranked based on the "Impact Index Per Article." The latest highlighted articles were selected for review. Only articles published in English were considered and the rest were rejected.

ARTIFICIAL INTELLIGENCE BASICS

Machine learning

Machine learning (ML) is a core AI branch that aims to provide computers with the ability to learn without being explicitly programmed[1]. ML focuses on developing algorithms that can analyze data and make predictions[2] (Figure 1).

Deep learning

Deep learning (DL) differs from ML in that DL uses neural networks for making predictions and decisions. These neural networks were inspired by the biological neural networks of animal brains. They use the statistical probability principle derived from large data volumes to learn how to improve their accuracy, making DL a valuable tool for aiding physicians in clinical practice.

Generative adversarial network

Generative adversarial networks (GANs) are paired neural networks used for unsupervised ML. They can generate images or other data for the discriminative neural network to evaluate the data and provide feedback to aid the learning process[3].

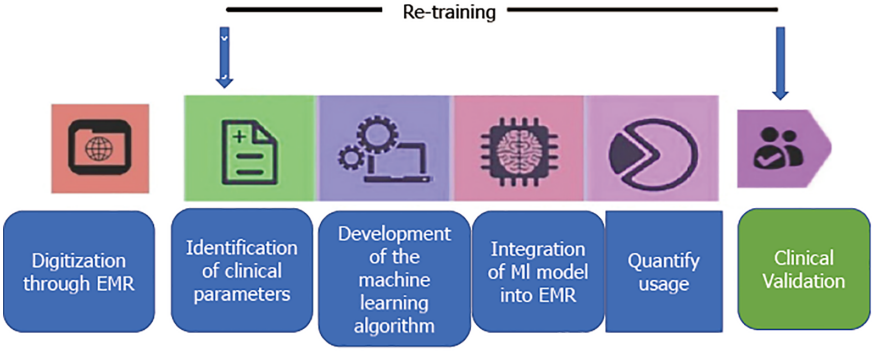

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Figure 1 Steps of machine learning algorithms that forms to analyze data and make predictions.

ARTIFICIAL INTELLIGENCE PLATFORMS

Algorithms resemble the AI software, whereas platforms resemble the computer hardware in which algorithms are installed and work to predict and make decisions. AI platforms simulate cognitive functions of the human mind including learning, reasoning, problem-solving, social intelligence and general intelligence[4].

Top Artificial intelligence platforms

The top AI platforms include Google, Microsoft Azure, TensorFlow, Railbird, Infosys Nia, Wipro HOLMES, Premonition, Dialogflow, Ayasdi, MindMeld, Meya, KAI and Vital A.I. Following the initial learning steps, the system or machine is taught to advance its initial learning for more accuracy and efficiency. This learning is further compounded by using complex mathematical equations to understand nonlinear relationships between different variables through an information flow called "neural networks." This "higher training" form enables AI to judge and weigh different outcome possibilities.

USE OF ARTIFICIAL INTELLIGENCE IN OPHTHALMOLOGY

From the back of the eye to the front, AI is expected to provide ophthalmologists with novel automated tools to diagnose and treat ocular diseases. Recently, the application of AI in medicine has garnered much attention from big players in the digital world, such as Google and IBM. This is expected to stimulate research and development for disease diagnosis and treatment. Researchers in the field of AI ophthalmology view computerized analytics as the path toward more efficient and objective ways of image interpretation compared with modern eye care practice.

Diabetic retinopathy

Patients with diabetes require regular and repetitive screening to detect and treat diabetic retinopathy (DR)[4,5]. Conventionally, this screening is performed by dilated fundus examination or color fundus photography using conventional fundus cameras (mydriatic or nonmydriatic). The primary issue in this screening is retinal image grading by retinal specialists or trained personnel, who are few compared with the patient load requiring screening. Another problem is that most patients reside in rural areas. Finally, constant follow-ups are needed for several years[4].

DR, a complication of chronic diabetes, is a vasculopathy affecting one-third of patients with diabetes and possibly leading to irreversible blindness[6,7]. Most AIs have been evaluated for their application in DR detection with the primary goal of assisting the development of a mass and rapid screening tool with high sensitivity and specificity. Considering the huge diversity in the clinical presentation of DR, it is essential for an AI neural network to be multilayered and extensively trained. This requires the use of multiple images evaluated against the ground truth.

Most studies have used the International Clinical Diabetic Retinopathy Disease severity scale, a 5-point scale [no apparent retinopathy, mild non-proliferative DR (NPDR), moderate NPDR, severe NPDR and proliferative diabetic retinopathy (PDR)]. Referable DR is defined as moderate or severe DR as disease management often changes from yearly screening to closer follow-up for moderate disease severity. A recent study by Shah *et al*[8] used an AI algorithm with a deep convolutional neural network (DCNN). It assessed its sensitivity and specificity with double validation, *i.e.* external and internal validation. External validation was performed using the Methods to Evaluate Segmentation and Indexing Techniques in the Retinal Ophthalmology dataset, *i.e.* the MESSIDOR dataset. In contrast,

internal validation was performed by two retinal specialists. The main advantage of this study was that 112489 images, acquired from various fundus cameras taking pictures of both mydriatic and nonmydriatic eyes, were fed into AI, thereby giving a multiethnicity advantage to the dataset. The agreement between AI and internal/external validation was high for ANY DR and REFERRAL DR, with a sensitivity of > 95%. The agreement for sight-threatening DR between AI and external validation was high but moderate between AI and internal validation. However, this did not affect the conclusion that AI proved to be a useful screening tool and detected referral DR cases with high specificity.

Valverde *et al*[9] reviewed the available algorithms and detailed the methods for segmenting exudates, red lesions and screening systems. These segmentation methods were used to develop a computer-aided diagnosis for automated DR detection, such as Retmarker DR, Retinalyze System, IDx-DR (first FDA-approved system), iGradingM and Telemedical Retinal Image Analysis and Diagnosis Network. Overall, all these systems achieved high sensitivity and specificity, provided that the segmentation of exudates was used to screen for DR rather than the segmentation of red lesions. Medios, an offline AI, was developed and studied by Sosale *et al*[10]. This offline algorithm was created because of Internet access limitations and the high computational power required for all cloud-based AIs in a developing country. Fundus photographs were captured using Remidio Non-Mydriatic Fundus on Phone 10 (NM FOP 10) and image processing was directly performed on the smartphone graphics processing unit. The sensitivity and specificity of the AI algorithm for detecting referral DR were 98% and 86%, respectively. For any DR, its sensitivity and specificity were 86% and 95%, respectively. Compared with other online cloud-based software, such as EyeArt and IDx-DR, Medios had better sensitivity and equivalent specificity (Figure 2). The specific abnormalities that can be detected using continuous machine learning (CML) are macular edema[11,12], exudates[13], cotton-wool spots[14], microaneurysms and optic disc neovascularization[15]. Commercially available DR detection and analysis technologies include Retinalyze System, IDx-DR, iGradingM and RetmarkerDR. The difference is that only a few modalities use lesion-based grading, whereas the others use image-based grading. The sensitivity of this system has reached around 80%, but its specificity remains lower than 90%.

A DL GAN can be trained to map anatomical features from different image modalities, *i.e.* fundus photographs and fluorescein angiography (FA) images, onto a shared feature manifold to generate one image modality from another[16]. Using GAN, detailed retinal vascular structures can be produced without the requirement of FA to avoid its potential side effects. The inferred structural measurements of retinal vasculature may allow clinicians to identify the natural history of changes in the retinal vasculature and the clinical outcomes of retinal diseases, as previously reported by direct fundus image analysis, but with the accuracy of FA or even optical coherence tomography angiography image analysis[17].

Morya *et al*[18] evaluated the first smartphone-based online annotation in the world, a tool for rapid and accurate image labeling, using AI-based DL for DR. This DL model evaluated its accuracy based on a binary referral DR classification system, depending on whether a retinal image had referral DR or not. A total of 32 ophthalmologists used the tool for over 55000 images. The data analysis proved considerable flexibility and portability with favorable grader variability in concurrence with image annotation. Table 1 demonstrates the collective data of various studies on DR-related AI. This table has been reproduced from the article by Padhy *et al*[19].

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the cause of approximately 9% of cases of blindness globally[20]. The worldwide number of people with AMD was projected to be 196 million in 2020, which is expected to substantially increase to 288 million in 2040[20]. The age-related eye disease study (AREDS) developed a simplified severity scale for AMD[22]. This scale combines the risk factors from both eyes to generate an overall score for the individual based on the presence of one or more large drusen (diameter of > 125 mm) or AMD pigmentary abnormalities in the macula of each eye. The simplified severity scale is also clinically useful because it allows ophthalmologists to predict an individual's 5-year risk of developing late AMD[23]. AMD detection and prediction are essential for individualized treatment. Using AI in cases of AMD could increase the detection rate of lesions such as drusen, with the presence of fluid and reticular pseudo-drusen and geographic atrophy.

Several DL systems have been developed for classifying color fundus photographs based on AMD severity scales. These severity scales include referable and non-referable AMD[22] and multiclass AMD classification systems (*e.g.*, 9-step AREDS severity scale and 4-class). Recent studies have shown the robust performance of automated AMD classification systems based on optical coherence tomography (OCT) scans[24].

DeepSeeNet is based on color fundus photography and uses three networks-Drusen-Net, Pigment-Net and Late AMD-Net (Figure 3). These three networks were designed as DCNNs, each with an Inception-v3 architecture and a state-of-the-art convolutional neural network (CNN) model for image classification. Similar to the study by De Fauw *et al*[25], DeepSeeNet includes two stages by design for improved performance and increased transparency. Images were obtained from the AREDS dataset,

Table 1 Comparative analysis of various studies done on artificial intelligence in diabetic retinopathy[19]

Ref.	Sensitivity, specificity or accuracy of the study	Total fundus images examined	Types of AI used	Main objective
Wong *et al*[20]	Area under the curve were 0.97 and 0.92 for microaneurysm and hemorrhages respectively	143 images	A three-layer feed forward neural network	Deals with detecting the microaneurysm and hemorrhages. Frangi filter used
Imani *et al*[57]	Sensitivity of 75.02%-75.24%; Specificity of 97.45%-97.53%	60 images	MCA	Detected the exudation and blood vessel
Yazid *et al*[58]	97.8% in sensitivity, 99% in specificity and 83.3% in predictivity for STARE database. 90.7% in sensitivity, 99.4% in specificity and 74% in predictivity for the custom database	30 images	Inverse surface thresholding	Detected both hard and soft exudates
Akyol *et al*[59]	Percentage accuracy of disc detection ranged from 90%-94.38% using different data set	239 images	Key point detection, texture analysis, and visual dictionary techniques	Detected the optic disc of fundus images
Niemeijer *et al*[13]	Accuracy in 99.9% cases in finding the disc	1000 images	Combined k-nearest neighbor and cues	Fast detection of the optic disc
Rajalakshmi *et al*[60], Smart phone based study	95.8% sensitivity and 80.2% specificity for detecting any DR. 99.1% sensitivity and 80.4% specificity in detecting STDR	Retinal images of 296 patients	Eye Art AI Dr screening software used	Retinal photography with Remidio 'Fundus on Phone'
Eye Nuk study	Sensitivity was 91.7%; Specificity was 91.5%	40542 images	Eye PAC Stelescreening system	Retinal images taken with traditional desktop fundus cameras
Ting *et al*[61]	Sensitivity and specificity for RDR was 90.5% and 91.6%; For STDR the sensitivity was 100% and the specificity was 91.1%	494661 retinal images	Deep learning system	Multiple Retinal images taken with conventional fundus cameras
IRIS	Sensitivity of the IRIS algorithm in detecting STDR was 66.4% with false-negative rate of 2% and the specificity was 72.8%. Positive Predictive value of 10.8% and negative predictive value 97.8%	15015 patients	Intelligent Retinal Imaging System (IRIS)	Retinal screening examination and nonmydriatic fundus photography

This table has been reproduced from the article by Padhy *et al*[19]. Citation: Padhy SK, Takkar B, Chawla R, Kumar A. Artificial intelligence in diabetic retinopathy: A natural step to the future. *Indian J Ophthalmol* 2019; 67: 1004-1009. Copyright© The Authors 2019. Published by *Indian Journal of Ophthalmology*. The authors have obtained the permission for table using from the Indian Journal of Ophthalmology Publishing Group (Supplementary material). AI: Artificial intelligence; MCA: Morphological component analysis.

comprising approximately 60000 retinal images. DeepSeeNet operates by first detecting individual risk factors (drusen and pigmentary abnormalities) in each eye and then combining values from both eyes to assign an AMD score for the patient. Therefore, DeepSeeNet closely matches the clinical decision-making process (Figure 3). The accuracy of Fine-Tuned DeepSeeNet (FT-DSN) was superior to that of human retinal specialists (67% *vs* 60%). On further analysis, the overall accuracy of FT-DSN was superior. However, subgroup analysis showed that FT-DSN correctly classified participants with severity scale scores of 0-4 more often than the retinal specialists. In contrast, the retinal specialists correctly classified those with late AMD more often than FT-DSN. Lee *et al*[26] developed an AMD screening system to differentiate between normal and AMD OCT images. They trained their CML using 48312 normal and 52690 AMD images. Their CML had a peak sensitivity and specificity of 92% and 93%, respectively. Treder *et al*[27] used OCT images (1112 images) to develop a CML that could differentiate a healthy macula from a macula showing exudative AMD, with a sensitivity of 100% and a specificity of 92%.

Bogunovic *et al*[28] developed a data-driven interpretable predictive model to predict the progression risk in those with intermediate AMD. Drusen regression, an anatomic intermediate AMD endpoint, and advanced AMD onset can be predicted using this specifically designed, fully automated, ML-based classifier. Treder *et al*[27] fed corresponding OCT images of patients with low or high anti-vascular endothelial growth factor (VEGF) injection requirements into a random forest (RF) classifier to develop a predictive model. The treatment requirement prediction showed an area under the curve (AUC) of 70%-80%. Prahs *et al*[29] trained a DCNN on OCT images to facilitate decision-making regarding anti-VEGF injection, and the outcomes were better than those using CML. These studies are an essential step toward image-guided prediction of treatment intervals in neovascular AMD or PDR management. In addition to screening, some studies have focused on grading AMD and predicting visual acuity from OCT images. This will aid clinicians in formulating a visual prognosis and support them in their

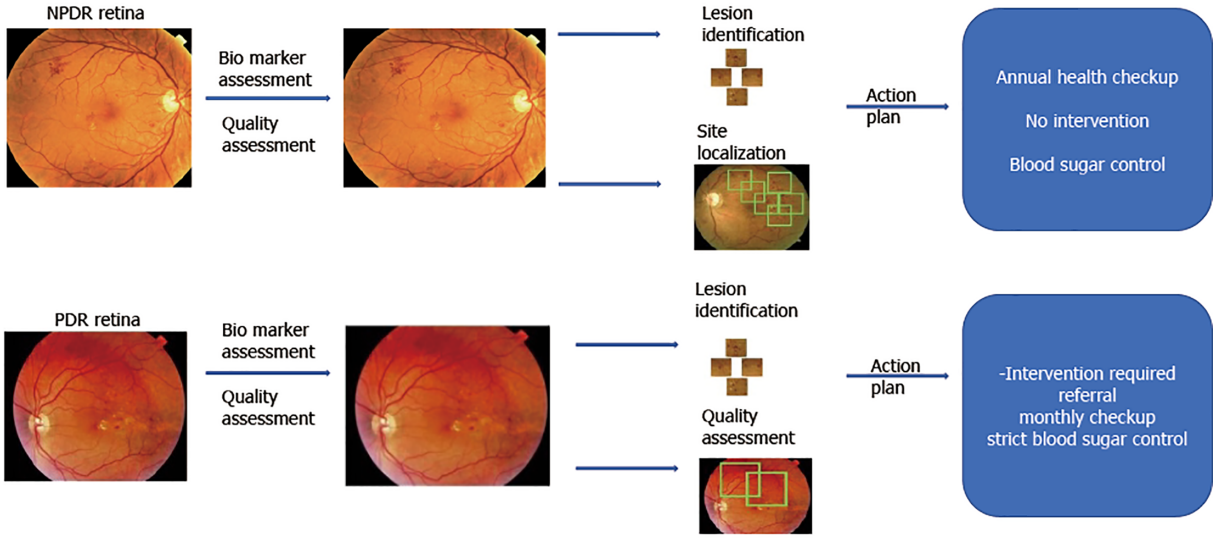

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Figure 2 AI software assess the diabetic retinopathy into referrable and non-referrable interventions.

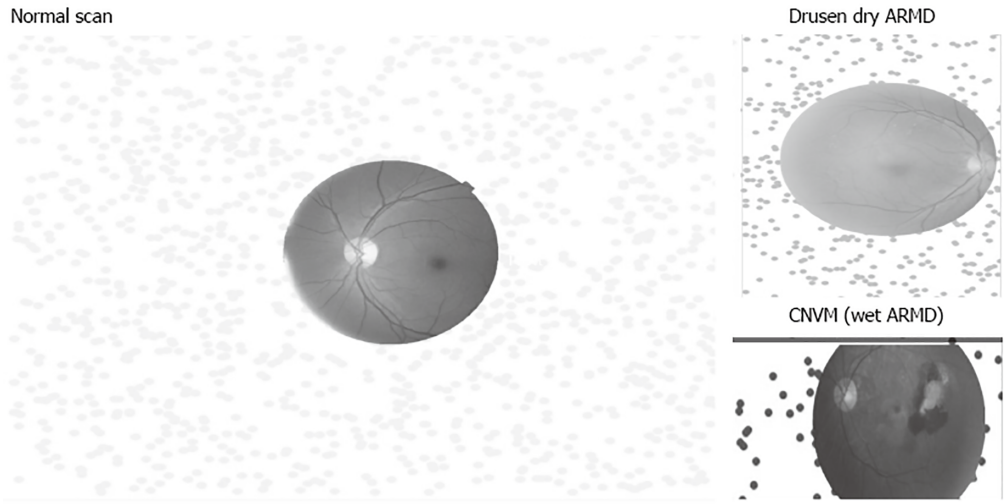

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Figure 3 Deep Sea net classify age-related macular degeneration (AMD) into dry AMD and wet AMD based on fundus photograph.

decision-making. Aslam *et al*[30] and Schmidt-Erfurth *et al*[31] developed CMLs that could estimate visual acuity. Aslam *et al*[30] trained their CML on data from 847 OCT scans, whereas Schmidt-Erfurth *et al*[31] trained their CML on data from 2456 OCT scans (from 614 eyes).

AI systems can be trained to perform segmentation, classification and prediction using retinal OCT images. Several AI systems were demonstrated to display high accuracy for segmentation which is essential to quantify intraretinal fluid, subretinal fluid and pigment epithelial detachment. Compared with noncomputerized segmentation techniques, the DL algorithm developed by Lee *et al*[26] accurately differentiated fluid accumulation from other abnormal retinal findings. Further, De Fauw *et al*[25] confirmed the ability of DL to detect > 50 retinal conditions and the robustness of the AI system in triaging the urgency of referrals for patients with retinal diseases. Table 2 is a summary of AI algorithms used for AMD.

Glaucoma

Glaucoma, also known as the silent sight killer, is the leading cause of preventable and irreversible blindness worldwide. The disease remains asymptomatic and an estimated 50%-90% of individuals with glaucoma remain undiagnosed. Thus, glaucoma screening is recommended for its early detection and treatment. Cup-disk ratio (CDR) can be calculated to assist early-stage glaucoma diagnosis using AI models[32]. After locating the coarse disk margin using a spatial correlation smoothness constraint, a support vector machine (SVM) model is trained to find the patches on OCT images to identify a

Table 2 Summary of artificial intelligence algorithm used in age-related macular degeneration

Ref.	Sensitivity	Specificity	Diagnostic accuracy	Output
Grassman *et al*[62]	84.20	94.30	63.3, Kappa of 92%	Final probability value for referable *vs* not referable
Ting *et al*[61]	93.20	88.70	Area under curve-0.932	Identifying referable AMD and advanced AMD
Lee *et al*[26]	84.60	91.50	87.60	Prediction of binary segmentation map
Treder *et al*[27]	100	92	96	AMD testing score-score of 0.98 or greater adequate for diagnosis of AMD

AMD: Age-related macular degeneration.

reference plane that can calculate the CDR[33].

In 2013, Yousefi *et al*[16] published an AI study on the progression of primary open-angle glaucoma (POAG) in 180 patients using many different CMLs and independent features. They found that retinal nerve fiber layer features provided sufficient information for CMLs to differentiate between stable POAG and progressing POAG at an early-moderate disease stage. RF tree and lazy K star were the most sensitive CMLs. Chen *et al*[34] developed a CNN using two different datasets [ORIGA dataset: 650 images (99 for training and 551 for validation) and SCES dataset: 1676 images (entirely used for validation as the images in the ORIGA set were used for training)] to detect POAG based on optic disk images. They reported the area under the receiver operating characteristic curve values of 0.831 and 0.887 for ORIGA and SCES datasets, respectively. Kim *et al*[35] and Raghavendra focused on detecting glaucoma *vs* normal fundus images. They reported an accuracy of 87.9%, equivalent to the accuracy of human experts, demonstrating an efficient method for glaucoma screening. Raghavendra *et al*[36] tested their CML on 589 normal and 837 glaucoma images and obtained a score of 0.98 for sensitivity, specificity and accuracy.

DL performs better than CML in detecting pre-perimetric open-angle glaucoma[36]. Holistic and local features of the optic disc on fundus images have been used to mitigate the influence of optic disk misalignment for glaucoma diagnosis[37]. Li *et al*[38] demonstrated that DL could be used to identify referable glaucomatous optic neuropathy with high sensitivity and specificity. Table 3 is a summary of studies using AI to detect progression in eyes with glaucoma.

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a leading cause of treatable childhood blindness, provided it is diagnosed timely[39]. This disease necessitates strict follow-up and screening which are very tedious and demanding. Repeated ROP screening and follow-up consume substantial manpower and energy. Therefore, the application of AI in ROP screening may improve the efficiency of care for ROP.

Wang *et al*[40] developed an automated ROP detection system called DeepROP using deep neural networks (DNNs). ROP detection was divided into ROP identification and grading tasks. Two specific DNN models–Id-Net and Gr-Net–were designed for the identification and grading tasks, respectively. Id-Net achieved a sensitivity of 96.62% and a specificity of 99.32% for ROP identification, whereas Gr-Net attained a sensitivity of 88.46% and a specificity of 92.31% for ROP grading. In another 552 cases, the developed DNNs outperformed some human experts[41].

A similar AI, developed by Tan Z, achieved similar accuracy for detecting plus ROP. They reported that this AI could distinguish the plus disease with 95% accuracy, comparable to the diagnoses of experts and much more precise than those of non-experts. Various studies have reported promising results, most of which were based on two-level sorting (plus or not plus disease).

Keratoconus

There are significant obstacles in distinguishing patients with very early keratoconus signs from the normal population. This is attributed to the limited availability of samples owing to low disease prevalence. For this purpose, the application of AI in corneal topography interpretation has been attempted. The methods used discriminative classifiers that, given a set of independent machine-derived variables from corneal topography (*e.g.*, simulated K readings and topographic asymmetries), can be trained to differentiate between two or more classes of topography (*e.g.*, normal, astigmatic and keratoconus).

AI has been used to detect keratoconus and forme fruste keratoconus[42] based on data from Placido topography, Scheimpflug tomography[43], anterior segment spectral domain OCT and biomechanical metrics (CorvisST and corneal hysteresis). Further, data from Pentacam[44], Sirius[45], Orbscan II, Galilei and TMS-1 have been studied using ML algorithms to detect early keratoconus.

The Pentacam RF index (PRFI) is an RF model built using data from Pentacam HR (Oculus, Wetzlar, Germany). It was the only model trained using the preoperative examination data of patients that developed ectasia. The index already available on the device (BAD-D) presented a sensitivity of 55.3%, whereas PRFI identified 80% of the cases correctly. In the external validation set, the model showed an

Table 3 Summary of studies using artificial intelligence to detect progression in Glaucomatous eyes

Ref.	No. of eyes	Instrument	Approach	Comments
Lin *et al*[63]	80	SAP	Supervised ML	Sensitivity-86%; Specificity-88%
Goldbaum *et al*[64]	478 suspects; 150 glaucoma; 55 stable glaucoma	SAP	Unsupervised ML	Specificity-98.4%, AROC not available; Use of variational Byesian. Independent component analysis mixture model in indentifying patterns of glaucomatous visual field defects and its validation
Wang *et al* [65]	11817 (method developing cohort) and 397 (clinical evaluation cohort)	SAP	Unsupervised ML	AROC of the archetype method 0.77
Yousefi *et al* [16]	939 Abnormal SAP and 1146 normal SAP in the cross section and 270 glaucoma in the longitudinal database	SAP	Unsupervised ML	Sensitivity 34.5%-63.4% at specificity 87% Comment: it took 3.5 years for ML analysis to detect progression while it took over 3.5 years for other methods to detect progression in 25% of eyes
Belghith *et al*	27- progressing; 26-stable glaucoma and 40 healthy controls		SD OCT Supervised ML	Sensitivity -78% Specificity in normal eyes-93%; 94% in non-progressive eyes

ML: Machine learning; OCT: Optical coherence tomography.

accuracy of 85% for detecting the normal topographic eye of very asymmetric cases (VAE-NT), reaching a specificity of 96.6%[46].

A single decision tree method was proposed based on the data obtained from the Galilei Dual Scheimpflug Analyzer (Ziemer Ophthalmic Systems AG, Port, Switzerland). This index showed a sensitivity of 90% and a specificity of 86% for detecting early disease forms[47]. Discriminant linear models were also successfully used to analyze the data obtained from Orbscan II (Technolas, Munich, Germany) with a sensitivity of 92% and a specificity of 96% in the first validation set and a sensitivity of 70.8%, and a specificity of 98.1% in a different ethnic background population[48].

Ambrósio *et al*[48] evaluated AI-based tomographic and biomechanical index (TBI), which combines Scheimpflug-based corneal tomography and biomechanics (Corvis ST) for improving ectasia detection. The Kerato Detect algorithm analyzes the corneal eye topography using a CNN that can extract and learn the features of a keratoconus eye. The results ensure high-level performance yielding an accuracy of 99.33% for the test dataset. Neural networks have been used to evaluate the waveform signals of the Ocular Response Analyzer (Reichert Ophthalmic Instruments, Buffalo, United States) yielding high accuracy for the study validation sample comprising early keratoconus forms (AUC, 0.978). The RF model called TBI achieved a sensitivity of 90.3% and a specificity of 96% for detecting VAE-NT. The combination of tomographic and biomechanical parameters was superior to either method used alone.

Sharif *et al*[49] showed that corneal images obtained *via* confocal microscopy could be assessed in detail using a committee machine developed from artificial neural networks and adaptive neuro-fuzzy inference systems that can detect abnormalities with high accuracy and enable 3D visualization. Nevertheless, considering that the research on these aspects is limited, there is a possibility that the characteristics learned in AI training may not be similar to those in another clinical population. When using tomographic data rather than Placido topographic data, researchers have found that combining biomechanical or additional imaging data is necessary to enhance the performance for detecting early keratoconus signs.

CORNEAL DYSTROPHIES AND DYSPLASIA

Eleiwa *et al*[50] used AI to differentiate Fuchs endothelial corneal dystrophy (FECD, without corneal edema) from late-stage FECD (with corneal edema) based on high-definition OCT images. The model they developed had a sensitivity of 99% and a specificity of 98% in differentiating normal cornea from FECD (early or late).

Gu *et al*[51] reported an AUC of 0.939 for detecting corneal dystrophy or degeneration using a slit-lamp photograph-based DL model. They included ocular surface disorders such as limbal dermoid, papilloma, pterygium, conjunctival dermolipoma, conjunctival nevus and conjunctival melanocytic tumors to differentiate ocular surface neoplasms. However, considering the limited existing evidence, the use of AI for detecting ocular surface neoplasms warrants further exploration. Kessel *et al*[52] created trained DL algorithms to detect and analyze amyloid deposition in corneal sections in patients with familial amyloidosis undergoing full-thickness keratoplasty.

Dry eye

Dry eye disease is a common condition that affects 8% of the global population and is caused by the reduced quantity or quality of tears. Left untreated, dry eye can result in pain, ulcers and even corneal

scars. Therefore, rapid diagnosis is essential and clinically based on tear production measurement and a tear film stability evaluation.

In a recent study, researchers used infrared thermal images of the eye along with the ML algorithms Gabor transform and Discrete Wavelet Transform (DWT) to detect dry eyes[53]. These ML methodologies were used to extract features from specific image frames, further segmented into eye regions, and the data were analyzed accordingly. Principal component analysis was ranked using a t-value and fed into the SVM classifier. Using the 1st, 5th, and 10th after the first blink, they achieved classification accuracies of: (1) 82.3%, 89.2% and 88.2% for the left eye; and (2) 93.4%, 81.5% and 84.4% for the right eye, respectively. Similarly, using the 1st, 5th, and 10th frames of the lower half of the ocular region, they achieved accuracies of: (1) 95.0%, 95.0% and 89.2%; and (2) 91.2%, 97.0% and 92.2% for the left and right eyes, respectively. This study showed that the lower half of the ocular region is superior to the upper half of the ocular region.

This method offers several advantages, such as being semiautomatic and making it less susceptible to interobserver variability. It is more accurate than standard clinical tools, more convenient for the patient and does not require a special dye. Gabor transform and DWT are methodologies for automatic feature extraction from biomedical images.

Cataract

Cataract refers to the clouding of the eye lens. It is the leading cause of blindness worldwide. Therefore, automatic detection for the diagnosis of this disease will be cost-effective.

Srivastava *et al*[54] proposed a system that automatically grades the severity of nuclear cataracts based on slit-lamp images. First, the lens region of interest is identified, following which the CNN filters randomly selected image patches, generating local representations *via* an iteration process with random weights. They named it ACASIA-NC_v0.10 (*i.e.*, Automatic Cataract Screening from Image Analysis-Nuclear Cataract, version 0.10) and specifically used the "visibility cue" for nuclear cataract grading C. Their system used visible features of the nucleus, such as sutures and demarcation lines, in greyscale. With the help of the software, they could analyze the number of visible features. ACASIA-NC_v0.10 achieved a similarity of > 70% against clinical grading and reduced the error by > 8.5%. Other studies similar to Liu *et al*[55] mainly focused on identifying pediatric cataracts. They reported exceptional accuracy and sensitivity for lens classification and density. In addition, cataract grading can also be achieved automatically based on lens OCT findings.

SMARTPHONE-BASED APPS USING AI IN OPHTHALMOLOGY

The advantages of using smartphones are many, including having built-in internal data storage and cloud storage capabilities[56]. Pegasus VISULYTIX, an inexpensive smartphone clip-on optic nerve scanner expected to aid the diagnosis and treatment of those with chronic blinding diseases, such as glaucoma, using AI, is being adapted. Pegasus could detect glaucomatous optic neuropathy with an accuracy of 83.4%, comparable to the average accuracies of ophthalmologists (80.5%) and optometrists (80%) using the same images.

CC-Cruiser was developed to study the application of AI in congenital cataracts (CC). CCs cause irreversible vision loss and breakthroughs in the research on CCs have substantially contributed to the field of medicine. Researchers have developed a three-fold AI system that includes identification networks for CC screening in populations, evaluation networks for risk stratification of patients with CC and strategist networks to assist ophthalmologists in making treatment decisions.

Shaw created a ComputeR Assisted Detector LEukocoia (CRADLE) app that uses AI to identify white eyes indicative of several serious eye diseases. The sensitivity of CRADLE for detecting white eyes in children aged ≤ 2 years surpassed 80%, which was substantially higher than the sensitivity of physical examination (8%). This new smartphone app takes advantage of parents' fondness for snapping pictures of their children to identify signs of a severe eye disease that the child might be developing. On average, the app detected white eyes in pictures collected 1.3 years before diagnosis.

FUTURE OF ARTIFICIAL INTELLIGENCE APPLICATION

The AI-based platform provides an intelligent diagnosis of eye diseases at present. It focuses on binary classification problems, whereas visiting patients suffer multi-categorical retinal disorders in clinical settings. Multimodal clinical images, such as OCTA, visual field and fundus images should be integrated to build a generalized AI system for more reliable AI diagnosis. The challenge is coordinating multicenter collaborations to build good quality and extensive data collection to train and improve AI models. AI is an instrument to upturn clinical decision power with many possible applications for ophthalmologists.

LIMITATIONS OF ARTIFICIAL INTELLIGENCE

Any software design is not perfect, and so artificial intelligence is also not bias-proof. Five distinct types of machine learning bias that we need to be aware of and guard against: (1) Sample bias: poor data collection for training. Example: Labeling other vascular retinopathies as DR; (2) Prejudice bias: Prejudice bias results from training data that is influenced by stereotypes. For example, a large cup is always glaucoma; (3) Measurement bias. For example, fundus photo color, different cameras give different color measurements; best avoided by having multiple or similar measuring devices and humans trained to compare the output of these devices when developing the algorithm; (4) Algorithm bias: Choosing the wrong software algorithm for a specific disease; and (5) The quality control of images for prediction.

CONCLUSION

With the substantial advances in AI in the field of ophthalmology, it can be assumed that now is the dawn of AI in ophthalmology. With the advent of technologies based on different AI modules, such as DL, ML and GAN, it can be assumed that AI has a promising role in the diagnosis of DR, ARMD, dry eye, glaucoma, keratoconus and cataracts. In particular, these AI-based applications are more relevant during the present coronavirus disease 2019 era and for serving the remotest of areas worldwide. Compared with conventional tests performed at tertiary ophthalmic centers, AI performs better in the screening and diagnosis of various eye diseases. After considering all the facts and overcoming challenges in its application, it can be said that AI in the field of ophthalmology is here to stay and revolutionize eye care in the 21st century. Nonetheless, researchers in the field of ophthalmology need to develop more robust AI modules with better verification and validation. Further, we must not rely only on near-real AI as no modality can possibly replace the level of affection, care and sensitivity as that provided by human caregivers.

FOOTNOTES

Author contributions: Morya AK and Janti SS contributed equally to this work; Sisodiya P, Tejaswini A, Gurnani B, Prasad R and Mali KR designed the research study; Morya AK and Janti SS performed the research; Sisodiya P, Tejaswini A, Gurnani B contributed new search and analytic tools; Morya AK, Janti SS,Tejaswini A and Gurnani B analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

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MINIREVIEWS

New therapeutic approaches for type 1 diabetes: Disease-modifying therapies

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Abstract

It has been 100 years since the first successful clinical use of insulin, yet it remains the only treatment option for type 1 diabetes mellitus (T1DM) patients. Advances in diabetes care, such as insulin analogue therapies and new devices, including continuous glucose monitoring with continuous subcutaneous insulin infusion have improved the quality of life of patients but have no impact on the pathogenesis of the disease. They do not eliminate long-term complications and require several lifestyle sacrifices. A more ideal future therapy for T1DM, instead of supplementing the insufficient hormone production (a consequence of β-cell destruction), would also aim to stop or slow down the destructive autoimmune process. The discovery of the autoimmune nature of type 1 diabetes mellitus has presented several targets by which disease progression may be altered. The goal of disease-modifying therapies is to target autoimmune mechanisms and prevent β-cell destruction. T1DM patients with better β-cell function have better glycemic control, reduced incidence of long-term complications and hypoglycemic episodes. Unfortunately, at the time symptomatic T1DM is diagnosed, most of the insulin secreting β cells are usually lost. Therefore, to maximize the salvageable β-cell mass by disease-modifying therapies, detecting autoimmune markers in an early, optimally presymptomatic phase of T1DM is of great importance. Disease-modifying therapies, such as immuno- and regenerative therapies are expected to take a relevant place in diabetology. The aim of this article was to provide a brief insight into the pathogenesis and course of T1DM and present the current state of disease-modifying therapeutic interventions that may impact future diabetes treatment.

Key Words: Type 1 diabetes; Mesenchymal stem cell; Immunotherapy; Islet cells; Autoimmunity; Regenerative medicine

Core Tip: Our knowledge is rapidly growing about the pathomechanism of type 1 diabetes mellitus, and new and improved therapies have emerged. However, the long-term complications and the required lifestyle changes cannot be eliminated. There is a growing number of research that aims to find specific immunological markers/targets that have a role in disease development. The ultimate goal is finding new therapeutic ways to treat the disease and to delay or even prevent its development. The aim of this review was to provide a brief insight into the current state of disease-modifying therapeutic interventions that may impact future diabetes care.

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INTRODUCTION

For many years it was accepted that type 1 diabetes mellitus (T1DM) starts with the classical triad of polyuria, polydipsia and polyphagia. However, it became clear that T1DM is a long standing, progressive disease with a preclinical phase without symptoms and with the appearance of multiple T1DM-associated autoantibodies. The preclinical phase is followed by a symptomatic clinical phase[1]. The burden of living with the chronic disease is considerable for the patient, the family and the society. This minireview focused on the clinical applications of novel disease-modifying therapeutic intervention options in early stages of T1DM that may prevent or reverse clinically overt symptomatic T1DM. The presentation of the latest improvements available for middle and late stage disease and diabetic complications is out of the scope of the current review.

Immunopathogenesis of T1DM

The pathogenesis of T1DM involves a complex interaction between pancreatic β cells and the innate and natural immune systems. The precise mechanism that leads to the loss of immune tolerance is still unclear. However, viral infections, nutritional factors and the perinatal environment have been associated with the disease[2-4]. It is assumed that the stability of the trimolecular complex (T cell receptor/human leukocyte antigen/peptide) during thymic selection plays a major role in the escape of autoimmune T cells[5].

In the development of T1DM, the initial step of the destructive process is considered to be the uptake and presentation of β-cell-derived peptides, autoantigens, by the antigen-presenting cells. Next, antigen-presenting cells, which can be both macrophages and dendritic cells, migrate to lymph nodes around the pancreas and activate $CD4^+$ helper T cells (Th)[6]. Th cells differentiate into Th1, which have a proinflammatory phenotype. Th1 cells are the key effector cells in the pathogenesis of T1DM and are capable of producing interferon-γ, tumor necrosis factor α, interleukin 1 (IL-1) and IL-2. These cytokines inhibit Th2 polarization, the cells responsible for the protection of islets[7]. Th1 cells are necessary for the activation and recruitment of other autoreactive cells, such as $CD8^+$ cytotoxic T lymphocytes (CTL), which are responsible for the lysis/apoptosis of β cells presenting the major histocompatibility complex I autoantigen complex. The cell-destructive effect of activated CTLs is due to macromolecules stored in granules (*e.g.*, perforin, granzyme), to the cytokines and to caspase-dependent apoptosis[8].

B cells are stimulated by Th1 cells and produce autoantibodies against β cells [islet cell antibody, glutamic-acid-decarboxylase antibody (GADA), islet tyrosine phosphatase 2 antibody, insulin autoantibody and zinc transporter 8 antibodies]. These antibodies have become the biomarkers of T1DM [9]. Furthermore, Th1 cells enhance antigen presenting, costimulatory and effector functions of macrophages and dendritic cells. Natural killer cells also contribute to β-cell destruction through their cytolytic effects and antibody-dependent cellular cytotoxicity. Th17, with strong inflammatory effects, is also involved in the inflammatory process: It secretes IL-17 family cytokines and plays an important role in neutrophil granulocyte recruitment and activation[10].

Under ideal conditions, regulatory T cells (Treg) inhibit the autoreactive lymphocytes. If Treg cells are deficient, the rate of T1DM progression is increased[11]. The above-mentioned immune cells infiltrate the islets (insulitis) and eventually cause β-cell death and reduced insulin levels (Figure 1).

New staging classification system of T1DM

T1DM is the result of a destructive autoimmune-mediated process in which insulin-producing β cells in the islets of Langerhans are damaged. According to the novel staging classification system proposed by

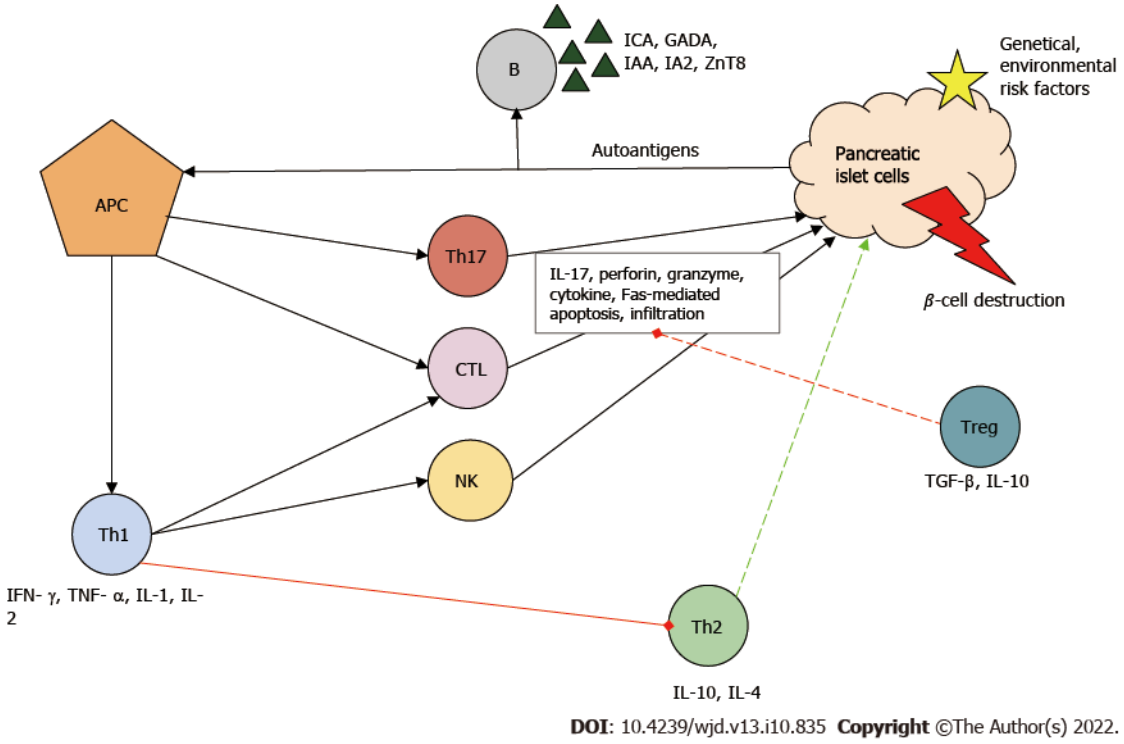

Figure 1 Immunopathogenesis of type 1 diabetes mellitus. Autoantigens from pancreatic islet β-cells are presented by antigen-presenting cells (APCs), thereby activating T cells including helper T (Th) cells type 1 and type 17 and cytotoxic T lymphocytes (CTLs). Th type 1 cells play a key role in the development of the autoimmune response. They stimulate the activity of inflammatory T cells, macrophages and natural killer (NK) cells by producing proinflammatory cytokines and stimulate B cells (B), which produces autoantibodies and inhibits the protective Th type 2 cell function. Together, these immune cells contribute to the destruction of pancreatic β-cells. Red line: Inhibition; Green line: Stimulation. GADA: Glutamic-acid-decarboxylase antibody; IA2: Islet tyrosine phosphatase 2 antibody; IAA: Insulin autoantibody; ICA: Islet cell antibody; IL: Interleukin; IFN-γ: Interferon γ; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α; Treg: Regulatory T cell; ZnT8: Zinc transporter 8 antibody.

the Juvenile Diabetes Research Foundation, the Endocrine Society and the American Diabetes Association, there are three distinct stages in T1DM[12]. Genetic predisposing factors are present from birth. The autoimmune reaction may be initiated in genetically susceptible individuals by environmental risk factors, which are not well understood[13]. People with a first- or second-degree relative with T1DM have a 15 times greater risk of developing the disease compared to the general population [14] (Figure 2).

Stage 1 is the critical point of no return since eventually the affected individuals will develop clinical diabetes. It is characterized by the presence of immune markers, two or more of the T1DM-associated islet antibodies, such as islet cell antibodies, GADA, Islet tyrosine phosphatase 2 antibodies and zinc transporter 8 antibodies, normoglycemia and absence of diabetic symptoms. In stage 2, the β-cell volume is critically decreased, and metabolic markers become detectable in asymptomatic patients. These individuals, besides being antibody positive, display impaired fasting glycemia, impaired glucose tolerance, abnormal oral glucose tolerance test or glycated hemoglobin (HbA_{1C}) ≥ 5.7%. Stage 3 represents the phase of clinical diagnosis and the manifestation of typical diabetic symptoms such as polyuria, polydipsia, weight loss, fatigue, diabetic ketoacidosis, *etc*[12]. Over time, most of the residual β cells are lost. However, sensitive C-peptide measurements have shown that 30%-80% of patients with long–standing T1DM are insulin microsecretors. This means that these patients have detectable stimulated C-peptide value of < 30 pmol/L (< 0.09 ng/mL)[15], an important consideration in therapeutic approaches targeting β-cell survival[16]. Shield *et al*[17] identified two clear phases of C-peptide decline after the diagnosis of T1DM: an exponential fall in the first 7 years (-47%/year) followed by a stable phase (-0.1%/year) (Figure 2).

Age has a major influence on the rate of disease progression. In children, the clinical stage develops more rapidly, and β-cell loss is more pronounced compared to adults. About 6 years to 9 years after the diagnosis of T1DM, 20% of those diagnosed in childhood and 60% of those diagnosed in adulthood had detectable C-peptide secretion, an indicator of endogenous insulin production[18]. In addition, the autoantibody titer and profile are also a determinant of β-cell loss. Most people will not develop diabetes if they have a single autoantibody. In contrast, the more autoantibodies a person has and the higher their serum concentration is, the rate of disease progression is greater[19]. Lately, stage-specific therapies have been the focus of clinical trials for modifying disease progression[13,19].

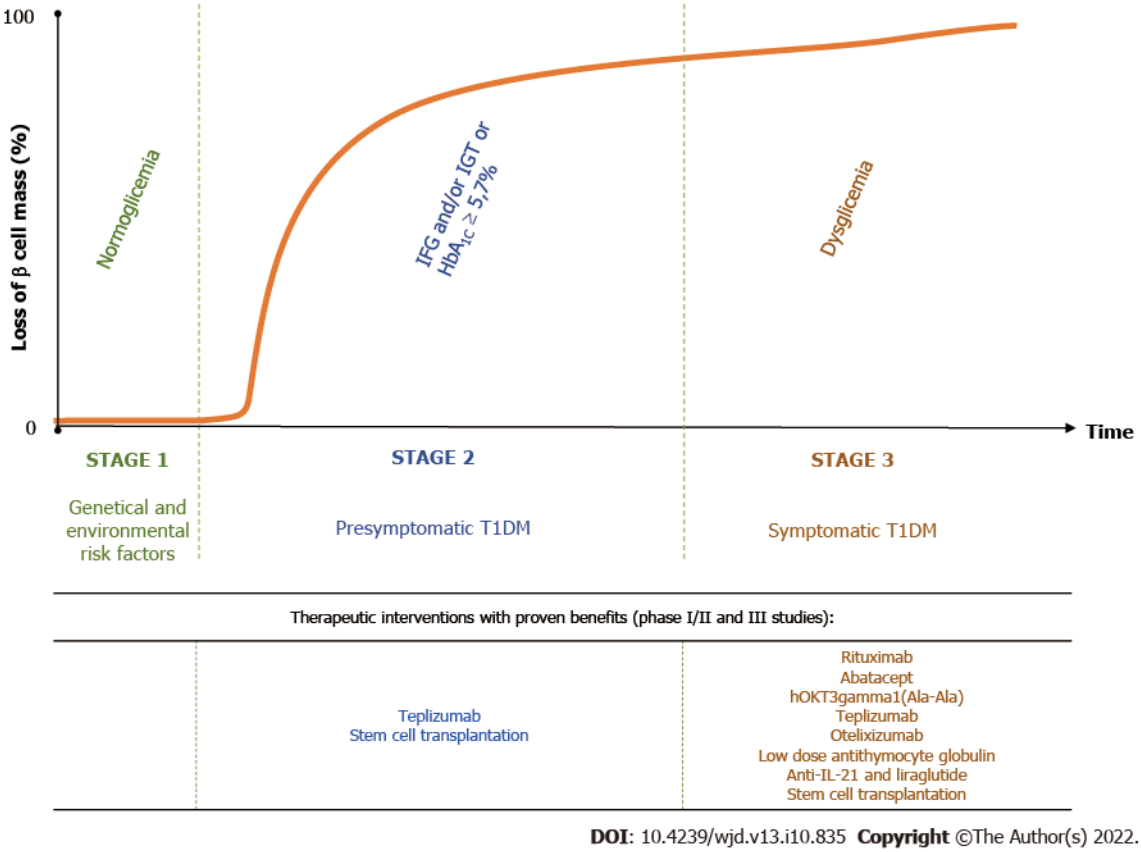

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Figure 2 Stages of type 1 diabetes mellitus and options of therapeutic interventions with proven therapeutic benefits in phase II and III clinical trials. The interaction between genetic predisposing factors, which are present from birth, and environmental factors trigger the autoreactive process. Stage 1 is characterized by individuals who exhibit at least two of the type 1 diabetes mellitus (T1DM)-associated antibodies (glutamic-acid-decarboxylase-, islet tyrosine phosphatase 2-, islet cell- and zinc transporter 8 antibodies). Stage 2 is characterized by dysglycemia due to reduced β-cell function. Stage 3 represents the onset of clinical T1DM and usually but not always the manifestation of typical symptoms. The application of the previously proposed staging system[12] in clinical trials, which even recognizes the earliest stages of T1DM, can improve the research and development of novel therapies that might delay/prevent the onset of the disease. IL: Interleukin; IFG: Impaired fasting glycemia; IGT: Impaired glucose tolerance; HBA_{1C}: Glycated hemoglobin. Figure adapted from Insel *et al*[12].

DISEASE-MODIFYING THERAPEUTIC OPTIONS

Groundbreaking studies with cyclosporin A in the 1980s showed that the disease course of T1DM can be altered with immune therapy[20] and gave rise to research in the field of definitive treatment of T1DM. Despite extensive efforts so far, immune-altering therapies could not reach approval for routine clinical use for several reasons. Some agents such as cyclosporin A have many side effects and lack long-term efficacy[21]. Other options with a more favorable tolerance profile such as the adjuvant-formulated GAD-alum vaccine, which incorporated recombinant human glutamic acid decarboxylase, had no effect on disease progression[22]. The recent development of more targeted immunotherapies, the advances in regenerative therapies and lessons learned about β-cell survival from type 2 diabetes mellitus studies gave rise to more promising therapeutic options. Of the immunotherapy agents, teplizumab has come closest to achieving success. In July 2021, the United States Food and Drug Administration considered the use of teplizumab in high-risk individuals but deemed further studies necessary before granting approval[23].

B-cell targeting agents

Even though T1DM is mainly a T cell-mediated autoimmune disease, antigen-presenting B lymphocytes can also play a pathogenic role by activating T-lymphocytes and triggering the autoimmune destruction of β cells. In animal models and triggered human studies, T1DM anti-B lymphocyte therapies have been shown to be effective[24].

Rituximab: Rituximab is an anti-CD20 monoclonal antibody known to cause B-cell depletion. It is widely used in clinical practice in malignant hematological diseases such as non-Hodgkins lymphomas and chronic lymphocytic leukemias. In a placebo controlled randomized trial, including 87 recent onset T1DM patients, it has been shown that rituximab treatment was associated with slower progression of β -cell dysfunction and better metabolic control during the 12-mo long study period[25]. Insulin dose requirements decreased during the study. However, none of the patients were able to become insulin

free. Extensive follow-up of the patients showed a constant decline in C-peptide production. It suggests that B-cell depletion by its own is not sufficient in restoring β-cell tolerance in the long run and does not fundamentally alter the course of overt T1DM[26].

It has been reported that rituximab can suppress insulin autoantibodies, but no such effect could be found in the case of GADA, islet tyrosine phosphatase 2 antibody and zinc transporter 8 antibody[27]. Compared to placebo controls, rituximab-treated T1DM patients, whose C-peptide response was significant, have shown increased proliferative responses to islet, neuronal and disease-relevant environmental antigens ultimately resulting in increasing insulin secretory function[28]. Moreover, the combination of rituximab with $CD4^+$ $CD25^{high}$ $CD127^-$ T regulatory cells[29] or therapy targeting $CD4^+$ T cells[30] can further improve treatment efficacy. One side effect of rituximab, reported by the study of Kroll *et al*[31], might be the reactivation of some asymptomatic polyomavirus infections. Rituximab is currently being tested in earlier stages of T1DM (NCT03929601[32]).

T-cell targeting agents

T-cell co-stimulation inhibition: Abatacept is a cytotoxic T lymphocyte-associated antigen 4 immunoglobulin fusion protein designed to selectively bind to CD80/86 to inhibit the early activation and proliferation of naïve T lymphocytes. Since effector memory T cells are less dependent on CD28 costimulation, abatacept is a more selective way to inhibit T cell activation compared to general immunosuppressants. In a phase II placebo-controlled study, Orban *et al*[33] investigated the effect of abatacept in a population with recent onset T1DM. Patients in the treatment group received a monthly dose of 10 mg/kg intravenous abatacept for 2 years. The authors found that cytotoxic T lymphocyte-associated antigen 4 inhibition slowed the decline of β-cell function during the 2 years of the treatment and had a beneficial effect during the 1-year follow-up period without active treatment[34]. However, the observed positive effect was only temporary and declined over time. Furthermore, it was found that abatacept does not change immunogenicity of other vaccines in T1DM patients[35], but different follicular Th and central memory $CD4^+$ T cell phenotypes might affect the efficacy of the treatment[36, 37]. There are still ongoing clinical trials (*e.g.*, NCT01773707[33], NCT04118153[38] and NCT03929601 [32]) investigating whether abatacept may have a more potent impact on the disease if administered at earlier stages (*e.g.*, at stage 2) of T1DM pathogenesis.

Anti-CD3 therapy: So far the most promising therapeutic target in modifying the course of T1DM is the ε chain of the CD3 receptor on the T cell surface, previously known as muromonab-CD3 (trade name: Orthoclone OKT3)[39]. Animal studies have shown that anti-CD3 therapy can induce diabetes remission in the models of T1DM[40]. This main effect is associated by the induction of Treg cells and immunosuppressive cytokines such as transforming growth factor β[41]. One of the first human trials reported significantly improved C-peptide response and other clinical parameters after a single shot of hOKT3gamma1(Ala-Ala)[42].

Teplizumab is a humanized anti-CD3 monoclonal antibody, which has been shown to be the most potent agent in slowing the progression of T1DM. In a series of human clinical trials, it was demonstrated that the treatment of teplizumab was a potent way to delay the decline of C-peptide production[43-45] and can help to preserve β-cell function[46], and its effect can be sustained by an average of 15.9 mo in T1DM[47]. Furthermore, the Protegé[48,49] study in which 516 T1DM patients were enrolled and treated with teplizumab has demonstrated that anti-CD3 therapy delayed the decline of insulin secretion and induced disease regression, and 5% of the patients became insulin independent. Even though teplizumab has shown promising results in preventing disease progression, it must be noted that significant metabolic benefits such as a significant reduction in HbA_{1C} could not be demonstrated.

These promising results led to further trials to evaluate the effect of anti-CD3 therapy in T1DM prevention. The recently published results of a phase III follow-up trial including non-diabetic patients at high risk of developing T1DM, defined as having impaired glucose tolerance and at least two diabetes-specific autoantibodies, demonstrated the efficacy of teplizumab in delaying the onset of T1DM by 48.4 mo compared to the placebo group (24.4 mo)[50]. C-peptide levels of those who responded to treatment remained significantly better even after a 7-year follow-up period[51]. Furthermore, clinical responders to teplizumab therapy have shown significant reduction in circulating $CD4^+$ effector memory T cells and decreased activation and regulatory gene expression in circulating $CD8^+$ central memory T cells[52]. Currently, studies are running to investigate teplizumab in at-risk individuals[53] and recent-onset T1DM patients[54,55].

Otelixizumab is another humanized anti-CD3 antibody that has been evaluated both in the treatment of overt[56] and new-onset[57,58] T1DM. Similarly to teplizumab, a 6 d treatment of otelixizumab preserved residual β-cell function for at least 18 mo in 40 patients with recent-onset T1DM. The protective effect of otelixizumab treatment appears to be dose dependent as studies attempting to lower adverse reactions by administering lower doses could not demonstrate a benefit in C-peptide preservation[59,60]. The protective effect of otelixizumab showed the highest benefit in insulin autoantibody-positive T1DM patients[61].

Anti-CD3 therapies have been overall well tolerated among patients. Adverse reactions that were significantly more prevalent in the treatment group included: vomiting, rash, chills, cytokine release syndrome, Epstein-Barr virus reactivation[57,62] and headache. Recently, a subcutaneous formulation was also introduced[63], which significantly reduced such undesirable effects. Adverse reactions were mostly mild to moderate and self-limited; 9% of patients were not able to complete all drug doses compared to a 2% dropout rate in the placebo group. The most common cause for treatment cessation was lymphopenia, neutropenia, elevated liver enzymes and reduced platelet counts.

Low dose antithymocyte globulin: Antithymocyte globulin (ATG) is a polyclonal immunoglobulin G antibody against multiple human T cell antigens and their precursors. Only a limited number of studies are available, and their results are somewhat controversial: 6.5 mg/kg ATG alone could not preserve β-cell function, but C-peptide secretion was preserved in older participants suggesting a possible age-specific action[64,65]. In contrast, low dose ATG treatment (2.5 mg/kg administered as 0.5 mg/kg on day 1 and 2 mg/kg on day 2) in combination with pegylated granulocyte colony-stimulating factor acts by decreasing the number of activated effector T cells while relatively preserving Treg cells. T1DM patients with a diabetes onset between 4 mo and 2 years receiving low dose ATG + granulocyte colony-stimulating factor have shown a benefit in disease progression[66,67]. Patients in the treatment group have had higher C-peptide production after a mixed meal test, and lower HbA_{1C} after 6 mo was also recorded in the treatment group compared to the placebo group.

A more recent study published by the same group indicated that low-dose ATG monotherapy without granulocyte colony-stimulating factor can delay the decline of C-peptide, can reduce the HbA_{1C} level and affect T cell phenotypes in new-onset T1DM[68,69]. The ongoing follow-up of this study is in progress along with two additional studies[70,71], and their results will help further evaluate the potential benefits of low-dose ATG and its therapeutic potential for preventing T1DM.

Anti-IL-21 and liraglutide: A new strategy to modify the disease course in T1DM is using a drug combination that not only halts or delays the progressive autoimmune process but aims at preserving and improving residual β-cell function. This approach may have the advantage over previous monotherapies in achieving disease modification with milder immunomodulation in a safer, more sustainable way. IL-21 plays a key role in the pathomechanism of T1DM by activating and leading $CD8^+$ T lymphocytes from lymph nodes and the exocrine pancreas to the pancreatic islets eventually leading to β-cell destruction[72,73]. Based on these findings, IL-21 inhibition has emerged as a potential disease-modifying target in preventing T1DM. 35-Liraglutide, a glucagon like peptide-1 analog that has routinely been used in type 2 diabetes therapy, has been proven to increase β-cell survival[74] and can improve glucose dependent insulin secretion not only in type 2 diabetes but in T1DM as well[75,76].

In a recent phase II clinical trial the effect of liraglutide and IL-21 inhibition was evaluated in 308 T1DM patients with recent onset disease and residual β-cell function[77]. The combination treatment was effective to preserve both fasting and postprandial endogenous insulin secretion resulting in a nonsignificant decrease in the number of hypoglycemic events and level of HbA_{1C} for 52 wk. During the follow-up period the combination treatment was considered safe, and there were no safety concerns raised. The study included a 26 wk off-drug observation period during which the effect of the treatment deteriorated rapidly, suggesting the need for continued treatment. Overall, this combination treatment seems to be a promising candidate for further evaluations in a phase III clinical trial.

MESENCHYMAL STEM CELL THERAPY IN T1DM

Characteristics of mesenchymal stem cells

Over the past two decades, stem cell transplantation has received increased attention in clinical trials as a promising therapy within regenerative medicine for T1DM. While the treatment of T1DM with hematopoietic stem cells was more typical in the 2000s and first half of the 2010s[78], the most recent studies focus more on the treatment with mesenchymal stem cells (MSCs)[13]. This modern approach to treat T1DM has several advantages over previous treatment options. MSC transplantation is hypo-immunogenic because the cells do not express costimulatory antigens (CD80, CD86, CD40, CD40L *etc*) nor major histocompatibility complex II and major histocompatibility complex I. MSCs allow both autologous and allogeneic transplantation, even without conditioning treatment[79].

MSCs can be easily cultured *in vitro* due to their high dividing capacity, and they can be isolated from many adult and perinatal sources (*e.g.*, bone marrow, adipose tissue, peripheral blood, dental pulp, skeletal muscle, liver, lung, umbilical cord blood, Wharton's jelly and placenta)[80]. Of these, the umbilical cord and its derivatives stand out as they can be obtained non-invasively, are considered as 'medical waste' and have an exceptional differentiation capacity towards insulin-secreting cells[81]. Unlike embryonic stem cell therapy, the use of these tissue sources does not raise special ethical issues.

In addition, MSCs have no known tumorigenic effect, whereas embryonic stem cells can form teratomas and teratocarcinomas *in vivo*[82]. However, tumorigenesis cannot be completely ruled out as a possible adverse effect: MSCs may be a direct source of malignant cells, may maintain various cancer processes (*e.g.*, breast and colon cancer) through paracrine factor secretion or may enhance tumor

growth and progression through their immunosuppressive effects[83-85]. However, to the best of our knowledge, no similar side effects have been reported in clinical trials using MSCs[86].

MSCs are capable of generating tissue types of mesodermal origin, such as musculoskeletal, cartilaginous and adipose tissue, and may cross the boundaries of germ layers and transdifferentiate into ectodermal neurons or even endodermal islet cells[87,88]. Low amounts of MSCs in the target tissue do not explain regeneration or even wound healing. However, *in vivo* experiences show that MSCs have other, more pronounced therapeutic effects, such as remodeling of the diabetic microenvironment[89, 90]. It should be noted that in systemic administration, MSCs are entrapped in capillaries, especially in the lungs, reducing the number of migrating cells towards the target tissue, suggesting that better outcome could be obtained through local injection[91].

Immunoregulatory function of MSCs in T1DM

The strong immunoregulatory function of MSCs plays a key role in the regeneration of β cells. This protective effect in T1DM is due to secretion of soluble factors and cell-cell interactions. Insulin deficiency and irreversible β-cell destruction are the consequences of the autoimmune reaction in T1DM, and MSCs are able to intervene at several points in this process, modulating immune cells. MSC transplantation with its paracrine effects, due to the production of cytokines, chemokines and growth factors, can affect the local environment, inhibit apoptosis and induce proliferation. The identified bioactive factors are: IL-6, IL-8, transforming growth factor β, vascular endothelial growth factor, hepatocyte growth factor and nitric oxide[13].

Two types of MSCs are known: proinflammatory MSCs (MSC1) and anti-inflammatory MSCs (MSC2). The type of polarization depends on the inflammatory milieu. In the absence of an inflammatory environment MSCs adopt a proinflammatory phenotype and amplify T cell responses. Conversely, in an inflammatory environment (high interferon-γ and tumor necrosis factor α levels), MSCs may adopt an immunosuppressive phenotype and suppress T cell proliferation *via* secreted soluble factors[92]. MSCs have a regulatory function against effector T cells. In the pathogenesis of T1DM, Th1 cells are the main effector cells, and Th2 cells have been shown to be protective.

Beneficial effects of MSCs in diabetes can be attributed to: (1) Secreted IL-4; (2) Altered Th1/Th2 ratio with a Th1 to Th2 shift; and (3) Promoted maturation of naïve T cells towards Th2[93]. Furthermore, MSCs can directly and indirectly inhibit through several pathways: (1) Th17 cell development and thus IL-17 production; (2) CTL function and thus Fas-mediated β-cell apoptosis; and (3) Both maturation and activation of antigen-presenting cells, principally dendritic cells, by secreting for example prostaglandin E2, IL-6 and macrophage colony-stimulating factor[13,94,95].

Two types of macrophages are known: M1 and M2 producing proinflammatory and anti-inflammatory cytokines, respectively. MSCs can modulate the phenotype shift, causing an M1 to M2 shift[96]. Treg cells are components of MSC-induced indirect immunosuppression. *In vivo* and *in vitro*, MSCs have been shown to enhance Treg proliferation through cell-cell interaction[13]. By producing IL-10 and transforming growth factor β, Treg cells downregulate Th1- and Th17-mediated inflammatory response and the cytotoxicity of CTLs, thereby leading to immune tolerance in the organism[97]. These mechanisms can contribute to both amelioration of auto-reactivity and of β-cell death (Figure 3).

Clinical application of MSC therapy for the treatment of T1DM

In recent years, MSCs have attracted the attention of many researchers and clinicians as a result of encouraging preclinical animal data in T1DM. The most important advantages are: (1) Wide range of sources; (2) Self-renewal capacity; (3) Multidifferentiation capacity; and (4) Strong immunomodulatory potential. MSCs are also immunoprivileged, well-tolerated and safe[98]. The clinical studies vary in MSC origin, dose, route of transplantation, administration frequency and in eligible patients' characteristics (Table 1).

Hu *et al*[99] studied the long-term effects of Wharton's jelly-derived MSC in newly diagnosed T1DM patients. Group 1 was treated with parenteral solution of Wharton's jelly-derived MSCs by intravenous delivery, while the control group received normal saline. In the treatment group HbA_{1C} reached its lowest value after half a year and then began to fluctuate. Fasting C-peptide showed a progressive increase, reaching its maximum after 1 year; 3/15 patients were insulin-free, and 8 had their insulin dose halved after 2 years. As the study follow-up period lasted 2 years, exceeding the average 1.5 year honeymoon period, the therapeutic effect was due to MSCs[99]. This was one of the first studies to prove the safety and effectiveness of MSCs.

Thakkar *et al*[100,101] used the combination of adipose-derived insulin-secreting mesenchymal stem cells and bone marrow-derived (BM-) hematopoietic stem cells, comparing autologous (group 1) and allogeneic (group 2) stem cells. The study procedure was as follows: Resection of adipose tissue from the abdominal wall, collected in proliferation medium, bone marrow aspiration, conditioning treatment with bortezomib, methylprednisolone, ATG, and finally injection of the mixed inoculum. Autologous stem cell therapy offered better long-term control of hyperglycemia, but the two groups fairly differed in baseline mean C-peptide levels[100]. Although the two treatment methods showed significant differences in carbohydrate metabolism, the results before and after stem cell therapy were not statistically analyzed within the groups, thus lacking conclusive information about the efficacy of MSCs. As an early-result, the group reported preliminary data of 10 patients[101]. After an approximately 3-year

Table 1 Summary of clinical trials using mesenchymal stem cells in type 1 diabetes mellitus

Ref.	Patient characteristics	Treatment	Therapeutic outcomes
Wu *et al*[105], 2022[1]	*n* = 14; aged 27-47 yr	Intrapancreatic: Allogeneic UC-MSC + autologous BM-MNC	Insulin independence: No
China, 8 yr	Duration of T1DM: 10-24 yr		Insulin requirement: Improvement at 1 yr but no difference at 8 yr
			FCP and HbA_{1C}: Significant improvement
			Significantly lower occurrence of diabetic complications
Izadi *et al*[108], 2022	*n* = 20; aged 8-40 yr	BM-MSC	Insulin independence: No
Iran, 12 mo	Duration of T1DM: < 1 yr (*n* = 11) and > 1 yr (*n* = 9)		Insulin requirement, FCP, HbA_{1C}: Significant improvement
	FCP (*n* = 11): 0.92 ± 0.57 ng/mL		Number of hypoglycemic events decreased
			Patients with early onset of T1DM benefit more
			Adverse effects: Possible mild injection site reactions
Lu *et al*[106], 2021	*n* = 27; aged 8-55 yr	IV (2x): Allogeneic UC-MSC	Insulin independence: 3 subjects
China, 12 mo	Median duration of T1DM: 2.3 mo		Insulin requirement, HbA_{1C}: No improvement
	FCP: 100 pmol/L (0.3 ng/mL)		SCP: improved in adult-onset T1DM subgroup
			Adverse effects: Mild fever
Dantas *et al*[103], 2021[2]	*N* = 7; Aged 16-35 yr	Allogenic AD-MSC + 2000 UI/d cholecalciferol	Insulin independence: 1 subject
Brazil, 6 mo	Duration of T1DM: ≤ 4 mo		Insulin requirement: Stable at 6 mo
	FCP: 0.80 ± 0.38 ng/dL		FCP and HbA_{1C}: Significant improvement
			Adverse effects: Transient headache, mild local reactions, immediate tachycardia, thrombophlebitis + other mild effects
Araujo *et al*[102], 2020	*n* = 8; aged 16-28 yr	Allogenic AD-MSC + 2000 UI/d cholecalciferol	Insulin independence: 2 subjects
Brazil, 3 mo	Duration of T1DM: ≤ 4 mo		Insulin requirement, HbA_{1C}: Decreased significantly at 3 mo
			FCP: Only initial improvements, with the same results at the 3-mo visit
			Adverse effects: Transient headache, mild local reactions, immediate tachycardia, thrombophlebitis + other mild effects
Cai *et al*[104], 2016	*n* = 21; aged 18-10 yr	Intra-pancreatic: Allogeneic UC-MSC + autologous BM-MNC	Insulin independence: No
China, 12 mo	Duration of T1DM: 2-16 yr		Insulin requirement, HbA_{1C}: Decreased significantly
	FCP: < 0.1 pmol/mL (< 0.3 ng/mL)		FCP: Markedly increased
			Adverse effects: Transient abdominal pain, bleeding
Carlsson *et al*[107], 2015	*n* = 9; aged 18-40 yr	IV: Autologous BM-MSC	Insulin independence: No
Sweden, 12 mo	Duration of T1DM: < 3 wk		Insulin requirement, HbA_{1C}, SCP: No significant improvement
	SCP: > 0.1 nmol/L (> 0.3 ng/mL)		Adverse effects: No
Thakkar *et al*[100], 2015	*n* = 20; aged 8-45 yr	Into portal + thymic circulation and subcutaneous tissue:	Insulin independence: No
India, 24 mo	Duration of T1DM: > 12 mo		Insulin requirement: Decreased

	2 groups with a mean C-peptide:	Group 1: Autologous IS-AD-MSC+ HSC	HbA_{1C}, C-peptide: Sustained improvement
	Group 1: 0.22 ng/mL	Group 2: Allogeneic IS-AD-MSC+ HSC	Adverse effects: No
	Group 2: 0.028 ng/mL		
Dave *et al*[101], 2015[3]	*n* = 10; aged 9-29 yr	Into portal + thymic circulation and subcutaneous tissue: autologous IS-AD-MSC+ HSC	Insulin independence: No
India, 27 mo	Duration of T1DM: 2-15 yr		Insulin requirement: Decreased
	Pre-IV C-peptide: 0.22 ng/mL		HbA_{1C}, C-peptide: Sustained improvement + significantly lower GADA levels
			Adverse effects: No
Hu *et al*[99], 2013	*n* = 15; aged < 25 yr	IV (2x): Allogeneic WJ-MSC	Insulin independence: 3 subjects
China, 24 mo	Duration of T1DM: < 6 mo	Control group: normal saline	Insulin requirement: 8 patients more than 50% reduction
	C-peptide: ≥ 0.3 ng/mL		HbA1C: Significantly decreased; FCP: Significantly increased
			Adverse effects: No

[1]Follow-up trial to Cai *et al*[104], 2016.
[2]Extension trial to Araujo *et al*[102], 2020.
[3]Preliminary data of Thakkar *et al*[100], 2015.
AD: Adipose-derived; BM: Bone marrow-derived; FCP: Fasting C-peptide; GADA: Glutamic-acid-decarboxylase antibody; HbA_{1C}: Glycated hemoglobin; HSC: Hematopoietic stem cell; IS-AD: Adipose-derived insulin-secreting; IV: Intravenous; MNC: Mononuclear cell; MSC: Mesenchymal stem cell; SCP: Stimulated C-peptide; T1DM: Type 1 diabetes mellitus; UC: Umbilical cord-derived; WJ: Wharton's jelly-derived.

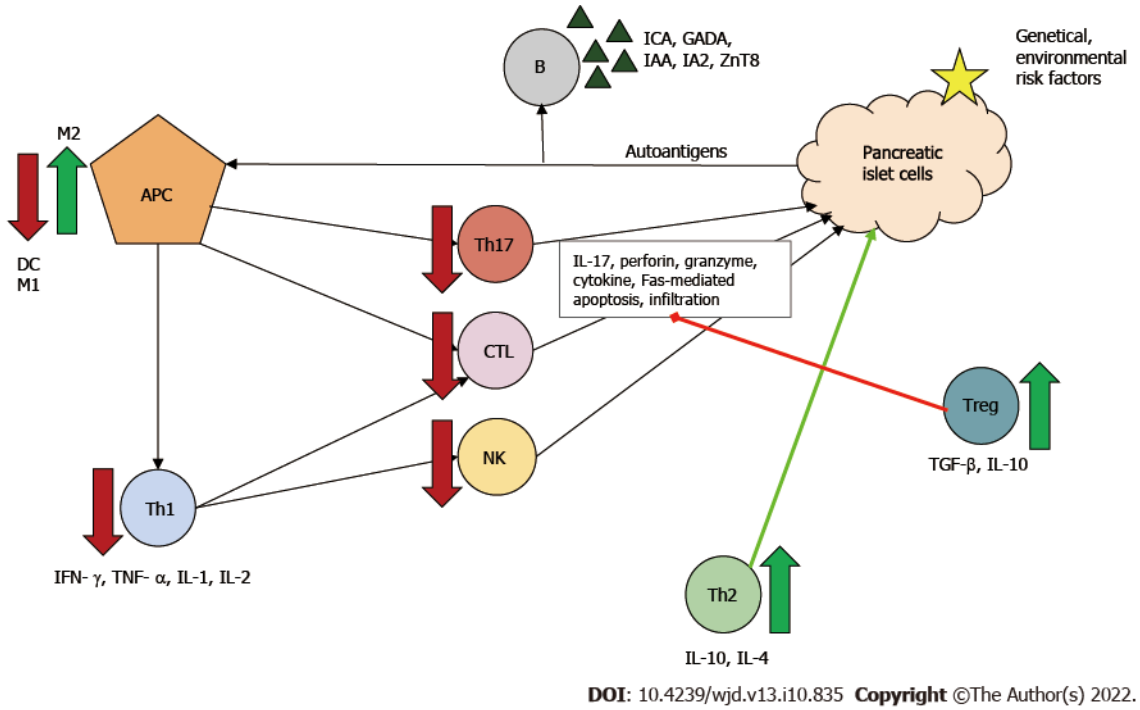

Figure 3 Immunoregulatory function of mesenchymal stem cells in type 1 diabetes mellitus. The effects of mesenchymal stem cells ultimately result in downregulation in the proliferation and function of dendritic cells, cytotoxic T lymphocytes (CTLs), helper T (Th) type 1 and type 17 cells, natural killer (NK) cells and type 1 macrophages (M1). Meanwhile, mesenchymal stem cells increase the number of type 2 macrophages (M2) and regulatory T cells (Tregs), which can inhibit effector T cells and stimulate Th type 2 protective cells. Red arrow/line: Downregulation/inhibition; Green arrow/line: Stimulation. APC: Antigen-presenting cell; B: B cell; DC: Dendritic cell; GADA: Glutamic-acid-decarboxylase antibody; IA2: Islet tyrosine phosphatase 2 antibody; IAA: Insulin autoantibody; ICA: Islet cell antibody; IL: Interleukin; IFN-γ: Interferon γ; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α; ZnT8: Zinc transporter 8 antibody.

follow-up, increased C-peptide secretion, decreased exogenous insulin requirement, improved HbA_{1C} and significantly lower GADA levels have been found.

Similar results were obtained in the studies of Araujo *et al*[102] and Dantas *et al*[103], where a single dose of adipose-derived MSC infusion were combined with daily 2000 IU vitamin D_3 supplementation. Compared to control subjects on traditional treatment, improved HbA_{1C} levels and reduced insulin doses have been found 3-mo after MSC infusion[102], while basal C-peptide levels remained the same at first but significantly improved for the 6-mo follow-up measurement[102,103]. It has to be mentioned though that most patients reported transient headache and local reactions, and further mild but resolving adverse events were also reported by a significant amount of the study population. Although the results of this study further strengthened the efficacy and safety of adipose-derived MSCs, all positive effects were somewhat overshadowed by the significant number of adverse events.

Cai *et al*[104] investigated the safety and efficacy of umbilical cord-derived MSC (UC-MSC) and autologous BM-mononuclear cell cotransplantation in adult patients. The treatment group received octreotide as a prophylaxis, followed by stem cell infusion into the dorsal pancreatic artery. After 1 year, the C-peptide area under the curve during a 3-h oral glucose tolerance test increased by 105.7% in 20 of 21 responders compared to a 7.7% decrease in the control group showing the robust effect of the treatment against disease progression. Further importance of this trial was that immunological parameters were also assessed. GADA positivity remained unchanged, while IL-10 levels increased, and interferon-γ levels and adenosine triphosphate levels in $CD4^+$ T cells decreased. While the effect of MSCs may be less pronounced in this study due to reduced inflammatory signals in long-standing disease[104], it has to be mentioned that the long-term follow-up analysis of the study population have shown a significantly decreased occurrence of various diabetic complications. Furthermore, the UC-MSC treated patients still had clinically better HbA_{1C} and C-peptide levels, 8 years after the UC-MSC treatment, but the initial difference in insulin requirement leveled off[105]. The combined results of the original and follow-up study[104,105] indicate that UC-MSCs are good candidates for slowing down the progression of T1DM.

Lu *et al*[106] assessed the repeated transplantation of allogeneic UC-MSC in T1DM. The primary efficacy endpoint was clinical remission, defined as a 10% increase from baseline in the levels of fasting and/or postprandial C-peptide. After 1 year, 11 out of 27 in the UC-MSC-treated group maintained clinical remission, whereas only 3 out of 26 in the control group maintained clinical remission. The UC-MSC-treated group showed a decreasing trend in fasting and postprandial C-peptide. Three UC-MSC-treated adults became insulin independent and started using insulin again in 3-12 mo. Among adult-onset T1DM (≥ 18 years of age), UC-MSC treatment showed a protective effect on β-cell function but failed to be protective in juveniles. Three recipients had mild fever after UC-MSC infusion; all of them recovered within 24 h[106]. It seems UC-MSC therapy might be more beneficial for patients with adult-onset T1DM.

Carlsson *et al*[107] tested the efficacy of autologous BM-MSCs in newly diagnosed T1DM patients. Stems cells were harvested from the aspiration of the iliac crest and subsequently administered to the MSC-treated group as an intravenous infusion without premedication. HbA_{1C}, fasting C-peptide and insulin requirement were not significantly different compared to the control group[107]. In contrast, Izadi *et al*[108] found improved HbA_{1C} and C-peptide levels, a reduced number of hypoglycemic events and increased anti-inflammatory patterns. Furthermore, early BM-MSC transplantation (< 1 year after disease onset) further improved HbA_{1C} levels and C-peptide levels compared to those who received the transplantation > 1 year after disease onset[108], similar to that of UC-MSC.

Summarizing the available clinical study results of the stem cell therapies, the results about BM-MSC and adipose-derived MSC are more controversial, suggesting that these two therapies may be less effective than UC-MSC therapy in T1DM. It should be noted, however, that based on the results of the studies so far it is recommended to apply these treatments as early as possible. The earlier these treatments are introduced, the greater the preservation of the remaining β cells, thereby the reduction of external insulin requirement and the development of long-term complications can be elongated. Adipose-derived MSCs and UC-MSCs are currently under further investigated in NCT05308836[109] and NCT04061746[110], respectively.

CONCLUSION

In the management of T1DM the focus remains on the challenges of glycemic control and long-term complications, which could not been fully overcome by new technological advances. Recently, there has been a paradigm change in the treatment of T1DM. The goal now is to cure rather than identify a lifelong 'symptomatic treatment' with insulin supplementation. The crucial future may lie in disease-modifying therapeutic options, which could be used to preserve β cells in the presymptomatic phase of the disease and to cease the destructive autoimmune process as well as to regenerate β-cell function in the clinical phase.

Immunotherapy appears to be a promising disease-modifying therapy in T1DM. Different agents have the potential to target the major autoreactive immune pathways leading to T1DM. Therapies interfering with T cell activation seem to be the most favorable. Regenerative therapy is developing parallel with immunotherapy. MSC therapy stands out from other cell therapies. It is safe, with its

beneficial effects due to immune regulation. However, the clinical trials are limited in their conclusions due to the small patient numbers and short follow-up times. Standardized stem cell processing, transplantation protocols and dosage will be essential for future randomized, double-blinded clinical trials with large patient cohorts. Combining disease-modifying therapies with glucagon like peptide-1 analogues seem to increase efficacy and increase tolerability of interventions.

So far, neither immunotherapies nor stem cell therapies, when used alone, have had ultimate successes in altering T1DM disease course. Their common disadvantage is that their short-term therapy effects are transient. The future for disease-modifying therapies might be the individualized, long-term multimodal approach combining immune, incretin based and regenerative therapeutic options potentially by identifying biomarkers of responders for it to be used in routine clinical treatment.

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MINIREVIEWS

Advances in traditional Chinese medicine as adjuvant therapy for diabetic foot

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Abstract

Diabetes mellitus (DM) is a complex disease that often causes multiple systemic complications that have become a major international public health problem. Diabetic foot (DF) is one of the severe and frequent chronic complications of DM due to vascular lesions and neuropathy. DF ulcers (DFU) affect approximately 15% of people with DM and are the leading cause of death and disability. The prevalence and recurrence of DF are worrisome, and morbidity and mortality are also on the rise, which poses a substantial socioeconomic burden. Treating DF is difficult for clinicians and requires multidisciplinary cooperation, combining local and systemic therapy to reduce amputation and case-fatality rates. Traditional Chinese Medicine (TCM) has received extensive attention due to noticeable therapeutic effects and few adverse reactions. In recent years, research on DF treatment by TCM has been increasing, and further progress has been made. TCM includes oral medication, injectable preparations, and adjuvant therapy. This article reviews the relevant research on TCM-related adjuvant therapy for DF. We describe current progress in TCM in terms of external application, acupuncture, massage, acupoint injection, foot bath, fumigation, and moxibustion, as well as the mechanisms involved.

Key Words: Diabetes Mellitus; Diabetic foot; Foot ulcers; Traditional Chinese medicine; Wound healing

Core Tip: Diabetic foot (DF) is a serious complication of diabetes and has become a major global health problem. Despite the emergence of many new therapies, amputation and mortality rates remain high. Traditional Chinese Medicine (TCM) has proved effective for various diseases, and more studies have observed its value of Traditional as adjuvant therapy. We review the role of TCM adjuvant therapy for DF, including external application, acupuncture, moxibustion, massage, acupoint injection, foot bath, and fumigation.

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INTRODUCTION

Diabetes mellitus (DM) is a complicated metabolic disorder caused by various pathogenic factors, and the main clinical feature is abnormally elevated blood glucose[1]. The American Diabetes Association divides diabetes into types 1 diabetes, type 2 diabetes, specific types of diabetes due to other causes, and gestational DM[2]. The prevalence of DM in virtually all world regions has increased significantly in recent decades. The estimated number of people with DM worldwide was 451 million in 2017. Approximately 1 in 11 adults has DM, and 90% of them have type 2 diabetes; that number is expected to increase to 693 million by 2045[3,4]. The main goal of therapy for type 2 diabetes is to prevent or delay complications and maintain quality of life[5]. There are many complications of DM, such as cardiovascular disorders[6], end-stage renal disease[7], retinopathy[8], neuropathy[9], mental illness[10], muscle atrophy[11], adhesive capsulitis[12] and even joint stiffness following surgery[13,14]. Diabetic foot (DF) is a frequent complication of DM due to vascular and neuropathological damage and is the main reason for amputation and death[15]. About 15% of people with DM suffer from DF ulcers (DFUs), and 14%-24% of those with DFU subsequently undergo lower limb amputation, which has led to DFU being the leading cause of non-traumatic lower limb amputations[16]. The 5-year mortality after amputation is 50%-59%[17], which is higher than the 5-year pooled mortality rate for cancer, which is 31.0%[18]. The global prevalence of DFUs is 6.3%, and in North America, this figure is 13.0%[19]. Moreover, DFU has a recurrence rate of 22.1% per person per year[20]. The direct cost of DM care in the USA in 2017 was US$237 billion, of which one-third was for lower extremity complications[18]. Healthcare expenditure for DF care is even more in the UK than for breast, prostate and lung cancer combined[21]. These data prove that DF has become a serious international medical and health problem. Therefore, understanding the pathogenesis of DF and developing targeted treatment is a major concern to clinicians.

DF is prone to ulceration and infection due to neuropathic edema and occlusive arterial disease[22]. DFU is caused by various factors, including peripheral neuropathy, foot deformity and trauma, and arterial disease[23]. In addition, DFU development was linked with a previous history of DFU and the male sex[24]. The precise mechanism of the delayed healing of DFU has not been fully elucidated. Wound healing is one of the most complex processes in the human body, mainly including four phases (hemostasis, inflammation, growth, re-epithelialization, and remodeling). Each stage has no recognizable boundaries and overlaps in time and space[25]. In DFU, however, extensive defects in the healing process result in ulcer healing delay and the occurrence of a highly pro-inflammatory chronic wound[26]. The major causes may be insufficient neovascularization, neuropathy, high probability of infection, tissue hypoxia[27], and nonphysiological inflammatory response[28]. They may also include an imbalance between metabolism and nutrient transport, abnormal cellular and gene expression, excessive formation of advanced glycation end products (AEGs)[29], and high concentrations of metalloproteases[30]. Pathologically, DFU has been found to decrease endothelial progenitor cell (EPC) recruitment due to reduced NO production[31]. Deficiency of cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, keratinocyte growth factor (KGF), and platelet-derived growth factor (PDGF) are also associated with delayed DFU wound healing[29]. Furthermore, the dysfunction of the immune system in DM increases the rate of ulcer infection[32], and the frequency and severity of the infection are associated with delay and failure of the healing process [33].

Standard therapy of DFU includes decompression and ulcer protection, tissue perfusion repair, infection and metabolic control, local ulcer care, and education of patients and families[34]. TCM is the main form of ancient Asian medicine and an essential element of the Chinese health system[35], which is commonly used in clinical work in China[36]. It has accumulated a solid theoretical foundation in practice for thousands of years[37]. Chinese herbal medicine decoction is the essence of TCM, which has the characteristics of a multitarget, fewer side effects, and significant therapeutic effects[38]. With the

advent of bioinformatics, the specific mechanism of TCM has been more scientifically explained[39]. Research on TCM for chronic non-communicable diseases has recently developed rapidly[40]. In particular, studies have reported that oral administration or injection of TCM herbal-based agents as an additional treatment to conventional therapies is beneficial to DFU healing[41,42]. Meanwhile, in recent years, complementary modalities have also demonstrated therapeutic potential. These methods include external application, acupuncture, massage, acupoint injection, foot bath, fumigation, and moxibustion. Therefore, a comprehensive search was conducted in the PubMed, Web of Science, and National Knowledge Infrastructure (CNKI) to investigate the value of TCM adjuvant therapy in DF. The electronic search was performed for articles published from inception to June 20, 2022. The search terms were used individually or combined: "Traditional Chinese Medicine," "Diabetic foot," "Diabetes Foot," "Diabetic Patients with DF," "Diabetes Feet," "DF," "External application," "Dressing," "Acupuncture," "Pharmacoacupuncture," "Moxibustion," "Massage," "Acupressure," "Knead," "Acupoint injection," "Acupuncture point injection," "hydro-acupuncture," "Foot bath," "Lavipeditum," "Soak," "Medicated bath," "Fumigation." Reference lists of relevant articles were also hand searched. In addition, we made appropriate modifications according to actual requirements.

EXTERNAL APPLICATON

Using plaster or compounds of TCM for external application is an efficient and straightforward treatment method. Compound Phellodendron liquid, which consists of Forsythia, Phellodendron, Honeysuckle, Dandelion, and Centipede, is one of the TCM for external application. Network pharmacology analysis shows that it contains 36 active ingredients related to DF. Functionally, the potential mechanisms of action are mainly related to inflammatory response and growth factor activity[43]. When DFU was treated with Compound Phellodendron liquid for four weeks, the ulcer area reduction, growth factor concentration, and total effective rate in the treatment group were higher than the standard nursing group[44]. Zhong *et al*[45] prepared a mixed ultramicro powder with Angelica, Calcined Gypsum, and Caleramide as raw materials, which promoted wound healing in DFU by accelerating wound closure and epithelialization, and inducing angiogenesis. Similarly, external application of the Chinese herbal medicine compound Tangzu Yuyang Ointment combined with standard wound treatment improved the rate of DFU healing. However, the healing time appeared to be prolonged[46].

In rats with DFU, Chinese medicine ulcer oi (Cortex Phellodendri and Angelica japonica as the main ingredients) upregulated the expression of VEGF and PDGF and downregulated protein tyrosine phosphatase 1B and AGEs in the wound tissue[47]. This indicated that Chinese medicine ulcer oil reduced local wound inflammation, promoted angiogenesis, and facilitated ulcer healing. Shixiang ointment promoted angiogenesis and accelerated ulcer healing by reducing AGEs and their receptors, activating nuclear factor kB p65, and upregulating VEGF, CD34, and endothelial NO synthase in the granulation tissue of DFU rats[48]. Wan *et al*[49] proved that San-huang-sheng-fu oil reduced cyclooxygenase-2 and upregulated VEGF and improved the decrease in plantar temperature and pain sensation in rats caused by the diabetic peripheral circulatory disorder. Similarly, another agent, Jing Wan Hong Ointment, elevated PDGF expression in a DFU murine model, enabling almost complete ulcer healing *via* retarding inflammation and promoting cell proliferation and angiogenesis[50].

The common factors involved in the beneficial effects of external application of TCM in DF treatment rely on VEGF and PDGF to promote angiogenesis, cell proliferation, and inhibition of local inflammatory response.

ACUPUNCTURE

Acupuncture is essential to TCM and has been used for thousands of years against many disorders, including vascular diseases. There are many modalities of acupuncture, such as encircling needling, Bangci (focal center-side needling), auricular acupuncture, pestle needling therapy, and traditional acupuncture[51]. After auricular acupuncture treatment in type 2 DM patients, the blood flow of the lower extremities is improved, and the temperature of the soles of the feet increases, showing a preventive effect against DF[52]. Pestle needling therapy can decrease the foot vibration perception threshold and improve the sensory nerve function of the foot and the quality of life in high-risk DF[53].

Wei *et al*[54] compared the efficacy of encircling needling and Bangci (focal center-side needling) in wound healing of mice with DM. Both promoted skin wound healing by increasing local blood perfusion, and the therapeutic effect of encircling needling was better than Bangci. Mechanistically, acupuncture may reduce the protein levels of proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β and increase neovascularization and fibroblasts in the wound[55]. These outcomes indicate that acupuncture can promote wound healing by reducing inflammation, promoting cell proliferation and angiogenesis, and inducing extracellular matrix remodeling.

Acupuncture benefits DF not only for its therapeutic effects but also demonstrates some preventive effects. It is important because reducing the incidence will significantly reduce the cost of DF treatment.

MOXIBUSTION

The mechanism of moxibustion-based therapy is similar to that of acupuncture, and it has complementary therapeutic effects to acupuncture. It has been verified that the smoke and heat of moxibustion have a role in promoting wound healing *via* inhibiting the inflammatory response and promoting the formation of collagen fibers, granulation tissues, and capillaries[56]. The expression of TGF-β in wound tissue is significantly increased after moxibustion intervention, indicating the promotion of fibroblast proliferation and rapid formation of granulation in the early stage[57]. After six moxibustion interventions in a rat model, Kan *et al*[58] found that fibroblasts and collagen fibers in the wound tissue were more closely arranged, and neovascularization was richer. They demonstrated that moxibustion ended the inflammatory stage by regulating proinflammatory cytokines and initiated the repair stage in advance. Moreover, the content of VEGF and VEGF in the serum of rats after the intervention was significantly increased[59]. However, some studies have raised doubts. Alonso *et al*[60] observed that acupuncture and moxibustion downregulated TGF-β1 and VEGF in adult female Wistar rats, but they still believed that moxibustion and acupuncture could stimulate fibroblast proliferation and neovascularization. Although many scholars have reported that moxibustion can promote wound healing, there is still no unified statement on its specific mechanism. Some scholars have even come to the opposite conclusion. Therefore, more basic and clinical research is needed to unveil the specific mechanism and justify the efficacy of moxibustion.

MASSAGE

Before the appearance of TCM decoction, ancient Chinese people started to use massage for disease prevention and treatment. Since massage needs to be administered at specific locations, when there are ulcers, massage will increase the pain and the risk of infection, so massage is mainly used for adjuvant treatment of diabetic peripheral neuropathy (DPN) and early DF. DPN is a significant risk factor for DF [61]. Nerve conduction studies are considered the gold standard in clinical research for DPN. Nerve conduction velocity (NCV) detects peripheral nerve conduction dysfunction caused by segmental demyelination and axonal damage and is usually slowed in DPN[62]. A recent meta-analysis of 3284 patients showed that TCM bath combined with acupoint massage improved the sensory and motor NCV and decreased neurological syndrome score in DPN[63].

Massage even improved the general condition of DM patients. Zarvasi *et al*[64] found that blood glucose significantly decreased and insulin levels increased after the self-acupressure intervention. After three years of acupressure treatment, the levels of total cholesterol, triglyceride, and low-density lipoprotein-cholesterol significantly decreased, and the level of high-density lipoprotein-cholesterol increased[65]. Massage appears to be beneficial not only for DF but also for the control of hyperlipidemia. It should be noted that since it is challenging to perform massage in animals, the specific mechanisms are hard to reveal.

ACUPOINT INJECTION

Acupoint injection is a common treatment method in TCM. Either injection of Chinese herbal extracts (*e.g.*, Danshen injection and Fufang Danggui injection) or conventional medicines (*e.g.*, mecobalamin, vitamin B1, and anisodamine) at specific acupoints are available[66]. Applying electroacupuncture after methylcobalamin injection at Sanyinjiao (SP6) can restore ulnar and tibial nerve motor NCV and sensory NCV in patients with DPN[67]. A systematic review of 1071 Chinese DPN patients showed that acupoint injection of Chinese herbal extracts at Zusanli (ST36) was safe and may reduce pain and improve nerve afferent velocity compared with intramuscular injection of the same drug[66]. However, the trials included in this review were of low quality. Therefore, higher quality clinical trials are necessary to delineate the safety and efficacy of acupoint injection as adjuvant therapy for DF.

FOOT BATH

TCM foot bath using decoctions increases blood circulation for Grade 0 DF[68]. Additionally, herbal foot baths can improve local microcirculation and regulate skin permeability to increase drug absorption, thus effectively increasing drug concentration[69,70]. Recently, clinical trials have been designed to

examine the efficacy and safety of TCM foot baths[68,71]. Nevertheless, foot baths can spread infection at the ulcer site in patients with chronic limb ulcers and increase the rate of toe loss (53%) and major amputation (30%)[72]. Consequently, the choice of foot bath treatment for DF needs to be carefully considered by clinicians due to its double-sided effect.

FUMIGATION

Chinese herbal fumigation is a kind of external treatment of TCM, which can relax muscles and tendons and remove obstructions from meridians, activating blood to eliminate stagnation[73]. Cuyuxunxi prescription is a Chinese herbal fumigant widely applied to wash surgical wounds after anal fistulotomy, potentially promoting wound healing and antagonizing infection[74]. Zhuyuan decoction fumigation is an effective treatment to relieve the symptoms of patients with chronic sinusitis[75]. In addition, fumigation reduces knee osteoarthritis swelling and pain by inhibiting the expression of pro-inflammatory factors, promoting blood reflux, and reducing skin sensory nerve excitability[76]. Meanwhile, fumigation smoke and heat can promote wound healing in rats by inhibiting inflammatory responses and ameliorating the formation of collagen fibers, granulation tissue, and capillary status[56]. An ongoing systematic review will evaluate the effectiveness and safety of TCM fumigation in DPN [77]. Fumigation may be an effective therapeutic measure for DFU due to its anti-infective, inflammation-inhibiting, and wound-healing effects.

PROSPECTIVE

DF can be divided into neurologic, ischemic, or neuroischemic according to the International Working Group on the Diabetic Foot (IWGDF)[78] while The Society for Vascular Surgery Lower Extremity Threatened Limb (SVS WIfI) classification system classifies DF into four grades: Grade 0, 1, 2, and 3 (Table 1)[79]. For grade 2 or 3 DF, amputation and hemodynamic reconstruction are often required. Therefore, TCM adjuvant therapy is mainly used for grades 0 and 1 DF. As shown in Figure 1, for grade 0 or 1 DF caused by neuropathy (usually DPN), foot bath, acupoint injection, and massage are optional treatment modalities because they can accelerate the sensory and motor NCV in the lower limbs. External application, moxibustion, fumigation, acupuncture, massage, and foot bath can increase blood flow in the lower extremities and promote neovascularization in the local wound for ischemic grade 0-1 DF. For neuroischemic grade 0 or 1 DF, massage, footbath, or a combination of other adjunctive therapies, can be chosen. For all DF (including grades 2 and 3), massage is an optional adjunctive therapy that regulates local and systemic metabolism (including blood glucose and lipids). In addition, topical application, moxibustion, and acupuncture can promote wound healing in grade 1 DF, and fumigation may be an effective anti-infection modality when local infection occurs. In conclusion, selecting appropriate TCM adjunctive therapy for early DF(grades 0 and 1) will positively affect patients, but it should be noted that foot baths may lead to skin maceration and increase the rate of amputation.

Chinese herbal medicine treatment is distinguished by its multi-target and multi-level nature. With the application of network pharmacology to the study of TCM herbal formulations in recent years, the active constituents of herbal medications and their unique targets of action have been discovered, providing a theoretical foundation for their clinical use[80]. Future studies may focus on new wound dressings utilizing medicinal plant extracts or their purified active components[81]. Nonetheless, we must not overlook the fact that the precise mechanism of action of TCM requires additional investigation. In addition, there is no research on the effectiveness of TCM in preventing DF. “Treating the untreated” has been a critical area of concern for TCM, and scientific randomized controlled trials (RCTs) can be used to confirm its risk-benefit ratio in the prevention of DF is also necessary.

CONCLUSION

DF is a common complication of diabetes. There are many adjunctive therapies in TCM that can be applied to DF. Some have been proven effective, while others require more research. Animal experiments have confirmed that TCM adjuvant therapy can promote DFU wounding healing by inhibiting nonphysiological inflammation *via* down-regulating AGEs, RAGE, TNF-α, and IL-1β, promoting neovascularization *via* up-regulating VEGF and PDGF, inducing extracellular matrix remodeling, improving local blood circulation, and accelerating the production of collagen fibers and granulation tissue. In the future, more high-quality research is needed to demonstrate and popularize the application of TCM adjuvant therapy in DF.

Table 1 Society for Vascular Surgery Lower Extremity Threatened Limb (SVS WIfI) classification system

Grade	Ulcer	Gangrene
0	No ulcer	No gangrene
1	Small, shallow ulcer(s) on distal leg or foot; no exposed bone, unless limited to distal phalanx	No gangrene
2	Deeper ulcer with exposed bone, joint or tendon; generally not involving the heel; shallow heel ulcer, without calcaneal involvement	Gangrenous changes limited to digits
3	Extensive, deep ulcer involving forefoot and/or midfoot; deep, full thickness heel ulcer ± calcaneal involvement	Extensive gangrene involving forefoot and/or midfoot; full thickness heel necrosis ± calcaneal involvement

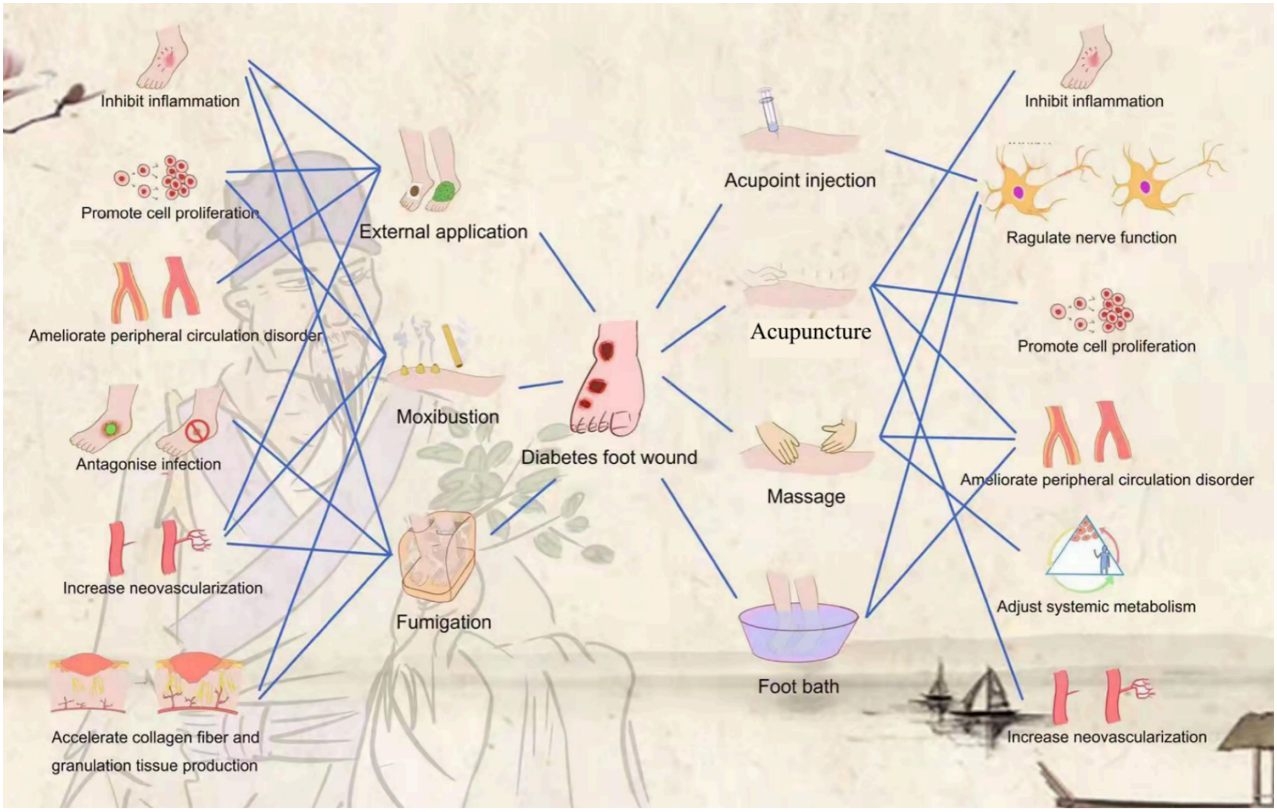

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Figure 1 Categories of traditional Chinese medicine as adjuvant therapy for diabetic foot wound and related mechanisms.

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FOOTNOTES

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Correlation between gut microbiota and glucagon-like peptide-1 in patients with gestational diabetes mellitus

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) places both the mother and offspring at high risk of complications. Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of GDM. However, it is still unclear whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients.

AIM

To explore the correlation between the gut microbiota and blood biochemical traits, particularly GLP-1, in GDM patients.

METHODS

The V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was sequenced

based on the fecal samples of 35 pregnant women with GDM and was compared to that of 25 pregnant women with normal glucose tolerance (NGT).

RESULTS

The results showed that *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Spearman's correlation analysis was performed to identify the relationships between microbiota genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were significantly negatively correlated with glucose. *Ruminococcaceae_UCG-002* was significantly negatively correlated with hemoglobin A1c. *Bacteroides* was significantly positively correlated with glucose. *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were significantly positively correlated with GLP-1. A random forest model showed that 20 specific genera plus glucose provided the best discriminatory power, as indicated by the area under the receiver operating characteristic curve (0.94).

CONCLUSION

The results of this study reveal novel relationships between the gut microbiome, blood biochemical traits, particularly GLP-1, and GDM status. These findings suggest that some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM.

Key Words: Gut microbiome; Glucagon-like peptide-1; Gestational diabetes mellitus; Glucose

Core Tip: Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of gestational diabetes mellitus (GDM). However, it is still unclear whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients. To the best of our knowledge, this is the first study to analyze the relationship between GLP-1 and the gut microbiota in patients with GDM, and this is the first report on the relationship between *Paraprevotella*, *Roseburia*, and *Faecalibacterium* and glucose in GDM, and the first report on the associations between GLP-1 and genera including *Sutterella, Oscillibacter*, and *Bifidobacterium* in GDM.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as abnormal glucose tolerance in pregnancy[1]. It is one of the most common complications of pregnancy. The incidence of GDM has increased due to lifestyle changes, increasing maternal age, and changes to the GDM diagnostic criteria. The incidence of GDM is reported to be 13.20%. GDM is closely related to the occurrence of perinatal maternal and neonatal complications, and also significantly increases the risk of long-term metabolic diseases in pregnant women and newborns. The pathogenesis of GDM is complex and has not yet been comprehensively elucidated. Timely diagnosis and intervention are of great significance to the long-term health of pregnant women and their fetuses.

In recent years, with increased research and understanding of gastrointestinal hormones, their roles in the occurrence and development of GDM have attracted growing attention. Research suggests that GDM patients exhibit insufficient glucagon-like peptide-1 (GLP-1) secretion during pregnancy and after delivery relative to their blood glucose level. Bonde *et al*[2] found that the postprandial GLP-1 level of pregnant women was decreased, especially in GDM patients. However, with the recovery of blood glucose homeostasis after delivery, postprandial GLP-1 secretion gradually returned to normal. Kosinski *et al*[3] confirmed that decreased GLP-1 in patients with GDM is reversible. Changes in GLP-1 levels may be related to insulin resistance (IR) as a result of high blood glucose levels. However, it

cannot be ruled out that changes in GLP-1 levels may be involved in the occurrence and development of GDM.

Evidence indicates that the gut microbiota is closely related to GDM[4]. The mechanism underlying the effect of probiotics in diabetes has not yet been fully elucidated, but it may be related to reductions in oxidative stress, regulation of the immune response, reductions in inflammation, and regulation of the gut microbiota[5,6]. In addition, probiotics can also reduce postprandial blood lipid levels and improve the absorption of antioxidants, which are related to oxidative stress[7]. Numerous studies have demonstrated that GLP-1 has insulin tropic and antioxidant effects[8-10]. Since GLP-1 and the gut microbiota each play roles in GDM, is there a correlation between the two?

Both clinical and animal studies have reported correlations between changes in the GLP-1 level and changes in the gut microbiota after gastrointestinal bypass surgery in type 2 diabetes mellitus (T2DM) patients or mice[11]. Therefore, it is speculated that GLP-1 may regulate blood glucose by regulating the number and structure of gut microbiota. Several authors have argued that GLP-1 may play a role in regulating blood glucose by increasing the diversity of the gut microbiota[12] and increasing the proportion of probiotics. Together, the current literature provides a comprehensive explanation of the hypoglycemic mechanism of GLP-1 and a reliable experimental basis for the study of GDM therapeutic targets and therapeutic drugs based on GLP-1. Accordingly, one study found that bifidobacteria improved insulin sensitivity by increasing the production of GLP-1[13].

The rapid increase in the prevalence of GDM in recent years cannot be easily explained by genetic factors; thus, it has been hypothesized that environmental factors may play a more important role. The gut microbiota constitutes an important environmental factor. GLP-1, as the most important representative of gastrointestinal hormones, may also be involved in the pathogenesis of GDM. The gastrointestinal microbiota and gastrointestinal hormones share the same root, are inseparable, influence and restrict each other, and jointly participate in the occurrence, development, and prognosis of GDM. Thus, a comprehensive study of the correlations between changes in the gut microbiota and GLP-1 will help to further clarify the pathogenesis of GDM. This is of great significance for the prevention, treatment, and prognosis of GDM, and may provide a novel and sensitive index for the clinical evaluation of GDM.

MATERIALS AND METHODS

Subjects

Patients were screened for GDM in the obstetric outpatient department according to the GDM diagnostic criteria (2014). Thirty-five patients with GDM were randomly selected from the patients who met the diagnostic criteria for GDM; these patients formed the GDM group. A further 25 pregnant women with normal glucose tolerance (NGT) were selected as the NGT group. Each subject provided written informed consent before inclusion in the study. The study was approved and carried out in accordance with the guidelines of the Ethics Committee of Nanhai District People's Hospital of Foshan.

The inclusion criteria were as follows: 18-45 years old, being female, and any education level. Based on the diagnostic criteria for gestational diabetes (2014), during the 24-28th week of gestation, the 75 g oral glucose tolerance test (OGTT) was used to measure each patient's blood glucose levels before, 1 h after, and 2 h after consuming sugar. If the patient's blood glucose level reached or exceeded 5.1, 10.0, or 8.5 mmol/L, respectively, they were deemed to have GDM and were eligible for inclusion in the GDM group.

The exclusion criteria were as follows: (1) History of chronic digestive system disorder; (2) history of treatment with GLP-1 analogues or GLP-1 receptor agonists; (3) history of cardiac, renal, or liver dysfunction; (4) multiple pregnancy; (5) pregnancy-induced hypertension syndrome, placental insufficiency, placenta previa, placental abruption, pelvic or soft birth canal abnormalities, or other pregnancy complications; (6) history of mental disorders; (7) exposure to a large amount of radiation, chemical poisons, or drugs that can affect the fetus during pregnancy; (8) tumor history or history of radiotherapy and chemotherapy within the past 6 mo; (9) participation in other research studies; (10) patients lost to follow-up due to various reasons, including the occurrence of other serious diseases during the study; and (11) consumption of antibiotics or probiotics within 1 mo prior to admission.

Sample collection and testing

Fresh fecal samples were collected from the participants and immediately frozen in a refrigerator at -80 °C. After collection of all samples, they were sent to the Treat Gut company for 16S rDNA sequencing.

Blood samples were collected after fasting and then 1 h and 2 h following consumption of sugar. Plasma glucose (Glu), glycosylated serum protein (GSP), low-density lipoprotein (LDL), uric acid (UA), hemoglobin (HB), total cholesterol (TCH), triglyceride (TG), and high-density lipoprotein (HDL) were determined with a Beckman AU5800 fully automatic biochemical analyzer. Glycosylated hemoglobin A1c (HbA1c) was determined using an ADAMS™ A1c HA-8180 automatic glycosylated hemoglobin analyzer. Insulin (INS), thyroid-stimulating hormone (TSH), and free tetraiodothyronine (FT4) were measured with a Maglumi2000plus automatic chemiluminescence immunoanalyzer.

The active forms of GLP-1 in the plasma samples of patients with and without GDM were measured using a GLP-1 (active) ELISA kit (ELabscience, Wuhan, Hubei Province, China).

Bacterial 16S rRNA gene sequencing

The total genomic DNA of each sample was extracted using a fecal genomic DNA extraction kit (Tiangen Company). Sixteen S rDNA sequencing was performed by PCR amplification of V4 variable regions (39 to 297 base pairs), and a purified product library was established. The library construction steps followed the library construction method of the Illumina sequencing platform. The sequencing analysis was as follows. First, the Illumina Miseq 2 × 300bp paired-end sequencing data were analyzed. According to the barcode information, the samples were distinguished. Then, the data were merged, spliced, and filtered, and quality control analysis was conducted, including Q20 and Q30 scores. The final clean data were analyzed by operational taxonomic unit (OTU) cluster analysis and species taxonomy.

Microbiome data

The data were filtered using Mothur software and clustered into OTUs (species) at a similarity level of 97% using Quantitative Insights into Microbial Ecology (QIIME) software version 1.80[14]. Based on the OTU analysis, the Ace, Shannon, observed species, Simpson, Chao1, and J indices were calculated as alpha diversity metrics. To compare the microbial composition between the samples, beta diversity analysis was performed using principal component analysis (PCA) and principal coordinate analysis (PCoA). Analysis of similarities (ANOSIM) was applied to evaluate the statistical significance of differences between the groups. A linear discriminant analysis (LDA) effect size (LEfSe) method was employed to evaluate any differences in the gut microbe between the groups.

Statistical analysis

GraphPad Prism (version 7.0) and R version 3.0.2 (R Foundation for Statistical Computing) were using for statistical analyses. The measurement data are expressed as the mean ± SD. Differences between groups were analyzed using oneway ANOVA. The differences were considered statistically significant at $P < 0.05$. Random-forest classification was performed for discriminating the samples from different groups using the R package "random forest". The model was employed for five-fold cross-validation of the relative species abundance profile. Case probabilities were calculated by drawing receiver operating characteristic (ROC) curves.

RESULTS

Characteristics of the study population

GDM was diagnosed in 35 women based on fasting or oral glucose-stimulated hyperglycemia, or a combination of the two. Markers of glucose and insulin homeostasis were higher in the GDM group compared with the NGT group (Table 1). Individuals with GDM also had higher hemoglobin A1C ($P = 0.003$) and fasting blood glucose levels ($P < 0.001$). There were no significant differences in pre-pregnancy body weight, BMI, UA, TCH, TG, HDL, LDL, TSH, or FT4 between the two groups.

OTU distributions

In this study, the OTUs annotated included 14 phyla, 62 families, and 214 genera of gut microbiota; the similarity among samples was 97% (Figure 1A). The total number of OTUs of the NGT group (at the 97% similarity level) was 652, and for the GDM group, it was 619. Venn diagram shows that 560 OTUs were shared by the NGT and GDM groups (Figure 1B).

Alpha and beta diversities

The observed species index of the GDM group was significantly difference from that of the NGT group (25; $P = 0.044$). The Chao1 richness index of the GDM group was significantly different from that of the NGT group (43; $P = 0.004$). The ACE index of the GDM group differed significantly from that of the NGT group (25; $P = 0.0055$) (Figure 2). There were no significant differences in the Shannon, Simpson, or J indices between the GDM group and NGT group (Shannon, $P = 0.65$; Simpson, $P = 0.9$; J, $P = 0.91$; Figure 2A). PCA and PCoA indicated that the gut microbiota in GDM patients differed significantly from that of the NGT subjects. There was no difference in the gut microbiota structure between the groups (ANOSIM, $r = 0.019$, $P = 0.2232$). NMDS cluster analysis indicated marked differences between the GDM patients and NGT subjects (Figure 2B).

Taxonomy

The composition of gut microbiota was different between the groups at the phylum, family, and genus levels. At the phylum level, *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and *Tenericutes* were common phyla in both the GDM group and NGT group,

Table 1 Clinical variables of gestational diabetes mellitus patients and healthy controls

Variable	Control (25)	GDM (31)	*P* value
Age (yr)	28.42 (3.11)	30.18 (3.26)	0.055
Pre-body weight (kg)	52.42 (7.68)	55.18 (6.48)	0.165
Height (cm)	160.13 (5.44)	157.88 (4.10)	0.063
Pre-BMI	20.73 (2.85)	22.2 (2.37)	0.054
GLU (mmol/L)	4.49 (0.38)	5.77 (0.95)	3.53×10^{-7}
GLP-1 0 h (ug/L)	67.72 (22.89)	75.45(23.23)	0.223
GLP-1 1 h (ug/L)	75.33 (26.14)	84.34 (19.84)	0.099
GLP-1 2 h (ug/L)	71.75 (23.83)	79.21 (24.20)	0.312
OGTT 0 h (mmol/L)	4.47 (0.39)	5.74 (0.99)	3.53×10^{-7}
OGTT 1 h (mmol/L)	7.77 (1.55)	11.23 (2.95)	3.00×10^{-6}
OGTT 2 h (mmol/L)	6.26 (0.87)	9.64 (3.12)	3.96×10^{-6}
Insulin 0 h (uIU/mL)	9.78 (3.41)	14.03 (15.93)	0.239
Insulin 1 h (uIU/mL)	85.85 (43.99)	64.52 (39.67)	0.061
Insulin 2 h (uIU/mL)	57.51 (39.36)	66.52 (45.21)	0.675
GSP (mmol/L)	1.68 (0.35)	1.97 (0.62)	0.054
HbA1c (%)	5.04 (0.30)	5.79 (1.12)	0.003
UA (umol/L)	279.52(68.47)	263.80(81.27)	0.463
TCH (mmol/L)	5.40 (1.06)	5.35 (1.08)	0.993
TG (mmol/L)	1.82 (0.72)	2.45 (1.54)	0.051
HDL (mmol/L)	2.11 (0.52)	2.08 (0.63)	0.946
LDL (mmol/L)	2.41 (0.89)	2.34 (0.82)	0.739
TSH (uIU/mL)	1.82 (3.43)	1.22 (1.24)	0.337
FT4 (pg/mL)	11.39 (3.27)	11.77 (1.96)	0.598

OGTT: Oral glucose tolerance test; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; Glu: Glucose; GSP: Glycosylated serum protein; HbA1c: Hemoglobin A1c; UA: Uric acid; TCH: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TSH: Thyroid-stimulating hormone; FT4: Free tetraiodothyronine.

accounting for 98.81% and 98.58% of the gut bacteria of each group, respectively (Figure 3). The GDM group had a lower abundance of *Firmicutes* (31.4% *vs* 33.2%), *Verrucomicrobia* (0.18% *vs* 1.01%, $P < 0.05$), *Synergistetes* (0.003% *vs* 0.110%, $P < 0.01$), and *Tenericutes* (0.05% *vs* 0.08%), and a higher abundance of *Bacteroidetes* (63.50% *vs* 60.81%), *Proteobacteria* (3.03% *vs* 2.56%), and *Fusobacteria* (0.33% *vs* 0.29%), compared to the NGT group. The ratio of *Firmicutes* to *Bacteroidetes* was decreased in the GDM group compared to the NGT group (0.49 *vs* 0.54).

At the family level, a greater number of different families were identified between the two groups (Figure 4). Fifty-five and 54 of the dominant families were detected in the GDM group and NGT group, respectively. *Bacteroidaceae* (phylum *Bacteroidetes*), *Prevotellaceae* (phylum *Bacteroidetes*), *Acidaminococcaceae* (phylum *Firmicutes*), *Veillonellaceae* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Ruminococcaceae* (phylum *Firmicutes*), *Enterobacteriaceae* (phylum *Proteobacteria*), and *Tannerellaceae* (phylum *Bacteroidetes*) had the highest relative abundance in the GDM group, while *Bacteroidaceae*, *Prevotellaceae*, *Acidaminococcaceae*, *Veillonellaceae*, *Lachnospiraceae*, *Enterobacteriaceae*, *Ruminococcaceae*, and *Rikenellaceae* were the eight most abundant families in the NGT group.

The bacterial taxa whose levels differed significantly between the two groups were identified by LEfSE analysis (Figure 4). At the family level, *Atopobiaceae*, *Eggerthellaceae*, *Streptococcaceae*, *Christensenellaceae*, *Clostridiaceae*, *Bifidobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were significantly more abundant in the NGT group than in the GDM group.

At the genus level, bacterial genera exhibited significant differences between the two groups (Figure 5). In the NGT group, *Bacteroides* (phylum *Bacteroidetes*), *Prevotella_9* (phylum *Bacteroidetes*), *Phascolarctobacterium* (phylum *Firmicutes*), *Megasphaera* (phylum *Firmicutes*), *Megamonas* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Escherichia-Shigella* (phylum *Proteobacteria*), and

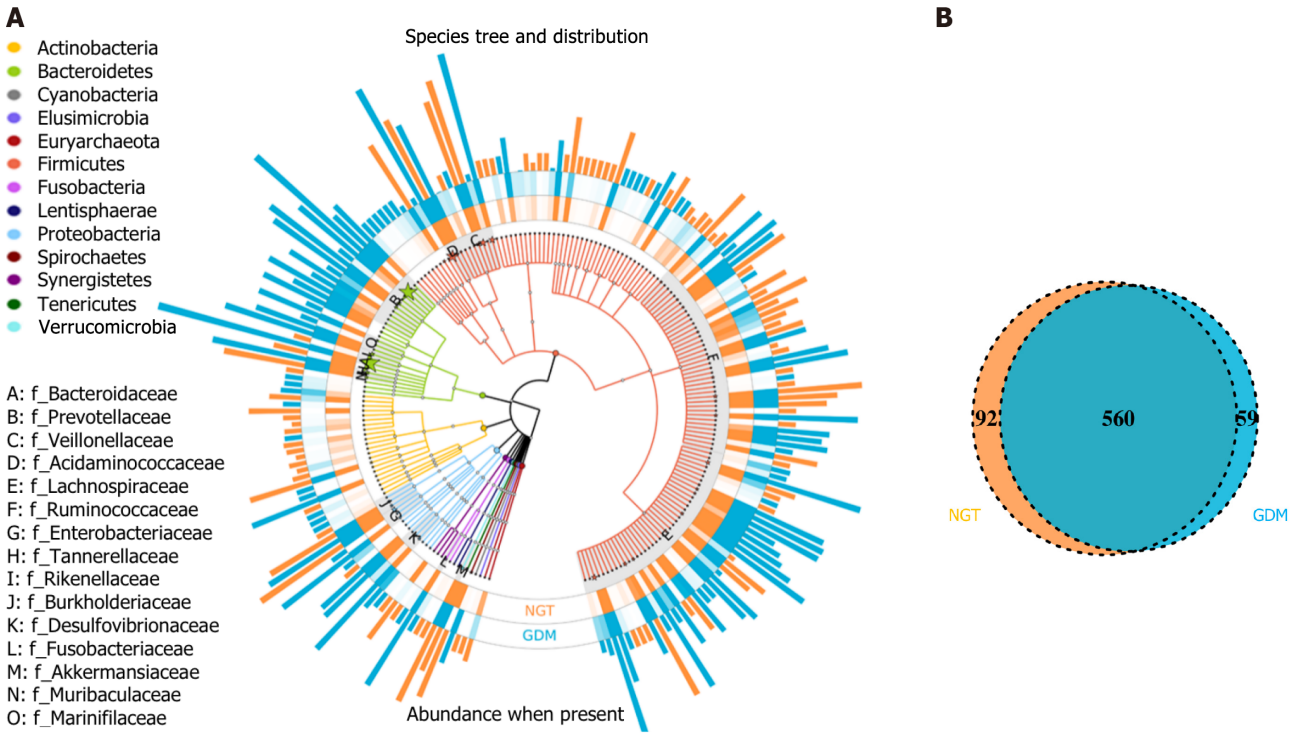

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Figure 1 Operational taxonomic units distributions. A: Species tree and distribution of the gut microbial community; B: Venn diagram showing the common or specific operational taxonomic units between the groups. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

Prevotella_2 (phylum *Bacteroidetes*) were the eight most dominant genera. The eight most dominant genera in the GDM group were *Bacteroides* (phylum *Bacteroidetes*), *Prevotella_9* (phylum *Bacteroidetes*), *Megamonas* (phylum *Firmicutes*), *Phascolarctobacterium* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Megasphaera* (phylum *Firmicutes*), *Prevotella_2* (phylum *Bacteroidetes*), and *Parabacteroides* (phylum *Bacteroidetes*).

Ruminococcaceae_UCG-002, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group ($P < 0.05$). *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group ($P < 0.05$). *Prevotella_9*, *Oscillibacter*, *Roseburia*, and *Faecalibacterium* were slightly more abundant in the NGT group than in the GDM group.

Functional profiling of the gut microbiome

The cluster of orthologous groups (COG) categories and KEGG pathways were compared between the GDM and NGT groups. Figure 6A shows that three functional KEGG pathways differed between the GDM group and NGT group, including the glycosphingolipid biosynthesis-globo series, synthesis and degradation of ketone bodies, and renal cell carcinoma pathways. Figure 6B shows that 20 COG categories differed between the GDM group and NGT group, including the phosphotransferase system, galactitol-specific IIC component, metal-dependent proteases with possible chaperone activity, uncharacterized protein, homolog of phage Mu protein gp30, uncharacterized protein conserved in bacteria, Acyl-CoA dehydrogenases, putative virion core protein (lumpy skin disease virus), predicted phosphohydrolase, large-conductance mechanosensitive channel, uncharacterized conserved protein, uncharacterized protein predicted to be involved in DNA repair, predicted permease, DMT superfamily, nicotinic acid mononucleotide adenylyltransferase, amidases related to nicotinamidase, histone acetyltransferase, plasmid maintenance system antidote protein, uncharacterized conserved protein, DNA polymerase III, alpha subunit, uncharacterized protein conserved in bacteria, antirestriction protein, NA polymerase III, and alpha subunit (Gram-positive type).

Correlations between blood biochemical traits and gut composition

Spearman's correlation analysis was performed to identify whether the different dominant genera were associated with blood biochemical traits in the second trimester of pregnancy (Figure 7). *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were negatively correlated with Glu ($P < 0.05$). *Ruminococcaceae_UCG-002* was negatively correlated with HbA1c ($P < 0.05$). *Clostridium_sensu_stricto_1*, *Desulfovibrio*, and (*Ruminococcus*)*_torques_group* were negatively correlated with pre-pregnancy body weight ($P < 0.05$). *Phascolarctobacterium* was negatively correlated with HDL ($P < 0.05$). *Ruminococ-*

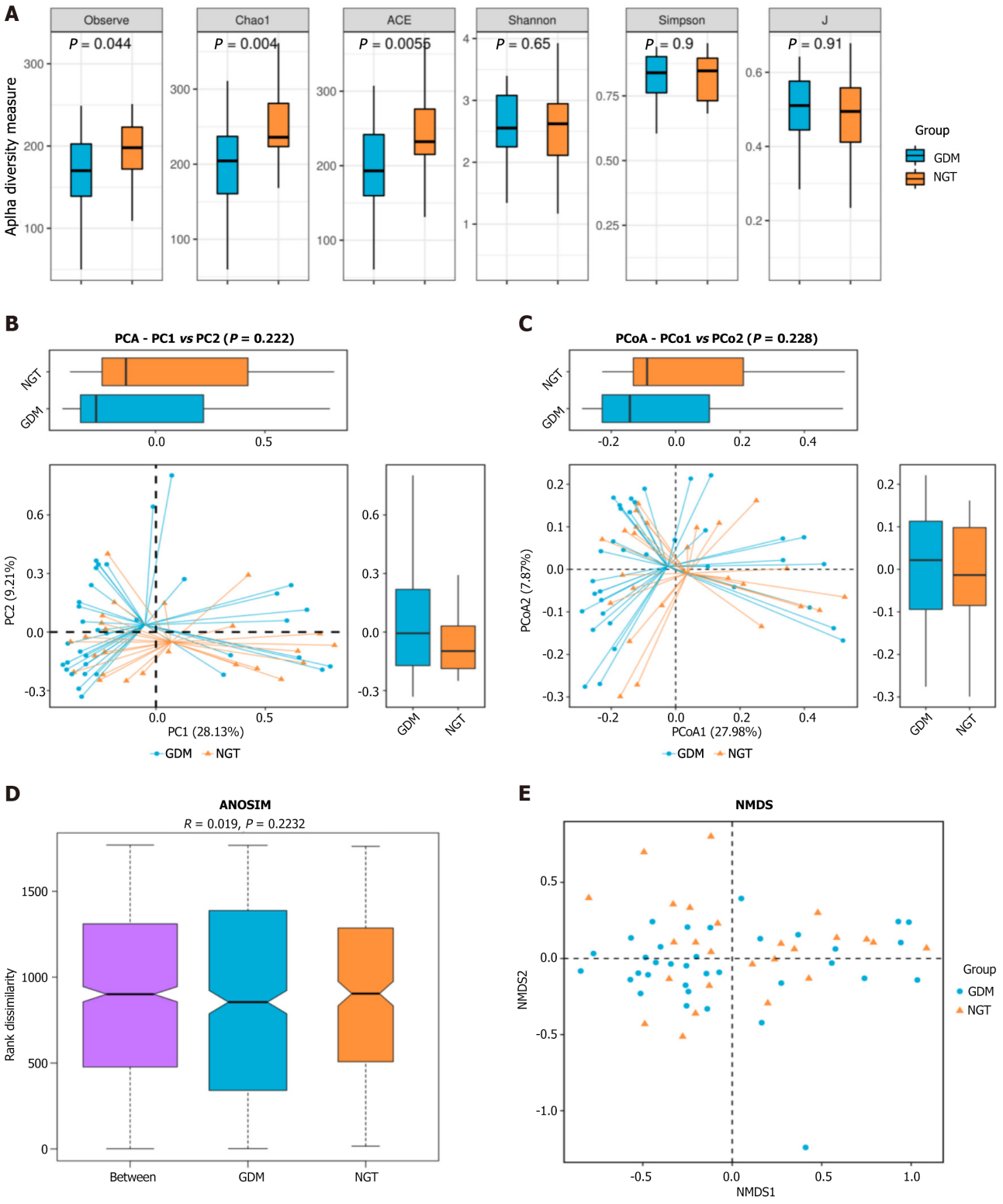

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Figure 2 Gut microbiota alpha and beta diversity indices in patients with gestational diabetes mellitus. A: Gut microbiota alpha diversity indices in patients with gestational diabetes mellitus. The Observed_species, ACE, Chao1, Simpson, Shannon, and J values are shown; B: Principal component analysis score plot based on the relative abundance of operational taxonomic units (97% similarity levels); C: Principal coordinate analysis; D: Similarities analysis; E: Non-metric multidimensional scaling. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; PCoA: Principal coordinate analysis; PCA: Principal component analysis.

caceae_UCG-003 and *Faecalibacterium* were negatively correlated with height ($P < 0.05$). *Lachnoclostridium* and *Lachnospiraceae_NK4A136_group* were positively correlated with age ($P < 0.05$). *Bacteroides* was significantly positively correlated with Glu ($P < 0.01$). *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were positively correlated with GLP-1 ($P < 0.05$).

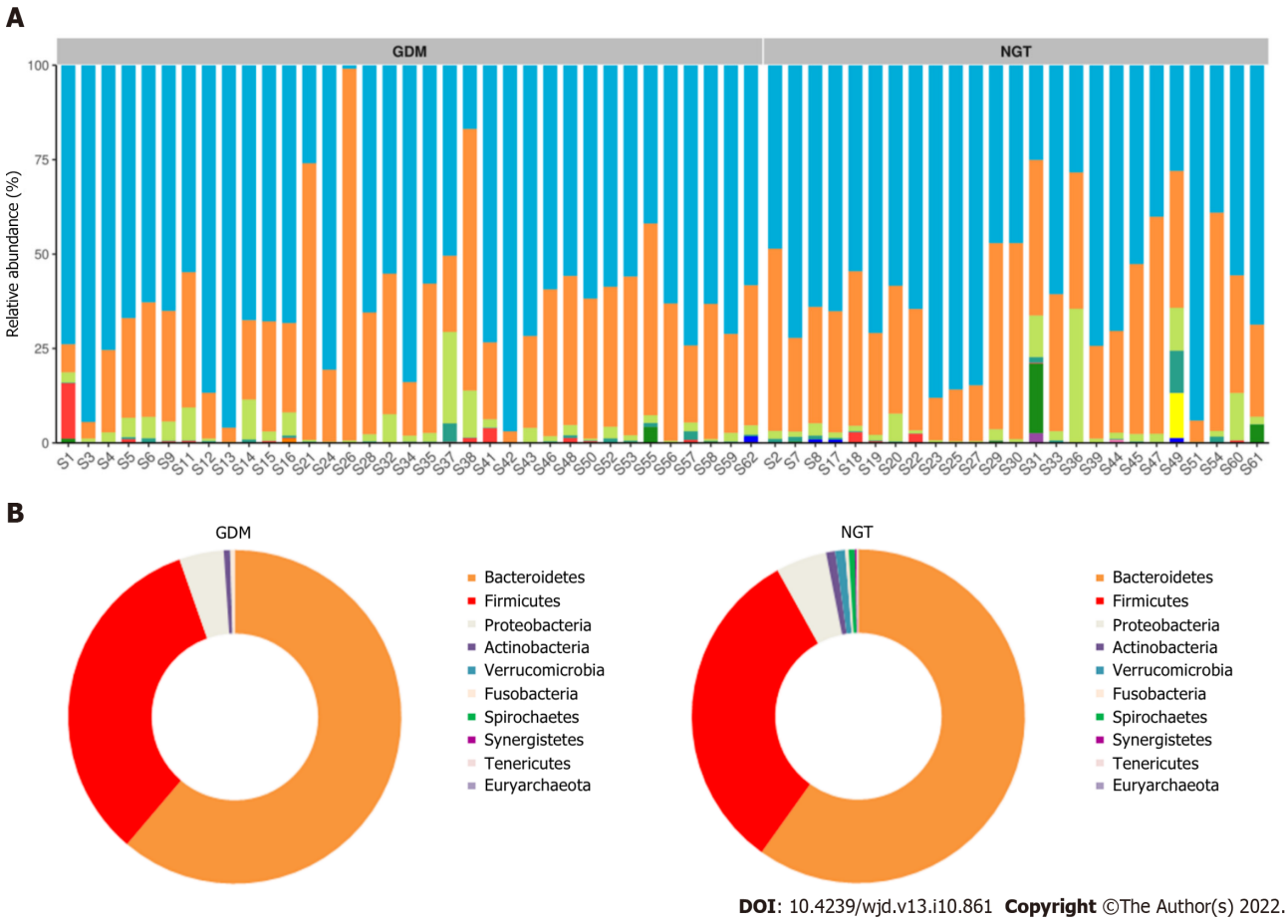

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Figure 3 Taxonomy. A: Top eight abundant species at the phylum level; B: Different bacteria were compared between each group at the phylum level. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

Roseburia was negatively correlated with OGTT (0 h), OGTT (1 h), and OGTT (2 h) ($P < 0.05$). *Faecalibacterium* was negatively correlated with OGTT (0 h) and OGTT (1 h) ($P < 0.05$). *Bacteroides* was positively correlated with OGTT (0 h), OGTT (1 h), OGTT (2 h), and GSP ($P < 0.05$). *Lachnoclostridium* was positively correlated with OGTT (1 h) and OGTT (2 h) ($P < 0.05$). *Sutterella* was positively correlated with GLP-1(0 h), GLP-1(1 h), GLP-1(2 h), and pre-pregnancy BMI ($P < 0.05$). *Oscillibacter* was positively correlated with GLP-1(0 h), GLP-1(1 h), and GLP-1(2 h) ($P < 0.05$). *Bifidobacterium* was positively correlated with GLP-1(0 h), GLP-1(1 h), OGTT (2 h), TG, and TCH ($P < 0.05$).

Gut microbiota–based prediction of GDM

Finally, random forest models were used to assess the ability of the genera abundance profiles to predict GDM status (Figure 8A). Twenty genera plus Glu provided the best discriminatory power, as indicated by the area under the receiver operating characteristic (AUROCC) value of 0.94. The value was higher than that achieved with a model including just 20 genera (the best AUC was 0.828) (Figure 8B). Further, models with 20 genera plus GLP-1, INS, or HbA1c had lower AUROCC values than the model with 20 genera plus Glu. The AUROCC values were 0.81, 0.8288, and 0.8502, respectively.

DISCUSSION

In recent years, the relationships between the gut microbiota and diabetes as well as other endocrine diseases have become research hotspots. Similarly, the characteristics of the gut microbiota among pregnant women with GDM have received widespread research attention. To date, research on GDM has focused on the correlation between the gut microbiota and blood glucose or insulin, but there is still a lack of research on the relationship between the gut microbiota and GLP-1. Many studies have reported that GLP-1 is closely related to the gut microbiota and short-chain fatty acids (SCFAs)[15-17], and changes in the gut microbiota directly affect the secretion of GLP-1, which, in turn, affects insulin and blood glucose. These are closely related to the occurrence of GDM. Therefore, we focused on the relationship between GLP-1 and the gut microbiota in GDM patients. To the best of our knowledge, it is the first report on the relationship between GLP-1 and the gut microbiota in patients with GDM.

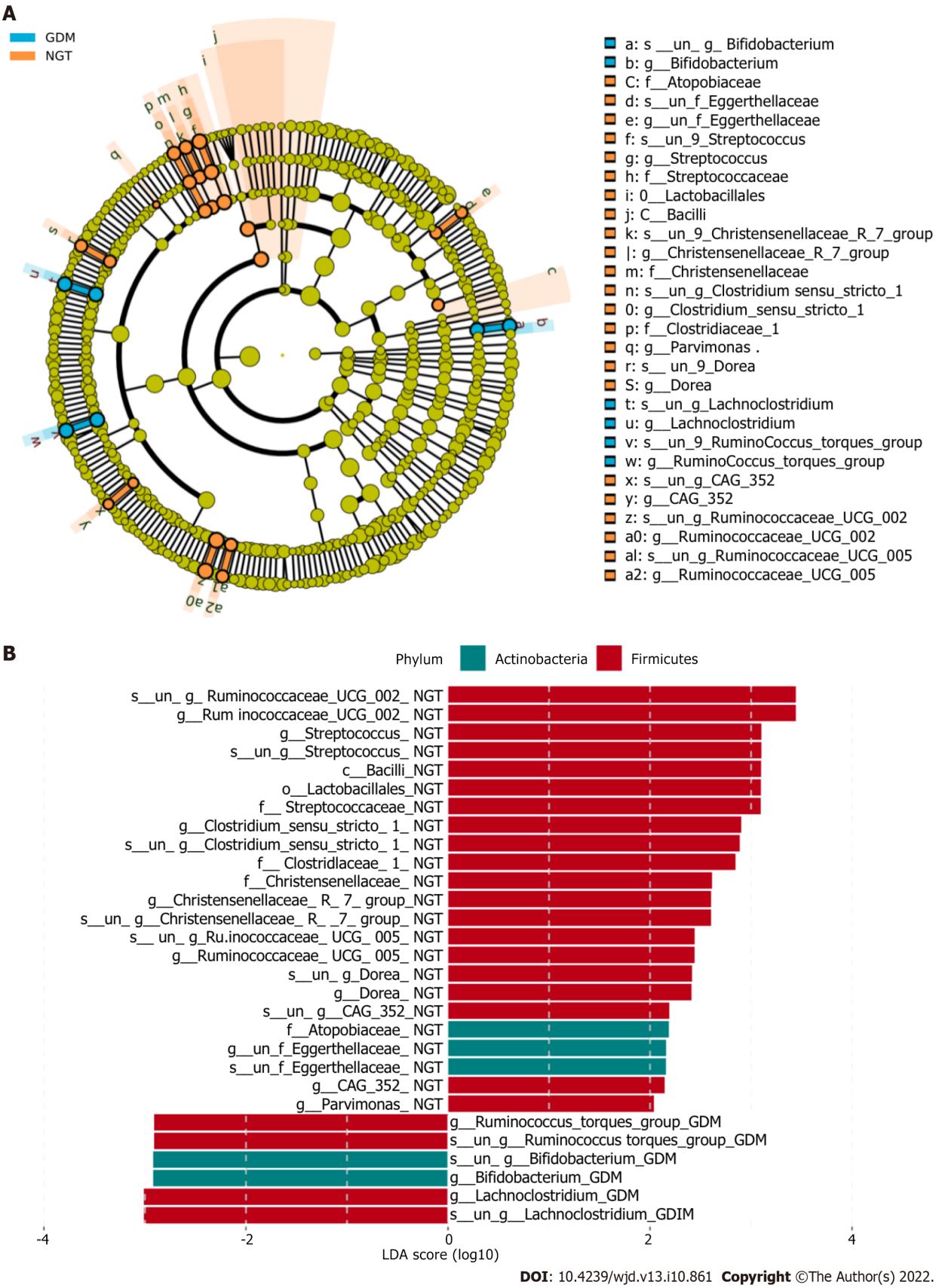

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Figure 4 LEfSE analysis to determine which bacterial taxa differ significantly between the groups. A: At the family level, a greater number of different families were identified between the normal glucose tolerance (NGT) and gestational diabetes mellitus groups (GDM); B: The bacterial taxa whose levels differed significantly between the NGT and GDM groups were identified by LEfSE analysis. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; LDA: Linear discriminant analysis.

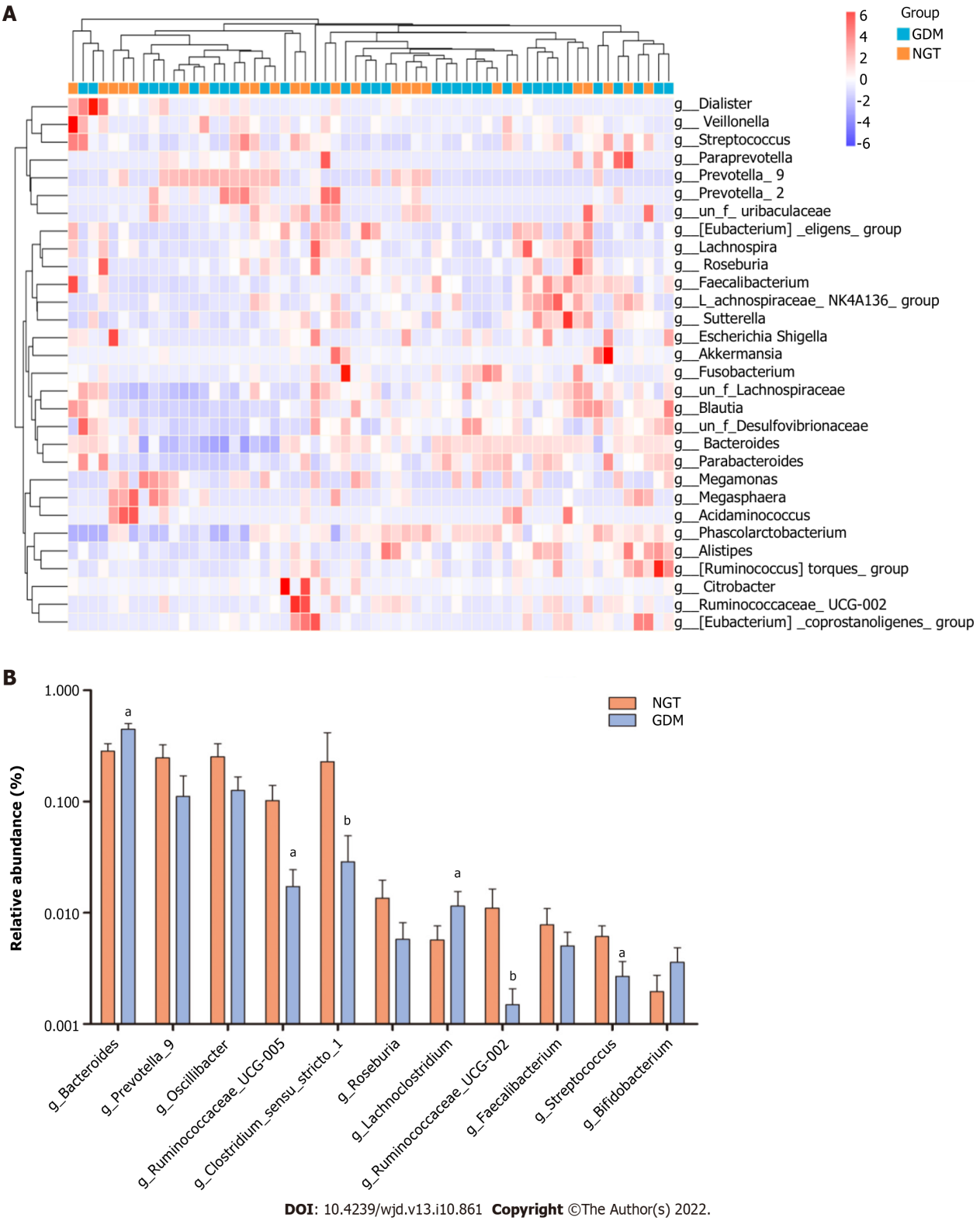

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Figure 5 Bacterial genera exhibit significant differences between the two groups. A: Heatmap showing the relative total abundance of the first 30 genera; B: Microbial community at the genus level between groups. $^{a}P < 0.05$, GDM group compared with NGT group. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

At the phylum level, the abundance of *Firmicutes* in the gut microbiota of the GDM group was lower than that in NGT group. *Firmicutes* are known to transform carbohydrates and undigested proteins into SCFAs, producing energy for the host organism. As a crucial SCFA, butyrate participates in the activation of multiple physiological signal pathways, including the proliferation and differentiation of regulatory T cells and anti-inflammatory activities[18,19]. Moreover, the GDM group exhibited reduced phylum levels of *Verrucomicrobia*, *Synergistetes*, and *Tenericutes* compared to the NGT group. Mucin-degrading bacteria *Verrucomicrobia* contribute to glucose homeostasis and intestinal health, and play a

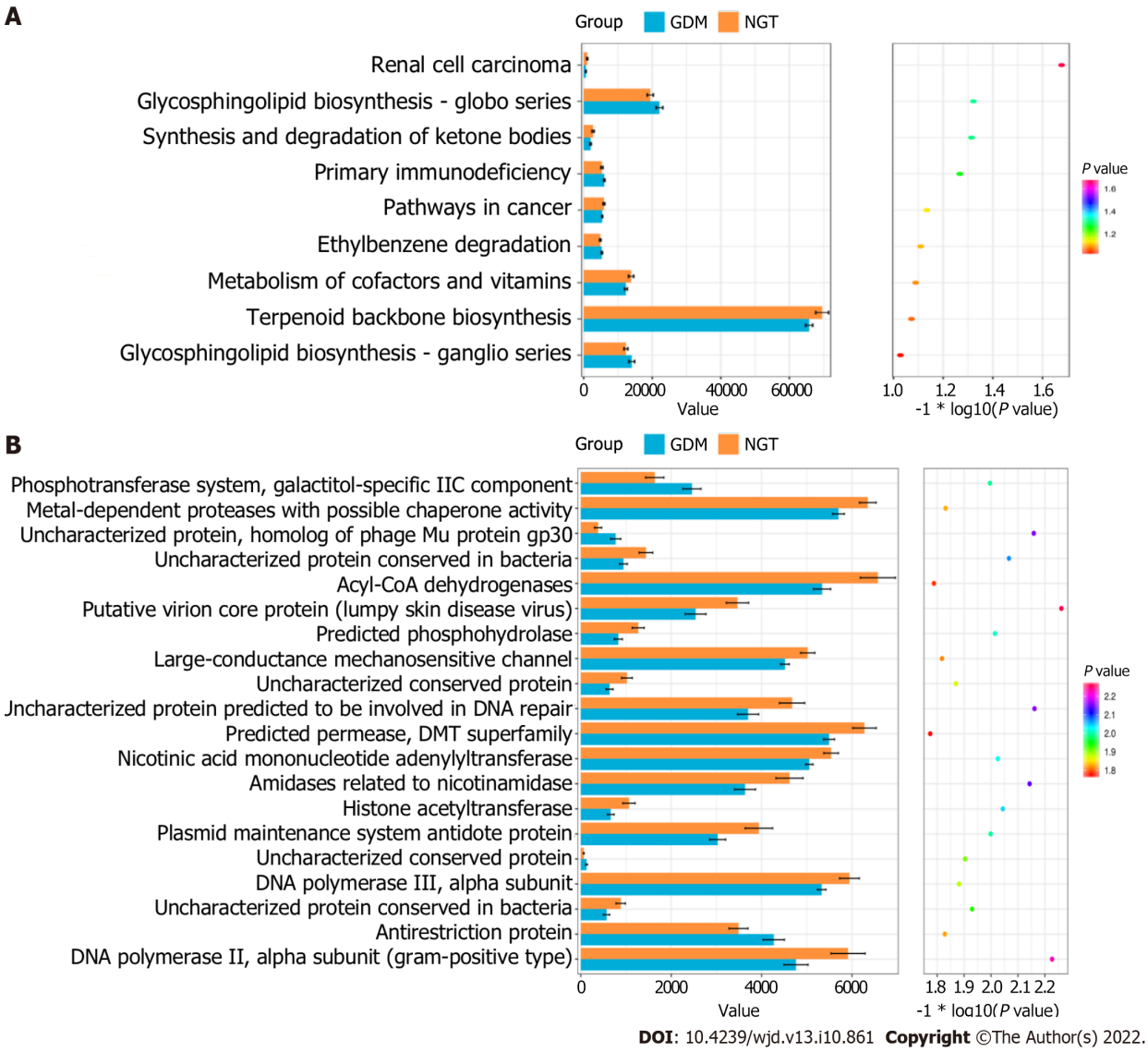

Figure 6 KEGG pathways and COG categories between the GDM and NGT groups. A: KEGG pathway; B: COG categories. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

key role in the interaction between the host tissues and gut microbiome[20]. The gut microbiota in the GDM patients also exhibited a higher abundance of *Bacteroidetes*, *Fusobacteria,* and *Proteobacteria* compared to healthy subjects. Proteobacteria is an opportunistic pathogen that creates a major structural imbalance in the gut microbiota of GDM patients. The ratio of *Firmicutes* to *Bacteroidetes* in GDM patients is lower than that of NGT individuals.

At the family level, *Atopobiaceae*, *Eggerthellaceae*, *Streptococcaceae*, *Christensenellaceae*, *Clostridiaceae*, *Bifidobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were more abundant in the NGT subjects than in the GDM patients. Zhang *et al*[21] reported that *Ruminococcaceae*, *Bifidobacteriaceae*, *Christensenellaceae*, *Erysipelotrichaceae*, *Peptostreptococcaceae*, and *Eggerthellaceae* were more abundant in the NGT subjects, which is consistent with the current study. In line with the study of Ma *et al*[22], the current results revealed that *Ruminococcaceae* were more abundant in the NGT group than in the GDM group. However, other studies have observed the opposite result[23,24]. The mechanisms remain unclear.

At the genus level, *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Kuang *et al*[25] found that the proportion of *Bifidobacterium* in the gut microbiota of GDM pregnant women was significantly reduced, while the proportions of *Bacteroides* and *Klebsiella* were significantly increased. Liu *et al*[26] found that compared with normal pregnant women, the proportion of *Faecalis* in GDM patients was significantly lower, while the proportion of *Prevotella* was significantly higher. In the present study, there were no significant differences in *Bifidobacterium*, *Klebsiella*, or *Prevotella* between the GDM group and NGT group. Ma *et al* found that *Ruminococcaceae_UCG-002* and *Ruminococcaceae_UCG-005* were reduced in women with GDM[22].

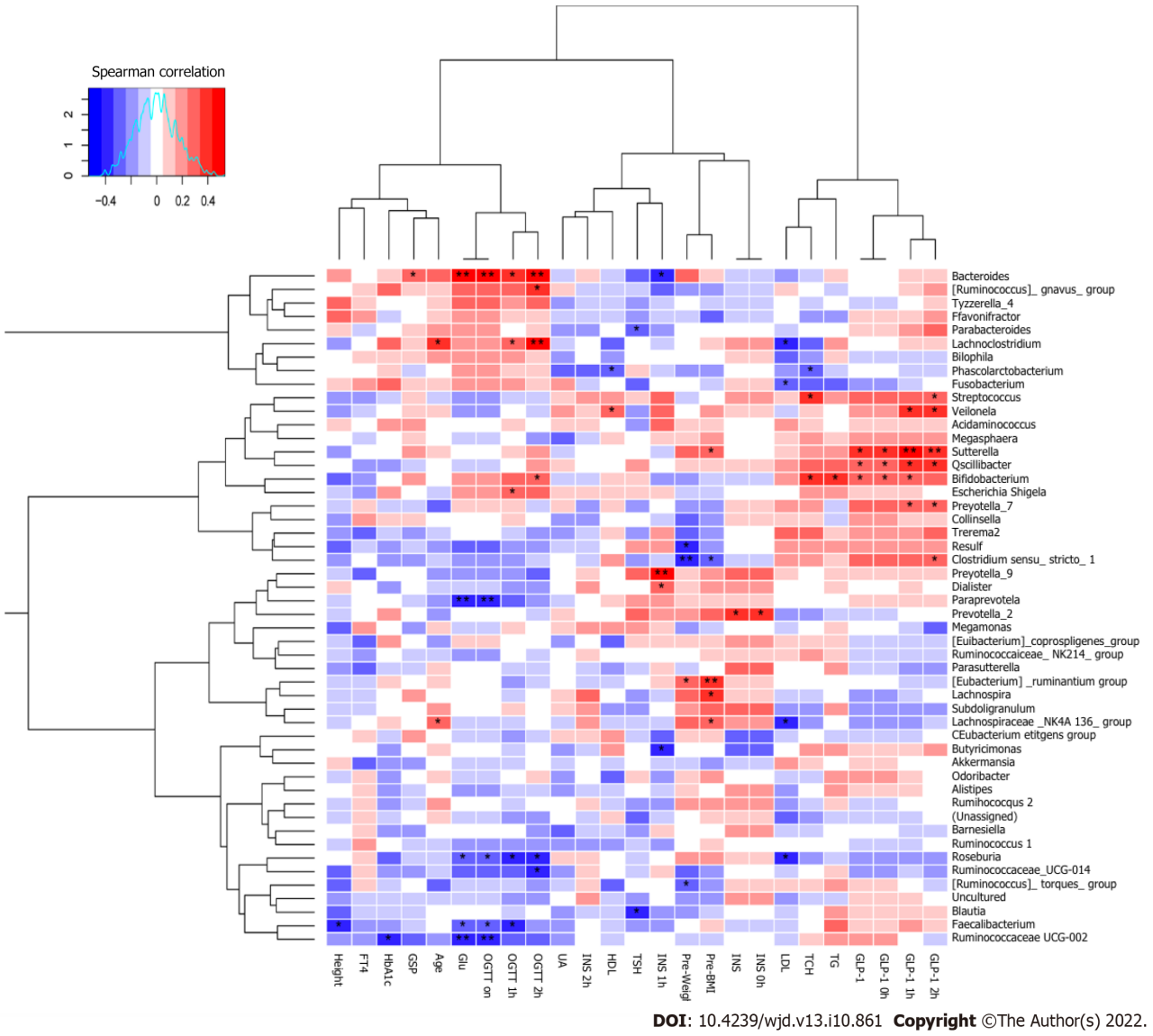

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Figure 7 Spearman's correlations between different dominant genera and blood biochemical traits. [a]*P* < 0.05, [b]*P* < 0.01. GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; GSP: Glycosylated serum protein; Glu: Glucose; HbA1c: Hemoglobin A1c; UA: Uric acid; TCH: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; INS: Insulin; OGTT: Oral glucose tolerance test; TSH: Thyroid-stimulating hormone; FT4: Free tetraiodothyronine.

One study found that supplementation with *Lactobacillus rhamnosus* in pregnant women may reduce the prevalence of GDM[27]. Another study showed that additional probiotic supplementation from pregnancy to 12 months post-delivery can reduce insulin levels and improve insulin sensitivity[28]. In this study, to identify beneficial bacteria for pregnant women, Spearman's correlation analysis was performed to identify the relationships between bacterial genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were negatively correlated with Glu. *Ruminococcaceae_UCG-002* was negatively correlated with HbA1c. *Clostridium_sensu_stricto_1*, *Desulfovibrio*, and (*Ruminococcus*)*_torques_group* were negatively correlated with pre-pregnancy body weight. *Phascolarctobacterium* was negatively correlated with HDL. *Ruminococcaceae_UCG-003* and *Faecalibacterium* were negatively correlated with height. *Lachnoclostridium* and *Lachnospiraceae_NK4A136_group* were positively correlated with age. Zhang *et al*[21] found that *Ruminococcaceae_UCG-002* was negatively correlated with fasting blood glucose levels. In the study of Crusell, *Clostridium (sensu stricto)* was positively correlated with gestational weight[29]. To the best of our knowledge, no studies have yet reported on the relationships between *Paraprevotella*, *Roseburia*, and *Faecalibacterium* and Glu in GDM. The current findings suggest that these genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment.

GLP-1 and its receptor agonist can promote insulin secretion only when the blood glucose level is elevated[30]. This safety feature makes GLP-1 and its agonist suitable for the treatment of GDM, which requires strict maintenance of blood glucose levels and stable, safe blood glucose regulation. Thus, the current study aimed to examine the correlations between the gut microbiota and GLP-1 levels, and

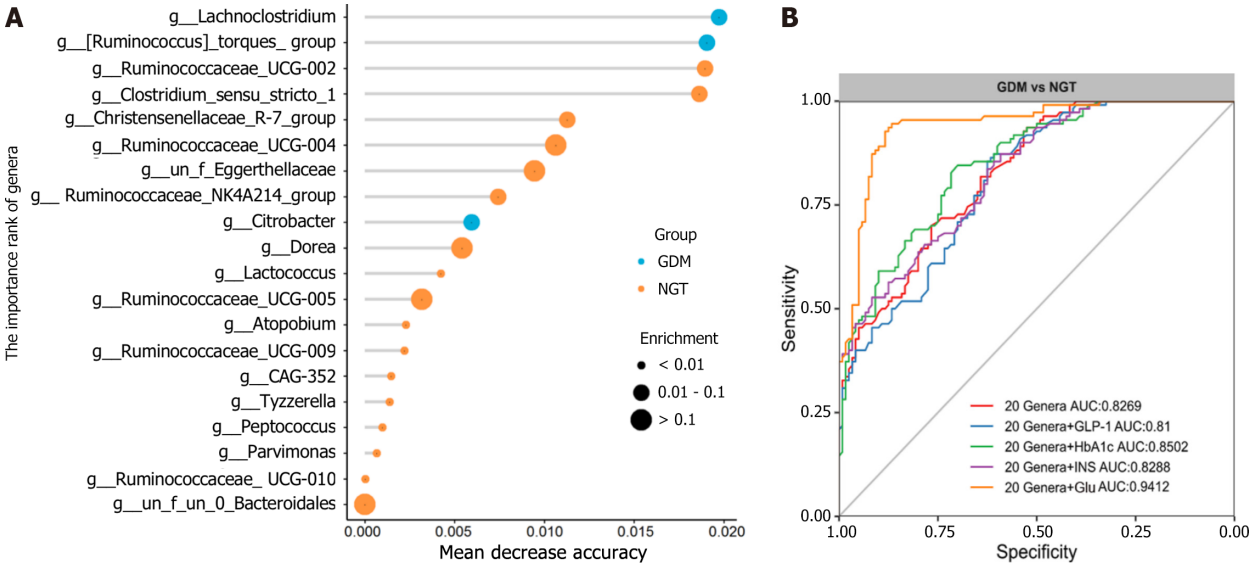

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Figure 8 Gut microbiota–based prediction of gestational diabetes mellitus. A: Identification of gestational diabetes mellitus (GDM) markers by random forest models; B: Receiver operating characteristic (ROC) curves of operational taxonomic units-based diagnostic biomarkers for GDM. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; INS: Insulin; Glu: Glucose; HbA1c: Hemoglobin A1c; GLP-1: Glucagon-like peptide-1.

identify beneficial bacteria that can improve the expression of GLP-1 in patients with GDM, so as to more safely control blood glucose. In the present study, *Sutterella* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), GLP-1 (2 h), and pre-pregnancy BMI. Wang *et al*[31] reported that subjects taking metformin exhibited a significantly increased relative abundance of *Sutterella*, whereas liraglutide dosing was associated with a significant increase in the genus *Akkermansia*. Another study showed that *Sutterella* was associated with C-reactive protein levels[32]. In the current study, *Oscillibacter* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), and GLP-1 (2 h). One study reported that *Cyclocarya paliurus polysaccharides* alleviated type 2 diabetic symptoms by increasing eleven SCFA-producing species, including *Oscillibacter_valericigenes* and *Oscillibacter_ruminantium*[33]. *Oscillibacter* belongs to the Clostridia class of Firmicutes, and in the human gut microbiota, this bacterium grow fermentatively, predominantly producing valerate when grown using glucose as a carbon source[34]. In the current study, *Bifidobacterium* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), TG, TCH, and OGTT (2 h). In the study of Zhao *et al*[35], *Bifidobacterium longum DD98* improved the serum and intestinal cell GLP-1 levels, which protected pancreatic β-islet cells from damage induced by type 2 diabetes. To the best of our knowledge, this is the first report on the associations between GLP-1 and genera such as *Sutterella*, *Oscillibacter*, and *Bifidobacterium* in GDM.

CONCLUSION

In summary, this study contributes to a better understanding of the relationships between the gut microbiota and blood biochemical traits, particularly GLP-1, in individuals with GDM. The current findings suggest that some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM. Future studies combining metagenomics and metabonomics would be of value for improving our understanding of the roles of specific strains and metabolites in patients with GDM and supporting precise prevention and intervention strategies for GDM.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) places both the mother and offspring at high risk of complications. Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of GDM.

Research motivation
To confirm whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients.

Research objectives
To explore the correlation between the gut microbiota and blood biochemical traits.

Research methods
The V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was sequenced based on the fecal samples of 35 pregnant women with GDM and was compared to that of 25 pregnant women with normal glucose tolerance (NGT).

Research results
The results showed that *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Spearman's correlation analysis was performed to identify the relationships between bacterial genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were significantly negatively correlated with glucose. *Ruminococcaceae_UCG-002* was significantly negatively correlated with HbA1c. *Bacteroides* was significantly positively correlated with glucose. *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were significantly positively correlated with GLP-1. A random forest model showed that 20 specific genera plus glucose provided the best discriminatory power, as indicated by the area under the receiver operating characteristic curve (0.94).

Research conclusions
The results of this study reveal novel relationships between the gut microbiome, blood biochemical traits, particularly GLP-1, and GDM status.

Research perspectives
These findings suggest some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM.

FOOTNOTES

Author contributions: Liang YY, Liu LY, and Jia Y designed the study, collected the samples, compiled the data, and drafted and critically revised the manuscript; Li Y, Cai JN, and Shu Y compiled the data and performed the statistical analysis and data interpretation; Tan JY collected the samples and compiled the data; Chen PY critically revised the manuscript; Cai HH and Cai XS designed this study, collected the samples, and drafted and critically revised the manuscript; all authors contributed to the article and approved the submitted version.

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Institutional review board statement: The study was approved and carried out in accordance with the guidelines of the Ethics Committee of Nanhai District People's Hospital of Foshan.

Conflict-of-interest statement: The authors declare that no competing interest exists.

Data sharing statement: Illumina sequencing reads were uploaded to the SRA under accession number PRJNA11381. The rest of the data that support the conclusions of this study are available from the corresponding author upon request.

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ORIGINAL ARTICLE

Clinical Trials Study

Effectiveness and safety of human umbilical cord-mesenchymal stem cells for treating type 2 diabetes mellitus

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Abstract

BACKGROUND

Progressive pancreatic β-cell dysfunction is a fundamental part of the pathology of type 2 diabetes mellitus (T2DM). Cellular therapies offer novel opportunities for the treatment of T2DM to improve the function of islet β-cells.

AIM

To evaluate the effectiveness and safety of human umbilical cord-mesenchymal stem cell (hUC-MSC) infusion in T2DM treatment.

METHODS

Sixteen patients were enrolled and received 1 × 10^6 cells/kg per week for 3 wk as intravenous hUC-MSC infusion. The effectiveness was evaluated by assessing fasting blood glucose, C-peptide, normal glycosylated hemoglobin A1c (HbA1c), insulin resistance index (homeostatic model assessment for insulin resistance), and islet β-cell function (homeostasis model assessment of β-cell function). The dosage of hypoglycemic agents and safety were evaluated by monitoring the occurrence of any adverse events (AEs).

RESULTS

During the entire intervention period, the fasting plasma glucose level was significantly reduced [baseline: 9.3400 (8.3575, 11.7725), day 14 ± 3: 6.5200 (5.2200, 8.6900); $P < 0.01$]. The HbA1c level was significantly reduced on day 84 ± 3 [baseline: 7.8000 (7.5250, 8.6750), day 84 ± 3: 7.150 (6.600, 7.925); $P < 0.01$]. The patients' islet β-cell function was significantly improved on day 28 ± 3 of intervention [baseline: 29.90 (16.43, 37.40), day 28 ± 3: 40.97 (19.27, 56.36); $P < 0.01$]. The dosage of hypoglycemic agents was reduced in all patients, of whom 6 (50%) had a decrement of more than 50% and 1 (6.25%) discontinued the hypoglycemic agents. Four patients had transient fever, which occurred within 24 h after the second or third infusion. One patient (2.08%) had asymptomatic nocturnal hypoglycemia after infusion on day 28 ± 3. No liver damage or other side effects were reported.

CONCLUSION

The results of this study suggest that hUC-MSC infusion can improve glycemia, restore islet β-cell function, and reduce the dosage of hypoglycemic agents without serious AEs. Thus, hUC-MSC infusion may be a novel option for the treatment of T2DM.

Key Words: Type 2 diabetes; Human umbilical cord mesenchymal stem cells; Blood glucose; Homeostasis model assessment of β-cell function; Hypoglycemic agents

Core Tip: Our article focused on the effectiveness and safety of human umbilical cord mesenchymal stem cell (hUC-MSC) infusion for treating type 2 diabetes. The results suggest that hUC-MSC infusion can improve glycemia, restore islet βcell function, and reduce the dosage of hypoglycemic agents without serious adverse events.

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INTRODUCTION

Diabetes has been a major public health problem worldwide in recent decades. Data from the International Diabetes Federation shows that the prevalence of diabetes among adults is 463 million globally. The estimated prevalence of diabetes and prediabetes among adults in China is 10.9% and 35.7% respectively[1], of which type 2 diabetes mellitus (T2DM) accounts for more than 90% of cases. In China, only 5.6% of T2DM patients achieved glycemic control in 2017[2].

T2DM is regarded as a chronic, progressive disease that arises from an impairment in the insulin-sensing mechanisms and culminates in insulin resistance (IR). Initially, the IR is compensated by increased insulin production; however, as the T2DM progresses over time, the general pancreatic dysfunction leads to increasingly lower insulin production. As glucose continues to accumulate in the bloodstream, chronic hyperglycemia promotes a chronic vicious cycle of metabolic decline[3]. In the first 10 years of T2DM, the β-cell function reduces by ~10%, but this is followed by a period of much more rapid decrease, of an additional ~10% every 2 years, until it eventually results in insulin-dependent diabetes[4].

Current treatments for diabetes include diet control, physical exercise, oral antidiabetic agents, and insulin therapy. Although novel medications and diet therapies continue to be developed, none has provided full protection against deterioration of β-cell function[5,6]. Islet/pancreas transplantation is an efficient way to restore islet β-cell function, but its clinical application is greatly restricted by the limited resource of donor tissues or organs, the immune rejection response, and the high cost and side effects of immunosuppressive drugs[7]. Therefore, the need for an effective and safe strategy to restore β-cell function in T2DM patients remains unmet.

In recent years, mesenchymal stem cell (MSC) therapy for diabetic patients has been extensively studied[8-10] as a novel therapeutic option for diabetes. MSCs are a population of multipotent stem cells from the mesoderm. Human umbilical cord-MSCs (hUC-MSCs) have been an important resource in clinical applications with many advantages including convenient material obtainability, less ethical controversy, great differentiation potential, robust multiplication capacity, low immunogenicity, and

less chance of virus infection. The transplantation of bone marrow MSCs reduced fasting blood glucose (FBG) and significantly increased serum C-peptide (CP) level in a macaque model study[11]. Liu *et al* [12] showed that injection of UC-MSCs with a 5-d interval decreased glycosylated hemoglobin A1c (HbA1c) levels and required insulin dose in patients with T2DM. In a relatively small T2DM patient study (n = 18), Kong *et al*[13] showed responsiveness to treatment of intravenous transfusion of UC-MSCs three times with 2-wk intervals, administered over a 6-mo period. Finally, in another small-size T2DM patient study (n = 6), Guan *et al*[14] showed that treatment with intravenous transfusion of UC-MSCs two times with 2-wk intervals led to one-half of the patients becoming insulin-free between treatment months 25 and 43.

We hypothesized that hUC-MSCs restore β-cell function by differentiating into β-cells. Animal studies have previously shown that the hUC-MSCs are able to restore β-cell function and insulin secretion in diabetic rats by differentiating into islet-like cells[15,16]. Later studies showed that the transplanted hUC-MSCs were also able to reduce IR by improving the microenvironment[17,18]. However, the effectiveness and safety of hUC-MSCs in clinical application have not been fully assessed, especially in a standardized clinical study for T2DM. To explore the therapeutic effectiveness and mechanism of hUC-MSC infusion, we conducted the present study to evaluate the effectiveness and safety of hUC-MSC infusion in treating T2DM. This is the first clinical trial of hUC-MSC infusion for T2DM treatment approved by the China Medical Biotech Association.

MATERIALS AND METHODS

Patients

The enrolled participants were patients admitted to Peking University Shenzhen Hospital (Shenzhen, China) for T2DM, and all provided signed informed consent. The study was conducted according to the Declaration of Helsinki and approved by the institutional review board of Peking University Shenzhen Hospital (IRB Approval No. [2018] 29th). The patient inclusion criteria were diagnosis with T2DM[19], age between 18 years and 70 years, and HbA1c level between 7% and 9.5% during the screening and follow-up periods. There were no restrictions on treatment of the T2DM patients. The exclusion criteria were: positivity for glutamic acid decarboxylase autoantibody; treatment with thiazolidinediones within 3 mo; history of severe drug allergy; neurological deficiency induced by severe brain injury; severe respiratory disease; severe cardiovascular disease (systolic blood pressure 180 mmHg and/or diastolic blood pressure 110 mmHg or refractory hypertension); severe hepatic dysfunction or uremia; other complications of uncontrollable diabetes, such as stage V and VI diabetic retinopathy and sustained hyperglycemia or catastrophic fluctuations; endocrine and metabolic disease other than diabetes; severe hematologic disease; any acute or chronic infection; any malignancies; human immunodeficiency virus infection; severe psychiatric disease; pregnancy, planned pregnancy, or lactation; taking drugs that affect glucose metabolism within 1 mo, such as glucocorticoid, thiazide diuretic, oral contraceptive, and tricyclic antidepressant; alcohol and drug abuse; participants of any other clinical trials within 3 mo; and, any other disease or status that may influence the patient's safety or adherence according to the investigators' assessment.

hUC-MSC preparation

The hUC-MSCs were provided by Beike Biotechnology (Shenzhen, China), and the preparation was performed as previously reported[20]. The prepared hUC-MSCs were analyzed for quality according to the standards of the International Society for Cellular Therapy and stored at -196 °C[21]. Briefly, the cells were adherent to plastic, positive for cluster of differentiation (CD) 105, CD73, and CD90, and negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and human leucocyte antigen DR. The hUC-MSCs were processed according to the workflow of Peking University Shenzhen Hospital.

Study design

Upon study enrollment, all participants were assessed for diabetes, complications, diet, and exercise in the Diabetic Out-Patient Clinic over a period of 16 wk prior to the initiation of intervention. The participants were recommended a daily diet that did not exceed 25-30 kcal/kg body weight and an exercise routine composed of walking or similar exercise for 30 min three times per week; these recommendations were provided throughout the study and followup periods. By the time of initiation of hUC-MSC therapy, all patients had already accepted treatments based upon diet, exercise, and prescribed medication (oral hypoglycemic agents and insulin injections); the latter had been administered as a baseline, at stable doses for at least 2 mo (day -56 ± 3 to day 0 ± 3).

During the follow-up period, the participants performed self-monitoring of their fasting plasma glucose (FPG) and 2-h postprandial plasma glucose (P2PG) 7 times per week. The dosages of oral hypoglycemic agents and insulin were adjusted according to the patient's blood glucose to keep the level stable, at FPG range of 79.2-126 mg/dL and P2PG range of 79.2-180 mg/dL. If the total daily insulin dose was ≤ 0.2 U/kg at any time during the study period, the administration of exogenous insulin was withdrawn; if the level of blood glucose was stable with the lowest dose of a single oral

hypoglycemic drug, the oral hypoglycemic drug was withdrawn.

All patients were assessed again after 16 wk and were administered hUC-MSC infusions. The infusion was administered at a dosage of 1 × 10^6 cells/kg per week for 3 wk. Considering the possible accidental episodes in the real-life that may interrupt the patients' follow-up visits plan in due time, we set a flexible time range (± 3 d) at the patient's discretion but which would not affect the safety and effectiveness of the study. This flexible schedule was structured for in-clinic evaluations to occur on day 14 ± 3, day 21 ± 3, day 28 ± 3 and day 84 ± 3 after the first dosage (Figure 1).

Effectiveness assessment

The effectiveness assessments were performed on day 14 ± 3, 21 ± 3, 28 ± 3, and 84 ± 3, including FBG, 2-h postprandial blood glucose, HbA1c, fasting CP (FCP), 2-h postprandial CP (P2CP), IR index [homeostatic model assessment for IR (HOMA-IR)] (by CP) = 1.5 + FCP ´ FBG/2800, islet β-cell function [HOMA of β-cell function (HOMA-β)] (by CP) = 0.27 ´ FCP/(FBG - 3.5), and hypoglycemic agent dosage. These dosages were adjusted by the treating physician according to standard clinical practice, based on blood glucose and A1c results.

Safety assessment

Any adverse event after receiving the hUC-MSC infusions would be reported and recorded. Routine safety assessment was conducted according to the visit schedule, including blood routine examination, hepatic function test, electrocardiogram, chest radiography, and tumor-associated antigen test.

Statistical analyses

All statistical analyses were analyzed with SPSS® 25.0 software (IBM Corp, Armonk, NY, United States). Quantitative data were analyzed with the two-samples Wilcoxon test. Differences in proportions were analyzed by the two-tailed test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 16 T2DM patients were enrolled. The clinical characteristics are shown in Table 1.

Intravenous infusion of hUC-MSCs significantly improved FG

FPG was significantly reduced on day 14 ± 3 without any alteration of hypoglycemic drug dosage, achieving the lowest level during the entire intervention period [baseline: 9.3400 (8.3575, 11.7725), day 14 ± 3: 6.5200 (5.2200, 8.6900); $P < 0.01$]. The FBG had a sustained decrease during the follow-up visit period, with a reduction of hypoglycemic agents for all patients. HbA1c level was significantly reduced on day 84 ± 3 [baseline: 7.8000 (7.5250, 8.6750), day 84 ± 3: 7.150 (6.600, 7.925); $P < 0.01$] (Figure 2). There was no significant difference in postprandial blood glucose level in the 75-g oral glucose tolerance test without hypoglycemic agents.

Intravenous infusion of hUC-MSCs improved islet β-cell function

The patients' islet β-cell function was significantly improved on day 28 ± 3 [baseline: 29.90 (16.43, 37.40), day 28 ± 3: 40.97 (19.27, 56.36); $P < 0.01$]. Islet β-cell function (HOMA-β) improvement was stably sustained during the following intervention period, with a reduction in hypoglycemic agents in all patients. The HOMA-IR decreased but not to a level that was statistically significant (Figure 2).

Intravenous infusion of hUC-MSCs decreased the dosage of hypoglycemic agents

After intravenous infusion of hUC-MSCs, the dosage of hypoglycemic agent was reduced in all patients on day 28 ± 3, of whom 12 (75%) had a decrement of 10%-50% and 4 (25%) had a decrement of 50%. On day 84 ± 3, the dosage was reduced in all patients, of whom 6 (50%) had a decrement of more than 50% and 1 (6.25%) discontinued the hypoglycemic agents (Figure 2).

Safety assessment

Four patients had transient fever (5 times in total), which occurred within 24 h after the second or third infusion. One patient (2.08%) had asymptomatic nocturnal hypoglycemia after infusion on day 28 ± 3. It did not recur after reducing the dosage of insulin in the following period. No patient had acute diabetic complications during the intervention period.

The leukocytes transiently increased significantly on day 14 ± 3 after the first dosage of hUC-MSCs, but there was no significant difference in leukocytes after the second dosage compared with baseline. The leukocyte level remained stable in the following period. There were no significant alterations in serum levels of alanine aminotransferase and creatinine (Figure 3).

Table 1 Baseline clinical characteristics of the patients

Characteristic	*n* = 16
Age (yr)	52.5 ± 7.91
Male	12
Female	4
Duration of diabetes (yr)	10.06 ± 5.74
BMI (kg/m^2)	24.47 ± 2.76
Glucose (mmol/L)	
FPG	9.66 ± 2.65
P2PG	16.32 ± 4.64
HbA1c (%)	8.01 ± 0.63
C peptide (pmol/L)	
FCP	741.56 ± 464.50
P2CP	1596.70 ± 989.65
HOMA-IR	4.22 ± 1.91
HOMA-β (%)	35.01 ± 24.35

BMI: Body mass index; FPG: Fasting plasma glucose; P2PG: 2-h postprandial plasma glucose; HbA1c: Glycosylated hemoglobin; FCP: Fasting c-peptide; P2CP: 2-h postprandial c-peptide; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-β: Homeostasis model assessment of β-cell function.

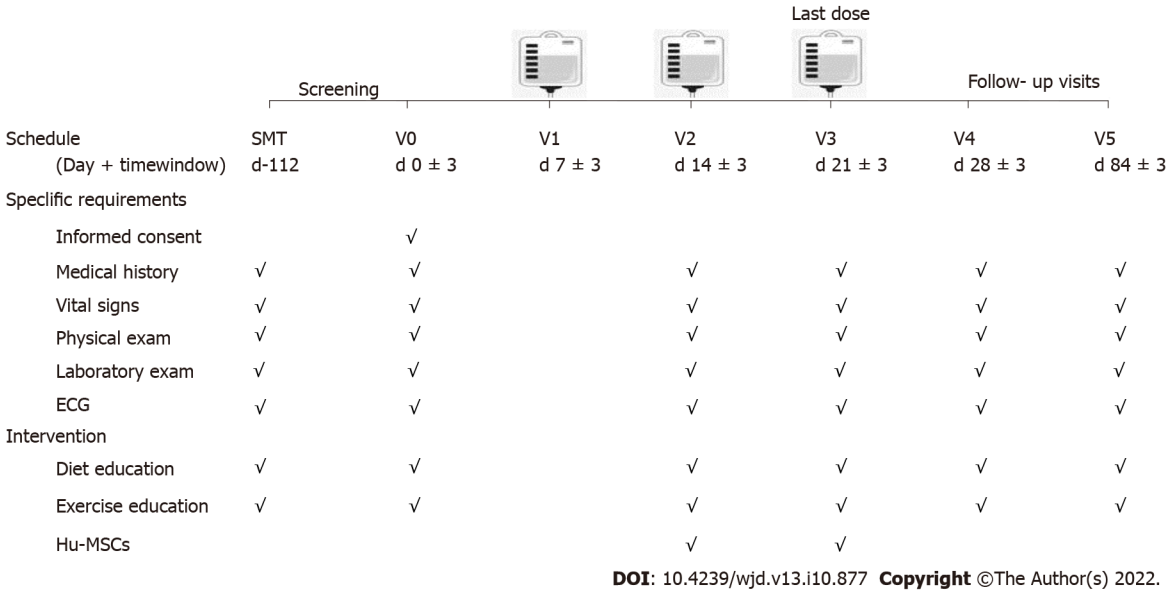

Figure 1 Flow chart for the study procedure. The patients enrolled in the present study received three infusions on days 7 ± 3, 14 ± 3, and 21 ± 3. There were three visits, on days 0 ± 3, 28 ± 3, and 84 ± 3. ECG: Electrocardiogram; hUC-MSCs: Human umbilical cord blood-mesenchymal stem cells.

After three doses of hUC-MSCs, carbohydrate antigen 199 and alpha fetoprotein decreased on day 28 ± 3. There was no significant alteration in carcinoembryonic antigen (Figure 3) or pancreatic autoantibody in the patients. All patients were negative for islet autoantibodies, with the exception of 1 who was positive for anti-islet cell antibody before and after the intervention.

DISCUSSION

Previous studies have demonstrated that hUC-MSCs are capable of decreasing the levels of FPG and

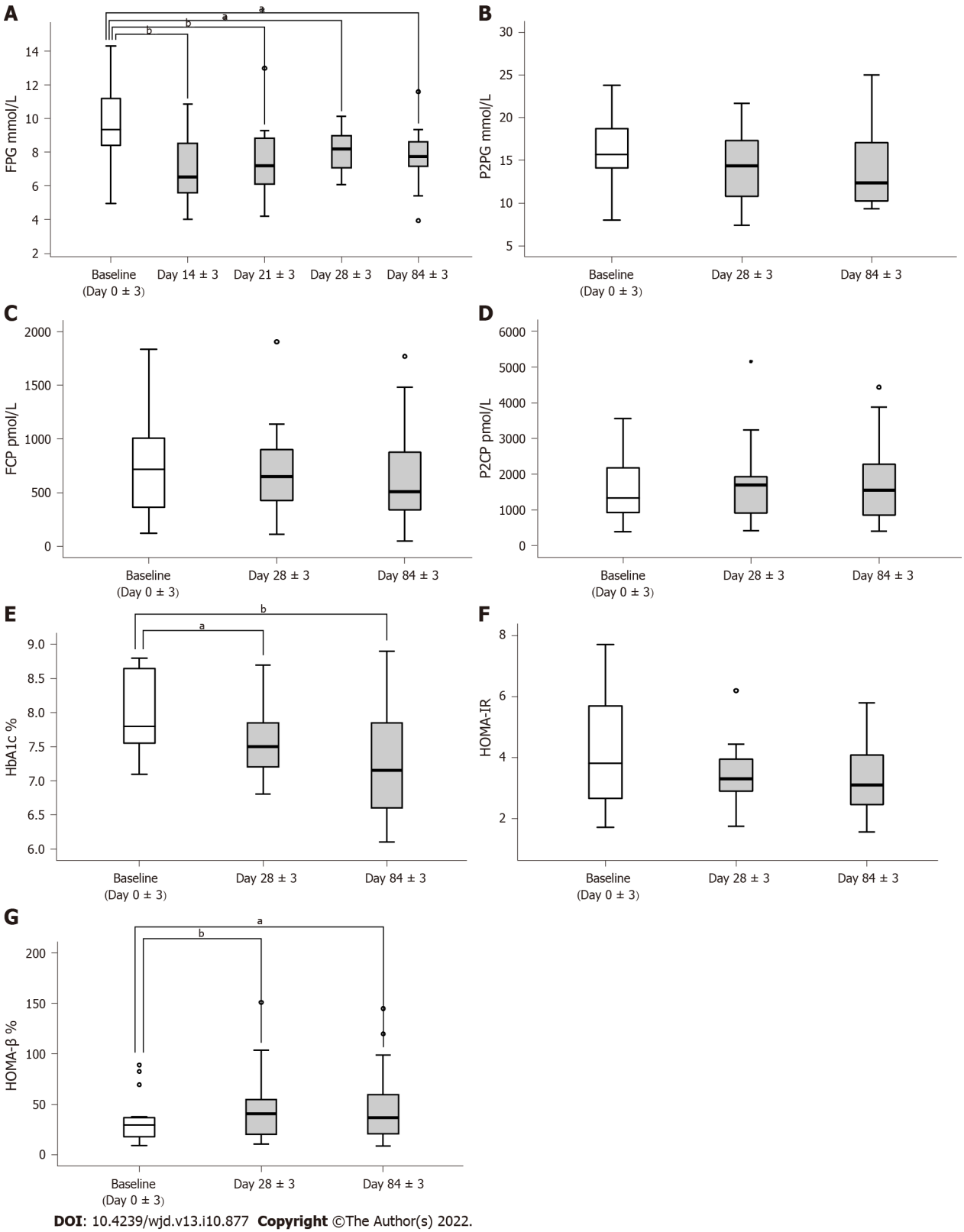

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Figure 2 Assessment of the effectiveness of human umbilical cord blood-mesenchymal stem cell treatment. A: Fasting plasma glucose tested on day 0 ± 3, day 14 ± 3, day 21 ± 3, day 28 ± 3, and day 84 ± 3; B-D: 2-h postprandial blood glucose, fasting C-peptide, and 2-h postprandial C-peptide tested on day 0 ± 3, day 28 ± 3, and day 84 ± 3; E: Glycosylated hemoglobin A1c levels tested on day 0 ± 3, day 28 ± 3, and day 84 ± 3; F and G: Homeostasis model assessment of insulin resistance and homeostasis model assessment of β-cell function calculated on day 0 ± 3, day 28 ± 3, and day 84 ± 3. $^{a}P < 0.05$, $^{b}P < 0.01$. FPG: Fasting plasma glucose; P2BG: 2-h postprandial blood glucose; FCP: Fasting C-peptide; P2CP: 2-h postprandial C-peptide; HbA1c: Glycosylated hemoglobin A1c; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-β: Homeostasis model assessment of β-cell function.

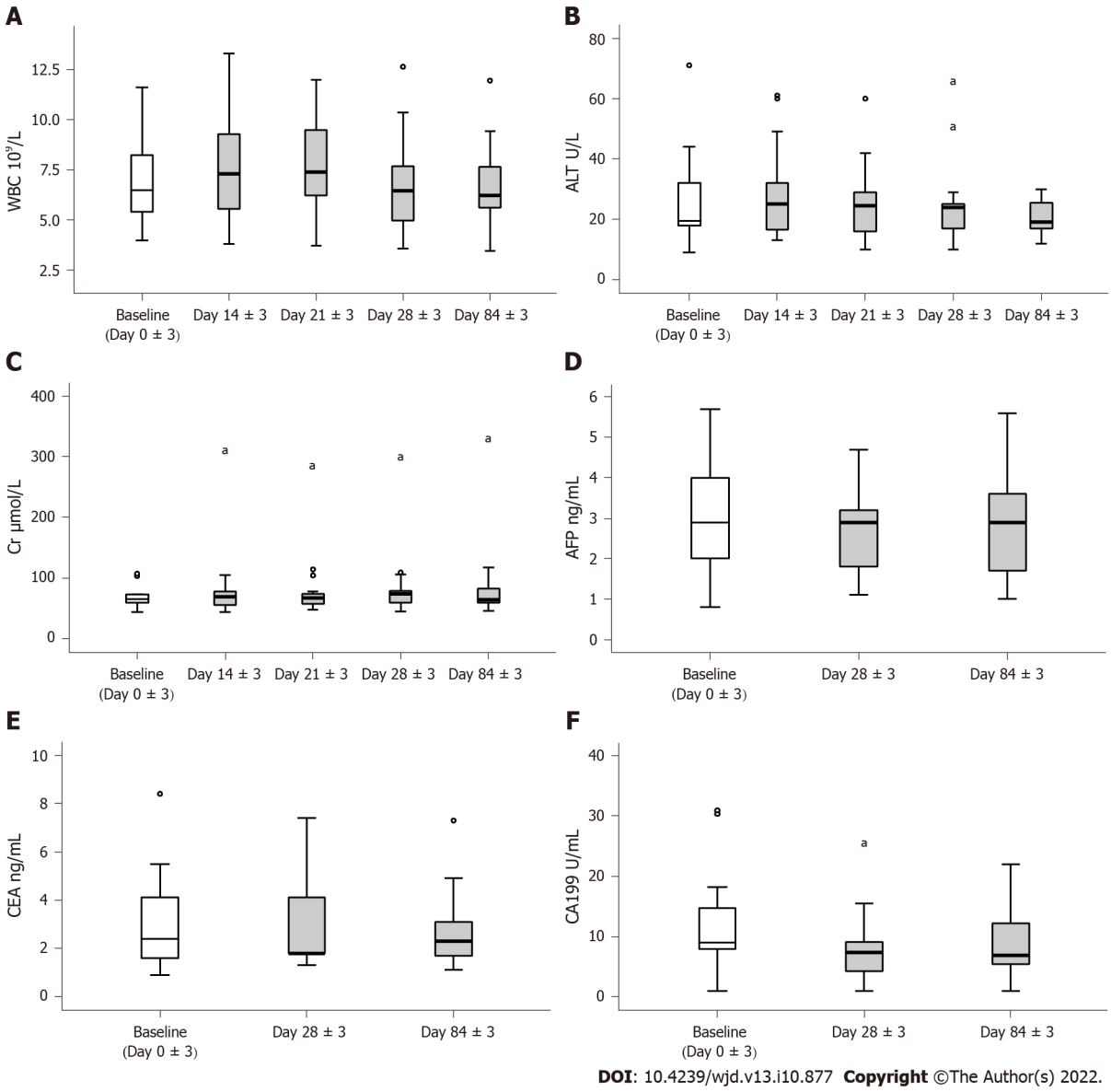

Figure 3 Assessment of the safety of human umbilical cord blood-mesenchymal stem cell treatment. A-C: Leukocytes, hepatic and renal function; D-F: Antigen associated with tumor. [a]*P* < 0.05.

HbA1c and reducing the dosage of hypoglycemic agents[13,22-24]. FBG was decreased and islet β-cell function was significantly improved after treatment with hUC-MSCs in our preliminary study in a diabetic rat model (data not shown). The purpose of this study was to evaluate the effectiveness and safety of hUC-MSC intravenous infusion in the short term for T2DM patients. The results demonstrated that hUC-MSCs could ameliorate hyperglycemia by decreasing FPG and HbA1c and reducing the dosage of hypoglycemic agents. It also improved islet β-cell function. Bell *et al*[25] observed the expression of pancreatic and duodenal homeobox 1 and islet cell differentiation gradually increased after hUC-MSC treatment of streptozotocin-treated NOD-SCID mice. MSC transplantation in streptozotocin-treated mice promoted the proliferation of endogenous islet cells and increased the amount of islet β-cells and insulin secretion[26]. Si *et al*[27] showed that the "increased" pancreatic islets and islet β-cells were not due to cell proliferation but to tissue repair and a decrease in apoptosis and damage in a rat model of T2D. Caplan *et al*[28] demonstrated that MSCs could ameliorate β-cell damage and restore islet β-cell function in a murine model in the early stage (7 d) of MSC treatment. Liu *et al*[12] reported that Wharton's jelly-derived MSC transplantation increased the HOMA-β from 65.99 ± 23.49 % to 98.86 ± 43.91%. The clinical trial results of Hu *et al*[22] also showed that the HOMA-β was significantly increased 1 mo after hUC-MSC treatment and was maintained for 18 mo. The results of the current study were consistent with these findings, indicating that improvement in glycemia in T2DM patients after hUC-MSC treatment was related to the repair of islet β-cells.

IR plays a critical role in the development of T2DM. It has been reported that MSC treatment in the early stage improved IR in animal experiments[27]. Chen *et al*[24] showed that hUC-MSC treatment could increase the area under curve of the CP and decrease the HOMA-IR. Hu *et al*[22] showed that

hUC-MSC treatment decreased the FCP and improved the HOMA-IR. However, the present study found no significant improvement of IR and no significant decrease in FCP and P2CP during the follow-up period. The islet β-cell function and IR state of patients in this study will be extensively followed for further analysis to elucidate the mechanisms underlying glycemia improvement.

Embryonic stem cells have the risk of teratoma formation, which limits their clinical application[29]. While the MSCs have been documented as having therapeutic efficacy for inflammation-related diseases, the concerns of possible tumorigenic effects are undeniable; although some studies have shown that MSCs do not undergo malignant transformation[30,31]. Guan *et al*[14] observed no immediate or delayed toxicity associated with MSC administration (within the followup period). In the present study, we observed no significant alterations in tumor-associated antigens (alpha-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 199) within the follow-up period. Because the follow-up time was short, we plan to follow up the participants for 3 years for further observations of possible transplant complications.

As this was a preliminary exploratory study, our sample size was limited; we plan to recruit more participants and include a healthy control group in our future study to evaluate the clinical utility of this therapy for T2DM.

CONCLUSION

The results of our study suggest that hUC-MSC treatment can improve glycemia, restore islet βcell function, and safely reduce the dosage of hypoglycemic agents required by the patient. Thus, hUC-MSC treatment could be a novel therapy for T2DM.

ARTICLE HIGHLIGHTS

Research background

Cellular therapies offer novel opportunities for the treatment of type 2 diabetes mellitus (T2DM) to improve the function of islet β-cells. However, the effectiveness and safety of human umbilical cord-mesenchymal stem cell (hUC-MSCs) in clinical application have not been fully assessed.

Research motivation

We conducted the present trial to explore the therapeutic effectiveness and mechanism of hUC-MSC infusion for treating T2DM.

Research objectives

We hypothesized that hUC-MSCs restore β-cell function by differentiating into β-cells. We conducted the present trial to treat T2DM with hUC-MSC infusion and evaluated the effectiveness and safety of hUC-MSC therapy.

Research methods

Patients were enrolled and received 1 × 10^6cells/kg per week for 3 wk of intravenous hUC-MSC infusion. The effectiveness was assessed by fasting blood glucose, C-peptide, normal glycosylated hemoglobin A1c level (HbA1c), insulin resistance (IR) index (homeostasis model assessment of insulin resistance), islet β-cell function (homeostasis model assessment of β-cell function), and dosage of hypoglycemic agents, and the safety was evaluated by monitoring the occurrence of any adverse events.

Research results

During the entire intervention period, the fasting plasma glucose level and HbA1c were significantly reduced. The patients' islet β-cell function was significantly improved, and the dosage of hypoglycemic agents was reduced in all patients without serious adverse events.

Research conclusions

We hypothesize that hUC-MSCs restore β-cell function by differentiating into β-cells. Our study suggests that hUC-MSC treatment can improve glycemia, restore islet βcell function, and safely reduce the dosage of hypoglycemic agents.

Research perspectives

Islet β-cell function and IR state of the patients in this study will be extensively followed for further analysis to elucidate the mechanisms underlying glycemia improvement.

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FOOTNOTES

Author contributions: Zhang F designed the report; Lian XF, Lu DH, Liu HL, Liu YL, Yang Y, Lin Y, Zeng QX, Huang ZJ, Xie F, Huang CH, Wu HM, Long AM, and Deng LP collected the patient's clinical data; Lian XF and Han XQ analyzed the data and wrote the paper.

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Institutional review board statement: This study was approved by the Ethics Committee of the Ethical Committee of the Peking University Shenzhen Hospital (IRB of Peking University Shenzhen Hospital [2018] 29th).

Clinical trial registration statement: This study is registered in the Chinese Clinical Trial Registry, Registration No. ChiCTR2200057370.

Informed consent statement: The participants were enrolled from patients admitted to Peking University Shenzhen Hospital for diabetes mellitus and all had signed informed consent.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: There are no additional data.

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ORIGINAL ARTICLE

Clinical Trials Study

Metabonomics fingerprint of volatile organic compounds in serum and urine of pregnant women with gestational diabetes mellitus

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) is a metabolic disease with an increasing annual incidence rate. Our previous observational study found that pregnant women with GDM had mild cognitive decline.

AIM

To analyze the changes in metabonomics in pregnant women with GDM and explore the mechanism of cognitive function decline.

METHODS

Thirty GDM patients and 30 healthy pregnant women were analyzed. Solid-phase microextraction gas chromatography/mass spectrometry was used to detect organic matter in plasma and urine samples. Statistical analyses were conducted using principal component analysis and partial least squares discriminant analysis.

RESULTS

Differential volatile metabolites in the serum of pregnant women with GDM included hexanal, 2-octen-1-ol, and 2-propanol. Differential volatile metabolites in the urine of these women included benzene, cyclohexanone, 1-hexanol, and phenol. Among the differential metabolites, the conversion of 2-propanol to acetone may further produce methylglyoxal. Therefore, 2-propanol may be a potential marker for serum methylglyoxal.

CONCLUSION

2-propanol may be a potential volatile marker to evaluate cognitive impairment in pregnant women with GDM.

Key Words: Gestational diabetes mellitus; Gas chromatography/mass spectrometry;

Humoral biomarkers; 2-propanol

Core Tip: Gas chromatography-mass spectrometry was used in a metabonomics analysis to determine the changes in volatile metabolites in pregnant women with gestational diabetes mellitus (GDM) and to explore the mechanism of cognitive function decline in these women. 2-propanol was identified as a potential volatile marker to evaluate cognitive impairment in pregnant women with GDM.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is a metabolic disorder in which hyperglycemia develops during pregnancy in women who did not previously have diabetes[1]. The incidence of GDM varies in different countries and regions, ranging from 1 to 30% of pregnancies, and is highest in Africa, Asia, and India [2]. Epidemiological evidence indicates a continuous increase in the incidence of GDM worldwide. The presence of hyperglycemia during gestation is often associated with various abnormalities, such as obesity, cardiovascular disease, preeclampsia, and even stillbirth. GDM diagnosed at 24 to 28 wk of gestation reportedly affects fetal development[3]. The substantial effect that GDM can have on maternal and fetal health necessitates the development of a screening method for predictive and diagnostic biomarkers of GDM in early stages of pregnancy[4].

Refinements of metabonomics research methods have led to the widespread use of the mature technology in various fields, including studies of disease mechanisms and diagnosis, treatment, treatment effects, and prevention. It is helpful to analyze the changes in metabolic substances caused by pathophysiological changes in diseases[5]. Metabonomics clinical research mainly obtains relevant differential substances by analyzing patients' serum, urine, and feces. There are many metabonomics technologies, and each has shortcomings. However, the use of the technology in combination with another technology, such as liquid/gas chromatography-mass spectrometry (LC/GC-MS), can improve their respective advantages and compensate for the shortcomings of each technology. This integration also improves the metabonomic method and can obtain more reliable clinical sample data. Sample analyses involved principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal projections to latent structures-DA. These analyses have identified differential metabolites. Bioinformatics database analysis of the relevant metabolic pathway can explain the possible metabolic mechanisms and pathophysiological changes and verify the biomarkers related to the disease mechanism.

We previously reported that patients with GDM have mild cognitive decline, but the underlying mechanism remains unclear[6]. A causative association of cognitive decline with metabolic abnormalities in patients with GDM is conceivable. To explore this speculation, we analyzed the blood and urine of GDM patients and normal pregnant women using solid-phase microextraction (SPME) GC/MS to observe abnormal metabolites in GDM and identify potential biomarkers for GDM.

MATERIALS AND METHODS

Subjects and protocol

Patients aged 18 to 35 years with American Society of Anesthesiologists physical status I-II were enrolled. Thirty women with GDM who were diagnosed, followed, and treated at the First Affiliated Hospital of Harbin Medical University were included in the study. Thirty age-matched pregnant women without diabetes constituted the normal pregnancy (NP) group. All patients and volunteers read and signed informed consent forms before enrollment in the study. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University and registered with the Chinese Clinical Trial Registry (registration number: ChiCTR2000038703).

GDM was diagnosed with at least one abnormal result during the oral glucose tolerance test: Plasma glucose during fasting ≥ 92 mg/dL (5.1 mmol/L) or ≥ 180 mg/dL (10.0 mmol/L) at 1 h or ≥ 153 mg/dL (8.5 mmol/L) at 2 h. Patients with pre-gestational type 1 (T1) or type 2 (T2) DM were not included in the

study. Patients with unnatural pregnancy or a gestational period of < 37 wk or > 41 wk were excluded. Subjects on medications affecting cognitive function, including corticosteroids, antidepressants, or antiepileptics, were also not included. Subjects with chronic metabolic, endocrine, inflammatory diseases, cancer, drug or alcohol dependency, history of major brain abnormalities (*e.g.*, tumors and hydrocephaly), epilepsy, or Parkinson's disease were excluded. The psychological status of pregnant women was assessed using the Hamilton Depression Scale; a score > 7 indicated the potential for depression and was the final exclusion criterion.

On the survey data, all the enrolled patients underwent routine medical history inquiries, physical examinations, and laboratory measurements. The clinical research coordinators used a standard questionnaire to collect information on demographic characteristics and medical history (Figure 1). All pregnant women were instructed to maintain their usual physical activity and diet for at least 3 d before the survey. After overnight fasting for ≥ 10 h, venous blood and urine samples were collected and stored at -80 °C. All parameters were measured within 6 mo of sample collection.

SPME

A 75 μM extraction head was used. The coating material was carbon molecular sieve of polydimethylsiloxane. An automatic sample injector was used for heating and extraction. A puncture made in the liquid sample bottle allowed injection. In the headspace extraction method, the extraction temperature was 40 °C, and the extraction time was 20 min. After the extraction and concentration of the samples were completed, the automatic sampling device inserted the extraction head into the GC-MS injection port for analysis.

GC/MS analysis

All analyses were performed on a model QP2010 GC/MS (Shimadzu) equipped with a DB-5MS PLOT column (length: 30 m; inner diameter: 0.250 μm; film thickness, 0.25 mm; Agilent Technologies). The injections were performed in splitless mode, with a splitless time of 1 min. The injector temperature was set at 200 °C, and helium was used as the carrier gas at a flow rate of 2 mL/min. The temperature in the column was maintained at 40 °C for 2 min to condense hydrocarbons. The temperature was then increased to 200 °C at a rate of 70 °C /min and held for 1 min. Subsequently, the temperature was increased to 230 °C at 20 °C/min and maintained for 3 min. MS analyses were performed in full-scan mode with an associated m/z range of 35–200 amu. An ionization energy of 70 eV was used for each measurement, and the ion source was maintained at 200 °C.

Statistical analysis

Statistical analyses were performed using the SIMCA-p + 11 software. Differences in volatile organic carbons (VOCs) between groups were tested using PLS-DA and PCA. SIMCA-p software was used to prevent overfitting by applying default seven-round cross-validation. Additionally, permutation tests using 200 iterations were performed to further validate the supervised model. Potential metabolic biomarkers were selected based on variable importance in the projection values calculated from the PLS-DA model. For all data analyses, $P < 0.05$ indicated statistical significance. The area under the curve (AUC) of the combined biomarkers and sensitivity and specificity calculations were performed using R language software 3.2 (R Development Core Team 2011).

RESULTS

Basic information of subjects

Sixty pregnant women participated in this study, including 30 pregnant women with GDM in the GDM group and 30 healthy pregnant women in the NP group. Body weight, blood glucose level, and hemoglobin A1c level in the GDM group were significantly higher than of those in the NP group ($P < 0.05$) (Table 1).

Blood sample analysis

Eighteen significant differential metabolites were evident between the GDM and NP groups (Table 2). Comparing the GDM and NP groups revealed a good separation trend in the two groups in the two-dimensional PCA score diagram (Figure 2A). When a single prediction component and three orthogonal components were used, the PLS-DA score map ($R^2X[1] = 0.203743$, $R^2X[2] = 0.123147$, $T^2 = 0.95$) revealed a good separation effect of the data of the GDM and NP groups (Figure 2B and C). Additionally, 200 iterations were conducted to test the supervision model. The R^2 and Q^2 values calculated from the converted data were lower than their original verification values [$R^2 = (0.0, 0.241)$, $Q^2 = (0.0, -0.269)$], which proved the effectiveness of the supervision model (Figure 2D). The receiver operating characteristic (ROC) curves showed that the AUC of the three VOCs was greater than 0.5; the closer it was to 1, the better was the diagnostic effect (Figure 2E and F).

Table 1 Demographic characteristics

	GDM	NP	*t*	*P* value
Sample	30	30		
Age (yr)	28.38 ± 2.52	29.14 ± 3.61	0.95	0.35
Height (cm)	162.34 ± 4.69	164.61 ± 5.36	1.75	0.09
Weight (kg)	76.33 ± 9.16	74.05 ± 8.97	0.98	0.33
Glucose (mmol/L)	4.825 ± 1.03	3.39 ± 0.56	6.70	< 0.001
Hba1c (%)	5.93 ± 0.73	4.86 ± 0.93	4.50	< 0.001

Data are expressed as the mean ± SD. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; HbA1c: Hemoglobin A1c.

Table 2 Differential metabolites in blood volatile organic carbons of the two groups

Differential metabolite	VIP	*P* value	Time	FC (GDM/NP)
1-Octyn-3-ol, 4-ethyl-	2.2718	0.0000	9.1501	-0.3956
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester	1.6588	0.0000	4.3833	-0.3885
Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-	2.11317	0.0000	8.6667	-0.4162
Isolongifolene-5-ol	1.85815	0.0000	18.1917	-0.6099
Oxime-, methoxy-phenyl-	1.06288	0.0001	6.3083	-0.2675
Trans-beta-Ocimene	2.01398	0.0000	6.9250	-0.3406
1H-Pyrazole-1-carbothioamide, 3,5-dimethyl-	1.66239	0.0000	4.7250	-0.5068
1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	1.38245	0.0000	18.8000	-0.9345
2-Octen-1-ol, (E)-	1.11297	0.0287	8.0417	0.5487
2-Propanol, 1,1,1-trichloro-2-methyl-	1.32854	0.0000	7.8167	-0.2366
3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-	1.51135	0.0000	18.7750	-0.5299
3-Heptanol, 5-methyl-	1.2384	0.0001	5.3235	-0.2684
5-Methyluridine, tris(trifluoroacetate)	2.04962	0.0000	10.6833	-0.2948
Hexanal	1.09786	0.0001	4.0858	0.3192
Malonic acid, bis (2-trimethylsilylethyl ester	1.59984	0.0000	19.5083	-0.3655
Oxalic acid, 2TMS derivative	1.13131	0.0097	16.6477	0.1542
Quinoxalin-2-one, decahydro-3-(3,3-dimethyl-2-oxobutenylideno)-	1.73099	0.0000	7.3270	-0.0682

VIP: Variable importance in the projection; FC: Fold change; GDM: Gestational diabetes mellitus; NP: Normal pregnancy.

Urine sample analysis

Eleven meaningfully differential metabolites were found between the GDM and NP groups (Table 3). Comparison between the GDM and NP groups revealed a good separation trend in the two-dimensional PCA score diagram (Figure 3A). When using a single prediction component and three orthogonal components, the PLS-DA score map ($R^2X[1] = 0.295851$, $R^2X[2] = 0.221649$, $T^2 = 0.95$) showed that the data from the GDM and NP groups also had a good separation effect (Figure 3B and C). Additionally, 200 iterative permutations were conducted to test the supervision model. The R^2 value and Q^2 value calculated from the converted data were lower than their original verification values [$R^2 = (0.0, 0.425)$, $Q^2 = (0.0, -0.33)$], which proved the effectiveness of the supervision model (Figure 3D). The ROC curves (Figure 3E and F) were the same as those described previously.

DISCUSSION

Investigations of metabolic substances in body fluids are an important supplement to the path-

Table 3 Differential metabolites in urine volatile organic carbons of the two groups

Differential metabolite	Similarity	VIP	*P* value	Time	FC (GDM/Ctrl)
Benzene, 1,3-bis(1,1-dimethylethyl)-	88	2.87589	0.0000	13.927	0.560880597
2-Pentanone	87	2.2746	0.0000	3.017	-0.077266923
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester	59	1.93204	0.0001	4.368	-0.339022989
Thiophene, 3,3'-(1,2-ethenediyl)bis-	58	1.62894	0.0000	4.371	-0.184457329
Cyclohexanone	95	1.13467	0.0081	6.018	0.935083138
.alpha.-Pinene	94	1.51705	0.0015	6.916	-0.221670672
Phenol	98	1.02127	0.0135	7.970	1.492584113
1-Hexanol, 2-ethyl-	96	1.16684	0.0444	9.118	0.693139386
Cyclododecanol	83	2.53908	0.0000	12.365	-0.41722675
3,4-Dimethylcyclohexanol	78	2.33881	0.0207	13.488	-0.094482061

VIP: Variable importance in the projection; FC: Fold change; GDM: Gestational diabetes mellitus.

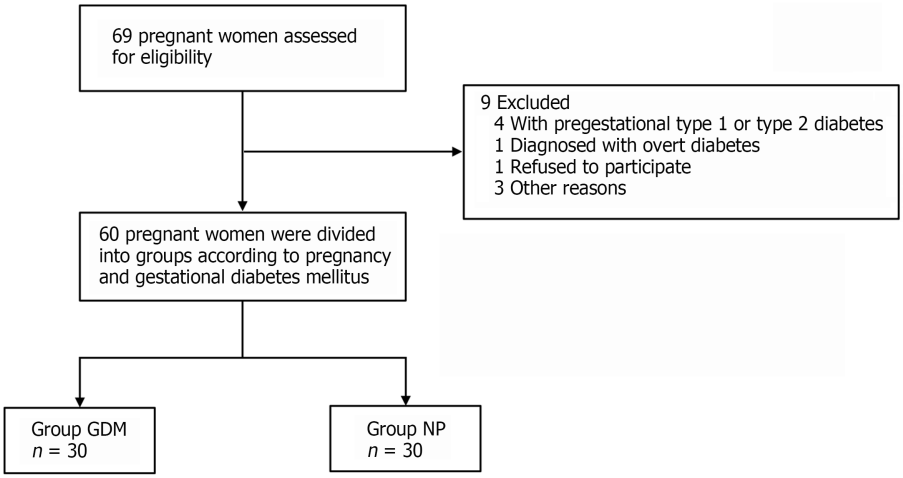

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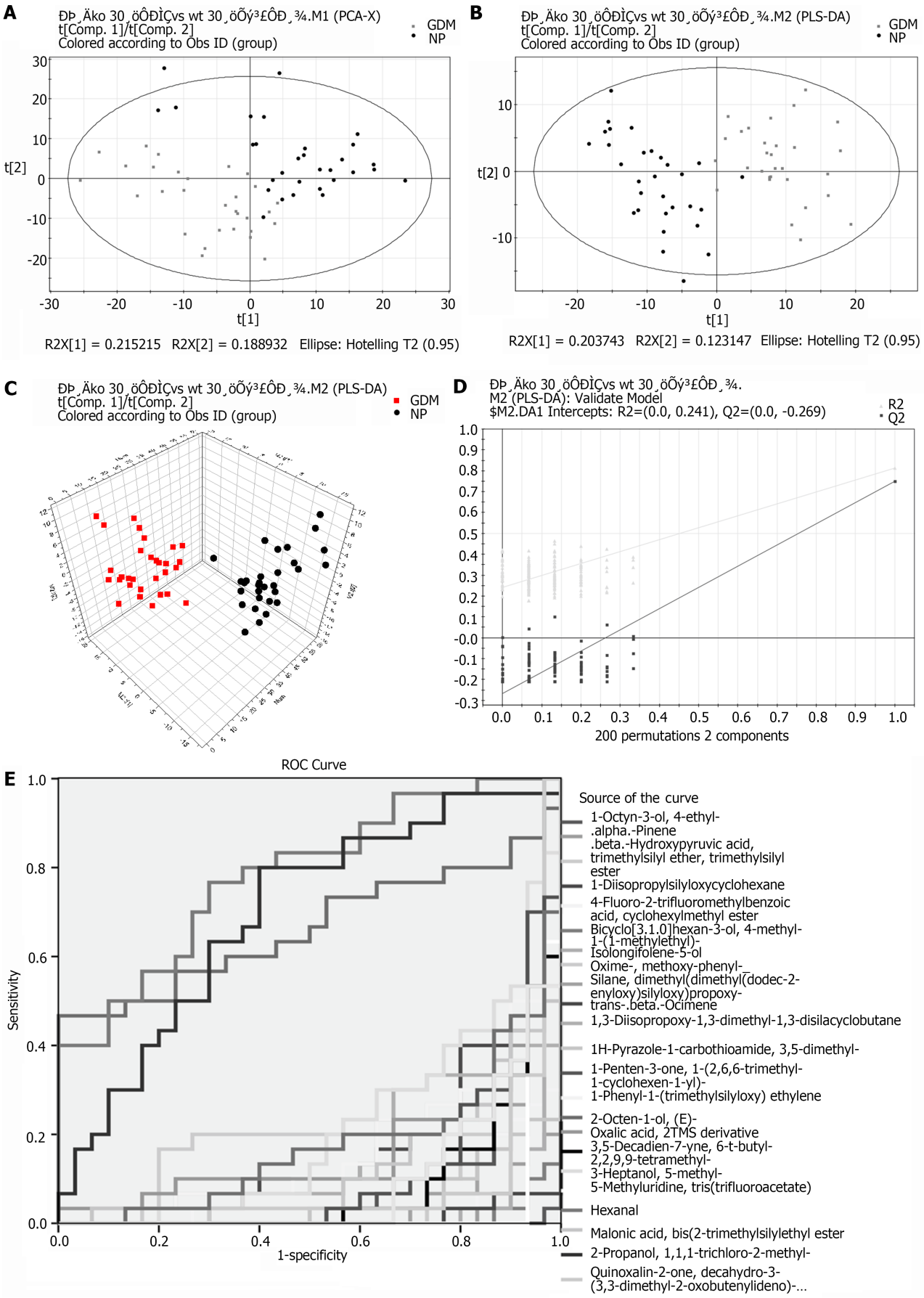
Figure 1 Patient recruitment flowchart. GDM: Gestational diabetes mellitus; NP: Normal pregnancy.

ophysiology of GDM and help identify new biomarkers and effective therapeutic targets. In this study, we observed VOCs in the blood and urine of GDM patients and compared them with age-matched normal patients. Abundant organic matter was detected in the blood and urine of GDM patients. Some of the compounds had diagnostic value.

The following five compounds were highly expressed in the GDM group: 2-octen-1-ol; 2-propanol, 1,1,1-trichloro-2-methyl; hexanal; oxalic acid, 2TMS derivative; and oxime-, methoxy-phenyl. Most of these compounds are alcohols or aldehydes.

Aldehydes are active carbonyl organic molecules that are widely present in the body. They have variable structures. The structures of over 20 types of active aldehydes have been determined and studied. These include hexanal found in this study[7]. ROC curves for hexanal correlated well with the specificity and sensitivity of GDM (AUC > 0.5). The findings implicate hexanal as a potential marker of GDM.

Active aldehydes are mainly produced during lipid and glucose metabolism (including enzymatic and non-enzymatic pathways). The enzyme pathway usually involves an aldehyde intermediate or by-product produced during glucose and lipid metabolism *in vivo*[8]. This is also consistent with the disorder of active aldehyde metabolism observed in pregnant women with GDM. Under pathological conditions, aldehyde metabolism is disordered, resulting in abundant accumulation of aldehyde and formation of an aldehyde microenvironment[9]. Aldehyde metabolism disorders are involved in the occurrence and development of various diseases. Active aldehydes are closely related to the pathogenesis of endocrine diseases. Our previous study found that serum methylglyoxal (MGO) levels in pregnant women with GDM were significantly higher than those in healthy pregnant women[6].


A
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.215215 R2X[2] = 0.188932 Ellipse: Hotelling T2 (0.95)
B
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.203743 R2X[2] = 0.123147 Ellipse: Hotelling T2 (0.95)
C
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
D
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.
M2 (PLS-DA): Validate Model
$M2.DA1 Intercepts: R2=(0.0, 0.241), Q2=(0.0, -0.269)
R2
Q2
200 permutations 2 components
E
ROC Curve
Sensitivity
1-specificity
Source of the curve
1-Octyn-3-ol, 4-ethyl-
.alpha.-Pinene
.beta.-Hydroxypyruvic acid, trimethylsilyl ether, trimethylsilyl ester
1-Diisopropylsilyloxycyclohexane
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester
Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-
Isolongifolene-5-ol
Oxime-, methoxy-phenyl-_
Silane, dimethyl(dimethyl(dodec-2-enyloxy)silyloxy)propoxy-
trans-.beta.-Ocimene
1,3-Diisopropoxy-1,3-dimethyl-1,3-disilacyclobutane
1H-Pyrazole-1-carbothioamide, 3,5-dimethyl-
1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-
1-Phenyl-1-(trimethylsilyloxy) ethylene
2-Octen-1-ol, (E)-
Oxalic acid, 2TMS derivative
3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-
3-Heptanol, 5-methyl-
5-Methyluridine, tris(trifluoroacetate)
Hexanal
Malonic acid, bis(2-trimethylsilylethyl ester
2-Propanol, 1,1,1-trichloro-2-methyl-
Quinoxalin-2-one, decahydro-3-(3,3-dimethyl-2-oxobutenylideno)-...

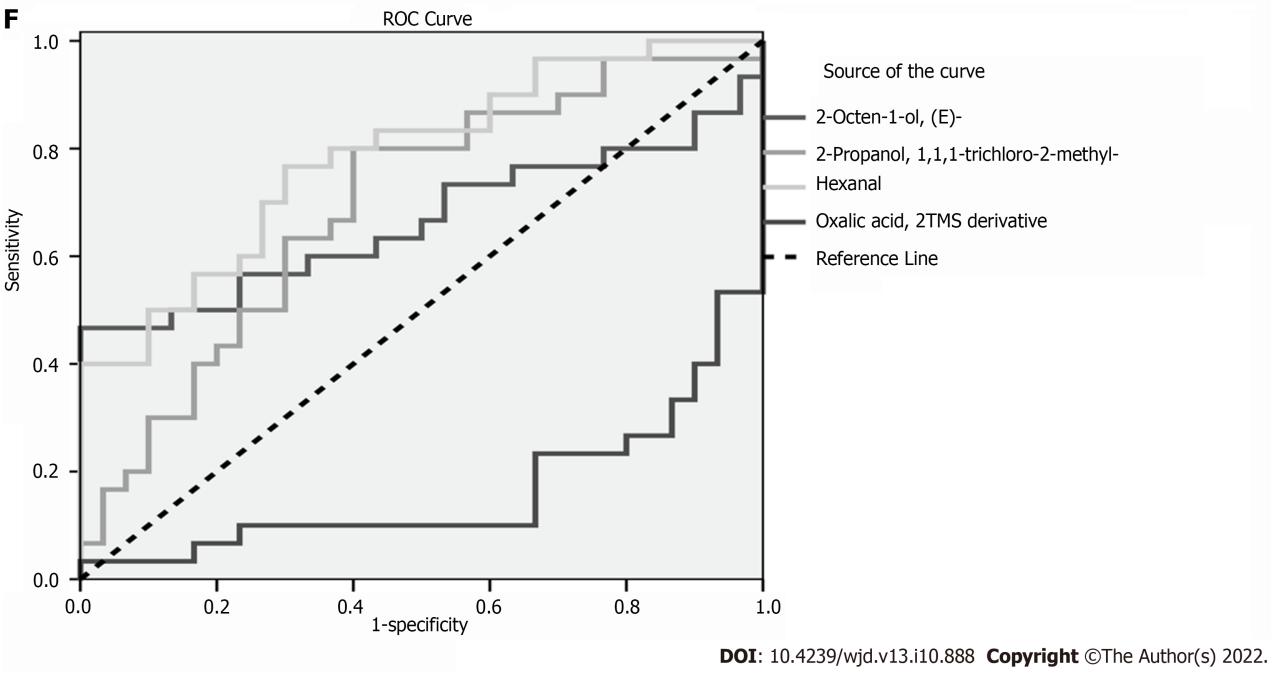


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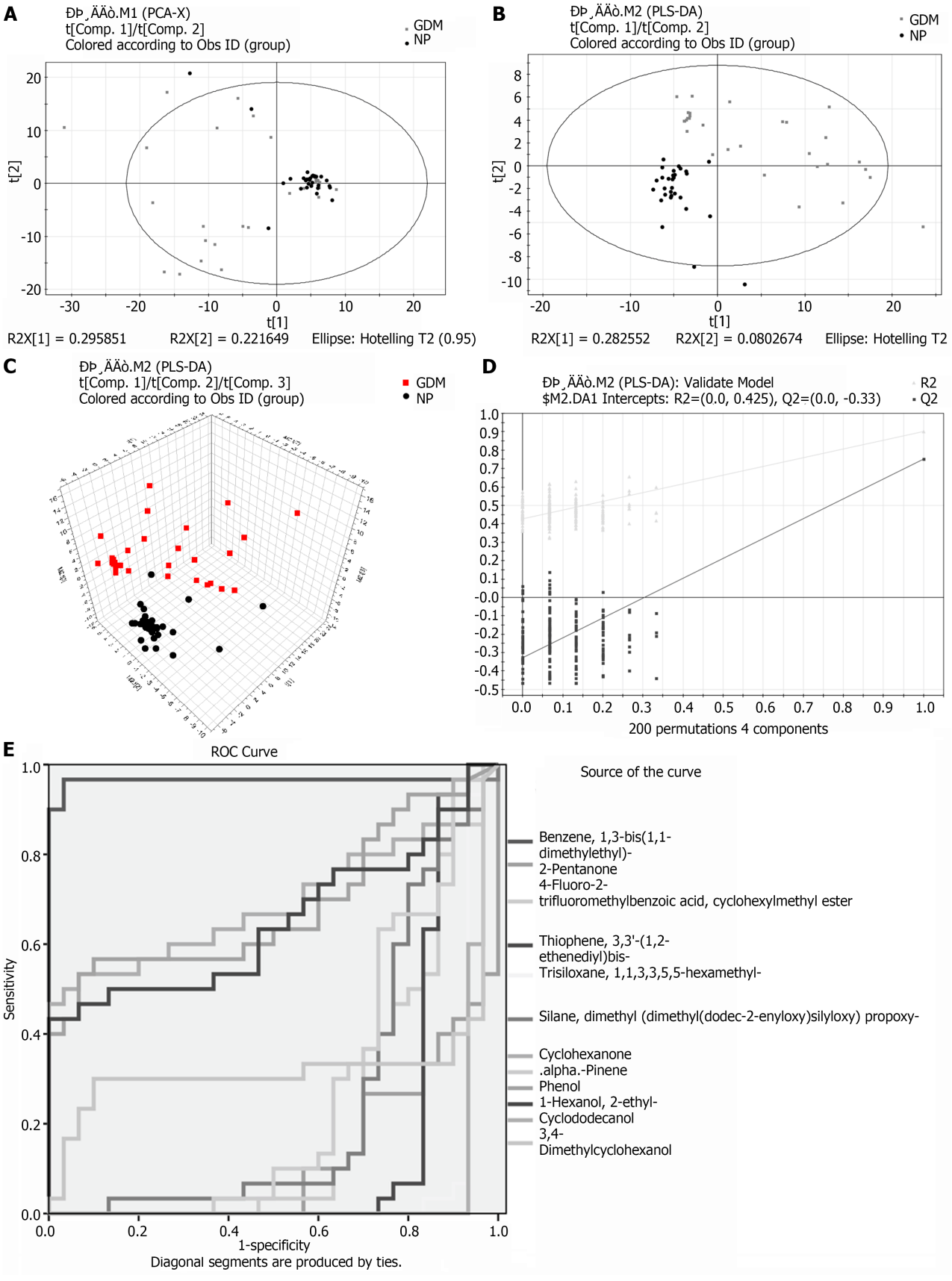
Figure 2 Metabolomic analysis of blood between groups. A: Principal component analysis score plot; B and C: Partial least squares-discriminant analysis (PLS-DA) score plots; (R^2X[1] = 0.203743, R^2X[2] = 0.123147, T^2 = 0.95); D: PLS-DA validation plot intercepts: R^2 = (0.0, 0.241), Q^2 = (0.0, -0.269); E and F: Receiver operating characteristic curves of differential substances from blood. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; ROC: Receiver operating characteristic.

MGO-induced insulin dysfunction (reduced secretion and increased resistance) may directly cause vascular dysfunction, which is a common complication of diabetes. In patients with neurodegenerative diseases, the levels of active aldehydes are also increased significantly[10]. The levels of 4-hydroxynonenal and acrolein in the brain of patients with mild cognitive impairment and early Alzheimer's disease are increased, and the neurotoxicity of acrolein is time- and concentration-dependent[11,12]. In addition, MGO can be detected in the arterial wall of a rat model of middle cerebral artery ischemia-reperfusion[13].

In a metabonomic study, GC-ion mobility spectrometry was used to analyze changes in exhaled VOCs in mild cognitive impairment, Alzheimer's disease, and normal control groups. Six compounds (tentatively acetone, 2-propanol, 2-butanone, hexanal, heptanaldehyde, and 1-butanol) play key roles in the diagnosis of mild cognitive impairment and Alzheimer's disease[14]. In addition to the detection of cognitive-related volatile substances, such as 2-propanol and hexanal, our analyses also revealed the metabolic pathways of 2-propanol and MGO (Figure 4). Therefore, the increase in serum 2-propanol levels in pregnant women with GDM may be a potential marker of MGO and cognitive decline.

In the urine analysis, 11 different metabolites were detected. Five of these were highly expressed in the GDM group: Benzene; 1,3-bis(1,1-dimethylethyl)-cyclohexanone; phenol; 1-hexanol, 2-ethyl; and 3,4-dimethylcyclohexanol. The ROC curves of these volatile substances correlated well, with AUCs > 0.5. The AUC for benzene was close to 0.9, and the AUCs for cyclohexanone, 1-hexanol, and phenol exceeded 0.5. Under the action of cytochrome P450 monooxygenase, human benzene is oxidized into toxic epoxy benzene, which combines with glutathione to form phenylmercaptouric acid. The latter is metabolized into phenol, catechol, hydroquinone, and other compounds and finally discharged from the body in the form of a sulfate conjugate or glucosidic acid. Cyclohexanone may be formed by oxidation of cyclohexane. Different amounts of cyclohexanone have been found in the exhaled breath of healthy individuals and patients with chronic obstructive pulmonary disease[15]. The levels of ethylhexyl alcohol in the blood and exhalate are reportedly reduced in patients with thyroid papillary cancer and colorectal cancer, respectively. This may be due to the consumption of ethylhexanol during tumor cell proliferation. Various lung cancer cells release 2-ethyl-1-hexanol[16]. These substances may also be related to the metabolic changes caused by oxidative stress and inflammatory changes in pregnant women with GDM.

An increasing number of metabonomic analyses of urine reflect the potential value of urine as an excreted product that can be collected non-invasively for analysis. Metabonomic methods for urinalysis can yield relatively complete metabonomic profiles and provide a new analytical approach for the diagnosis and mechanistic analysis of diseases. Metabolism of oral hypertension drugs, such as losartan, in patients with T2DM studied by GC has shown that plasma metabolites do not change, whereas urine metabolites (sorbitol and inositol) change significantly[17]. Diaz *et al*[18] analyzed the changes in urine metabolism in pregnant women in early, middle, and late pregnancy and found 21 different metabolites,


A
ÐÞ¸ÄÄò.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.295851
R2X[2] = 0.221649
Ellipse: Hotelling T2 (0.95)
B
ÐÞ¸ÄÄò.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.282552
R2X[2] = 0.0802674
Ellipse: Hotelling T2
C
ÐÞ¸ÄÄò.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]/t[Comp. 3]
Colored according to Obs ID (group)
GDM
NP
D
ÐÞ¸ÄÄò.M2 (PLS-DA): Validate Model
$M2.DA1 Intercepts: R2=(0.0, 0.425), Q2=(0.0, -0.33)
R2
Q2
200 permutations 4 components
E
ROC Curve
Sensitivity
1-specificity
Diagonal segments are produced by ties.
Source of the curve
Benzene, 1,3-bis(1,1-dimethylethyl)-
2-Pentanone
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester
Thiophene, 3,3'-(1,2-ethenediyl)bis-
Trisiloxane, 1,1,3,3,5,5-hexamethyl-
Silane, dimethyl (dimethyl(dodec-2-enyloxy)silyloxy) propoxy-
Cyclohexanone
.alpha.-Pinene
Phenol
1-Hexanol, 2-ethyl-
Cyclododecanol
3,4-Dimethylcyclohexanol

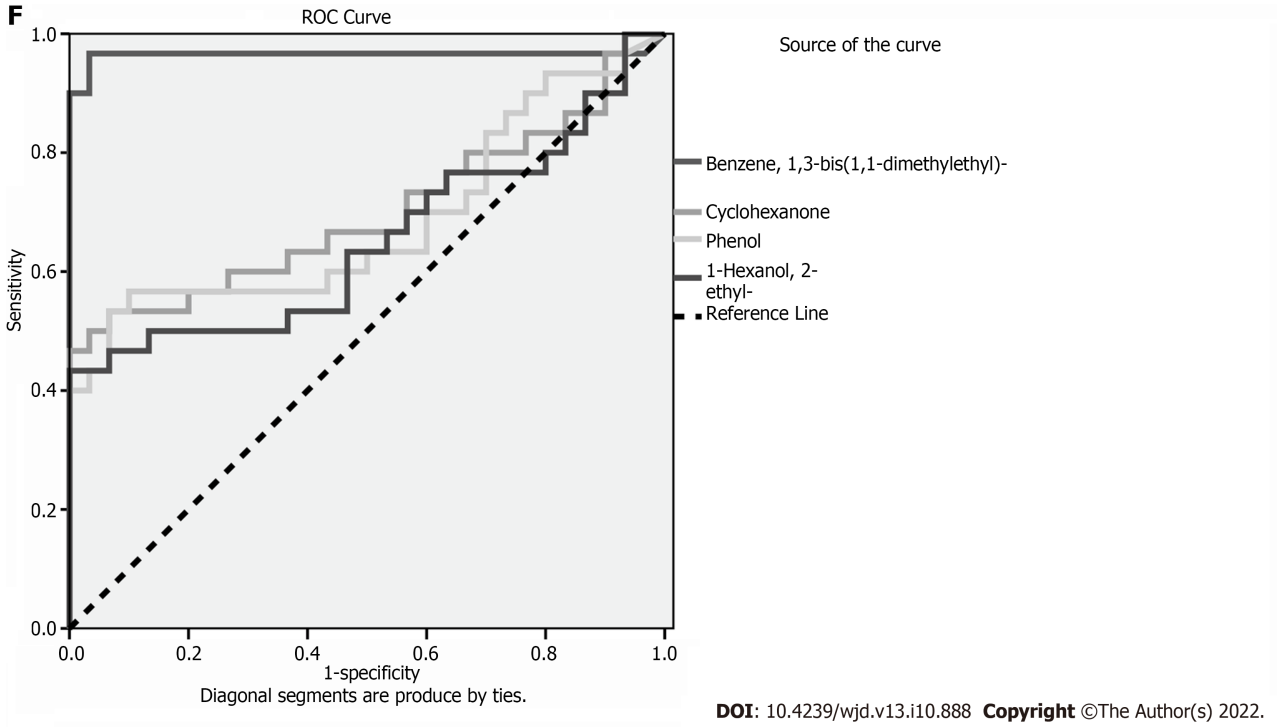


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Figure 3 Metabolomic analysis of urine between groups. A: Principal component analysis score plot; B and C: Partial least squares-discriminant analysis (PLS-DA) score plots ($R^2X[1]$ = 0.295851, $R^2X[2]$ = 0.221649, T^2 = 0.95); D: PLS-DA validation plot intercepts: R^2 = (0.0, 0.425), Q^2 = (0.0, -0.33); E and F: Receiver operating characteristic curves of differential substances from urine. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; ROC: Receiver operating characteristic.

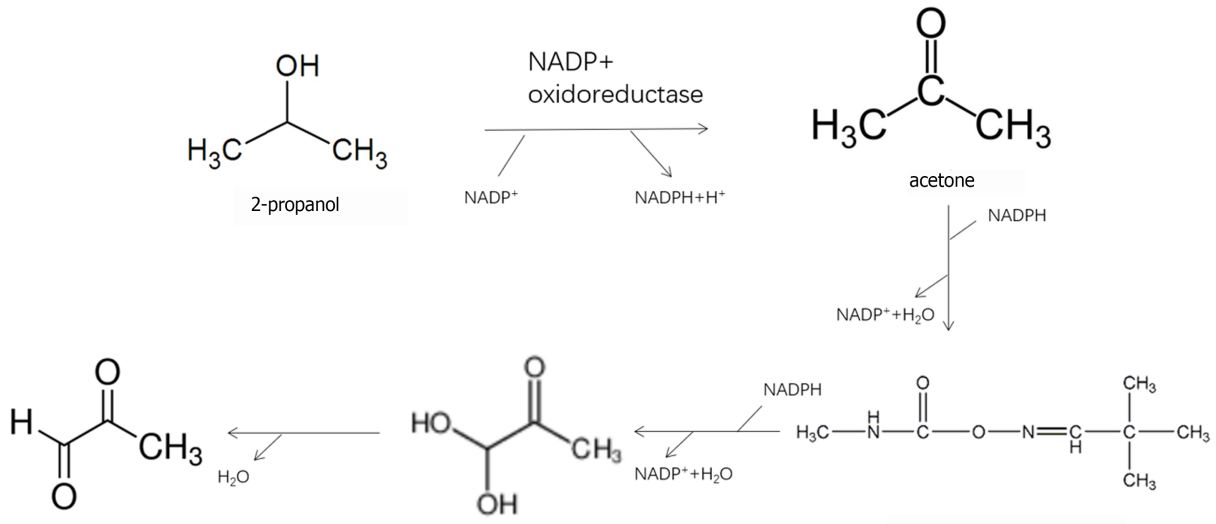

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Figure 4 Pathway of 2-propanol conversion to methylglyoxal.

including choline, creatinine, and lactate[18]. Other metabonomics analyses of the urinary metabolites of GDM pregnant women correlated p-inositol phosphate polysaccharide (P-IPG) with maternal blood glucose and pointed out that P-IPG may be a potential marker of insulin resistance in pregnant women with GDM[19]. Changes in urinary metabolites were observed in GDM patients from 8 to 16 wk postpartum[20]. The authors found that the longer the pregnancy cycle, the higher the lactose content in the urine samples of GDM pregnant women. However, lactose decreased rapidly after termination of pregnancy; in contrast, the blood concentrations of glucose and citric acid increased[20].

In this study, differential volatile metabolites in the serum and urine of pregnant women with GDM were detected by SPME. Volatile substances, such as hexanal, 2-octen-1-ol, and 2-propanol, were found in the serum of pregnant women with GDM. ROC curves indicated that they had a good correlation

with GDM, which may be potential markers. Additional analyses demonstrated the metabolic conversion of 2-propanol in GDM serum to MGO, which could cause systemic damage. Thus, 2-propanol may be a potential marker of MGO. This should be investigated further. Volatile substances, such as benzene, cyclohexanone, 1-hexanol, and phenol, were found in the urine of pregnant women with GDM. However, their metabolic sources require further study.

CONCLUSION

Differential volatile metabolites in the serum of pregnant women with GDM mainly include hexanal, 2-octen-1-ol, and 2-propanol. The differential volatile metabolites in the urine of pregnant women with GDM include benzene, cyclohexanone, 1-hexanol, and phenol.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) is a metabolic disorder in which hyperglycemia develops during pregnancy in non-diabetic women.

Research motivation

Gas chromatography-mass spectrometry (GC-MS) was used to analyze changes in metabonomics in pregnant women with GDM and to explore the mechanism of cognitive function decline in pregnant women with GDM.

Research objectives

To study the cognitive function of pregnant women with GDM and to identify potential volatile markers to evaluate the cognitive impairment of pregnant women with GDM.

Research methods

Solid-phase microextraction GC-MS analysis was used to detect organic matter in plasma and urine samples. The statistical methods used were principal component analysis and partial least squares-discriminant analysis.

Research results

Differential volatile metabolites in the serum of pregnant women with GDM mainly included hexanal, 2-octen-1-ol, and 2-propanol. The differential volatile metabolites in the urine of pregnant women with GDM included benzene, cyclohexanone, 1-hexanol, and phenol.

Research conclusions

Of 2-propanol may be a potential volatile marker to evaluate the cognitive impairment of pregnant women with GDM.

Research perspectives

The study of perinatal cognitive decline is worthwhile, especially in women with GDM. The key is the prevention and treatment of the disease. Whether 2-propanol can be used as a therapeutic target requires further investigation.

FOOTNOTES

Author contributions: Sana SRGL, Chen GM, Lv Y, Guo L, and Li EY designed the research study; Sana SRGL, Chen GM, Lv Y, and Guo L performed the research, and contributed new reagents and analytic tools; Sana SRGL analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

Clinical trial registration statement: This study is registered with the Chinese Clinical Trial Registry (No. ChiCTR2000038703).

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at lienyou_1111@163.com.

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