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Diabetes and aging: Glycemic control, insulin regulation, and the subsequent effects

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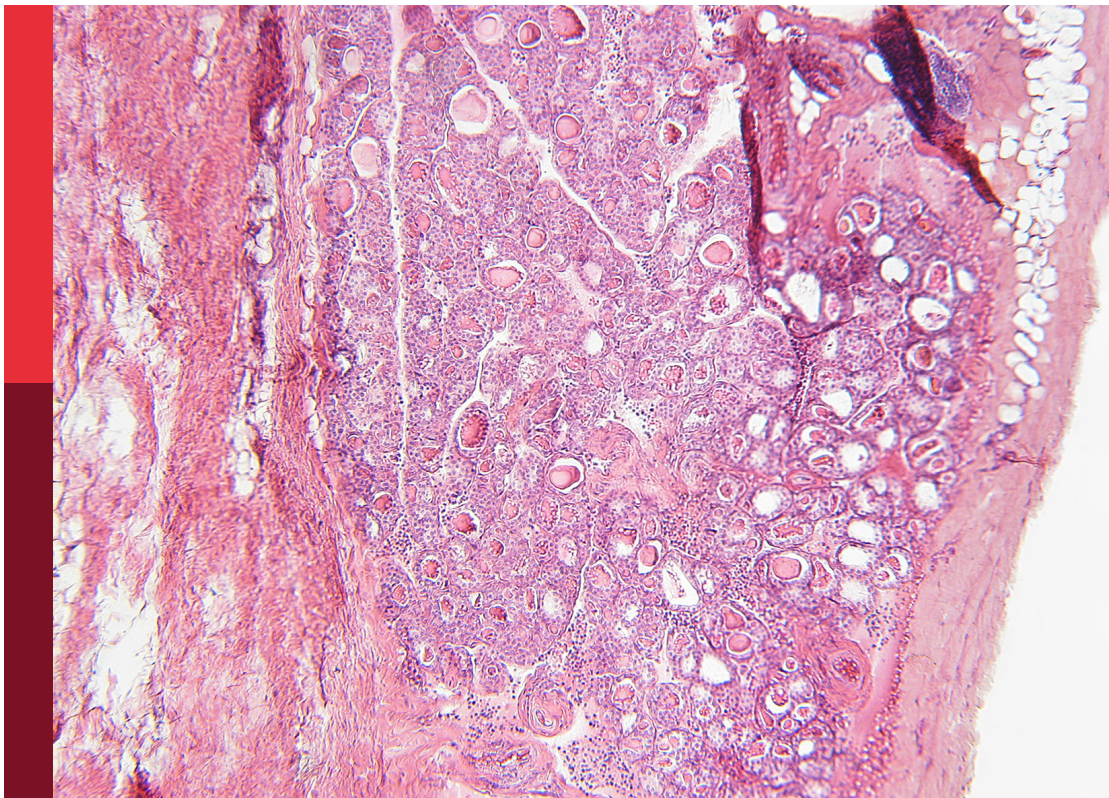

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Diabetes and aging: Glycemic control, insulin regulation, and the subsequent effects

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Editorial: Diabetes and aging

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Editorial on the Research Topic

Diabetes and aging: Glycemic control, insulin regulation, and the subsequent effects

Diabetes mellitus is one of the major cause of morbidity and mortality, and it is a major risk factor for early onset of several disfunction. The present Research Topic has been designed to publish original articles and reviews highlighting recent advances in our understanding of diabetes and the importance of glycemic control in elderly. Emphasis has been given on the underlying molecular mechanisms, the new technologies that have been introduced to facilitate early diagnosis or prevention, and the new potential therapies for the associated complications.

In a paper of this Research Topic entitled "*LC-MS-Based Untargeted Metabolomics Reveals Early Biomarkers in STZ-Induced Diabetic Rats With Cognitive Impairment*", Chen et al. performed a non-targeted metabolomics approach based on liquid chromatography-mass spectrometry (LC-MS), to screen out the serum biomarkers of diabetic mild cognitive impairment (DMMCI) in rats. Differentially expressed metabolites could provide a novel strategy for the early diagnosis of DMMCI and give new insights into the pathophysiological changes and molecular mechanisms of disease in the future. In this study, the authors used a combination of low-dose streptozotocin and a high-fat diet to establish a rat model mimicking human the T2DM model, and observed its cognitive deficit. They showed that an LC-MS-based metabolomics technology has potential value in identifying DMMCI biomarkers for the early detection and provides a novel avenue for effective therapeutic intervention in DCD, as detected in serum, sphingolipid (SP) metabolism, tryptophan (Trp) metabolism, glycerophospholipid (GP) metabolism, these metabolites may be used as the most critical biomarkers for early diagnosis of DMMCI.

In the article "*Aging Reduces Insulin Clearance in Mice*" Marmentini et al. investigated whether the effects of aging upon hepatic insulin clearance were related to changes in the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and insulin-degrading enzyme (IDE) expression, as well as IDE activity, in the liver of old mice. The authors pointed that although several studies have considered IDE as the major enzyme involved with hepatic insulin clearance, recent studies suggest that other molecular mechanisms must be more important to modulate hepatic insulin clearance, such as CEACAM1 expression. To achieve that, the authors evaluated the glucose

homeostasis, insulin secretion and hepatic insulin clearance in 3- and 18-month-old mice. Insulin clearance reduces with age and this may contribute to age-related hyperinsulinemia. Although previous studies suggest that IDE is not involved in the modulation of hepatic insulin clearance, in control and obese mice, Marmentini et al. suggest that during aging this enzyme might have a role in this modulation, as well as, the CEACAM1. Thus, the authors suggested that to investigate the molecular mechanisms whereby aging reduces IDE and CEACAM1 function in the liver might be helpful to understand how insulin clearance is affected by age.

In another article, entitled "*Glycemic Dysregulations Are Associated With Worsening Cognitive Function in Older Participants at High Risk of Cardiovascular Disease: Two-Year Follow-up in the PREDIMED-Plus Study*" the authors aimed to examine 2-year associations between baseline diabetes/glycemic status and changes in cognitive function in older participants at high risk of cardiovascular disease. As pointed, meta-analyses and longitudinal studies of population-based cohorts have shown an increased risk of cognitive dysfunction in people with metabolic syndrome, prediabetes and diabetes. Thus, Gómez-Martínez et al. evaluated longitudinal associations between glycemic status (diabetes status, control/treatment, and related biomarkers) and cognitive decline and impairment using 2 years of follow-up data, within the framework of the PREDIMED-Plus, which is a multicenter, randomized, parallel-group clinical trial conducted in Spain for primary cardiovascular disease prevention. The author pointed that the work is the first prospective study investigating associations between glycemic status (diabetes status/control/treatment, and HOMA-IR and HbA1c biomarkers) and cognitive function in a large cohort of older adults at risk high cardiovascular disease in a short period. Eligible participants were community-dwelling adults with overweight/obesity (27≤ BMI <40 kg/m2) who met at least three criteria of metabolic syndrome. The study suggested larger risk of cognitive decline in participants with type 2 diabetes. Also, they showed that, compared to participants without diabetes, those with diabetes had a borderline increased risk of developing cognitive impairment, even when the period of follow-up was only 2 years. Besides, no associations between metformin treatment and cognition were observed by the authors, as well this was not observed for IDDP-4 or sulfonylureas use. However, insulin-treated participants showed larger cognitive decline than those not treated with insulin. Thus, Gómez-Martínez et al. concluded that several glycemic dysregulations and insulin treatment were associated with greater cognitive decline in older individuals with overweight/obesity at high cardiovascular disease risk in a short time period, pointing the clinical relevance to assess novel effective strategies at the initial stages of diabetes-related alterations.

In their study titled "*Comparative Cardiovascular Outcomes of SGLT2 Inhibitors in Type 2 Diabetes Mellitus: A Network Meta-Analysis of Randomized Controlled Trials*", Jiang et al. performed an network meta-analysis (NMA) of randomized controlled trials (RCTs) for the first time to explore cardiovascular outcomes of different kind and dosages of sodium-glucose cotransport-2 (SGLT2) inhibitors in T2DM patients, including dapagliflozin 2.5mg/5mg/10mg, empagliflozin 10mg/25mg, and canagliflozin 100mg/300mg. The authors searched for studies to compare the therapeutic effects of different SGLT2 inhibitors in T2DM patients, using Cochrane Library, PubMed, and Embase databases. As suggested by the authors, empagliflozin 10mg/25mg, and canagliflozin 100mg was associated with significantly lower risks of all-cause mortality compared with placebo, according to NMA. Their study also suggested that empagliflozin 10mg/25mg was leaded to significantly lower risks of all-cause mortality compared with dapagliflozin 10mg. Dapagliflozin 10mg, empagliflozin 10mg and 25mg displayed the lower risks for cardiovascular events compared with placebo. In addition, they pointed that canagliflozin 100/300mg showed significantly higher risks of cardiovascular events compared with empagliflozin 10mg/25mg according to NMA. Moreover, their analysis suggested that treatment with canagliflozin 100/300mg were associated with significantly increased risks of volume depletion compared with placebo by NMA. The authors concluded that empagliflozin 10mg/25mg once daily might be better than other SGLT2 inhibitors with low risks of all-cause mortality and cardiovascular events in patients with T2DM suggesting the need for *ad hoc* RCTs.

As part of this Research Topic, also figured the article "*Hyperglycemia and Physical Impairment in Frail Hypertensive Older Adults*", where Pansini et al. aimed at investigating the impact of hyperglycemia (HG) on physical impairment in frailty, as HG is frequently observed in frail older adults, and represents an independent predictor of worst outcomes, with or without diabetes mellitus. The authors mentioned that the results refer to a frail hypertensive population of older adults, in which physical performance affects functional decline, loss of independence, and cognitive impairment. Their interesting study suggested that HG drives physical impairment independently of DM and the authors speculated that glycemic control appears to be the best way to attempt to reverse physical impairment, with or without DM. Pansini et al. pointed that the study population was relatively small, therefore, further studies are necessary to confirm their results, ideally in large randomized trials.

In the article "*Association Between Long-Term HbA1c Variability and Functional Limitation in Individuals Aged Over 50 Years: A Retrospective Cohort Study*" Shao et al. explored the longitudinal association between long-term glycemic variability, represented by visit-to-visit HbA1c variability and functional limitations. They analyzed adults aged over 50 years who participated in the 2006 to 2016 waves of the Health and Retirement Study. The authors pointed that limitation of physical functioning threatens independence and is an independent risk factor for impaired quality of life,

institutionalization, further functional decline, and premature mortality in older adults. They indicated that the association between diabetes and functional limitation and disability is well documented, but mean HbA1c provides incomplete information regarding glycemic variability. Thus, the authors explored whether glycemic variability in individuals without diabetes is an independent risk factor for functional limitation, which is currently unknown, using data from the 2006 to 2016 waves of the Health and Retirement Study. Shao et al. found that HbA1c variability was associated with more difficulties in functional activities over time, indicating that HbA1c variability was a superior predictor of functional decline over mean HbA1c. Their results showed the association between glycemic variability, as measured by variability score in visit-to-visit HbA1c over time, and the number of physical functioning difficulties independent of mean HbA1c in individuals aged over 50 years. The authors also pointed that more trials are needed to establish glycemic variability as an independent risk factor for functional decline and diabetes complications, and regarding the importance to confirm whether strategies to reduce glycemic variability in HbA1c can effectively reduce the incidence or progression of physical functioning impairment.

Turning their attention to the complex link between type 2 diabetes, cognition, and neurovascular coupling, Barloese et al. worked in the review "*Neurovascular Coupling in Type 2 Diabetes With Cognitive Decline. A Narrative Review of Neuroimaging Findings and Their Pathophysiological Implications*". In the article they discuss how the disease-related pathology changes neurovascular coupling (NVC) in the brain from the organ to the cellular level. The authors pointed that a clinical manifestation cognitive impairment or so-called diabetic "cogno-pathy" is receiving increasing attention. As mentioned by the authors, the identification of neurovascular abnormalities that are attributable to diabetes and precede structural and clinical changes, holds the potential to guide personalized preventive interventions. In this line, Barloese et al. focused on how NVC is impaired by T2DM and how it is possible to measure T2DM-related neurovascular dysfunction in humans. The authors pointed modalities that are used to measure NVC in humans, and that each of them has its limitations, however, there is converging evidence for an independent effect of the T2DM-state on NVC with cognitive decline as a possible progressing clinical correlate. Thus, the authors suggest the importance of early detection of impaired NVC in T2DM patients and preventive treatment before irreversible damage occurs.

Kim et al. in their paper titled "*Tolerability and Effectiveness of Switching to Dulaglutide in Patients With Type 2 Diabetes Inadequately Controlled With Insulin Therapy*" conducted a retrospective, observational study, to investigate whether switching to dulaglutide, a GLP-1 receptor agonist, would improve glycemic control of patients with T2DM inadequately controlled with conventional insulin treatment. As the authors pointed, it is common the use of insulin as an adjunct to oral hypoglycemic agents (OHAs). Thus, they analyzed the human subjects' medical record and laboratory data of patients with T2DM whose HbA1c levels were 7.6% or higher when treatment was switched from insulin to dulaglutide. Replacing insulin therapy with a combination of a GLP-1 receptor agonist and OHAs could be effective in patients with uncontrolled T2DM receiving insulin therapy. They showed that 20 patients with T2DM (approximately 14.5%) could not tolerate or did not prefer weekly dulaglutide administrations (reasons included cost, gastrointestinal side effects, dissatisfaction with the drug), and 56 (approximately 40.6%) could successfully discontinue insulin and use either weekly dulaglutide or OHAs and presented glycemic effectiveness after the switch. The mean HbA1c value in that group significantly reduced from 8.7% to 7.8%, and of the 56 patients, 23 (16.7%) patients could completely cease all injection therapies including dulaglutide and maintained stable glycemia over the 6-month period. They also found that older age, a higher dose of insulin at the time of switching to dulaglutide, and a low level of postprandial glucose were significant predictive factors for insulin resumption after switching from insulin to weekly dulaglutide. Kim et al. concluded that dulaglutide can be used for glycemic control in patients with T2DM with glucose levels inadequately controlled by insulin regimens. The authors pointed several limitation of the work, as was an uncontrolled, open-label, longitudinal, retrospective study, which is limited in its applicability and clinical relevance to generalization and broader clinical practice. Besides, the short follow-up period is an other limitation of their study. However, they highlight that the findings show the natural results of real-world practice that did not involve any interventions.

In a cross-sectional study, tilted "*Influence of circulating nesfatin-1, GSH and SOD on insulin secretion in the development of T2DM*", Huang et al. aimed to evaluate the correlation of nesfatin-1, GSH and SOD levels with beta cell insulin secretion and their influence on insulin secretion in the development of T2DM. They analyzed serum levels of nesfatin-1, GSH and SOD from 75 patients with T2DM, 67 with prediabetes and 37 healthy participants, that were recruited in this study. The author pointed that in face of multiple explanations proposed in the development of T2DM, oxidative stress is considered to be pivotal in this process, and evaluation of glutathione (GSH) or superoxide dismutase (SOD) are important indicative of oxidative stress. Also, they highlight nesfatin-1, a newly identified peptide with 82 amino acids, that has been found to be functional in anti-inflammation and antioxidation. Thus, evaluating important factors involving insulin secretion in the development of T2DM, the authors aimed to provide new ideas for forthcoming investigations on the roles of these factors in pathogenesis of T2DM. They divided T2DM and prediabetes patients into subgroups by HOMA-β with the cut-off value of 62.9 for male and 60.6 for female. Also, to assess whether beta

cell insulin secretion varies in prediabetes, three subgroups of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and IFG combined IGT were divided, according to the American Diabetes Association classification, and their HOMA-β values were compared. In a scenario, where there were no significant differences in gender, age and BMI among the three studied groups, the authors showed that serum GSH levels in T2DM were significantly reduced than that in prediabetes or the control, and this significant reduction was also confirmed in prediabetes vs. the control. Besides, further comparisons revealed that the difference of GSH levels among prediabetes subgroups of IGT, IFG and IFG+IGT was insignificant. Also, they showed that GSH levels in either subgroup of T2DM or prediabetes with impaired HOMA-β values were overwhelmingly dropped, in contrast to the counterparts with normal HOMA-β. Besides, they found that SOD levels in T2DM and prediabetes were remarkably decreased compared with the healthy control, and also a significant reduction of the SOD level in T2DM vs. prediabetes. Moreover, they observed that serum SOD levels in subgroup of IFG or IFG combined IGT displayed a marked reduction compared to the IGT subgroup. Their results of GSH and SOD reduction in T2DM and prediabetes suggest that in the condition of T2DM or prediabetes, the anti-oxidation capacity in the body may be partly damaged. In that way, they suggest that fortifying the antioxidative defense system of the patients with prediabetes may help regress or alleviate the progression of the disease toward T2DM. In addition, they found that serum nesfatin-1 levels in T2DM were obviously reduced compared to that in prediabetes or healthy subjects, and his reduction still presented when they compared prediabetes to the control. Besides, they found that difference of serum nesftain-1 levels in IGT were insignificant compared to either in IFG or in IFG+IGT, and that nesfatin-1 levels were significantly correlated with GSH and SOD, indicating a high probability of nesfatin-1 exerting antioxidative effects in the development of T2DM. In conclusion, despite the limitations of the study, as pointed by the authors, Huang et al. study managed to identify the correlation of nesfatin-1, GSH and SOD levels with beta cell dysfunction in T2DM, implicating their roles in beta cell toxicity as a result of oxidative stress.

In the study "*Relationship between physical performance and mild cognitive impairment in elderly hemodialysis patients is modified by the presence of diabetes: A multicenter cross-sectional study*", Zhao et al. aimed to explore the relationship between physical performance and mild cognitive impairment (MCI) in elderly hemodialysis patients with and without diabetes. They hypothesized that the presence of diabetes would lead to poorer physical performance and high prevalence of MCI, and different conditions may influence the association between physical performance and MCI, and also investigated the association between physical performance and specific cognitive functions in the presence or absence of T2DM in hemodialysis patients. To achieve that, Zhao et al. performed a multicenter cross-sectional study recruiting patients, aged 60 years or older, who underwent hemodialysis in dialysis units. They formed four groups: non-diabetes non-MCI, non-diabetes MCI, Diabetes non-MCI, and Diabetes MCI, a total of 396 patients. The authors found that diabetic hemodialysis patients with MCI performed worse mobility than the non-diabetes group, and that, whether compared with MCI in the non-diabetes group or non-MCI in the diabetes group, diabetic patients with MCI have poor mobility. Also, they found that the prevalence of MCI in diabetic hemodialysis patients was high (20.6%), thus they pointed that diabetes in end-stage renal disease patients receiving hemodialysis may be an important risk factor for the development of MCI. Besides, in face of a significant interaction found between mobility and diabetes in hemodialysis patients in the study, they suggested that poor physical performance due to diabetes may be an important risk factor for the development of MCI. However, they also pointed the need of future studies focused on the different cognition changes in the weak physical population, in more well-designed cohort studies to verify the relationship between physical performance and different cognitive functions. In conclusion, their study study provides considerations for physicians that poor mobility in diabetic hemodialysis patients are more associated with MCI.

In the last study, titled "*The clinical characteristics of Chinese elderly patients with different durations of type 2 diabetes mellitus*" Yu et al. explored the clinical characteristics among 3840 elderly (aged ≥60 years) patients, diabetes duration and the comprehensive management of T2DM as well as diabetic vascular complications in Chinese elderly patients with T2DM. The authors studied 972, 896, 875 and 1097 patients, that were respectively divided into four groups, according to diabetic duration: < 1 year (Group 1), 1~5 years (Group 2), 5~10 years (Group 3), and ≥ 10 years (Group 4). They found that compared to group 1, the level of HbA1c was significantly higher in group 4, but was significantly lower in group 2 and group 3. Also, they observed that group 4 had a significantly higher control rate of total cholesterol (TC) when compared with groups 1, similarly for the control rates of low density lipoprotein cholesterol (LDL-C). Besides, patients of group 4 were more likely to be higher control rate of triglyceride (TG) and body mass index (BMI) when compared with other groups. They also found that elderly T2DM patients with a duration of diabetes of ≥ 10 years were more likely to achieve the comprehensive control targets for TC, LDL-C and TG, while elderly T2DM patients with a duration of diabetes of 1~5 years were more likely to achieve the HbA1c control target than elderly T2DM patients with a duration of diabetes of < 1 year. The authors pointed that the higher control rates for TC, LDL-C, TG and BMI were observed in elderly T2DM patients with a duration of diabetes of ≥ 10 years than that in patients who had a duration of diabetes less than 1 year. Their study also suggest that elderly T2DM patients with a duration of diabetes of 5~10 years or ≥ 10 years were more likely to develop diabetic macrovascular complications than those with a duration of diabetes of <1 year. In addition, they indicated that the duration of diabetes was significantly associated with microvascular complications.

Yu et al. concluded that the clinical characteristics of elderly patients with T2DM in different durations of diabetes are different.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aging Reduces Insulin Clearance in Mice

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Hyperinsulinemia is frequently associated with aging and may cause insulin resistance in elderly. Since insulin secretion and clearance decline with age, hyperinsulinemia seems to be maintained, primarily, due to a decrease in the insulin clearance. To investigate these aging effects, 3- and 18-month-old male C57BL/6 mice were subjected to intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT) and, during the ipGTT, plasma c-peptide and insulin were measure to evaluate *in vivo* insulin clearance. Glucose-stimulated insulin secretion in isolated pancreatic islets was also assessed, and liver samples were collected for molecular analyses (western blot). Although insulin sensitivity was not altered in the old mice, glucose tolerance, paradoxically, seems to be increased, accompanied by higher plasma insulin, during ipGTT. While insulin secretion did not increase, insulin clearance was reduced in the old mice, as suggested by the lower c-peptide:insulin ratio, observed during ipGTT. Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) and insulin-degrading enzyme (IDE), as well as the activity of this enzyme, were reduced in the liver of old mice, justifying the decreased insulin clearance observed in these mice. Therefore, loss of hepatic CEACAM1 and IDE function may be directly related to the decline in insulin clearance during aging.

Keywords: CEACAM1, hepatic insulin clearance, hyperinsulinemia, insulin-degrading enzyme, insulin secretion, insulin sensitivity

INTRODUCTION

Aging is commonly associated with insulin resistance and hyperinsulinemia (1, 2). Although it is hypothesized that insulin resistance may cause a compensatory hyperinsulinemia (3), it has been demonstrated that hyperinsulinemia downregulates insulin receptors at the cellular membrane and disrupts post-receptor intracellular signaling in its target cells, inducing insulin resistance (4, 5). Thus, it remains unclear whether insulin resistance or hyperinsulinemia comes first during the aging process.

In mice, genetic ablation of insulin gene (*Ins2* $^{+/-}$) reduced the circulating levels of this hormone, and this reduction preserved their insulin sensitivity as they aged, compared with their controls (6). It suggests that hyperinsulinemia might induce insulin resistance during aging. Therefore, to

investigate the mechanisms whereby circulating insulin levels increase with age it is important to find new strategies to counteract this age-related disorder.

Plasma insulin levels are determined by insulin secretion, and its removal from the circulation, known as insulin clearance. Thus, increased insulin secretion and/or decreased insulin clearance could contribute to hyperinsulinemia during aging. While several studies have reported decreased insulin secretion in aged rodents and humans (7, 8), others have reported decreased insulin clearance in elderly (9, 10). These latter data suggest that age-related hyperinsulinemia could be explained, primarily, by a reduction in the insulin clearance. Therefore, to better understand the effects of aging upon insulin clearance, the molecular mechanisms involved in this reduction should be investigated.

Insulin clearance has, basically, two components: hepatic and extrahepatic clearance. Since the hepatic insulin clearance can remove about 50 to 80% of insulin secreted, during its first passage through the liver (11), we focus on this component. In the liver, this process is initiated when insulin binds to its receptor (IR). After IR is activated by insulin, an important protein that promotes receptor-mediated insulin internalization, namely carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), is activated and it associates with insulin-IR complex, targeting this complex to clathrin-coated pits/vesicles, triggering the endocytosis process. In the early endosome, insulin-IR complex is destabilized and the IR may be recycled to the cellular membrane, *via* retro-endocytosis, while insulin is cleaved by the major enzyme responsible for its degradation, the insulin-degrading enzyme (IDE) (11–13). Although IDE have been considered an important enzyme involved with insulin clearance, recent studies have demonstrated that liver-specific ablation of IDE (L-IDE-KO) did not affect insulin clearance in mice (14, 15), suggesting that other molecular mechanisms may play an important role in this process. Indeed, mice with global null mutation or with liver-specific inactivation of Ceacam1 gene display hyperinsulinemia due to their impaired insulin clearance, which in turn induces insulin resistance in these mice (16, 17).

Here, we evaluated the glucose homeostasis, insulin secretion and hepatic insulin clearance in 3- and 18-month-old mice. We also investigated whether the effects of aging upon hepatic insulin clearance were related to changes in the CEACAM1 and IDE expression, as well as IDE activity, in the liver of these mice.

MATERIAL & METHODS

Animals

Twenty male C57BL/6 mice from the University of Campinas (UNICAMP) facilities were housed collectively (5 animals per cage) and maintained under a light-dark cycle (12 h light and 12 h dark) with a controlled humidity and temperature until 3- (control group, CTL, n=10) or 18-months-old (old group, OLD, n=10). These mice were allowed to freely drink tap water and feed a standard chow diet. The described experimental procedures were approved by the Committee on Ethics in the Use of Animals of the UNICAMP (CEUA-UNICAMP, approval number 4659-1/2017), and were conduct in accordance with the last revision of the National Institutes of Health (NIH) guide for the care and use of laboratory animals.

Intraperitoneal Glucose and Insulin Tolerance Tests (ipGTT and ipITT)

To test glucose tolerance, mice were restricted to food during 10 h before they receive an intraperitoneal administration of 1 g × kg^{-1} glucose load. Their blood glucose was measured before (0 min) and 15, 30, 60 and 120 min after glucose load administration, from the tip of their tails using a blood glucose meter (Accu-chek®, Roche, Basileia, Switzerland). To test insulin tolerance, mice were restricted to food during 2 h before they receive an intraperitoneal administration of 0.75 U × kg^{-1} insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA), and their blood glucose was measured before (0 min) and 5, 10, 15, 20, 25, 30 and 60 min after insulin administration.

In Vivo Insulin Clearance

The insulin clearance of mice was evaluated calculating plasma c-peptide:insulin ratio, during the ipGTT, as previously described (18). To this purpose, blood samples were collected from the tip of the tail before (0 min) and after 15 and 60 min glucose load administration. The blood samples were centrifuged (1100 g, during 15 min at 4°C) to obtain plasma, which were stored at -80°C to posterior c-peptide and insulin measurements. These hormones were measured using specifics enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Mouse C-Peptide ELISA Kit Catalog # 90050 and Ultra-Sensitive Mouse Insulin ELISA Kit Catalog # 90080, Crystal Chem, Elk Grove Village, IL, USA).

Glucose-Stimulated Insulin Secretion in Isolated Pancreatic Islets

All mice were anesthetized with isoflurane and killed by decapitation to dissect and collect tissues, such as the pancreas, which were digested with collagenase to isolate pancreatic islets, as described before (19). Five islets from each mouse were used to assess the glucose-stimulated insulin secretion as previously described (20) with minor modifications. After 1 h preincubation in Krebs-Ringer bicarbonate (KRB) buffer containing 0.3% bovine serum albumin (BSA) and 5.6 mmol × l^{-1} glucose (95% O2, 5% CO2, pH 7.4, at 37°C), the islets were incubated for an additional hour in the same buffer containing 0.3% BSA and 2.8 or 11.1 mmol × l^{-1} glucose. After this incubation, the supernatants were collected to access insulin secretion and the remaining islets were homogenized in an alcohol-acid solution to measure total insulin content using the Ultra-Sensitive Mouse Insulin ELISA Kit (Catalog # 90080, Crystal Chem, Elk Grove Village, IL, USA).

Western Blot Analyses

Liver samples were also collected to evaluate protein expression by western blot as previously described (21). In this study, the primary antibodies and their respective dilutions used, in this study, were as follow: anti-IDE 1:500 (Catalog ab32216, Abcam, Cambridge, UK); anti-CEACAM1 1:500 (Catalog 14771, Cell

Signaling, Danvers, MA, USA); and anti-α-Tubulin 1:30000 (Catalog T5168, Sigma-Aldrich, St Louis, MO, USA).

IDE Activity Measurements

Liver IDE activity was measured using the SensoLyte 520 IDE Activity Assay Kit according to the manufacturer's instructions (Catalog AS-72231; AnaSpec, Fremont, Canada). Total IDE activity was calculated as described before (18) and normalized per μg of total protein content determined using the Bio-Rad Protein Assay Dye Reagent Concentrate (Catalog #5000006, Bio-Rad, Hercules, CA, USA).

Statistics

Normal distribution of the data and homogeneity of variance were tested, and to compare data from CTL and OLD groups (CTL vs OLD) Student's unpaired t-test was applied. These statistical analyses were performed using Prism software version 8.0.1 for Windows (GraphPad Software, La Jolla, CA, USA). The sample size (n) used for the statistical analysis of each group was described in the figure's legends. All data were presented as the mean ± standard deviation (SD) and were considered significantly different if the p-value was equal or lower than 0.05 ($p \leq 0.05$).

RESULTS

Aging Did Not Change Fasting Blood Glucose and Plasma Insulin Levels

Eighteen-month-old (OLD) mice had increased body weight and reduced gastrocnemius muscle pad without change in the perigonadal fat pad, compared with 3-month-old (CTL) mice, as shown in the **Table 1**. In addition, fasting blood glucose and plasma insulin levels were not different between the groups.

Aging Increased Glucose Tolerance Without Changing Insulin Sensitivity

To evaluate glucose homeostasis, intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT) were performed. During the ipGTT, OLD mice presented decreased blood glucose levels at 15 and 30 min (**Figure 1A**). Also, the area under the curve (AUC) was lower, compared with CTL mice (**Figure 1B**). Although the OLD mice displayed increased glucose tolerance, their insulin sensitivity was similar to that observed in the controls (**Figures 1C, D**).

Aging Decreased Hepatic Insulin Clearance

During the ipGTT, blood samples were collected and the plasma was used to measure c-peptide and insulin levels at 0, 15 and 30 min after the glucose load (**Figures 2A, B**). Although plasma c-peptide levels were similar between groups, plasma insulin levels were significantly higher in the OLD at 15 min, compared with CTL group, provoking a reduction in the c-peptice:insulin ratio at this time point (**Figure 2C**). It seems that insulin secretion was not altered, since plasma c-peptide was similar between groups, but the hepatic insulin clearance was reduced in the OLD group, as judged by their lower AUC of plasma c-peptide:insulin ratio, compared with the CTL's ratio (**Figure 2D**).

Aging Did Not Alter Glucose-Stimulated Insulin Secretion in Isolated Pancreatic Islets

Corroborating the similar plasma c-peptide levels between the groups, during the ipGTT, glucose-stimulated insulin secretion was not significantly different in isolated pancreatic islets (**Figure 3A**), although insulin content was higher in the OLD, compared with CTL group (**Figure 3B**).

Aging Decreased Hepatic CEACAM1 and IDE Expression

To investigate the molecular mechanism whereby aging decreases hepatic insulin clearance, we evaluate the expression of proteins involved with this process. The expression of the transmembrane protein involved with the endocytosis of the insulin-IR complex, CEACAM1, was decreased in the liver from the OLD mice compared with controls (**Figure 4A**). Also, IDE, an important enzyme that degrades insulin, had its expression (**Figure 4B**) and activity (**Figures 4C, D**) reduced in the liver from the OLD, compared with CTL mice.

DISCUSSION

Hyperinsulinemia is related to aging and may be the consequence of an increase in insulin secretion and/or a decrease in its clearance. In our previous study, while insulin secretion was increased, insulin clearance did not change in 10-month-old mice compared with 3-month-old mice (22). Here, 18-month-old mice displayed similar insulin secretion, whereas hepatic insulin clearance was lower to that found in the 3-month-old mice. These data suggest that with advancing age, β-cells from pancreatic islets may lose their ability to maintain a higher insulin

TABLE 1 | Metabolic parameters of control and old mice.

Metabolic parameters (units)	CTL	OLD
Body weight (g)	23.81 ± 0.642 (n=10)	29.47 ± 1.173 (n=10)*
Skeletal muscle pad (% of body weight)	0.578 ± 0.041 (n=10)	0.470 ± 0.023 (n=10)***
Fat pad (% of body weight)	1.058 ± 0.160 (n=10)	1.121 ± 0.352 (n=10)
Fasting glycemia (mg × dl^{-1})	107.1 ± 10.52 (n=10)	99.7 ± 15.85 (n=10)
Fasting insulinemia (ng × ml^{-1})	0.172 ± 0.029 (n=10)	0.175 ± 0.057 (n=10)

*$p \leq 0.05$ and ***$p \leq 0.001$ vs CTL (Student's unpaired t-test).*

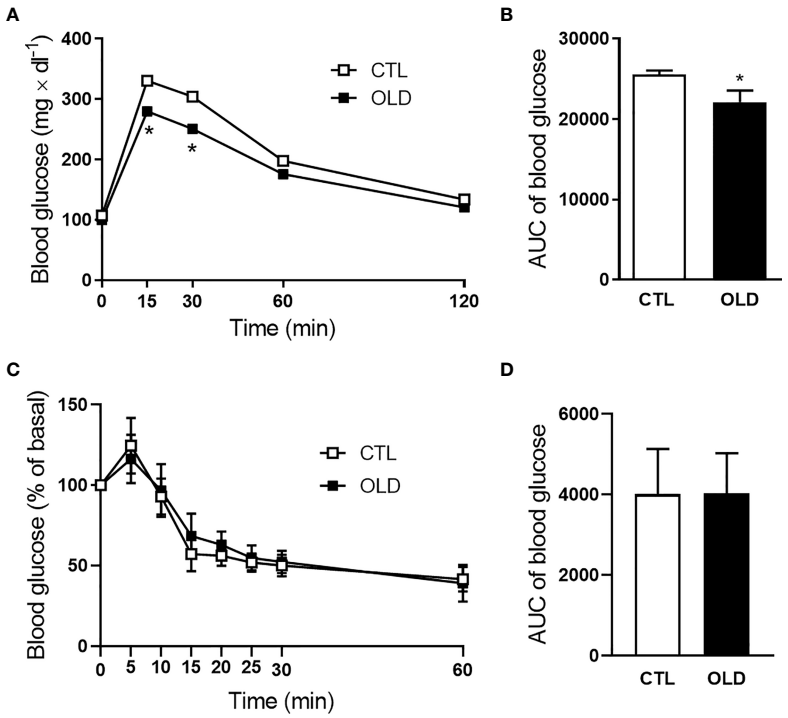

FIGURE 1 | Effect of aging upon glucose and insulin tolerance. **(A)** Blood glucose and **(B)** its AUC before (0 min) and 15, 30, 60 and 120 min after 1g × kg^{-1} intraperitoneal glucose administration (ipGTT, n = 10 CTL and 10 OLD). **(C)** Blood glucose and **(D)** its AUC before (0 min) and 5, 10, 15, 20, 25, 30 and 60 min after 0.75U × kg^{-1} intraperitoneal insulin administration (ipITT, n = 9 CTL and 9 OLD). CTL, 3-month-old mice; and OLD, 18-month-old mice. Data are presented as the mean ± standard deviation (SD). Student's unpaired t-test was used to compare the groups (*p ≤ 0.05 *vs* CTL).

secretion. To compensate, hepatic insulin clearance is reduced, probably due to a lower expression of CEACAM1 and IDE, associated with a decreased IDE activity, in the liver.

Although several studies have demonstrated impairment on glucose tolerance with age (23, 24), here, the OLD mice had improved glucose tolerance (which might be explained by the elevated plasma insulin level, as shown in **Figure 2B**), and had no change in the insulin sensitivity (**Figures 1C, D**). These data contrast to those reported in our previous study using 10- and 3-month-old mice (22). Ten-month-old mice displayed glucose intolerance, insulin resistance and hyperinsulinemia, compared with their controls. Thus, it seems that 18-month-old mice are metabolic different from 10-mont-old mice. Indeed, the body weight of 10-mont-old mice is higher than 18-month-old mice (36.05 ± 1.546 g *vs* 29.47 ± 1.173 g). Also, the perigonadal fat pad weight (% of body weight) seemed to be increased in the 10-month-old mice compared with their controls in the previous study (CTL = 1.738 ± 0.238 g *vs* OLD = 2.861 ± 0.495 g, p = 0.075), whereas here, this increase was not observed (CTL = 1.058 ± 0.160 g *vs* OLD =1.121 ± 0.352 g, p = 0.610). These differences may explain the glucose intolerance and insulin resistance observed in the 10-month-old mice used in our previous study, compared with the 18-month-old mice used here, since the increase in visceral fat pad may raise the risk for insulin resistance (25, 26).

The paradoxical normal insulin sensitivity, found in the OLD mice, led us to ask whether age-insulin resistance is an obligatory finding. We found the answer in studies with centenarians (90-100 years old) that have a preserved insulin action compared with aged subjects (<80 years old) (27). These studies show that age-related insulin resistance is not an obligatory finding in the elderly, and this may be found in other species, including rodents, as we described here.

Although age-related hyperinsulinemia was previously associated with increased insulin secretion (22, 28), here, insulin secretion in the OLD mice was similar to that found in their controls. It is possible that, in these 18-month-old mice, β-cells are in decline of their function, and the compensatory hypersecretion of insulin, that probably have occurred earlier, may not be observed at this stage. Decreased expression of the glucose transporter 2 (GLUT2) (29), decreased Ca^{2+} influx (18), mitochondrial dysfunction (30) and chronic low-grade inflammation (31), observed in aged β-cells, might be the molecular mechanisms involved with the decline in insulin secretion that occurs with age.

Since insulin secretion was not altered in the OLD mice compared with their controls (**Figure 3A**), the hyperinsulinemia observed in the former, after a glucose load (**Figure 2B**), could be due to an impaired hepatic insulin clearance as suggested by the lower c-peptide:insulin ratio, during the ipGTT (**Figures 2C, D**), in the OLD mice, compared with controls.

It is important to be aware that the c-peptide:insulin ratio can be used to measure hepatic insulin clearance when the c-peptide clearance does not change between the experimental groups.

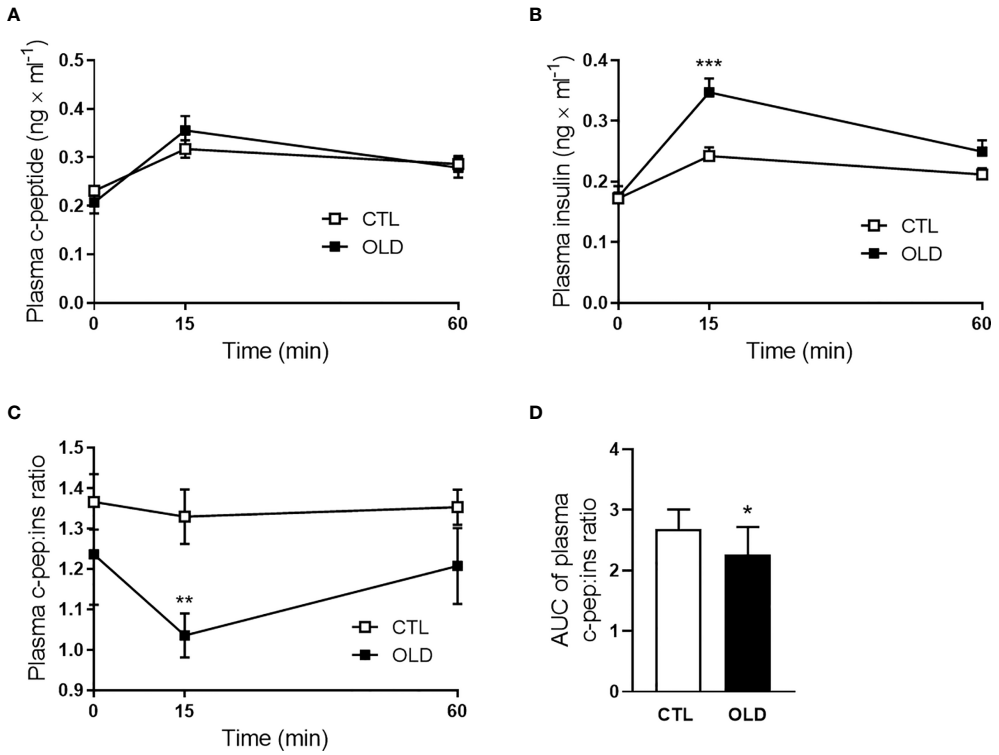

FIGURE 2 | Effect of aging upon plasma c-peptide:insulin ratio (insulin clearance) during the ipGTT. **(A)** Plasma c-peptide, **(B)** insulin, and **(C)** c-peptide:insulin ratio at 0, 30 and 60 min after 1g × kg^{-1} glucose load administration, and **(D)** AUC of plasma c-peptide:insulin ratio (n = 10 CTL and 9 OLD). CTL, 3-month-old mice; and OLD, 18-month-old mice. Data are presented as the mean ± standard deviation (SD). Student's unpaired t-test was used to compare the groups (*$p \leq 0.05$, **$p \leq 0.00$ and ***$p \leq 0.001$ *vs* CTL).

As observed in isolated pancreatic islets, insulin secretion in the OLD was not different from that found in the CTL group (**Figure 3A**). Since c-peptide is co-secreted with insulin at 1:1 molar ratio, the secretion of this hormone was not different between the groups. Considering this similar secretion of c-peptide, and the similar c-peptide kinetic, observed during the ipGTT (**Figure 2A**), we can assume that the c-peptide clearance does not change between the groups, validating our hepatic insulin clearance measurements.

During the ipGTT (**Figure 2B**), we observed lower hepatic insulin clearance only 15 min after the glucose load. This data suggests that this impairment only emerges during a glucose stimulation. We believe that in the fasting state, the liver of the OLD mice can properly handle a small amount of insulin secreted by the pancreas. However, when glucose stimulates insulin secretion, the liver of the OLD mice cannot handle the excess of insulin that reaches this organ, as the liver of the CTL mice.

Although several studies have considered IDE as the major enzyme involved with hepatic insulin clearance, recent studies using L-IDE-KO mice suggest that other molecular mechanisms must be more important to modulate hepatic insulin clearance, such as CEACAM1 expression (14–16). Here, 18-month-old mice that displayed lower hepatic insulin clearance, had a decreased CEACAM1 expression in the liver, compared with their controls (**Figure 4A**), similar to the data found in 18-month-old rats (**Supplementary Figure S1**). Corroborating these data, the hepatic expression of CEACAM1 did not decrease when insulin clearance was not significantly changed in the 10-month-old mice (**Supplementary Figure S2**).

During the process of hepatic insulin clearance, CEACAM1 is phosphorylated at specific tyrosine residue (Tyr 488) by the activated insulin receptor. This phosphorylation allows CEACAM1 to associate with insulin-IR complex, *via* Shc (SH2-containing adapter protein), targeting this complex to clathrin-coated pits/vesicles by interaction with the adaptor protein-2 (AP2) complex (13, 32), thereby triggering the endocytosis process. Therefore, although we evaluated CEACAM1 expression, it is important that further studies also investigate its activation by measuring the tyrosine phosphorylation of this protein in the liver of aged rodents.

In addition to changes in CEACAM1, changes in IDE function might be also associated with alterations in hepatic insulin clearance. Previously, in 10-month-old mice, lower hepatic IDE activity was compensated by the higher expression of this enzyme, maintaining insulin clearance similar to that found in the 3-month-old mice (33). However, considering that 12-month-old rats (older than 10-month-old) (34) showed a decrease in the hepatic IDE expression compared with their young controls, we speculated that this could also occur in the 18-month-old mice. As expected, we confirmed this effect of aging (**Figure 4B**), which might contribute to the decreased hepatic insulin clearance observed in these OLD mice.

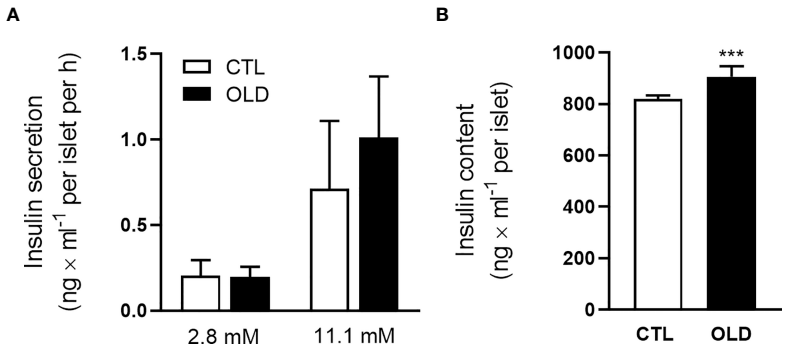

FIGURE 3 | Effect of aging upon glucose-stimulated insulin secretion in isolated pancreatic islets. **(A)** Insulin secretion per islet after 1 h incubation with 2.8 or 11.1 mmol × l^{-1} glucose. **(B)** Total insulin content per islet (n = 10 CTL and 10 OLD). CTL, 3-month-old mice; and OLD, 18-month-old mice. Data are presented as the mean ± standard deviation (SD). Student's unpaired t-test was used to compare the groups (***p ≤ 0.001 *vs* CTL).

Even though, the contribution of IDE for the modulation of insulin clearance remains controversial. It was suggested that this enzyme in the liver contributes to modulate insulin sensitivity (14, 15). Indeed, pathological conditions related with insulin resistance, such as obesity and type 2 diabetes, are frequently associated with lower hepatic IDE expression and activity (18, 35, 36), while physical exercise, which improves insulin sensitivity, is associated with higher hepatic IDE expression and activity (37–39). In line with these data, insulin resistance observed in 10-month-old mice was accompanied by a lower hepatic IDE activity compared with their young controls (22). However, in the present study, the reduction in the IDE activity in the liver from 18-month-old mice (**Figures 4C, D**), was not associated with insulin resistance. It is possible that the impairment on

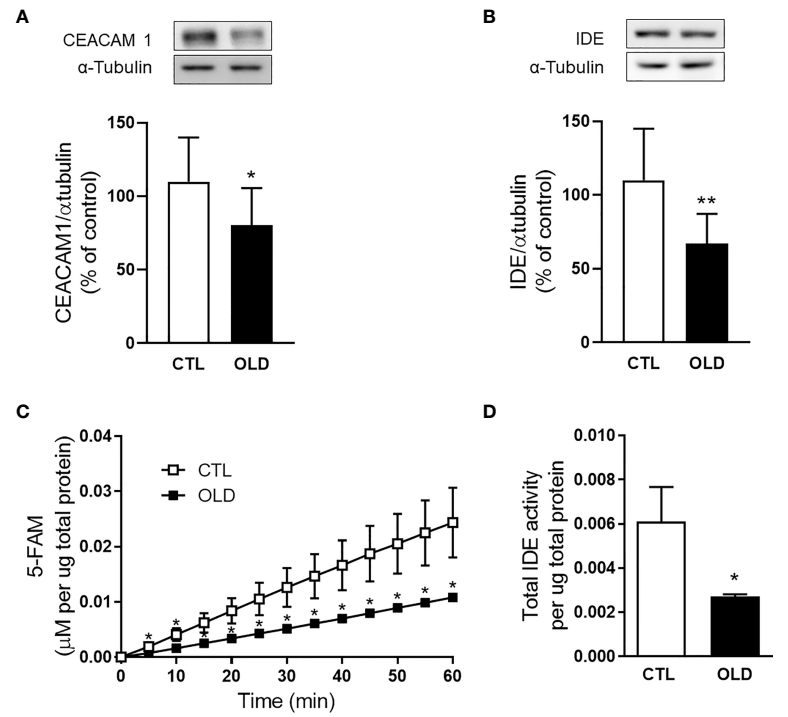

FIGURE 4 | Effect of aging upon hepatic CECAM1 and IDE expression, and upon hepatic IDE activity. Protein expression of **(A)** CEACAM1 and **(B)** IDE in the liver from the mice and its representative immunoblotting images (CEACAM1, n = 10 CTL and 10 OLD; IDE, n = 10 CTL and 10 OLD). **(C)** Kinetics of the IDE activity assay in liver of mice. Fluorescent intensity at Ex/Em = 490/520 nm was continuously recorded, every 5 min, during 60 min. 5-FAM concentration was calculated using a standard curve and normalized per μg of total protein. **(D)** IDE activity was calculated as previously described (18) and normalized per μg of total protein in the liver (n = 10 CTL and 10 OLD). CTL, 3-month-old mice; and OLD, 18-month-old mice. Data are presented as the mean ± standard deviation (SD). Student's unpaired t-test was used to compare the groups (*p ≤ 0.05 and **p ≤ 0.001 *vs* CTL).

hepatic IDE activity might precede insulin resistance, but to confirm this hypothesis a time-course study is necessary.

Taking into account all data from 10- and 18-month-old mice, one effect of aging is consistent, hepatic IDE activity reduces with age. This effect was also observed in 18-month-old rats (**Supplementary Figure S3**) and this may be involved with an impaired glucose homeostasis, frequently observed in aged subjects. Previously, we suggested that an increased expression of the inducible nitric oxide synthase (iNOS), observed in the liver from 10-month-old mice, should be linked to the reduction in the hepatic IDE activity, because it was reported that nitric oxide (NO) inhibits insulin degradation by IDE (40, 41). Here, the expression of iNOS was not increased, in fact, it was decreased in the liver from 18-month-old mice compared with controls (**Supplementary Figure S4**), suggesting that other molecular mechanisms must be involved in the impairment on IDE function in the liver of these OLD mice (35, 42).

In summary, insulin clearance reduces with age and this may contribute to age-related hyperinsulinemia. Although previous studies suggest that IDE is not involved in the modulation of hepatic insulin clearance, in control and obese mice, our finds suggest that during aging this enzyme might have a role in this modulation, as well as, the CEACAM1. Therefore, to investigate the molecular mechanisms whereby aging reduces IDE and CEACAM1 function, in the liver, might be helpful to understand how insulin clearance is affected by age.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Ethics in the Use of Animals of the UNICAMP (CEUA-UNICAMP, approval number 4659-1/2017), Sao Paulo, Brazil.

AUTHOR CONTRIBUTIONS

Conceptualization, MK. Methodology, MK, CM, and GS. Formal Analysis, MK and CM. Investigation, MK, CM, GS, GB, and SP. Resources, CM-C, AB, and EC. Data Curation, CM. Writing – Original Draft Preparation, MK. Writing – Review & Editing, MK and AB. Visualization, MK. Supervision, MK. Project administration, MK. Funding Acquisition, AB, EC, and CM-C. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.679492/full#supplementary-material

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LC-MS-Based Untargeted Metabolomics Reveals Early Biomarkers in STZ-Induced Diabetic Rats With Cognitive Impairment

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Diabetes in the elderly increases cognitive impairment, but the underlying mechanisms are still far from fully understood. A non-targeted metabolomics approach based on liquid chromatography-mass spectrometry (LC-MS) was performed to screen out the serum biomarkers of diabetic mild cognitive impairment (DMMCI) in rats. Total 48 SD rats were divided into three groups, Normal control (NC) group, high-fat diet (HFD) fed group and type 2 diabetes mellitus (T2DM) group. The T2DM rat model was induced by intraperitoneal administration of streptozotocin (STZ, 35 mg/kg) after 6 weeks of high-fat diet (HFD) feeding. Then each group was further divided into 4-week and 8-week subgroups, which were calculated from the time point of T2DM rat model establishment. The novel object recognition test (NORT) and the Morris water maze (MWM) method were used to evaluate the cognitive deficits in all groups. Compared to the NC-8w and HFD-8w groups, both NOR and MWM tests indicated significant cognitive dysfunction in the T2DM-8w group, which could be used as an animal model of DMMCI. Serum was ultimately collected from the inferior vena cava after laparotomy. Metabolic profiling analysis was conducted using ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) technology. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to verify the stability of the model. According to variable importance in the project (VIP > 1) and the p-value of t-test ($P < 0.05$) obtained by the OPLS-DA model, the metabolites with significant differences were screened out as potential biomarkers. In total, we identified 94 differentially expressed (44 up-regulated and 50 down-regulated) endogenous metabolites. The 10 top up-regulated and 10 top down-regulated potential biomarkers were screened according to the FDR significance. These biomarkers by pathway topology analysis were primarily involved in the metabolism of sphingolipid (SP) metabolism, tryptophan (Trp) metabolism, Glycerophospholipid (GP) metabolism, *etc.* Besides, SP metabolism, Trp metabolism and GP metabolism mainly belonging to the lipid metabolism showed marked perturbations over DMMCI and may contribute to the development of disease. Taken collectively, our results revealed that

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T2DM could cause cognitive impairment by affecting a variety of metabolic pathways especially lipid metabolism. Besides, serum PE, PC, L-Trp, and S1P may be used as the most critical biomarkers for the early diagnosis of DMMCI.

Keywords: mild cognitive impairment (MCI), serum metabolomics, streptozotocin (STZ), biomarkers, diabetes mellitus (DM)

INTRODUCTION

Increasing numbers of people are suffering from diabetes mellitus (DM), with the improvement of living standards and lifestyle changes. According to the eighth edition of the International Diabetes Federation (IDF), Diabetes Atlas in 2017, about 425 million people worldwide have diabetes, and the number is expected to rise to 700 million by 2045 (1). Besides, DM is considered to be a major disease associated with cognitive decline and dementia, another most common chronic disabling disease among the elderly, with a 1.5–2.5-fold higher risk of dementia than the general population (2, 3). So the high prevalence of diabetes-related cognitive dysfunction (DCD) will become a serious public health burden globally following significant financial and social implications. As dementia is an irreversible disease, early diagnosis and detection of dementia are critical for its prevention and treatment. However, there is still a lack of accurate and reliable diagnostic criteria for DCD, making early detection of diabetic cognitive impairment more difficult.

Growing studies have consistently proposed that Alzheimer's disease (AD) is fundamentally a metabolic disease defined as "T3DM", which has specific metabolic changes similar to the pathological characteristics of DM during the development of DCD (4). Recently, metabolomics as a powerful systematic approach born and defined in 1999 has been used frequently to evaluate global changes of disease-specific metabolites in biological samples (5). Compared with proteomics and genomics, metabonomics is characterized by high accuracy, high resolution, high sensitivity and small sample size, which is very helpful for discovering the pathophysiological changes of cells, body fluids, and tissues. As a result, it is an effective means of finding disease-related biomarkers that are more reliable and secure than genomics and proteomics (6). The most extensively applied techniques consist of nuclear magnetic resonance (NMR), gas chromatography (GC), and liquid chromatography-mass spectrometry (LC-MS) (7). In recent years, ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) has shown significant advantages in the accurate and rapid determination of metabolite activities (8).

In this paper, we used a combination of low-dose streptozotocin (STZ, 35 mg/kg body weight) and a high-fat diet (HFD, 60% of energy as fat) to establish a rat model mimicking human the T2DM model based on the previous study and observe its cognitive deficit (8). In this study, we aimed to primarily screen out the serum biomarkers for the early diagnosis of diabetic mild cognitive impairment (DMMCI) and explore its potential pathophysiological mechanism by analyzing the characteristics of the serum metabolomics in rats based on untargeted LC-MS technology. These differentially expressed metabolites could provide a novel strategy for the early diagnosis of DMMCI and give new insights into the pathophysiological changes and molecular mechanisms of disease in the future.

EXPERIMENT

Chemicals and Solutions (Chemicals and Reagents)

Streptozotocin (STZ, NO. S0130) was purchased from Sigma Corporation (St. Louis Missouri, USA). HFD (NO. D12492) containing 20% protein, 20% carbohydrate, and 60% fat was supplied by Research Diets, Inc. (New Brunswick, Canada). Blood glucose meter and test strip (GA-3, Sinocare Inc., China) were used to determine the random blood glucose (RBG) of tail venous blood in rats. Rat Insulin Elisa Kit (NO. 10-1250-01) was provided by Mercodia Inc. (Uppsala, Sweden). Acetonitrile, formic acid, methanol, and 2-Propanol using in High Pressure Liquid Chromatography (HPLC)-grade were purchased from Fisher Chemical (China).

Animal Experiment

The work-flow of the study process was shown in **Figure 1**.

Animals, Diets, and Treatments

Total 48 healthy male Sprague–Dawley (SD) rats (aged 6–7 weeks) weighing 260 ± 20 g were purchased separately and reared in a specific pathogen-free (SPF) animal laboratory at the Experimental Animal Center of Central South University, China. All rats were maintained under controlled conditions (12 h light/dark cycles, 25°C, 50–60% room humidity) with food and water. To minimize the possible effects of circadian rhythm changes, all experiments were conducted at the same time in the morning. All research protocols were conducted according to the guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Central South University Xiangya School of Medicine.

HFD/STZ Induced T2DM Rat Model

For the experimental design, 48 rats were randomly divided into three equal groups as follows: Group I: normal control rats (NC, n = 16). Group II: HFD fed rats (HFD, n =16). Group III: T2DM rats (n = 16). The NC was fed a normal diet. The other group (HFD and T2DM) rats were fed with an HFD throughout the whole study containing 20% protein, 20% carbohydrate, and 60%

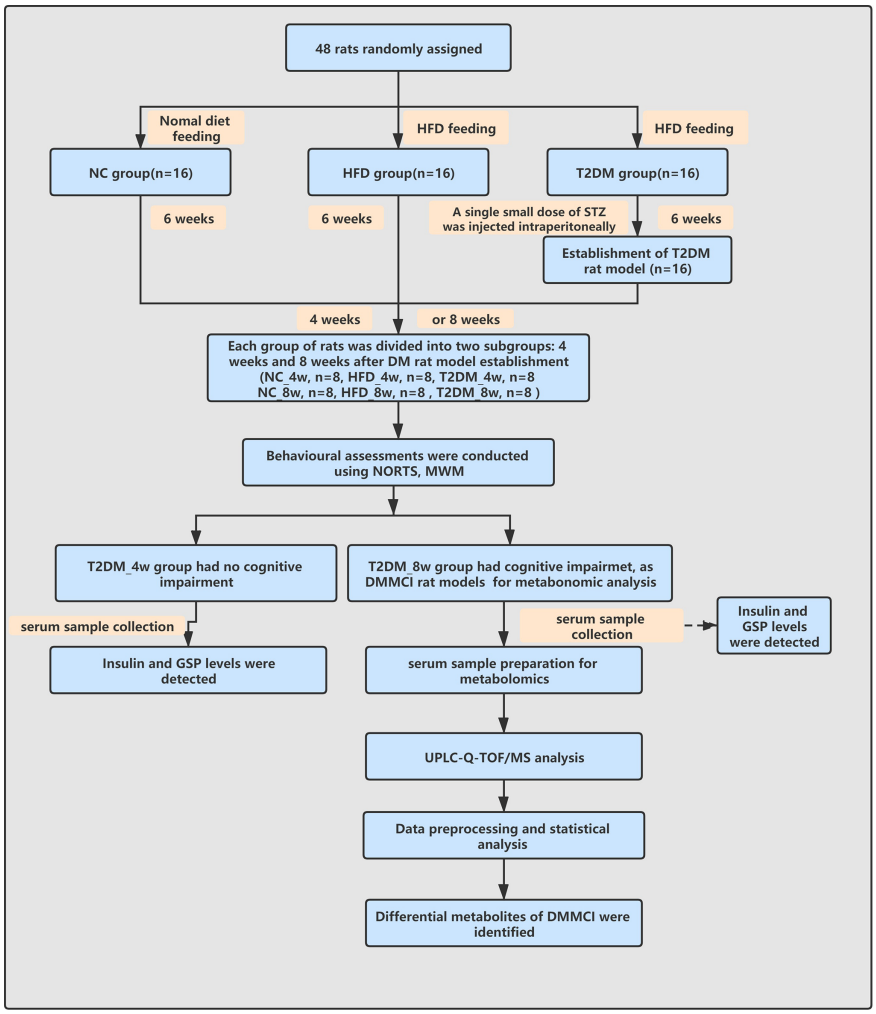

FIGURE 1 | Work-flow diagram of the study process. NC, nomal control; HFD, high-fat diet; T2DM, type 2 diabetes mellitus; NORT, novel object recognition test MWM, Morris water maze; GSP, glycosylated serum protein; UPLC-Q-TOF-MS, ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; DMMCI, diabetic mild cognitive impairment.

fat (Research Diets, D12492, Canada) for 6 weeks. Then after 12 h of fasting, the rats of the T2DM group were injected with 35 mg/kg of streptozotocin (STZ; Sigma-Aldrich, USA) dissolved in a 0.1 M citric acid/sodium citrate buffer at pH 4.5 intraperitoneally (9). Two days after injection, rats with a constant RBG level ≥16.7 mmol/L were considered T2DM model rats and selected in further experiments. Then each group was further divided into 4-week (NC-4w, HFD-4w, and T2DM-4w) and 8-week (NC-8w, HFD-8w, and T2DM-8w) subgroups, which were calculated from the time point of T2DM rat model establishment. During each week, the body weights (BW) of the rats were measured until the end of the experiment, and the diabetes onset of the STZ injection group and RBG levels of all groups were determined using a blood glucose meter (GA-3 type Lifescan, Sinocare, China) with 2 μl blood collected from the tail veins.

Assessment of the HFD/STZ-Induced Diabetic Cognitive Impairment in the Diabetic Rat Model

All rats were assessed for cognitive behavioral deficits using the Morris water maze (MWM) test and novel object recognition test (NORT) at 11 or 15 weeks of the study (**Figure 1**).

NORT Task

The NORT relies on the rats' innate tendency for investigating more novelty compared to a familiar object, which can be used to test rats' non-spatial memory performances. As the procedure of NOR task previously reported (10), a rat was initially placed into an arena (50 cm long, 60 cm wide, 60 cm high) without objects for 2 min per day for 3 consecutive days. On the 4th day, each rat received two trials for a total duration of 15 minutes (min). The first trial (10 min) was the sample exploration which contained two identical objects placed in the left and right corners of the

testing box. The second trial (5 min) was the testing trial when one of the two familiar objects presented during exploration was replaced with a new object after 1 h inter-trial interval. A video camera mounted above the testing box recorded the animal's behavior once the rats were placed into the box for the object recognition test. The walls and floor of the testing box were cleaned with 70% isopropyl alcohol prior to each test, and the rats were returned to their home cages after each trial. Exploration behaviors were defined as a rat touching the object with its nose and/or directing towards the object within 2 cm. Each object exploration time was measured with a stopwatch and a discrimination ratio (DR) = [TN/(TF + TN), TF = time spent exploring familiar sample, TN = time spent exploring the novel object; DR was calculated to evaluate the recognition memory. A recognition DR significantly above 0.50 illustrates a novelty preference and positive recognition memory (11).

MWM Test

After the NOR test, the rats were subjected to 5 days of trial in the MWM tests to investigate their spatial learning ability and memory function after the object recognition test as described previously (12), which was developed by Richard Morris (13). In brief, the test was conducted in a round opaque pool (170 cm in diameter) filled with water (temperature 26 ± 1° C), virtually divided into four quadrants. The escape platform is a clear platform with a diameter of 10 cm, submerged about 1 cm beneath the surface of the water and located in the fixed target quadrant. The maze was surrounded by blue curtains, with visual stimuli of various shapes placed. Hidden platform test: each rat was trained for four consecutive days, four times one day by placing the animal into each quadrant as a starting point. Animals were given the 90 s per trial to locate the hidden platform, and any animal that did not find the platform within the 90 s was guided to the platform with sticks. Then they were set to remain on the platform for 15 s, regardless of where it was located. From the second day of training, behavioral parameters were recorded using an online image video tracking system (Stoelting Co., USA) within a maximum of 90 s as the escape latency in each trial. Spatial probe test: on the 5th day, the platform was removed from the pool. Each rat was left to the farthest quadrant of the pool from the primary platform. The probe time and the percentage of time spent in the target quadrant were tracked and analyzed by the tracking system.

Sample Preparation and Determination of Hormonal and Biochemical Parameters

After 12 h of fasting, animals were anesthetized with chloral hydrate. The blood sample was immediately collected from the inferior vena cava after laparotomy. Following centrifugation at 3,000 g for 10 min, the serum was collected and stored at 80°C until use. Serum insulin levels were measured with the Rat Insulin Mercodia (Mercodia AB, Uppsala, Sweden) by enzyme-linked immunosorbent assay (ELISA) (Multiskan MK3, Thermo Scientific, USA). Glycated serum protein (GSP) was measured with a biochemical analyzer (Rayto Chemray 800, Shenzhen, China).

Serum Sample Preparation for Metabolomics

A 100 ul liquid sample placed in a 1.5 ml centrifuge tube was added with 400 ul extract (acetonitrile: methanol = 1:1). The mixture was then injected with a 20 ul internal standard (IS, 0.3 mg/ml, containing L-2-chloro-phenylalanine and acetonitrile) and blended by vortex for 30 s and ultrasound (40 kHz, 5°C) for 30 min. The samples were settled at −20°C for 30 min to precipitate and obtained by centrifugation (13,000 g, 4°C) for 15 min; the supernatant was transferred, dried with nitrogen, and stored at −80°C for LC-MS/MS analysis.

Besides, as a necessary part of the quality control and system conditioning process, the quality control (QC) sample was made by mixing equal volumes of each sample. Resolution with 100 ul complex solution (acetonitrile: water = 1:1) was followed by low temperature ultrasonic extraction for 5 min (5°C, 40 k Hz). The mixture was centrifuged for 5 min (13,000 g, 4°C), and the supernatant was transferred to a sample injection vial with an inner cannula for analysis on the machine; 20 ul of the supernatant for each sample was transferred and mixed it as a QC sample. It was injected at regular intervals (every 9–10 samples) to minimize the carryover and monitor the stability of the experiment.

UPLC-Q-TOF/MS Analysis

The metabolites were separated by chromatography on an ExionLTMAD system (AB Sciex, USA) which is equipped with an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, USA). The mobile phases contained two solvents [A: 0.1% formic acid in water with formic acid (0.1%), B: 0.1% formic acid in acetonitrile: isopropanol (1:1, v/v)]. The solvent gradient varies with the following conditions: a) 0–3 min, 95% (A): 5% (B) changed to 80% (A): 20% (B); b) 3–9 min, 80% (A): 20% (B) changed to 5% (A): 95% (B); c) 9–13 min, 5% (A): 95% (B) changed to 5% (A): 95% (B); d) 13–13.1 min, 5% (A): 95% (B) changed to 95% (A): 5% (B); e) 13.1–16 min, 95% (A): 5% (B) changed to 95% (A): 5% (B) for the systems equilibration. The injection volume of the sample was 20 ul with the flow rate at 0.4 ml/min, and the column temperature was set to hold at 40°C. All these samples were stored at 4°C during the period of analysis.

The positive and negative ion scanning modes were processed on the UPLC system to collect the quality spectrum signal of the sample, which was coupled to a quadrupole-time-of-flight mass spectrometer (Triple TOFTM5600+, AB Sciex, USA) equipped with an electrospray ionization (ESI) source. The detection was conducted over a mass range of 50–1,000 m/z. The optimal conditions included: ion-spray voltage floating (ISVF), 5,000 V in positive mode, −4000 V in negative mode; curtain gas (CUR), 30 psi; source temperature, 500°C; both ion source GS1 and GS2, 50 psi; declustering potential, 80 V; collision energy (CE), 20–60 V cyclic impact energy.

Data Preprocessing and Annotation

Based on UPLC-Q-TOF/MS analysis, this paper imports the original data into Progenesis QI 2.3 (Nonlinear Dynamics,

Waters, USA) for peak detection and calibration. The preprocessing results generated a data matrix, including retention time (RT), mass charge ratio (M/Z) values, and peak intensity. At least 80% of the metabolic features detected in any set of samples were retained. After screening, the minimum metabolic value was calculated for the specific samples whose metabolic level was lower than the quantitative lower limit, and the sum of all metabolic characteristics was normalized. The IS was used to evaluate the stability of the instrument. The pooled QC was not only used for the conditioning of the LC-MS system to ensure its stability before starting the analysis sequence, but also used as a powerful approach to tracking the intrabatch analytical variability with principal component analysis (PCA) plot visualization and setting standard deviation limits for selected features. The metabolic characteristics of QC greater than 30% relative standard deviation (RSD) are abandoned. After normalization and imputation, statistical analysis of log10-converted data was performed to determine significant differences in metabolite levels between the comparison groups. These metabolic characteristics were identified by precise mass spectrometry. Searching a reliable biochemical database such as the human metabolome database (HMDB) (http://www.hmdb.ca/) and Metlin database (https://metlin.scripps.edu/) MS/MS fragments' spectra, accurate mass, and isotope ratio difference were obtained. For MS/MS confirmed metabolites, only metabolites with MS/MS fragment score greater than 50 are considered to be positively identified.

Statistical Analysis

A multivariate statistical analysis including PCA and orthogonal least partial square discriminant analysis (OPLS-DA) was conducted using ropls (Version1.6.2, http://bioconductor.org/packages/release/bioc/html/ropls.html) R package. The stability of the model was assessed using seven cyclic interaction validations. Besides, a two-tailed student's t-test combined with the multivariate analysis of OPLS-DA was conducted. The significantly different metabolites were selected based on the variable importance in the project (VIP) obtained by the OPLS-DA model and p-value of the student's t-test. The metabolites with VIP >1 and p <0.05 (after Benjamini–Hochberg false discovery rate correction) were significantly different metabolites. Correlation analysis was performed using Pearson correlation test coefficient, and p-value <0.05 was considered significant between each comparison. Differential metabolites were mapped into the metabolic enrichment and pathway analysis through the KEGG database (https://www.kegg.jp/kegg/pathway.html). The Python package Scipy. stats (https://docs.scipy.org/doc/scipy/) performed a pathway enrichment analysis, and the biological pathway most relevant to the experimental treatment was identified using Fisher's exact test. Significantly altered metabolite data were introduced for metabolic analysis 5.0 (https://www.metaboanalyst.ca) to investigate the DMMCI metabolic mechanisms.

Other statistical analyses were performed using Prism 5.0 (GraphPad) or the SPSS 11.0 software package. Data were expressed as the mean ± SEM. For the repeated-measures data such as weight, RBG levels, MWM data, a two-way repeated-measure (RM) ANOVA was performed. The remaining biochemical data such as insulin levels were analyzed by using the one-way ANOVA test or t-test. $P < 0.05$ was considered statistically significant.

RESULTS

The Establishment of the Animal Model for the Diabetic Cognitive Dysfunction

T2DM Rat Model Induced by HFD and STZ

Diabetic SD rat models induced by the administration of STZ in the 6th week were confirmed through monitoring BW, RBG levels, GSP levels, and insulin levels.

The RBG and GSP levels of T2DM group (both in T2DM-4w and T2DM-8w) were significantly higher than those of the NC and HFD rats [RBG-4w: $F(18, 150) = 104.1$, $P < 0.0001$; RBG-8w: $F(26, 210) = 56.82$, $P < 0.0001$; GSP: T2DM-4w *vs* NC-4w, $P < 0.001$; T2DM-4w *vs* HFD-4w, $P < 0.01$; T2DM-8w *vs* NC-8w, $P < 0.001$; T2DM-8w *vs* HFD-8w, $P < 0.0001$]. The HFD fed rats gained more weight in the first 6 weeks than the NC group. After STZ administration, the body weight and insulin levels of the diabetic rats were significantly decreased compared to those of other groups [BW-4w: $F(10, 110) = 10.14$, $P < 0.0001$; BW-8w: $F(14, 150) = 31.99$, $P < 0.0001$; Insulin: T2DM-4w *vs* NC-4w, $P < 0.01$; T2DM-4w *vs* HFD-4w, $P < 0.001$; T2DM-8w *vs* NC-8w, $P < 0.001$; T2DM-8w *vs* HFD-8w, $P < 0.0001$]. Besides, the decrease of insulin levels was more obvious in the T2DM-8w group than in the T2DM-4w group. Persistent high glucose and GSP, as well as low insulin levels suggested the establishment of a diabetic rat model (**Figure 2**).

Results of DMMCI Assessments

In the NOR tests (**Figure 3A**), the results of one-way analysis of variance showed that the average total exploration time (ATET) and DR of rats had no significant difference among the groups at 4w [ATET-4w, $F(2, 9) = 0.3666$, $P > 0.05$, DR-4w, $F(2, 9) = 0.4388$, $P > 0.05$], but had significant difference among the three groups at 8w [ATET-8w, $F(2, 9) = 101.4$, $P < 0.0001$, DR-8w, $F(2, 9) = 26.93$, $P < 0.001$]. The ATET and DR of the T2DM-8w group were significantly lower than those of the other two groups. Two-way ANOVA showed that, except for T2DM-8w rats, there were significant differences in the exploration time of familiar and novel things in other groups. Although T2DM-8w rats spent more time exploring novelty than familiarity, the difference was not significant ($P > 0.05$).

As shown in **Figure 3B**, the mean escape latency for the trained rats significantly decreased over the 4 days, and the total time spent in the target quadrant in the spatial probe trials without the platform on day 5 showed no significance in all 4w groups. It suggested no learning and memory deficits in T2DM-4w rats. However, in the 8w groups, the T2DM-8w rats performed significantly worse than the NC and HFD in the hidden platform trials ($p < 0.0001$, $p < 0.01$) and the probe trials ($p < 0.01$, $P < 0.05$) (**Figure 3C**). Though escape latency decreased significantly across the four days of training, there

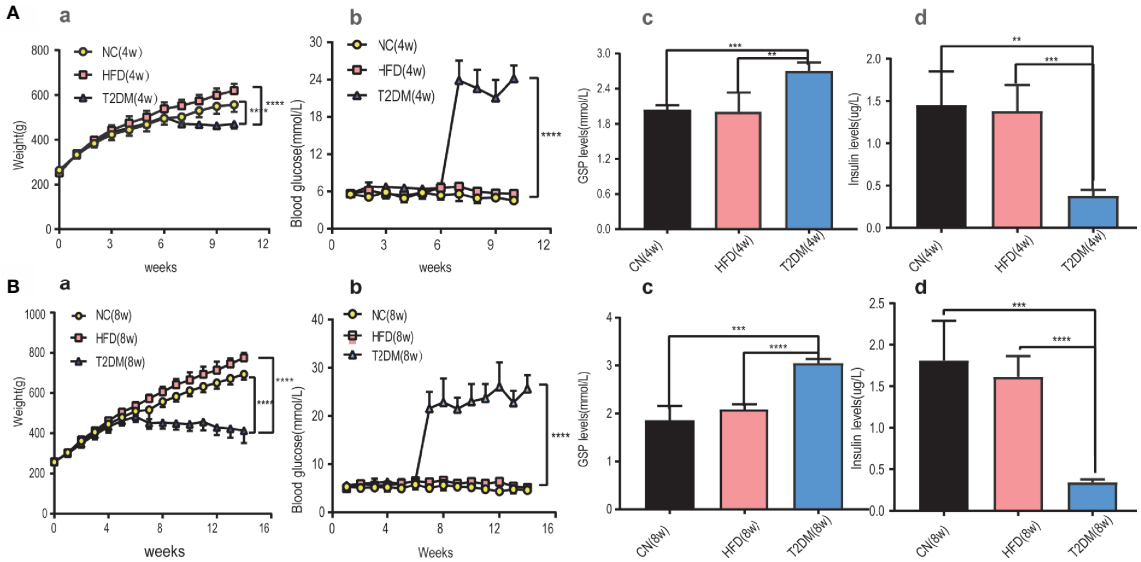

FIGURE 2 | Diabetic rats was set up and treated with HFD and STZ. **(A)** Basic characteristics of rats in the 4-week subgroup, **(B)** Basic characteristics of rats in the 8-week subgroup. (a) Body weight, (b) Random blood glucose levels were measured at the indicated times each week in NC, HFD, and T2DM rats. (c) GSP levels, (d) insulin levels were determined 4 or 8 weeks after STZ injection. Mean ± S.E.M, n = 8. *p < 0.05; **p < 0.01;***p < 0.001;****p < 0.0001. NC, nomal control; HFD, high-fat; T2DM, type 2 diabetes mellitus; GSP, glycosylated serum protein.

were significant differences between the three groups [F (3, 40) = 3.311, P < 0.05]. A Bonferroni *post-hoc* test revealed that the T2DM-8w group took longer to find the platform than the other two groups on both day 3 and day 4 (both, P < 0.0001). In the probe trials, we found a significant difference in the time spent in the target quadrant among the three groups [F (2, 15) = 23.03, P < 0.0001]. Turkey's test indicated that the T2DM-8w group spent less time in this quadrant than the NC and HFD groups (P < 0.0001, P < 0.001), but there was no difference between the NC and HFD groups in swimming time (P > 0.05).

Both NOR and MWM tests indicated significant cognitive dysfunction in the T2DM-8w group, which could be used as an animal model of DMMCI for subsequent metabolomic studies to search for potential metabolomic markers.

Serum Metabolic Profiling by UHPLC-Q-TOF/MS in DMMCI Rats

Identification of Potential Biomarkers of DMMCI

The UPLC-Q-TOF/MS in metabolomics was applied to detect and collect the metabolic profiles of serum samples in positive and negative ion modes between the three groups. PCA method was used to find abnormal samples and evaluate the repeatability of experimental data. PCA score chart results show a high degree of QC polymerization, indicating good QC repeatability and a stable analysis system (**Figure S1**). Multivariate statistical analysis by OPLS-DA supervised pattern recognition method was adopted to identify the metabolomic differences of serum in three rat groups. As shown in **Figure 4**, significantly separated clusters appeared between every two groups (DMMCI *vs* NC, DMMCI *vs* HFD) in both positive ion and negative ion modes, respectively, which indicated that the serum metabolic profiles were different at baseline. High statistical values of R2Y and Q2 in the OPLS-DA score plots indicated the fitness and the prediction ability of our model [DMMCI *vs* NC, positive-ion, R2 = (0, 0.8406), Q2 = (0, −0.5128), negative-ion, R2 = (0, 0.8081), Q2 = (0, −0.3076); DMMCI *vs* HFD, positive-ion, R2 = (0, 0.9882), Q2 = (0, −0.2121), negative-ion, R2 = (0, 0.9595), Q2 = (0, −0.1083)]. Subsequently, potential markers of DMMCI (DM-8w) were screened for further study based on the ions with VIPs >1.0 and P <0.05 after OPLS-DA analysis by comparing them with those of NC and HFD groups, respectively (shown in **Figure 5A**). A total of 94 differentially expressed (44 up-regulated and 50 down-regulated) endogenous metabolites were discovered as shown in **Figure 5A**, **Tables 1**, **2**. In the positive ion mode, 43 (24 up-regulated/19 down-regulated) differential endogenous metabolites were putatively identified. In the negative ion mode, 51 (20 up-regulated/31 down-regulated) differential metabolites were detected. Hierarchical cluster analysis was used to further characterize the specific and unique expression patterns of these differentially expressed metabolites in serum of NC, HFD, and DMMCI rats (**Figure 5B**), showing a global profile of all serum metabolites that have been detected and visualized. Cluster heat map analysis of 94 differential metabolites showed clear separation for each alignment. Interestingly, differences in metabolite heat maps between groups of rats based on DMMCI and NC/HFD showed clear clustering. This study indicated the reliability of the OPLS-DA model for distinguishing different disease-specific metabolic phenotypes (**Figure 5C**). The metabolites with similar variation trends in abundance were located closer, indicating that the metabolites of DMMCI were clustered closely and separated from other groups. The 44 up-regulated and 50 down-regulated differential metabolites were ranked according to the FDR

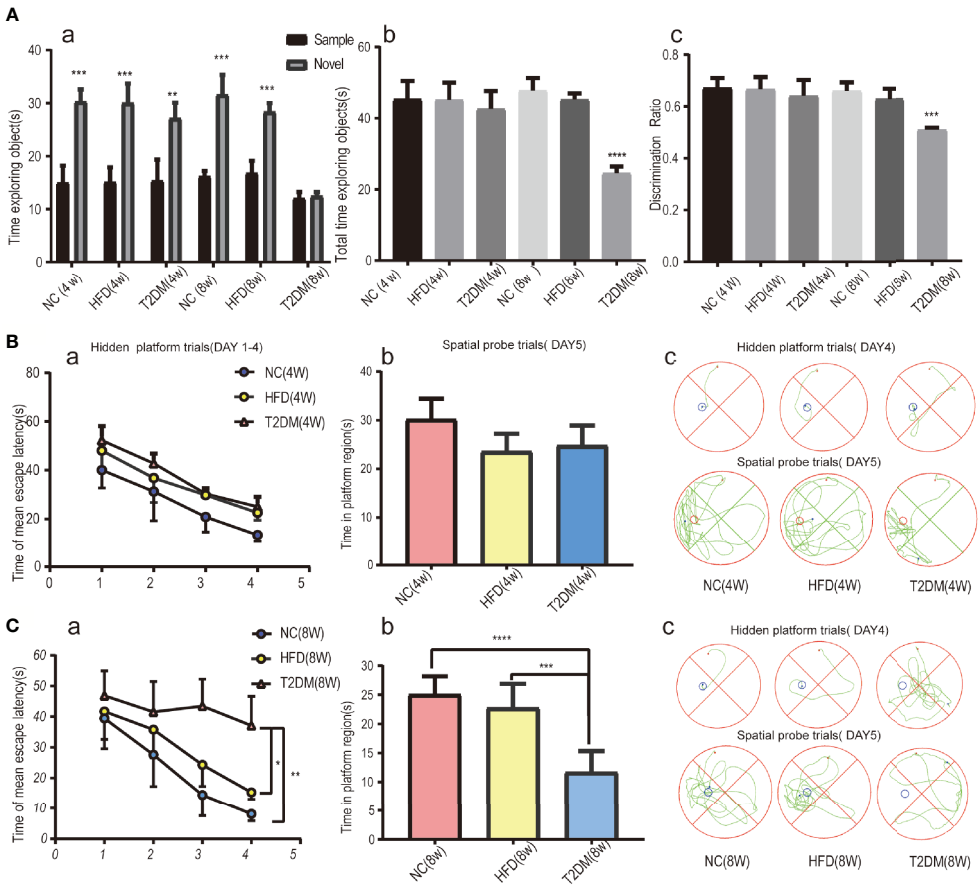

FIGURE 3 | Assessments of mild cognitive impairment using NORT and MWM tests. Novel object recognition (NOR) test analysis revealed no evidence of deficits in short-term recognition memory in the T2DM-4w group of rats and significant impairment of cognitive function in the T2DM-8w group of rats; (a) TN(time spent exploring the novel object) vs TF(time spent exploring familiar sample) in each group, **p < 0.01; ***p < 0.001; (b) the average total exploration time(ATET) compared between T2DM-8w with NC-8w and HFD-8w group, ****p < 0.0001 (n = 4/group). **(A)** Spatial learning and memory evaluated by the MWM test in all 4w subgroups showed that T2DM-4w group had no cognitive impairment; **(B)** Spatial learning and memory evaluated by the MWM test in all 8w subgroups showed that T2DM-8w group had significant cognitive impairment; (**B,C**) (a) Mean escape latency during the hidden platform tests (DAYs 1–4); (b) The time in the target quadrant during the spatial probe tests(day5); (c) Representative searching strategy of rats on day 4 and day 5. *p < 0.05; **p < 0.01; ***p < 0.001;****p < 0.0001 (n = 4/group).

(corrected P-value) of significance and the top 10 significant metabolites were selected as the potential biomarkers, separately. *Up-regulated* markers included PE [15:0/22:1 (13Z)], 3-[8-hydroxy-2-methyl-2-(4-methylpent-3-en-1-yl)-2H-chromen-5-yl]propanoic acid, Sphingosine-1-phosphate (S1P), PE [15:0/24:1 (15Z)], 3,4,5-trihydroxy-6-[(3-methylbutanoyl)oxy]oxane-2-carboxylic acid, Agavoside A, 2-Hydroxyacetaminophen sulfate, Propylene glycol alginate, Glycocholic Acid, Sphingofungin A. *Down-regulated* markers included PC [14:0/22:5 (4Z,7Z,10Z,13Z,16Z)], LysoPC [20:5(5Z,8Z,11Z,14Z,17Z)], N-Arachidonoyl-L-Serine, Sagittariol, (±)12-HEPE, LysoPE [0:0/20:1(11Z)], (±)12,13-DiHOME, LysoPC [18:3(9Z,12Z,15Z)], LysoPC [16:1(9Z)/0:0], LysoPE [0:0/20:2(11Z,14Z)].

KEGG Pathway Enrichment Analysis

Pathway classification analysis of the 94 differential metabolites by KEGG showed 12 metabolites annotated lipid metabolism (including Glycocholic Acid, S1P, Aldosterone, L-tryptophan, Acetylcholine, Cholic acid, LysoPC [20:5(5Z,8Z,11Z,14Z,17Z)], LysoPC [20:3(5Z,8Z,11Z)], LysoPC [20:2(11Z,14Z)], SM [d18:1/18:1(11Z)], LysoPC [20:1(11Z)], LysoPC [16:1(9Z)/0:0, Prostaglandin F2a], six metabolites annotated cancers: overview (L-tryptophan), LysoPC [20:5(5Z,8Z,11Z,14Z,17Z)], LysoPC [20:3(5Z,8Z,11Z)], LysoPC [20:2(11Z,14Z)], LysoPC [20:1(11Z)], LysoPC [16:1(9Z)/0:0)], six metabolites annotated digestive system (Glycocholic acid, L-tryptophan, Acetylcholine, Cholic acid, Prostaglandin F2a, P-cresol), four metabolites annotated signal transduction, four metabolites annotated amino acid metabolism [Phenylacetylglycine, 5-Methoxyindoleacetate, L-tryptophan, (S)-3-Hydroxyisobutyric acid], three metabolites annotated signaling molecules and interaction (S1P, Acetylcholine, Prostaglandin F2a), three metabolites annotated endocrine system (Aldosterone, Acetylcholine, Prostaglandin F2a), three metabolites annotated

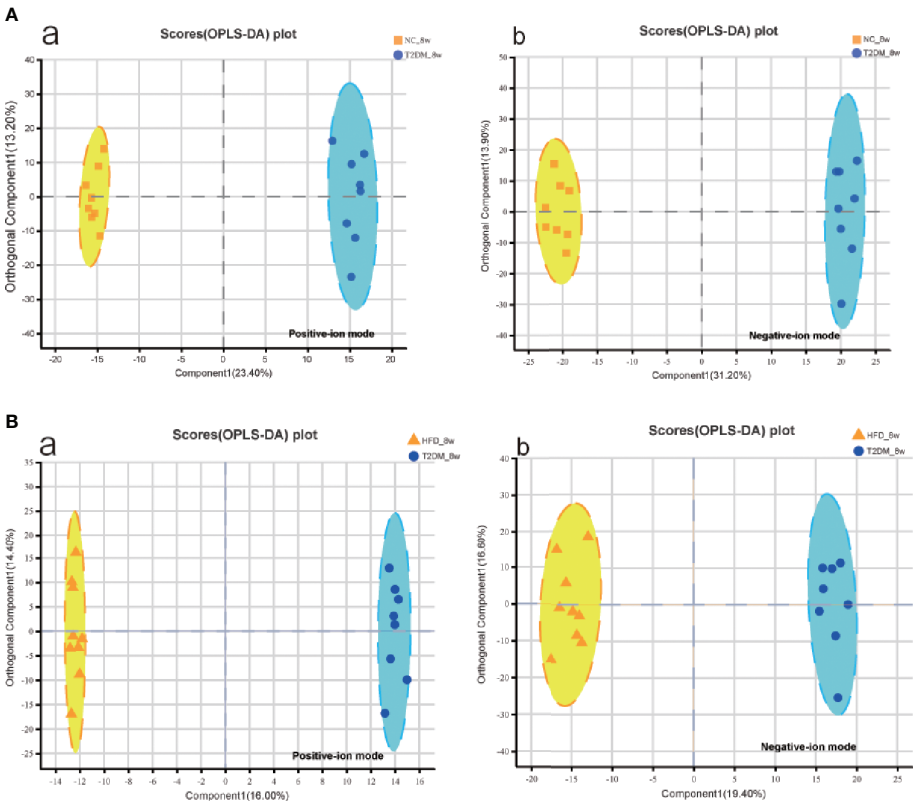

FIGURE 4 | Multivariate statistical analysis of serum metabolomics of the 8w subgroups of rats after the establishment of T2DM rat model. **(A, B)** OPLS-DA score plots between every two groups(T2DM *vs* NC, T2DM *vs* HFD) in positive- and negative-ion modes, respectively.

nervous system (including L-tryptophan, Acetylcholine, Prostaglandin F2a) (**Figure 6A**). Pathway annotation analysis by KEGG revealed the pathways where the p-value is in the top 20 (bile secretion: OS, Phospholipase D signaling pathway: EIP, sphingolipid signaling pathway: EIP, sphingolipid metabolism: M, Serotonergic synapse: OS, Neuroactive ligand–receptor interaction: EIP, primary bile acid biosynthesis: M, protein digestion and absorption: OS, Glycerophospholipid (GP) metabolism: M, regulation of actin cytoskeleton: CP, nicotine addiction: HD, Aldosterone-regulated sodium reabsorption: OS, Fc gamma R-mediated phagocytosis: OS, tuberculosis: HD, Apelin signaling pathway: EIP, African trypanosomiasis: HD, calcium signaling pathway: EIP, tryptophan metabolism: M, choline metabolism in cancer: HD, cholinergic synapse: OS (**Figure 6B**) Pathway topology analysis highlighted the following pathways: sphingolipid (SP) metabolism, tryptophan (Trp) metabolism, Glycerophospholipid (GP) metabolism, primary bile acid biosynthesis, folate biosynthesis, Aminoacyl-tRNA biosynthesis, Valine, leucine, and isoleucine degradation, phenylalanine metabolism, glycine, serine and threonine metabolism, Butanoate metabolism, arachidonic acid metabolism, Steroid hormone biosynthesis, phenylalanine, tyrosine, and Trp biosynthesis, *etc.* (**Figure 6C** and **Table 3**) Besides, SP metabolism, Trp metabolism and GP metabolism were the most important pathways according to the P-value corrected (**Table 3**).

DISCUSSION

In the present study, a non-targeted metabolomics approach based on LC-MS was performed to explore the characteristics of blood metabolism in rats with DMMCI. Diabetes was induced by intraperitoneal administration of streptozotocin (STZ, 35 mg/kg) after 6 weeks of HFD feeding. The NORT and MWM tests were used to evaluate cognitive deficits in rats at 4 weeks or 8 weeks after DM rat model establishment. Compared to the NC and HFD 8w groups, both NOR and MWM tests indicated significant cognitive dysfunction in the DMMCI group, which could be used as an animal model of DMMCI. In metabolic profiling analysis, we identified 94 differentially expressed (44 up-regulated and 50 down-regulated) endogenous metabolites. The 10 top up-regulated and 10 top down-regulated potential biomarkers were screened according to the FDR of significance. These biomarkers by pathway topology analysis were primarily involved in the metabolism of GP metabolism, Linoleic acid metabolism, arachidonic acid metabolism, Trp metabolism, primary bile acid biosynthesis, alpha-Linolenic acid

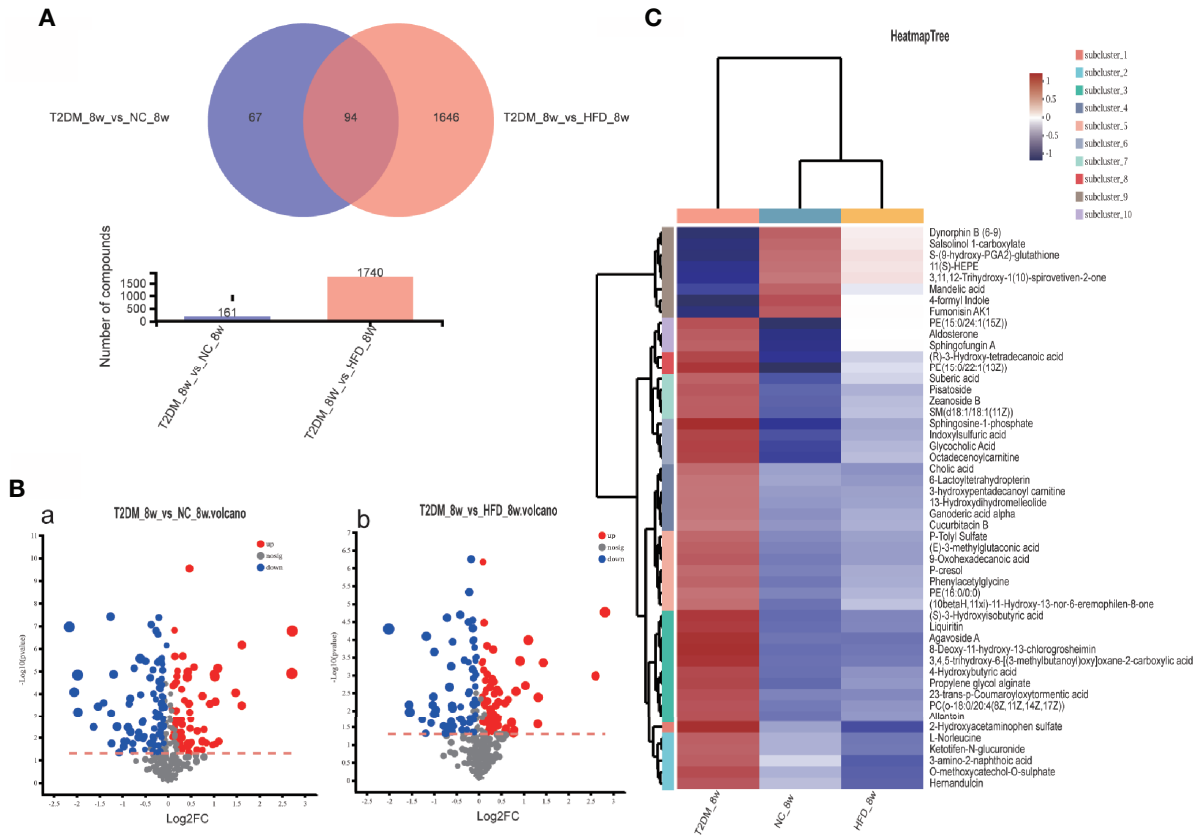

FIGURE 5 | Venn diagram, volcanic plot and heat map of the identified differentially expressed metabolites. **(A)** Venn diagram of 94 differentially expressed metabolites through the comparison between T2DM with groups NC and HFD; **(B)** Volcano plot of differentially expressed metabolites in T2DM group compared with NC and HFD groups, respectively. Volcano plots were constructed using fold-change values and p-values. The vertical lines correspond to 2.0 fold-up and down-regulation between each group (T2DM *vs*. NC, T2DM *vs* HFD), and the horizontal lines represent p-values. Red plot points represent up-regulated metabolites with statistical significance. Blue plot points represent down-regulated metabolites with statistical significance. Gray plot points represent no significant metabolites; **(C)** Heat map analysis of 94 differential metabolites identified between T2DM, NC, and HFD groups. The blue band indicates a decreased level of metabolite, and the red band indicates an increased level of metabolite.

metabolism, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, SP metabolism, Folate biosynthesis, Valine, leucine and isoleucine degradation, Aminoacyl-tRNA biosynthesis, Steroid hormone biosynthesis. Therefore, our results revealed that DM could cause cognitive impairment by affecting a variety of metabolic pathways especially lipid metabolism. Besides, GP metabolism and Trp metabolism showed marked perturbations over DMMCI and could contribute to the development of disease.

DCD with cognitive impairment as the main clinical manifestation, such as learning and memory deficit, and even dementia, is a common complication of DM (1, 14). Our study observed significant cognitive decline accompanied by hyperglycemia and weight loss in HFD-fed and STZ-treated diabetic rats at 8 weeks in animal models, which is in agreement with those of previous studies (15, 16). Therefore, T2DM-8w rats were selected as the DMMCI rat model for further serum metabolomics analysis. However, it is interesting that different experiments reported different times of cognitive impairment in rats or mice (4 to 12 weeks or more). It may be due to different experimental designs, such as T1DM or T2DM, and different study specimens, such as cerebrospinal fluid, hippocampus, and urine (15–19). This animal model has also been established to explore its potential metabolic mechanisms based on the metabonomic approach between STZ-induced diabetic rats with cognitive impairment (DMMCI) and age-matched groups (NC) when they focused on changes in cerebrospinal fluid, brain tissue, or urine metabolites (19–21), but to our knowledge serum metabolomics has been rarely reported.

According to our results, T2DM induced cognitive dysfunction and significant lipid perturbations in the blood, especially in GP, SP, and Trp metabolisms which may be integral to the evolution of DMMCI neuropathology.

GPs are crucial structural components of neural membranes (predominantly including GPs, SPs, and cholesterol), which not only constitute the backbone but also maintain the membrane with a fluidity, suitable environment and ion permeability (22). The five

TABLE 1 | List of differentially expressed (*up-regulated*) endogenous metabolites detected by UHPLC-QTOF/MS in the T2DM(8W) group compared with NC(8w) and HFD(8w) groups.

	Metabolite	Library ID	Mode	NC(8w) mean ± SD	HFD(8w) mean ± SD	T2DM(8w) mean ± SD	P-value	FDR
1	PE [15:0/22:1(13Z)]	HMDB0008908	neg	4.148 ± 0.09274	4.326 ± 0.128	4.564 ± 0.0801	6.164E-07	0.00002617
2	3-[8-hydroxy-2-methyl-2-(4-methylpent-3-en-1-yl)-2H-chromen-5-yl] propanoic acid	HMDB0134704	neg	0.6685 ± 0.4295	1.634 ± 0.4523	2.042 ± 0.1598	0.000008904	0.0002114
3	Sphingosine-1-phosphate	LMSP01050001	pos	4.164 ± 0.1283	4.284 ± 0.1252	4.607 ± 0.1008	0.000004242	0.0006402
4	PE [15:0/24:1(15Z)]	HMDB0008915	neg	3.209 ± 0.1809	3.453 ± 0.1975	3.694 ± 0.09474	0.00004521	0.0006738
5	3,4,5-trihydroxy-6-[(3-methylbutanoyl)oxy]oxane-2-carboxylic acid	HMDB0130798	pos	2.026 ± 0.1621	2.04 ± 0.2671	2.525 ± 0.086	0.00000554	0.000785
6	Agavoside A	HMDB0034391;LMST01080006	pos	0.8463 ± 0.3096	0.8432 ± 0.4648	1.796 ± 0.2367	0.00001012	0.001119
7	2-Hydroxyacetaminophen sulfate	HMDB0062547	neg	0.6808 ± 0.4383	0.2935 ± 0.4622	2.075 ± 0.7116	0.0001569	0.001652
8	Propylene glycol alginate	HMDB0039860	pos	1 ± 0.3304	1.141 ± 0.633	2.009 ± 0.288	0.00004428	0.002477
9	Glycocholic acid	HMDB0000138;HMDB0000331; LMST05030001	pos	3.361 ± 0.4004	3.691 ± 0.508	4.492 ± 0.306	0.00005172	0.002637
10	Sphingofungin A	LMSP01080061	pos	0.3903 ± 0.7135	1.488 ± 1.261	2.54 ± 0.5903	0.00006245	0.002737
11	Suberic acid	LMFA01170001;HMDB0000893	neg	2.522 ± 0.149	2.73 ± 0.414	3.091 ± 0.2445	0.0003707	0.003135
12	Aldosterone	HMDB0000037;LMST02030026	pos	1.298 ± 0.4807	1.96 ± 0.7199	2.634 ± 0.3457	0.00008335	0.003312
13	(R)-3-Hydroxy-tetradecanoic acid	HMDB0010731	neg	1.878 ± 0.2754	2.35 ± 0.3804	3.152 ± 0.6299	0.0004312	0.003472
14	Liquiritin	LMPK12140021;HMDB0029520	pos	4.194 ± 0.1063	4.216 ± 0.1664	4.512 ± 0.1121	0.000107	0.003839
15	Pisatoside	HMDB0039127	pos	2.157 ± 0.09419	2.223 ± 0.2149	2.447 ± 0.09779	0.0001321	0.004438
16	(S)-3-Hydroxyisobutyric acid	HMDB0000023	pos	2.363 ± 0.06227	2.388 ± 0.1694	2.694 ± 0.1328	0.0001327	0.004438
17	8-Deoxy-11-hydroxy-13-chlorogrosheimin	HMDB0041037	pos	2.776 ± 0.08043	2.786 ± 0.1374	3.131 ± 0.1458	0.0001349	0.004444
18	Octadecenoylcarnitine	HMDB0094687	pos	4.24 ± 0.0814	4.374 ± 0.176	4.664 ± 0.186	0.0002363	0.006075
19	SM [d18:1/18:1(11Z)]	HMDB0012100	neg	1.805 ± 0.5173	2.021 ± 0.4085	2.547 ± 0.1286	0.001258	0.008047
20	Zeanoside B	HMDB0038844	neg	1.688 ± 0.3638	1.865 ± 0.4262	2.338 ± 0.215	0.001518	0.009299
21	O-methoxycatechol-O-sulphate	HMDB0060013	neg	2.081 ± 0.7052	1.731 ± 0.6763	3.247 ± 0.7041	0.001631	0.009696
22	Blepharin	HMDB0029344	neg	1.313 ± 0.5386	2.06 ± 0.2259	2.469 ± 0.4154	0.001885	0.01074
23	Indoxylsulfuric acid	HMDB0000682	neg	4.131 ± 0.2206	4.268 ± 0.1585	4.684 ± 0.2737	0.002276	0.01251
24	4-Hydroxybutyric acid	HMDB0000710	pos	1.933 ± 0.2757	2.009 ± 0.3993	2.641 ± 0.3109	0.0007537	0.01308
25	3-amino-2-naphthoic acid	–	neg	1.008 ± 0.6616	0.6332 ± 0.4615	1.701 ± 0.5239	0.002568	0.01366
26	PC [o-18:0/20:4(8Z,11Z,14Z,17Z)]	LMGP01020247;HMDB0013420	neg	2.846 ± 0.1666	2.872 ± 0.1676	3.145 ± 0.1503	0.003204	0.01611
27	Allantoin	HMDB0000462	neg	2.451 ± 0.1714	2.487 ± 0.1535	2.757 ± 0.1545	0.003315	0.01651
28	Hernandulcin	HMDB0037906	neg	1.876 ± 0.1966	1.754 ± 0.2666	2.207 ± 0.2097	0.003415	0.0169
29	23-trans-p-Coumaroyloxytormentic acid	HMDB0040682	pos	2.174 ± 0.2028	2.18 ± 0.2143	2.569 ± 0.2203	0.003532	0.03302
30	L-Norleucine	HMDB0001645	pos	3.127 ± 0.2053	3.051 ± 0.3139	3.458 ± 0.1724	0.003686	0.03413
31	(E)-3-methylglutaconic acid	LMFA01170068	pos	1.697 ± 0.1063	1.718 ± 0.1751	1.92 ± 0.1195	0.004254	0.03736
32	Cholic acid	LMST04010001;HMDB0000619	neg	4.675 ± 0.4112	4.64 ± 0.5403	5.249 ± 0.3615	0.01501	0.04857
33	Ketotifen-N-glucuronide	HMDB0060596	pos	1.708 ± 0.223	1.621 ± 0.355	2.088 ± 0.2461	0.008069	0.05475
34	9-Oxohexadecanoic acid	HMDB0030973	neg	2.275 ± 0.1388	2.376 ± 0.5223	3.179 ± 0.7307	0.02066	0.06055
35	PE (16:0/0:0)	LMGP02050002;HMDB0011503	pos	4.191 ± 0.2451	4.242 ± 0.2388	4.472 ± 0.09897	0.01097	0.06678
36	Ganoderic acid alpha	HMDB0033024	pos	2.223 ± 0.8058	2.313 ± 0.6968	3.007 ± 0.1928	0.0116	0.06964
37	P-Tolyl sulfate	–	neg	3.999 ± 0.6002	4.066 ± 0.4533	4.817 ± 0.6236	0.03111	0.07997
38	P-cresol	HMDB0001858	neg	2.463 ± 0.7798	2.619 ± 0.6048	3.514 ± 0.768	0.03305	0.08354
39	(10betaH,11xi)-11-Hydroxy-13-nor-6-eremophilen-8-one	HMDB0037605	neg	2.586 ± 0.2696	2.71 ± 0.2906	3.049 ± 0.348	0.03411	0.08534
40	3-hydroxypentadecanoyl carnitine	HMDB0061641	pos	1.504 ± 0.9274	1.526 ± 1.032	2.536 ± 0.4998	0.01631	0.08538
41	6-Lactoyltetrahydropterin	HMDB0002065	pos	2.081 ± 0.28	2.062 ± 0.3702	2.437 ± 0.247	0.02741	0.1156
42	Phenylacetylglycine	HMDB0000821	pos	3.015 ± 0.4174	3.142 ± 0.3587	3.717 ± 0.5212	0.0045	0.122
43	Cucurbitacin B	HMDB0034927;LMST01010104	pos	1.761 ± 0.7552	1.828 ± 0.515	2.418 ± 0.4032	0.03453	0.1356
44	13-Hydroxydihydromelleolide	HMDB0036929	pos	2.415 ± 0.6791	2.454 ± 0.7022	3.241 ± 0.6334	0.04166	0.1539

The "-" indicates that the corresponding metabolite did not pass through the screening process. FDR represents the P-value corrected. Mean represents the average relative abundance of metabolites in different groups; SD represents standard deviation; one-way ANOVA was used to compare the three groups. P-value <0.05 is significant.

TABLE 2 | List of differentially expressed (*down-regulated*) endogenous metabolites detected by UHPLC-QTOF/MS in the T2DM-8w group compared with NC-8w, and HFD-8w groups.

	Metabolite	Library ID	Mode	NC-8w mean ± SD	HFD-8w mean ± SD	T2DM-8w mean ± SD	P-value	FDR
1	PC [14:0/22:5(4Z,7Z,10Z,13Z,16Z)	LMGP01012130;HMDB0007890	neg	4.148 ± 0.1066	4.086 ± 0.1375	3.597 ± 0.09977	6.05E-08	0.000004795
2	LysoPC [20:5(5Z,8Z,11Z,14Z,17Z)]	HMDB0010397	neg	4.499 ± 0.1749	4.381 ± 0.2234	3.743 ± 0.1392	2.62E-07	0.00001466
3	N-Arachidonoyl-L-Serine	–	neg	3.118 ± 0.1499	2.855 ± 0.1767	2.409 ± 0.1316	2.80E-07	0.00001527
4	Sagittariol	HMDB0036835	neg	2.039 ± 0.284	1.708 ± 0.4858	0.8501 ± 0.1322	3.79E-07	0.00001851
6	(±)12-HEPE	–	neg	3.901 ± 0.1387	3.705 ± 0.1464	3.341 ± 0.09963	0.000000608	0.00002608
7	LysoPE [0:0/20:1(11Z)]	LMGP02050046;HMDB0011482	neg	3.558 ± 0.1086	3.44 ± 0.1151	3.233 ± 0.05414	0.000007621	0.0001873
5	(±)12,13-DiHOME	–	pos	2.232 ± 0.3281	2.024 ± 0.6836	0.4983 ± 0.3734	4.11E-07	0.0002344
8	LysoPC [18:3(9Z,12Z,15Z)]	HMDB0010388	neg	4.304 ± 0.09767	4.244 ± 0.1491	3.891 ± 0.1256	0.00001294	0.0002819
9	LysoPC [16:1(9Z)/0:0]	HMDB0010383	neg	4.919 ± 0.1613	4.749 ± 0.2127	4.458 ± 0.06338	0.00001682	0.0003518
10	LysoPE [0:0/20:2(11Z,14Z)]	LMGP02050047;HMDB0011483	neg	2.797 ± 0.1183	2.67 ± 0.1648	1.986 ± 0.2883	0.00002515	0.0004517
11	PC [16:0/18:3(6Z,9Z,12Z)]	LMGP01010598;HMDB0007974	neg	3.909 ± 0.1288	3.821 ± 0.1233	3.544 ± 0.1034	0.00004042	0.0006245
12	16-Hydroxy-10-oxohexadecanoic acid	HMDB0041287	neg	1.872 ± 0.1299	1.842 ± 0.2167	1.117 ± 0.2812	0.00004262	0.000649
13	LysoPC [20:1(11Z)]	HMDB0010391	neg	3.336 ± 0.1033	3.251 ± 0.08911	3.061 ± 0.08274	0.0000953	0.001158
14	LysoPC[20:2(11Z,14Z)]	HMDB0010392	neg	4.331 ± 0.2004	4.212 ± 0.2266	3.843 ± 0.1405	0.0001242	0.001381
15	Dynorphin B (6-9)	HMDB0012937	neg	2.649 ± 0.1651	1.995 ± 0.793	1.157 ± 0.6269	0.0001412	0.001521
16	LysoPC [20:3(5Z,8Z,11Z)]	HMDB0010393;LMGP01050139	neg	4.73 ± 0.1409	4.753 ± 0.1737	4.31 ± 0.1777	0.0001842	0.001853
17	Fumonisin AK1	HMDB0033397	neg	3.418 ± 0.1705	3.201 ± 0.2073	2.975 ± 0.1387	0.000234	0.00221
18	PC [17:1(9Z)/0:0]	LMGP01050126	pos	3.962 ± 0.1284	3.845 ± 0.1359	3.642 ± 0.03797	0.00003614	0.00226
19	Phytolaccinic acid	HMDB0034640	pos	4.158 ± 0.2234	4.029 ± 0.2471	3.626 ± 0.1074	0.00005294	0.002637
20	4-formyl Indole	–	pos	2.016 ± 0.09908	1.779 ± 0.178	1.537 ± 0.1904	0.00007242	0.003018
21	Prostaglandin F2a	HMDB0001139;LMFA03010002	neg	1.84 ± 0.06677	1.619 ± 0.2139	1.211 ± 0.3284	0.0004464	0.003581
22	1-(8Z,11Z,14Z-eicosatrienoyl)-sn-glycero-3-phosphocholine	–	pos	4.889 ± 0.209	4.949 ± 0.1933	4.466 ± 0.1559	0.00009844	0.003692
23	({6-[(E)-2-methoxyethenyl]-2-oxo-2H-chromen-7-yl}methoxy)sulfonic acid	HMDB0135800	neg	1.634 ± 0.1493	2.054 ± 0.4748	0.9036 ± 0.403	0.0004906	0.003854
24	2-O-beta-D-Glucopyranuronosyl-D-mannose	HMDB0039722	pos	2.707 ± 0.255	2.012 ± 1.022	0.6856 ± 0.8457	0.0001568	0.004829
25	Undecylenic acid	HMDB0033724;LMFA01030036	neg	1.398 ± 0.4536	1.491 ± 0.1991	0.9852 ± 0.2044	0.0007431	0.005375
26	1-Heptadecene-4,6-diyne-3,9-diol	LMFA05000599;HMDB0038782	neg	1.489 ± 0.4433	1.139 ± 0.4965	0.5592 ± 0.3104	0.0007936	0.005653
27	S-(9-hydroxy-PGA2)-glutathione	HMDB0013060	neg	2.483 ± 0.1517	2.07 ± 0.5766	1.396 ± 0.583	0.0009696	0.006584
28	N-(1-Deoxy-b-D-fructopyranosyl) (R)C(S)S-alliin	HMDB0040829	pos	1.63 ± 0.6413	1.161 ± 0.6927	0.3917 ± 0.02535	0.0003851	0.008527
29	5-Tetradecenoic acid	HMDB0000499	neg	1.782 ± 0.1411	1.734 ± 0.09607	1.464 ± 0.1496	0.001364	0.00858
30	Salsolinol 1-carboxylate	HMDB0013068	neg	2.107 ± 0.4538	1.524 ± 0.6139	0.7277 ± 0.7035	0.001511	0.009284
31	Artabsin	HMDB0036641	neg	2.099 ± 0.1712	2.026 ± 0.2375	1.763 ± 0.1536	0.002986	0.01525
32	LysoPE[0:0/18:1(11Z)]	HMDB0011475;LMGP02050039	neg	2.77 ± 0.2145	2.693 ± 0.2234	2.277 ± 0.2685	0.003575	0.01741
33	3,4,5-trihydroxy-6-[2-(3-oxoprop-1-en-1-yl)phenoxy]oxane-2-carboxylic acid	HMDB0134044	neg	2.123 ± 0.295	2.3 ± 0.2116	1.484 ± 0.5169	0.004692	0.02112
34	L-tryptophan	HMDB0000929	pos	4.831 ± 0.0709	4.782 ± 0.1133	4.6 ± 0.1235	0.001951	0.0227
35	Mandelic acid	HMDB0000703	pos	1.664 ± 0.7677	0.9643 ± 0.6709	0.4234 ± 0.2473	0.002352	0.02551
36	N-(1-Deoxy-1-fructosyl)threonine	HMDB0037843	neg	1.653 ± 0.7827	1.283 ± 0.6896	0.5343 ± 0.4483	0.006621	0.02742
37	6-Hydroxy-1H-indole-3-acetamide	HMDB0031173	neg	1.872 ± 0.7703	1.948 ± 0.6647	0.9572 ± 0.5212	0.007763	0.03038
38	LysoPE(0:0/20:0)	HMDB0011481;LMGP02050045	pos	3.014 ± 0.2419	2.846 ± 0.1636	2.567 ± 0.2013	0.004034	0.0361
39	Methyl N-methylanthranilate	HMDB0034169	pos	2.226 ± 0.1137	2.148 ± 0.2091	1.951 ± 0.1518	0.004539	0.0393
40	Baicalin	HMDB0041832;LMPK12111081	neg	2.626 ± 0.227	2.86 ± 0.368	1.802 ± 0.7688	0.01399	0.04611
41	3,11,12-Trihydroxy-1(10)-spirovetiven-2-one	HMDB0038154	pos	1.94 ± 0.2031	1.707 ± 0.2802	1.315 ± 0.4251	0.007232	0.05121
42	Isoquinoline	HMDB0034244	pos	1.952 ± 0.549	1.795 ± 0.6743	0.9008 ± 0.6406	0.009656	0.06099
43	Nopalinic acid	HMDB0029437	neg	2.7 ± 0.3405	2.642 ± 0.3953	1.732 ± 0.7764	0.02197	0.06305
44	Acetylcholine	HMDB0000895	pos	2.43 ± 0.1702	2.461 ± 0.1738	2.104 ± 0.2401	0.01098	0.06678
45	5-Methoxyindoleacetate	HMDB0004096	pos	1.982 ± 0.4658	1.658 ± 0.3664	1.08 ± 0.5623	0.01453	0.08067
46	Ganglioside GT3 (d18:1/20:0)	HMDB0012073	neg	2.608 ± 0.2643	2.482 ± 0.3186	2.066 ± 0.4347	0.03383	0.08495
47	11(S)-HEPE	–	pos	3.407 ± 0.1163	3.323 ± 0.0596	3.194 ± 0.1272	0.01611	0.08509

(Continued)

TABLE 2 | Continued

	Metabolite	Library ID	Mode	NC-8w mean ± SD	HFD-8w mean ± SD	T2DM-8w mean ± SD	P-value	FDR
48	Nicotyrine	–	pos	1.521 ± 0.7153	1.522 ± 0.5714	0.7747 ± 0.451	0.01724	0.08768
49	Arginyl-Cysteine	HMDB0028706	pos	2.391 ± 0.4847	2.37 ± 0.4952	1.567 ± 0.5999	0.01841	0.09109
50	Phe4Cl-Phe-OH	–	pos	1.916 ± 0.1608	1.81 ± 0.6848	1.084 ± 0.7053	0.02812	0.118

The "-" indicates that the corresponding metabolite did not pass through the screening process. FDR represents the P-value corrected. Mean represents the average relative abundance of metabolites in different groups; SD represents standard deviation; one-way ANOVA was used to compare the three groups. P-value < 0.05 is significant.

prominent classes of GPs include phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) (23). Plasma lipidomics studies in humans have also revealed a significant association between PE (consequently, a decreased PC : PE ratio) and obesity (24), prediabetes, and type 2 diabetes (25). This rearrangement can radically alter membrane potential and permeability to proteins such as cytokines. Maintaining this balance seems to have an important impact on health. Besides, they also act as a storage depot for lipid mediators derived from GPs which have been suggested to be involved in abnormal signal transduction processes, oxidative stress, neuroinflammation and neurodegeneration of AD (22). Similar to previous studies (26, 27), our results found that the levels of PE (increased), PC (increased), and their metabolites LysoPC, LysoPE (decreased) were significantly disturbed in serum compared with the control groups, suggesting that they may participate in the pathological process of cognitive impairment in diabetic rats.

Compared with GPs, SPs (such as sphingomyelins, gangliosides and ceramides) which constitute membrane microdomain "lipid rafts", appear very low in abundance, usually being present in the body less than 20% of the level of their glycerolipid (28, 29). These lipids belong to a family of lipid molecules, circulate in the serum and accumulate in the skeletal muscle and associate with insulin resistance and glucose homeostasis. Ceramides and related sphingolipids, as mediators of insulin resistance, cell death, and inflammation (30), can interfere with insulin signaling (31), suggesting that they play an important role in DMMCI. The previous study has used quantitative and targeted metabolomics to identify a group of SPs, demonstrating that their concentrations in brain tissue correlate with neuropathological severity of AD, and in blood with measurements of pre-clinical and pro-clinical AD progression (32). In addition, more and more evidence shows that the metabolism of GPs, SPs, and cholesterol are closely interconnected and interrelated. For example, GP-derived lipid mediators (arachidonic acid) regulate SP metabolism by regulating sphingolipase, and SP-derived lipid mediators (ceramide, ceramide-1-phosphate) modulate GP metabolism by regulating the isomer of phospholipase A2 (PLA2) (33). The interaction between their metabolites may act an important role in the initiation and maintenance of oxidative stress related to neurological diseases (such as stroke, AD, and Parkinson's disease) as well as in the proliferation, differentiation, and apoptosis of nerve cells (34). Some recently discovered SP mediators contain S1P as shown in our results (up-regulated markers) and ceramine-1-phosphate, which are key mediators of cellular reactions. S1P is a strong signaling molecule that, in addition to regulating essential physiological processes such as blood vessels, bone formation (35, 36) and inflammatory response (37), also regulates many molecular events critical to brain development and neuronal survival (38, 39). In cells, S1P may play different roles according to its subcellular localization, normally regulating mitochondrial function (40), gene expression (41), and endoplasmic reticulum (ER) stress (42); extracellularly, S1P has been shown to influence cell proliferation and migration, cell differentiation and survival, and neurite growth and neurogenesis by regulating five known G-protein-coupled

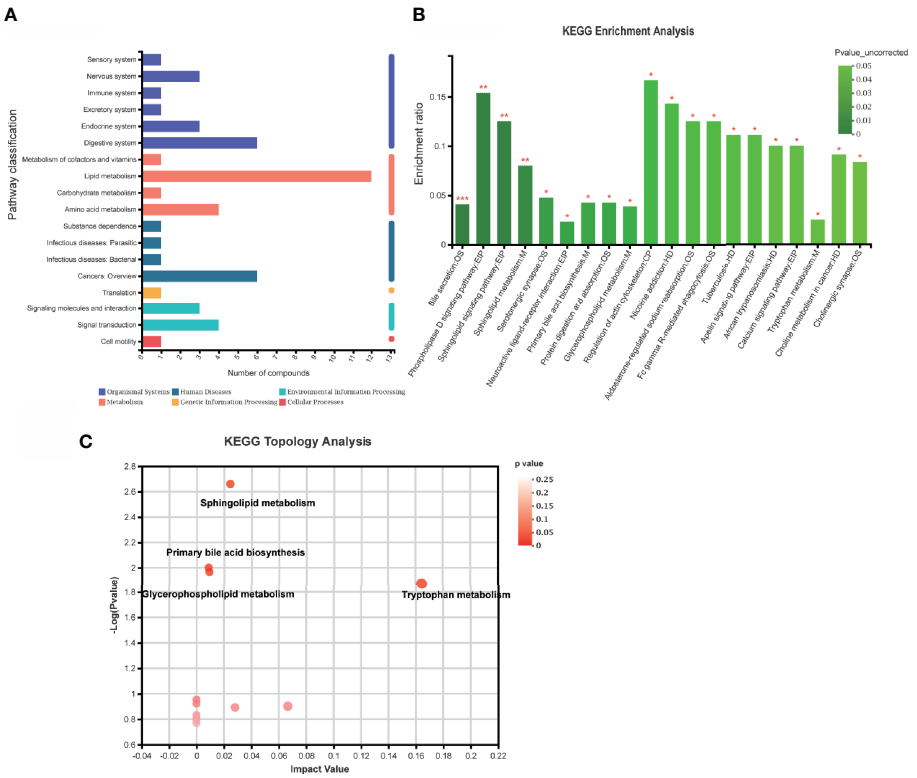

FIGURE 6 | KEGG enrichment analysis and pathway analysis of the identified 94 differentially expressed metabolites. **(A)** Pathway classification analysis by KEGG revealed showed 12 metabolites annotated lipid metabolism, six metabolites annotated cancers: Overview, six metabolites annotated digestive system, four metabolites annotated signal transduction, four metabolites annotated amino acid metabolism, three metabolites annotated signaling molecules and interaction, three metabolites annotated endocrine system, three metabolites annotated nervous system, *etc.* **(B)** Pathway annotation analysis by KEGG revealed the pathways where p-value is in the top 20 including bile secretion: OS, Phospholipase D signaling pathway: EIP, sphingolipid signaling pathway: EIP, sphingolipid metabolism: M, Serotonergic synapse: OS, neuroactive ligand–receptor interaction: EIP, primary bile acid biosynthesis: M, protein digestion and absorption: OS, Glycerophospholipid (GP) metabolism: M, regulation of actin cytoskeleton: CP, nicotine addiction: HD, Aldosterone-regulated sodium reabsorption: OS, Fc gamma R-mediated phagocytosis: OS, tuberculosis: HD, Apelin signaling pathway: EIP, African trypanosomiasis: HD, calcium signaling pathway: EIP, tryptophan metabolism: M, choline metabolism in cancer: HD, cholinergic synapse: OS. **(B, C)** Pathway topology analysis highlighted the following pathways: sphingolipid (SP) metabolism, tryptophan (Trp) metabolism, Glycerophospholipid (GP) metabolism, primary bile acid biosynthesis, folate biosynthesis, Aminoacyl-tRNA biosynthesis, Valine, leucine, and isoleucine degradation, phenylalanine metabolism, glycine, serine, and threonine metabolism, Butanoate metabolism, arachidonic acid metabolism, steroid hormone biosynthesis, phenylalanine, tyrosine, and Trp biosynthesis, etc. *p < 0.05; **p < 0.01; ***p < 0.001.

TABLE 3 | Pathway topological characteristics of 94 differentially expressed metabolites.

Pathway_ID	Pathway Description	Match_status	Num	Impact_value	P value_uncorrected	P value_corrected
map00600	Sphingolipid metabolism	2|21	2	0.024390244	0.002186261	0.0142107
map00380	Tryptophan metabolism	2|54	2	0.164478114	0.013490228	0.035074593
map00564	Glycerophospholipid metabolism	2|48	6	0.009100322	0.010828049	0.035191159
map00120	Primary bile acid biosynthesis	2|46	2	0.008546673	0.009995385	0.043313334
map00790	Folate biosynthesis	1|56	1	0	0.169623492	0.169623492
map00970	Aminoacyl-tRNA biosynthesis	1|52	1	0	0.159718126	0.17302797
map00280	Valine, leucine, and isoleucine degradation	1|40	1	0.028084852	0.128096085	0.185027678
map00360	Phenylalanine metabolism	1|51	1	0	0.157193065	0.185773623
map00260	Glycine, serine, and threonine metabolism	1|47	1	0	0.146894973	0.190963465
map00650	Butanoate metabolism	1|39	1	0.066598709	0.125328146	0.203658237
map00590	Arachidonic acid metabolism	1|37	1	0	0.119729446	0.222354685
map00140	Steroid hormone biosynthesis	1|89	1	0.0096509	0.240179458	0.223023783
map00400	Phenylalanine, tyrosine, and tryptophan biosynthesis	1|34	1	0	0.111172765	0.240874324

Pathway_ID: represents the KEGG Pathway number; Match_status: represents the metabolites participating in the pathway. The data before the "/" represents the number of metabolites participating in the pathway in the current metabolism concentration; the number behind the "/" is the total number of metabolites in the current pathway; Pathway description: represents the name of the path; IMPACT VALUE: represents the overall importance score of the pathway, with a total score of 1, which can be calculated, according to the relative position of metabolites in the pathway.

receptors, S1PR1–S1PR5 (43, 44). It is hypothesized that regulation of SP metabolism and its associated signaling pathways may be a potential treatment for these devastating diseases.

Trp is a significant biosynthetic precursor of neurotransmitters, which is closely related to attention, memory, and reaction ability as a monoamine neurotransmitter (45). Trp metabolic routes consist of the two branches of the serotonin (5HT) and kynurenine pathway (KP). Trp could be metabolized into 5-HT, which promotes the formation and maintenance of synapses and affects cerebral cortex maturation. On the other hand, Trp can be metabolized to 3-HK and QUIN through the KP route, which has toxic effects on the nervous system. 3-HK could accelerate the generation of free radicals and mediates the death of neurons (21).

It was found that the content of neuroprotective 5-HT in the striatum and cortex of the aged rats decreased (46), while the contents of neurotoxic 3-HK in serum and hippocampal pyramidal neurons increased in AD patients (47). In this study, we found a similar change in the levels (decreased) of Trp in the blood of the DMMCI rats. Therefore, there is no doubt that the development of an effective means to explore the dynamics of Trp metabolic pathways in the central and peripheral systems may benefit the discovery of biomarkers for clinical treatment and pathological features of cognitive dysfunction.

Similar serum analysis to establish the link between DM and MCI has also been reported in some patient studies (48, 49). Zhang et al.'s study in 2015 on the plasma metabolomic *Profiling of Patients* has also found the disorders of sphingolipid metabolism and bile acid metabolism both happened in T2DM and diabetes-associated cognitive decline (DACD) (48). Morris et al.'s study in 2018 observed lower abundances of Trp, phosphatidylcholines (PCs), and sphingomyelins in cognitive healthy subjects with T2DM compared with those without T2DM and suggested that AD may obscure the typical metabolic phenotype of T2DM (49). These prior studies indicated that there was certain similarity/link in identified pathways/metabolites between our developed rat model and patients, which further validated the developed DMMCI rat model.

However, our study still has some limitations. First, a major "limitation" of untargeted metabolic phenotyping, which is also a major strength, is that as an unbiased metabolomic analysis, it would identify a wide range of metabolites and pathways. However, non-targeted LC-MS approaches have been proved useful and effective in the biomarker discovery stages of numerous metabolic phenotypic studies (48, 49). Differential expression of or modifications to these metabolites can provide a more reliable source of potential diagnostic biomarkers for DMMCI, which was also our main purpose. Once potential biomarkers are identified from the findings of either untargeted metabotyping studies, these can be confirmed through targeted approaches, using specific, fully validated, quantitative methods and this will be our next study direction. Second, the relatively small number of serum samples in our primary non-target metabolomics analysis may have limited our ability to probe substantial associations with other metabolites. Future studies will need more serum samples to validate and confirm our findings. Third, it should be noted that our main analysis was only based on serum metabolites, and these metabolites represent only a small part of the organism metabolome. Future analyses will expand our study framework to contain more classes of metabolites.

CONCLUSION

Our study indicated that alterations in serum metabolites of lipid metabolism such as up-regulation of PE and S1P and down-regulation of PC and L-Trp may contribute to the underlying mechanisms of DMMCI by affecting GP metabolism, Trp metabolism, and SP metabolism pathways, respectively. Serum PE, PC, L-Trp, and S1P may be used as the most critical biomarkers for early diagnosis of DMMCI. An LC-MS-based metabolomics technology has potential value in identifying DMMCI biomarkers for the early detection and provides a novel avenue for effective therapeutic intervention in DCD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Central South University Xiangya School of Medicine.

AUTHOR CONTRIBUTIONS

YS determined the structure of the review. RC and YZ selected the references and contributed to the writing. WX and LZ contributed to the revision and finalization of the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.665309/full#supplementary-material

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Glycemic Dysregulations Are Associated With Worsening Cognitive Function in Older Participants at High Risk of Cardiovascular Disease: Two-Year Follow-up in the PREDIMED-Plus Study

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Introduction: Type 2 diabetes has been linked to greater cognitive decline, but other glycemic parameters such as prediabetes, diabetes control and treatment, and HOMA-IR and HbA_{1c} diabetes-related biomarkers have shown inconsistent results. Furthermore, there is limited research assessing these relationships in short-term studies. Thus, we aimed to examine 2-year associations between baseline diabetes/glycemic status and changes in cognitive function in older participants at high risk of cardiovascular disease.

Methods: We conducted a 2-year prospective cohort study (n=6,874) within the framework of the PREDIMED-Plus study. The participants (with overweight/obesity and metabolic syndrome; mean age 64.9 years; 48.5% women) completed a battery of 8 cognitive tests, and a global cognitive function Z-score (GCF) was estimated. At baseline, participants were categorized by diabetes status (no-diabetes, prediabetes, and <5 or ≥5-year diabetes duration), and also by diabetes control. Furthermore, insulin resistance (HOMA-IR) and glycated hemoglobin (HbA_{1c}) levels were measured, and antidiabetic medications were recorded. Linear and logistic regression models, adjusted by potential confounders, were fitted to assess associations between glycemic status and changes in cognitive function.

Results: Prediabetes status was unrelated to cognitive decline. However, compared to participants without diabetes, those with ≥5-year diabetes duration had greater reductions in GCF (β=-0.11 (95%CI -0.16;-0.06)], as well as in processing speed and executive function measurements. Inverse associations were observed between baseline HOMA-IR and changes in GCF [β=-0.0094 (95%CI -0.0164;-0.0023)], but also between HbA_{1c} levels and changes in GCF [β=-0.0085 (95%CI -0.0115, -0.0055)], the Mini-Mental State Examination, and other executive function tests. Poor diabetes control was inversely associated with phonologic fluency. The use of insulin treatment was inversely related to cognitive function as measured by the GCF [β=-0.31 (95%CI -0.44, -0.18)], and other cognitive tests.

Conclusions: Insulin resistance, diabetes status, longer diabetes duration, poor glycemic control, and insulin treatment were associated with worsening cognitive function changes in the short term in a population at high cardiovascular risk.

Clinical Trial Registration: http://www.isrctn.com/ISRCTN89898870, identifier ISRCTN: 89898870.

Keywords: cognitive function, diabetes duration, glycated (glycosylated) hemoglobin, insulin resistance, type 2 diabetes, prediabetes

INTRODUCTION

Type 2 diabetes is an important public health problem worldwide. In 2019, the International Diabetes Federation estimated that ∼463 million people were living with diabetes (and 374 million had prediabetes), of whom one-third were >65 years old, and this figure is expected to rise to 700 million by 2045 (1). Diabetes mellitus is not only among the top 10 causes of death worldwide (2), but is also a risk factor for blindness, renal failure, and lower limb amputation, overall decreasing quality of life (2). As well, over 50 million people worldwide live with dementia, a form of cognitive impairment, and this number is expected to triple by 2050 (3). Cognitive impairment, characterized by loss of memory, concentration and reduced ability to learn new things, affecting everyday life, is relatively common and is a costly condition for the health system (3).

Meta-analyses and longitudinal studies of population-based cohorts have shown an increased risk of cognitive dysfunction in people with metabolic syndrome, prediabetes and diabetes (4–6). Specifically, type 2 diabetes has been related to deficits in different cognitive domains (7) and to accelerated cognitive decline, especially in psychomotor speed, memory and executive functions (8). However, some prospective studies have failed to confirm these associations (9, 10). Also, the relationship between cognitive decline and metabolic syndrome, prediabetes, insulin resistance and glycemic control is less well understood (4, 6, 11). Therefore, more studies are warranted to determine if glycemic dysregulations before diabetes onset may affect cognition in order to establish early strategies of prevention-focused on these populations.

Risk factors for cognitive decline when type 2 diabetes has been already established are also of great interest because consideration of these could help screen individuals with diabetes who may particularly benefit from intensive and suitable treatment strategies. The risk of accelerated cognitive decline in type 2 diabetes has been reported by some studies to be dependent on both disease duration and glycemic control (5, 12). Glucose-lowering treatments have also been related to cognitive function in a few epidemiologic studies with moderate-quality evidence (6, 13). Therefore, more studies are required to increase the strength of the evidence for these associations.

Furthermore, there is a gap in the research relating to shorter follow-up studies assessing the aforementioned relationships. Majority of the research to date has been conducted with medium to long-term duration (from 4 to more years of follow-up) (5, 9). The PREDIMED-Plus study offers an unprecedented opportunity to evaluate cognitive changes, using a battery of cognitive tests, and several measurements of glycemic status in a large population at high cardiovascular disease risk in the shorter term (2 years).

The objectives of the present study were to examine longitudinal associations between glycemic status (diabetes status, control/treatment, and related biomarkers) and cognitive decline and impairment. We hypothesized that glycemic dysregulations would be negatively associated with changes in cognitive function.

Abbreviations: BMI, Body Mass Index; CDT, Clock Drawing Test; DST-b, Digit Span Test backward section; DST-f, Digit Span Test forward section; GCF, Global Cognitive Function; HbA_{1c}, glycated hemoglobin; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; IDDP-4, dipeptidyl peptidase-4 inhibitors; MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test A section; TMT-B, Trail Making Test B section; VFT-a, verbal Fluency Test animals category; VFT-p, Verbal Fluency Test letter p category.

MATERIALS AND METHODS

The present study is based on an observational prospective cohort design conducted within the framework of the PREDIMED-Plus study using 2 years of follow-up data. The PREDIMED-Plus study is a multicenter, randomized, parallel-group clinical trial conducted in Spain for primary cardiovascular disease prevention. Participants were randomized to an intensive weight loss intervention program based on an energy-restricted traditional Mediterranean diet, physical activity promotion and behavioral support (intervention group) or usual care consisting of general recommendations to follow an energy-unrestricted Mediterranean diet (control group). The study protocol has been described extensively elsewhere (14) and can be found at http://www.predimedplus.com. The trial was registered in 2014 at the International Standard Randomized Controlled Trial (http://www.isrctn.com/ISRCTN89898870).

Study Population

Eligible participants were community-dwelling adults (55–75 years) with overweight/obesity (27≤ BMI <40 kg/m^2) who met at least three criteria of metabolic syndrome (15). Exclusion criteria are reported elsewhere (14).

Participant recruitment was conducted between October 2013 and December 2016 in 23 Spanish health centers. A total of 6,874 candidates met eligibility criteria and were randomly allocated in a 1:1 ratio to the intervention or control groups, using a centrally controlled, computer-generated random-number internet-based system with stratification by center, sex, and age. Couples sharing the same household were randomized together, using the couple as unit of randomization. The flow-chart of the studied PREDIMED-Plus population is shown in **Supplementary Figure 1**.

All participants provided written informed consent, and the study protocol and procedures were approved by all the ethical committees of all participating institutions.

Diabetes Status and Glycemic Measurements

At baseline fasting blood samples were collected and biochemical analyses were performed to determine fasting plasma glucose and glycated hemoglobin (HbA_{1c}) by routine laboratory methods. Insulin was centrally measured by an electrochemiluminescence immunoassay using an Elecsys immunoanalyzer (Roche Diagnostics, Meylan, France). Insulin resistance was estimated at baseline using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index (16).

Prediabetes and diabetes were defined following the American Diabetes Association criteria (17). Diabetes was defined as a previous diagnosis of diabetes, HbA_{1c} ≥48 mmol/mol (6.5%), use of antidiabetic medication, or having fasting plasma glucose >126 mg/dl in both the screening and baseline visits. Self-reported diabetes duration was categorized in <5-year and ≥5-year diabetes duration. Prediabetes status was defined as HbA_{1c} being between 39 mmol/mol (5.7%) and 46 mmol/mol (6.4%), or having fasting plasma glucose between ≥100 mg/dl and ≤125 mg/dl. Participants who did not meet any of these parameters were categorized into the no-diabetes category. Furthermore, we categorized diabetes status in participants with diabetes (participants with <5-year and ≥5-year diabetes duration) and no-diabetes (participants with prediabetes and no-diabetes).

Glycated hemoglobin was used to categorize participants into those having "good" or "poor" diabetic control [HbA_{1c} <57 mmol/mol or ≥57 mmol/mol (7.4%)], respectively (17). Diabetes treatment was assessed at baseline using self-reported data on insulin, sulfonylureas, metformin or dipeptidyl peptidase-4 inhibitors (IDPP-4) use.

Covariates

Covariates were evaluated at baseline by trained staff in a face-to-face interview using self-reported general questionnaires on socio-demographics (sex, age, level of education, and civil status), lifestyle (alcohol intake, smoking habits, physical activity, and Mediterranean diet adherence), and disease history. Baseline anthropometric variables (weight and height) were determined to estimate body mass index (BMI). Adherence to an energy-reduced Mediterranean diet was assessed using a 17-point diet score, adapted from a previously validated one (18). Leisure-time physical activity was estimated using a validated short version of the Minnesota Leisure-Time Physical Activity Questionnaire (19, 20). The depressive status risk was evaluated using the Beck Depression Inventory-II (21).

Neuropsychological Assessment

A battery of 8 cognitive tests was administered at baseline and 2 years of follow-up by trained staff. The tests performed, Mini-Mental State Examination (MMSE), Clock Drawing Test (CDT), Digit Span Test forward (DST-f) and backward (DST-b) section, Verbal Fluency Test animals (VFT-a) and "p" (VFT-p) version, and Trail Making Test part A (TMT-A) and B (TMT-B) are described in **Supplementary Material 1**.

Statistical Analyses

We used the December 2020 PREDIMED-Plus database. Descriptive variables are reported as means and standard deviation (SD) for continuous variables or numbers and percentages (%) for qualitative variables. Differences between diabetes status and baseline characteristics were examined using chi-square and one-way ANOVA, for qualitative and quantitative variables, respectively.

For longitudinal analysis, linear and logistic regression models were used, including only participants with complete cognitive data at baseline and 2 years of follow-up for each cognitive test analyzed. To facilitate comparisons across cognitive tests, Z-scores were generated for each cognitive score at baseline and after 2 years using the mean and SD of baseline data, as previously reported (5, 12). A global cognitive function Z-score (GCF) was obtained averaging all cognitive Z-scores at each time point, standardizing by the mean and SD of cognitive Z-scores at baseline.

Using linear regression analyses we examined the associations between baseline status and 2-year changes in cognitive Z-scores in relation to: a) HOMA-IR levels; b) diabetes status, no diabetes being the reference group; c) HbA_{1c} levels; d) glycemic control measured by HbA_{1c} in participants with diabetes, good glycemic control being the reference group; e) diabetes treatment in participants with diabetes, no treatment being the reference group. Two models were fitted to adjust linear and logistic regression analyses. Model 1 was adjusted for baseline sex, age (years), intervention group, and center size (with <250; 250-300, 300-400; >400 randomized participants). Model 2 was additionally adjusted for baseline education level (primary school; high school; college), civil status (single, divorced or separated; married; widower), physical activity (MET min/week), smoking habits (smoker; former smoker; never smoker), alcohol intake (g/day), 17-point Mediterranean diet score, BMI (kg/m^2), hypertension (yes/no), hypercholesterolemia (yes/no), and depression (yes/no). Furthermore, Model 3 was fitted exclusively for antidiabetic treatments to further adjust for baseline diabetes control (good/poor) and diabetes duration (<5-year diabetes duration/≥5-year diabetes duration).

Logistic regression analyses were used to estimate odds ratios (OR) and 95% confidence intervals (95% CI), examining the 2-year risk for cognitive impairment in participants with normal cognitive performance at baseline by diabetes status, with no diabetes being the reference group. Cognitive function cut-offs were defined by the dichotomization of neuropsychological assessments at the respective visits. Cognitive impairment was defined as GCF ≤10^{th} percentile, MMSE ≤24 punctuation, CDT ≤4 punctuation, and VFT-a, VFT-p, DST-d, DST-b ≤ respective mean - 1.5*SD and TMT-A, TMT-B ≥ respective mean + 1.5*SD (22–25).

Interaction analyses between glycemic status (diabetes status, HOMA-IR, HbA_{1c}, and glycemic control and treatment) and sex, age, hypertension and BMI for the GCF were performed by comparing the model with and without the interaction product using the likelihood ratio test.

Participants with missing data on covariables (always <1% missing) were imputed as either the mean of the group or into the subcategory with the highest frequency (26).

All analyses were conducted with robust estimates of the variance to correct for intracluster correlation. The data were analyzed using the Stata-14 software program (StataCorp). Statistical significance was set using the Benjamini-Hochberg false discovery rate correction procedure (27) at a Q-value <0.05.

RESULTS

Descriptive Results

Table 1 shows the baseline characteristics of the study population (n=6,874) according to diabetes status. A total of 20.9% of participants were classified as having no-diabetes, 48.6% prediabetes, 14.8% with <5-year diabetes duration, and 15.6% with ≥5-year diabetes duration. The mean age of the total population was 64.9 ± 4.9 years and 48.5% were women. Participants with ≥5-year diabetes duration were older, had lower education level and alcohol consumption, greater adherence to the Mediterranean diet and higher HbA_{1c} levels. They were also more likely to have hypertension, hypercholesterolemia and depressive symptoms. Participants with <5-year diabetes duration had greater prevalence of obesity and higher HOMA-IR levels, and were less likely to be a woman. Participants without diabetes were more likely to have a higher education level. All cognitive assessments showed significant differences across diabetes status and participants with ≥5-year diabetes duration with lower scores.

TABLE 1 | Baseline characteristics by diabetes status.

Characteristics	Diabetes status				P-value
	No-Diabetes (n=1440)	Prediabetes (n=3341)	<5y Diabetes (n=1020)	≥5y Diabetes (n=1073)	
Age (years)	64.5 ± 4.92	65.0 ± 4.91	64.7 ± 4.98	65.5 ± 4.81	<0.001
Sex (women)	706 (49.03)	1703 (50.97)	435 (42.65)	491 (45.76)	<0.001
Intervention group	730 (50.69)	1632 (48.85)	503 (49.31)	541 (50.42)	0.623
Education level					<0.001
Primary school or less	653 (45.35)	1627 (48.70)	489 (47.94)	593 (55.27)	
High school	417 (28.96)	976 (29.21)	302 (29.61)	291 (27.12)	
College	370 (25.69)	738 (22.09)	229 (22.45)	189 (17.61)	
Civil status					0.803
Single, divorced or separated	199 (13.82)	440 (13.17)	123 (12.06)	135 (12.58)	
Married	1097 (76.18)	2546 (76.20)	797 (78.14)	821 (76.51)	
Widower	144 (10.00)	355 (10.63)	100 (9.80)	117 (10.90)	
Physical activity (MET min/week)	2508 ± 2433	2493 ± 2264	2344 ± 2140	2420 ± 2378	0.236
Current smoker					0.195
Smoker	170 (11.81)	418 (12.51)	138 (13.53)	131 (12.21)	
Former smoker	602 (41.81)	1434 (42.92)	463 (45.39)	484 (45.11)	
Never smoker	668 (46.39)	1434 (44.57)	419 (41.08)	458 (42.68)	
Alcohol consumption (g/day)	11.0 ± 14.2	11.6 ± 15.9	11.7 ± 15.6	9.8 ± 14.6	0.004
17-point Mediterranean diet score	8.51 ± 2.71	8.37 ± 2.70	8.64 ± 2.60	8.72 ± 2.55	0.001
BMI (kg/m²)	32.2 ± 3.46	32.6 ± 3.41	32.9 ± 3.49	32.6 ± 3.52	<0.001
HOMA-IR	3.91 ± 2.61	5.08 ± 3.14	6.65 ± 4.19	6.30 ± 4.45	<0.001
HbA_{1c} (mmol/mol)	36.4 ± 4.7	40.5 ± 3.5	49.3 ± 10.2	54.7 ± 13.1	<0.001
HbA_{1c} (%)	5.48 ± 0.43	5.86 ± 0.32	6.66 ± 0.93	7.16 ± 1.20	<0.001
Hypertension	1192 (82.78)	2764 (82.73)	855 (83.82)	947 (88.26)	<0.001
Hypercholesterolemia	966 (67.08)	2281 (68.27)	755 (74.02)	811 (75.58)	<0.001
Depressive symptomatology	281 (19.51)	667 (19.96)	226 (22.16)	253 (23.58)	0.029
Cognitive assessments	**Diabetes status**				
	No-Diabetes	**Prediabetes**	**<5y Diabetes**	**≥5y Diabetes**	
MMSE (n=6654)	28.3 ± 1.85	28.3 ± 1.86	28.2 ± 1.95	28 ± 2.10	<0.001
CDT (n=6659)	5.95 ± 1.29	5.96 ± 1.21	6.02 ± 1.12	5.76 ± 1.34	<0.001
DST-f (n=5867)	8.95 ± 2.59	8.78 ± 2.39	8.87 ± 2.48	8.52 ± 2.48	<0.001
DST-b (n= 5864)	5.28 ± 2.36	5.11 ± 2.20	5.19 ± 2.19	4.93 ± 2.15	0.043
VFT-a (n=6816)	16.4 ± 5.00	16.1 ± 4.75	16.1 ± 4.84	15.2 ± 4.65	<0.001
VFT-p (n=6816)	12.6 ± 4.62	12.4 ± 4.53	12 ± 4.35	11.4 ± 4.39	<0.001
TMT-A (n=6802)§	50.9 ± 28.0	52.3 ± 27.5	52.7 ± 30.2	56.2 ± 30.2	<0.001
TMT-B (n=6783)§	121.6 ± 68.6	128.0 ± 70.2	130.1 ± 72.3	144.2 ± 79.6	<0.001

<5y diabetes, less than 5 years diabetes duration; ≥5y diabetes, more than 5 years diabetes duration; GCF, Global Cognitive Function; MMSE, Mini-Mental State Examination; CDT, Clock Drawing Test; DST-f, Digit Span Test forward section; DST-b, Digit Span Test backward section; VFT-a, Verbal Fluency Test animal category; VFT-p, Verbal Fluency Test letter "p"; TMT-A, Trail Making Test part A; TMT-B, Trail Making Test part B.

§ Inverse neuropsychological assessment score.

Data are n (%) or mean ± SD for categorical and quantitative variables, respectively.

Only the participants reported in each neuropsychological assessment are available.

Chi-square is used for categorical variables and One-way ANOVA for quantitative variables.

Diabetes Status and Related Biomarkers

Table 2 shows the associations between baseline diabetes status and 2-year changes in cognitive Z-scores. Compared to participants without diabetes, no significant differences in the associations between prediabetes and cognitive tests were observed. Compared to participants without diabetes, those with <5-year diabetes duration displayed larger decrements in cognitive Z-scores measured by the GCF, VFT-a, VFT-p and TMT-B tests in model 1, but these associations were attenuated in model 2. Compared to participants without diabetes, those with ≥5-year diabetes duration displayed larger reductions in all cognitive assessments in model 1, except in the case of the CDT test (**Table 2**). These associations remained significant for the GCF score, and the VFT-a, VFT-p, TMT-A and TMT-B tests in model 2. Similar results were found when comparing participants with diabetes and no-diabetes, finding a larger 2-year decrease with the presence of type 2 diabetes in the MMSE score (**Supplementary Table 1**).

Supplementary Table 2 shows the odds ratio (95% CI) for cognitive impairment incidence after 2 years of follow-up in participants with normal cognitive performance at baseline. Compared with participants without diabetes, those with diabetes had a borderline significant 34% (95% CI 0.96;1.87) higher risk of cognitive impairment when assessed by the GCF

TABLE 2 | Association between baseline diabetes status and changes in cognitive Z-scores.

Z-scores	Diabetes status	Model 1		Model 2	
		β (95% CI)	P-value	β (95% CI)	P-value
GCF	No-Diabetes (n=1023)	Ref.		Ref.	
	Prediabetes (n=2429)	-0.04 (-0.10, 0.03)	0.277	-0.01 (-0.04, 0.03)	0.756
	<5y Diabetes (n=667)	-0.12 (-0.20, -0.03)	0.008*	-0.04 (-0.09, 0.01)	0.109
	≥5y Diabetes (n=684)	-0.27 (-0.36, -0.18)	<0.001*	-0.11 (-0.16, -0.06)	<0.001*
MMSE	No-Diabetes (n=1187)	Ref.		Ref.	
	Prediabetes (n=2786)	-0.01 (-0.07, 0.05)	0.749	0.01 (-0.05, 0.06)	0.865
	<5y Diabetes (n=847)	-0.08 (-0.16, 0.01)	0.054	-0.05 (-0.13, 0.03)	0.209
	≥5y Diabetes (n=865)	-0.11 (-0.19, -0.02)	0.011*	-0.06 (-0.14, 0.02)	0.134
CDT	No-Diabetes (n=1189)	Ref.		Ref.	
	Prediabetes (n=2788)	0.01 (-0.06, 0.07)	0.874	0.01 (-0.05, 0.07)	0.780
	<5y Diabetes (n=846)	-0.01 (-0.09, 0.08)	0.843	0.01 (-0.08, 0.09)	0.847
	≥5y Diabetes (n=866)	-0.09 (-0.18, -0.01)	0.031	-0.06 (-0.14, 0.03)	0.171
DST-f	No-Diabetes (n=1072)	Ref.		Ref.	
	Prediabetes (n=2526)	-0.03 (-0.10, 0.05)	0.474	-0.01 (-0.08, 0.06)	0.725
	<5y Diabetes (n=702)	-0.08 (-0.17, 0.01)	0.087	-0.06 (-0.15, 0.03)	0.198
	≥5y Diabetes (n=716)	-0.12 (-0.21, -0.03)	0.012*	-0.07 (-0.16, 0.02)	0.126
DST-b	No-Diabetes (n=1072)	Ref.		Ref.	
	Prediabetes (n=2525)	-0.04 (-0.11, 0.03)	0.293	-0.02 (-0.09, 0.04)	0.528
	<5y Diabetes (n=702)	-0.07 (-0.16, 0.02)	0.116	-0.04 (-0.13, 0.04)	0.349
	≥5y Diabetes (n=716)	-0.11 (-0.20, -0.02)	0.014*	-0.05 (-0.14, 0.04)	0.251
VFT-a	No-Diabetes (n=1226)	Ref.		Ref.	
	Prediabetes (n=2866)	-0.07 (-0.13, -0.01)	0.033	-0.05 (-0.11, 0.01)	0.101
	<5y Diabetes (n=870)	-0.14 (-0.22, -0.05)	0.001*	-0.10 (-0.17, -0.02)	0.018
	≥5y Diabetes (n=889)	-0.25 (-0.33, -0.16)	<0.001*	-0.18 (-0.26, -0.10)	<0.001*
VFT-p	No-Diabetes (n=1227)	Ref.		Ref.	
	Prediabetes (n=2865)	-0.05 (-0.12, 0.02)	0.149	-0.03 (-0.09, 0.03)	0.348
	<5y Diabetes (n=870)	-0.13 (-0.21, -0.04)	0.005*	-0.08 (-0.16, 0.01)	0.060
	≥5y Diabetes (n=889)	-0.23 (-0.32, -0.14)	<0.001*	-0.15 (-0.23, -0.07)	<0.001*
TMT-A§	No-Diabetes (n=1226)	Ref.		Ref.	
	Prediabetes (n=2862)	-0.02 (-0.08, 0.04)	0.512	-0.03 (-0.09, 0.03)	0.323
	<5y Diabetes (n=869)	0.08 (0.01, 0.16)	0.037	0.05 (-0.02, 0.13)	0.185
	≥5y Diabetes (n=886)	0.20 (0.11, 0.29)	<0.001*	0.15 (0.06, 0.23)	0.001*
TMT-B§	No-Diabetes (n=1221)	Ref.		Ref.	
	Prediabetes (n=2859)	0.01 (-0.05, 0.07)	0.690	0.01 (-0.06, 0.06)	0.994
	<5y Diabetes (n=866)	0.11 (0.03, 0.20)	0.006*	0.08 (0.01, 0.16)	0.039
	≥5y Diabetes (n=883)	0.24 (0.15, 0.32)	<0.001*	0.17 (0.09, 0.25)	<0.001*

<5y diabetes, less than 5 years diabetes duration; ≥5y diabetes, more than 5 years diabetes duration; GCF, Global Cognitive Function; MMSE, Mini-Mental State Examination; CDT, Clock Drawing Test; DST-f, Digit Span Test forward section; DST-b, Digit Span Test backward section; VFT-a, Verbal Fluency Test animal category; VFT-p, Verbal Fluency Test letter "p"; TMT-A, Trail Making Test part A; TMT-B, Trail Making Test part B.

§ Inverse neuropsychological assessment score.

Model 1: adjusted for sex, age (in years), intervention group, and center size (<250; 250-300, 300-400; ≥400).

Model 2: further adjusted for baseline education level (primary school; secondary school; college), civil status (single, divorced or separated; married; widower), physical activity (MET min/week), smoking habits (smoker; former smoker; never smoker), alcohol intake (g/day, adding the quadratic term), 17-point Mediterranean diet score, BMI (kg/m²), hypertension (yes/no), hypercholesterolemia (yes/no), and depressive symptomatology (yes/no).

Beta coefficients were estimated using linear regression models with robust standard errors to account for intracluster correlations.

**Significant association after Benjamini-Hochberg correction.*

Z-score, and a non-significant 30% (95%CI 1.01;1.68) higher risk of impairment based on the VFT-a test after the false discovery rate correction. No significant associations were found between diabetes status and cognitive impairment incidence in the rest of the cognitive tests.

Table 3 shows the association between baseline HOMA-IR (per one unit increment) and changes in cognitive Z-scores after 2 years of follow-up after excluding those participants with insulin treatment. Significant inverse associations between HOMA-IR and changes in cognitive Z-scores measured by GCF and the DST-f and DST-b tests were found (model 2). No significant associations between insulin resistance and changes in cognitive Z-scores were found for the MMSE, CDT, VFT-a, VFT-p, TMT-A and TMT-B tests. Furthermore, a sensitivity analysis was conducted excluding those participants with insulin or sulfonylurea treatment (n=596). Compared with the results of **Table 3**, no changes in the direction of β coefficients or significances after the Benjamini-Hochberg correction were shown.

Table 4 presents the association between baseline HbA_{1c} levels (per one mmol/mol increment) and 2-year changes in cognitive Z-scores. An inverse association was observed between baseline HbA_{1c} levels and the GCF score, as well as the MMSE, VFT-a, VFT-p, TMT-A and TMT-B tests. No significant associations were found for the CDT, DST-f and DST-b tests.

There were no significant interactions by sex, age, hypertension or BMI between the glycemic status (HOMA-IR, HbA_{1c} and glycemic control/treatment) and changes in the GCF score (all p>0.05). However, an interaction by age was found between diabetes status and changes in the GCF score (P=0.046). Compared to participants without diabetes, a larger decline in the GCF score was shown in those participants aged ≤65 years and presenting with prediabetes and <5-year and ≥5-year of diabetes duration, whereas participants aged >65 years with prediabetes showed increased performance in the GCF score. No associations were found between diabetes duration and the GCF score in participants aged >65 years.

Diabetes Control and Treatment

Supplementary Table 3 shows the association between baseline glycemic control (HbA_{1c} ≥57 mmol/mol or <57 mmol/mol) in participants with diabetes and 2-year changes in cognitive Z-scores. Compared to participants with good diabetes control, those with poor control showed a larger decrement in the VFT-p [β= -0.13 (95%CI -0.22;-0.04)] test (model 2). No associations between glycemic control and the rest of the cognitive tests were observed.

Supplementary Table 4 shows the association between baseline insulin treatment in participants with diabetes and changes in cognitive Z-scores. Compared to participants without insulin treatment, those with insulin treatment showed a significantly greater decrease in cognitive function measured by the GCF score and the DST-f, DST-b, VFT-a, VFT-p, TMT-A and TMT-B tests. No associations were observed for the remaining cognitive tests assessed (MMSE and CDT). Concerning oral glucose medication use, sulfonylurea treatment was not significantly associated with an increase in the TMT-A (β= 0.22 [95%CI 0.07;0.38]) Z-score after the Benjamini-Hockberg correction (**Supplementary Table 5**). No significant associations were shown between the use of metformin or IDDP-4 and changes in cognitive Z-scores (**Supplementary Tables 6**, **7**, respectively). When the associations between diabetes treatment and cognitive function were further adjusted by diabetes duration or glycemic control, the results remained similar (model 3).

No significant interactions by sex, age, hypertension, and BMI were observed between diabetes control or treatment and changes in the GCF score.

TABLE 3 | Association between baseline HOMA-IR levels (per one unit increment) and changes in cognitive Z-scores.

Z-scores	Model 1		Model 2	
	β (95% CI)	P-value	β (95% CI)	P-value
GCF (n=4377)	-0.0140 (-0.0217, -0.0061)	<0.001*	-0.0094 (-0.0164, -0.0023)	0.009*
MMSE (n=5180)	-0.0040 (-0.0120, 0.0039)	0.322	-0.0006 (-0.0087, 0.0075)	0.884
CDT (n=5183)	-0.0006 (-0.0075, 0.0064)	0.868	-0.0006 (-0.0077, 0.0065)	0.862
DST-f (n=4560)	-0.0116 (-0.0195, -0.0037)	0.004*	-0.0091 (-0.0170, -0.0013)	0.023
DST-b (n=4559)	-0.0106 (-0.0184, -0.0028)	0.007*	-0.0082 (-0.0157, -0.0006)	0.035
VFT-a (n=5319)	-0.0072 (-0.0144, 0.0001)	0.051	-0.0050 (-0.0121, 0.0020)	0.163
VFT-p (n=5319)	-0.0065 (-0.0146, 0.0015)	0.111	-0.0042 (-0.0115, 0.0030)	0.249
TMT-A (n=5311)§	0.0070 (-0.0008, 0.0147)	0.077	0.0040 (-0.0037, 0.0117)	0.306
TMT-B (n=5301)§	0.0087 (0.0014, 0.0159)	0.019	0.0060 (-0.0007, 0.0127)	0.079

GCF, Global Cognitive Function; MMSE, Mini-Mental State Examination; CDT, Clock Drawing Test; DST-f, Digit Span Test forward section; DST-b, Digit Span Test backward section; VFT-a, Verbal Fluency Test animal category; VFT-p, Verbal Fluency Test letter "p"; TMT-A, Trail Making Test part A; TMT-B, Trail Making Test part B.

§ Inverse neuropsychological assessment score.

Participants with insulin treatment were excluded (n=320) from the analysis.

Model 1: adjusted for sex, age (in years), intervention group, and center size (<250; 250-300, 300-400; ≥400).

Model 2: further adjusted for baseline education level (primary school; secondary school; college), civil status (single, divorced or separated; married; widower), physical activity (MET min/week), smoking habits (smoker; former smoker; never smoker), alcohol intake (g/day, adding the quadratic term), 17-point Mediterranean diet score, BMI (kg/m²), hypertension (yes/no), hypercholesterolemia (yes/no), and depressive symptomatology (yes/no).

Beta coefficients were estimated using linear regression models with robust standard errors to account for intracluster correlations

**Significant association after Benjamini-Hochberg correction.*

TABLE 4 | Association between baseline HbA_{1c} levels (per one mmol/mol increment) and cognitive Z-scores changes.

Z-scores	Model 1		Model 2	
	β (95% CI)	P-value	β (95% CI)	P-value
GCF (n=4406)	-0.0085 (-0.0115, -0.0055)	<0.001*	-0.0056 (-0.0081, -0.0030)	<0.001*
MMSE (n=5162)	-0.0043 (-0.0071, -0.0015)	0.002*	-0.0029 (-0.0055, -0.0002)	0.035*
CDT (n=5166)	-0.0017 (-0.0043, 0.0009)	0.210	-0.0007 (-0.0032, 0.0019)	0.615
DST-f (n=4601)	-0.0030 (-0.0061, 0.0001)	0.058	-0.0015 (-0.0045, 0.0015)	0.330
DST-b (n=4600)	-0.0042 (-0.0072, -0.0013)	0.005*	-0.0023 (-0.0051, 0.0005)	0.114
VFT-a (n=5316)	-0.0071 (-0.0099, -0.0043)	<0.001*	-0.0051 (-0.0078, -0.0024)	<0.001*
VFT-p (n=5316)	-0.0087 (-0.0118, -0.0056)	<0.001*	-0.0063 (-0.0091, -0.0035)	<0.001*
TMT-A (n=5307)§	0.0074 (0.0045, 0.0103)	<0.001*	0.0053 (0.0025, 0.0081)	<0.001*
TMT-B (n=5296)§	0.0072 (0.0043, 0.0100)	<0.001*	0.0045 (0.0019, 0.0072)	0.001*

GCF, Global Cognitive Function; MMSE, Mini-Mental State Examination; CDT, Clock Drawing Test; DST-f, Digit Span Test forward section; DST-b, Digit Span Test backward section; VFT-a, Verbal Fluency Test animal category; VFT-p, Verbal Fluency Test letter "p"; TMT-A, Trail Making Test part A; TMT-B, Trail Making Test part B.

§ Inverse neuropsychological assessment score.

Missing data on HbA_{1c} (n=633).

Model 1: adjusted for sex, age (in years), intervention group, and center size (<250; 250-300, 300-400; ≥400).

Model 2: further adjusted for baseline education level (primary school; secondary school; college), civil status (single, divorced or separated; married; widower), physical activity (MET min/week), smoking habits (smoker; former smoker; never smoker), alcohol intake (g/day, adding the quadratic term), 17-point Mediterranean diet score, BMI (kg/m²), hypertension (yes/no), hypercholesterolemia (yes/no), and depressive symptomatology (yes/no).

Beta coefficients were estimated using linear regression models with robust standard errors to account for intracluster correlations.

**Significant association after Benjamini-Hochberg correction.*

DISCUSSION

To the best of our knowledge, this is the first prospective study investigating associations between glycemic status (diabetes status/control/treatment, and HOMA-IR and HbA_{1c} biomarkers) and cognitive function in a large cohort of older adults at risk high cardiovascular disease in a short period (2-year). In this community-based population, compared to participants without diabetes, those with diabetes showed a larger decline in several cognitive performance measurements. Additionally, longer duration of diabetes was associated with greater decreases in the scores of tests measuring processing speed and executive functions. Furthermore, poor diabetes control, the use of insulin treatment, and increases in HOMA-IR and HbA_{1c} levels were inversely associated with cognitive functioning.

Our results concur with those of meta-analyses of prospective studies, suggesting larger risk of cognitive decline in type 2 diabetes (6–8). The mechanisms explaining these associations remain largely unknown. Several risk factors for cognitive dysfunction in diabetes have been reported, such as hypertension or depression, but each of them appear to have weak isolated effects (28, 29). In order to control for these potential confounding factors, we have adjusted our statistical models for several recognized confounders.

Our findings are similar to those reported in other studies, suggesting a greater risk of cognitive decline in participants with type 2 diabetes, especially in relation to executive functions (5, 8, 30). Similarly, we found inverse associations in participants with diabetes and all the executive function-related tests, except in the case of the DST-b test, which measures working memory. Concerning memory function, we also assessed immediate verbal memory using the DST-f test, which was borderline inversely associated with the presence of diabetes. These results concur with those reported in a recent meta-analysis in which immediate (measured by the DST-f) and working memory (measured by the DST-b) were not associated in type 2 diabetes, while the other memory and executive function abilities assessed were reduced (8). Regarding visuospatial function, discrepancies in longitudinal studies have been reported in individuals with type 2 diabetes (31, 32). However, a small effect size in this function was reported in a meta-analysis conducted in 2014 (30). In our study, a non-significant inverse association between diabetes and the CDT test was observed, and longer follow-up of our population may be needed to observe a significant decline in this cognitive function.

Our results also showed that, compared to participants without diabetes, those with diabetes had a borderline increased risk of developing cognitive impairment as measured by the GCF score, even when the period of follow-up was only 2 years. Meta-analyses including prospective studies have shown an incidence of cognitive impairment in participants with type 2 diabetes (6, 33). However, the assessment of short-time periods were not commonly reported in regard to the association between type 2 diabetes and cognitive function, and it may be the reason for the discrepancies observed between the aforementioned meta-analyses and our study.

As far as we know, no longitudinal studies have been conducted assessing associations between diabetes status and cognitive decline, while also considering both the prediabetes status and the duration of diabetes. Longitudinal cohort studies have shown contradictory results regarding the association of prediabetes with cognition (5, 12, 31, 34), which can be explained by the different range of ages and sample sizes, the tests and cognitive domains assessed, and the length of follow-up. Concerning diabetes duration, our results are in line with other longitudinal studies in which higher rates of cognitive decline

were described in individuals with longer diabetes duration (5, 12).

The observed interaction of the GCF score with age in prediabetes has not been previously reported in the literature and cannot be explained by a specific mechanism. We cannot rule out that this interaction was a random finding and it is a result that requires further investigation.

Several mechanisms have been suggested to explain the association between diabetes status and control with changes in cognitive functioning. Among them, insulin resistance, hyperglycemic excursions and glycemic control have received much attention. Insulin resistance linked to low-grade inflammation is a factor contributing to the onset of diabetes, that appears to play a key role in the cognitive impairment associated with obesity and diabetes, given the role that insulin has in the brain promoting neuronal survival and synaptic plasticity and inhibiting apoptosis and neuroinflammation (35). In the case of peripheral insulin resistance and type 2 diabetes, a decrease in insulin permeation through the blood-brain barrier was observed, leading to a smaller amount of insulin reaching the brain, thus impairing neuronal activation and inducing changes in synaptic plasticity, neuronal apoptosis and neuroinflammation, all responsible for cognitive deterioration (35).

Longitudinal studies linking insulin resistance, as measured by HOMA-IR, and cognitive decline have shown discrepancies. In an older U.S. population with 8 years of follow-up, baseline HOMA-IR was not associated with changes in global cognitive function (36). However, in surviving patients with coronary heart disease, baseline HOMA-IR was associated with subsequent poorer cognitive performance on the composite cognitive score over 15 years (37). Our results were in line with those of the latter study, as we also observed an inverse association between baseline HOMA-IR and changes in cognitive performance using a global cognitive function score.

Additional mechanisms explaining the deleterious association of diabetes on cognitive functioning include hyperglycemic status and glycemic excursions. Increased HbA_{1c} levels or high levels of repeated glucose measurements over time have been linked to cognitive decline and an increased risk of dementia in people without diabetes (38). In our study, no associations between HbA_{1c} levels and changes in cognitive function were observed in participants without diabetes (data not shown). Nevertheless, when HbA_{1c} was measured as a continuous variable, we found negative associations between high baseline values in HbA_{1c} levels and all the cognitive tests measured, except in the case of the CDT and the DSTs, thus aligning with findings from recent studies (34, 36).

When diabetes is established, increased HbA_{1c} levels have been linked to diabetes-associated cognitive decline and dementia, but the strength of these relationships is weak (11). In our study, compared to participants with good diabetes control, those with poor control showed a larger 2-year decrease in cognitive performance measured by the VFT-p test, but this association was not observed in the case of the GCF score and other cognitive assessments. Unlike other typical diabetic end-organ complications, no clear evidence exists that the increased risk of cognitive impairment can be attributed solely to hyperglycemic excursions and glycemic control (11). For example, the ACCORD MIND trial (39), which compared intensive with standard treatment with the aim to lower HbA_{1c} in people with long-standing type 2 diabetes, found no association between the intervention and cognitive function.

Several other mechanisms have been implicated in diabetes-related cognitive decline and dementia. For example, type 2 diabetes has substantial adverse effects on blood vessels and the heart (40), leading to an increased risk of stroke and small cerebral vessel disease. Indeed, neuropathological studies also report an increased burden of cerebrovascular lesions, especially of lacunar type, in people with diabetes (41).

Observational studies have reported that some glucose-lowering medications may have a potential beneficial or deleterious relationship with cognition (6, 13). In our study, contrary to other results showing improved cognitive function (13), no associations between metformin and cognition were observed, as well this was not observed for IDDP-4 or sulfonylureas use. However, in line with findings of recent meta-analyses, insulin-treated participants showed larger cognitive decline than those not treated with insulin (6, 13). This could be explained by the fact that these individuals tend to have worse glycemic control and larger risk of hypoglycemia, a condition that has been linked to cognitive decline and dementia risk (42, 43).

It is worth mentioning a strength of the present study is the novelty of being one of the largest population-based studies longitudinally and concurrently exploring relationships between glycemic status (diabetes status, markers of glucose metabolism, and diabetes control and treatment) and cognitive function in an older individuals at high cardiovascular risk. Moreover, this study suggests that larger follow-up periods are not required to observe associations between glycemic status and cognitive function. Nevertheless, the present findings should be considered in the context of some limitations. Firstly, although we adjusted the models for many potential confounding factors, there may be residual confounding factors not assessed, such as genetic susceptibility (APOE genotype). Unfortunately, genetic data was not available in all the PREDIMED-Plus population. Secondly, the PREDIMED-Plus study did not contemplate the use of neuroimaging, such as magnetic resonance imaging (MRI). Finally, our study has been conducted in older Mediterranean individuals with overweight/obesity at high risk of cardiovascular disease, and therefore we cannot extrapolate our results to other populations.

In conclusion, several glycemic dysregulations, such as insulin resistance measured by HOMA-IR, diabetes status, longer duration of diabetes, poor glycemic control and higher levels of HbA_{1c}, and insulin treatment were associated with greater cognitive decline in older individuals with overweight/obesity at high cardiovascular disease risk in a short time period. We also reported that participants with type 2 diabetes had a borderline increased risk of developing cognitive impairment as measured by the GCF score, compared to those without diabetes.

Therefore, it is clinically relevant to assess novel effective strategies at the initial stages of diabetes-related alterations in order to reduce the impact of cognitive dysfunction when these glycemic dysregulations are more pronounced.

DATA AVAILABILITY STATEMENT

There are restrictions on the availability of data for the PREDIMED-Plus trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Requestors wishing to access the PREDIMED-Plus trial data used in this study can make a request to the PREDIMED-Plus trial Steering Committee chair: predimed_plus_scommitte@googlegroups.com. The request will then be passed to members of the PREDIMED-Plus Steering Committee for deliberation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CEI Provincial de Málaga-Servicio Andaluz de Salud O01_feb_PR2 - Predimedplus nodo 1 CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío-Servicio Andaluz de Salud PI13/00673 CEIC Universidad de Navarra 053/2013 CEI de las Illes Balears - Conselleria de Salut Direcció General de Salut Publica i Consum IB 2242/14 PI CEIC del Hospital Clínic de Barcelona HCB/2016/0287 CEIC Parc de Salut Mar y IDIAP Jordi Gol PI13/120 CEIC del Hospital Universitari Sant Joan de Reus y IDIAB Jordi Gol 13-07-25/7proj2 CEI de la Provincia de Granada- Servicio Andaluz de Salud MAB/BGP/pg CEIC de la Fundacion Jiménez Díaz EC 26-14/IIS-FJD CEIC Universidad de Navarra 053/2013 CEIC Euskadi PI2014044 CEIC Corporativo de Atención Primaria de la Comunitat Valenciana 2011-005398-22 CEI Humana de la Universidad de las Palmas de Gran Canaria CEIH-2013-07 CEIC del Hospital de Bellvitge PR240/13 CEI de Cordoba-Junta de Salud 3078 CEI de la Fundación IMDEA Alimentación PI-012 CEIC Hospital Clínico San Carlos de Madrid-Piloto-CEIC Servicio Madrileño de salud-General 30/15 CEI Provincial de Málaga-Servicio Andaluz de Salud CEI de las Illes Balears - Conselleria de Salut Direcció General de Salut Publica i Consum IB 2251/14 PI CEIC del Hospital Clínic de Barcelona HCB/2017/0351 CEIC del Hospital General Universitario de Alicante CEIC PI2017/02 CEIC de la Investigación Biomédica de Andalucía (CCEIBA) CEI de la Universidad de León ÉTICA-ULE-014-2015. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The principal PREDIMED-Plus investigators (MM, JS-S, DC, JM, AA, JW, JVio, DR, JL-M, RE, FT, JL, LS-M, AB-C, JT, VM-S, XP, PM-M, JVid, CV, LD, and ER) contributed to study concept and design and to data extraction from the participants. CG, NBT, NB, JJ, and JS-S performed the statistical analyses. CG and JS-S drafted the manuscript. All authors reviewed the manuscript for important intellectual content and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.754347/full#supplementary-material

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Comparative Cardiovascular Outcomes of SGLT2 Inhibitors in Type 2 Diabetes Mellitus: A Network Meta-Analysis of Randomized Controlled Trials

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Background: A network meta-analysis of randomized controlled trials (RCTs) was conducted to explore the cardiovascular outcomes of all the kind and dosages of sodium-glucose cotransport-2 (SGLT2) inhibitors in type 2 diabetes mellitus (T2DM) patients.

Method and Result: The Cochrane Library, PubMed, and Embase databases were systematically searched for studies to compare the therapeutic effects of different SGLT2 inhibitors in T2DM patients. The effect measurements estimate chosen were odds ratios (ORs) and their corresponding 95% confidence interval (CI). Forty-seven RCTs involving a total of 70574 participants were eligible for direct and indirect comparisons. In direct comparison, treatment with dapagliflozin 5mg showed significantly lower risk of all-cause mortality compared with treatment with dapagliflozin 2.5mg (OR 0.09, 95% CI 0.01-0.70). According to NMA, interestingly, empagliflozin 10mg/25mg, and canagliflozin 100mg was associated with significantly lower risks of all-cause mortality compared with placebo (OR of 0.70, 95% CI 0.58-0.85; 0.69, 95% CI 0.57-0.84; and 0.83, 95% CI 0.73-0.95, respectively). Compared with placebo, dapagliflozin 10mg, empagliflozin 10mg and 25mg displayed the lower risks for cardiovascular events (OR 0.78, 95% CI 0.44-1.00; OR 0.47, 95% CI 0.22-0.93; and 0.43, 95% CI 0.24-0.74, respectively) by direct comparison. Moreover, canagliflozin 100/300mg showed significantly higher risks of cardiovascular events compared with empagliflozin 10mg (OR of 4.83, 95% CI 1.14-20.46 and 5.31, 95% CI 1.26-22.34, respectively) and empagliflozin 25mg (4.23, 95% CI 1.13-15.83 and 4.65, 95% CI 1.25-17.27, respectively) according to NMA. There were non-significant differences among all interventions in volume depletion in traditional pairwise meta-analysis. While in NMA, canagliflozin 100/300mg were associated with significantly increased risks of volume depletion compared with placebo (OR of 1.47, 95% CI 1.08-1.99 and 2.19, 95% CI 1.66-2.90, respectively).

Conclusion: In the limitations of the NMA, this study showed that empagliflozin might be better than other SGLT2 inhibitors with low risks of all-cause mortality and cardiovascular events in patients with T2DM suggesting the need for *ad hoc* RCTs.

Keywords: type 2 diabetes mellitus, SGLT2 inhibitors, cardiovascular events, meta-analysis, empagliflozin

1 INTRODUCTION

Type 2 diabetes mellitus (T2DM) affects roughly 451 million adults in 2017 worldwide, these figures were expected to increase to almost 700 million by 2045 (1). T2DM is one of the most important risk factors of cardiovascular disease (CVD) (2, 3), the present of both T2DM and CVD is correlated with higher mortality rate despite advances in treatment (4). Enormous studies have shown that glucose lowing therapy failed to reduce the rates of death, although metabolism benefits were shown in these studies (5). In addition, some antihypergycemic agents increase the risk of all-cause mortality and major adverse cardiovascular events (MACEs) in T2DM patients with established CVD or CVD risk factors (6–8). Thus, novel strategies to improve prognosis and reduce mortality in T2DM patients are needed.

Sodium-glucose cotransport-2 (SGLT2) inhibitors, which reduce blood glucose levels in an insulin-independent manner in T2DM patients (9), are correlated with improvement of many metabolic and hemodynamic abnormalities (10). Moreover, it is important to note that SGLT2 inhibition is associated with reduced aortic stiffness (11) and cardiac structure and function improvement (12). Because of the beneficial cardiometabolic/hemodynamic profile induced by SGLT2 inhibitors treatment, clinical studies investigated the efficacy and safety of this class of drugs in T2DM patients (13–15). However, debate continues as to whether all SGLT2 inhibitors that exert similar cardioprotective effects.

Network meta-analysis (NMA) offers the potential to assess multiple therapeutic strategies simultaneously within a single framework and to rank treatments based on efficacy and safety (16). In the current paper, we conducted an NMA of randomized controlled trials (RCTs) for the first time to explore cardiovascular outcomes of different kind and dosages of SGLT2 inhibitors in T2DM patients.

2 METHODS

2.1 Data Sources and Search Strategy

We conducted a systematic search up to October 1, 2020, without any language restriction, using PubMed, Embase, the Cochrane Library, and Clinical trials. We searched studies with key words and Medical Subject Headings that covered "diabetic" or "diabetes" or "Type 2 Diabetes Mellitus" or "T2MD" and "sodium-glucose co-transporter 2 inhibitors", or "SGLT 2 inhibitors" or "SGLT2 inhibitors" or "sodium-glucose transporter inhibitors" or "canagliflozin" or "dapagliflozin" or "empagliflozin" or "ipragliflozin" or "remogliflozin" or "tofogliflozin" or "sergliflozin". We also reviewed the corresponding reference list of each retrieved article to identify any relevant studies that may be neglected. The meta-analysis was conducted and reported according to the Preferred Reporting Items of Systematic Reviews and Meta-Analyses (PRISMA) guidelines (17). The search strategies are provided in **Supplementary Table S1**.

2.2 Selection Criteria

We collected all RCTs to compare the therapeutic effects of different SGLT2 inhibitors in T2MD patients in this network meta-analysis. Inclusion criteria of the studies were as follows: (a) T2MD patients treatment with SGLT2 inhibitors, (b) study design was an RCT of the treatment group (SGLT2 inhibitors) and control group, (c) studies with outcomes of "all-cause death", "all-cause mortality", "myocardial infarction", "nonfatal myocardial infarction", "nonfatal stroke", "cardiovascular death", "hypertension", "hypotension", "volume depletion", "dehydration", or "hypovolemia". The detail was shown in the **Table 1**.

The criteria for exclusion were as follows: (a) studies such as systemic reviews, comments, case reports, conference abstracts, and editorials, (b) subjects with an eGFR level lower than 30 mL/min per 1.73m (2), and (c) articles that had no data on T2DM patients.

Included trials reported comparisons of 10 interventions (placebo, dapagliflozin 2.5mg, 5mg, 10mg; empagliflozin 10mg, 25 mg; and canagliflozin 100mg, 300 mg). NMA integrates data from direct comparisons of treatments within trials and from indirect comparisons of interventions assessed against a common comparator in separate trials to compare all investigated treatments.

2.3 Data Extraction and Quality Assessment

Two authors (J-Y and Y-PP) extracted data and accessed quality independently in an electronic database. The investigators cross-checked the data and reached a consensus on any discrepancies through discussion. Disagreements were resolved through discussions or referral to other authors (S-W and W-QH). Reference lists of identified trials and review articles were manually scanned to identify related research references at the same time as indicated in **Figure 1**.

The extracted data included the first author's name, year of publication, clinical characteristics, HbA1C% level, sample size, the number of males, doses of treatment, control, combined

Abbreviations: RCTs, Randomized controlled trials; SGLT2, Sodium-glucose cotransport-2; T2DM, Type 2 diabetes mellitus; ORs, Odds ratios; CVD, Cardiovascular disease; MACEs, Major adverse cardiovascular events; NMA, Network meta-analysis; SUCRA, The surface under the cumulative ranking area; CI, Confidence interval; CANVAS, Canagliflozin cardiovascular assessment study.

TABLE 1 | Inclusion and exclusion criteria.

Category	Inclusion criteria	Exclusion criteria
Patient population	T2DM	T1DM, any other disease and non-human-studiese, GFR < 30 mL/min per 1.73m^2
Intervention/ comparator	SGLT2 inhibitors and control group	Other oral hypoglycemic drugs vs SGLT2 inhibitors
Outcome	"All-cause mortality", "cardiovascular events" , "volume depletion"	No "all-cause mortality", "cardiovascular events" , and "volume depletion" outcomes reported
Study design	RCT	Not-RCTs: systemic reviews, comments, case reports, conference abstracts, and editorials
Language	English	Non-English language publications

T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; SGLT2, sodium-glucose cotransport-2; RCT, randomized controlled trials.

drugs, follow-up duration, the outcomes of all-cause mortality, cardiovascular events, volume depletion. Cardiovascular events included "myocardial infarction", "nonfatal myocardial infarction", "nonfatal stroke", "cardiovascular death", and "hypertension".

2.4 Risk of Bias Assessment

Two independent reviewers (J-Y and Y-PP) assessed the methodological quality of included trials using a slightly adapted version of the risk of bias approach by using Review Manager 5.3 (Cochrane Collaboration, Oxford, UK) risk of bias tool including four sections: selection, performance, detection, attrition, reporting, and other bias. The publication bias assessment was performed *via* Deek's funnel plot asymmetry.

2.5 Statistical Analysis

The data were abstracted and analyzed by STATA (version 14.0, Stata MP) and Review Manager (version 5.3, Cochrane Collaboration, Copenhagen, Denmark). The odds ratios (ORs) and their corresponding 95% confidence interval (CI) were used to compare different medications with respect to various clinical outcomes. For each analysis, we generated 50000 simulations for each of the 2 sets of different initial values and discarded the first 20000 simulations as the burn-in period. The stability of the results was obtained by sensitivity analyses by discarding each study sequentially. Convergence was checked using trace plots and the Brooks-Gelman-Rubin (18). To rank the treatments for an outcome, we used the surface under the cumulative ranking area (SUCRA) probabilities (19). Thus, a larger SUCRA score might indicate a higher probability of the end point event. We also used Loop-specific inconsistency (used in Stata and R software) to assess the inconsistency that is the actual difference between direct and indirect comparisons (20).

3 RESULTS

3.1 Description of Included Studies

We identified 3787 unique records from our searches. Forty-seven RCTs involving a total of 70574 participants were eligible for this NMA. The selection process details are shown in **Figure 1**. The trials included were issued up to September 2020. **Table 2** summarizes the essential baseline characteristics of these included studies (detail in **Supplementary Table S2**). Of 43 studies, all studies reported the end point event of all-cause death, 25 studies submitted data on cardiovascular events, and 30 studies provided data on volume depletion. The number of

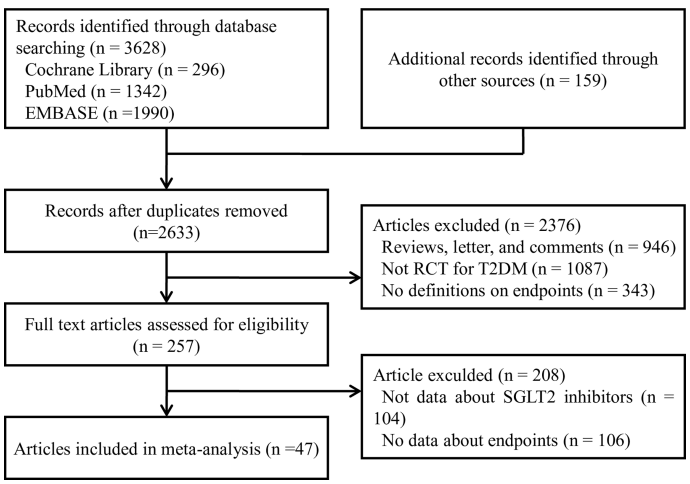

FIGURE 1 | PRISMA diagram of the study selection process for the meta-analysis.

TABLE 2 | Baseline characteristics of included RCTs.

Study	Disease	N	Male	Mean age	HbA1c	SGLT2 inhibitors	Control	Combined drugs	Follow-up	Outcomes
Bailey (21)	T2DM	546	292	–	8.06%	Dapagliflozin 2.5mg, 5mg, 10mg	Placebo	Metformin	102 weeks	Deaths, CV events, VD
Bailey (22)	T2DM	274	132	52.2	7.91%	Dapagliflozin 2.5mg, 5mg, 10mg	Placebo	Metformin	102 weeks	Deaths, VD
Barnett (23)	T2DM+CKD	738	430	63.9	8.00%	Empagliflozin 5mg, 10mg	Placebo	–	52 weeks	Deaths, CV events, VD
Bolinder (24)	T2DM	180	100	60.7	7.17%	Dapagliflozin 10mg	Placebo	Metformin	24 weeks	Deaths, VD
Bode (25)	T2DM	714	396	63.6	7.77%	Canagliflozin 100mg, 300mg	Placebo	–	104 weeks	Deaths, CV events, VD
Davies (26)	T2DM+CVD	2313	1146	55.9	8.00%	Canagliflozin 100mg, 300mg	Placebo	–	26 weeks	Deaths
DeFronzo (27)	T2DM	674	362	56.2	7.98%	Empagliflozin 10mg, 25mg	Metformin	Linagliptin	52 weeks	Deaths
Fioretto (28)	T2DM+CKD	321	182	65.8	8.18%	Dapagliflozin 10 mg	Placebo	–	24 weeks	Deaths, VD
Ferrannini (29)	T2DM	326	172	58.0	7.85%	Empagliflozin 5mg, 10mg, 25mg	Placebo	–	12 weeks	CV events, VD
Frías (30)	T2DM	685	328	54.3	9.30%	Dapagliflozin 10 mg	Placebo	Exenatide	28 weeks	Deaths, CV events, VD
Fulcher (31)	T2DM	411	273	62.5	8.09%	Canagliflozin 100mg, 300mg	Placebo	DPP-4i, GLP-1RA	18 weeks	Deaths, VD
Haring (32)	T2DM	666	390	57.1	8.1%	Empagliflozin 10 mg, 25 mg	Placebo	metformin + sulphonylurea	76 weeks	Deaths, VD
Henry (33)	T2DM	200	102	56.9	8.34%	Dapagliflozin 10mg	Placebo	insulin + metformin	4 weeks	Deaths
Inagaki (34)	T2DM	146	93	59.2	8.87%	Canagliflozin 100mg	Placebo	–	18 weeks	Deaths
Jabbour (35)	T2DM	685	328	54.3	9.31%	Dapagliflozin 10mg	Placebo	Exenatide	52 weeks	Deaths, CV events, VD
Kadowaki (36)	T2DM	547	410	57.5	7.95%	Empagliflozin 5 mg, 10 mg, 25 mg, 50 mg	Placebo	–	12 weeks	Deaths, VD
Kaku (37)	T2DM	261	155	58.8	7.50%	Dapagliflozin 5 mg, 10 mg	Placebo	–	24 weeks	CV events,
Kaku (38)	T2DM with CVD	1517	1118	61.0	8.07%	Empagliflozin 10 mg, 25 mg	Placebo	–	48 weeks	Deaths, CV events
Kohan (39)	T2DM and MRI	252	164	67.0	8.35%	Dapagliflozin 5mg, 10mg	Placebo	–	104 weeks	Deaths, CV events, VD
Kovacs (40)	T2DM	498	241	54.5	8.09%	Empagliflozin 10 mg, 25 mg	Placebo	–	76 weeks	Deaths, VD
Lavalle-González (41)	T2DM	1971	930	55.7	8.1%	Canagliflozin 100 mg, 300 mg	Placebo	Metformin, sulfonylurea	52 weeks	Deaths, VD
Leiter (42)	T2DM and CVD	1924	1288	63.8	8.05%	Dapagliflozin 10mg	Placebo	–	52 weeks	Deaths, CV events, VD
Mahaffey (43)	T2DM and CKD	4401	2907	63.0	8.3%	Canagliflozin 100mg	Placebo	–	126 weeks	Deaths, CV events
Merton (44)	T2DM	2313	1146	55.9	8.0%	Canagliflozin 100 mg, 300 mg	Placebo	–	26 weeks	Deaths, CV events, VD
Perkovic (45)	T2DM and CKD	4041	2547	63.0	8.3%	Canagliflozin 100 mg	Placebo	–	168 weeks	Deaths, CV events
Roden (46)	T2DM	899	551	55.0	7.88%	Empagliflozin 10 mg, 25 mg	Placebo	Sitagliptin	76 weeks	Deaths, CV events
Romera (47)	T2DM and obese	439	247	52.5	8.7%	Empagliflozin 10 mg, 25 mg	Placebo	–	24 weeks	Deaths, VD
Rosenstock (48)	T2DM	1186	569	54.9	8.8%	Canagliflozin 100mg, 300mg	Placebo	Metformin	26 weeks	Deaths, VD
Rosenstock (49)	T2DM	424	250	58	7.9%	Empagliflozin 5 mg, 10 mg, 25 mg, 50mg	Placebo	sitagliptin	12 weeks	CV events
Rosenstock (50)	T2DM	494	276	58.8	8.2%	Empagliflozin 10 mg, 25 mg	Placebo	–	78 weeks	Deaths, CV events
Schumm-Draeger (51)	T2DM	399	179	57.7	7.8%	Dapagliflozin 2.5mg, 5mg, 10mg	Placebo	Metformin	16 weeks	Deaths, VD

(Continued)

TABLE 2 | Continued

Study	Disease	N	Male	Mean age	HbA1c	SGLT2 inhibitors	Control	Combined drugs	Follow-up	Outcomes
Sinclair (52)	T2DM	4058	2364	58.2	8.1%	Canagliflozin 100mg, 300mg	Placebo	–	52 weeks	Deaths, VD
Søfteland (53)	T2DM	327	191	55.2	7.97%	Empagliflozin 10 mg, 25 mg	Placebo	Metformin+ Linagliptin	24 weeks	Deaths
Stenlo¨f (54)	T2DM	584	258	55.4	8.0%	Canagliflozin 100mg, 300mg	Placebo	–	52 weeks	Deaths, VD
Strojek (55)	T2DM	592	285	59.8	8.11%	Dapagliflozin 2.5mg, 5mg, 10mg	Placebo	Glimepiride	48 weeks	Deaths, CV events, VD
Tikkanen (56)	T2DM+ hypertension	823	495	60.2	7.90%	Empagliflozin 10 mg, 25 mg	Placebo	–	12 weeks	Deaths VD
Tinahones (57)	T2DM	467	254	56.6	7.96%	Empagliflozin 10 mg, 25 mg	Placebo	Metformin+ Linagliptin	24 weeks	Deaths
Weber 2016 (58)	T2DM+ hypertension	449	247	56.6	8.05%	Dapagliflozin 10mg	Placebo	–	12 weeks	VD
Wilding (59)	T2DM	469	239	56.8	8.10%	Canagliflozin 100mg, 300mg	Placebo	–	52 weeks	Deaths VD
Wiviott (15)	T2DM	17160	10738	63.7	8.30%	Dapagliflozin 10mg	Placebo	–	201 weeks	Deaths, CV events, VD
Yale (60)	T2DM+CKD	269	163	68.5	8.0%	Canagliflozin 100mg, 300mg	Placebo	–	52 weeks	Deaths, VD
Yale (61)	T2DM	146	79	65.1	8.2%	Canagliflozin 100mg, 300mg	Placebo	–	52 weeks	Deaths, VD
Yang (62)	T2DM	1453	801	54.7	8.12%	Dapagliflozin 5mg, 10mg	Placebo	–	24 weeks	Deaths, VD
Yang (63)	T2DM	272	110	57.5	8.56%	Dapagliflozin 10mg	Placebo	Insulin	24 weeks	Deaths, VD
Zinman (13)	T2DM	7020	5016	63.1	8.7%	Empagliflozin 10mg, 25mg	Placebo	–	136 weeks	Deaths, CV events, VD
Mordi (64)	T2DM+HF	23	17	69.8	7.9%	Empagliflozin 25mg	Placebo	furosemide	8 weeks	CV events VD
Eickhoff (65)	T2DM	36	89	64.0	7.5%	Dapagliflozin 10 mg	Placebo	–	24 weeks	Deaths, CV events, VD

RCTs, randomized controlled trial; N, mumble; HbA1c, hemoglobin A1c; SGLT2, Sodium glucose co-transporter 2; T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; CVD, cardiovascular disease; CV, cardiovascular; VD, volume depletion.

patients included in every study ranged from 35 to 17160, and the follow-up for patients ranged from 4 to 201 weeks. The risk of bias in studies contributing to the primary outcomes was generally low (**Supplementary Table S3**).

A network plot of treatment comparisons for NMA is shown in **Figure 2**. There are 8 interventions for all-cause death, cardiovascular events and volume depletion. The size of the nodes (blue circles) corresponds to the sample size of the interventions. The comparisons are linked by a straight line, of which the thickness corresponds to the number of trials that assessed the comparison. As shown in the network plot, the number of interventions varied in different subjects.

3.2 The Outcome

3.2.1 All-Cause Mortality

We performed a series of traditional pairwise meta-analysis and NMA to summarize the results of trials directly and indirectly comparing the same classes of SGLT2 inhibitors. In direct comparison, there were non-significant differences among all interventions in all-cause mortality, expected Dapagliflozin 5mg vs 2.5mg (OR 0.09, 95% CI 0.01-0.70). According to NMA, interestingly, empagliflozin 10mg/25mg, and canagliflozin 100mg was associated with significantly lower risks of all-cause mortality compared with placebo (OR of 0.70, 95% CI 0.58-0.85; 0.69, 95% CI 0.57-0.84; and 0.83, 95% CI 0.73-0.95, respectively). Moreover, empagliflozin 10mg/25mg was leaded to significantly lower risks of all-cause mortality compared with dapagliflozin 10mg (OR of 0.75, 95% CI 0.60-0.95; 0.74, 95% CI 0.59-0.93, respectively), as depicted in **Table 3A**.

The comparative effects of different class and doses of SGLT2 inhibitors in reducing mortality by SUCRA probabilities and incidence rate of each intervention was shown in **Table 4**. The NMA suggested that higher dosage of empagliflozin (25 mg once daily) was associated with the lowest probability of achieving at all-cause mortality (SUCRA, 23.9%), followed by canagliflozin 300 mg (SUCRA, 24.4%), and empagliflozin 10 mg (SUCRA, 26.3%). However, dapagliflozin 2.5mg was associated with the highest probability of all-cause death (SUCRA, 89.7%).

3.2.2 Cardiovascular Events

In traditional pairwise meta-analysis, compared with placebo, dapagliflozin 10mg, empagliflozin 10mg and 25mg displayed the lowest risks for cardiovascular events (OR 0.78, 95% CI 0.44-1.00; OR 0.47, 95% CI 0.22-0.93; and 0.43, 95% CI 0.24-0.74, respectively). According to NMA, canagliflozin 100/300mg was associated with significantly higher risks of cardiovascular events

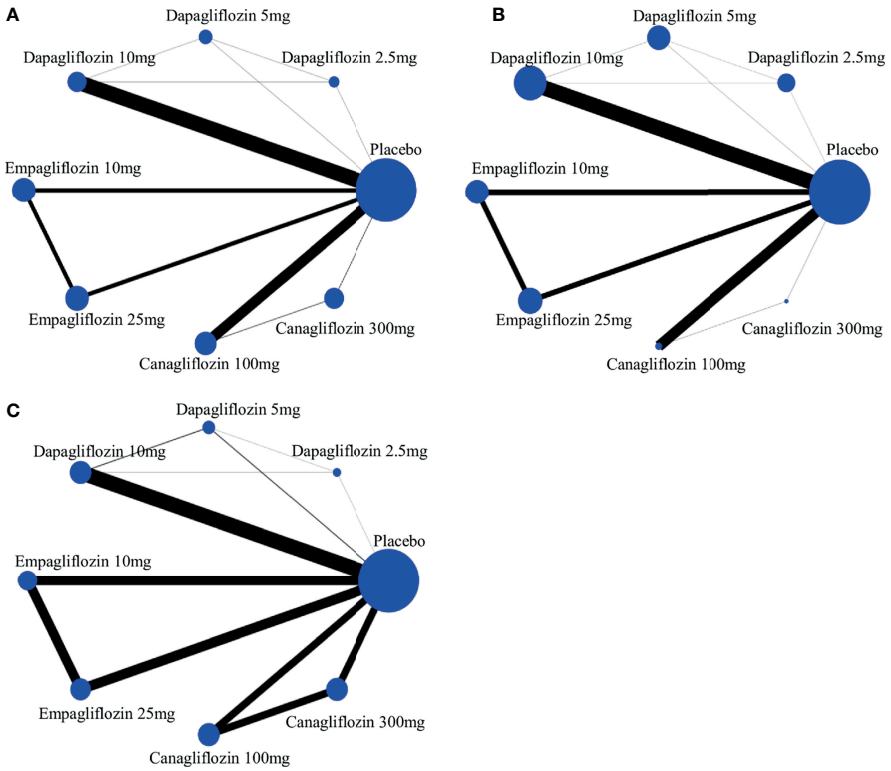

FIGURE 2 | The evidence network of studies reporting **(A)** all-cause death, **(B)** cardiovascular event and **(C)** volume depletion. The size of the nodes (blue circles) corresponds to the overall sample size of the corresponding intervention. Each line represents the direct comparison between the two interventions, and its thickness corresponds to the number of trials that assessed the comparison.

compared with empagliflozin 10mg (OR of 4.83, 95% CI 1.14-20.46 and 5.31, 95% CI 1.26-22.34, respectively) and empagliflozin 25mg (4.23, 95% CI 1.13-15.83 and 4.65, 95% CI 1.25-17.27, respectively) (**Table 3B**). Empagliflozin 25mg and dapagliflozin 5mg ranked the best and second (SUCRA of 21.8% and 23.9%, respectively), followed by empagliflozin 10mg (SUCRA of 30.9%). In addition, canagliflozin 300 mg was ranked the least effective treatment in reducing cardiovascular events (**Table 4**).

3.2.3 Volume Depletion

There were non-significant differences among all interventions in volume depletion in traditional pairwise meta-analysis. While in

TABLE 3A | Summary of results from network meta-analysis and traditional pairwise meta-analysis on all-cause death.

OR 95% CI	Placebo	Dapagliflozin 2.5mg	Dapagliflozin 5mg	Dapagliflozin 10mg	Empagliflozin 10mg	Empagliflozin 25mg	Canagliflozin 100mg	Canagliflozin 300mg
Placebo		3.10 (0.26, 47.00)	0.27 (0.04, 2.70)	0.94 (0.55, 1.70)	0.60 (0.22, 1.70)	0.70 (0.32, 1.70)	0.82 (0.53, 1.20)	0.56 (0.12, 2.20)
Dapagliflozin 2.5mg	1.67 (0.56, 5.01)		**0.09 (0.01, 0.70)**	0.41 (0.01, 4.30)				
Dapagliflozin 5mg	0.66 (0.24, 1.81)	0.40 (0.10, 1.50)		1.50 (0.13, 17.00)				
Dapagliflozin 10mg	0.93 (0.83, 1.04)	0.56 (0.19,1.67)	1.40 (0.51, 3.85)					
Empagliflozin 10mg	**0.70 (0.58, 0.85)**	0.42 (0.14, 1.28)	1.06 (0.38, 2.96)	**0.75 (0.60, 0.95)**				
Empagliflozin 25mg	**0.69 (0.57, 0.84)**	0.41 (0.14, 1.26)	1.04 (0.37, 2.91)	**0.74 (0.59, 0.93)**	0.98 (0.79, 1.22)			
Canagliflozin 100mg	**0.83 (0.73, 0.95)**	0.50 (0.16,1.50)	1.25 (0.45, 3.46)	0.89 (0.75, 1.06)	1.18 (0.93, 1.50)	1.20 (0.95, 1.53)		
Canagliflozin 300mg	0.66 (0.40,1.08)	0.39 (0.12,1.31)	0.99 (0.32, 3.05)	0.71 (0.43, 1.18)	0.94 (0.55, 1.60)	0.95 (0.56, 1.63)	0.79 (0.48, 1.31)	

TABLE 3B | Summary of results from network meta-analysis and traditional pairwise meta-analysis on cardiovascular events.

OR 95% CI	Placebo	Dapagliflozin 2.5mg	Dapagliflozin 5mg	Dapagliflozin 10mg	Empagliflozin 10mg	Empagliflozin 25mg	Canagliflozin 100mg	Canagliflozin 300mg
Placebo		0.67 (0.23, 2.00)	0.51 (0.19, 1.40)	0.78 (0.44, 1.00)	0.47 (0.22,0.93)	0.43 (0.24, 0.74)	0.75 (0.53, 1.40)	4.0 (0.77,21.00)
Dapagliflozin 2.5mg	0.30 (0.03, 2.57)		0.55 (0.10, 3.10)	0.64 (0.12, 3.30)				
Dapagliflozin 5mg	1.19 (0.30, 4.76)	4.04 (0.42, 39.15)		0.87 (0.22, 3.50)				
Dapagliflozin 10mg	1.14 (0.42, 3.09)	3.86 (0.44, 33.53)	0.96 (0.24, 3.81)					
Empagliflozin 10mg	0.66 (0.31, 1.40)	2.24 (0.23, 22.01)	0.55 (0.12, 2.61)	0.58 (0.17, 2.01)				
Empagliflozin 25mg	0.76 (0.47, 1.21)	2.56 (0.28, 23.37)	0.63 (0.15, 2.71)	0.66 (0.22, 2.00)	1.14 (0.60, 2.19)			
Canagliflozin 100mg	3.20 (0.93, 10.98)	10.81 (0.90, 130.36)	2.68 (0.42, 17.10)	2.80 (0.57, 13.70)	**4.83 (1.14, 20.46)**	**4.23 (1.13, 15.83)**		
Canagliflozin 300mg	3.51 (1.03, 11.97)	11.89 (0.99, 142.75)	2.94 (0.46, 18.70)	3.08 (0.63, 14.97)	**5.31 (1.26, 22.34)**	4.65 (1.25, 17.27)	1.10 (0.44, 2.75)	

NMA, canagliflozin 100/300mg were associated with significantly increased risks of volume depletion compared with placebo (OR of 1.47, 95% CI 1.08-1.99 and 2.19, 95% CI 1.66-2.90, respectively). The incidence of volume depletion induced by canagliflozin 300mg was significantly higher than that induced by dapagliflozin 5/10mg, empagliflozin 10/25mg and canagliflozin 100mg. These NMA results are illustrated in **Table 3C**.

Canagliflozin 300mg had the highest probabilities of being ranked first with respect to volume depletion (SUCRA 95.5%), whereas canagliflozin 100mg had the second highest probability (SUCRA 75.1%). Both dapagliflozin 5mg and empagliflozin 10mg shown smallest cumulative probabilities for volume depletion, with all values lower 30% (SUCRA 29.9% and 30.5%, respectively), as depicted in **Table 4**.

3.3 Exploration of Inconsistency, Sensitivity Analysis, and Publication Bias

Treatment from network meta-analysis evidence in general did not demonstrate evidence of statistical inconsistency (**Supplementary Figure S1**). As shown in **Supplementary Figure S1**, the

TABLE 3C | Summary of results from network meta-analysis and traditional pairwise meta-analysis on volume depletion.

OR 95% CI	Placebo	Dapagliflozin 2.5mg	Dapagliflozin 5mg	Dapagliflozin 10mg	Empagliflozin 10mg	Empagliflozin 25mg	Canagliflozin 100mg	Canagliflozin 300mg
Placebo		0.027 (0.01, 67.00	1.60 (0.39, 6.80)	1.20 (0.84, 2.30)	1.10 (0.45, 2.60)	1.30 (0.62, 2.70)	1.70 (0.78, 3.90)	2.50 (1.30, 4.90)
Dapagliflozin 2.5mg	1.36 (0.24, 7.55)		0.01 (0.00, 14.00	1.20 (0.03, 42.00)				
Dapagliflozin 5mg	0.94 (0.44,2.00)	0.69 (0.12, 4.18)		1.20 (0.37, 3.70)				
Dapagliflozin 10mg	1.06 (0.89,1.27)	0.78 (0.14, 4.34)	1.12 (0.53, 2.36)					
Empagliflozin 10mg	1.02 (0.81, 1.28)	0.75 (0.13, 4.26)	1.08 (0.49, 2.38)	0.96 (0.72, 1.29)		1.10 (0.48, 2.40)		
Empagliflozin 25mg	1.13 (0.90, 1.41)	0.83 (0.15, 4.71)	1.20 (0.55, 2.62)	1.07 (0.80, 1.42)	1.10 (0.89, 1.38)			
Canagliflozin 100mg	**1.47 (1.08, 1.99)**	1.08 (0.19, 6.19)	1.56 (0.69, 3.50)	1.39 (0.97, 1.97)	1.44 (0.98, 2.10)	1.30 (0.89, 1.89)		
Canagliflozin 300mg	**2.19 (1.66, 2.90)**	1.62 (0.28, 9.22)	**2.33 (1.04, 5.19)**	**2.07 (1.49, 2.89)**	**2.15 (1.50, 3.08)**	**1.94 (1.36, 2.78)**	**1.50 (1.16, 1.92)**	

Summary of results from network meta-analysis and traditional pairwise meta-analysis on all-cause death.On the lower triangle, the column-defining treatment is compared to the row-defining treatment, and odds ratios (OR) < 1 favor the column-defining treatment. On the upper triangle, the row-defining treatment is compared to the column-defining treatment, and OR < 1 favor the row-defining treatment. To obtain ORs for comparisons in the opposite direction, reciprocals should be taken. To obtain ORs for comparisons in the opposite direction, reciprocals should be taken. Significant results are in bold.

TABLE 4 | Incidence rate and SUCRA for the efficacy of treatments to induce end points in the T2DM patients.

End event / Treatment	All-cause death				Cardiovascular events				Volume depletion			
	Event (n)	Total (n)	Incidence rate (%)	SUCRA (%)	Event (n)	Total (n)	Incidence rate (%)	SUCRA (%)	Event (n)	Total (n)	Incidence rate (%)	SUCRA (%)
Placebo	1027	20916	4.91	83.0	1814	16162	11.22	80.0	284	15524	1.83	25.0
Dapagliflozin 2.5mg	4	456	0.87	89.7	12	456	2.63	46.9	1	456	0.22	55.0
Dapagliflozin 5mg	3	1020	0.29	33.7	13	615	2.11	23.9	11	1020	1.08	29.9
Dapagliflozin 10mg	539	10484	5.14	68.4	27	1822	1.48	45.7	245	11207	2.19	38.6
Empagliflozin 10mg	17	2534	0.67	26.3	24	902	2.66	30.9	29	1287	2.25	30.5
Empagliflozin 25mg	21	2692	0.78	23.9	35	1108	3.16	21.8	41	1488	2.76	50.5
Canagliflozin 100mg	347	8128	4.27	50.6	559	4645	12.03	63.1	41	2814	1.46	75.1
Canagliflozin 300mg	5	3648	0.14	24.4	14	236	5.93	87.7	62	2814	2.20	95.5

The graphs display the distribution of probabilities of treatment ranked from best to worst for each outcome. The ranking indicates the probability that the drug class is first "best," second "best", etc. For example, the ranking suggests that dapagliflozin 2.5mg posed the highest risk for incurring all-cause death (worst), while empagliflozin 25mg incurred the lowest probability of all-cause death (best).

inconsistency plot consists of two triangular loops and four quadrangular loops. The IF values of all loops were truncated at zero, and P value > 0.05 verified their consistency statistically.

A sensitivity analysis was conducted to examine the impact of studies according to the treatment effects on the outcomes of all-cause death, cardiovascular events, and volume depletion. We performed an analysis in T2DM used Bayesian, and there was no significant difference in the different methods (**Supplementary Table S3**). No significant publication bias was detected in the funnel plot (**Supplementary Figure S2**).

4 DISCUSSION

In recent years, clinical trials revealed the cardioprotective effects of SGLT2 inhibitors in T2DM patients (13–15). Moreover, SGLT-2 inhibitors were most likely to rank best for all-cause/cardiac mortality and the outcomes of heart failure and myocardial infarction compared with dipeptidyl peptidase 4 inhibitors and glucagon-like peptide 1 agonists (66). However, no studies have directly or simultaneously compared the efficacy and safety of all the kind and dosages of SGLT2 inhibitors. In the present meta-analysis, we grouped all available SGLT2 inhibitors together, including dapagliflozin 2.5mg/5mg/10mg, empagliflozin 10mg/25mg, and canagliflozin 100mg/300mg. Since only few studies allowed direct comparisons, indirect comparisons were further conducted to identified the effects of different SGLT2 inhibitors on all-cause mortality, cardiovascular events and volume depletion, respectively. We demonstrated that dose of dapagliflozin 5mg was associated with all-cause mortality reduction, rather than other SGLT2 inhibitors by direct comparison. According to NMA, empagliflozin 10mg/25mg, and canagliflozin 100mg was associated with significantly lower risks of all-cause mortality compared with placebo. Moreover, empagliflozin 10mg/25mg was leaded to significantly lower risks of all-cause mortality compared with dapagliflozin 10mg. Dapagliflozin 10mg, empagliflozin 10mg and 25mg displayed the lower risks for cardiovascular events compared with placebo. In addition, it seems likely that canagliflozin 100/300mg showed significantly higher risks of cardiovascular events compared with empagliflozin 10mg/25mg according to NMA. Finally, we suggested that treatment with canagliflozin 100/300mg were associated with significantly increased risks of volume depletion compared with placebo by NMA.

In recent years, numerous studies investigated the role of SGLT2 inhibitor on mortality reduction. For instance, In EMPA-REG OUTCOME trial, Zinman and coworkers revealed that the rate of all-cause mortality was significantly lower in patients who received empagliflozin 10mg/25mg than controlled group (13). More recently, however, CANVAS (Canagliflozin Cardiovascular Assessment Study) Program which is an integrated analysis of CANVAS and CANVAS-R (Canagliflozin Cardiovascular Assessment Study-Renal) (14), and DECLARE–TIMI 58 trial (15) failed to show the positive effect of canagliflozin 100mg/300 mg and dapagliflozin 10mg on reducing the rate of all-cause death, respectively. The major limitations of CANVAS Program are lack of events number and discontinuation of randomized therapy, which may result in underestimation of benefits effect of canagliflozin on all-cause mortality reduction. The present meta-analysis grouped all available SGLT2 inhibitors for direct and indirect comparison. Since only few studies allowed direct comparisons between SGLT2 inhibitors, NMA were conducted and found that dapagliflozin 5mg was associated with lower risk of all-cause mortality. Moreover, the positive role of empagliflozin 10mg/25mg and canagliflozin 100mg on reducing all-cause mortality was demonstrated by NMA (as shown in **Table 3**). Although the potential factors that responsible for mortality reduction of canagliflozin are need to

be further investigated, it may be relevant to identify that canagliflozin has less SGLT2 selectively and may induce more glucosuria (67), suggesting great volume depletion by canagliflozin and rise in hematocrit and hemoconcentration could increase blood viscosity. On the other hand, it has been reported that canagliflozin impacts activation of AMP-kinase in mitochondrial function, which is helpful to keep the balance of cellular energy metabolism (68). As an alternative explanation, we selected 43 studies investigating the efficacy of SGLT2 inhibitors on mortality rate in 66819 T2DM patients and included a total of 2470 deaths, data reporting may have differed between these studies. These finding highlighted that more clinical trials are urgently needed to explore the specific cardioprotective role of SGLT2 inhibitors in T2DM patients, even though they have similar effects.

For patients with T2DM, an important goal of currently treatment is reducing the rate of cardiovascular events. In recent years, clinical trials investigated the role of SGLT2 inhibitors on MACE and suggested that the particular clinical benefits of SGLT2 inhibition may rely on the baseline characteristics of patient population. For instance, the positive effect of empagliflozin on cardiovascular mortality and MACEs rate was revealed in EMPA-REG OUTCOME trial which included T2DM patients with established CVD (13). However, dapagliflozin treatment resulted in lower rate of cardiovascular death rather than total MACEs in DECLARE-TIMI 58 trial which included 41.64% T2DM with CVD patients (15). Moreover, canagliflozin significantly reduce the rate of MACEs in CANVAS Program which included 66% T2DM with CVD patients (14). Recently, a meta-analysis included these three clinical trials and their secondary analyses revealed that SGLT2 inhibitors reduce MACEs by 11% in patients with established CVD. Moreover, they found that the effect of empagliflozin on cardiovascular death was more pronounced than that of canagliflozin or dapagliflozin (69). In currently paper, we suggested that dapagliflozin 10mg, empagliflozin 10mg/25mg and canagliflozin 100mg could exert similar role in reducing the risk of cardiovascular events in T2DM patients, highlighting that SGLT2 inhibitors may be considered to manage T2DM in patients not only with established CVD but also in patients without CVD but at elevated risk. Additionally, we found that empagliflozin 10mg/25mg rank the best and second choice in regarding to cardiovascular events risk reduction by SUCRA probabilities.

It has been reported that SGLT2 inhibition decrease sodium reabsorption and increases urinary sodium excretion (70). In addition to exert multiple metabolic effects including reduce HbA1c, change caloric balance and weight loss, the glucosuria/natriuretic effects of SGLT2 inhibitors are also account for, at least in part, the positive role of it on reducing the rate of hospitalization for heart failure. However, it is important to recognize that these effects may have protective and injurious potential. For instance, natriuresis may also result in increasing risk of volume depletion, such as hypotension and syncope, and promoting neurohormonal activation and tissue ischemia in the periphery (71). According to our analysis, we found that higher dosage of canagliflozin (300 mg once daily) was associated with increased the rate of volume depletion among all SGLT2 inhibitors included in this study, suggesting that the potential of higher dosage of canagliflozin induced volume depletion may account for the neutralize results in all-cause mortality.

We acknowledge several limitations of our study. First, we used aggregated study-level data rather than individual participant data, and the different studies have been conducted in different population. Second, we could not include data from several trials such as DECLARE-TIMI 58, CANVAS Program, EMPA-REG OUTCOME due to data was limited in these studies. Third, we did not perform a subgroup analysis of cardiovascular mortality caused by SGLT2 inhibitors in T2DM patients. Then, although the heterogeneity in the network analysis was low, it is likely that the low power to detect heterogeneity is due to limited data for some dosage SGLT2 inhibitors. Finally, the NMA results do not meet the assumptions of homogeneity of direct evidence and/or transitivity, therefore, several larger multi-center RCTs which investigated effects between SGLT2 inhibitors including patients with similar clinical characteristics need to be implemented to achieve more robust results.

5 CONCLUSION

In the limitations of the NMA, this study showed that empagliflozin 10mg/25mg once daily might be better than other SGLT2 inhibitors with low risks of all-cause mortality and cardiovascular events in patients with T2DM suggesting the need for *ad hoc* RCTs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YJ, PY, and LF performed the meta-analysis. LS was responsible for the statistical analysis. WS provided editing assistance. QW prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.802992/full#supplementary-material

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Hyperglycemia and Physical Impairment in Frail Hypertensive Older Adults

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Background: Frailty is a multidimensional condition typical of elders. Frail older adults have a high risk of functional decline, hospitalization, and mortality. Hypertension is one of the most common comorbidities in elders. Hyperglycemia (HG) is frequently observed in frail older adults, and represents an independent predictor of worst outcomes, with or without diabetes mellitus (DM). We aimed at investigating the impact of HG on physical impairment in frailty.

Methods: We studied consecutive older adults with frailty and hypertension at the ASL (local health unit of the Italian Ministry of Health) of Avellino, Italy, from March 2021 to September 2021. Exclusion criteria were: age <65 years, no frailty, no hypertension, left ventricular ejection fraction <25%, previous myocardial infarction, previous primary percutaneous coronary intervention and/or coronary artery bypass grafting. Blood glucose, Hb1Ac, and creatinine were measured in all patients. Physical frailty was assessed applying the Fried Criteria; we performed a 5-meter gait speed (5mGS) test in all patients.

Results: 149 frail hypertensive older adults were enrolled in the study, of which 82 had normoglycemia (NG), and 67 had HG. We observed a significantly slower 5mGS in the HG group compared to the NG group (0.52 ± 0.1 *vs*. 0.69 ± 0.06; p<0.001). Moreover, we found a strong and significant correlation between 5mGS and glycemia (r: 0.833; p<0.001). A multivariable linear regression analysis using 5mGS as a dependent variable revealed a significant independent association with glycemia (p<0.001) after adjusting for likely confounders.

Conclusions: HG drives physical impairment in frail hypertensive older adults independently of DM.

Keywords: aging, blood glucose, cognitive impairment, COPD, diabetes, elderly, gait speed, MoCA score

BACKGROUND

Frailty is a multidimensional condition typical of elders that determines physical decline. Frail older adults have a high risk of functional decline, hospitalization, and mortality (1–4). Hence, a careful geriatric evaluation is one of the best strategies to obtain an early diagnosis of physical impairment, and managing comorbidities and complications is fundamental to counteract it (5–11). Hypertension is one of the most common comorbidities in elders, affecting endothelial function, leading to oxidative stress, inflammation, and atherosclerosis (12–19).

Hyperglycemia (HG) is frequently observed in frail hypertensive older adults, and we and others have shown that it represents an independent predictor of worst outcomes, even if diabetes mellitus (DM) is not present (20–23). Indeed, HG drives inflammation and oxidative stress, leading to endothelial dysfunction, with a negative impact on frail patients (7, 24–28).

In this context, reaching and maintaining an optimal glycemic control may be crucial to reduce the incidence of functional decline and avoid complications (11, 29–32). On these grounds, we investigated the impact of HG on physical impairment in frail hypertensive older adults.

METHODS

We studied consecutive older adults with frailty and hypertension at the ASL (local health unit of the Italian Ministry of Health) of Avellino and Caserta, Italy, from March 2021 to September 2021.

Inclusion criteria were: Age ≥65 years; frailty; primary hypertension. Exclusion criteria were: Age <65 years; absence of frailty; secondary hypertension or absence of hypertension; previous myocardial infarction, left ventricular ejection fraction <25%, and previous cardiac revascularization.

HG was defined as blood glucose level ≥140 mg/dL according to previous investigations that evaluated HG in complex patients, both diabetic and non-diabetic (33–37), and following ADA recommendations, which refer to this value for hospitalized patients (38) and/or subjects with impaired glucose tolerance (39).

Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg on repeated measurements, or as a previously diagnosed hypertension (40). Blood samples to measure glycemia, HbA1c, hyperlipidemia, and creatinine were taken from all patients. The study was approved by the Campania Nord Ethical Committee. A written informed consent was signed by all patients.

Assessment of Physical Frailty

A diagnosis of frailty status was made according to the Fried Criteria, as we previously reported (19, 41):

- Weight loss (unintentional loss ≥4.5 kg in the past year);
- Weakness (handgrip strength in the lowest 20% quintile at baseline, adjusted for sex and body mass index);
- Exhaustion (poor endurance and energy, self-reported);

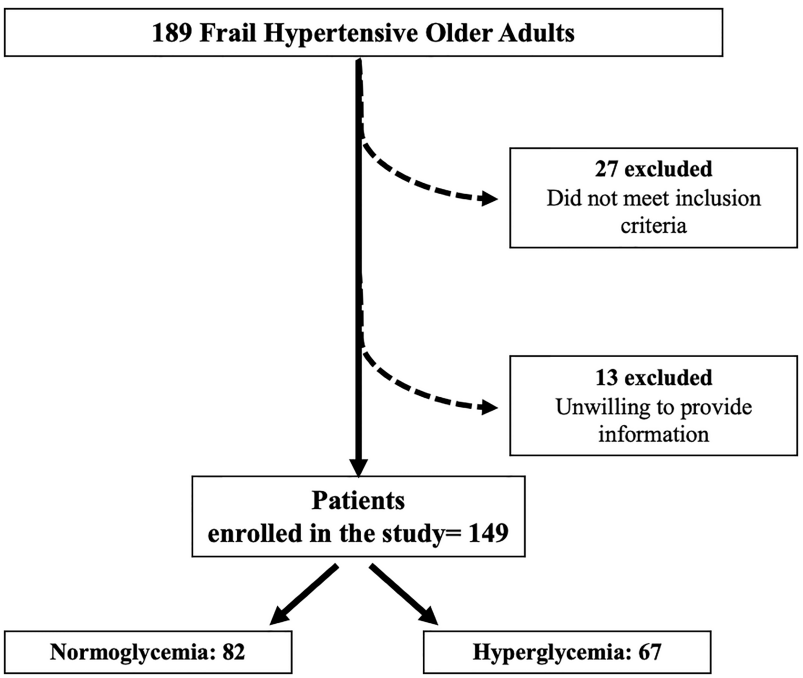

FIGURE 1 | Study flow diagram.

TABLE 1 | Clinical characteristics of the patients.

N	NG 82	HG 67
Sex (M/F)	36/46	29/38
Mean age (years)	84.62 ± 6.1	84.48 ± 6.3
BMI (kg/m^2)	27.7 ± 1.6	27.9 ± 1.6
SBP (mmHg)	118.7 ± 7.4	119.0 ± 7.8
DBP (mmHg)	79.6 ± 6.7	79.4 ± 6.3
Heart rate (bpm)	87.3 ± 9.8	87.1 ± 8.8
5mGS (m/s)	0.69 ± 0.06	0.52 ± 0.1*
Comorbidities, n (%)		
Diabetes	32 (39.0)	54 (80.6)*
COPD	38 (46.3)	33 (49.3)
CKD	39 (47.6)	35 (52.2)
CVD	44 (53.7)	34 (50.7)
Hyperlipidemia	43 (52.4)	37 (55.2)
Laboratory analyses		
Plasma glucose (mg/dl)	100.1 ± 19.6	231.5 ± 71.4*
HbA1c, mmol/mol (%)	57 ± 5.5 (7.4 ± 0.5)	58 ± 5.5 (7.5 ± 0.5)
Serum creatinine (mg/dl)	1.1 ± 0.5	1.2 ± 0.5
Global Cognitive Evaluation		
MoCA	19.5 ± 3.6	19.1 ± 3.9

*Data are means ± SD or n (%). 5mGS, 5 m gait speed; BMI, body mass index; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HF, heart failure; HG, hyperglycemic; MoCA, Montreal Cognitive Assessment; NG, normoglycemic; SBP, systolic blood pressure. *p < 0.001.*

- Slowness (walking speed under the lowest quintile adjusted for sex and height);
- Low physical activity level (lowest quintile of kilocalories of physical activity during the past week).

Frailty was diagnosed with at least 3 criteria out of 5.

A 5-meter gait speed (5mGS) test was performed in all patients, as we previously described (42). 5mGS was advocated as a reliable measure of physical capacity in frail patients with cardiovascular diseases (43). Indeed, this test evaluates lower extremity muscle function, neurological and cardiopulmonary capacity (44, 45).

Statistical Analysis

Data are presented as mean ± SD or percentage. We developed a dispersion model using Pearson analysis to assess the correlation between glycemia and 5mGS. To explore the impact of comorbidities, we carried out a multivariable linear regression model with a 5mGS test as a dependent variable. All calculations were performed using the software Statistical Product and Service Solutions (SPSS) version 26.

RESULTS

We screened 189 frail hypertensive patients. Since 13 patients did not give their consent and 27 subjects did not meet inclusion criteria, 149 patients were enrolled in the study, of which 82 had normoglycemia (NG) and 67 had HG (**Figure 1**).

Patients were similar in age, BMI, sex distribution, and comorbidities (**Table 1**). We found a strong and significant correlation between 5mGS and glycemia (r: 0.833; 95% C.I.: -0.8766 to -0.7765; p<0.001) in all patients (**Figure 2**).

We observed a significantly slower 5mGS in the HG group compared to the NG group (0.52 ± 0.1 *vs.* 0.69 ± 0.06; p<0.001) (**Figure 3**). A multivariable linear regression analysis with 5mGS as a dependent variable (**Table 2**) confirmed the significant

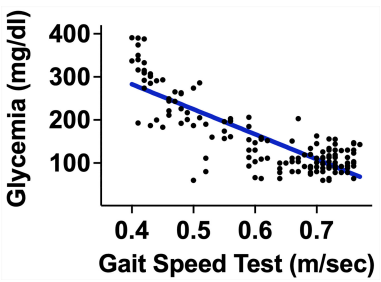

FIGURE 2 | Dispersion model correlating glycemia and 5-meter gait speed.

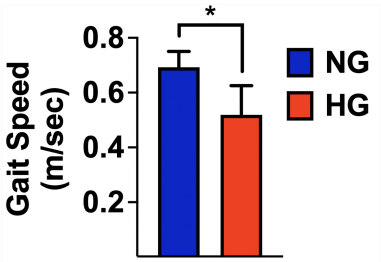

FIGURE 3 | Gait speed measured in normoglycemic (NG) and hyperglycemic (HG) patients; mean±SD; *p < 0.001.

TABLE 2 | Linear regression analysis with 5mGS as a dependent variable.

	B	Standard Error	Beta	t	p	95% Confidence Interval	
						Lower Bound	Upper Bound
Age	0.002	0.001	0.091	1.312	0.192	-0.001	0.004
Diabetes	-0.006	0.017	-0.025	-0.355	0.723	-0.040	0.028
CVD	-0.017	0.017	-0.072	-1.042	0.299	-0.050	0.015
Hyperlipidemia	0.014	0.016	0.060	0.922	0.358	-0.016	0.045
CKD	0.019	0.015	0.080	1.242	0.216	-0.011	0.049
COPD	0.029	0.014	0.121	2.038	0.043	0.001	0.058
Glycemia	-0.001	0.000	-0.854	-14.672	<0.001	-0.002	-0.001
Serum creatinine	-0.026	0.015	-0.124	-1.679	0.095	-0.056	-0.006
HbA1c	-0.008	0.011	-0.035	-0.714	0.476	-0.029	0.014
BMI	-0.003	0.003	-0.045	-0.957	0.340	-0.011	0.003
SBP	0.001	0.001	0.049	0.806	0.421	-0.001	0.002
DBP	0.001	0.001	0.036	0.731	0.466	-0.001	0.002
HR	-0.001	0.001	-0.074	-1.150	0.136	-0.002	0.000

BMI, body mass index; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; DBP, diastolic blood pressure; Hb1Ac: glycated hemoglobin; HR, heart rate; SBP, systolic blood pressure.

impact of glycemia (p<0.001) and revealed also an association with COPD (p: 0.043).

DISCUSSION

Our study indicates that frail hypertensive elders with HG have a significantly lower 5mGS compared to NG subjects. It is important to emphasize the fact that these results refer to a frail hypertensive population of older adults, in which physical performance affects functional decline, loss of independence, and cognitive impairment (30, 46).

Glucose levels may increase the risk of frailty in older adults without DM (31). It is interesting to observe that these findings are independent of a previous diagnosis of DM as well as from HbA1c values. In this scenario, HG drives physical impairment independently of DM and we speculate that glycemic control appears to be the best way to attempt to reverse physical impairment, with or without DM.

Our study does have some limitations. First, the study population is relatively small; second, there is no follow-up. Therefore, further studies are necessary to confirm our results, ideally in large randomized trials. We also reckon that a majority of our study population is represented by women; this finding is in agreement with the REPOSI Study on elderly people (47). Consistent with our observations, HG is associated with the development of frailty and lower extremity mobility limitations in older women (48, 49). Furthermore, a previous study had suggested to consider functionally independent women with osteoporosis and arthritis as a different cluster of frailty (50).

CONCLUSIONS

Taken together, our data indicate that HG drives physical impairment in frail and hypertensive older adults independently from DM and HbA1c values.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Campania Nord Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP, AL, GS, and PM designed the study, contributed to drafting the manuscript, approved its final version, and made the decision to submit and publish the manuscript. MM, MR, GM, ADL, and PM analyzed data, revised the manuscript's intellectual content, and approved the final version. EB, SF, and AM acquired the data, revised the manuscript's intellectual content, and approved the final version. PM is the guarantor of this work and, as such, had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of data analysis. All authors contributed to the article and approved the submitted version.

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Association Between Long-Term HbA1c Variability and Functional Limitation in Individuals Aged Over 50 Years: A Retrospective Cohort Study

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Background: As mean HbA1c provides incomplete information regarding glycemic variability, there has been considerable interest in the emerging association between glycemic variability and macrovascular events and with microvascular complications and mortality in adults with and without diabetes. However, the association between long-term glycemic variability, represented by visit-to-visit HbA1c variability, and functional limitations has not been clarified in previous literature. The present study aimed to explore the longitudinal association between long-term glycemic variability, represented by visit-to-visit HbA1c variability and functional limitations.

Methods: This cohort study included adults aged over 50 years who participated in the 2006 to 2016 waves of the Health and Retirement Study. Physical functions, including mobility, large muscle function, activities of daily living (ADLs), and instrumental ADLs (IADLs), were assessed at baseline and every 2 years, and HbA1c levels were assessed at baseline and every 4 years. Visit-to-visit HbA1c variability was calculated using the HbA1c variability score (HVS) during the follow-up period. Generalized estimating equation models were used to evaluate the longitudinal association between HbA1c variability and functional limitations with adjustment for a series of confounders.

Results: A total of 5,544 participants having three HbA1c measurements from 2006 to 2016, having two or more physical function measures (including one at baseline), and age over 50 years were included in this analysis. The mean age at baseline was 66.13 ± 8.39 years. A total of 916 (16.5%) participants had an HVS = 100, and 35.1% had an HVS = 50. The highest HVS category (HVS =100) was associated with increased functional status score (β = 0.093, 95% CI: 0.021–0.165) in comparison with the lowest HVS category (HVS = 0). Sensitivity analyses using the CV and SD of HbA1c as measures of variability showed similar associations between HbA1c variability and functional limitation. An incremental increase in HbA1c-CV (β = 0.630, 95% CI: 0.127–1.132) or HbA1c-SD (β = 0.078, 95% CI: 0.006–0.150) was associated with an increase in functional limitation in the fully adjusted model.

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Conclusions: HbA1c variability was associated with heightened difficulty in performing functional activities over time after adjusting for mean HbA1c levels and multiple demographics and comorbidities. This study provides further evidence regarding the detrimental effect of HbA1c variability and highlights the significance of steady glycemic control.

Keywords: functional limitation, glycated hemoglobin A1c, glycemic variability, HbA1c, mobility, physical functioning

INTRODUCTION

Physical functioning is a multidimensional concept encompassing mobility, large muscle functioning, gross motor skills, fine motor skills, and the ability to perform activities of daily living (ADLs) and instrumental ADLs (IADLs) (1–3). It is an essential aspect of daily life and enables autonomy and participation in meaningful physical, social, and cultural activities. Limitation of physical functioning threatens independence and is an independent risk factor for impaired quality of life, institutionalization, further functional decline, and premature mortality in older adults (3–5). Accordingly, identification of risk factors for physical function limitations in middle-aged and elderly individuals may provide insights into appropriate clinical practice and public health interventions to inform optimal self-management and clinical management of adults with these conditions (2, 3).

Hemoglobin A1c (HbA1c) is the current gold standard for monitoring blood glucose control and is now recommended for use in diagnosing diabetes and identifying individuals at risk of developing diabetes (6). The association between diabetes and functional limitation and disability is well documented in literature (7–10). Previous population-based longitudinal studies have also indicated that impaired fasting glucose, impaired glucose tolerance, and newly diagnosed diabetes are associated with reduced of health-related functioning, and that this is evident before the onset of these conditions (11–13). People with insulin resistance, and as they age, they are significantly more likely to have a deterioration in their quality of life in the areas of physical functioning, emotional role limitations, social functioning, pain and general health perception (14). Evidence for the association between diabetes management using glycemic markers and physical function limitations has been inconsistent. Some studies have reported that poor glycemic control is associated with decreased physical function, and others have indicated a significant association between tight (lower) glycemic control and physical disability; however, some studies reported that there was no significant association (15–17). A prospective cohort study indicated a nonmonotonic longitudinal relationship between HbA1c levels and the physical functioning decline in later life, however the HbA1c was assessed only at baseline (18).

In this context, whether an average glycemic measure is most appropriate for assessing the risk of complications is currently under debate. The concept of glycemic variability, which is related to fluctuations in glycemia, has recently emerged as another measure of glycemic control, which might constitute an additive or even better predictor of diabetic complications compared to mean HbA1c levels (19). Two components of glycemic variability have been recognized: short-term glycemic variability over days to weeks, and long-term glycemic variability ascertained by calculating visit-to-visit fluctuations of HbA1c over periods of follow-up lasting months to years (19). Although it remains controversial, some reviews and meta-analyses have shown significant associations between HbA1c variability and all-cause mortality, renal disease, and cardiovascular disease in type 2 diabetes and retinopathy, renal disease, and cardiovascular disease in type 1 diabetes (20–22).

There is also considerable interest in the emerging association between glycemic variability and decline in cognitive function and the increased level of symptoms of depression (23, 24). However, most of the included studies had limitations such as little adjustment for key confounders, concentration on secondary care patients with diabetes, and high levels of heterogeneity between studies, possibly related to different definitions and measurements of variability (20). Furthermore, glycemic variability seems to have an effect in individuals without diabetes (19, 25). A study including 6,756 individuals without diabetes indicated an association between high HbA1c variability and increased risks of incident major adverse cardiovascular events and death from all causes (25).

In conclusion, although some studies have examined HbA1c and its association with functional disability, these studies examined cross-sectional data or assessed functional decline over a brief period of time and have reported controversial results (16–18, 26). As mean HbA1c provides incomplete information regarding glycemic variability, there has been considerable interest in the emerging association between visit-to-visit glycemic variability and macrovascular events and with microvascular complications and mortality in adults with and without diabetes (19). However, the breadth of information on the longitudinal association between HbA1c variability and functional limitations is limited. Whether glycemic variability in individuals without diabetes is an independent risk factor for functional limitation is currently unknown.

This study aimed to determine the association between HbA1c variability and functional limitations across a wide range of physical tasks, after accounting for a series of sociodemographic confounders and comorbidities, in a nationally representative sample of middle-aged and elderly

Abbreviations: ADLs, activities of daily living; DBS, dried blood spot; HRS, Health and Retirement Study; HVS, HbA1c variability score; IADLs, instrumental activities of daily living; SD, standard deviation; CV, coefficient of variation.

adults. We hypothesized that a higher variability in HbA1c, represented as the higher HbA1c variability score (HVS) and the intra-individual SD and coefficient of variation (CV) of HbA1c value across visits, would be associated with more difficulties in performing functional activities in this population after adjustment for potential confounders and mean HbA1c.

MATERIALS AND METHODS

Data Source and Study Population

We used data from the 2006 to 2016 waves of the Health and Retirement Study (HRS) (27). The HRS is a longitudinal cohort study of health and retirement among American adults aged 50 years and older which collects data on demographics, socioeconomic factors, health conditions, and behavioral indicators biennially. HRS began to collect dried blood spot (DBS) blood-based biomarkers from half of the sample population in 2006, and the other half of the population provided DBS biomarker data in 2008 (28). The first group provided blood samples again in 2010 and 2014, and the second group provided repeat blood samples in 2012 and 2016, creating a 4-year interval between the biomarker blood collections. The time of the first HbA1c measurement was considered the baseline for all participants.

The RAND HRS Longitudinal File is an easy-to-use dataset based on the HRS core data, and it was used for analyses (29, 30). This file was developed at RAND with funding from the National Institute on Aging and the Social Security Administration. The HRS is sponsored by the National Institute on Aging (grant number NIA U01AG009740) and is conducted by the University of Michigan.

Inclusion and Exclusion Criteria

The inclusion criteria for this study included the following: participants having three HbA1c measurements from 2006 to 2016, having two or more physical function measures (including one at baseline), and age over 50 years. A total of 5,796 respondents who had three HbA1c measurements from 2006 to 2016 were identified, after excluding those with missing physical function measures (n=48) and those aged less than 50 years (n=204), a total of 5,544 participants were included in this analysis.

Measurement of HbA1c and HbA1c Variability

Blood sample collection and HbA1c measurement in the HRS were conducted every 4 years. The details of this process have been described elsewhere (28). The National Health and Nutrition Examination Survey-equivalent assay values of HbA1c in the HRS were used for analysis in our study, as recommended (28). Mean HbA1c values were calculated based on mean values of all visits for each participant. To better fit clinical practice, the visit-to-visit variability in HbA1c was defined as the HbA1c variability score (HVS), calculated by the number of successive measurements which differed by 0.5% (5.5 mmol/mol) or more divided by the number of comparisons and then multiplied by 100 (31, 32). Due to the lack of an appropriate gold-standard measurement for HbA1c variability, we calculated two other metrics, including the intra-individual SD and the coefficient of variation (CV) across visits as additional measures of glycemic variability (19).

Functional Limitation

Physical function was assessed every 2 years in the HRS (33). We used several summary measures, including measures for mobility, large muscle function, ADLs, and IADLs for functional limitations located in the biennial core interview (34). Physical function was measured using 17 distinct physical tasks derived from well-validated questionnaires and were categorized into four functional domains according to published definitions: mobility (five tasks, namely, walking one block, walking several blocks, walking across a room, climbing one flight of stairs, and climbing several flights of stairs), large muscle limitation (four tasks, namely, sitting for 2 h, getting up from a chair, stooping or kneeling or crouching, and pushing or pulling a large object), ADLs (ADLs, three tasks, namely, bathing, eating, and dressing), and IADLs (IADLs, five tasks, namely, using a telephone, taking medication, handling money, shopping, and preparing meals) (33, 34).

For each task, a code of 0 indicated that the respondent did not report any problems with the activity. A code of 1 indicated that the respondent reported some difficulty with the activity or could not perform the activity. We used a composite score of the 17 items summed to obtain a disability score, with higher scores indicating greater disability (range, 0–17). This composite measure, which captures a broad range of disability from early or "preclinical" disability to later personal care disability, has the advantage of capturing finer graduations in limitations and reducing ceiling or flooring effects (33, 35).

Demographic and Clinical Covariates

Covariates shown by previous studies to be associated with HbA1c levels and physical function were selected for analyses. The demographic covariates included age (continuous variable), sex (male or female), race (Hispanic, non-Hispanic White, non-Hispanic Black, others), marital status (married or partnered, separated or divorced, widowed, never married), and current smoking (yes or no). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2) and treated as a continuous variable. Depressive symptoms were measured using an 8-item version of the Center for Epidemiologic Studies Depression Scale, with higher scores indicating more depressive symptomology (36). Cognitive function included immediate and delayed word recall, the serial 7s test, counting backward, naming tasks (e.g., date naming), and vocabulary questions, resulting in a score range of 0–35 (37). Comorbidities included dichotomous measures (yes or no) of self-reported physician's diagnosis of (1) high blood pressure or hypertension; (2) diabetes or high blood sugar; (3) cancer or a malignant tumor of any type except skin cancer; (4) chronic lung disease other than asthma, such as chronic bronchitis or emphysema; (5) heart problems; (6) stroke or

transient ischemic attack; (7) emotional and nervous disorders or psychiatric problems; and (8) arthritis or rheumatism (30). Sex and race were adjusted using baseline data, whereas other confounder variables were included as time-variants and adjusted using multi-wave data.

Statistical Analyses

Continuous variables are described using median values (lower and upper quartiles), and categorical variables are presented as numbers (proportions). HVS was calculated as a measure of glycemic variability. Participants were grouped in terms of HVS, and baseline characteristics were compared using Kruskal-Wallis H test or Pearson χ^2 tests, when appropriate.

Generalized estimating equations with a negative binomial distribution and an unstructured covariance matrix were used to evaluate the longitudinal association between the long-term HbA1c variability and functional status. Negative binomial regression was used to account for the over-dispersion of the functional scores. The effect of variability in HbA1c was calculated by modeling the HVS as a category variable (0, 50, 100, with HVS=0 as reference). Model 1 was adjusted for age, sex, race, and marital status, and mean HbA1c value. Model 2 was additionally adjusted for current smoking status, BMI, depression, cognitive function, hypertension, diabetes, cancer, lung disease, heart disease, stroke, arthritis, and psychiatric disorders. Sensitivity analyses were performed for outcome by using the SD and CV of the HbA1c instead of the HVS. To examine potential modification effects, interactions between HVS and age, sex and BMI were investigated. Whenever there was evidence of interaction ($p < 0.05$ for interaction term), stratified analyses were performed.

All significance tests were two-tailed, and a p-value of less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SAS 9.4 software.

RESULTS

Baseline Demographics and Clinical Characteristics

Mean age at baseline was 66.13 ± 8.39 years. A total of 3,349 (60.41%) participants were women. The participants were categorized into three groups according to HbA1c variability score (HVS = 0, 50, 100). Thirty-five percent of the patients had an HVS = 50; 16.5% had an HVS = 100. **Table 1** presents the characteristics of the included participants across the HbA1c variability score categories. There were significant differences in baseline characteristics, including race, marital status, BMI, depression symptoms, cognitive function, self-reported doctor diagnosis of diabetes, hypertension, heart disease, arthritis, and psychiatric disorder between groups (p s < 0.05).

Association Between HbA1c Variability and Functional Limitations

Table 2 provides the results of the generalized estimating equation models to understand the influence of glycemic variability on functional limitations. After adjusting for demographics, in comparison with the reference (lowest HVS category, HVS =0), the highest HVS category (HVS =100) and medium HVS category (HVS =50) were associated with increased functional limitation score (β = 0.168, 95% CI: 0.089–0.247; β = 0.102, 95% CI: 0.041–0.162) in model 1. In the fully adjusted model, the highest HVS category was associated with increased functional status score (β = 0.093, 95% CI: 0.021–0.165) in comparison with the lowest HVS category.

An incremental increase in the mean HbA1c value was associated with an increased functional status score (β = 0.153, 95% CI: 0.120–0.186) in Model 1, but there was no significant association after further adjustment for comorbidity covariates in Model 2.

Interaction Analyses and Subgroup Analyses

The results indicated a significant interaction between HVS category and sex (ps for interaction <0.05). We found no statistically significant effect modifications of age (highest HVS category × age: $p = 0.927$; medium HVS category × age: $p = 0.720$), and BMI (highest HVS category × BMI: $p = 0.291$; medium HVS category × BMI: $p = 0.453$).

Subgroup analyses based on sex and baseline diabetes diagnosis were shown in **Table 3**. The association between the highest HbA1c variability category (HVS=100) and functional limitation showed a similar pattern in male participants (β = 0.215, 95% CI: 0.089–0.342) and non-diabetes subgroup (β = 0.107, 95% CI: 0.022–0.193). However, this association between HbA1c variability and functional limitation in female subgroup lost significance in the fully adjusted model. We found no significant association of HbA1c variability with functional decline among individuals with diabetes.

Sensitivity Analyses

The sensitivity analysis based on the CV and SD of HbA1c showed a similar association between HbA1c variability and functional limitation. As shown in **Table 4**, an incremental increase in the HbA1c-CV was associated with an increase in functional limitation (β = 0.630, 95% CI: 0.127–1.132) in the fully adjusted model. An incremental increase in the HbA1c-SD value was associated with an increase in functional limitation (β = 0.078, 95% CI: 0.006–0.150) in the fully adjusted model.

DISCUSSION

To the best of our knowledge, this is the first study to examine the association between visit-to-visit HbA1c variability and functional limitations across a wide range of physical functional domains, and which analyzes data over a long-term 10-year follow-up period in a large population-based prospective cohort study. Potential confounders including mean HbA1c were comprehensively considered. We also considered the SD and CV of the annual mean HbA1c as an additional measure of glycemic variability.

TABLE 1 | Baseline Characteristics of adults aged over 50 years of age by HbA1c variability score (N=5544).

Characteristics	Total	HbA1c variability score (HVS) categories			*H* or $\chi 2$	*p*
	N = 5544	0 (n = 2683)	50 (n = 1945)	100 (n = 916)		
Range of HbA1c CV	0.05 (0.03, 0.08)	0.03 (0.02, 0.04)	0.07 (0.06, 0.10)	0.11 (0.08, 0.15)	3900.063	**<0.001**
Range of HbA1c SD	0.31 (0.19, 0.49)	0.18 (0.13, 0.24)	0.43 (0.34, 0.56)	0.70 (0.50, 1.05)	3989.302	**<0.001**
HbA1c mean,%	5.69 (5.41, 6.11)	5.56 (5.35, 5.81)	5.77 (5.46, 6.26)	6.29 (5.71, 7.19)	746.676	**<0.001**
Age (years)	66.00 (59.00,72.00)	66.00 (59.00,72.00)	66.00 (59.00,72.00)	66.00 (59.00,72.00)	1.332	0.514
Sex, n (%)					1.406	0.495
Male	2195 (39.59)	1041 (38.80)	787 (40.46)	367 (40.07)		
Female	3349 (60.41)	1642 (61.20)	1158 (59.54)	549 (59.93)		
Race, n (%)					107.038	**<0.001**
Hispanic	440 (7.94)	193 (7.19)	143 (7.35)	104 (11.35)		
Not Hispanic white	4325 (78.01)	2201 (82.04)	1508 (77.53)	616 (67.25)		
Not Hispanic black	651 (11.74)	222 (8.27)	260 (13.37)	169 (18.45)		
Not Hispanic other	128 (2.31)	67 (2.50)	34 (1.75)	27 (2.95)		
Marital status, n (%)					26.588	**<0.001**
Married or partnered	3768 (67.97)	1894 (70.59)	1294 (66.53)	580 (63.32)		
Separated or divorced	753 (13.58)	353 (13.16)	261 (13.42)	139 (15.17)		
Widowed	841 (15.17)	370 (13.79)	315 (16.20)	156 (17.03)		
Never married	182 (3.28)	66 (2.46)	75 (3.86)	41 (4.48)		
Current smoker, n (%)	630 (11.45)	291 (10.94)	231 (11.95)	108 (11.88)	1.327	0.515
BMI, kg/m^2	29.00 (25.60,32.90)	28.30 (25.10,32.00)	29.10 (25.90,33.20)	30.50 (27.10,34.80)	106.941	**<0.001**
Depression symptoms	0.00 (0.00, 2.00)	0.00 (0.00, 2.00)	0.00 (0.00, 2.00)	1.00 (0.00, 2.00)	32.335	**<0.001**
Cognition	24.00 (21.00, 26.00)	24.00 (21.00, 26.00)	24.00 (21.00, 26.00)	23.00 (20.00, 26.00)	14.465	**0.001**
Diabetes, n (%)	951 (17.17)	192 (7.16)	385 (19.82)	374 (40.83)	559.021	**<0.001**
Hypertension, n (%)	2910 (52.53)	1278 (47.65)	1061 (54.63)	571 (62.34)	64.376	**<0.001**
Cancer, n (%)	653 (11.80)	316 (11.80)	244 (12.57)	93 (10.18)	3.427	0.180
Lung disease, n (%)	366 (6.61)	171 (6.38)	125 (6.44)	70 (7.66)	1.956	0.376
Heart disease, n (%)	1013 (18.29)	449 (16.75)	373 (19.18)	191 (20.90)	9.415	**0.009**
Stroke, n (%)	275 (4.96)	123 (4.59)	103 (5.30)	49 (5.35)	1.561	0.458
Arthritis, n (%)	3158 (56.97)	1484 (55.31)	1117 (57.46)	557 (60.81)	8.705	**0.013**
Psychiatric disorder, n (%)	742 (13.40)	321 (11.99)	278 (14.31)	143 (15.63)	9.904	**0.007**
Functional total score	1.00 (0.00, 3.00)	1.00 (0.00, 3.00)	1.00 (0.00, 3.00)	2.00 (0.00, 4.00)	51.272	**<0.001**

*Values are median (lower quartile, upper quartile) for continuous variables and number (%) for continuous variables. *Calculated using Kruskal-Wallis H test or Pearson $\chi 2$ test. p values less than .05 are in bold.*

Overall, this study found that HbA1c variability was associated with more difficulties in functional activities over time. After adjusting for multiple demographics, comorbidities, and mean HbA1c levels, HbA1c variability maintained an independent longitudinal association with more difficulties in functional activities. Sensitivity analyses using SD and CV of HbA1c instead of HVS did not materially change our results. This study provides further evidence for the detrimental effect of HbA1c variability and highlights the significance of steady glycemic control.

Going "beyond the mean HbA1c level" is an important focus of the current study. A cross-sectional study with the use of the ADA-recommended definition based on HbA1c measurement found that individuals with prediabetes had more physical function limitations than those with normoglycemia (11). A prospective cohort study revealed a U-shaped association of HbA1c levels and physical functioning impairment, and indicated that both high and low HbA1c levels were associated with a faster rate of decline in objectively measured physical functioning (18). In addition to average HbA1c measurement, previous meta-analysis showed that HbA1c variability was positively associated with adverse outcomes in micro- and macro-vascular outcomes and mortality independently of mean HbA1c, and HbA1c variability was more predictive of adverse outcomes than mean HbA1c in the majority of studies (20). The results of the present study add to those of previous studies, indicating that HbA1c variability was a superior predictor of functional decline over mean HbA1c.

The lack of a significant association of HbA1c variability with functional decline among individuals with diabetes may be due to a number of factors. A pooled analysis of two prospective population-based cohorts observed a significant association between long-term HbA1c variability and cognitive decline among the non-diabetic population but not among individuals with diabetes (23). The relatively small number of participants with diabetes in the present study (n = 951) may restrict the power to detect a positive association. Several studies have also reported that long-term HbA1c variability has a greater impact among individuals without diabetes, while short-term variability is a predictor among those with diabetes (38). The association of HbA1c variability with functional limitation among female subgroup lost significance in the fully adjusted model, suggesting that the effects of HbA1c variability may be explained by the preceding confounders including BMI, depressive symptoms, cognition, and comorbidities in females. Future studies are still needed to verify these observed associations.

TABLE 2 | Association between HbA1c variability and functional limitation using GEE models (N=5544).

		β	SE	z	*p*	95% CI lower	95% CI Upper	OR	95% CI lower	95% CI Upper
Model 1	Intercept	-2.011	0.156	-12.92	**<0.001**	-2.315	-1.705	0.134	0.099	0.182
	HVS=100	0.168	0.040	4.18	**<0.001**	0.089	0.247	1.183	1.093	1.280
	HVS=50	0.102	0.031	3.29	**0.001**	0.041	0.162	1.107	1.042	1.176
	HVS=0	Ref.								
	HbA1c Mean	0.153	0.017	8.99	**<0.001**	0.120	0.186	1.165	1.127	1.204
	Age	0.028	0.001	20.26	**<0.001**	0.025	0.030	1.028	1.025	1.030
	Female	0.332	0.029	11.34	**<0.001**	0.275	0.390	1.394	1.317	1.477
	Male	Ref.								
	Not Hispanic Other	-0.041	0.117	-0.35	0.723	-0.270	0.187	0.960	0.763	1.206
	Not Hispanic Black	-0.013	0.058	-0.21	0.830	-0.126	0.101	0.987	0.882	1.106
	Not Hispanic White	-0.266	0.048	-5.59	**<0.001**	-0.359	-0.173	0.766	0.698	0.841
	Hispanic	Ref.								
	Never Married	0.111	0.053	2.08	**0.038**	0.006	0.215	1.117	1.006	1.240
	Widowed	0.042	0.022	1.88	0.061	-0.002	0.086	1.043	0.998	1.090
	Separated or divorced	0.169	0.031	5.49	**<0.001**	0.109	0.229	1.184	1.115	1.257
	Married or partnered	Ref.								
Model 2	Intercept	-2.218	0.219	-10.12	**<0.001**	-2.648	-1.789	0.109	0.071	0.167
	HVS=100	0.093	0.037	2.53	**0.011**	0.021	0.165	1.097	1.021	1.179
	HVS=50	0.050	0.028	1.82	0.069	-0.004	0.104	1.051	0.996	1.110
	HVS=0	Ref.								
	HbA1c Mean	-0.016	0.020	-0.82	0.414	-0.056	0.023	0.984	0.946	1.023
	Age	0.024	0.002	12.96	**<0.001**	0.020	0.027	1.024	1.020	1.027
	Female	0.225	0.028	8.14	**<0.001**	0.171	0.279	1.252	1.186	1.322
	Male	Ref.								
	Not Hispanic Other	-0.087	0.109	-0.79	0.427	-0.301	0.127	0.917	0.740	1.135
	Not Hispanic Black	-0.141	0.055	-2.56	**0.011**	-0.249	-0.033	0.868	0.780	0.968
	Not Hispanic White	-0.164	0.046	-3.56	**0.004**	-0.255	-0.074	0.849	0.775	0.929
	Hispanic	Ref.								
	Never Married	0.069	0.069	1.00	0.317	-0.066	0.205	1.071	0.936	1.228
	Widowed	-0.053	0.028	-1.89	0.059	-0.107	0.002	0.948	0.899	1.002
	Separated or divorced	0.050	0.037	1.33	0.183	-0.024	0.123	1.051	0.976	1.131
	Married or partnered	Ref.								
	Current smoker	0.207	0.042	4.92	**<0.001**	0.124	0.289	1.230	1.132	1.335
	BMI	0.035	0.002	18.20	**<0.001**	0.031	0.039	1.036	1.031	1.040
	Depression	0.132	0.005	24.29	**<0.001**	0.122	0.143	1.141	1.130	1.154
	Cognition	-0.022	0.003	-8.14	**<0.001**	-0.027	-0.017	0.978	0.973	0.983
	Diabetes	0.114	0.032	3.62	**<0.001**	0.053	0.176	1.121	1.054	1.192
	Hypertension	0.058	0.027	2.14	**0.032**	0.005	0.111	1.060	1.005	1.117
	Cancer	0.073	0.028	2.60	**0.009**	0.018	0.128	1.076	1.018	1.137
	Lung disease	0.298	0.032	9.45	**<0.001**	0.236	0.360	1.347	1.266	1.433
	Heart disease	0.205	0.024	8.45	**<0.001**	0.158	0.254	1.228	1.171	1.289
	Stroke	0.225	0.037	6.10	**<0.001**	0.152	0.297	1.252	1.164	1.346
	Arthritis	0.614	0.034	18.20	**<0.001**	0.548	0.681	1.848	1.730	1.976
	Psychiatric disorder	0.143	0.030	4.70	**<0.001**	0.083	0.202	1.154	1.087	1.224

HVS, HbA1c variability score. Model 1, adjusted for age, sex, race, marital status, and mean HbA1c; Model 2, further adjusted for current smoking, BMI, depressive symptoms, cognition, hypertension, diabetes, cancer, lung disease, heart disease, stroke, arthritis and psychiatric disorder. p values less than .05 are in bold.

To the best of our knowledge, this is the first study assessing the association between long-term HbA1c variability and functional decline that analyzes data from more than three physical functioning measurements over time. Many studies have been restricted to individuals diagnosed with diabetes, whereas others have included both diabetics and non-diabetics with stratification by diabetes diagnosis (20, 39). Our study further extends the findings of a significant association between long-term HbA1c variability and functional decline in a community-dwelling population. Recent systematic reviews have identified a range of potential risks associated with HbA1c variability but have had great difficulty in reaching clear conclusions (19, 20). This uncertainty may be due to the lack of a standard approach to summarizing HbA1c variability or agreement about how much might be clinically significant. Many studies use a relative measure (e.g., using quartiles of HbA1c variability), but this is difficult to compare across studies and even within the same study. The present study has mainly focused on the metrics of glycemic variability that are based on the HbA1c variability score (as it can be interpreted as the percentage of total HbA1c measures that vary by >0.5%), while omitting discussion of the more complicated computations to simplify the message, as a prerequisite for healthcare providers to be able to easily calculate and interpret in clinical practice.

The reasons for intraindividual variability in HbA1c are largely unknown. A previous study identified the patient characteristics associated with raised visit-to-visit glycemic variability in people

TABLE 3 | Subgroup analyses of association between HbA1c variability and functional limitation. (N=5544).

		β	SE	z	*p*	95% CI lower	95% CI Upper	OR (95% CI)
Male (n=2195)								
Model 1 [a]	HVS=100	0.261	0.072	3.65	**<0.001**	0.121	0.401	1.298 (1.129, 1.493)
	HVS=50	0.149	0.056	2.65	**0.008**	0.039	0.260	1.161 (1.040, 1.297)
Model 2 [b]	HVS=100	0.215	0.064	3.35	**<0.001**	0.089	0.342	1.240 (1.093, 1.408)
	HVS=50	0.055	0.049	1.13	0.257	-0.040	0.150	1.057 (0.961, 1.162)
Female (n=3349)								
Model 1 [a]	HVS=100	0.120	0.049	2.47	**0.014**	0.025	0.215	1.127 (1.025, 1.240)
	HVS=50	0.083	0.036	2.28	**0.023**	0.012	0.153	1.087 (1.012, 1.165)
Model 2 [b]	HVS=100	0.034	0.044	0.77	0.443	-0.052	0.119	1.035 (0.949, 1.126)
	HVS=50	0.050	0.033	1.54	0.123	-0.014	0.114	1.051 (0.986, 1.121)
Diabetes (n=951)								
Model 1 [c]	HVS=100	0.136	0.075	1.80	0.073	-0.012	0.283	1.146 (0.988, 1.327)
	HVS=50	0.021	0.073	0.28	0.778	-0.122	0.163	1.021 (0.885, 1.177)
Model 2 [d]	HVS=100	0.057	0.075	0.76	0.445	-0.089	0.203	1.059 (0.915, 1.225)
	HVS=50	0.009	0.070	0.13	0.895	-0.128	0.146	1.009 (0.880, 1.157)
No diabetes (n=4588)								
Model 1 [c]	HVS=100	0.133	0.050	2.68	**0.007**	0.036	0.231	1.142 (0.996, 1.260)
	HVS=50	0.100	0.034	2.91	**0.004**	0.033	0.167	1.105 (1.034, 1.182)
Model 2 [d]	HVS=100	0.107	0.043	2.47	**0.013**	0.022	0.193	1.113 (1.022, 1.213)
	HVS=50	0.056	0.030	1.84	0.066	-0.004	0.115	1.058 (0.996, 1.122)

HVS, HbA1c variability score. [a], adjusted for age, race, marital status, and mean HbA1c; [b], further adjusted for current smoking, BMI, depressive symptoms, cognition, hypertension, diabetes, cancer, lung disease, heart disease, stroke, arthritis and psychiatric disorder. [c], adjusted for age, sex, race, marital status, and mean HbA1c; [d], further adjusted for current smoking, BMI, depressive symptoms, cognition, hypertension, cancer, lung disease, heart disease, stroke, arthritis and psychiatric disorder. p values less than .05 are in bold.

with Type 2 diabetes, and thus the association of HbA1c variability with risk may not be a feature of the HbA1c variability per se but, rather, a marker of this baseline difference in patient characteristics (40). The current study adjusted comprehensively for baseline characteristics although we acknowledge that there could be residual confounding. Another study suggested that HbA1c variability is associated with the quality of care, indicated that intraindividual variability in HbA1c can be derived from poor quality of care or poor compliance with medical recommendations (41).

The pathophysiological mechanisms involved in the observed association between visit-to-visit glycemic variability and functional limitations remain unclear. Glycemic variability is a measure that accounts for the amplitude, frequency, and duration of glycemic oscillations around the average blood glucose level and an integral component of glucose homoeostasis (19, 42). Glycemic variability may be associated with functional disability through mechanisms associated with oxidative stress, chronic systemic inflammation, extremes of blood glucose, decreased muscle strength, lower muscle quality, and accelerated loss of muscle mass (7, 8, 38, 42–45). Oxidative stress is suggested to explain the association between short-term glycemic variability and adverse outcomes (41), but it is not clear whether this is increased in patients with high visit-to-visit HbA1c variability. Glycemic variability can represent the presence of excess glycemic excursions and, consequently, the risk of hyperglycemia or hypoglycemia (43). High concentrations of glucose might lead to systemic, chronic inflammation, which is part of a multifactorial process that eventually results in frailty and disability (8, 45). Further studies are necessary to clarify the mechanisms underlying the association between glycemic variability and functional limitations.

TABLE 4 | Association between CV or SD of HbA1c variability and functional limitation score. (N=5544).

		β	SE	z	*p*	95% CI lower	95% CI Upper	OR (95% CI)
Model 1								
	HbA1c CV	1.263	0.264	4.79	**<0.001**	0.747	1.779	3.536 (2.111, 5.924)
	mean HbA1c	0.141	0.019	7.63	**<0.001**	0.105	0.177	1.151 (1.111, 1.194)
Model 2								
	HbA1c CV	0.630	0.257	2.45	**0.014**	0.127	1.132	1.878 (1.135, 3.102)
	mean HbA1c	-0.022	0.021	-1.06	0.288	-0.063	0.019	0.978 (0.093, 1.019)
Model 1								
	HbA1c SD	0.146	0.036	4.03	**<0.001**	0.075	0.218	1.157 (1.078, 1.244)
	mean HbA1c	0.135	0.021	6.51	**<0.001**	0.095	0.176	1.145 (1.100, 1.192)
Model 2								
	HbA1c SD	0.078	0.037	2.12	**0.034**	0.006	0.150	1.081 (1.006, 1.162)
	mean HbA1c	-0.028	0.023	-1.21	0.227	-0.072	0.017	0.972 (0.931, 1.017)

Model 1, adjusted for age, sex, race, marital status, and mean HbA1c; Model 2, further adjusted for current smoking, BMI, depressive symptoms, cognition, hypertension, diabetes, cancer, lung disease, heart disease, stroke, arthritis and psychiatric disorder. p values less than .05 are in bold.

The findings of the present study should be considered in the context of some potential limitations, including the observational study design which does not allow for casual inference and self-reported measures of comorbidities, which may underestimate the true prevalence of these conditions. Difficulties in physical functioning were also based on self-reported measures. Although self-reports provide valuable information about the person's own perception of their functioning in the living environment, replication using objective physical performance measures would alleviate concerns regarding potential self-reported bias. Although we adjusted for many potential confounding factors, there remains the possibility that residual confounding factors were not measured in this association, which could have influenced the variability observed in the study.

Despite these limitations, the strength of the present study is the use of a large, representative, longitudinal cohort of middle-aged and community-dwelling elderly and large data of multiple visit-to-visit HbA1c measures, enabling us to accurately calculate long-term HbA1c variability over a long-term 10-year follow-up period. The outcome measure was based on difficulty in 17 physical functioning tasks across different physical functional domains, covering not only ADLs and IADLs but also other clinically relevant disability domains, such as mobility (e.g., walking several blocks), and general physical activities (e.g., stooping, bending, and pulling a large object). Many previous studies have focused on single disability domains or items; however, it is common for older people to have difficulties in multiple areas of physical functioning (35). This comprehensive assessment allowed us to explore the association between HbA1c variability and composite functional limitations across multiple physical functional domains.

CONCLUSION

In conclusion, data from a population-based sample of US adults indicate the association between glycemic variability, as measured by variability score in visit-to-visit HbA1c over time, and the number of physical functioning difficulties independent of mean HbA1c in individuals aged over 50 years. This association remained significant even after adjusting for sociodemographic and clinical factors. Further well-controlled randomized controlled trials are needed to establish glycemic variability as an independent risk factor for functional decline and diabetes complications and to confirm whether strategies to reduce glycemic variability in HbA1c can effectively reduce the incidence or progression of physical functioning impairment. Future studies are also needed to investigate the potential of using HbA1c variability in assessing risk in older people and to inform optimal approaches to achieving a safe and stable glycemic level.

AUTHORS CONTRIBUTIONS

DS: Conception and design, acquisition of data, analysis, drafting. SSW, JWS, HPWand QS: Conception and design, analysis and interpretation of data, critical revision of the manuscript. All authors contributed to the article, approved the version to be published and agreed to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All participants provided informed consent, and the HRS is approved by the Institutional Review Board at the University of Michigan.

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Tolerability and Effectiveness of Switching to Dulaglutide in Patients With Type 2 Diabetes Inadequately Controlled With Insulin Therapy

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Aims: Glucagon-like peptide 1 (GLP-1) receptor agonists have demonstrated strong glycemic control. However, few studies have investigated the effects of switching from insulin to GLP-1 receptor agonists. We aimed to investigate, using real-world data, whether switching to dulaglutide improves glycemic control in patients with type 2 diabetes mellitus (T2D) inadequately controlled with conventional insulin treatment.

Materials and methods: We retrospectively evaluated 138 patients with T2D who were switched from insulin to dulaglutide therapy. We excluded 20 patients who dropped out during the follow-up period. The participants were divided into two groups according to whether they resumed insulin treatment at 6 months after switching to a GLP-1 receptor agonist (group I) or not (group II). A multiple logistic regression analysis was performed to evaluate the parameters associated with the risk of resuming insulin after replacement with dulaglutide.

Results: Of 118 patients initiated on the GLP-1 receptor agonist, 62 (53%) resumed insulin treatment (group I), and 53 (47%) continued with GLP-1 receptor agonists or switched to oral anti-hypoglycemic agents (group II). Older age, a higher insulin dose, and lower postprandial glucose levels while switching to the GLP-1 receptor agonist were associated with failure to switch to the GLP-1 receptor agonist from insulin.

Conclusions: A considerable proportion of patients with T2D inadequately controlled with insulin treatment successfully switched to the GLP-1 receptor agonist. Younger age, a lower dose of insulin, and a higher baseline postprandial glucose level may be significant predictors of successful switching from insulin to GLP-1 receptor agonist therapy.

Keywords: GLP-1 receptor agonist, insulin therapy, type 2 diabetes, switching to GLP-1 receptor agonist, type 2 diabetes inadequately controlled with insulin therapy

INTRODUCTION

For patients with type 2 diabetes (T2D) having uncontrolled glucose levels, insulin therapy has been traditionally considered as the most effective treatment available for managing hyperglycemia, and, more commonly, as an adjunct to oral hypoglycemic agents (OHAs) (1–3). However, approximately 40% of patients treated with insulin ultimately fail to achieve their target HbA1c levels and require insulin intensification (4). Although insulin intensification is theoretically the best treatment option for glycemic control (5, 6), in clinical practice, it does not successfully maintain glycemic control. Moreover, insulin intensification, such as that by a multiple daily injection regimen, frequently increases the risks of adverse events such as weight gain and hypoglycemia (7, 8). Consequently, it may lead to overall dissatisfaction with the treatment and poor compliance with therapy in patients with T2D (8) Moreover, HbA1c goals are often unmet even after increasing the number and dose of insulin injections in real practice.

As per recent research, the intestine, brain, kidney, and immune system are emerging targets for the treatment of diabetes (9); therefore, glucagon-like peptide-1 (GLP-1) receptor agonists that target pancreatic beta and alpha cells, the intestine, and the brain have been developed and are widely used to regulate glucose metabolism (10). Several recent studies have demonstrated that GLP-1 receptor agonists are as effective as insulin regimens in lowering HbA1c (11, 12).

However, few studies have been conducted on whether patients with uncontrolled T2D could be successfully switched from insulin therapy to GLP-1 receptor agonist therapy. Moreover, there is limited data on the clinical characteristics that predict the successful continuation of GLP-1 receptor agonists after switching from an insulin regimen. Therefore, the present study aimed to investigate whether switching to dulaglutide, a weekly injectable GLP-1 receptor agonist, from insulin improves glycemic control in patients with T2D inadequately controlled with conventional insulin treatment.

MATERIALS AND METHODS

Study Design and Data Source

In this retrospective, observational study, we analyzed the human subjects' medical record and laboratory data of 138 patients with T2D whose HbA1c levels were 7.6% or higher when treatment was switched from insulin to dulaglutide with OHAs between July 2017 and March 2021. Although this study is retrospective, the researcher's own supervision and the Institutional Review Board's deliberation were conducted on the data processing. The protocol of this study adhered to the tenets of the Declaration of Helsinki and Korean Good Clinical Practice and was approved by the Institutional Review Board (IRB No. 4-2021-1639) of Severance Hospital. The requirement of written informed consent was waived because the data were accessed only for analysis, and personal information was not used. We reviewed the electronic medical records to assess whether the subjects who stopped using dulaglutide later resumed insulin therapy or switched to OHAs after discontinuing dulaglutide over the 6 months. For the effectiveness of analysis, 20 patients who dropped out for various reasons were excluded, and the remaining study subjects were first classified into two groups according to the resumption of insulin therapy during the 6 months: the resumption-to-insulin group (group I, n= 62) and the continued-dulaglutide-or-changed-to-

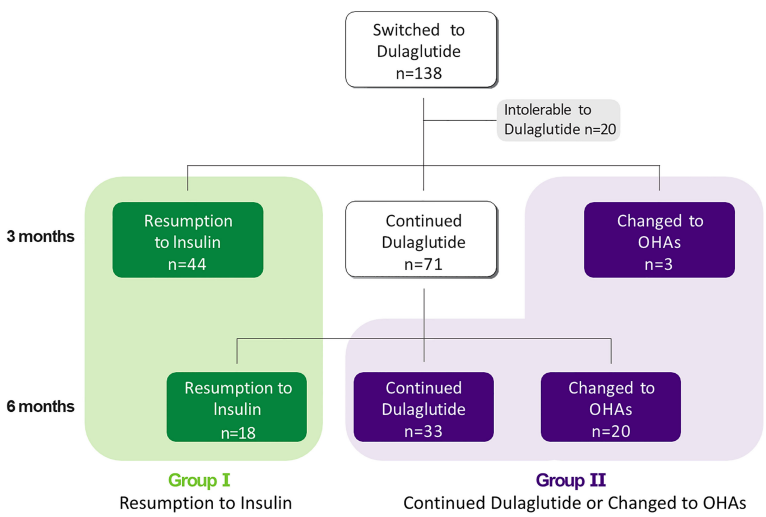

FIGURE 1 | A total of 138 patients switched from insulin to dulaglutide. 20 patients were not resistant to Dulaglutide and 118 patients were followed for six months after conversion. 44 patients resumed insulin, 71 patients continued to use dulaglutide. And three patients switched from dulaglutide to oral hypoglycemic agents after three months. Additional 18 patients resumed insulin, 33 patients continued using dulaglutide and 20 patients switched to OHA after six months.

OHAs group (group II, n=56) (**Figure 1**). Group I was further divided into group Ia (n=44), in which insulin was replaced before or at the first visit, and group Ib (n=18), in which insulin was replaced six months after switching to dulaglutide.

Because this was a retrospective and real-world data study, the decisions of switching to and continuing with dulaglutide, switching to only OHAs, or resuming insulin were fully at the discretions of the physicians based on their clinical judgments and the patients' blood glucose parameters. However, OHAs were usually given in combination with metformin and sulfonylurea, according to the health insurance policy, and there were rare exceptions.

Clinical and Laboratory Measurements

The baseline demographic and clinical characteristics of the patients were included. Body mass index (BMI) was defined as body weight divided by the square of the height in meters (kg/m^2). Hypertension and dyslipidemia were confirmed by diagnoses and prescription medications present in the medical records.

The baseline laboratory parameters were also included. Fasting and postprandial glucose levels were measured using the hexokinase method, and enzyme colorimetry was used to measure total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels. The HbA1c level was determined by high-performance liquid chromatography using Variant™ II Turbo (Bio-Rad Laboratories, Her-clusters, CA, USA). Serum C-peptide levels were measured in duplicates using immunoradiography (Beckman Coulter, Fullerton, CA, USA). Glycated hemoglobin, fasting glucose, and postprandial glucose levels were checked at the first and second visits to assess the glycemic efficacy after switching from insulin to dulaglutide therapy.

Statistical Analysis

Continuous variables are expressed as mean ± standard deviation, and categorical variables are expressed as percentages. Data are presented as the mean ± standard error (SE). Differences between the two groups were analyzed using Student's t-test for continuous variables and chi-square test for categorical variables. A logistic regression analysis was performed to assess whether continuing with dulaglutide/changing to OHAs or resumption of insulin treatment after the switch to dulaglutide were associated with clinical and laboratory parameters at the baseline. The receiver operating characteristic curve of the total insulin dose was used to determine the optimal cut-off value for the prediction of successful continuation of dulaglutide therapy by using the area under the curve with a maximum Youden index (sensitivity+specificity-1). Statistical significance was set at $p<0.05$. Statistical analyses were performed using PASW Statistics version 26.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Flow and Classification

Of the 293 patients with T2D who switched to weekly dulaglutide from insulin, 138 patients were finally enrolled in this study. During the follow-up period, 20 patients stopped using dulaglutide for various reasons, such as gastrointestinal disturbances and patient preferences. Approximately 86% of the enrolled subjects tolerated weekly dulaglutide treatment. Thereafter, 118 subjects were included for the effectiveness and predictive factor analyses. Among them, 62 (53%) resumed insulin by the end of the follow-up period (group I). Of them, 44 patients resumed insulin at the first visit at 3 months and 18 patients resumed insulin at the second visit at 6 months. Fifty-six (47%) patients continued with dulaglutide or switched to OHAs without restarting insulin (group II) (**Figure 1**). All patients started with dulaglutide at 0.75 mg for initial 1 month and then increased to 1.5 mg if tolerated. 96.6% of patients included in the study maintained 1.5 mg of dulaglutide during the maintenance period, except for the first adaptation period.

Baseline Clinical and Laboratory Characteristics of the Patients

The baseline clinical and laboratory characteristics of the patients in the effectiveness analysis set are shown in **Table 1**. The presence of glutamic acid decarboxylase (GAD) antibody was not found in enrolled subjects. The mean age and the mean duration of T2D were 60.4 ± 11.7 and 14.2 years, respectively. The proportion of male were 59%, 63% and 55% in all enrolled patients, group 1, and group 2, respectively. The average BMI was 27.42 ± 3.50 kg/m^2. The duration of insulin use was > 6 months in all the patients. HbA1c and fasting and postprandial glucose were measured in all patients at every visit. We adopted HbA1c and fasting and postprandial glucose as patient's blood sugar levels in this study. The baseline HbA1c level (group I vs. group II, 8.56 ± 0.78% vs. 8.69 ± 1.08%, p= 0.802), the fasting (140.8 ± 50.2 mg/dL vs. 148.1 ± 53.8 mg/dL, p=0.351) and postprandial (213.5 ± 65.7 mg/dL vs. 246.1 ± 97.9 mg/dL, p=0.166) blood glucose levels showed no significant differences between the two groups. We analyzed the C-peptide levels measured within one year of enrollment of this study. All enrolled patients were checked with fasting and postprandial C-peptide. The fasting C-peptide (2.38 ± 1.80 mg/dL vs. 2.29 ± 1.26 mg/dL, p=0.630) and postprandial C-peptide (3.83 ± 2.19 mg/dL vs. 4.14 ± 1.64 mg/dL, p=0.137) showed no significant differences between the two groups, but there was a significant difference in the total daily insulin doses (55.7 ± 23.6 U/day vs. 40.7 ± 20.8 U/day $p< 0.05$). There were no differences in baseline eGFR (82.74 ± 19.2 vs. 88.6 ± 20.9) and the prevalence of the hypertension (85% vs. 84%) or dyslipidemia (90% vs. 95%). There were no significant differences in the type of insulin or OHAs used in combination with insulin between the two groups. However, the frequency of basal insulin uses in group II seemed to be higher (16.1% vs. 26.8%, p=0.055).

Changes in Glycemic Parameters During the Follow-Up Period

The changes in the HbA1c levels during the follow-up period are shown in **Figure 2A**. In contrast to the HbA1c of group I, which increased at three months or six months, the HbA1c of group II was decreased and sustained after switching to weekly dulaglutide from insulin. In group Ia, the HbA1c level increased by 20% from baseline at 3 months (ΔHbA1c =1.7, $p <0.05$). In group Ib, the HbA1c level decreased by 4.5% (ΔHbA1c = -0.42) from baseline at 3 months, but increased by 5.6% (ΔHbA1c = 0.54) from baseline and 10.6% compared to 3 months (ΔHbA1c = 0.96, $p<0.05$), at 6 months. In group II, the HbA1c level decreased from 8.7% at baseline to 7.8% at 6 months after switching from insulin to dulaglutide (ΔHbA1c =

TABLE 1 | Clinical and laboratory characteristics of patients at baseline.

	Total	Group I	Group II	P-value
Patient Number	118	62	56	–
Age (yr)	60.4 ± 11.7	61.6 ± 9.7	59.1 ± 13.6	0.639
Sex (% male)	59%	63%	55%	0.407
BMI (kg/m2)	27.42 ± 3.50	27.60 ± 3.52	27.21 ± 3.50	0.590
Duration of Diabetes (yr)	14.2 ± 8.0	15.3v8.3	12.9 ± 7.5	0.103
Total Insulin Dose (Units)*	***48.5 ± 22.4***	***55.7 ± 23.6***	***40.7 ± 20.8***	***<0.05***
HbA1c (%)	8.62 ± 0.93	8.56 ± 0.78	8.69 ± 1.08	0.802
Fasting plasma glucose (mg/dL)	144.2 ± 51.9	140.8 ± 50.2	148.1 ± 53.8	0.351
Postprandial glucose (mg/dL)	229.4 ± 84.2	213.5 ± 65.7	246.1 ± 97.9	0.166
Fasting C-peptide (ng/mL)	2.34 ± 1.55	2.38 ± 1.80	2.29 ± 1.26	0.630
Postprandial C-peptide (ng/mL)	3.98 ± 1.94	3.83 ± 2.19	4.14 ± 1.64	0.137
eGFR (ml/min/1.73 m²)	85.5 ± 20.1	82.74 ± 19.2	88.6 ± 20.9	0.155
Hypertension (%)	85	85	84	0.815
Dyslipidemia (%)	92	90	95	0.379
Insulin				0.055
Basal insulin	25 (21.2%)	10 (16.1%)	15 (26.8%)	
Premixed insulin	89 (75.4%)	48 (77.4%)	41 (73.2%)	
MDI	4 (3.4%)	4 (6.5%)	0 (0%)	
OHAs with Insulin (%)				
DDP4i	53.3	50	57.1	0.136
Sulfonylurea	20	20.3	19.6	0.077
Metformin	70	73.4	66.1	0.398
SGLT2i	19.2	18.8	19.6	0.230
Etc.	5	3.1	7.1	0.542

*BMI, body mass index; eGFR, Estimated glomerular filtration rate; MDI, Multiple daily injection; OHAs, oral hypoglycemic agents. Data are expressed as mean ± standard deviation. Sex (% male), OHAs with insulin (%), Hypertension (%), Dyslipidemia (%) analyzed by Chi-square test; other baseline characteristics analyzed by T-test. *P-value < 0.05, Group I vs Group II. Bold values for Statistically significant values (P-value < 0.05).*

-0.93, p <0.05). **Figures 2B, C** show the changes in fasting and postprandial blood glucose levels during the follow-up period, the trends in the change of postprandial and fasting plasma glucose levels during the follow-up period were similar to that of HbA1c.

Figure 3 demonstrates the change in the rate of insulin resumption during the follow-up period based on the baseline HbA1c categories. There was no significant relationship between insulin resumption and the baseline HbA1c levels (p=0.737). This shows that the possibility of insulin resumption is low in patients with relatively high postprandial blood glucose levels at baseline, which is consistent with the results of the logistic regression analysis described hereafter.

Predictive Parameters for the Resumption of Insulin Therapy After Initial Insulin Discontinuation and Failure of Treatment With Dulaglutide

A logistic regression analysis was performed to identify the predictive parameters for the resumption of insulin therapy for glycemic control in patients who switched to dulaglutide from insulin therapy, and the results are shown in **Table 2**. We included clinically significant traditional factors and established parameters that were significantly different between groups I and II based on the results in **Table 1**. The results showed that younger age, a lower total daily dose of insulin, and higher postprandial plasma glucose levels were associated with lower risks of the resumption of insulin use after switching to dulaglutide from insulin as shown in **Table 2**.

DISCUSSION

Individuals with T2D each have different pathogenic and clinical conditions in glucose metabolism (13). Interactions between genetic, environmental, and behavioral factors lead to considerable phenotypic variability, and this variability is reflected by heterogeneous responses to different drugs (8, 14). Considering both the limitations of insulin usage and the advantages of weekly GLP-1 receptor analog administration with respect to the ease of injection and therapeutic targeting toward the intestine, brain, and pancreas (15), we hypothesized the applicability of switching to weekly GLP-1 receptor analogs from insulin in patients with T2D whose HbA1c levels were 7.6% or higher. In this retrospective study of 138 subjects with T2D, there were two main findings. First, 20 patients with T2D (approximately 14.5%) could not tolerate or did not prefer weekly dulaglutide administrations (reasons included cost, gastrointestinal side effects, dissatisfaction with the drug), and 56 (approximately 40.6%) could successfully discontinue insulin and use either weekly dulaglutide or OHAs and demonstrated glycemic effectiveness after the switch. The mean HbA1c value in group II significantly reduced from 8.7% to 7.8%, and of the 56 group II patients, 23 (16.7%) patients could completely cease all injection therapies including dulaglutide and maintained stable glycemia over the 6-month period. Second, we found that older age, a higher dose of insulin at the time of switching to dulaglutide, and a low level of postprandial glucose were significant predictive factors for insulin resumption after switching from insulin to weekly dulaglutide.

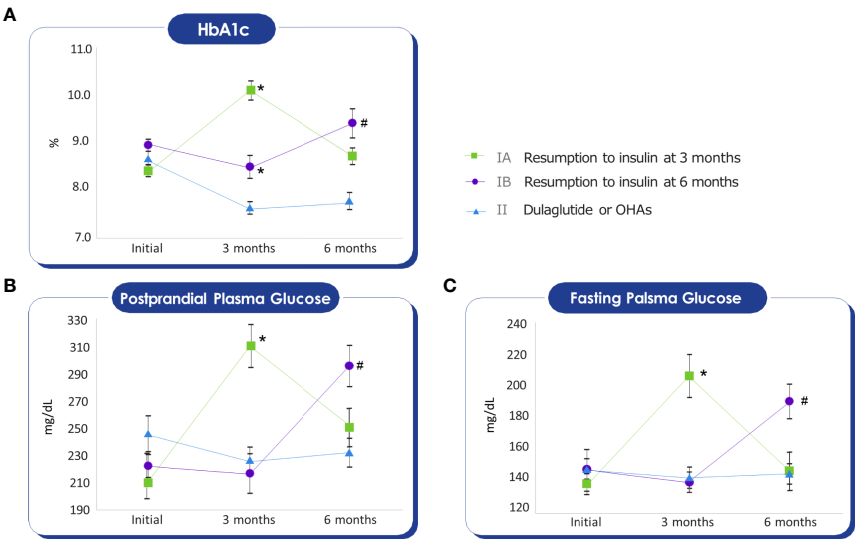

FIGURE 2 | Mann-Whitney test was conducted to compare the average of the glycemic parameters with Group II. Initial HbA1c, Fasting glucose, Postprandial glucose values do not show differences between groups. **(A)** HbA1c of Group IA, IB at three months, and HbA1c of Group IB at six months were higher than Group II. *p-value < 0.05, #p-value < 0.05. **(B)** Postprandial glucose of Group IA at three months, and Postprandial glucose of Group IB at six months were higher than Group II. *p-value < 0.05, #p-value < 0.05. **(C)** Fasting glucose of Group IA at three months, and Fasting glucose of Group IB at six months were higher than Group II. *p-value < 0.05, #p-value < 0.05.

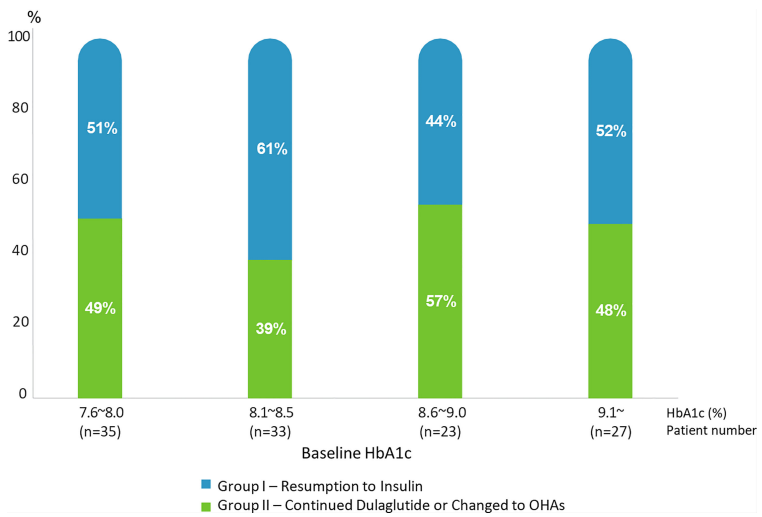

FIGURE 3 | Rates of Resumption to Insulin (Group I) and Continued Dulaglutide/Changed to OHAs (Group II) according to baseline HbA1c range.

Previous studies have investigated the effectiveness of switching from insulin to dulaglutide in reducing HbA1c levels and body weight in patients with T2D (16). In contrast to our study, the patients used a lower dose of insulin (about 20U/day), and approximately 94% of the enrolled patients used only basal insulin with OHAs. However, in our study, the average insulin dose was higher (mean total daily insulin dose, 48U/day), and approximately 78.8% of the patients were using premixed or basal–bolus insulin with prandial short-acting regimens. Moreover, the HbA1c level was also higher in our study compared to that in a previous study (8.6% vs. 8.2%). Regarding replacing preprandial short-acting insulin analogs

TABLE 2 | Logistic regression analysis of resumption to insulin after change from insulin to dulaglutide.

	Univariate				Multivariate			
	P-value	β[a] (standardized)	Confidence interval		P-value	β[a] (standardized)	Confidence interval	
Age	0.262	1.018	0.987	1.050	***0.035****	***1.044***	***1.003***	***1.086***
Sex	0.405	0.731	0.350	1.528	0.446	–	–	–
BMI	0.547	1.033	0.930	1.146	0.710	–	–	–
Duration of Diabetes	0.115	1.309	0.991	1.090	0.569	–	–	–
Total Insulin Dose	***0.001†***	***1.032***	***1.013***	***1.052***	***0.001****	***1.036***	***1.015***	***1.059***
HbA1c	0.457	0.861	0.581	1.277	0.532	–	–	–
Fasting plasma glucose	0.447	0.997	0.990	1.004	0.922	–	–	–
Postprandial glucose	***0.047†***	***0.995***	***0.990***	***1.000***	***0.022****	***0.993***	***0.988***	***0.999***
Fasting C-peptide	0.773	1.036	0.816	1.314	0.911	–	–	–
Postprandial C-peptide	0.395	0.920	0.759	1.115	0.397	–	–	–
eGFR	0.114	0.985	0.967	1.004	0.420	–	–	–
Hypertension	0.815	1.128	0.413	3.007	0.943	–	–	–
Dyslipidemia	0.384	0.528	0.126	2.221	0.592	–	–	–
Type of Insulin	0.473	–	–	–	0.572	–	–	–

*BMI, body mass index; eGFR, Estimated glomerular filtration rate † P-value < 0.05, Group I vs. Group II (Univariate logistic regression analysis), *P-value < 0.05, Group I vs. Group II (Multivariate logistic regression analysis). [a]Results expressed as standardized β coefficient.*
Bold values for Statistically significant values (P-value < 0.05).

with GLP-1 receptor agonists in poorly controlled glycemia despite intensive insulin regimens, the FLAT-SUGAR study indicated that basal insulin plus the mealtime administration of exenatide can be as effective in reducing HbA1c levels as basal–bolus insulin therapy (17). Kim et al. also reported that the dulaglutide and basal insulin combination therapy was as effective as basal–bolus insulin therapy in kidney transplant recipients with T2D (12). These studies indicated that basal insulin with GLP-1 receptor agonist therapy reduced the overall insulin dose and lowered body weight as compared to basal–bolus insulin therapy alone. This effect can be explained by the established glucose-dependent, glucose-lowering, and appetite-decreasing effects of GLP-1 receptor agonists that consequently result in marked reductions in postprandial glucose levels (15). However, few studies have investigated whether replacing insulin therapy with a combination of a GLP-1 receptor agonist and OHAs could be effective in patients with uncontrolled T2D receiving insulin therapy.

With respect to tolerability and the effectiveness of switching to weekly dulaglutide from insulin therapy, approximately 40.6% of the patients did not resume insulin treatment in this study. This unexpected high success rate of switching from insulin treatment to weekly GLP-1 receptor agonist therapy might be explained by the GLP-mediated improvement in insulin resistance and secretion, and its strong beneficial effect in controlling glycemic excursion (15). Low postprandial glucose levels at the time of switching were associated with a higher risk of insulin resumption after switching to dulaglutide. This can support the effectiveness of GLP-1 receptor agonists on postprandial glucose levels. Moreover, the frequency of injections dramatically reduced from more than one insulin injection per day to a weekly GLP-1 receptor agonist; therefore, this might lead to satisfaction and preference for the GLP-1 receptor agonist treatment in patients, and it may consequently enhance treatment compliance. Despite the effectiveness of insulin in controlling hyperglycemia, it has limitations as patients and physicians are reluctant to intensify insulin treatment due to side effects such as hypoglycemia, weight gain, and the inconvenience of frequent injections in clinical practice.

The predictive clinical factors for the resumption of insulin therapy to maintain the optimal glucose control in Korean patients with T2D were as follows: 1) total daily insulin dose at baseline, regarding daily insulin doses, we noted that using the receiver-operating characteristic analysis with a total daily insulin dose of >44U was the cutoff value for predicting insulin resumption, 2) postprandial glucose level, and 3) older age. It is expected that the baseline HbA1c and C-peptide levels may be the key predictive factors to resume insulin therapy after failure of change from insulin to dulaglutide or continue changing to dulaglutide. Interestingly, neither the initial HbA1c levels (when patients shifted from insulin to dulaglutide) nor C-peptide levels were independent factors predicting the resumption of insulin therapy in this study. Even in individuals with HbA1c levels > 9%, 48% of the patients were able to continue GLP-1 receptor agonist treatment and did not resume insulin treatment. We postulated that some enrolled patients may be poorly compliant with the insulin injections and lifestyle modification. Additionally, high insulin dosage and multi-daily insulin injections may contribute to poor compliance. Although information on the frequency or severity of hypoglycemia after treatment change is lacking, Dulaglutide may improve compliance by reducing unfavorable hypoglycemia, compared to insulin. Moreover, dulaglutide's beneficial effects on satiety factored into its effects in glycemic control independent from the baseline parameters. Finally, most patients that were included in this study still had sufficient insulin secretion and average HbA1c was approximately 8.5%. In this study, those who were successfully shifted to weekly dulaglutide had improved glycemic control. Based on the postulations, the baseline HbA1c and C-peptide levels were not the predictive factors in resuming insulin therapy after failure of changing from insulin to dulaglutide or continuing the switch to dulaglutidein this study.

The finding suggests that the switch from insulin therapy to GLP-1 receptor agonist therapy can be done in patients with T2D who are younger and have a relatively low dose of insulin and with relatively high postprandial glucose levels even if they have higher HbA1c levels upon changing to dulaglutide. However, we have come up with a few reasons concerning that they did not have great influence in our study.

The present study has several limitations that should be addressed in further studies. First, this study was designed as an uncontrolled, open-label, longitudinal, retrospective study, which is limited in its applicability and clinical relevance to generalization and broader clinical practice. However, our findings show the natural results of real-world practice that did not involve any interventions. Second, as the proposed switch therapy was not a guideline-based accepted approach, a relatively small number of patients were enrolled in this study. Furthermore, the short follow-up period is a limitation of our study, potentially limiting the generalization of our results. Moreover, the change in anti-diabetes medications before and after initiating dulaglutide treatment may also influence the final results. Third, we could not collect data on patient satisfaction, compliance or adverse events including hypoglycemia with dulaglutide use,. Nevertheless, the present study clearly demonstrated that the proposed switch method may benefit a significant number of patients even when hyperglycemia is uncontrolled with high doses and multiple injections of insulin.

In conclusion, dulaglutide, a weekly GLP-1 receptor agonist, can be used for glycemic control in patients with T2D with glucose levels inadequately controlled by insulin regimens. The tolerability and effectiveness of dulaglutide were not dependent on HbA1c level at the time of switching to dulaglutide from insulin. Older age, a relatively high daily insulin dose (> 44U/day), and a lower level of postprandial glucose at baseline were clinical predictive characteristics for the resumption of insulin after switching to dulaglutide. Large-scale, long-term, randomized controlled studies are needed to generalize our findings and to accurately analyze the efficacy of the present approach.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YK and JH contributed equally to this work. YK, study design, data acquisition, data analysis and interpretation, and manuscript writing. JH and B-WL, study design, data analysis and interpretation, and manuscript writing. ML, EK, and B-SC, data analysis and interpretation. All authors have read and approved the final manuscript.

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REVIEW
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Neurovascular Coupling in Type 2 Diabetes With Cognitive Decline. A Narrative Review of Neuroimaging Findings and Their Pathophysiological Implications

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Type 2 diabetes causes substantial long-term damage in several organs including the brain. Cognitive decline is receiving increased attention as diabetes has been established as an independent risk factor along with the identification of several other pathophysiological mechanisms. Early detection of detrimental changes in cerebral blood flow regulation may represent a useful clinical marker for development of cognitive decline for at-risk persons. Technically, reliable evaluation of neurovascular coupling is possible with several caveats but needs further development before it is clinically convenient. Different modalities including ultrasound, positron emission tomography and magnetic resonance are used preclinically to shed light on the many influences on vascular supply to the brain. In this narrative review, we focus on the complex link between type 2 diabetes, cognition, and neurovascular coupling and discuss how the disease-related pathology changes neurovascular coupling in the brain from the organ to the cellular level. Different modalities and their respective pitfalls are covered, and future directions suggested.

Keywords: type 2 diabetes (T2D), cognitive decline, neurovascular coupling (NVC), neuroimaging, alzheimer's disease

INTRODUCTION

Driven by changing demographics and lifestyle factors, diabetes mellitus will affect half a billion people worldwide within a few decades, with severe economic and personal consequence (1). A recent study estimated the projected number of adults with diagnosed diabetes to increase from 22 million to 61 million in 2060 in The United States (2). In the setting of strained health care provision

with exigent, concurrent demands for efficiency and quality, providing optimum care and preventing comorbidity in diabetes is challenging.

Type 2 diabetes (T2DM) is by far is the most prevalent of the two subtypes of diabetes, making up 90-95% of cases (3). It develops as a result of impaired beta-cell function in combination with insulin resistance in the tissues. The resulting hyperglycemia, along with dyslipidaemia and hypertension, has detrimental effects on many organ systems (4). T2DM causes substantial long-term morbidity with late diabetic complications from the eyes, kidney and nervous system as well as increased risk of arteriosclerosis (5). One clinical manifestation which is receiving increasing attention is cognitive impairment or so-called diabetic "cogno-pathy" (6). T2DM has been identified as an independent risk factor for cognitive decline evolving into manifest Alzheimer's disease (AD) (7–9). T2DM patients with elevated HbA1c levels (10), and intriguingly non-diabetics with acute elevated blood glucose levels (11) as well as cognitively intact adults with pre-diabetes (12), have decreased metabolism in brain regions characteristic for AD. Furthermore, possible effects on AD of anti-diabetic drugs such as pioglitazone, which reduce insulin resistance or the Glucagon-Like Polypeptide-1 receptor agonist (GLP-1 RA), with an effect on low-grade inflammation, are being investigated (13). These findings motivate the search for biomarkers that are sensitive to early functional brain changes on which prognosis and intervention can be based.

Decades of intense research, including studies on patients with stroke or headache, have advanced our understanding of the physiological regulation of the brain's blood supply and its pathophysiological relevance (14). However, an obstacle in understanding the physiology is that with decreasing vessel diameter, it becomes increasingly difficult to probe regulatory mechanisms and study the tight functional interactions between vessels, neurons and glia (15).

The concept of neurovascular coupling (NVC) describes a cellular mechanism by which neuronal activation induces concurrent local increases in cerebral blood flow (CBF). These local increases of blood supply are critical to brain function, and impaired NVC may play an early role in triggering cognitive dysfunction in T2DM (16). In diabetes, cognitive ability is influenced by multiple factors at the systemic level, such as the degree of extra- and intra-cranial atherosclerosis, dysfunction of glymphatic tissue clearance (17), and cellular dysfunction, such as altered receptor expression. The identification of neurovascular abnormalities that are attributable to diabetes and precede structural and clinical changes, holds the potential to guide personalized, preventive interventions (18).

In this narrative review, we focus on how NVC is impaired by T2DM and how we can measure T2DM-related neurovascular dysfunction in humans (**Figure 1**). We will first provide a brief introduction to diabetes, cognitive decline, and the neurovascular architecture. We will then discuss current concepts of how diabetes affects NVC and in what way this relates to cognitive decline. In the last section, we will review commonly employed methodology that has contributed to our understanding of NVC and its alteration in T2DM, with a focus on magnetic resonance imaging (MRI). It bears mentioning that while a detailed understanding of individual cellular mechanisms is within reach in bench models, translating and relating this to clinical or pre-clinical observations is not always possible.

COGNITIVE DECLINE IN THE SETTING OF DIABETES

The relative general prevalence of dementia in individuals aged over 60 years is 6-7% (19). Diabetes confers a 1.3 to 1.9-fold increased risk of cognitive impairment, but even pre-diabetes and diabetes-associated biochemical changes (fasting glucose,

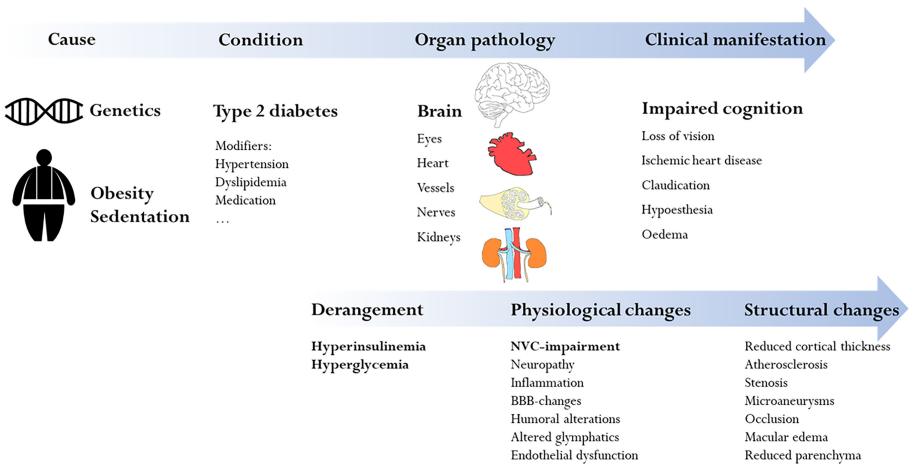

FIGURE 1 | Overview of the complex and multifactorial mechanisms which lead to impaired cognition in type 2 diabetes. The focus of this review is the specific diabetic influence on neurovascular coupling and how this leads to impaired cognition (bold in the figure).

postload glucose, glycosylated hemoglobin, insulin) predict cognitive decline (20). Also, diabetes is one of nine potentially modifiable risk factors modelled by the 2017 Lancet Commission on dementia prevention, intervention and care (21). Cognitive decline encompasses subjective cognitive decline [reviewed in (22)], mild cognitive impairment and manifest dementia with AD being the most frequent underlying disease (**Table 1**). AD can be divided into AD-pathophysiological process (AD-P), which precedes the clinical phase (AD-C), and may include patients with cognitive impairment due to AD-P before clinical onset (25). The pathology behind AD is complex, involving neuroinflammation and accumulation of β-amyloid and tau protein leading to neuronal death and atrophy in specific cortical areas (26). Thus, risk factors include T2DM and genetics, among others, but the single most relevant is age (27).

DEFINITION OF COGNITIVE DECLINE

The mechanism by which diabetes induces cognopathy was originally ascribed to vascular changes but this model is too simple as multiple vascular and non-vascular processes act in concert (28) (**Figure 2**). It has been demonstrated that cognition can be affected by hyperglycemia, changes to insulin secretion and sensitivity, T2DM complications, comorbidity as well as certain medications. New findings also show that the diabetes-induced metabolic milieu is specifically conducive to AD-P processes with greater β-amyloid plaque and tau deposition, advanced glycation end products and activated microglia in diabetic AD compared to non-diabetic AD (29, 30). At the pathophysiological level, multiple mechanisms have been implicated, including impaired NVC, a malfunction of cerebrovascular autoregulation (31) and glucose transport (32), neuroinflammation (33) and insulin resistance (9). Of note, insulin crosses the blood-brain barrier (BBB) and acts as a neuropeptide in the central nervous system having distinct neuromodulatory effects on key brain structures (34). Animal studies have shown that insulin targets astrocytes (35) and has trophic actions promoting synapse growth, neuron maintenance and repair as well as improving hippocampal synaptic plasticity (36). These findings indicate an intimate relationship between diabetes and AD which led to the proposal to consider AD as "type 3 diabetes" (37), and prompted clinical trials testing the efficacy of anti-T2DM drugs such as liraglutide (38), thiazolidinediones (39), intranasal insulin (40) and metformin (41) in AD with positive preliminary results (13).

TABLE 1 | Operational definitions of cognitive decline.

Diabetic "cognopathy" (6)	Research term referring to cognitive impairment (e.g., memory impairment, reduced psychomotor speed, affected executive function, verbal fluency and attention) that is attributable to diabetes mellitus, typically associated with functional and structural changes in the brain
Subjective cognitive decline (23)	1. Self-experienced persistent decline in cognitive capacity in comparison with a previously normal status and unrelated to an acute event. 2. Normal age-, gender-, and education-adjusted performance on standardized cognitive tests, which are used to classify MCI or prodromal AD. 1 and 2 must be present Exclusion criteria: • Mild cognitive impairment, prodromal AD, or dementia • Can be explained by a psychiatric or neurologic disease, medical disorder, medication, or substance use
Mild cognitive impairment (MCI) (24)	Measurable cognitive impairment without effect on activities of daily living. This diagnostic label is applied if there is no disease to which MCI can be attributed. Term of exclusion.
Alzheimer's disease (AD) (24)	Progressive cognitive decline (i.e., impaired memory) Preserved consciousness Disrupted emotional control Duration of at least 6 months *In-vivo* markers of Alzheimer's pathology: Corticospinal fluid (CSF): amyloid β, total tau, and phospho-tau Positron emission tomography (PET): Regional accumulation of amyloid and tau tracers, reduced mid-temporal and mid-parietal glucose metabolism Structural magnetic resonance imaging (MRI): Atrophy of medial temporal lobe, medial parietal cortex

AUTOREGULATION AND NEUROVASCULAR COUPLING

Precise spatial and temporal titration of CBF supply is critical to brain function. Cerebrovascular autoregulation stabilizes regional cerebral blood flow by sheltering it from fluctuations in systemic perfusion pressure. This mechanism is partly intrinsic to smooth muscle cells at the pial arteriole/parenchymal section, which relax and contract according to the transmural pressure, referred to as the myogenic response (42). NVC refers to a separate mechanism ensuring that perfusion is adjusted to the neuro-metabolic demands at the cellular level. NVC can be thought of as a variable resistor that works at the level of parenchymal arterioles "in series" with, and at the level of pial vessels, "in parallel" with cerebral autoregulation.

Innervation of Cerebral Vasculature

The density and nature of regional innervation of the cerebral vasculature varies considerably depending on the lobe and segment of the vascular tree. Based on fiber origin, the innervation can be divided into intrinsic and extrinsic projections. The perivascular nerves of the adventitia of pial arteries and arterioles are external, while microvessels along with interneurons and astrocytes receive intrinsic innervation (43).

The intrinsic neurovascular supply originates from the locus coeruleus (noradrenaline), the raphe nuclei (serotonin), the ventral tegmental area and the nucleus basalis (acetylcholine). These projections from the basal nuclei innervate the vasculature and the cells of the neurovascular unit, particularly astrocytes, without leaving the brain (43). Although its role in NVC is poorly understood, relevant receptors are present on the

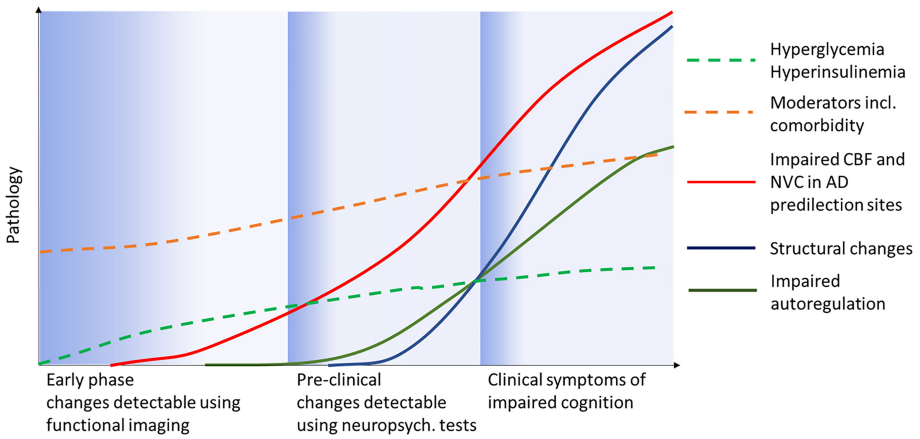

FIGURE 2 | Possible scenario for clinical progression (x-axis) from pre-diabetes to manifest clinical diabetic "cognopathy". Moderators and comorbidity represent all diseases and factors present before and after T2DM onset such as sleep apnea, obesity, hypertension etc. Adapted from Jessen and colleagues (27).

involved cells and CBF changes can be invoked in response to stimulation of the mentioned nuclei (43).

The extrinsic neurovascular supply system is composed of sympathetic, parasympathetic and sensory nerve fibers (44) running in the adventitia of pial arteries and arterioles (45). These fibers, which predominantly originate in the superior cervical ganglion (sympathetic), otic and sphenopalatine ganglia (parasympathetic) and the trigeminal ganglion (sensory), follow various paths including the ethmoidal nerve to re-enter the cranial cavity. These systems are controlled by brainstem and mesencephalic circuits and seemingly have no major function in autoregulation during physiological conditions (46). However, in certain states such as hypercapnia-induced vasodilation or chronic conditions they do exert influence (47). In hypertension, sympathetic innervation extends autoregulation to higher pressures and may protect the brain against pressure surges whereas sensory innervation may serve a protective role restoring vessel tone after constriction (48, 49).

The Neurovascular Unit

The neurovascular unit (NVU) consists of a set of cells that intimately interact to enable a temporal and spatial NVC and secures that local blood supply is rapidly aligned to moment-to-moment changes in regional neural activity and associated fluctuations in metabolic demand and waste production (45). The cells involved are neurons (pyramidal cells and interneurons), astrocytes, smooth muscle cells, endothelial cells, and pericytes (45). Depending on the ongoing level of regional activity, neurons and astrocytes release vasodilatory factors that act directly on the perivascular cells to induce vasodilation and increase local arterial blood supply.

NVC involves five consecutive steps: Initiation, modulation and spatial shaping, neurovascular transmission, retrograde propagation and implementation (**Figure 3**) (45). It is noteworthy that influences at the arteriolar and capillary level differ since arterioles, as opposed to capillaries, are not only subject to locally mediated vasodilation in response to neuronal activation, but also to retrograde propagation from capillaries which also reaches pial arteries (45). NVC employs both feed-forward (glutamate receptors, Ca^{2+}, nitric oxide (NO), eicosanoids) and feed-backward (adenosine, lactate and CO_2) signaling mechanisms, and the many messengers involved provide redundancy and condition-dependent signaling reflecting the previous and current state of the system (50).

Neurons. About eighty percent of the brain's energy expenditure is attributed to the generation of action potentials in neurons, maintenance of ion-concentrations and postsynaptic effects (51). Any perturbation of cellular supply compromises the signaling function of neurons. To secure sufficient neuronal blood supply, neurons directly control their own homeostatic environment through glutamate actions on N-methyl-D-aspartate (NMDA)-receptors which, *via* downstream signaling and nitric oxide synthase (NOS)-activation with subsequent increase in NO-synthesis, induces vasodilation (52). The same glutamate signal also activates receptors on neighboring astrocytes.

Although most cortical neurons in the cortex are glutamatergic pyramidal cells, gabaergic inhibitory interneurons are also relevant. The gabaergic interneurons project to microvessels influencing the release of NO, prostanoids, endothelin among others (53). The relative contribution of pyramidal cells or interneurons to NVC likely depends on multiple factors including location and the neuronal circuit in question and requires further investigation (54).

Astrocytes are glial cells of key importance to NVC by virtue of their perisynaptic and endfeet processes extending from their soma (55, 56). They exert differential control according to the metabolic state of the tissue through constriction and dilation control pathways (57). *In vitro* studies have cast light on the multiple factors influencing the balance between these

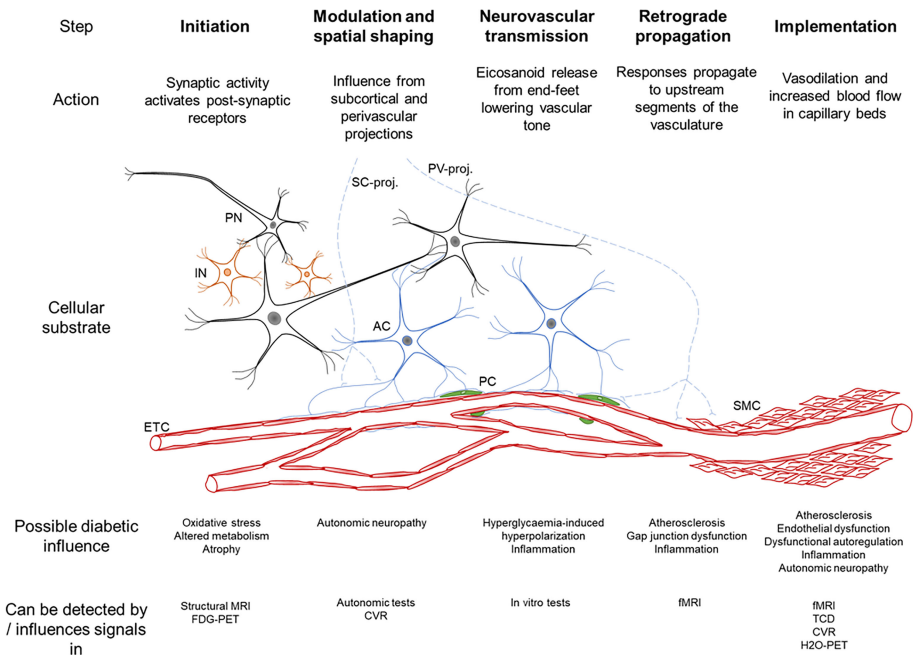

FIGURE 3 | Schematic summary of the neurovascular coupling cascade (46). The cellular substrate for each step is seen, PN – principal neuron, IN – interneuron, AC – astrocyte, PC – pericyte, SMC – smooth muscle cell, ETC – endothelial cells, SC-proj. – subcortical projections from locus coeruleus, basal forebrain, raphe nuclei, PV-proj. – perivascular projections from cranial autonomic ganglia. The bottom rows describe the possible influence of diabetes on each step and how this has been detected.

vasoconstrictors and -dilators including previous vascular tone (58), NO (59), O_2, lactate and adenosine (57). As the primary neurotransmitter, glutamate in itself activates specific astrocyte group 1 metabotropic glutamate receptors (mGluR) leading to increasing calcium concentrations which in turn forces release of vasoactive substances (dilator and constrictor eicosanoid gliotransmitters) from astrocyte endfeet proximate to the smooth muscle cells (SMC) lining the vessels (60). Phospholipase A_2 activation induces release of arachidonic acid which is converted to prostaglandins (for example PGE_2, PGI_2) and epoxyeicosatrienoic acids (EETs). These reduce vascular tone *via* prostaglandin receptor activation and TRPV4- and BK_{Ca}-channels. Detrimental increase of vascular tone happens when arachidonic acid is converted to 20-hydroxyeicosatetraenoic acid (20-HETE) which may occur pathologically (61). It has been observed that under physiological conditions simultaneous activation of both neurons and astrocytes induces a 4-fold increase in local CBF than the increase in ATP (60 vs 15%) which is indirectly supportive of a feed-forward mechanism (62).

Smooth muscle cells, pericytes and endothelium. Smooth muscle cells and pericytes make up the vasomotor apparatus of the NVU (45). These cells ultimately determine vascular tone on the basis of neuronal, astrocytic and possibly intrinsic system influence (43). In cerebral capillaries, pericytes replace smooth muscle cells and also serve to maintain structure and BBB (63). Pericytes likely exist in both contractile and non-contractile variations (64) and are interspersed at regular intervals along these vessels (65). Although they have been shown to dilate and constrict in response to various stimuli (66), including amyloid β (67) and during mild CO_2-challenges (68), results are divergent and their contribution to NVC is debated (69, 70). Recent results suggest a substantial but slow regulation of capillary diameter by pericytes, again introducing a serial layer of control of tissue perfusion (68).

The endothelium itself possesses strong intercellular vasodilators including NO and endothelin and has gap junctions with vasomotor cells ensuring retrograde propagation (45). The labile NO itself seems to exert influence dependent on its concentration dynamics but *in vivo* studies are sparse (71). Recent research has also identified a caveolae-mediated pathway in arteriolar endothelial cells as a major mechanism of neurovascular coupling. Caveolae are invaginations of the plasma membrane that are specifically abundant in arteriolar endothelial cells and mediate NVC independently of endothelial NOS (72). It has been hypothesized that caveolae in the arteriolar endothelial cells may serve as local clusters for ion channels and receptors that convey vasodilatory signals to adjacent smooth muscle cells (72). A recent study in mice found that arteriolar endothelial cells are unique in that they possess abundant caveolae (72). It seems this caveola-specific function in NVC acts independently of the NOS-pathway described above as partial ablation of either NOS or caveolae both partially impair NVC. Ablation of both pathways induces complete decoupling (72).

THE INFLUENCE OF DIABETES ON CEREBRAL BLOOD FLOW

While it is now generally accepted that diabetes mellitus affects NVC, it remains a challenge to dissect the contributions of chronic hyperglycemia, dysinsulinemia and other modifiers such as hypertension, aging and still other variables (**Figure 1**, also, see section on investigations in humans below). This has been exemplified in the attempt to disentangle diabetic and pre-diabetic vascular complications from the associated (sub-) clinical manifestations (73).

Hyperglycemia itself has acute and chronic adverse effects on NVC. In humans, acute hyperglycemia reduces light-flicker induced vasodilation of retinal arteries (74). Such impairment has been confirmed in animal models pointing to hyperpolarization at the gliovascular interface as the possible mechanism (75–77). This would occur when neuronal activation and subsequent Ca-increase in the endfeet result in potassium-increase and Kir-channel activation in adjacent smooth muscle cells (76). Implicating the NOS-pathway, administration of sodium nitroprusside (an NO donor), seems effective in ameliorating such hyperglycemia-induced decoupling (77). Whether manipulating NO-pathways in humans represents a valid therapeutic avenue remains to be seen.

Increased glucose concentrations also induce oxidative stress and compromise the function of gap junctions of *in vitro* astrocytes (78). Oxidative stress represents an important pathogenetic factor that is shared between T2DM and AD, contributing to endothelial and microvascular dysfunction with neurovascular uncoupling in T2DM (79) and increased amyloid-β deposition in AD (80). Hyperglycemia has also been shown to increase tau phosphorylation in hippocampal neurons of diabetic rats, involving a reduced expression of caveolin-1, the essential structure protein of caveolae, mentioned above, and activation of the mTOR/S6K signaling pathway (81). Namely caveolae represent a recently discovered research target with particular relevance for NVC.

Transcranial doppler (TCD) and functional MRI studies dominate the available clinical knowledge but findings are not entirely congruent (16). Phase-contrast MR measurements did not identify global CBF differences between T2DM patients and controls although it did correlate with cognitive ability (82). Also, global CBF did not predict changes over time suggesting that deteriorating cerebral perfusion does not drive cognitive decline (83). Regional blood flow assessed using arterial spin-labeling (ASL)-MRI confirmed these findings to a degree with comparable CBF reductions in patients with T2DM with subjective cognitive decline, vascular dementia and AD compared to controls (84). However, impaired glycemic control was related to reduced CBF hinting at a possible specific diabetes mechanism.

Overall, these results indicate that CBF-changes and cognitive decline in T2DM are determined by tertiary risk factors and not a particular T2DM pathology. Conversely, other studies have found compelling evidence for T2DM specific changes. Cerebral hypoperfusion has been shown with ASL-MRI in individuals with T2DM (85–87). While the magnitude of hypoperfusion varies, it correlates with cognitive declineClick or tap here to enter text.. In one study, an interaction between hypoperfusion and hypertension suggests that increased blood pressure may precipitate the CBF-decrease, possibly involving compromised autoregulation (87). In these populations, brain atrophy was comparable to controls suggesting that altered perfusion precedes structural changes. Another ASL-study investigated healthy controls, patients with insulin resistance (but not diabetes) and T2DM patients (88). Here, CBF fluctuated with spontaneous end-tidal CO_2 indicating intact cerebrovascular reactivity (CVR) in manifest T2DM and healthy controls, but not in unmedicated patients with insulin resistance. This was speculated to be attributable to glucose-lowering medications, statins and antihypertensives, thought to increase NVC in the diabetics but not the unmedicated insulin resistance group.

Regional low-grade neuroinflammation represents another overlap between neurodegeneration and diabetes (89)It is likely that the mechanism leading to CNS insulin resistance in Alzheimer's disease, diabetes and obesity in general is the same and involves such persisting low-grade neuroinflammation. This is likely caused by recruitment of macrophages and secretion of an "inflammatory soup" with cytokines such as TNF-α, IL-1β and IL-6 (90). The initiation of the inflammation cascade can be ascribed to various elements including toxic metabolites, ischemia, infection, trauma. This is a difficult research target as it likely occurs over several years and before symptom onset. Neuroinflammation may induce exaggerated vasoconstriction and diminished vascular reactivity (89). In humans, a prospective ASL-study in T2DM patients supported this view (91). Using CO_2-rebreathing to assess CVR, prospective rCBF measurements showed diminished reactivity after just two years which was associated with a decrease in cognitive ability (91). This T2DM group also had higher inflammation markers, the levels of which corresponded with decreases in vasoreactivity independently of glycemic and blood pressure control.

While ASL provides measures of regional blood flow, functional MRI (fMRI) maps regional changes in the blood oxygen level-dependent (BOLD) signal (92). The regional BOLD signal, with some caveats (see below), can be used to specifically assess regional NVC at the tissue level (93). BOLD fMRI in early T2DM revealed changes in the hemodynamic response function were observed indicating deterioration of NVC (94). Using similar methods in a breath-hold paradigm, Tschistiakova and colleagues showed that T2DM patients with hypertension had decreased CVR and cortical thickness compared to patients with only hypertension, again suggesting a specific T2DM effect on NVC (95). Hu and colleagues pioneered co-analyzing resting state fMRI and ASL data, an elaboration upon methods previously applied in schizophrenia and depression, to develop specific NVC biomarkers (96, 97). They found that several of these hybrid markers were decreased regionally in T2DM patients without cognitive impairment which might identify patients where early intervention would arrest a pathological cerebrovascular trajectory.

Astrocyte involvement in maintaining BBB also deserves mention in this context. White matter lesions (WML) are

associated with increased risk of dementia and cognitive decline as well as stroke (98). Their cause is disputed but may relate to BBB dysfunction (99). In T2DM, cognitive decline is associated with WMLs, atrophy, infarcts and HbA_{1c} and BBB permeability may be increased in these patients (100, 101). Thus, there is an indirect connection between WMLs and BBB disruption in the setting of T2DM with cognitive decline, however, findings are not homogenous and further studies are needed as the specificity of these changes is debatable (102).

Effect of Antidiabetic Treatment on NVC

Therapeutic manipulation of impaired NVC is in its infancy. Aside from improving vascular health, other interventions may protect against cognitive decline (103). Resveratrol may acutely enhance cerebrovascular responsiveness, as measured by TCD, and possibly also clinically measures of cognition but findings need to be reproduced (104, 105). In diabetic mice, empagliflozin, a sodium glucose transporter inhibitor, ameliorated detrimental structural changes in the NVU (106), possibly through a specific action on astrocyte foot process detachment (107). Other drugs may have detrimental effects, for example, non-steroidal anti-inflammatory drugs including indomethacin and naproxen have been shown to attenuate NVC (108). Semaglutide, a long-acting GLP-1 analogue, which is very effective in T2DM, is entering phase 3 development for the indication of Alzheimer's disease. Both clinical and preclinical studies have shown promising results with regards to this drug's effect on cognitive decline (109, 110). Several mechanisms have been suggested and it is particularly interesting that these drugs may have anti-inflammatory properties (111).

INVESTIGATING NEUROVASCULAR COUPLING IN HUMANS

In humans, NVC is studied non-invasively using MRI, positron emission tomography (PET) and TCD. NVC is typically studied as the regional or global response in blood flow to various forms of stimulation. In the following section, we discuss the strengths and weaknesses of relevant modalities to probe NVC.

Transcranial Doppler Ultrasound

TCD is highly accessible, non-invasive, safe and provides measurements in real-time with high temporal resolution (112). Blood flow velocities in the major arteries are measured as regional CBF surrogates and evoked changes are typically in the range of 10-20% in the posterior and 5-8% in the middle cerebral artery (50, 113). Vessel diameter changes with blood gas composition and during hypercapnia, metabolic regulation of the NVC is reduced during passive flexion of the arm (114). Consequently, changes are reliable only if vessel diameter is unchanged which may be fair to assume when looking at rapid responses (115). However, end-tidal CO_2 does fluctuate on a breath-by-breath basis and CBF measurements by TCD may be falsely lowered during hypoxia and hypercapnia (116). Also, reliable measurements require user experience and while being highly accessible, portable and non-invasive this method's sensitivity and reproducibility may be lower and is highly user-dependent.

Positron Emission Tomography and Single-Photon Emission Computed Tomography

The tracer employed defines the usefulness of PET to study biological processes. With regards to studying metabolism and blood-flow ^{18}F-fluro-deoxy-glucose (FDG) and ^{15}O-H_2O water are gold standard. Amyloid tracers are available and used clinically in the diagnosis of AD. Cellular FDG-uptake is representative of glucose metabolism in the 20-30 minutes following tracer injection with a theoretical spatial resolution of around 4-6 mm. While the time resolution is low, this method allows investigation of *neurometabolic* coupling (change in cerebral metabolic rate vs. blood flow ($\Delta CMR_{Glu}/\Delta CBF$)). NVC *per se* is not always defined in the same manner and FDG-PET as well as calibrated BOLD (see below) allows for more stringent measurement of the neuronally-induced change in CBF at an arteriolar and capillary level whether this is defined as neuro-metabolic or neurovascular coupling.

The freely diffusible ^{15}O-H_2O, as detected by PET, is the gold standard for minimally-invasive measurement of blood flow and can detect transient phenomena of around 30 sec. Academically, it has been discussed whether glucose metabolism is an accurate proxy for neuronal activation. However, at least in health, perfusion in the CNS is closely coupled to metabolism. Indeed, the case has been made that regional CBF increases during activation is driven primarily by coupling to glucose metabolism whereas oxygen consumption increases are less pronounced (117). The PET-modality can be combined with CT or MR to give hybrid measurements of CBF and brain anatomy and function. The major drawbacks are the use of ionizing radiation and limited availability.

Magnetic Resonance Imaging

Compared to PET, fMRI has the advantage of excellent spatial resolution and not using ionizing radiation although sometimes contrast agents are required. Several MRI methods are relevant in the study of NVC including BOLD, ASL and phase-contrast fMRI. Gadolinium has been used to evaluate the intactness of the BBB. Cardiovascular reactivity has been assessed using ASL and BOLD fMRI during breathing of CO_2-enriched gas, breath-holding or rebreathing.

Arterial Spin Labeling

ASL quantitates regional CBF without use of contrast or radiation. The method labels blood water molecules in a slab and tracks them circulating the brain. Clinically, ASL can distinguish normal brains from AD (118) and in the research setting it directly allows evaluation of NVC during a neurostimulation paradigm. Discussion is ongoing whether regional CBF, measured by ASL, correlates with oxygen and glucose consumption. However, FDG-PET and ASL-MRI correlate in CBF and the cerebral metabolic rate of oxygen (CMR_{O2}) and glucose metabolism ($r=0.54$, $p<0.0001$ and

r=0.31, p=0.005 respectively) indicating that ASL-CBF reflects oxygen and glucose metabolism (119, 120). Combining fMRI and ASL, functional and CBF maps can be assessed together and may provide more specific markers for neurovascular decoupling (96, 97).

Blood Oxygen Level Dependent MRI

BOLD fMRI can capture the regional vascular response to neuronal activation with high temporal and spatial resolution in heavily T2*-weighted sequences (**Figure 4**). To use this signal as a measure for NVC requires factoring in dynamics of CBF, volume and cerebral metabolic rate for oxygen ($CMRO_2$). It is a proxy for neuronal activation and its validity relies on intact physiological cascades. The BOLD-signal reflects the uniformity of the magnetic field in response to paramagnetic deoxyhemoglobin washout from the capillary bed. Thus, deoxyhemoglobin can be thought of as an endogenous contrast agent. With no NVC, neuronal activity would result in increased deoxyhemoglobin and a decreased BOLD signal. However, NVC induces an overcompensating flow increase leading to a relative *decrease* of deoxyhemoglobin and an *increase* in the BOLD signal. That NVC is likely initially driven by glutamate-release and not O_2 consumption, means that theoretically BOLD is a measure of the intactness of NVC as induced by synaptic activity (15). Thus, reduced BOLD signals can indicate decreased neuronal activation or dysfunctional NVC at some point in the cascade. To mitigate other influences BOLD signals can be evaluated in conjunction with ASL and a vascular challenge, a combination also called calibrated BOLD (121). This combination allows disentanglement of the neuro-metabolic response from the vascular CBF response to a stimulus, that is $\Delta CMRO_2/\Delta CBF$. Other methods have been used to disentangle vascular and neural factors including normalization to baseline CBF or CVR; comparison with other neuronal markers such as electro-encephalogram, magnetoencephalography and PET; and statistical modelling (122). Event-related approaches and performance-matched stimuli are likely preferable in group comparisons (94, 96, 97).

Phase-Contrast MRI

Phase contrast MRI has been used to assess blood velocity or bulk flow in supplying vessels and is based on the principle that hydrogen nuclei moving through a magnetic field gradient will acquire a velocity-dependent phase shift. Together with brain volume, acquired from a structural scan, CBF per ml brain tissue per minute can be obtained and corrected for brain tissue density. The method provides absolute measures of global blood flow with a high temporal resolution and without requiring a contrast agent.

Dynamic Contrast-Enhanced MRI

Lastly, pericyte control of microcirculation may be affected and a measure for their function may be capillary transit time heterogeneity which can be measured with dynamic contrast-enhanced MRI (123). The biological basis is an increase in transit time heterogeneity following neuronal activity and consequent capillary recruitment. Theoretically, compromised regulation of this capillary dilation, as would be expected to be present in T2DM-mediated neurovascular uncoupling, would manifest as increased capillary transit time heterogeneity. This has not yet been investigated in T2DM to our knowledge.

Technical Considerations

Technical limitations need to be considered when measuring regional cerebral activation and blood flow simultaneously and contribute to the heterogeneity of NVC investigations. Aging has its own detrimental effects on cerebral hemodynamics (124) and increasing age is associated with increasing prevalence of

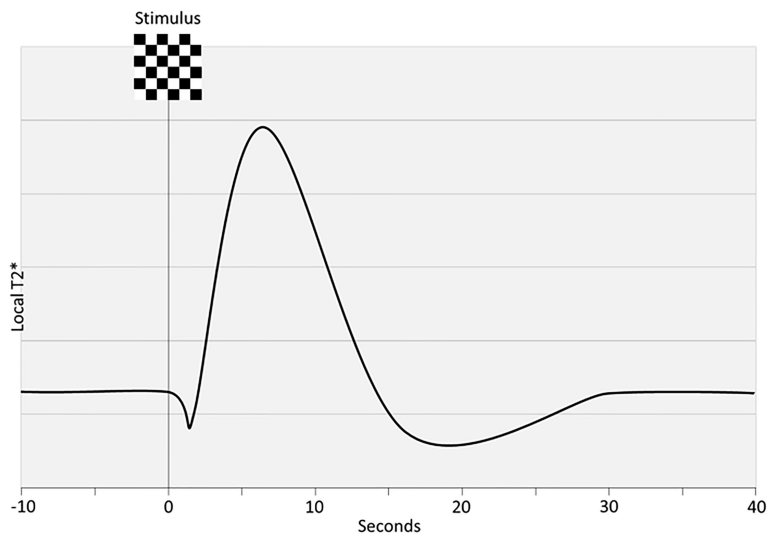

FIGURE 4 | Idealized BOLD response. Steady-state is disrupted by a stimulus which results in an initial dip, an overshoot and lastly an undershoot before steady-state is re-acquired. The rising curve reflects an increase in magnetic field uniformity due to washout of paramagnetic deoxyhemoglobin.

comorbid conditions such as hypertension (125) and obesity (126). Arterial vascular pathology produces multiple secondary effects, lowering capillary density, disrupting the BBB, damaging the endothelia, reducing contractility, increasing pulsatility and compromising retrograde propagation (122). In some of these cases, CO_2-enriched air may trigger vascular steal phenomena which necessitates evaluation of the global vascular haemodynamics. Other variables such as time of day, level of arousal, alcohol, caffeine, exercise, menstrual phase and medications also affect measurements (127). Age, sex and body-mass index influence cardiac output distribution to the brain but complete correlation between neurocognitive and neurovascular ageing is not given and reduced CBF in the elderly does not seem to result from age-related decreases in cardiac output (128). Further, age likely affects glia and neurons differently and changes may be driven more by one group of cells.

Lastly, as stated above, each modality has distinct characteristics with regards to temporal and spatial resolution. With regards to temporal resolution, ultrasound has the highest and PET the lowest. With regards to spatial resolution, with some variation, fMRI and ASL are likely superior. However, a typical fMRI pixel size of around 3-4 mm is still far from the approximately 200 μm at which some mechanisms have been described for NO-mediation of NVC between neurons and arterioles in the rat hippocampus (71). The anatomical substrate is present since both neurons and smooth muscle cells can co-inhabit a space of this size (129). While higher-field systems may provide greater spatial resolution they are still no substitute for the insights which invasive animal studies can provide.

CONCLUSIONS AND FUTURE RESEARCH TRAJECTORIES

The cellular mechanisms regulating NVC are complex and still incompletely understood. Each modality used to measure NVC in humans has its limitations, and the multiple confounding variables need to be considered in the population of interest. Despite of these limitations, there is converging evidence for an independent effect of the T2DM-state on NVC with cognitive decline as a possible progressing clinical correlate. Potentially, all steps of the NVC-cascade may be affected by separate diabetes-induces changes and currently it is impossible to discern which are clinically relevant. Further, how the induced pathological changes precisely affect measurements of the discussed modalities needs clarification.

Early detection of impaired NVC in T2DM patients could represent an opportunity for initiation of preventive treatment before irreversible damage occurs, especially since it is plausible that novel therapeutics may directly or indirectly involve NVC. Future studies could explore subgroups of T2DM where specific aspects of CBF control may be compromised such as those with autonomic neuropathy. NVC effects of medications such as pioglitazone and GLP-1 receptor agonists with effects on insulin sensitivity and low-activity inflammation commonly used in diabetes also need further exploration.

AUTHOR CONTRIBUTIONS

MB wrote the first draft of the manuscript. EP, SM and HS wrote sections of the manuscript. All authors contributed to conception and design of the review, manuscript revision, read, and approved the submitted version.

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The clinical characteristics of Chinese elderly patients with different durations of type 2 diabetes mellitus

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Aims: To explore the clinical characteristics among elderly (aged ≥60 years) patients with type 2 diabetes (T2DM) of different durations.

Methods: Clinical characteristics were investigated in 3840 elderly T2DM patients according to their different durations of diabetes (< 1 year, 1~5 years, 5~10 years, and ≥ 10 years). Kruskal-Wallis and Dunn tests were used to assess the differences among groups for continuous variables. The chi-square and *post hoc* tests were carried out for dichotomous variables. The logistic regression was adopted to investigate the relationships between various durations of diabetes and the control rates of achieving the control targets for T2DM as well as diabetic vascular complications.

Results: There were 972, 896, 875 and 1097 patients with a duration of diabetes of <1, 1~5, 5~10 and ≥10 years, respectively. In logistic regression models adjusted for age, sex, education, BMI, smoking and family history of diabetes, elderly T2DM patients with a duration of diabetes of ≥10 years were more likely to reach the comprehensive control targets for TC (OR_{TC} = 1.36, 95% CI =1.14-1.63), LDL-C ($OR_{LDL\text{-}C}$ = 1.39, 95% CI =1.17-1.66), TG (OR_{TG} = 1.76, 95% CI =1.46-2.12) and BMI (OR_{BMI} = 1.82, 95% CI =1.52-2.18). Elderly T2DM patients with a duration of diabetes of 1~5 years were more likely to achieve the HbA1c control target (OR_{HbA1c} = 1.92, 95% CI = 1.59-2.31) than elderly T2DM patients with a duration of diabetes of <1 year. Furthermore, in elderly T2DM patients with a duration of diabetes of 5~10 years or ≥ 10 years, the duration of diabetes was positively associated with diabetic macrovascular complications (coronary heart disease and peripheral artery disease). In elderly T2DM patients with a duration of diabetes of over 10 years, the duration of diabetes was associated with diabetes kidney disease (all $P < 0.05$).

Conclusions: It is worth noting that the clinical characteristics of elderly patients with type 2 diabetes in different durations of diabetes are different.

KEYWORDS

clinical characteristics, elderly patients, type 2 diabetes mellitus, duration of diabetes, control targets

Introduction

Diabetes is a metabolic disease characterized by chronic hyperglycaemia that is prevalent in China and globally. The global diabetes map (Ninth Edition), released by the International Diabetes Federation, shows that the number of patients with diabetes worldwide is 463 million currently and will increase to 700 million by 2045. China has the largest number of patients with diabetes and the largest elderly diabetic population (1). The ageing population and increasing elderly diabetic population have placed a heavy burden on health systems and the social economy (2, 3).

Type 2 diabetes mellitus (T2DM) is the most common clinical type of diabetes, accounting for nearly 90% of all diabetic cases (4). In addition to the damage resulting from hyperglycaemia and diabetic macrovascular and microvascular complications, the harmful effects of T2DM are mainly caused by comorbidities such as hypertension, dyslipidaemia, overweight and obesity. These conditions could result in a low quality of life and shortened life expectancy (5).

According to the guidelines of prevention and treatment for Chinese T2DM (2020 edition), in addition to achieving glycosylated haemoglobin (HbA1c) < 7% and maintaining glucose levels within the target range, the comprehensive management of T2DM also includes the control of other risk factors, such as hypertension, dyslipidaemia, overweight and obesity (6). Good T2DM comprehensive management can reduce the incidence of diabetic complications and comorbidities (7). Our previous research found that elderly patients (aged ≥ 80 years) with T2DM were more likely to achieve HbA1c and lipid profile targets than younger patients (aged < 60 years) (8). However, the impact of diabetes duration on the comprehensive management of T2DM remains to be explored. Furthermore, since age and diabetic duration are both major risk factors for diabetic complications (9), it is crucial to understand the differences in clinical characteristics in T2DM patients with different durations of diabetes, especially in elderly individuals.

In the present cross-sectional study, we aimed to explore the relationships between diabetes duration and the comprehensive management of T2DM as well as diabetic vascular complications in Chinese elderly patients with T2DM.

Methods

Study population

A population of 5516 elderly patients with T2DM (aged ≥ 60 years) visited the Geriatric Hospital of Nanjing Medical University in Nanjing, China, between Jan 2013 and Dec 2020. The diagnosis of T2DM was based on the diagnostic criteria of the World Health Organization in 1999 (10). After excluding those who had a history of type 1 diabetes, latent autoimmune diabetes in adults (LADA), or secondary diabetes or those who had any missing data for key variables including blood pressure, HbA1c, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) triglyceride (TG) and body mass index (BMI), a total of 3840 elderly patients with T2DM were finally eligible for analysis. Patients were divided into four groups according to diabetic duration: < 1 year (Group 1), 1~5 years (Group 2), 5~10 years (Group 3), and ≥ 10 years (Group 4). The Ethics Committee of the Geriatric Hospital of Nanjing Medical University approved the study protocol. Each patient signed written informed consent.

Data sources

The clinical data of all elderly patients with T2DM were collected from the electronic medical record system. These information included diabetes duration, age, sex, education level, smoking status, family history of diabetes, systolic pressure (SBP), diastolic pressure (DBP), BMI, HbA1c, TG, TC, LDLC, HDL-C, therapeutic regimens, such as diabetes treatment (diet and exercise alone and anti-hyperglycaemic agents), and antihypertensive agents. Education level was defined as ≥ 9 years or < 9 years. Smoking status was divided into two groups, current smoking and non-current smoking. BMI was calculated as weight (kg)/height $(m)^2$. Overnight fasting

venous blood samples were obtained to measure HbA1c. All blood samples were measured at the laboratory of the Geriatric Hospital of Nanjing Medical University.

The data of macrovascular and microvascular complications of diabetes were also collected based on the available information. The diagnosis of coronary heart disease (CHD) was based on a history of angina pectoris, myocardial infarction, percutaneous coronary intervention or coronary artery bypass. The diagnosis of peripheral artery disease (PAD) was based on an ankle-brachial pressure index (ABI) < 0.9. The estimated glomerular filtration rate (eGFR) was calculated by the CKD-EPI formula (11), and the diagnosis of diabetes kidney disease (DKD) was based on the KDIGO clinical practice guidelines (12, 13). Albuminuria was defined as a urine albumin/creatinine ratio (ACR) > 30 mg/gCr. Patients with eGFR < 60 ml·min^{-1}·1.73m^2 or albuminuria were defined as having DKD.

Comprehensive control targets for T2DM

According to the guidelines for Chinese T2DM (2020 edition) (6), the T2DM control targets of comprehensive management include BP<130/80 mmHg, HbA1c < 7%, TC < 4. 5 mmol/L, LDL-C < 2. 6 mmol/L, HDL-C >1.0 mmol/L (males) or >1.3 mmol/L(females), TG< 1. 7 mmol/L, and BMI < 24.0 kg/m^2.

Statistical analysis

Our clinical characteristics were non-normally distributed, descriptive information was presented as median (interquartile range [IQR]) for continuous variables or numbers and percentages for categorical variables. For continuous variables, Kruskal-Wallis and *post hoc* multiple comparison Dunn's tests were used to test for the differences (R package FSA). For categorical variables, $\chi 2$ tests and *post hoc* tests were carried out to examine the significant differences (R package companion). Logistic regression analysis was used to calculate the odds ratios (ORs) and 95% confidence intervals (95% CIs) for the T2DM achieved control targets or the associations of different diabetic duration groups with the risks of diabetic macrovascular and microvascular complications. The unadjusted ORs and 95% CIs were calculated in Model 1. Two other models were progressively adjusted for age, sex, education, BMI (Model 2), smoking and family history of diabetes (Model 3). Patients with a diabetes duration < 1 year were the reference group. *P* values for trend were calculated by modeling ordinal categories as continuous variables. A two-tailed threshold of $P < 0.05$ was considered statistically significant. Missing data was presented as not available and were not taken into consideration during analysis. The statistics were calculated using R statistical software version 4.1.0.

Results

Baseline characteristics of elderly patients with T2DM among different diabetic duration groups

The characteristics of elderly patients with T2DM according to different diabetic duration groups are described in ###$###

Table 1. A total of 3840 elderly patients with T2DM were classified as group 1 (n = 972), group 2 (n = 896), group 3 (n = 875) and group 4 (n = 1097). Between the four groups, there were no significant differences in age, SBP, the proportion of higher education, current smoking and patients who used antihypertensive agents (all $P > 0.05$). In group 4, compared with groups 1 and 2, there was a significantly decreased percentage of male ($P_{group\ 4\ vs\ group\ 1} = 0.013$, $P_{group\ 4\ vs\ group\ 2} = 0.035$) and lower levels of DBP ($P_{group\ 4\ vs\ group\ 1} < 0.001$, $P_{group\ 4\ vs\ group\ 2} = 0.014$), BMI ($P_{group\ 4\ vs\ group\ 1} <0.001$, $P_{group\ 4\ vs\ group\ 2} < 0.001$), TG ($P_{group\ 4\ vs\ group\ 1} < 0.001$, $P_{group\ 4\ vs\ group\ 2} < 0.001$) and TC ($P_{group\ 4\ vs\ group\ 1} < 0.001$, $P_{group\ 4\ vs\ group\ 2} = 0.018$), whereas the increased percentage of family history of diabetes ($P_{group\ 4\ vs\ group\ 1} < 0.001$, $P_{group\ 4\ vs\ group\ 2} < 0.001$). With prolonged diabetic status, there was a significantly decreasing trend in the proportion of patients who used diet and exercise alone to control blood glucose (28.70%, 16.96%, 10.06%, 3.92%, $P < 0.001$). Compared to group 1, the level of HbA1c was significantly higher in group 4 (7.40% *vs* 7.10%, $P = 0.015$), but was significantly lower in group 2 (6.70% *vs* 7.10%, $P < 0.001$) and group 3 (7.00% *vs* 7.10%, $P = 0.002$).

Control rates of T2DM targets among elderly patients with different durations

The control rates of T2DM targets in elderly patients with T2DM between different groups are shown in Table 2. Compared to group 1, the control rate of HbA1c was higher in group 2 (61.61% vs 45.86%, $P < 0.001$), whereas the rate was significantly lower in group 4 (36.55% *vs* 45.86%, $P < 0.001$). Group 4 had a significantly higher control rate of TC when compared with groups 1 (47.77% *vs* 41.15%, $P = 0.018$). The similar results were observed for the control rates of LDL-C (47.86% *vs* 39.92%, $P = 0.002$). Patients of group 4 were more likely to be higher control rate of TG and BMI when compared with other groups (all $P < 0.05$). Additionally, no significant differences in the control rates of blood pressure and HDL-C were found across the groups with various durations of diabetes (all $P > 0.05$).

TABLE 1 Characteristics of elderly T2DM patients with different diabetic durations.

Characteristics	Diabetic duration				*P* value
	Group 1 (< 1 year)	Group 2 (1-5 years)	Group 3 (5-10 years)	Group 4 (≥10 years)	
Number of patients	972	896	875	1097	
Age (years)	70 (65 - 77)	70 (64 - 76)	71 (65 - 77)	70 (65 - 76)	0.297
Sex					0.002
Male	602 (61.93)[a]	550 (61.38)[a]	489 (55.89) [a,b]	605 (55.15) [b,c]	
Female	370 (38.07)	346 (38.62)	386 (44.11)	492 (44.85)	
Education [n (%)]†					0.570
≥9 years	658 (67.70)	591 (65.96)	567 (64.80)	719 (65.54)	
<9 years	313 (32.20)	305 (34.04)	308 (35.20)	378 (34.46)	
Current smoking [n (%)]					0.758
Yes	188 (19.34)	168 (18.75)	157 (17.94)	217 (19.78)	
No	784 (80.66)	728 (81.25)	718 (82.06)	880 (80.22)	
Family history of diabetes [n (%)]					< 0.001
Yes	298 (30.66)[a]	296 (33.04)[a,b]	332 (37.94)[b,c]	516 (47.04)[d]	
No	674 (69.34)	600 (66.96)	543 (62.06)	581 (52.96)	
SBP (mmHg)	130 (120 - 140)[a]	130 (120 - 140)[a]	130 (120 - 140)[a]	130 (120 - 140)[a]	0.045
DBP (mmHg)	80 (70 - 80)[a]	80 (70 - 80)[a]	78 (70 - 80)[a,b]	77 (70 - 80)[b]	< 0.001
BMI (kg/m^2)	25.2 (23.4 - 27.4)[a]	25.1 (23.0 - 27.3)[a]	25.0 (22.9 - 27.0)[a]	24.2 (22.3 - 26.5)[b]	< 0.001
HbA1c (%)	7.10 (6.40 - 9.13)[a]	6.70 (6.20 - 7.60)[b]	7.00 (6.40 - 8.00) [c]	7.40 (6.60 - 9.00)[d]	< 0.001
TG (mmol/L)	1.50 (1.09 - 2.03)[a]	1.40 (1.00 - 1.96)[b]	1.31 (0.95 - 1.90)[b]	1.26 (0.91 - 1.76)[c]	< 0.001
TC (mmol/L)	4.74 (4.10 - 5.44)[a]	4.71 (3.99 - 5.40) [a]	4.66 (3.96 - 5.35) [a,b]	4.54 (3.93 - 5.23) [b,c]	< 0.001
LDL - C (mmol/L)	2.81 (2.21 - 3.42)[a]	2.74 (2.16 - 3.28)[a,b]	2.70 (2.19 - 3.30)[a,b]	2.63 (2.10 - 3.20) [b]	< 0.001
HDL - C (mmol/L)	1.12 (0.96 - 1.35)[a]	1.15 (0.99 - 1.39)[a]	1.17 (0.98 - 1.39)[a]	1.17 (0.97 - 1.40)[a]	0.019
Diabetes treatment [n (%)]					
Diet and exercise alone	279 (28.70)[a]	152 (16.96)[b]	88 (10.06)[c]	43 (3.92)[d]	< 0.001
Others*	693 (71.30)	744 (83.04)	787 (89.94)	1054 (96.08)	
Antihypertensive agents [n (%)]					0.068
Yes	538 (55.35)	544 (60.71)	492 (56.23)	648 (59.07)	
No	434 (44.65)	352 (39.29)	383 (43.77)	449 (40.93)	

Data are expressed as median (Q1-Q3) or numbers and percentages, n (%); P values for comparison over all 4 categories.
[a,b,c,d] Groups with the same superscript letters are not significantly different.
†Education information is not available in one patient.
*Others include anti-hyperglycemic agents, such as insulin and oral antidiabetic drugs.

As shown in Table 3, after adjusting for age, sex, education, BMI, smoking and family history of diabetes (model 3), T2DM patients in group 2 had significantly increased OR for achieving the control target for HbA1c (OR_{HbA1c} = 1.92, 95% CI = 1.59-2.31, $P < 0.001$), while elderly T2DM patients in group 4 showed significantly decreased OR for achieving the control target for HbA1c (OR_{HbA1c} = 0.65, 95% CI = 0.54-0.78, $P < 0.001$). The significantly increased ORs for achieving the control targets for TC (OR_{TC} = 1.36, 95% CI =1.14-1.63, $P < 0.001$), LDL-C (OR_{LDL-C} = 1.39, 95% CI =1.17-1.66, $P < 0.001$) and BMI (OR_{BMI} = 1.82, 95% CI =1.52-2.18, $P < 0.001$) were observed in group 4. Interestingly, we observed the gradually increased ORs for achieving the control targets for TC with the prolonged duration of diabetes ($OR_{group\ 2}$ = 1.75, 95% CI =1.45-2.12; $OR_{group\ 3}$ = 1.38, 95% CI =1.13-1.68; $OR_{group\ 4}$ = 1.76, 95% CI =1.46-2.14; $P < 0.001$) in model 3.

Macrovascular and microvascular complications of diabetes among the groups

The prevalence of diabetic macrovascular and microvascular complications among the groups are presented in Table 4. Notably, there are 2157 (56.18%) patients with missing PAD data, 1214 (31.61%) with missing albuminuria, and 114 (2.97%) with missing eGFR. Between the four groups, there was no significant difference in the percentage of patients with eGFR<60 ml·min-1 ·1.73m^2 ($P = 0.420$). Compared to group1, group 4 had the higher percentage of patients with CHD, PAD and albuminuria (all $P < 0.05$). As shown in Table 5, after adjusting for potential confounders, we observed elderly T2DM patients in groups 3 and 4 had significantly increased ORs for CHD ($OR_{group\ 3}$ = 1.43, 95% CI =1.07-1.89; $OR_{group\ 4}$ =

TABLE 2 Control rates of elderly T2DM patients with different diabetic durations.

Characteristics	Diabetic duration				*P* value
	Group 1 (< 1 year)	Group 2 (1-5 years)	Group 3 (5-10 years)	Group 4 (≥ 10 years)	
Number of patients	972	896	875	1097	
Blood pressure (mmHg)					0.333
SBP<130 and DBP<80	266 (27.37)	258 (28.79)	273 (31.20)	324 (29.54)	
≥ 130/80	706 (72.63)	638 (71.21)	602 (68.80)	773 (70.46)	
HbA1c (%)					< 0.001
< 7	444 (45.68)[a]	552 (61.61)[b]	433 (49.49)[a]	401 (36.55)[c]	
≥ 7	528 (54.32)	344 (38.39)	442 (50.51)	696 (63.45)	
TC (mmol/L)					0.010
< 4.5	400 (41.15)[a]	374 (41.74)[a,b]	384 (43.89)[a,b]	524 (47.77)[b,c]	
≥ 4.5	572 (58.85)	522 (58.26)	491 (56.11)	573 (52.23)	
LDL-C (mmol/L)					0.003
< 2.6	388 (39.92)[a]	394 (43.97)[a,b]	400 (45.71)[a,b]	525 (47.86)[b,c]	
≥ 2.6	584 (60.08)	502 (56.03)	475 (54.29)	572 (52.14)	
HDL-C (mmol/L)					0.083
Achieved target*	513 (52.78)	523 (58.37)	487 (55.66)	592 (53.97)	
Did not achieve target*	459 (47.22)	373 (41.63)	388 (44.34)	505 (46.03)	
TG (mmol/L)					< 0.001
< 1.7	580 (59.67)[a]	577(64.40)[a,b]	587 (67.09)[b]	800 (72.93)[c]	
≥ 1.7	392 (40.33)	319(35.60)	288 (32.91)	297 (27.07)	
BMI (kg/m^2)					< 0.001
< 24	310 (31.89)[a]	313 (34.93)[a]	314 (35.89)[a,b]	503 (45.85)[c]	
≥ 24	662 (68.11)	583 (65.07)	561 (64.11)	594 (54.15)	

Data are expressed as numbers and percentages, n (%); P values for comparison over all 4 categories.
[a,b,c] Groups with the same superscript letters are not significantly different. *Achieved target means the levels of HDL-C >1.0 mmol/L in males or >1.3 mmol/L in females; Did not achieve target means the levels of HDL-C≤1.0 mmol/L in males or ≤1.3 mmol/L in females.

1.56, 95% CI =1.19-2.04; P_{trend}< 0.001) and for PAD ($OR_{group\ 3}$ = 1.82, 95% CI =1.10-3.01; $OR_{group\ 4}$ = 1.60, 95% CI =1.00-2.55; P_{trend}= 0.022) in model 3. Patients in group 4 had significantly increased ORs for albuminuria ($OR_{albuminuria}$ = 1.96, 95% CI =1.55-2.48, P_{trend} < 0.001; OR_{eGFR} = 1.36, 95% CI =1.05-1.75, P_{trend} =0.030).

Discussion

In the current study, we evaluated the comprehensive control of T2DM and diabetic complications in elderly patients stratified by different durations of diabetes. Elderly T2DM patients with a duration of diabetes of ≥ 10 years were more likely to achieve the comprehensive control targets for TC, LDL-C and TG, while elderly T2DM patients with a duration of diabetes of 1~5 years were more likely to achieve the HbA1c control target than elderly T2DM patients with a duration of diabetes of < 1 year. In elderly T2DM patients with a duration of diabetes of 5~10 years or ≥ 10 years, the duration of diabetes was independently associated with diabetic macrovascular complications (CHD and PAD). In addition, in patients with a duration of diabetes of ≥ 10 years, the duration of diabetes was independently associated with the risk of DKD.

HbA1c is typically used as the gold standard for evaluating glycaemic control and is a clinical indicator for predicting diabetic complications (4). In addition, HbA1c < 7% is the standard for good glycaemic control in most adults with T2DM that is recommended by most guidelines (4, 6). Previous studies have shown that the ORs of poor glycaemic control increase with diabetes duration in T2DM (14, 15). Interestingly, in the current study, elderly T2DM patients with a duration of diabetes of 1~5 years were more likely to achieve the HbA1c control target than those who had a duration of diabetes less than 1 year, and the control target rate of HbA1c gradually decreased with the extension of the duration. The reasons for this finding are still unclear but may be caused by differences in study design, the deterioration of islet function with diabetes progression (16), or the clinical criteria for glycaemic control (17).

Dyslipidaemia is one of the most important risk factors for CHD, and optimal lipid control can improve cardiovascular outcomes (18). Our study found that the higher control rates for TC, LDL-C, TG and BMI were observed in elderly T2DM patients with a duration of diabetes of ≥ 10 years than that in

TABLE 3 Odds ratios (95% CI) for achieved comprehensive control targets by different diabetic duration groups among elderly patients with T2DM (Ref. < 1 year).

Characteristics	Diabetic duration			P_{trend}
	Group 2 (1-5 years)	Group 3 (5-10 years)	Group 4 (≥ 10 years)	
HbA1c < 7%				
Model 1	1.91 (1.59,2.29)	1.16 (0.97,1.40)	0.69 (0.57,0.82)	< 0.001
Model 2	1.91 (1.59,2.30)	1.14 (0.94,1.37)	0.64 (0.53,0.76)	< 0.001
Model 3	1.92 (1.59,2.31)	1.15 (0.95,1.38)	0.65 (0.54,0.78)	<0.001
BP < 130/80 mmHg				
Model 1	1.07 (0.88,1.31)	1.20 (0.98,1.47)	1.11 (0.92,1.35)	0.187
Model 2	1.06 (0.86,1.3)	1.15 (0.94,1.42)	1.00 (0.82,1.21)	0.921
Model 3	1.06 (0.86,1.3)	1.16 (0.94,1.42)	1.00 (0.82,1.22)	0.844
TC < 4.5 mmol/L				
Model 1	1.02 (0.85,1.23)	1.12 (0.93,1.35)	1.31 (1.10,1.56)	0.001
Model 2	1.03 (0.86,1.24)	1.15 (0.96,1.39)	1.37 (1.15,1.64)	< 0.001
Model 3	1.03 (0.86,1.24)	1.15 (0.95,1.39)	1.36 (1.14,1.63)	< 0.001
LDL-C < 2.6 mmol/L				
Model 1	1.18 (0.98,1.42)	1.27 (1.05,1.52)	1.38 (1.16,1.65)	< 0.001
Model 2	1.18 (0.98,1.42)	1.27 (1.05,1.53)	1.39 (1.16,1.65)	< 0.001
Model 3	1.18 (0.98,1.42)	1.27 (1.06,1.53)	1.39 (1.17,1.66)	< 0.001
HDL-C Achieved target*				
Model 1	1.25 (1.04,1.51)	1.12 (0.93,1.35)	1.05 (0.88,1.25)	0.932
Model 2	1.26 (1.04,1.52)	1.17 (0.97,1.42)	1.04 (0.87,1.24)	0.928
Model 3	1.25 (1.04,1.52)	1.18 (0.97,1.43)	1.06 (0.88,1.27)	0.742
TG < 1.7 mmol/L				
Model 1	1.22 (1.01,1.47)	1.38 (1.14,1.67)	1.82 (1.51,2.19)	< 0.001
Model 2	1.22 (1.01,1.48)	1.37 (1.13,1.67)	1.75 (1.45,2.11)	< 0.001
Model 3	1.22 (1.01,1.48)	1.38 (1.13,1.68)	1.76 (1.46,2.14)	< 0.001
BMI < 24 (kg/m^2)†				
Model 1	1.15 (0.95,1.39)	1.20 (0.99,1.45)	1.81 (1.51,2.16)	< 0.001
Model 2	1.15 (0.95,1.40)	1.19 (0.98,1.44)	1.80 (1.51,2.16)	< 0.001
Model 3	1.16 (0.95,1.40)	1.19 (0.98,1.45)	1.82 (1.52,2.18)	< 0.001

Model 1: unadjusted model.
Model 2: adjusted for age, sex, education and BMI.
Model 3: adjusted for age, sex, education and BMI, smoking and family history of diabetes.
*Achieved target means the levels of HDL-C >1.0 mmol/L in males or >1.3 mmol/L in females.
†Adjusted for age, sex and education in mode 2, and adjusted for age, sex, education, smoking and family history of diabetes in model 3.

patients who had a duration of diabetes less than 1 year. A possible reason is that since the similar prevalence of CHD and PAD was observed in patients with a duration of diabetes of ≥ 10 years, elderly patients with T2DM may pay more prone to it. Another reason is that the elderly patients with long duration may use lipid-lowering or antiplatelet agents to relieve symptoms. Further studies with more detail information should be performed to explore the underlying reasons.

Previous cohort studies have reported that the duration of diabetes is associated with the risk of developing diabetes-related complications in aged T2DM patients (19, 20). One study in the USA showed that the prevalence of CHD, PAD and cerebrovascular disease was significantly higher in elderly T2DM patients (aged ≥ 60 years) with a duration of diabetes ≥ 10 years than in those with a shorter duration (19). Furthermore, another study in Australia reported that the incidences of myocardial infarction and stroke-related death increased along with an increase in the duration of diabetes in 1433 aged male patients with diabetes (aged ≥ 65 years) (20). Our study also found that elderly T2DM patients with a duration of diabetes of 5~10 years or ≥ 10 years were more likely to develop diabetic macrovascular complications (CHD and PAD) than those with a duration of diabetes of <1 year, which was consistent with the abovementioned studies. Moreover, the present study also suggested that the duration of diabetes was significantly associated with microvascular complications (albuminuria), which is in accordance with a previous study (21).

There were several limitations in our study. First, there is missing data on outcomes of interest including PAD and DKD, and our study was a cross-sectional and single-center study. Future

TABLE 4 Characteristics of elderly T2DM patients with diabetic complications.

Characteristics	n (%)	Diabetic duration				*P* value
		Group 1 (< 1 year)	Group 2 (1-5 years)	Group 3 (5-10 years)	Group 4 (≥10 years)	
Total number of patients	3840 (100)	972	896	875	1097	
Macrovascular complications						
CHD						
Yes	504 (13.13)	101 (10.39)[a]	102 (11.38)[a,b]	128 (14.63)[b,c]	173 (15.77)[c]	0.001
No	3336 (86.87)	871 (89.61)	794 (88.62)	747 (85.37)	924 (84.23)	
PAD*						
Yes	181 (4.71)	29 (2.98)[a]	25 (2.79)[a,b]	47 (5.37)[b]	80 (7.29)[b]	0.001
No	1502 (39.11)	398 (40.95)	284 (31.70)	313 (85.37)	507 (46.22)	
Microvascular complications						
DKD (Albuminuria)[†]						
Yes	786 (20.47)	150 (15.43)[a]	148 (16.52)[a]	173 (19.77)[a]	315 (28.71)[b]	< 0.001
No	1840 (47.92)	501 (51.54)	403 (44.98)	426 (48.69)	510 (46.49)	
DKD (eGFR<60 ml·min^{-1} ·1.73m^2)[‡]						0.420
Yes	668 (17.40)	156 (16.05)	153 (17.08)	154 (17.60)	205 (18.69)	
No	3058 (79.64)	794 (81.69)	711 (79.35)	694 (79.31)	859 (78.30)	

CHD, Coronary heart disease. PAD, Peripheral vascular disease. DKD, diabetes kidney disease.
Data are expressed as numbers and percentages, n (%); P values for comparison over all 4 categories.
*PAD information is available in 1683 (43.83%) patients.
[†]Albuminuria (ACR > 30 mg/gCr). ACR information is available in 2626 (68.39%) patients.
[‡]eGFR is available in 3726 (97.03%) patients.
[a,b,c]Groups with the same superscript letters are not significantly different.

TABLE 5 Odds ratios (95% CI) for macrovascular and microvascular complications by different diabetic duration groups among elderly patients with T2DM (Ref. < 1 year).

Characteristics	Diabetic duration			P_{trend}
	Group 2 (1-5 years)	Group 3 (5-10 years)	Group 4 (≥ 10 years)	
Macrovascular complications				
CHD				
Model 1	1.11 (0.83,1.48)	1.48 (1.12,1.95)	1.61 (1.24,2.10)	< 0.001
Model 2	1.13 (0.84,1.51)	1.44 (1.09,1.91)	1.60 (1.23,2.09)	< 0.001
Model 3	1.12 (0.84,1.51)	1.43 (1.07,1.89)	1.56 (1.19,2.04)	< 0.001
PAD				
Model 1	1.21 (0.69,2.11)	2.06 (1.27,3.35)	2.17 (1.39,3.38)	< 0.001
Model 2	1.12 (0.63,1.98)	1.83 (1.11,3.03)	1.64 (1.03,2.60)	0.016
Model 3	1.11 (0.63,1.97)	1.82 (1.10,3.01)	1.60 (1.00,2.55)	0.022
Microvascular complications				
DKD (Albuminuria)				
Model 1	1.23 (0.94,1.59)	1.36 (1.05,1.75)	2.06 (1.64,2.6)	< 0.001
Model 2	1.21 (0.93,1.57)	1.29 (1.00,1.67)	1.96 (1.55,2.47)	< 0.001
Model 3	1.21 (0.93,1.58)	1.29 (1.00,1.67)	1.96 (1.55,2.48)	< 0.001
DKD (eGFR < 60 ml·min^{-1} ·1.73m^2)				
Model 1	1.10 (0.86,1.40)	1.13 (0.88,1.44)	1.21 (0.97,1.53)	0.097
Model 2	1.16 (0.88,1.51)	1.11 (0.85,1.46)	1.32 (1.03,1.71)	0.047
Model 3	1.16 (0.89,1.52)	1.12 (0.86,1.47)	1.36 (1.05,1.75)	0.030

Model 1: unadjusted model.
Model 2: adjusted for age, sex, education and BMI.
Model 3: adjusted for age, sex, education, BMI, smoking and family history of diabetes.

large and longitudinal studies with these outcomes are needed to confirm the associations. Second, the preference of doctors on medication regiment and drug choice might influence the analytic data on characteristics of the elderly T2DM patients based on diabetes duration. Data on the detail medication and medication adherence of patients should be considered into further studies.

In summary, elderly T2DM patients with a duration of diabetes of 1~5 years were more likely to achieve the HbA1c control target, while patients with a duration of diabetes of ≥ 10 years were more likely to achieve the comprehensive control targets for the lipid profile. In addition, the duration of diabetes was independently associated with diabetic macrovascular complications (CHD and PAD) in elderly T2DM patients with a duration of diabetes of 5~10 years or ≥ 10 years and was significantly associated with DKD in patients a duration of diabetes of with ≥ 10 years.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

Author contributions

WT and YY conceived and design the research. YY and KX analyzed the data. QL, HX, DW, LD, CH, SS, and KW collected the data. YY and KX wrote and revised the initial manuscript. WT supervised the whole study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Influence of circulating nesfatin-1, GSH and SOD on insulin secretion in the development of T2DM

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Aims: To evaluate the correlation of nesfatin-1, GSH and SOD levels with β-cell insulin secretion and their influence on insulin secretion in the development of type 2 diabetes mellitus (T2DM).

Materials and methods: 75 patients with T2DM, 67 with prediabetes and 37 heathy participants were recruited in this study. Serum levels of nesfatin-1, GSH and SOD were quantified and statistically analyzed.

Results: The levels of nesfatin-1, GSH and SOD in T2DM were significantly decreased ($P < 0.001$) compared to either in prediabetes or in healthy control, and significant reduction of these biomarkers was also observed in prediabetes when compared to the control ($P < 0.001$). Circulating nesfatin-1, GSH and SOD were not only strongly correlated with β-cell insulin secretion, but also exerted remarkable influence on the secretion.

Conclusion: Serum nesfatin-1, GSH and SOD are important factors involving insulin secretion in the development of T2DM, which may help provide new ideas for forthcoming investigations on the roles of these factors in pathogenesis of T2DM, as well as for active prediction and prevention of prediabetes before it develops into overt T2DM.

KEYWORDS

type 2 diabetes mellitus, prediabetes, GSH, SOD, insulin secretion

Introduction

Up to 2021, the global prevalence of diabetes reached 10.5% (536.6 million people) and the number is estimated up to 12.2% (783.2 million people) by 2045 (1); among them, 90% to 95% are type 2 diabetes mellitus (T2DM) (2). Prediabetes is a risky stage before T2DM, characterized by metabolic abnormality of the body, such as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). A meta-analysis concluded that the hazard ratios for IFG, IGT and IFG+IGT developing into T2DM are 4.32, 3.61 and 6.90, respectively (3).

Although resistance of peripheral tissues to insulin or β-cell dysfunction is common in T2DM, the exact mechanism of T2DM remains to be clarified. Multiple explanations

have been proposed in the development of T2DM, of them, oxidative stress is considered to be pivotal in this process (4). Free radicals, including reactive oxygen species (ROS), and some metal ions (such as iron and copper) can be generated through metabolic pathways or immune cells (5, 6), and play key roles in many physiological activities such as cell signaling, growth, apoptosis and aging (7–9). When free radicals are accumulated, they will overcome the anti-oxidative effects in the cell, initiated by such as glutathione (GSH) or superoxide dismutase (SOD), resulting in oxidative stress (9, 10). Pancreatic β-cells heavily rely on oxidative metabolism to synthesize adenosine triphosphate, especially when the glucose level is high (11, 12). In spite of the fact that pancreatic β-cells actively function in metabolic process, which leads to ROS accumulation as ROS is an inevitable byproduct of mitochondrial respiration during glucose stimulation (13), enzymes involved in anti-oxidative defenses are present at very low levels in β-cells and they are prone to be inactivated by disallowed genes (11); in this regard, protecting pancreatic β-cells from the destructive free radicals is expected to be a potential strategy for preventing and controlling T2DM.

Besides insulin, many different peptide hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) can affect the balance of glucose metabolism in the body (14). GLP-1 and GIP are released into the circulation from gut endocrine cells in response to food digestion and facilitate insulin secretion in a glucose-dependent manner (15, 16). However, the very short half-lives (1–7 min) of GLP-1 and GIP in plasma represent a major limitation for their use in the clinical setting (17). Nesfatin-1 is a newly identified peptide with 82 amino acids; in addition to nesfatin-1, cleavage of prohormone convertase on NEFA/nucleobindin2 (NUCB2) yields fragments of nesfatin-2 and nesfatin-3 (18). Although its receptor is still unclear, nesfatin-1 has been found to be functional in anti-inflammation (19), antioxidation (20), appetite suppression (21) and insulin resistance (22). Importantly, researches on the variation of serum nesfatin-1 levels in T2DM have so far proved inconclusive. Some studies reported elevated serum nesfatin-1 levels (23, 24), while others showed the contrary results (25–27). Another meta-analysis concluded that serum nesfatin-1 upregulated in newly diagnosed T2DM patients but decreased after drug therapy (28).

Homeostatic model assessment (HOMA) is a convenient and economic method to quantify β-cell function of insulin secretion (HOMA-β), insulin resistance (HOMA-IR) and insulin sensitivity (HOMA-IS) with measurement of fasting blood glucose and insulin (29).

We conducted this cross-sectional study to assess the correlation of nesfatin-1, GSH and SOD levels with β-cell insulin

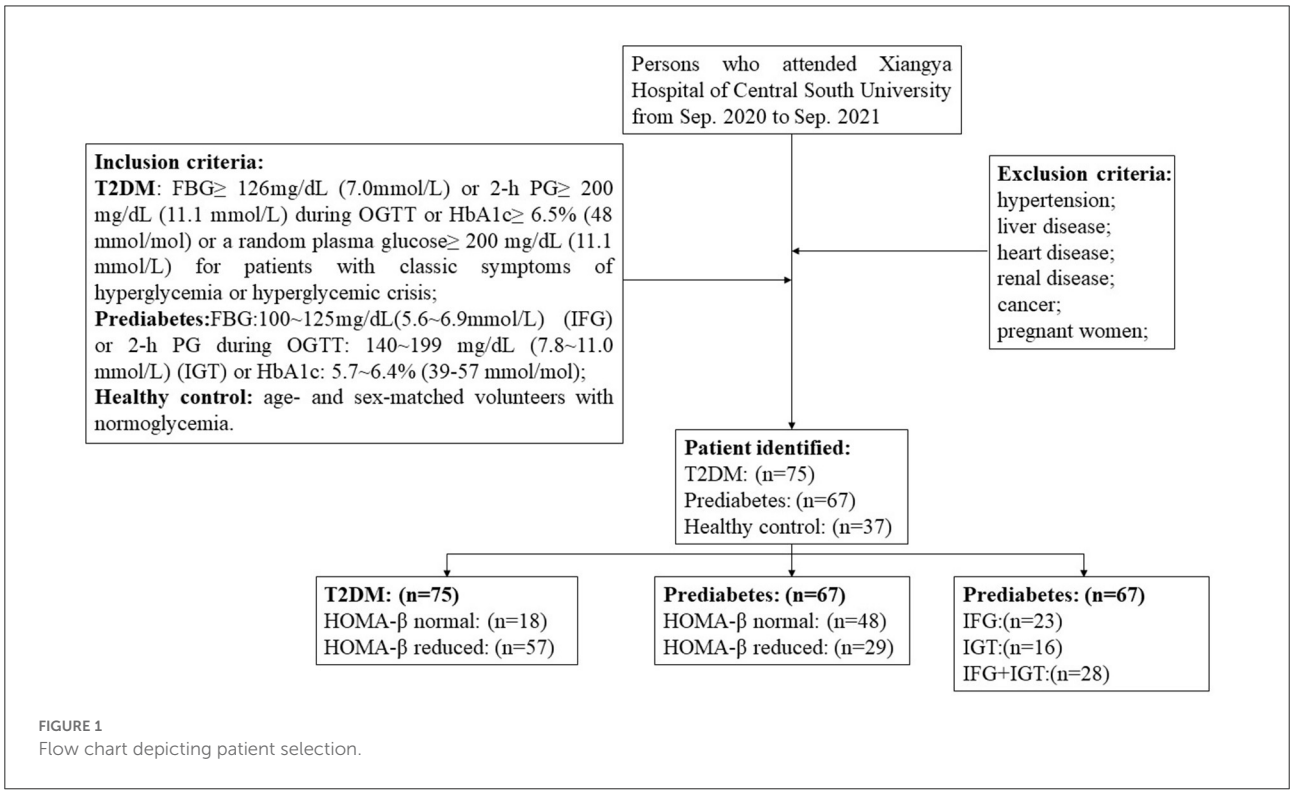

FIGURE 1
Flow chart depicting patient selection.

secretion, and to explore their influence on insulin secretion in the development of T2DM through prediabetes.

Materials and methods

Participants

This cross-sectional study recruited 75 T2DM patients, 67 prediabetes who attended in Xiangya Hospital of Central South University from Sep. 2020 to Sep. 2021. According to the American Diabetes Association (ADA) guideline for diabetes (30), the inclusion criteria for T2DM include the following: FBG $\geq$ 126 mg/dL (7.0 mmol/L) or 2-h PG $\geq$ 200 mg/dL (11.1 mmol/L) during oral glucose tolerance test (OGTT) or HbA1c $\geq$ 6.5% (48 mmol/mol) or a random plasma glucose $\geq$200 mg/dL (11.1 mmol/L) for patients with classic symptoms of hyperglycemia or hyperglycemic crisis; the criteria required for prediabetes inclusion contain FBG: 100∼125 mg/dL (5.6∼6.9 mmol/L), IFG or 2-h PG during 75-g OGTT: 140∼199 mg/dL (7.8∼11.0 mmol/L) (IGT) or HbA1c: 5.7∼6.4% (39–57 mmol/mol). 37 age- and sex-matched volunteers with normoglycemia were introduced as the healthy controls. Subjects with hypertension, liver disease, heart disease, renal disease, cancer, or other chronic diseases as well as pregnant women were excluded. The procedure of patient selection was depicted in a flowchart (Figure 1). All participants were given informed consent and this study was permitted by the ethics committee of Xiangya Hospital of Central South University (No. 202109180).

Blood samples were drawn between 08:00 a.m. and 10:00 a.m. from each participant after fasting food for at least 8 h. Body weight and height were assessed and body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. The collected venous blood samples were centrifuged at 3,600 rpm for 10 min to isolate sera and stored at −20 °C until they were required for testing. Routine tests for biochemistry indicators such as high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total bile acid (TBA), uric acid (UA), serum creatinine (Scr), blood urea nitrogen (BUN), glycosylated hemoglobin (HbA1c), fasting blood-glucose (FBG), insulin, urine creatinine (Ucr) and urine microalbumin (UmALB) were measured on an AU5800 automatic analyzer (Beckman Coulter, CA, USA). HOMA-β, HOMA-IR and HOMA-IS were calculated by the following equations (29): HOMA-β = 20 * insulin (μU/ml)/(FBG(mmol/L)-3.5); HOMA-IR = insulin (μU/ml) * FBG(mmol/L)/22.5 and HOMA-IS = 100 * 22.5/insulin (μU/ml) * FBG(mmol/L).

Serum levels of nesfatin-1, GSH and SOD were determined using commercially available kits in accordance with the manufacturers' instructions. Nesfatin-1 was measured by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA), supplied by Jiangsu Meimian Industrial, Jiangsu, China. In brief, 50 μL of the 1:5 diluted serum specimens were added to each microplate well pre-coated with purified human nesfatin-1 antibody, and incubated at 37°C for 30 min; after washed 5 times with washing buffer, 50 μL of HRP-conjugated nesfatin-1 antibody was added and kept at 37°C for another 30 min; following 5 repeatedly washing steps, 50 μL of the TMB substrate solution A and 50 μL of the substrate B were pipetted to each well and preserved at 37°C from light for 10 min; finally, 50 μL of stop solution were added to terminate the reaction. The absorbance at 450 nm (A_{450}) of each well was read within 15 min on an automatic microplate reader and the concentration of nesfatin-1 is quantified by comparing the A_{450} of the samples to the standard curve.

Detection of GSH is based on an enzymatic cycling method in the presence of GSH and a chromophore, and the assay kit was provided by Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The reduction of the chromophore produces a stable product, which can be followed by measuring A_{405}, therefore, the A_{405} is directly proportional to the amount of GSH in the sample. The procedure began with adding 50 μL of the serum into 200 μL of the precipitant working solution, followed by centrifugation at 3,500 rpm for 10 min before 100 μL of the supernatant were collected, then 100 μL of the GSH assay buffer and 25 μL of the chromogenic agent were added to the supernatant with sufficient mixing, kept at room temperature from light for 5 min, after that, the A_{405} of each well was read within 10 min on an automatic microplate reader and the levels of GSH was derived from the prepared standard curve.

The SOD WST-1 assay kit (Nanjing Jiancheng Bioengineering Institute, China) allows a very convenient and highly sensitive SOD measurement by utilizing WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion, and the reduction is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the IC50 (50% inhibition concentration) of SOD can be determined using colorimetric methods. In brief, 20 μL of the serum sample and 20 μL of the enzyme working solution were pipetted to the sample well, followed by adding 200 μL of the WST working solution, then incubated at 37°C for 20 min. Meanwhile, blank 1 (coloring without inhibitor), blank 2 (sample blank) were prepared as indicated in the manufacturer's manual. The absorbance at 450 nm of each well was read within 10 min on a microplate reader, and the activity of SOD was calculated with the following equation: SOD activity (U ml^{-1}) = ($A_{blank1} - A_{sample}$)/($A_{blank1} - A_{blank2}$) × 40.

Appropriate kits for testing serum adiponectin (ADPN) (Guangdong Uniten Biotechnology, Guangdong, China), retinol binding protein (RBP) (Aucher, Hunan, China), total iron binding capacity (TIBC) (Beijing Strong Biotechnologies, Beijing, China), neutrophil gelatinase-associated lipocalin

(NAGL) (Aucher, Hunan, China) and cystatin C (CysC) (Aucher, Hunan, China) were adopted for quantification of the above indicators.

Statistical analysis

Statistical analysis was implemented using SPSS version 26 (SPSS Inc., IL, USA). The results for continuous variables were presented as mean ± standard deviation (SD) and underwent normal distribution test, while the parameter of age was shown as median. Differences among groups were calculated with ANOVA, meanwhile, differences between groups were evaluated with SNK test. Independent Samples *t*-Test was used to determine the differences between two unpaired subgroups. Gender as categorical data was coded as male = 1 and female = 0. Differences of gender and age were acquired by Chi-Square test. Correlations between HOMA-β and other indexes were analyzed with Pearson correlation test. The impact factors of HOMA-β were assessed with multiple linear regression analysis (α in = 0.05, α out = 0.10). $P < 0.05$ (two-tailed) was regarded as statistically significant.

Results

General characteristics and parameter comparisons

A total of 179 participants were recruited in this study, consisting of three groups: T2DM, prediabetes, and the healthy control. The anthropometric and clinical characteristics of the subjects were shown in Table 1 and there were no significant differences in gender, age and BMI among the three groups. After normality of the continuous variables was tested and validated, differences of the serum indicators were compared. The results showed that serum levels of nesfatin-1, GSH, SOD, ADPN and NAGL in T2DM were significantly decreased compared to either in prediabetes ($P < 0.001$) or in healthy controls ($P < 0.001$); in contrast, RBP levels in T2DM were significantly elevated ($P < 0.001$) compared to either in prediabetes or in healthy controls, and this significant elevation exhibited in the prediabetes vs. the healthy ($P < 0.001$). In addition, TIBC levels in T2DM were distinctly high ($P < 0.01$) in comparison with either in prediabetes or in healthy controls.

Correlation between HOMA-β and other indexes

Pearson correlation test disclosed that HOMA-β was closely correlated with serum GSH ($r = 0.4307$, $P < 0.001$), SOD ($r = 0.5140$, $P < 0.001$), nesfatin-1 ($r = 0.6342$, $P < 0.001$), ADPN (r

TABLE 1 Anthropometric and clinical characteristics of the study subjects in different groups.

Parameters	T2DM	Prediabetes	Healthy control
Gender (M/F)*	41/34	33/34	23/14
Age (years)[a]*	54 (51–61)	55 (52–59)	52 (47.5–57.5)
BMI (Kg/m^2)[b]	23.01 ± 3.89	23.15 ± 2.76	22.94 ± 2.18
GSH (μmol/L)[b]	8.70 ± 3.60$^{\#\#\#\&\&\&}$	12.04 ± 6.62$^{\&\&\&}$	14.97 ± 6.93
SOD (U/ml)[b]	1,577.12 ± 180.67$^{\#\#\#\&\&\&}$	1,976.14 ± 234.00$^{\&\&\&}$	2,089.95 ± 190.66
Nesfatin-1 (pg/ml)[b]	622.94 ± 218.28$^{\#\#\#\&\&\&}$	875.88 ± 578.71	1,060.43 ± 823.72
ADPN (μg/ml)[b]	17.76 ± 12.64$^{\#\#\#\&\&\&}$	64.50 ± 12.84$^{\&\&\&}$	90.22 ± 7.21
RBP (mg/L)[b]	88.29 ± 31.83$^{\#\#\#\&\&\&}$	32.89 ± 17.59$^{\&\&\&}$	14.39 ± 3.27
TIBC (μmol/L)[b]	67.30 ± 61.85$^{\#\#\&\&}$	39.72 ± 8.39	42.42 ± 28.79
NAGL (ng/ml)[b]	450.47 ± 232.53$^{\#\#\#\&\&\&}$	1,344.44 ± 365.46$^{\&\&\&}$	2,512.84 ± 654.34
CysC (mg/L)[b]	0.88 ± 0.76	0.70 ± 0.16	0.67 ± 0.16
HDL-C (mmol/L)[b]	1.16 ± 0.29$^{\#\&}$	1.32 ± 0.27	1.30 ± 0.24
LDL-C (mmol/L)[b]	3.30 ± 0.99	3.58 ± 0.74	3.35 ± 0.72
HDL-C/LDL-C[b]	0.39 ± 0.20	0.38 ± 0.11	0.41 ± 0.12
TBA (μmol/L)[b]	3.48 ± 3.25	3.53 ± 3.92	2.89 ± 2.35
UA (μmol/L)[b]	349.12 ± 114.25	364.57 ± 87.24	373.45 ± 82.19
Scr (μmol/L)[b]	1.37 ± 1.80	0.92 ± 0.17	0.94 ± 0.16
BUN (mmol/L)[b]	8.48 ± 16.94	5.31 ± 1.13	6.48 ± 8.89
HbA1c (%)[b]	7.83 ± 1.92$^{\#\#\&\&}$	5.88 ± 0.32	5.56 ± 0.32
FBG (mmol/L)[b]	8.36 ± 3.45$^{\#\#\&\&}$	5.86 ± 0.42	5.26 ± 0.53
2h-PG (mmol/L)[b]	13.62 ± 4.44$^{\#\#\&\&}$	8.66 ± 1.55$^{\&\&}$	6.44 ± 0.90
Insulin (μIU/ml)[b]	6.87 ± 3.74$^{\#}$	9.50 ± 6.45$^{\&}$	6.46 ± 3.26

(Continued)

TABLE 1 Continued

Parameters	T2DM	Prediabetes	Healthy control
HOMA-IR[b]	2.41 ± 1.33[&]	2.48 ± 1.66[&]	1.55 ± 0.88
HOMA-β[b]	38.62 ± 28.49[###&&&]	83.29 ± 62.61	74.87 ± 34.00
HOMA-IS[b]	0.58 ± 0.45	0.55 ± 0.29	0.89 ± 0.58

[a]Median (interquartile range), [b]mean ± SD, [*]Chi-Square $P < 0.05$. BMI, body mass index; GSH, glutathione; SOD, superoxide dismutase; ADPN, adiponectin; RBP, retinol binding protein; TIBC, total iron binding capacity; NAGL, neutrophil gelatinase-associated lipocalin; CysC, cystatin C; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TBA, total bile acid; UA, uric acid; Scr, serum creatinine; BUN, blood urea nitrogen; HbA1c, glycosylated hemoglobin; FBG, fasting blood-glucose; 2h-PG, two-hour post glucose; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell; HOMA-IS, homeostasis model assessment of insulin sensitivity. [#]: vs. prediabetes $P < 0.05$, [##]: vs. prediabetes $P < 0.01$, [###]: vs. prediabetes $P < 0.001$; [&]: vs. healthy control $P < 0.05$, [&&]: vs. healthy control $P < 0.01$, [&&&]: vs. healthy control $P < 0.001$.

$= 0.3517$, $P < 0.001$), RBP ($r = -0.2355$, $P = 0.005$), NAGL ($r = 0.3505$, $P < 0.001$), UA ($r = 0.2338$, $P = 0.005$) and TBA ($r = 0.1675$, $P = 0.046$), as shown in Figure 2. Relevance analysis between HOMA-β and the glucose metabolism indexes (Figure 3) found that HOMA-β was significantly relevant to FBG ($r = -0.3909$, $P < 0.001$), HbA1c ($r = -0.2786$, $P < 0.001$), 2h-PG ($r = -0.3222$, $P < 0.001$), insulin ($r = 0.9016$, $P < 0.001$), HOMA-IR ($r = 0.6755$, $P < 0.001$) and HOMA-IS ($r = -0.4083$, $P < 0.001$).

Impact factors on HOMA-β level

Multiple linear regression analysis was performed to evaluate the impact factors on β-cell insulin secretion, where HOMA-β was set as the dependent variable while the independent variables included GSH, SOD, nesfatin-1, ADPN, RBP, NAGL, UA, TBA, FBG, HbA1c, 2h-PG, insulin, HOMA-IR and HOMA-IS (α in = 0.05 and α out = 0.10 with backward selection). Factors of GSH, SOD, nesfatin-1, FBG, insulin, HOMA-IR and HOMA-IS were introduced to the equation as $Y = 0.729X_1+0.012X_2+0.007X_3+4.752X_4+18.518X_5-38.817X_6+6.512X_7-67.357$, $R^2 = 0.951$ (Y: HOMA-β, X1: GSH, X_2: SOD, X_3: nesfatin-1, X_4: FBG, X_5: insulin, X_6: HOMA-IR, X_7: HOMA-IS, $P < 0.001$), with the adjusted $R^2 = 0.948$. The standardized regression coefficients of GSH, SOD, nesfatin-1, FBG, insulin, HOMA-IR and HOMA-IS were 0.076, 0.066, 0.056, 0.254,1.882, −1.099, 0.047, respectively (Table 2).

Interestingly, when we excluded glucose metabolism indexes of FBG, HbA1c, 2h-PG, insulin, HOMA-IR and HOMA-IS, and included GSH, SOD, nesfatin-1, ADPN, RBP, NAGL, UA and TBA as independent variables (α in = 0.05 and α out = 0.10 with backward selection), and then we introduced GSH, SOD, nesfatin-1, UA and TBA to the new equation of $Y = 2.437X_1+0.045X_2+0.025X_3+0.062X_4+1.658X_5$, $R^2 = 0.458$ (Y: HOMA-β, X_1: GSH, X_2: SOD, X_3: nesfatin-1, X_4: UA, X_5: TBA, $P < 0.001$), with the adjusted $R^2 = 0.442$ (Supplementary Table 1), the results from this new equation showed that the standardized regression coefficients of GSH, SOD, nesfatin-1, UA and TBA were 0.300, 0.271, 0.284, 0.123, 0.112, respectively.

Comparisons between subgroups of T2DM and prediabetes divided by HOMA-β

To further explore whether serum metabolic indexes could be affected by β-cell insulin secretion, we divided T2DM and prediabetes patients into subgroups by HOMA-β with the cut-off value of 62.9 for male and 60.6 for female (31). The characteristics of T2DM and prediabetes subgroups and the differential analyses were described in Table 3. The differences of age, gender and BMI for T2DM and prediabetes subgroups were non-significant. Further analysis revealed that serum levels of GSH, SOD and nesfatin-1 in T2DM or prediabetes with impaired HOMA-β values (under the cut-off) were apparently low ($P < 0.001$, $P = 0.006$, $P < 0.001$, respectively, in T2DM; $P < 0.001$, $P < 0.001$, $P < 0.001$, respectively, in prediabetes), compared to those patients with normal HOMA-β values (equal to or above the cut-off). Moreover, RBP and TIBC levels in T2DM subgroup with normal HOMA-β were obviously reduced ($P = 0.05$, $P < 0.001$, respectively), compared to those with impaired HOMA-β, but this reduction was not observed between the two prediabetes subgroups.

Comparisons of HOMA-β among subgroups of IFG, IGT and IFG+IGT in prediabetes

To assess whether β-cell insulin secretion varies in prediabetes, three subgroups of IFG, IGT and IFG combined IGT were divided, according to the ADA classification, and their HOMA-β values were compared. The basic characteristics of the subgroups and the comparisons were summarized in the Supplementary Table 2. HOMA-β values in the subgroup of IGT seemed higher (113.52 ± 100.03) than that in IFG (75.00 ± 37.41) or IFG+IGT (74.08 ± 48.55), but the difference among the three subgroups was non-significant ($P = 0.096$). Serum levels of SOD in the IGT subgroup (2,090.95 ± 154.00 U/ml) were significantly higher than that in the IFG combined IGT subgroup (1,869.69 ± 330.96 U/ml), but no apparent difference was found when compared to the IFG subgroup (1,999.77 ±

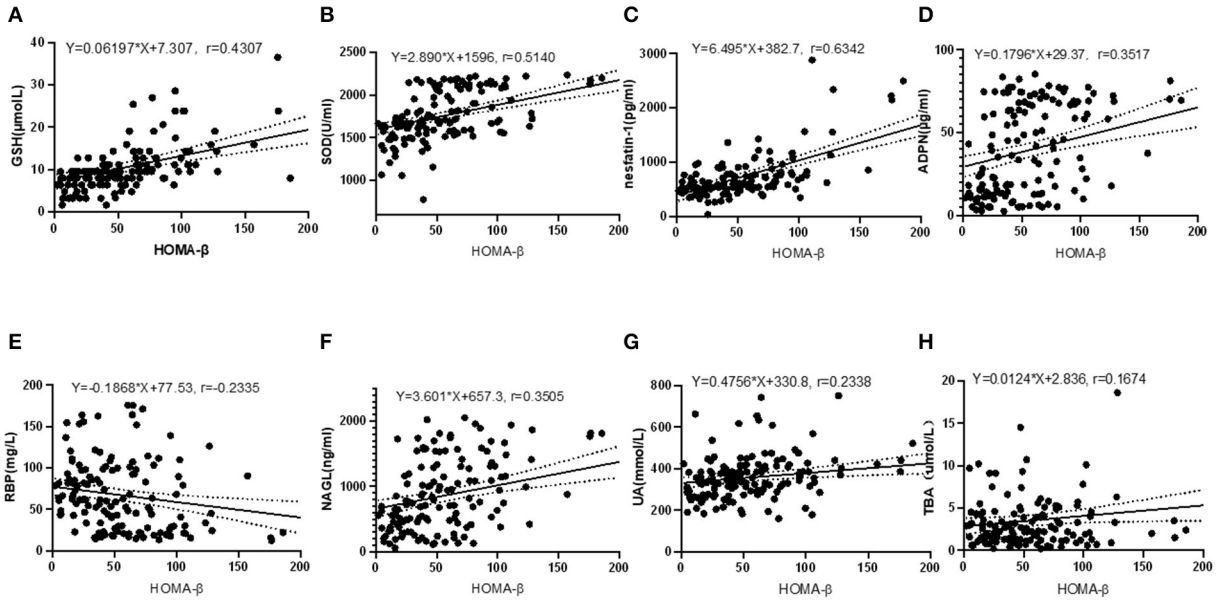

FIGURE 2
Correlations between HOMA-β and GSH, SOD, nesfatin-1, ADPN, RBP, NAGL, UA and TBA. **(A)** correlation between HOMA-β and GSH (R^2 = 0.1855, F = 31.88, $P < 0.001$); **(B)** correlation between HOMA-β and SOD (R^2 = 0.2642, F = 50.26, $P < 0.001$); **(C)** correlation between HOMA-β and nesfatin-1(R^2 = 0.4022, F = 94.18, $P < 0.001$); **(D)** correlation between HOMA-β and ADPN (R^2 = 0.1237, F = 19.77, $P < 0.001$); **(E)** correlation between HOMA-β and RBP (R^2 = 0.0545, F = 8.070, P = 0.005); **(F)** correlation between HOMA-β and NAGL (R^2 = 0.1299, F = 19.61, $P < 0.001$); **(G)** correlation between HOMA-β and UA (R^2 = 0.0547, F = 8.097, P = 0.005); **(H)** correlation between HOMA-β and TBA (R^2 = 0.0280, F = 4.034, P = 0.046).

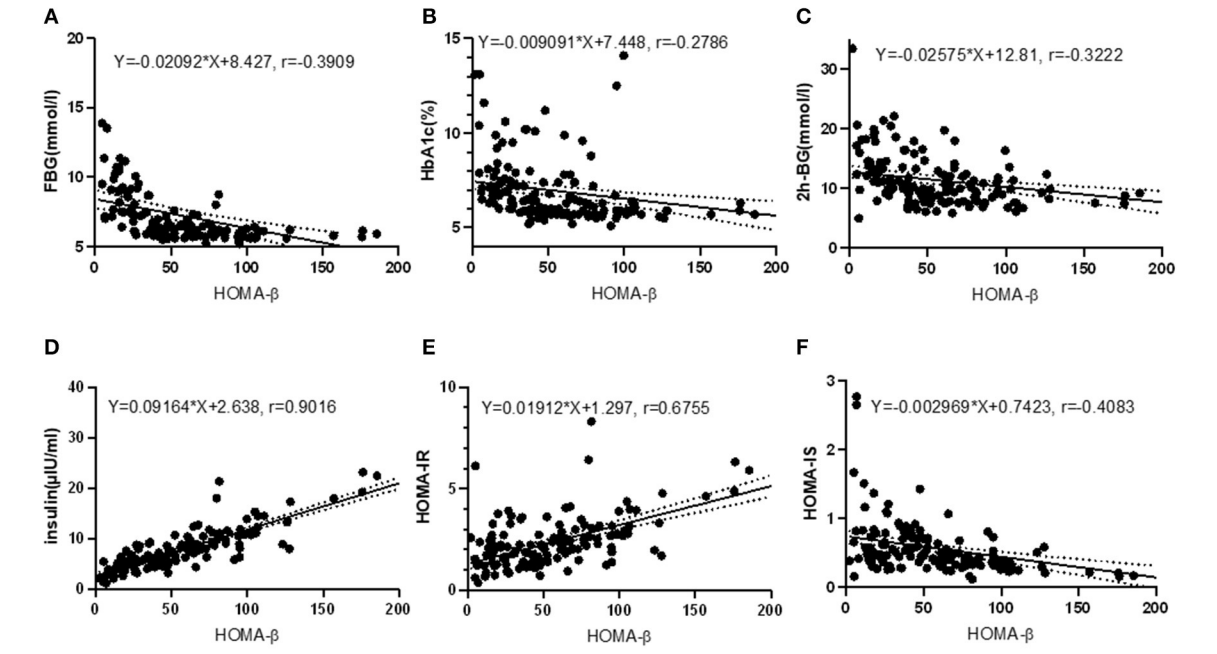

FIGURE 3
Correlations between HOMA-β and glucose homeostasis indexes. **(A)** correlation between HOMA-β and FBG (R^2 = 0.1528, F = 25.25, $P <$ 0.001); **(B)** correlation between HOMA-β and HbA1c (R^2 = 0.0800, F = 11.78, $P < 0.001$); **(C)** correlation between HOMA-β and 2h-BG (R^2 = 0.1038, F = 16.21, $P < 0.001$); **(D)** correlation between HOMA-β and insulin (R^2 = 0.8128, F = 608.0, $P < 0.001$); **(E)** correlation between HOMA-β and HOMA-IR (R^2 = 0.4563, F = 117.5, $P < 0.001$); **(F)** correlation between HOMA-β and HOMA-IS (R^2 = 0.1667, F = 28.01, $P < 0.001$).

TABLE 2 Independent variables introduced to multiple liner regression.

Parameters	*b*	S_b	b'	|t|	*P*
Constant	−67.36	9.36	/	7.195	0.000
GSH	0.73	0.21	0.076	3.413	0.001
SOD	0.012	0.004	0.066	2.941	0.004
Nesfatin-1	0.007	0.003	0.056	2.044	0.043
FBG	4.752	0.697	0.254	6.813	<0.001
Insulin	18.518	1.006	1.882	18.399	<0.001
HOMA-IR	−38.817	3.288	−1.099	11.805	<0.001
HOMA-IS	6.512	3.590	0.047	1.814	0.072

GSH, glutathione; SOD, superoxide dismutase; FBG, fasting blood-glucose; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-IS, homeostasis model assessment of insulin sensitivity.

195.08 U/ml). In contrast, levels of GSH ($P = 0.502$), nesfatin-1 ($P = 0.793$), ADPN ($P = 0.724$), TIBC ($P = 0.263$) and NAGL ($P = 0.808$) were insignificant among the subgroups of IGT, IFG and IFG+IGT.

Discussion

To the best of our knowledge, this research is the first to reveal the differences of circulating levels of nesfatin-1, GSH and SOD in a progressive direction from the healthy condition to T2DM patients through prediabetes. We also disclosed the correlation between HOMA-β and the biomarkers of nesfatin-1, GSH and SOD, and found that these factors could exert influence on β-cell secretion.

Comprised of glutamate, cysteine and glycine, GSH is a ubiquitous thiol tripeptide which could consume hydroxyl, peroxynitrite and superoxide radicals through interacting with ROS (32). Glutathione peroxidases are prominent enzymes in protecting cells against oxidative stress by oxidizing GSH to glutathione and depleting the radicals (33). A previous study observed GSH deficiency in T2DM patients (34), while another report unfolded slightly higher GSH levels in IFG than in the control (35). In the present research, we found that serum GSH levels in T2DM were significantly reduced than that in prediabetes or the control, and this significant reduction was also confirmed in prediabetes vs. the control; further comparisons revealed that the difference of GSH levels among prediabetes subgroups of IGT, IFG and IFG+IGT was insignificant ($P = 0.502$). Notably, GSH levels in either subgroup of T2DM or prediabetes with impaired HOMA-β values were overwhelmingly dropped, in contrast to the counterparts with normal HOMA-β. As a substrate of glutathione peroxidase, GSH is of great importance in human metabolic activities for it constitutes the anti-oxidative defensive system *in vivo*. SOD is an antioxidant enzyme, capable of catalyzing superoxide to hydrogen peroxide and oxygen molecules (36). There are three isoforms of mammal SOD: SOD1 in cytosolic (such as Cu and Zn-SOD), SOD2 in mitochondrion (such as Mn-SOD) and SOD3 in extracellular matrix (such as EC-SOD) (37). In this study, the SOD detected in serum mainly belongs to SOD3. Our results demonstrated that SOD levels in T2DM and prediabetes were remarkably decreased compared with the healthy control, and we also observed a significant reduction of the SOD level in T2DM vs. prediabetes. GSH and SOD are the classical component of the cell anti-oxidation system. Indeed, our results revealed that GSH and SOD levels in the subgroup of T2DM or prediabetes with impaired β-cell insulin secretion were significantly low in comparison to the counterpart with normal insulin action; in addition, serum SOD levels in subgroup of IFG or IFG combined IGT displayed a marked reduction compared to the IGT subgroup. Our results of GSH and SOD reduction in T2DM and prediabetes suggest that in the condition of T2DM or prediabetes, the anti-oxidation capacity in the body may be partly damaged, which was in consistent with previous studies (2, 38, 39).

Elevated blood glucose is essential for the formation of advanced glycation end products (AGEs), a group of modified proteins and/or lipids with damage potential, which contribute to the progression of T2DM. For one thing, AGEs could increase the formation of ROS and undermine the anti-oxidative defense mechanism of human body; for another, the generation of AGEs is enhanced under oxidative stress conditions (39). Abnormal glycometabolism is the major hallmark for the pathogenesis and development of T2DM, which is currently controllable but irreversible in most cases. However, prediabetes is a reversible state that could be transited from disturbance of carbohydrate metabolism to normoglycaemia; therefore, fortifying the antioxidative defense system of the patients with prediabetes may help regress or alleviate the progression of the disease toward T2DM.

Compared to IFG, IGT presents severe transitory hyperglycemia, which may explain the higher GSH, SOD, nesfatin-1, insulin and HOMA-β levels in IGT than IFG in our study. Investigation on nesfatin-1 provided evidences that its circulating level correlated with T2DM and elevated in newly diagnostic T2DM patients (24), but decreased in those patients who received antidiabetic treatment (28). In our study, serum nesfatin-1 levels in T2DM were obviously reduced compared to that in prediabetes or healthy subjects, which was supported by other evidences (40, 41), and this reduction still presented when comparing prediabetes to the control, which has not been reported so far. Furthermore, we found that difference of serum nesftain-1 levels in IGT were insignificant compared to either in IFG or in IFG+IGT. A few studies (20, 42, 43) declared the antioxidant function of nesfatin-1; noteworthy, we observed that nesfatin-1levels were significantly correlated with GSH ($r = 0.222$, $P = 0.003$) and SOD ($r = 0.287$, $P < 0.001$) (refer to the Supplementary Figure), indicating a high probability of

TABLE 3 Anthropometric and clinical characteristics of the subgroups divided by HOMA-β.

Parameters	T2DM		|t|	*P*	Prediabetes		|t|	*P*
	HOMA-β reduced	HOMA-β normal			HOMA-β reduced	HOMA-β normal		
Age (years)[a]	54 (51–61)	55 (51–61)	0.152	0.880	55 (51.50–57)	57 (51.75–62)	1.595	0.116*
Gender (M/F)	32/25	9/9	0.653	0.419*	10/19	33/15	0.015	0.903*
BMI (Kg/m^2)[b]	23.16 ± 4.31	22.51 ± 2.02	0.617	0.539	22.97 ± 2.85	23.37 ± 2.66	0.585	0.561
GSH (μmol/L)[b]	7.54 ± 2.90	14.02 ± 5.42	4.854	<0.001	8.70 ± 3.02	14.58 ± 7.47	4.404	<0.001
SOD (U/ml)[b]	1,557.35 ± 181.60	1,695.31 ± 171.13	2.847	0.006	1,846.28 ± 257.13	2,075.24 ± 156.00	4.237	<0.001
Nesfatin-1 (pg/ml)[b]	564.55 ± 171.70	863.40 ± 299.85	4.025	<0.001	598.41 ± 227.24	1,087.64 ± 672.25	4.184	<0.001
ADPN (μg/ml)[b]	16.79 ± 12.20	23.06 ± 19.17	1.64	0.105	65.86 ± 11.85	63.46 ± 13.60	0.757	0.452
RBP (mg/L)[b]	87.74 ± 33.38	106.69 ± 40.59	1.991	0.05	33.18 ± 14.77	32.67 ± 19.67	0.116	0.908
TIBC (μmol/L)[b]	77.98 ± 67.46	33.48 ± 8.14	4.869	<0.001	38.49 ± 8.93	40.66 ± 7.94	1.046	0.299
NAGL (ng/ml)[b]	443.84 ± 230.19	471.46 ± 245.38	0.437	0.663	1,296.21 ± 347.49	1,381.25 ± 379.01	0.943	0.349
CysC (mg/L)[b]	0.78 ± 0.62	1.19 ± 1.05	1.577	0.130	0.68 ± 0.14	0.71 ± 0.17	0.949	0.346
HDL-C (mmol/L)[b]	1.13 ± 0.30	1.27 ± 0.24	1.801	0.076	1.41 ± 0.28	1.24 ± 0.24	2.552	0.013
LDL-C (mmol/L)[b]	3.36 ± 0.93	3.13 ± 1.17	0.832	0.488	3.79 ± 0.63	3.43 ± 0.78	2.038	0.046
HDL-C/LDL-C[b]	0.36 ± 0.13	0.49 ± 0.34	1.545	0.139	0.38 ± 0.09	0.38 ± 0.11	0.047	0.963
TBA (μmol/L)[b]	3.12 ± 2.38	4.62 ± 5.07	1.213	0.240	3.07 ± 3.26	3.88 ± 4.38	0.834	0.407
UA (μmol/L)[b]	342.12 ± 95.51	371.31 ± 161.53	0.728	0.475	328.98 ± 72.86	391.73 ± 88.37	3.102	0.003
Scr (μmol/L)[b]	1.17 ± 1.49	2.01 ± 2.51	1.352	0.191	0.87 ± 0.12	0.96 ± 0.19	2.562	0.013
BUN (mmol/L)[b]	8.34 ± 19.07	8.92 ± 7.15	0.125	0.901	5.40 ± 1.04	5.24 ± 1.21	0.584	0.561
HbA1c (%)[b]	7.93 ± 1.75	7.49 ± 2.42	0.850	0.850	5.89 ± 0.31	5.87 ± 0.33	0.237	0.813
FBG (mmol/L)[b]	9.00 ± 3.70	6.32 ± 0.99	4.949	<0.001	5.93 ± 0.36	5.80 ± 0.46	1.325	0.190
2h-BG (mmol/L)[b]	14.40 ± 4.64	11.45 ± 2.90	2.450	0.017	8.44 ± 1.47	8.82 ± 1.61	0.983	0.329
Insulin (μIU/ml)[b]	5.49 ± 2.29	11.22 ± 4.14	5.598	<0.001	5.24 ± 1.46	12.75 ± 6.89	6.520	<0.001
HOMA-IR[b]	2.12 ± 1.02	3.30 ± 1.77	2.665	0.015	1.39 ± 0.45	3.30 ± 1.77	6.386	<0.001
HOMA-β[b]	25.34 ± 15.09	80.68 ± 17.89	12.967	<0.001	43.23 ± 9.69	113.87 ± 68.58	6.268	<0.001
HOMA-IS[b]	63.00 ± 49.00	39.00 ± 21.00	2.082	0.041	0.79 ± 0.25	37.00 ± 16.00	8.188	<0.001

[a]Median (interquartile range), [b]mean ± SD, *Chi-Square $P < 0.05$. BMI, body mass index; GSH, glutathione; SOD, superoxide dismutase; ADPN, adiponectin; RBP, retinol binding protein; TIBC, total iron binding capacity; NAGL, neutrophil gelatinase-associated lipocalin; CysC, cystatin C; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TBA, total bile acid; UA, uric acid; Scr, serum creatinine; BUN, blood urea nitrogen; HbA1c, glycosylated hemoglobin; FBG, fasting blood-glucose; 2h-PG, two-hour post glucose; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell; HOMA-IS, homeostasis model assessment of insulin sensitivity.

nesfatin-1 exerting antioxidative effects in the development of T2DM.

Our exploration of impact factors on β-cell secretory action revealed that FBG, insulin and HOMA-IR could significantly affect insulin secretion, as well as the factors of GSH, nesfatin-1 and SOD. β-cell viability and insulin release could be crippled as a consequence of hyperglycaemia and glucotoxicity in human body (44, 45). Oxidative stress has been widely accepted as a major causative factor responsible to increase the production of ROS and impede the antioxidant pathway combined with glucotoxicity and/or lipotoxicity, ultimately leading to β-cell dysfunction and overt T2DM (45, 46). GSH and SOD were recognized as vital components of intrinsic defense mechanism involving anti-oxidative activity (10), and higher circulating GSH and SOD levels were believed to be able to protect β-cells from damage of free radicals, including ROS, superoxide, hydrogen peroxide and hydroxyl (6).

To make a long story short, our study successfully managed to identify the correlation of nesfatin-1, GSH and SOD levels with β cell dysfunction in T2DM, implicating their roles in β cell toxicity as a result of oxidative stress. However, this study is limited firstly in that it is cross-sectional in nature and unable to determine causality between the disease and its risky factors in diabetic patients. Secondly, due to our relatively small sample size of only 75 T2DM and 67 prediabetes individuals, further investigation with enlarged samples is needed to make the conclusion more convincible. Thirdly, the cutoffs used for HOMA-β evaluation were adopted from a previous study based on population in Tehran, and the impact of the ethnic variations on glycemic indices has to be considered when apply the data to different population. Fourthly, medication histories of the studied subjects were not obtained because of the unavailability of sufficient clinical information of the patients at hand. To remedy this, we plan to record the medication history details

in our future cohort study on exploring whether insulin or other anti-diabetic agents can impose effects on nesfatin-1 level in serum. Last but not least, despite the fact that we revealed that serum nesfatin-1, GSH and SOD levels correlated with and affected insulin secretion, more efforts should be made to unveil the effects of these factors on insulin function.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KH collected the data and wrote the main manuscript draft. YL and KW made the statistical analysis and the figures. JW and HL prepared the tables. BY designed the investigation, modified the original draft, and approved the final version. All authors reviewed the manuscript and agreed on this submission.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.882686/full#supplementary-material

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Relationship between physical performance and mild cognitive impairment in elderly hemodialysis patients is modified by the presence of diabetes: A multicenter cross-sectional study

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Objective: The purpose of this study was to observe the relationship between physical performance and mild cognitive impairment (MCI) in the presence or absence of type 2 diabetes in elderly hemodialysis patients.

Methods: In this multicenter cross-sectional study, 396 clinically stable and aged ≥60 years hemodialysis patients (255 men; mean age: 68.3 ± 5.9 years) were included from seven dialysis units in Shanghai, China. The Chinese version of the Modified Mini-Mental State Examination (MMSE) and the Instrumental Activities of Daily Living (IADL) scale were utilized to assess MCI. The performance-based assessments consisted of three physical tests, grip strength (GS), Timed Up and Go Test (TUGT), and 4-m walking test, which respectively represent muscle strength, mobility, and walking speed (WS). Logistic regression and multivariate linear regression were used for analysis.

Results: Hemodialysis patients with diabetes had a high prevalence of MCI (20.6%). The odds ratio (OR) of MCI for the interacted items [(TUGT) * (diabetes) and (WS) * (diabetes)] was significant ($p < 0.05$). In diabetes patients, TUGT was positively associated with MCI, and WS was negatively associated with MCI after

adjusting covariates [OR = 0.129; 95% confidence interval (CI) = 0.028–0.704, p = 0.021]. However, no significant association was found between physical performance and MCI in the non-diabetes hemodialysis patients (p > 0.05). Further analysis showed that TUGT was negatively associated with attention and calculation and language. WS was positively associated with recall and language in diabetic hemodialysis patients.

Conclusions: Physical performance was associated with MCI in diabetic hemodialysis patients rather than the non-diabetes group. Whether increasing mobility or WS can positively influence MCI in individuals with type 2 diabetes requires further study.

KEYWORDS

mild cognitive impairment, diabetes, physical performance, walking speed, hemodialysis

Introduction

Mild cognitive impairment (MCI) represents a transitional stage between normal age-related decline in cognitive function and dementia and is more prevalent in the elderly population and hemodialysis patients than the general population (1). As a therapeutic window, the latest guidelines show that MCI patients are still more likely to improve or maintain cognitive function (2). The decline in cognitive function is often influenced by many factors, such as age, education, vascular diseases, and chronic diseases (diabetes, hypertension) (3). Considering that chronic kidney disease (CKD) patients are usually accompanied by the protein-energy wasting and metabolic disorders that lead to impaired muscle mass and a decline in physical performance (4), the relationship between physical performance and MCI in elderly hemodialysis patients deserves further in-depth study.

Diabetes is considered to be a major cause of end-stage renal disease and appears to be increasing rapidly (5). Having prediabetes and diabetes was significantly associated with lower health-related quality of life relative to normal glucose tolerance (6). Data from a well-functioning population showed that compared with those without diabetes, those with diabetes exhibited lower performance on objective measures of lower-extremity function (7). The latest study showed a strong and significant correlation between 5-m gait speed and glycemia (8). Physical activity and sedentary behavior are associated with biomarkers of endothelial dysfunction, and the associations were stronger in (pre)diabetes than in normal glucose metabolism (9). In addition, a previous study has indicated a link of diabetes to an increased risk of MCI (10) and shown that the risk of incident MCI is higher in people with type 2 diabetes than that in those without diabetes (11). Pasquale et al. found a significant correlation between 5-m gait speed test and Montreal Cognitive Assessment (MoCA) score in frail diabetic older adults (12). Due to insufficient insulin secretion or insulin resistance, insulin-stimulated glucose uptake is markedly reduced in skeletal muscle and a hyperglycemic condition leads to endothelial and cerebral microvascular dysfunction (13–15), which may affect both physical performance and cognitive function. Previous and our studies have reported that poor physical performance is significantly associated with MCI in community-dwelling older adults (16–18). However, whether the presence of diabetes alters the relationship between physical performance and MCI is not yet known.

Therefore, this study aimed to explore the relationship between physical performance and MCI in elderly hemodialysis patients with and without diabetes. According to the above indications, we hypothesized that the presence of diabetes would lead to poorer physical performance and high prevalence of MCI, and different conditions may influence the association between physical performance and MCI. Moreover, it also investigated the association between physical performance [muscle strength, mobility, and walking speed (WS)] and specific cognitive functions in the presence or absence of type 2 diabetes to provide evidence for clinicians to effectively manage MCI in hemodialysis patients.

Methods

Study participants

The multicenter cross-sectional study recruited patients who underwent hemodialysis in seven dialysis units in Shanghai, China [ChiCTR1900027039] between July 2020 and April 2021. Hemodialysis is a process in which blood is drained outside the

body through a circulatory line, exchanged through a dialyzer, and the purified blood is returned to the body. Vascular access modalities for hemodialysis included fistulas and catheters, and dialyzer models included F14, LOPS15, FX80, etc. Patients aged 60 years or older who were on hemodialysis for 4 h per session, three times a week, and for more than 3 months were included in the study. Participants with the following conditions were excluded: 1) unable to communicate with interviewers or grant informed consent; 2) unable to complete the physical performance test; 3) had a known diagnosis of dementia, psychiatric disorders, or other neurodegenerative diseases; and 4) no blood sample collection. Following these exclusions, the final analyzed population comprised 396 subjects (255 men, 141 women). All participants are required to complete an annual health screening and a detailed questionnaire on lifestyle and disease history. The study was approved by the Ethics Committee of the Shanghai University of Medicine and Health Sciences, and the methods were carried out in accordance with the principles of the Declaration of Helsinki. All participants were informed and signed consent prior to enrollment in the study.

Baseline variables

Demographic characteristics (including age, gender, education level, registered residence, and marital status) and health behaviors (including smoking, drinking, and sleep duration) were obtained from a standardized questionnaire by face-to-face interview. Physical activity was assessed using the short form of the International Physical Activity Questionnaire (IPAQ) (19), and depressive symptoms were assessed using the Patient Health Questionnaire 9 (PHQ9) (20). Nutritional status was assessed using the Malnutrition Inflammation Score (MIS) (21). Charlson Comorbidity Index (CCI) was used to assess the comorbidity risk associated with several conditions (22). We collected biochemical data including serum albumin, hemoglobin, calcium, phosphate, and parathyroid hormone (PTH) within 3 months of physical assessment. Dialysis adequacy was defined as the total fractional clearance index for urea (Kt/V) and urea reduction ratio (URR).

Diabetes information

Access to diabetes information was based on subjects' self-reports, and we again carefully checked the fasting plasma glucose (FPG) data through electronic medical records. According to the American Diabetes Association 2021 criteria, FPG level ≥7.0 mmol/L or 2-h plasma glucose ≥11.1 mmol/L during an oral glucose tolerance test or HbA1c ≥6.5% was considered as diabetes (23).

Physical performance

Performance-based assessment consisted of grip strength (GS), Timed Up and Go Test (TUGT), and 4-m WS test. GS was measured using a dynamometer (GRIP-D; Takei Ltd., Niigata, Japan). Participants were allowed to exert maximum efforts twice using the dominant hand, and the average value was calculated from two attempts. TUGT assessed the seconds of standing up from a chair, walking 3 m at usual pace past a line on the floor, turning around, walking back to the chair, and then sitting down on the chair. The WS test consists of participants being timed while walking 4 m at their usual pace and they were allowed to use a gait-assistive device. Participants completed the test twice, and the mean gait speed (m/s) was calculated (17). Higher GS values, shorter TUGT, and faster WS represent better physical performance. All tests were monitored by corresponding professional physical therapists.

MCI and cognitive function

This study adopted the MCI diagnostic criteria based on Petersen's definitions with modifications (24): 1) memory complaints (self-reported or reported by family members or caregivers); 2) objective cognitive impairment, as assessed by the Mini-Mental State Examination (MMSE); 3) intact or only mildly impaired daily living ability, as assessed by the Instrumental Activities of Daily Living (IADL) Scale; 4) no clear dementia, as evaluated by the Chinese version of the Dementia Rating Scale (CDRS); 5) no abnormal memory impairment for age. The MMSE score ranges from 0 to 30 points, with the higher scores indicating better cognitive performance. It has been reported that the Chinese version of the MMSE indicates MCI for scores ≤17, 20, and 24 in people with the educational level of illiteracy, primary school, and middle school or higher, respectively (25). The IADL Scale includes eight items, and the score ranges from 0 to 8 points, with the higher scores indicating better daily living ability. IADL scored ≥6 indicates intact or only mildly impaired daily living ability (26). The MMSE includes a broad set of cognitive functions that measure the following: orientation (10 points), registration (3 points), attention and calculation (5 points), recall (3 points), and language (9 points).

Statistical analysis

The baseline characteristics of participants were presented according to the classification of diabetes and MCI. Continuous variables were presented as means ± standard deviation (SD), and categorical variables were expressed as numbers and percentages. Baseline sociodemographic characteristics were

analyzed using t test, Pearson's chi-square test, and Mann–Whitney U test. The interaction effect between the component of physical performance and diabetes was tested by adding three interacted items (GS * diabetes; TUGT * diabetes; WS * diabetes) in the logistic regression analysis. Binary logistic regression analysis was used to analyze the relationship between physical performance and MCI in hemodialysis patients in the non-diabetic and diabetic groups. MCI was used as the dependent variable, each component of physical performance (GS, TUGT, WS) was used as an independent variable, and several confounding factors [age, gender, body mass index (BMI), year, widowhood, living alone, illiteracy, smoking, alcohol consumption, sleep duration, IPAQ, depression, number of medications, and CCI] were adjusted as covariates. Linear regression models were used to analyze the relationship between GS, TUGT, WS, and various cognitive functions. All of the statistical analyses were performed with the SPSS V26.0 software, and differences were defined as significant when $p < 0.05$.

Results

Participant characteristics

Figure 1 shows the flow of hemodialysis participants with subgroups. The analysis sample consisted of 396 study participants (255 men; mean age: 68.3 ± 5.9 years). Baseline characteristics of the subjects were presented in Table 1. Among all participants, 204 (51.5%) reported diabetes and 74 (18.7%) had MCI. Compared to non-MCI, MCI patients with or without diabetes were prone to be widowed ($p < 0.05$). Compared to non-diabetes, hemodialysis patients with diabetes were prone to be men, have a shorter vintage, and have a higher CCI level. As shown in Figure 2, it is noteworthy that in the diabetes group, MCI patients' physical performance (TUGT and WS) was significantly worse than that of the cognitively normal group. The TUGT of patients with diabetes was significantly longer than that of those without diabetes ($p < 0.05$), indicating poorer mobility. However, in the non-diabetes group, there was no significant difference in physical performance between the MCI group and the cognitively normal group.

Associations between physical performance and MCI in the non-diabetic or diabetic hemodialysis patients

As the main findings, we investigated the association between physical performance and MCI, and the interactive effects of physical performance and diabetes were evaluated by adding the interacted items using logistic regression analysis (Table 2). The odds ratio (OR) of MCI for the interacted items [(TUGT) * (diabetes) and (WS) * (diabetes)] were significant (1.044, 95% confidence interval [CI] 1.002–1.087, $p = 0.040$; 0.905, 95% CI 0.826–0.991, $p = 0.032$; Table 2), suggesting a diabetes-dependent effect of mobility and WS. In the subgroup analysis, the crude model showed that TUGT and WS were associated with the risk of MCI in the diabetes group, and ORs (and 95% CIs) were 1.077 (1.005–1.155) and 0.181 (0.048–0.681), respectively ($p < 0.05$), indicating that longer TUGT was associated with a higher risk of MCI, and faster WS was

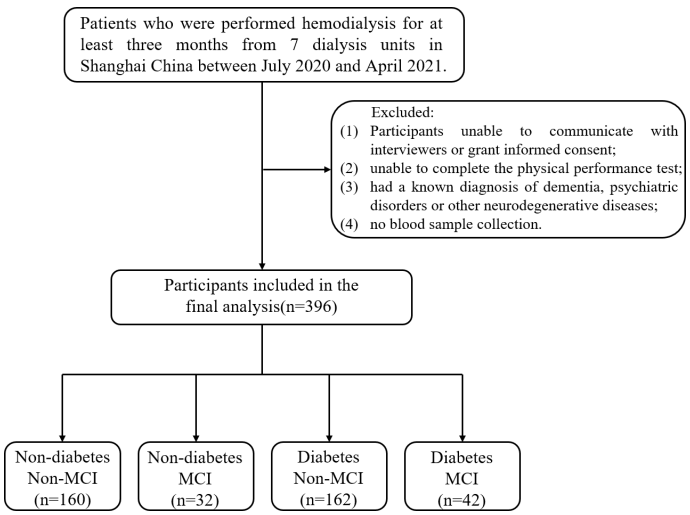

FIGURE 1
Flow diagram of the study. MCI, mild cognitive impairment.

TABLE 1 Baseline data classified by diabetes and MCI in the elderly hemodialysis patients.

Characteristics	Non-diabetes			Diabetes		
	Non-MCI (n=160)	MCI(n=32)	P	Non-MCI(n=162)	MCI(n=42)	P
Age (years)	68.58 ± 6.32	68.81 ± 6.63	0.852	67.70 ± 5.35	68.57 ± 5.82	0.359
Men (%)	97 (60.6)	13 (40.6)	0.037	118 (72.8) a	27 (64.3) b	0.276
Vintage (months)	58.6 (35.5,121.4)	46.7 (31.9,101.2)	0.226	37.1 (15.9,72.1) a	35.1 (22.2,55.2) b	0.741
BMI (kg/m^2)	22.95 ± 3.32	22.46 ± 3.38	0.447	23.63 ± 3.48	23.97 ± 3.92	0.581
Widowed (%)	10 (6.3)	8 (25.0)	0.001	10 (6.2)	9 (21.4)	0.002
Living alone (%)	6 (3.8)	2 (6.3)	0.518	8 (4.9)	5 (11.9)	0.100
Illiterate (%)	8 (5.0)	4 (12.5)	0.110	7 (4.3)	3 (7.1)	0.450
Drinking (%)	20 (12.5)	4 (12.5)	1.000	19 (11.7)	3 (7.1)	0.393
Smoking (%)	31 (19.4)	4 (12.5)	0.358	43 (26.5)	9 (21.4)	0.498
GS (kg)	23.67 ± 7.35	21.29 ± 7.29	0.096	24.71 ± 6.89	23.08 ± 5.92	0.167
TUGT (s)	9.20 ± 3.53	8.41 ± 1.81	0.220	9.51 ± 4.24	11.15 ± 4.56 b	0.029
WS (m/s)	0.99 ± 0.27	0.98 ± 0.25	0.771	1.01 ± 0.26	0.89 ± 0.27	0.010
IPAQ (Met/week)	1882 (693,3066)	2299 (1105,4198)	0.249	1386 (693,3079) a	1473 (635,3796)	0.875
Depression (%)	65 (40.6)	17 (54.8)	0.143	63 (38.9)	11 (26.8) b	0.152
MIS score	4.09 ± 2.38	4.22 ± 2.21	0.787	4.12 ± 2.92	4.48 ± 2.68	0.471
Sleep duration (h)	8.05 ± 1.81	8.63 ± 1.87	0.106	8.35 ± 2.26	8.89 ± 1.84	0.155
MMSE score	27.02 ± 1.98	20.50 ± 3.89	<0.001	26.95 ± 2.28	21.07 ± 3.99	<0.001
CCI	3.49 ± 1.45	3.69 ± 1.42	0.491	4.38 ± 1.73 a	4.50 ± 1.61 b	0.677
Laboratory parameters						
Glucose (mmol/L)	5.64 ± 0.86	5.39 ± 0.76	0.132	9.82 ± 4.10 a	10.44 ± 3.88 b	0.383
Hemoglobin (g/L)	114.22 ± 13.89	113.48 ± 15.22	0.786	109.46 ± 14.00 a	112.16 ± 14.83	0.274
Albumin (g/L)	39.74 ± 3.27	39.67 ± 3.54	0.919	39.27 ± 3.24	39.41 ± 2.74	0.794
Creatinine (μmol/L)	951.46 ± 279.14	956.51 ± 176.90	0.922	931.80 ± 264.39	958.64 ± 235.90	0.554
Uric acid (μmol/L)	434.11 ± 106.91	461.45 ± 90.11	0.268	451.73 ± 81.14	446.96 ± 94.69	0.774
Calcium (mmol/L)	2.30 ± 0.26	2.25 ± 0.29	0.404	2.27 ± 0.28	2.21 ± 0.22	0.226
Phosphate (mmol/L)	1.93 ± 0.63	1.92 ± 0.52	0.945	1.87 ± 0.62	1.90 ± 0.67	0.826
PTH (pg/ml)	381.13 ± 354.86	371.62 ± 375.72	0.891	286.06 ± 238.17 a	250.83 ± 173.14	0.381
Kt/V	1.40 ± 0.31	1.44 ± 0.21	0.525	1.36 ± 0.38	1.38 ± 0.24	0.773
URR	0.68 ± 0.09	0.70 ± 0.07	0.219	0.67 ± 0.08	0.68 ± 0.06	0.632

MCI, mild cognitive impairment; BMI, body mass index; GS, grip strength; TUGT, Timed Up and Go Test; WS, walking speed; IPAQ, International Physical Activity Questionnaire; MIS, Malnutrition Inflammation Score; MMSE, Mini-Mental State Examination; CCI, Charlson Comorbidity Index; PTH, parathyroid hormone; Kt/V, fractional clearance index for urea; URR, urea reduction ratio.
Data are presented as mean ± SD or n (%).
a In non-MCI patients, the Diabetes group vs. the Non-Diabetes group, $p < 0.05$.
b In MCI patients, the Diabetes group vs. the Non-Diabetes group, $p < 0.05$.

associated with a lower risk of MCI, respectively. In the adjusted model (age, sex, BMI, vintage, widowed, living alone, illiteracy, smoking, drinking, sleep duration, IPAQ, depression, number of drugs, and CCI), only WS was negatively associated with MCI ($p = 0.021$). However, whether crude or adjusted, this association did not exist in the non-diabetes group (all $p > 0.05$).

Associations between physical performance and specific cognitive functions in the hemodialysis patients

Then, we performed multivariate linear regression analysis of the association between different physical performance components and cognitive functions in the non-diabetes and diabetes hemodialysis patients (Table 3). In the fully adjusted model, the TUGT was negatively associated with overall cognition, attention and calculation, and language, and the WS was positively associated with overall cognition, recall, and language in the diabetes group ($p < 0.05$), while only WS was positively associated with attention and calculation in the non-diabetes group ($p = 0.044$). Whether in the non-diabetes or diabetes group, GS was not associated with any of the cognitive functions (all $p > 0.05$).

Discussion

The main findings of our current study showed that diabetic hemodialysis patients with MCI performed worse mobility than the non-diabetes group. Further analysis found that the

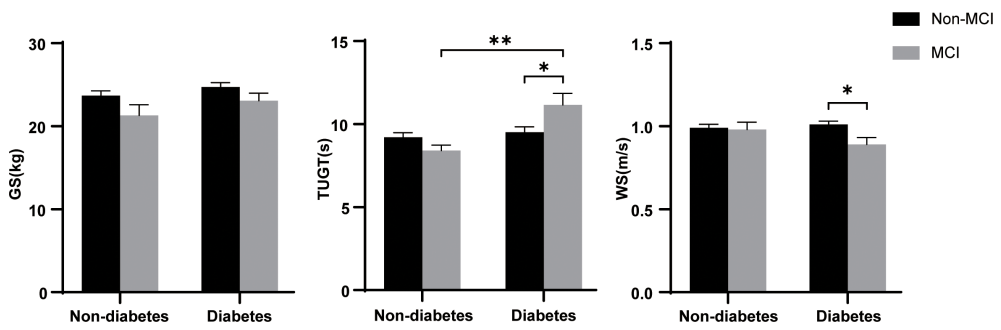

FIGURE 2
Difference of physical performance between the different groups. GS, grip strength; TUGT, Timed Up and Go Test; WS, 4-m walking speed; MCI, mild cognitive impairment. Data are presented as mean ± SD using t test. * represents $p < 0.05$, ** represents $p < 0.01$.

interaction between mobility/WS and diabetes is significant. In hemodialysis patients with diabetes, those with MCI performed worse WS than those without MCI, whereas no association was found for patients without diabetes. Moreover, multivariate linear regression analysis showed that TUGT was negatively associated with attention and calculation and language. WS was positively associated with recall and language in diabetic hemodialysis patients.

Our previous studies have shown that physical performance was significantly different based on MCI status in Chinese older adults with an average age of 72.6 years (17). Poor health outcomes in diabetes are closely linked to physical activity and dietary patterns, which are also risk factors for CKD (27–29). Therefore, we compared physical performance in hemodialysis populations grouped by diabetes and MCI and explored the relationship between physical performance and MCI in hemodialysis patients with and without diabetes in our study. It is worth noting that we found, whether compared with MCI in the non-diabetes group or non-MCI in the diabetes group, diabetic patients with MCI have poor mobility (Figure 2, $p < 0.05$). This finding is unprecedented. Patients with CKD experience substantial loss of muscle mass, and skeletal muscle dysfunction leads to mobility limitation (30). Kestenbaum et al. (31) demonstrated a 25% reduced leg muscle mitochondrial oxidative capacity in participants with CKD and that leg muscle oxidative capacity is a significant predictor of mobility. Moreover, a history of diabetes also imparted nearly the same magnitude of reduction in mitochondrial function (31). Therefore, altered metabolic transcriptional networks and defective mitochondrial function are likely to be major mechanistic factors in the progression of CKD caused by diabetes that impairs physical function (32). In a slight

TABLE 2 Logistic regression analysis of physical performance and MCI in the non-diabetic and diabetic hemodialysis patients.

Variables	Crude		Adjusted Model	
	OR (95%CI)	p	OR (95%CI)	p
Non-Diabetes				
GS	0.955 (0.904,1.009)	0.908	0.981 (0.902,1.071)	0.733
TUGT	0.914 (0.791,1.057)	0.225	0.863 (0.706,1.055)	0.132
WS	0.806 (0.190,3.416)	0.770	0.825 (0.113,5.731)	0.845
Diabetes				
GS	0.964 (0.914,1.016)	0.168	0.973 (0.906,1.048)	0.428
TUGT	1.077 (1.005,1.155)	0.036	1.084 (0.993,1.187)	0.059
WS	0.181(0.048,0.681)	0.011	0.129 (0.028,0.704)	0.021
Interacted items				
GS*diabetes			1.003 (0.984,1.023)	0.754
TUGT*diabetes			1.044 (1.002,1.087)	0.040
WS*diabetes			0.905 (0.826,0.991)	0.032

The interacted items were included in the above model, and the p-values for [(TUGT) * (diabetes) and (WS) * (diabetes)] were significant. Adjusted model is adjusted with age, sex, BMI, vintage, widowed, living alone, illiteracy, smoking, drinking, sleep duration, IPAQ, depression, number of drugs, and CCI.
MCI, mild cognitive impairment; BMI, body mass index; IPAQ, International Physical Activity Questionnaire; CCI, Charlson Comorbidity Index; GS, grip strength; TUGT, Timed Up and Go Test; WS, walking speed; CI, confidence interval.

TABLE 3 Multivariate linear regression analysis of the association between physical performance and cognitive functions in the non-diabetic and diabetic hemodialysis patients.

Variables	Non-diabetes				Diabetes			
	Crude		Adjusted Model		Crude		Adjusted Model	
	β	p	β	p	β	p	β	p
GS								
MMSE score	0.070 (0.005,0.136)	0.036	0.043 (-0.032,0.114)	0.264	0.126 (0.055,0.197)	0.001	0.041 (-0.036,0.125)	0.275
Orientation	0.017 (-0.006,0.041)	0.150	0.016 (-0.015,0.040)	0.368	0.040 (0.016,0.064)	0.001	0.019 (-0.0080.050)	0.146
Registration	0.003 (-0.003,0.009)	0.288	0.001 (-0.007,0.009)	0.783	0.006 (-0.003,0.014)	0.183	0.003 (-0.007,0.015)	0.471
Attention and calculation	0.031 (0.002,0.060)	0.035	0.026 (-0.014,0.060)	0.229	0.034 (0.005,0.064)	0.023	0.005 (-0.033,0.039)	0.859
Recall	-0.005 (-0.026,0.017)	0.666	-0.005 (-0.031,0.025)	0.819	0.006 (-0.018,0.030)	0.638	0.004 (-0.025,0.036)	0.713
Language	0.024 (0.002,0.046)	0.031	0.013 (-0.014,0.033)	0.421	0.041 (0.016,0.065)	0.001	0.009 (-0.019,0.039)	0.482
TUGT								
MMSE score	-0.058 (-0.205,0.089)	0.439	-0.046 (-0.198,0.101)	0.527	-0.240 (-0.351,-0.130)	<0.001	-0.172 (-0.284,-0.064)	0.002
Orientation	-0.004 (-0.057,0.049)	0.887	-0.015 (-0.067,0.046)	0.718	-0.042 (-0.080,-0.004)	0.030	-0.018 (-0.055,0.025)	0.467
Registration	-0.002 (-0.015,0.011)	0.720	0.006 (-0.012,0.020)	0.622	-0.013 (-0.028,0.001)	0.064	-0.011 (-0.029,0.003)	0.114
Attention and calculation	-0.011 (-0.077,0.054)	0.731	-0.041 (-0.119,0.032)	0.257	-0.065 (-0.111,-0.019)	0.006	-0.057 (-0.108,-0.010)	0.017
Recall	0.016 (-0.031,0.064)	0.505	0.037 (-0.022,0.090)	0.236	-0.039 (-0.075,-0.002)	0.041	-0.039 (-0.077,0.006)	0.092
Language	-0.054 (-0.103,-0.006)	0.029	-0.035 (-0.080,0.016)	0.182	-0.080 (-0.118,-0.043)	<0.001	-0.049 (-0.090,-0.012)	0.014
WS								
MMSE score	1.861 (0.033,3.689)	0.046	1.261 (-0.497,3.026)	0.161	3.635 (1.834,5.435)	<0.001	2.827 (1.030,4.634)	0.002
Orientation	0.274 (-0.391,0.939)	0.418	0.169 (-0.499,0.832)	0.625	0.809 (0.195,1.423)	0.010	0.469 (-0.179,1.125)	0.155
Registration	0.077 (-0.086,0.239)	0.352	-0.002 (-0.192,0.187)	0.976	0.084 (-0.148,0.317)	0.474	0.034 (-0.233,0.298)	0.817
Attention and calculation	0.781 (-0.028,1.589)	0.058	0.927 (0.036,1.812)	0.044	0.738 (-0.016,1.492)	0.055	0.567 (-0.248,1.375)	0.170
Recall	0.148 (-0.447,0.743)	0.625	0.013 (-0.656,0.683)	0.962	0.950 (0.360,1.541)	0.002	1.107 (0.436,1.768)	0.001
Language	0.562 (-0.051,1.175)	0.072	0.165 (-0.400,0.735)	0.558	1.053 (0.437,1.668)	0.001	0.658 (0.017,1.306)	0.042

IPAQ, International Physical Activity Questionnaire; BMI, body mass index; CCI, Charlson Comorbidity Index; GS, grip strength; TUGT, Timed Up and Go Test; WS, walking speed; CI, confidence interval; MMSE, Mini-Mental State Examination.
Adjusted model is adjusted with age, sex, BMI, vintage, widowed, living alone, illiteracy, smoking, drinking, sleep duration, IPAQ, depression, number of drugs, and CCI.

departure from our previous study, we did not find an association between GS and MCI; the reason may be that our hemodialysis population cohort is relatively younger (with an average age of 68.3 years) than the elderly population cohort. Although another comparison of physical performance between the groups according diabetes showed lower physical performance in the diabetes group than the non-diabetes group of hemodialysis and peritoneal dialysis patients (33), however, so far, no research has shown that physical activity is significantly worse in the coexistence of diabetes and MCI diseases.

Furthermore, results of our study found that the prevalence of MCI in diabetic hemodialysis patients was high (20.6%). This finding was similar to the AGES–Reykjavik study (34) that showed that individuals with type 2 diabetes had poorer performance on cognitive tests than individuals without type 2 diabetes. There are several possible mechanisms for the result. First, the accumulation of glycosylation end products triggers vascular endothelial dysfunction (35), and multiple risk factors including oxidative stress, inflammation, vascular calcification, and insulin-like growth factor-1 also play roles in the development and progression of MCI (36). Second, neurodegenerative mechanisms have been proposed for the association of diabetes with MCI. The hippocampus, entorhinal formation, and frontal cortex are potential target regions in the brain that are known to have insulin receptors through which insulin-related effects may affect cognitive function (37). Diabetes may adversely affect amyloid processing and increase brain intraneuronal β-amyloid deposition (38) and tau hyperphosphorylation (39) in target regions, which is a sign of cognitive impairment. Therefore, it is reasonable to believe that diabetes in end-stage renal disease patients receiving hemodialysis may be an important risk factor for the development of MCI.

In previous studies, the relationship between physical performance and MCI in hemodialysis patients has not been fully established. In the current study, we found a significant interaction between mobility/WS and diabetes in hemodialysis patients, while the interaction between GS and diabetes was not significant. It is possible that the mechanism underlying this interaction is multifactorial. For instance, diabetes and its primary risk factors (hypertension, heart disease, and obesity)

are both strongly associated with impaired mobility function and WS. Secondly, WS is associated with factors such as inflammation, neuropathy, and vascular function, which are common pathways to cognitive and physical function. However, current evidence of the association between GS and diabetes is controversial, and a study has shown no significant association between them (40). In addition, WS in physical performance was negatively associated with MCI in diabetic hemodialysis patients; however, no significant association was found in the non-diabetic group. The possible reasons are as follows: for patients with diabetes, they are significantly associated with poor physical performance, the mean TUGT was also longer in the diabetes group than that in the non-diabetes group (11.15 vs. 8.41, $p < 0.05$), and the mean WS was slower in the diabetes group than that in the non-diabetes group in our study (0.89 vs. 0.98) but did not show a significant difference probably due to the small sample size. Moreover, a study has shown that hyperglycemia is associated with the development of frailty and incident mobility limitations, potentially mediated by loss of muscle (41). This is also consistent with previous findings supporting a role of specific cardiovascular risk factor contributors in the association between physical performance and cognitive decline (42). Therefore, it is reasonable to believe that poor physical performance due to diabetes may be an important risk factor for the development of MCI. This finding takes our pinpointing of amenable factors for MCI a step further, and physical performance interventions in more precise populations may be useful for early prevention and control of MCI progression.

Moreover, we found that TUGT was negatively associated with not only global cognitive function but also several specific functions, including attention and calculation and language. WS was positively associated with recall and language even after adjusting for potential confounding factors. Recent studies have revealed a strong relationship between gait and executive functions in healthy and pathological aging. The main negative correlations were found between time of TUGT and total score ($r = -0.476$) and language domain ($r = -0.448$) in the MCI group (43). McGough etal. (44) found that slow gait was associated with registration, attention and calculation, and executive performance. This is consistent with our findings that showed that WS was positively associated with attention and calculation ($p = 0.044$) in the non-diabetes group, and TUGT was negatively associated with language in the diabetes group. The following clinically relevant links can explain our results: cognitive function is related to the dorsolateral frontal cortex and hippocampus, which affect the executive function, attention and calculation, and recall of individuals. On the flip side, gait decline increases the risk of cognitive decline and dementia, and poor mobility outcomes were reliably associated with reduced gray and white matter volume (45). At present, although many consistent studies showed the relationship between physical activity and cognitive functions, there are still some inconsistent results (16, 44). Future studies should focus on the different cognition changes in the weak physical population, and more well-designed cohort studies need to be carried out to verify the relationship between physical performance and different cognitive functions. Generally, our finding gives us some inspiration on how to manage physical activity and interfere with MCI in hemodialysis patients, especially those with diabetes.

The strengths of our study included the following: It is the first multicenter study to examine the relationship between physical performance and MCI among hemodialysis patients across different diabetic states. Secondly, the study assessed the association between physical performance and multiple cognitive functions in hemodialysis patients with and without diabetes. Furthermore, most recognized confounders were taken into account in regression models to analyze the independent association of physical performance and MCI in this study. However, some limitations also exist. First, all participants in the present study come from one city, which means that this study has a certain degree of regional limitation. Second, this study is based on a cross-sectional design, so it is not possible to determine causal relationships. To clarify this issue, a further longitudinal study with a large sample size is needed to explore the new onset risk of MCI in the hemodialysis population with diabetes.

Conclusion

In this study, we found that physical performance was associated with MCI in diabetic hemodialysis patients rather than the non-diabetes group. Further analysis showed the relationship between physical performance and specific cognitive functions. This study provides some key considerations for physicians that poor mobility and WS in diabetic hemodialysis patients are more associated with MCI. Further research is required to confirm the direction of causality.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was reviewed and approved by the Ethics Committee of Shanghai University of Medicine and Health Sciences and the methods were carried out in accordance with the principles of the

Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YZ, PS and CZ contributed equally to this work and should be considered as the co-first authors. XC, PS and YZ conceived the concept and design of the study. WD, JN, JZ, XS, LMZ, CY and JX provided the study materials or patients. CZ, LYZ and YZ collected and assembled the data. PS, CF, PH and HZ analyzed and interpreted the data. YZ, PS and CZ drafted the article or revising it critically for important intellectual content. QG provided administrative support. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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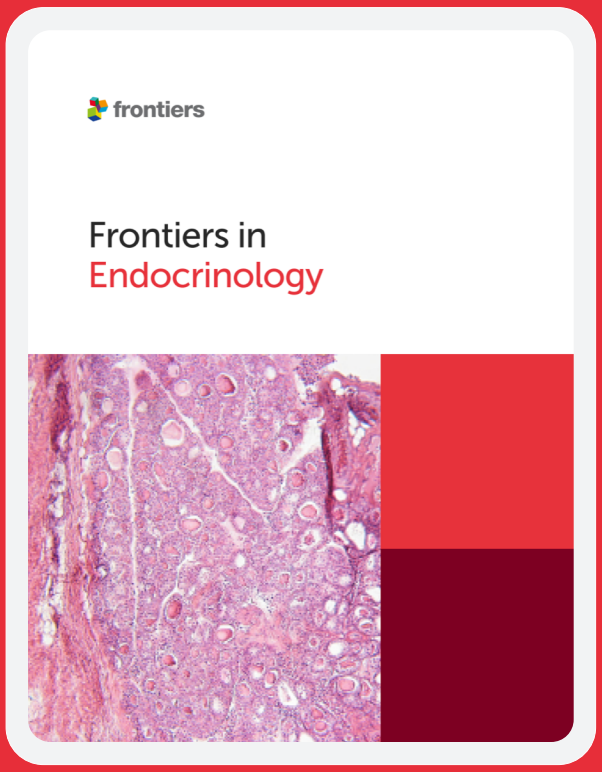

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