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Disease-modifying approaches in type 1 diabetes

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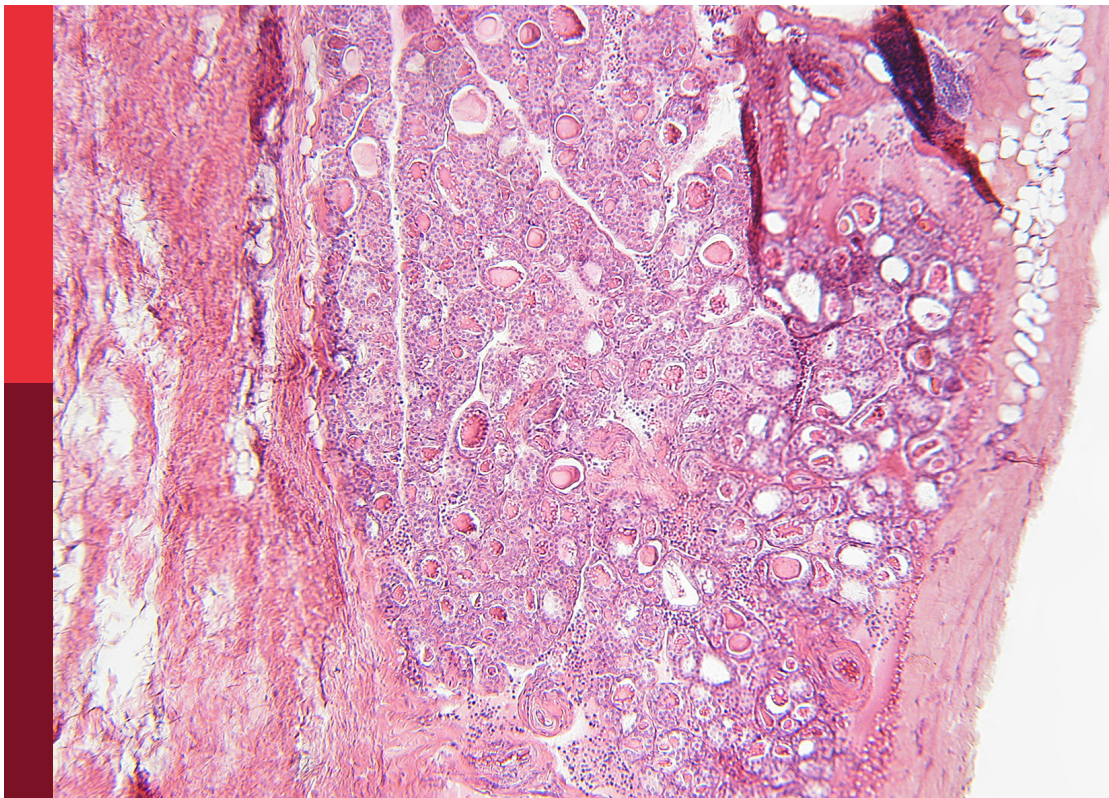

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Disease-modifying approaches in type 1 diabetes

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Editorial: Disease-modifying approaches in type 1 diabetes

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Editorial on the Research Topic
Disease-modifying approaches in type 1 diabetes

Type 1 diabetes (T1D) is a disorder that is marked by persistent hyperglycemia due to autoimmune destruction of pancreatic β-cells, necessitating lifelong dependence on exogenous insulin for survival. TID places a heavy burden on the individual and the national healthcare budgets because of its short- and long-term complications, such as severe hypoglycemia, diabetic ketoacidosis, blindness, diabetic neuropathy, amputations, kidney failure, and atherosclerotic cardiovascular disease. As a result, there have been calls for disease-modifying therapies that can target the root cause of T1D, preserve endogenous insulin production, and ultimately alter the trajectory of T1D and reduce complications. Initial efforts have centered on immunosuppression and immunotherapy. However, the failure of these approaches to completely protect the β-cells has led to more critical thinking in the field.

This Research Topic aimed to capture these new ideas in the field. Reports for this Research Topic range from interventions to prolong the partial clinical remission (PR) phase of T1D, new theoretical frameworks such as the use of high-dose gamma-aminobutyric acid (GABA) molecule to prolong PR, generation of insulin-secreting cell lines using CRISPR technology, and the exploration of new lipid-based pathways via the theory of hyperlipidemic memory for disease modification. These innovative works will shape the future of diabetology. O'Donovan et al., kicked off the Research Topic by providing a robust review of the current disease-modifying therapies in T1D. Teplizumab is the first FDA-approved therapy to delay T1D, but it only postpones disease progression by a median of about 2 years in high-risk individuals. They suggested that prolonging residual β-cell function (RBCF) by these therapies could increase life expectancy by up to 14 years in children diagnosed with T1D at a young age. An original research article from Li et al. reported on developing engineered cells that can secrete endogenous insulin as a promising therapeutic approach to T1D, evading autoimmune attacks, and reducing reliance on exogenous insulin administration. Using CRISPR/Cas9 gene editing and homology-directed repair, they precisely integrated a promoter-free EMCVIRES-insulin cassette into the 3' untranslated region of the *GAPDH* gene in human HEK-293T cells. The investigators demonstrated in mouse studies that the subsequent Cytopore 1 microcarriers are biocompatible and promote the long-term survival of insulin-producing cells *in vivo*. By

inserting the insulin gene into a housekeeping gene locus without using an external promoter, the insulin can be expressed constitutively along with an essential gene, reducing the risk of silencing and ensuring stable insulin production. These non-endocrine cells secreted functional insulin and reduced hyperglycemia. This promoter-free genetic engineering strategy for insulin secretion and efficient cell transplantation could enhance disease-modifying therapeutic approaches in T1D.

The failure of immunosuppressants and immunomodulators to completely protect the β-cells has led to a newer focus on augmenting intrinsic β-cell health versus protection from autoimmune attacks to ensure prolonged RBCF. In this regard, Jing et al. propose oral adjunctive therapies that focus on β-cell health as candidates of interest for disease modification in T1D. They reviewed agents that target thioredoxin-interacting protein (TXNIP), especially TIX100, an oral antidiabetic drug that inhibits TXNIP. Verapamil, a calcium channel blocker, was previously shown to improve β-cell survival by suppressing TXNIP; TIX100 is a next-generation compound designed for this pathway. However, compared to verapamil, TIX100 has a reduced side effect profile, higher specificity, potency, and effectiveness, and reduces hyperglucagonemia and hepatic fat. By improving β-cell health without immunosuppression, a TXNIP inhibitor like TIX100 could potentially be repurposed to preserve β-cells in T1D, although it has so far been studied as an attractive agent for managing patients with type 2 diabetes. Along the lines of newer agents to promote intrinsic β-cell health and prolong PR, Mick and McCormick. explored the role of GABA molecule in patients with T1D regarding its known actions, such as the augmentation of pancreatic β-cell content, reduction of excess glucagon secretion, and the mitigation of T-cell-mediated immune destruction. They proposed that given the depletion of GABA in islets of patients with T1D, the repletion of GABA may have pharmacologic applications in these patients. This suggests that a threshold level of GABA might be necessary to exert therapeutic effects, potentially by more robustly activating GABA receptors on islet and immune cells. They made an important observation that high-dose GABA therapy would be more likely to elicit a positive metabolic outcome than regular supplementation in a similar approach to high-dose vitamin D supplementation to prolong PR in patients with T1D (1).

Given the rising prevalence of childhood obesity in children and adolescents with T1D, Resnick et al. recommended that glucagon-like peptide-1 receptor agonists (GLP-1RAs) be used to reduce the prevalence of obesity in patients withT1D and thus modify or blunt the trajectory of adiposity-driven cardiovascular complications. They reviewed the impact of insulin resistance (IR) in these patients and the practical steps to introduce GLP-1RAs in individuals with T1D. Addressing double diabetes (T1D with IR) with GLP-1RA class of drugs could also reduce hypoglycemia risk by markedly lowering total insulin requirement in such individuals. Along the same lines, Lei et al., reported in their meta-analysis on the safety and efficacy of Sotagliflozin, a dual inhibitor of sodium-dependent glucose transporter-1 and 2, in patients with T1D, that adjunctive Sotagliflozin could reduce the risk for cardiovascular disease, end-stage kidney disease, and fractures by improving metabolic profiles. However, it is important to note that SGLT inhibitors in T1D come with an increased risk of diabetic ketoacidosis; the meta-analysis suggests that with careful patient selection and monitoring, the benefits might outweigh the risks, suggesting a potential adjunctive role for Sotagliflozin in T1D management.

Mittal et al. focused on the paucity of data on the gene-environment interactions for the pathogenesis of T1D. They published an integrative perspective article aimed at characterizing gene-environment interactions in patients with T1D. They proposed using 'omics' (i.e., combine genomics, metabolomics, microbiome analysis, and exposomics) technology to determine the impact of environmental factors such as viruses, pesticides, gut dysbiosis, genetic, and epigenetic changes in triggering autoimmune response against pancreatic β-cells. They further called for investigations into 'epidrugs', which they described as agents that modify epigenetic changes, as novel therapies for T1D. Such epigenetic therapies (for example, DNA methylation or histone modification inhibitors) could potentially reprogram immune or β-cell gene expression profiles to a less auto-aggressive state. While this concept is in its infancy for T1D, the authors believe that targeting the epigenome could interrupt the disease process in ways traditional drugs have not done. They believe that this precision medicine approach could modify the trajectory of T1D and reduce the complications of the disease.

In a 23-year prospective, population-based, cohort study of 391 women with gestational diabetes mellitus (GDM), Luiro et al. showed that women with GDM who possessed 3 diabetes-associated autoantibodies in their first-trimester blood samples developed T1D within 7 years from the GDM pregnancy. They added that the progression to T1D was associated with a diagnosis of GDM at <30 years, lower BMI, and insulin requirements during GDM. This study suggests a trial of disease-modifying therapies for these women during their preclinical phase of T1D. In their view, Tandel et al. proposed that using multiplex antibody-detection-by-agglutination-PCR (ADAP) assay could be an ideal tool for T1D risk testing for large-scale stages 1 and 2 T1D testing in the general population. The ADAP technology allows highly sensitive and simultaneous detection of multiple autoantibodies with a minimal sample, which could make broad population screening for early T1D risk feasible. By identifying at-risk individuals (such as those with multiple autoantibodies) in the general population, one could intervene earlier with disease-modifying therapies.

Liu et al. reported that in Chinese adults with a 1-5-year history of T1D, RBCF was associated with higher time in range or near normoglycemia, suggesting that disease-modifying therapy could improve outcomes for these patients by prolonging their RBCF. Even a small amount of preserved endogenous insulin production can significantly stabilize blood glucose levels, reducing glycemic variability and dangerous extremes in glycemia. This underscores the clinical importance of therapies that preserve β-cell function: patients with preserved C-peptide tend to experience fewer hypoglycemic episodes and fewer complications, as seen in prior diabetes studies. Another publication by Xiong et al. reported on a predictive model for personalized postprandial glycemic response

(PPGR) in Chinese patients with T1D, given the complexity of the Chinese diet compared to the Western diet. They found that the key predictors of PPGR were the premeal blood glucose level, blood glucose trend 30 minutes before a meal, and the carbohydrate-to-protein ratio of the meal. They recommended lower pre-prandial blood glucose and lower carbohydrate intake to maintain normal PPGR. Such a model could help tailor meal planning and insulin dosing for individuals, which is especially relevant as dietary patterns vary globally. By better predicting blood sugar excursions after meals, clinicians can personalize nutrition therapy in T1D, a strategy that, while not directly altering the autoimmune process, can mitigate marked glycemic variability and thereby reduce glucotoxicity or other metabolic stresses on the body.

Another study from China by Zhang et al. explored the dynamics of stimulated C-peptide concentrations and fasting and postprandial glucagon concentrations using a steamed bread meal tolerance test. They found that as T1D progresses, C-peptide levels decrease, and postprandial glucagon levels rise. They suggested that reducing postprandial hyperglucagonemia could be a disease-modifying therapy in T1D. In practice, this could mean developing treatments to suppress inappropriate glucagon release or action in T1D. For instance, adjunct therapies like GLP-1 agonists or glucagon receptor antagonists that specifically target α-cell activity. By curbing excessive glucagon release (which exacerbates hyperglycemia), one could improve overall glycemia and decrease the glucotoxic burden on surviving β-cells. In a review article, Nwosu expanded on his theory of hyperlipidemic memory of T1D, which explains the dichotomy in atherosclerotic cardiovascular (ASCVD) risk based on the presence or absence of PR. In this article, he proposes two fundamental ideas for disease-modifying therapies. The first is that any effort at complete β-cell protection must include lipid pathways to ensure a significant reduction in ASCVD risk. In other words, focusing only on glycemic control and autoimmunity is not sufficient; controlling dyslipidemia early in the course of T1D is crucial to prevent long-term cardiovascular complications. This idea arises from observations that some youth with T1D develop adverse lipid profiles very soon after diagnosis (especially if they did not experience a remission phase), which may set the stage for future cardiovascular disease. Secondly, PR is an imprimatur and not a process, suggesting that strategies to ensure the occurrence of PR in individuals with preclinical T1D will lead to more robust long-term outcomes than interventions to prolong the duration of PR following stage 3 T1D. This means that inducing a remission (even a short one) around the time of diagnosis or in the late preclinical phase might confer lasting metabolic benefits, perhaps by instilling a healthier metabolic memory, whereas trying to extend an established remission later may be less impactful. This concept challenges researchers to prioritize therapies that trigger remission in new-onset T1D (or prevent symptomatic onset altogether) as a strategy to imprint a lower-risk metabolic profile from the start.

In conclusion, this Research Topic provides a tour de force of the current strategies to protect the β-cells in T1D by reducing autoimmune attacks, augmenting intrinsic β-cell health, and exploring physiological, genetic, epigenetic, environmental, bioengineering, and population-based approaches to preserve β-cells, prolong RBCF, and reduce the medical and financial burdens of T1D around the world. The innovative concepts highlighted here will undoubtedly shape the future of diabetology and inspire further research into state-of-the-art disease-modifying therapies for T1D.

Author contributions

BN: Resources, Writing – original draft, Validation, Visualization, Methodology, Conceptualization. JP: Visualization, Validation, Writing – review & editing, Software, Supervision. AA: Validation, Writing – review & editing, Visualization, Supervision, Software, Formal analysis.

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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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Autoantibodies predict type 1 diabetes after gestational diabetes – a 23-year cohort study

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Objective: To study the predictive value of autoantibodies for type 1 (T1DM) and type 2 (T2DM) diabetes morbidity after gestational diabetes (GDM) in a 23-year follow-up study.

Design: Prospective population-based cohort study.

Methods: We studied 391 women with GDM, and 391 age- and parity-matched controls, who delivered in 1984–1994. Four autoantibodies were analysed in first-trimester blood samples: islet cell autoantibodies (ICAs), glutamic acid decarboxylase autoantibodies (GADAs), insulin autoantibodies (IAAs) and insulinoma-associated antigen-2 autoantibodies (IA-2As). Two follow-up questionnaires (1995–1996, 2012–2013) were sent to assess development of T1DM and T2DM. Predictive value of autoantibodies and clinical factors were analysed by conditional linear regression and ROC analyses.

Results: Single autoantibody positivity was detected in 12% (41/342) of the GDM cohort and in 2.3% (8/353) of the control cohort. In the GDM cohort, 2.6% (9/342) tested positive for two autoantibodies and 2.3% (8/342) for three autoantibodies, whereas only one subject in the control cohort had two autoantibodies. ICA positivity was found in 12.5% of the cases, followed by GADA (6.0%), IA-2A (4.9%) and IAA (1.2%). In the control cohort, GADA positivity was found in 1.4%, IA-2A in 0.8%, IAA in 0.6%, and ICA in 0.3% of

the subjects. Detection of ICA, GADA and/or IA-2A autoantibodies decreased T1DM-free survival time and time to diagnosis. All subjects with three positive autoantibodies developed T1DM within seven years from the GDM pregnancy. Development of T2DM after GDM occurred independent of autoantibody positivity.

Conclusion: Development of T1DM can be reliably predicted with GADA and ICA autoantibodies during early pregnancy.

KEYWORDS

autoantibody, GDM, insulin, ICA, OGTT, prediction, type 1 diabetes, type 2 diabetes

1 Introduction

Insulin sensitivity decreases during pregnancy along with increasing weight, adiposity and placental hormones, inducing insulin resistance to favour foetal growth. Gestational diabetes mellitus (GDM) develops when compensatory hyperinsulinaemia, that normally maintains an euglycemic state during pregnancy, can no longer counteract the increasing insulin resistance, and blood glucose levels rise (1). The prevalence of GDM is increasing worldwide and varies between 2 and 17% depending on the diagnostic criteria and genetic background of the studied population (2). The affected women are at high risk of developing type 2 diabetes (T2DM), and also type 1 (T1DM), later in life. Autoantibody positivity is a known risk factor for progression to T1DM (3), and autoantibodies predicting T1DM have been detected variably in 1-35% of women with GDM (4). However, long, prospective controlled studies aimed at assessing their role in the prediction of morbidity in both T1DM and T2DM after GDM, are lacking (5–25).

We have previously reported a prospective, 6-year cohort study of women with GDM and healthy controls, showing that positivity for islet cell autoantibodies (ICAs) and glutamic acid decarboxylase autoantibodies (GADAs), as well as GDM below the age of 30 years and the need for insulin treatment during pregnancy are associated with a high risk of subsequent progression to T1DM (15). Recently, we reported that during a 23-year follow-up of the same cohort, 5.7% of them developed T1DM and they were all diagnosed within 7 years after the GDM pregnancy, and their disease progression was predictable with high oral glucose tolerance test (OGTT) 2-h glucose levels, and associated with insulin treatment for GDM (16). Moreover, type 2 diabetes (T2DM) was diagnosed in 50.4% of the women with GDM, and the incidence remained linear until the end of the follow-up period.

Here we report the analysis of four autoantibodies; ICAs, GADAs, insulin autoantibodies (IAAs) and insulinoma-associated antigen 2 autoantibodies (IA-2As), evaluated during the first trimester of pregnancy from women with GDM and healthy controls in relation to the progression of T1DM and T2DM during a 23-year follow-up. Combined with the demographic and clinical data, we calculated the cumulative risk of one or more positive autoantibodies in disease progression and developed prediction models to assess the significance of independent clinical risk factors.

2 Methods

2.1 Study population

The study population has been previously described (16). This cohort study included 435 women with a singleton pregnancy and GDM, who delivered in the Oulu University Hospital, Finland, in 1984–1994. The control cohort of 435 women was pair-matched by age (± 2 years), parity (nulliparous, 1–3, or more than three deliveries) and date of delivery (± 2 days). All women were white. GDM was diagnosed by OGTT (n=363) or by insulin treatment (n=28). Subjects with a diagnosis based on multiple glucose measurements, or on abnormal HbA1c values, were excluded (n=44), and subsequently, 391 women with GDM, and 391 matched controls were included in the analyses.

Indications for OGTT included glucosuria, BMI ≥25 kg/m^2, previous delivery of a macrosomic infant (≥4500 g) or expected macrosomic infant in the current pregnancy. A standard 2-h OGTT (75 g glucose load in 250 mL water) was performed after a 12-h overnight fasting. Three capillary whole blood samples were drawn: at baseline, at 60 min and 120 min. The cut-off values for the glucose concentrations were set according to the recommendation of the Finnish Diabetes Association: fasting, ≥4.8 mmol/L; 1-hour, ≥10.0 mmol/L; and 2-hour, ≥8.7 mmol/L. The blood samples were analysed

Abbreviations: GADA, GAD antibody; GDM, gestational diabetes mellitus; IA-2A, protein tyrosine phosphatase–related IA-2 molecule antibody; IAA, insulin autoantibody, ICA, islet cell antibody; OGTT, oral glucose tolerance test; LADA, latent autoimmune diabetes in adults.

using the HemoCue® System (AB Leo Diagnostics, Helsingborg, Sweden) (1). The inter-assay coefficient of variation of the method was 3.8-4.0% at glucose concentration of 4.5-17.6 mmol/l. Any single abnormal value in the OGTT was considered diagnostic.

All women diagnosed with GDM were given nutritional advice. Insulin treatment was initiated, if at least two glucose values (fasting or preprandial) were ≥5.5 mmol/l or when one fasting or preprandial value was ≥5.5 mmol/l and one postprandial value was ≥7.8 mmol/l 1.5 hours after a meal in a 24-hour glucose profile.

All study participants signed an informed consent form. The Ethics Committee of the Northern Ostrobothnia Hospital District approved the study protocol.

2.2 Autoantibody analyses

A serum sample was taken during the first trimester of pregnancy for routine rubella screening. Diabetes-associated autoantibodies were analysed using a standard immunofluorescence method (ICA) or specific radiobinding assays (IAA, GADA and IA-2A) as previously described (18). All four autoantibodies were analysed successfully in 342 cases and 353 controls. ICA was analysed successfully in 352 cases and 354 controls, GADA in 350 cases and 354 controls, IA-2A in 344 cases and 353 controls, and IAA in 340 cases and 353 controls.

The cut-off level for ICA positivity was set at 2.5 Juvenile Diabetes Foundation units (JDFU) and for IAA, GADA, and IA-2A the cut-off levels were based on the 99th percentile in nondiabetic Finnish subjects (N=105, 772 and 374, respectively). The cut-off limit for IAA positivity was set at specific binding of 54 nU/ml, for GADA 6.5 relative units (RU) and for IA-2A 0.43 RU. The disease sensitivity of the assays for ICA, IAA, GADA, and IA-2A were 100%, 78%, 79%, and 62%, respectively. The corresponding disease specificity was 98%, 100%, 97%, and 97%, respectively. All samples with IAA, GADA, or IA-2A levels between the 97th and 99.5th percentiles were reanalysed to confirm their status.

2.3 Questionnaire-based follow-up

Two questionnaires were sent to the study participants, first an invitation to participate in this study in 1995–1996 (1–11 years after pregnancy) with the first follow-up questionnaire and an informed consent form. Second follow-up questionnaire was sent in 2012–2013. 297 women with GDM and 297 control subjects (76%) took part in the study. Thirteen women in the GDM cohort (3.3%) and six women in the control cohort (1.5%) had died. The mean post-delivery follow-up time was 23.1 (range 18.7-28.8) years in the GDM cohort and 23.3 (range 18.9-30.1) years for the control cohort.

The questionnaires included questions about GDM treatment (diet or insulin), pre-pregnancy weight and height, progression to clinical diabetes, the type of diabetes, the time of diagnosis and diabetes medication.

2.4 Statistical analysis

Baseline demographic characteristics were analysed by one-way ANOVA. Development of T1DM and T2DM after pregnancy was assessed by Kaplan–Meier survival curves regarding (1) individual autoantibody positivity, and (2) number of positive autoantibodies. The time between blood sampling (taken in the first trimester) to the diagnosis of diabetes or to the end of follow-up was used as survival time (time-to-event). Subjects who did not answer the second questionnaire or who had died were censored at the end of their follow-up time or at the time of death. To evaluate the independent associations of each risk factor and to find the best predictive model for disease progression to diabetes, conditional logistic regression analysis and receiver operating characteristic (ROC) curves were constructed. AUC was used in the classification analysis. In model 1, the number of positive autoantibodies (0, 1, 2, or 3–4), age at the time of pregnancy (≤ 30 years vs. > 30), and non-insulin vs. insulin treatment for GDM were included as contributing factors. In model 2, positivity vs. negativity for each autoantibody, age at the time of pregnancy (≤ 30 years vs. > 30), and non-insulin vs. insulin treatment for GDM were included as contributing factors. The analyses were performed with IBM SPSS Statistics for Windows (versions 21 and 25, IBM, Armonk, NY) and RStudio (Boston, MA) software. The figures were produced using the ggplot2 (R package version 0.4.6., https://CRAN.R-project.org/package=survminer) and Adobe Illustrator (Adobe Systems, San Jose, CA).

3 Results

The demographic characteristics of the study population have been previously described (16). In brief, mean body weight and mean BMI (± SD) were higher in the GDM group than in the control group at the first trimester (69.5 ± 14.5 kg vs 61.7 ± 10.4 kg, P<0.001; 26.3 ± 5.2 kg/m^2 vs 22.8 ± 3.5 kg/m^2, P<0.001), as expected. Within the GDM cohort, women who later reported T1DM had lower first trimester mean BMI compared to those who later reported T2DM (24.2 ± 3.4 kg/m^2 vs. 27.9 ± 5.7 kg/m^2; P<0.001). The mean age of the GDM cohort at the time of second follow-up (± SD) was 54.7 (± 6.4) years, and that of the control cohort was 55.3 (± 6.4) years.

3.1 Autoantibody analyses

At least one autoantibody was found positive in 12% (41/342) of the GDM cohort and in 2.3% (8/353) of the controls (Table 1). Only one control subject (0.3%) had two positive autoantibodies, whereas in the GDM cohort, 2.6% (9/342) tested positive for two autoantibodies and 2.3% (8/342) for three autoantibodies. ICA positivity was found in 12.5% of the GDM cohort, followed by GADA (6.0%), IA-2A (4.9%) and IAA (1.2%). In the control cohort, GADA positivity was found in 1.4% of the subjects, IA-2A in 0.8%, IAA in 0.6%, and ICA in 0.3% of the subjects.

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TABLE 1 Prevalence of the autoantibodies in the GDM and control cohort.

	Cases N=391*	Controls N=391*
	% (N)	% (N)
Positivity of autoantibodies*		
ICA	12.5 (44)	0.3 (1)
GADA	6.0 (21)	1.4 (5)
IA-2A	4.9 (17)	0.8 (3)
IAA	1.2 (4)	0.6 (2)
No. of positive autoantibodies†		
0	83.0 (284)	97.5 (344)
1	12.0 (41)	2.3 (8)
2	2.6 (9)	0.3 (1)
3	2.3 (8)	0 (0)
4	0 (0)	0 (0)

*ICA was analysed successfully from 352 cases and 354 controls, GADA from 350 cases and 354 controls, IA-2A 344 cases and 353 controls and IAA 340 cases and 353 controls.
†All four autoantibodies were analysed successfully from 342 cases and 353 controls.

Positivity for ICA, GADA and/or IA-2A, but not for IAA, decreased T1DM-free survival time and time to diagnosis (Figure 1). All women who tested positive for three autoantibodies developed T1DM (Figure 2A). In contrast, T2DM-free survival rate and time to diagnosis were not significantly related to autoantibody positivity or negativity (Figure 2B).

Among women who later reported being diagnosed with T2DM (N=197), nine had two, and eight had three positive autoantibodies. Compared to the women reporting later T2DM but no autoantibodies detected, their first trimester BMI was lower (22.8 ± 5.1 vs 27.9 ± 6.2 kg/m^2, p=0.306), however this, or the time to diagnosis (12.9 ± 6.8 vs 13.1 ± 7.2 years, p=0.408), did not reach statistical significance.

3.2 Prediction of diabetes progression after GDM

To analyse the influence of independent factors for T1DM or T2DM progression after GDM, two conditional logistic regression models were developed (Table 2). The highest risk of developing T1DM was associated with three positive autoantibodies, insulin treatment for GDM, and inversely associated with age under 30 years at the time of the GDM pregnancy. In terms of the individual autoantibodies, positivity for ICA was associated with the highest risk for T1DM progression, followed by GADA and IA-2A. This finding was supported by the ROC analyses, in which ICA positivity was the most predictive autoantibody regarding T1DM development (Table 3). The best predictive value was achieved by the combination of ICA and GADA positivity. Combination of ICA positivity and insulin treatment for GDM resulted in a highly sensitive, but less specific, prediction for T1DM. Despite some positive autoantibodies among those women who later developed T2DM, seropositivity was not significantly associated with the development of T2DM (Table 2).

4 Discussion

This 23-year prospective cohort study showed that T1DM can be reliably predicted with ICA and GAD autoantibodies during pregnancy, and that progression to T1DM occurs during the first decade after GDM.

Development of T1DM results from the immune-mediated destruction of the pancreatic ß-cells. Presence of circulating autoantibodies produced by the B-lymphocytes is a well-characterized phenomenon, and they can be detected in the serum months to years before the onset of diabetes (26). Prevalence of autoantibodies in women with GDM has been previously described in several studies, including our own 6-year follow-up study of the same study population (15). Most studies have investigated the autoantibodies during pregnancy (7, 11, 13, 15, 17, 20, 21, 23–25), however, some studies investigated them after pregnancy (5, 14, 22) and one study both during and after the GDM pregnancy (12). Overall, GADA has been the most frequently assessed autoantibody, however, its prevalence (0-10.8%) and association to the progression to T1DM has varied considerably in different populations (5, 6, 8–10, 12, 13, 19, 21, 27–32), which probably at least partly reflects the differences of β-cell autoimmunity in various ethnic groups. Similarly, ICA prevalence has been variable (1-44%), but it may partly be due to technical issues regarding the standardization of the assays (33). The ICA assay applied in this study is highly sensitive (100%), adding to the reliability of our results. Here, IAA and IA-2A were not useful in predicting later T1DM after GDM, and this may reflect that they are more commonly found in young children and rarely in adults (34, 35). A novel β-cell autoantibody, ZnT8A, has been introduced since the initiation of our study, and initially, it was reported to have a prevalence of 4.8% in a GDM cohort (31). A subsequent study reported a lower prevalence 3.2%, while overall 6.8% of GDM women were autoantibody positive (32), and it seemed that ZnT8A provided no additional benefit above GADA positivity in terms of T1DM prediction.

In the present study, all women with three positive autoantibodies developed T1DM, which is in line with previous findings estimating that positivity for two autoantibodies increases the risk for developing T1DM to 63%, and up to 84%, when three autoantibodies are present (12). Here, the combination of ICA and GADA predicted T1DM with a 70.6% sensitivity and 88.0% specificity, and the prediction did not improve with an additional antibody analysed. Combination of any autoantibody and insulin treatment for GDM was very sensitive, but not a very specific predictor of T1DM progression, as it is also associated with later T2DM progression. We had as well eight women who had tested initially positive for three autoantibodies, yet self-reported being subsequently diagnosed with T2DM. Positivity for three autoantibodies strongly indicates that these patients do have autoimmune diabetes and not T2DM. In our view, these women most likely represent latent autoimmune diabetes in adults (LADA)

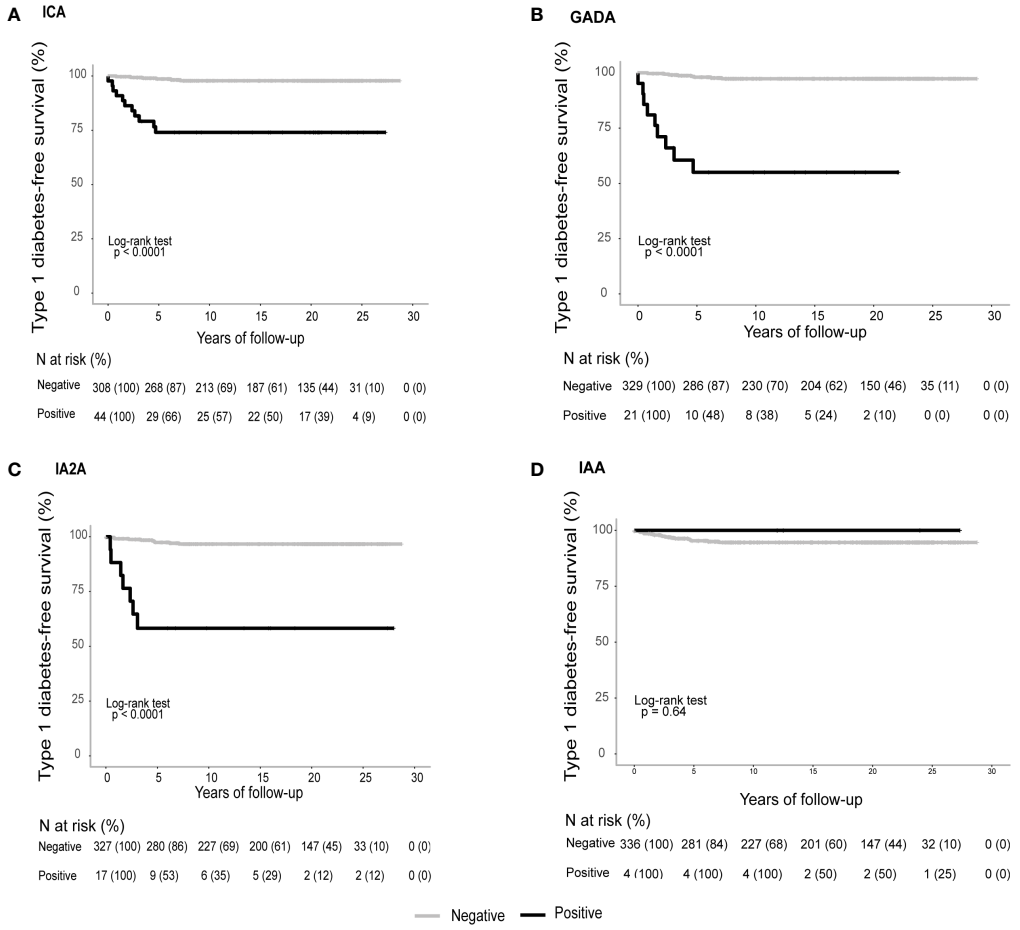

FIGURE 1
Mean (95% CI) T1DM -free survival time of women with vs without autoantibody positivity for **(A)** ICA 21.8 (18.3-25.4) vs 28.2 (27.8-28.7) years; **(B)** GADA 16.6 (10.7-22.6) vs 28.1 (27.7-28.6) years; **(C)** IA-2A 17.5 (11.1-23.9.) vs 27.9 (27.4-28.4) years; **(D)** IAA 28.8 (28.8-28.8) vs 27.4 (26.7-28-0). Log rank for a-c) *P*<0.001; **(D)** *P*<0.64.

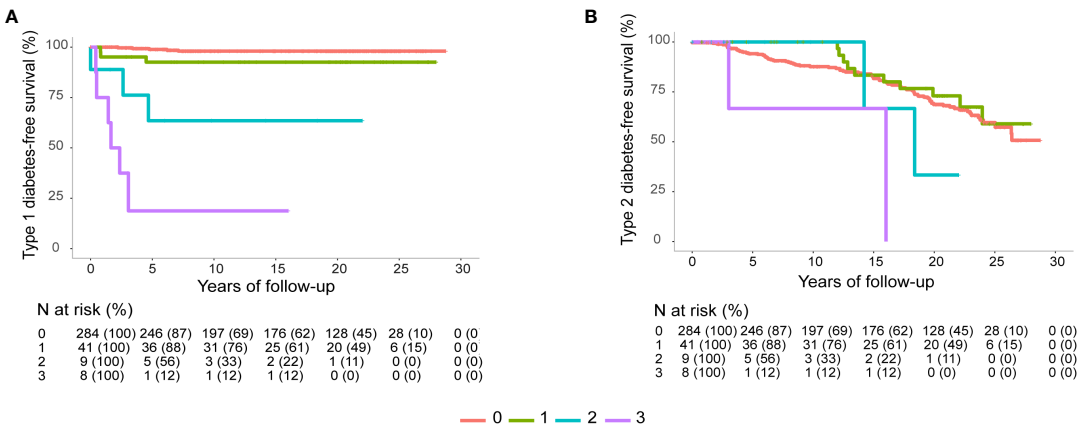

FIGURE 2
(A) Mean (95% CI) T1DM free survival time of women with no autoantibodies, 28.3 (27.9-28.7) years; one positive autoantibody, 26.8 (24.6-29.0) years; two positive autoantibodies, 19.2 (10.5-27.9) years; and three autoantibodies, 6.76 (-1.5-15.1) years. **(B)** Mean (95% CI) T2DM free survival time of women with no autoantibodies, 22.8 (21.7-23.8) years; one positive autoantibody, 24.2 (21.8-26.5) years; two positive autoantibodies, 20.5 (13.5-27.4) years; and three autoantibodies, 11.7 (4.8-18.6) years. Log rank *P*<0.0001 in both figures.

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that have been misdiagnosed in the primary care setting, where T2DM typically is treated in Finland. LADA may exhibit prolonged preservation of insulin secretion, and therefore a variable progression to insulin dependence, thus in the absence of antibody testing at the primary care setting, a misdiagnosis of T2DM is highly likely. The fact that they were slimmer supports this finding, although this difference did not reach statistical significance, most likely, due to a small sample size.

While results presented here and in previous studies seem conclusive that autoantibodies can effectively predict future T1DM, the main clinical question of whom to test for autoantibodies remains. In our population-based cohort, 5.7% of women with

TABLE 2 "Prediction of disease progression to T1 or T2 diabetes after GDM by independent factors using two logistic regression models.

	Type 1 diabetes	Type 2 diabetes
	OR (95% CI)	OR (95% CI)
Model 1		
No. of positive autoantibody types		
0	1.00	1.00
1	6.56 (1.52-28.34)	1.07 (0.55-2.08)
2	15.19 (3.54-65.15)	1.37 (0.33-5.59)
3	33.93 (8.95-128.66)	3.01 (0.72-12.49)
Age at time of GDM*		
≤ 30 years	1.00	1.00
> 30 years	0.24 (0.07-0.77)	1.65 (1.06-2.56)
Insulin for GDM		
No	1.00	1.00
Yes	13.44 (2.73-66.07)	3.74 (2.46-5.69)
Model 2		
ICA		
Negative	1.00	1.00
Positive	13.08 (3.60-47.56)	0.96 (0.45-2.02)
GADA		
Negative	1.00	1.00
Positive	5.21 (1.17-23.22)	1.38 (0.41-4.69)
IA-2A		
Negative	1.00	1.00
Positive	0.57 (0.12-2.76)	1.33 (0.40-4.41)
IAA		
Negative	NA[$]	1.00
Positive	NA[$]	2.36 (0.73-7.63)
Age at time of GDM		
≤ 30 years	1.00	1.00
> 30 years	0.47 (0.13-1.76)	1.91 (1.18-3.07)
Insulin for GDM		
No	1.00	1.00
Yes	28.37 (5.50-146.38)	3.73 (2.44-5.71)

*Age at the time of blood sampling during pregnancy.
[$]Not applicable due to small sample size.

TABLE 3 Autoantibody positivity or combination of autoantibodies and individual clinical factors in prediction of disease progression to T1DM by receiver operating characteristic (ROC) analyses; area under curve (AUC), sensitivity and specificity.

	Type 1 diabetes mellitus		
	AUC	Sensitivity (%)	Specificity (%)
Positivity of autoantibodies			
ICA	**0.77**	**64.7**	**90.1**
GADA	0.75	52.9	96.4
IA-2A	0.69	41.2	96.9
IAA	0.51		
Combinations of positive autoantibodies			
ICA + GADA	**0.82**	**70.6**	**88.0**
ICA + IA-2A	0.78	64.7	87.5
ICA + IAA	0.78	64.7	90.1
GADA + IA-2A	0.78	58.8	95.1
GADA + IAA	0.75	52.9	96.6
IA-2A + IAA	0.70	41.2	97.2
ICA + GADA + IA-2A	0.82	70.6	88.0
ICA + IAA + GADA	0.82	70.6	88.2
ICA + IAA + IA- 2A	0.79	64.7	87.8
GADA + IAA + IA-2A	0.78	58.8	95.3
Combinations of autoantibodies and insulin			
ICA + insulin	0.90	100.0	56.8
IAA + insulin	0.77	88.2	66.0
GADA + insulin	0.87	94.1	64.4
IA-2A + insulin	0.82	88.2	64.0
ICA + IAA + insulin	0.90	100.0	57.6
ICA + GADA + insulin	0.91	64.7	96.4
ICA + IA-2A + insulin	0.89	100.0	56.6
IAA + GADA + insulin	0.88	94.1	65.0
IAA + IA-2A + insulin	0.83	88.2	64.8
GADA + IA-2A + insulin	0.88	94.1	64.2
ICA + IAA + GADA + insulin	0.92	64.7	96.6

(Continued)

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TABLE 3 Continued

	Type 1 diabetes mellitus		
	AUC	Sensitivity (%)	Specificity (%)
ICA + IAA + IA-2A + insulin	0.89	100.0	57.2
ICA + GADA + IA-2A + insulin	0.92	64.7	97.5
IAA + GADA + IA-2A + insulin	0.88	94.1	64.7

The most predictive values are marked in bold.

GDM developed T1DM (16), and therefore it is hardly clinically or economically sensible to consider autoantibody testing for all women with GDM, although that has been suggested (36). In this study, progression to T1DM was associated with GDM at the age below 30 years, insulin therapy and lower BMI, and these clinical factors would probably be most useful in the clinical decision making. In addition, presence of ketones and co-morbidity with other autoimmune diseases (such as hypothyroidism) have been proposed (37). In clinical practice, an atypical response to GDM treatment, e.g. no/little response to diet or metformin treatment, but strong response to insulin treatment indicates low insulin resistance, and is suggestive of insulin deficiency, thus justifying autoantibody testing.

Strengths of this study include a remarkably high participation rate (76%), and to our knowledge, the longest follow-up period to date. In addition, the GDM diagnosis was mainly (92.8%) based on OGTT, the gold standard for GDM diagnostics. We also investigated all four autoantibodies associated with diabetes progression instead of one or two typically seen in previous reports and were able to integrate significant clinical factors such as maternal age and BMI into the prediction models. However, self-reported data on disease progression is a weakness of this study, and a systematic OGTT on follow-up would have probably increased the prevalence of T2DM in both GDM and control cohorts. At the time of the study, a risk-based screening for GDM was used in Finland, which compared to the current nearly universal screening, may also underestimate the incidence of GDM. It is also noteworthy that the incidence of T1DM among young adults is higher in Finland than in other countries, which may diminish the generalisability of these results (38).

In conclusion, the presence of autoantibodies in first trimester samples of women with GDM predicts well later T1DM progression. The combination of ICA and GADA seems to be particularly sensitive and specific for this. Investigation of autoantibodies should be considered if GDM includes T1DM-like features, such as young age, low BMI or an atypical response to common GDM treatment.

Data availability statement

The datasets presented in this article are not readily available because current GDPR legistlation does not allow transfer of data without the consent of the individuals who participated in the study. Requests to access the datasets should be directed to Juha S. Tapanainen, juha.tapanainen@helsinki.fi.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Northern Ostrobothnia Hospital District. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KL: Data curation, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing, Supervision. AA: Data curation, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. JA: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – review & editing. JJ: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – review & editing, Visualization. IJ: Investigation, Writing – review & editing. MK: Investigation, Methodology, Resources, Writing – review & editing. JT: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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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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Gene-environment interaction in the pathophysiology of type 1 diabetes

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Type 1 diabetes (T1D) is a complex metabolic autoimmune disorder that affects millions of individuals worldwide and often leads to significant comorbidities. However, the precise trigger of autoimmunity and disease onset remain incompletely elucidated. This integrative perspective article synthesizes the cumulative role of gene-environment interaction in the pathophysiology of T1D. Genetics plays a significant role in T1D susceptibility, particularly at the major histocompatibility complex (MHC) locus and cathepsin H (CTSH) locus. In addition to genetics, environmental factors such as viral infections, pesticide exposure, and changes in the gut microbiome have been associated with the development of T1D. Alterations in the gut microbiome impact mucosal integrity and immune tolerance, increasing gut permeability through molecular mimicry and modulation of the gut immune system, thereby increasing the risk of T1D potentially through the induction of autoimmunity. HLA class II haplotypes with known effects on T1D incidence may directly correlate to changes in the gut microbiome, but precisely how the genes influence changes in the gut microbiome, and how these changes provoke T1D, requires further investigations. These gene-environment interactions are hypothesized to increase susceptibility to T1D through epigenetic changes such as DNA methylation and histone modification, which in turn modify gene expression. There is a need to determine the efficacy of new interventions that target these epigenetic modifications such as "epidrugs", which will provide novel avenues for the effective management of T1D leading to improved quality of life of affected individuals and their families/caregivers.

KEYWORDS

type 1 diabetes, genetics, gene-environment interaction, epigenetics, viral infections, pesticide exposure, pathological mechanisms

1 Introduction

Type 1 diabetes (T1D) is a complex metabolic disorder characterized by the destruction of pancreatic β-cells due to autoimmunity leading to insulin deficiency and consequent

hyperglycemia (1, 2). T1D is associated with a significant disease burden and its prevalence is increasing gradually (3). In 2021, there were an estimated 8.4 million people worldwide living with T1D (4). The prevalence of T1D has been reported to increase by 0.34% every year (4–6). By the year 2040, it is projected that the worldwide prevalence of T1D will potentially reach up to 17.4 million individuals (4–6). This represents a more than twofold increase within a span of 19 years (4). Furthermore, T1D has been associated with serious long-term complications, shortened life expectancy, and reduced quality of life (1, 2). In addition, T1D is a substantial economic burden on the healthcare system. In 2020, the lifetime economic burden of 1,630,317 patients with T1D in the United States was found to be $813 billion higher than an equal number of patients without T1D (7). In 2022 alone, the total estimated cost of diagnosed diabetes mellitus in the U.S. was $412.9 billion, including $306.6 billion in direct medical costs and $106.3 billion in indirect costs attributable to diabetes (8). The high disease burden and substantial healthcare costs associated with T1D underscore the urgent necessity to understand the precise molecular mechanisms underlying its pathophysiology, with the aim of developing effective prevention strategies, or ultimately cure for this disease.

Despite advances in the field of T1D, the precise trigger of autoimmunity and disease onset remain incompletely elucidated. A better understanding of the underlying pathophysiology will help in the identification of potential biomarkers and risk factors associated with T1D. This information will lead to the early detection of T1D and the development of preventive interventions to delay or even prevent its onset.

Genetics plays a crucial role in the pathophysiology of T1D (9–16). Individuals with a family history of the disease are at a higher risk, highlighting the hereditary nature of T1D. The primary genetic association is with specific human leukocyte antigen (HLA) genes, particularly those within the HLA-DR and HLA-DQ loci (15). Besides HLA, other genes such as cathepsin H (CTSH), *INS*, *GLIS3*, *CCR5*, and *BAD* have been implicated in predisposition to T1D (9–15). While genetic susceptibility has long been recognized as a key factor in T1D development, it is increasingly evident that environmental influences can play a pivotal role in shaping disease risk (17). Environmental factors such as viral infections and pesticide exposure have been shown to increase susceptibility to T1D (18–22). Although genetics and environmental factors individually have been associated with T1D, limited information is available regarding their cumulative contribution in the disease process. The interplay between genetic predisposition and environmental triggers is a dynamic and complex process, which may contribute to the heterogeneous nature of T1D.

This perspective article discusses the cumulative role of gene-environment interaction in the pathophysiology of T1D. We also discussed the potential molecular mechanisms through which this gene-environment interplay can trigger autoimmunity and predisposition to T1D. By synthesizing the latest research findings, we aim to elucidate the intricate mechanisms through which genetics and the environment converge to impact the risk and onset of T1D, ultimately paving the way for more targeted preventive and therapeutic strategies.

2 Genetic etiology of T1D

Genetics plays a significant role in T1D susceptibility, particularly at the major histocompatibility complex (MHC) locus, in addition to 59 other susceptibility loci (9–15, 23–30) (Table 1). These T1D risk variants are frequently found in regions that control gene activity across various cell types, including those within the exocrine pancreas (13).

2.1 Human leukocyte antigen

There is an increased risk of developing T1D in individuals having mutations in the human leukocyte antigen (HLA) class II genes on chromosome 6, which contributes about 50% of the lifetime risk of this disease (15, 38). In particular, 90% of children with T1D possess either the DR4-DQ8 (DQA1*03:01 – DQB1*03:02) or the DR3-DQ2 (DQA1*05:01 – DQB1*02:01) haplotype. The combination of these two haplotypes in an individual's genotype represents the highest risk factor for developing the disease (12). The relationship between *HLA* gene variants and T1D risk is a focus of extensive research. These genetic associations not only help in understanding the pathophysiology of T1D but also have implications for disease prediction and prevention strategies. For instance, HLA typing is used in risk stratification and in identifying individuals who may benefit from early interventions in T1D prevention trials (39).

2.2 Cathepsin H

Besides HLA, other gene loci have also been implicated in the development of T1D, namely the susceptibility locus of cathepsin H (CTSH). Genome-Wide Association Studies (GWAS) have associated CTSH with increased risk of developing T1D (40). A study determined the potential pathogenic mechanisms of the *CTSH* gene in T1D using integrated data from quantitative trait locus (eQTL) with GWAS (41). A marked overexpression of the *CTSH* gene in acinar cells was observed in pancreas from T1D patients compared to control group using single cell RNA sequencing (scRNA). Furthermore, utilizing single-cell weighted gene co-expression network analysis (WGCNA), a set of genes co-expressed with *CTSH* were identified that have a strong positive correlation with T1D. Based on functional enrichment analysis, it was hypothesized that the *CTSH* gene within the exocrine pancreas amplifies the antiviral response. This amplification leads to an increased expression of pro-inflammatory cytokines and the creation of an inflammatory microenvironment. Such a process is likely to cause injury to β cells, ultimately contributing to the development of T1D. Another study observed that the incidence of T1D was found to correlate with high CTSH expression, which itself is modified by other environmental factors such as epigenetics and post-translational modifications (42). Taken together, these studies highlight the role of *CTSH* in increased susceptibility of developing T1D.

TABLE 1 A summary of genes associated with the development of type 1 diabetes (T1D).

Gene	SNP	Function	Reference
HLA Class II	rs6927022 rs2157051 rs9275184 rs7744001	Antigen presenting complex for recognition by CD4+ T-cells	(31)
CTLA4	rs11571316 rs3087243	Protein receptor that downregulates immune reaction	(12, 32)
CCR5	rs113010081	Impacts immune cell function	(9)
TLR7/8	rs5979785	Receptor important for pathogen recognition and immune response activation	(33)
AFF3	rs9653442	Activates transcription, involved in oncogenesis and lymphoid development	(26)
INS	rs7111341	Insulin production; decreases blood glucose concentration	(27)
GLIS3	rs7020673 rs10758593	Participates in β-cell generation and insulin gene expression	(28)
BAD	rs694739	Initiates apoptosis and promoting cell death	(34)
IL7R	rs11954020	Involved in binding to antigens, production of immunoglobulins, and executing cell-mediated cytotoxic functions.	(9)
IL10	rs3024505	Anti-inflammatory cytokine	(32)
IL27	rs151234	Cytokine that regulates helper T-cell development and suppresses T-cell proliferation	(9)
WFS1	rs1046322	Mitigates endoplasmic reticulum stress in β-cells and allocortex of brain	(35, 36)
CTSB	rs1296023	Lysosomal enzyme necessary for protein degradation	(34)
CTSH	rs3825932	Lysosomal enzyme necessary for protein degradation	(32)
GPX7		Proliferation and apoptosis of pancreatic islet beta cells	(37)
GSTT1		Proliferation and apoptosis of pancreatic islet beta cells	(37)
SNX19		Proliferation and apoptosis of pancreatic islet beta cells	(37)

2.3 Other genes

Other candidate genes such as *INS*, *GLIS3*, *CCR5*, *BAD*, *GPX7*, *GSTT1*, and *SNX19* have been shown to increase susceptibility to T1D (9–15, 23–30). Some of these genes directly affect the proliferation and apoptosis of pancreatic β-cells. A comprehensive list of genes associated with increased predisposition to T1D along with their function has been summarized in Table 1.

Although genetics have been found to play an integral role in the pathophysiology of T1D, recent studies have shown that development of T1D is multifactorial. Studies with identical twins have shown that if one twin develops T1D, the other twin may not show any susceptibility to the disease, suggesting that genetic factors alone cannot completely explain the development of T1D.

3 Environmental factors in the pathophysiology of T1D

Besides genetic etiology, environmental factors such as viral infections, pesticide exposure, lifestyle and dietary factors as well as vitamin D deficiency have all been individually associated with the development of T1D (17–19) (Supplementary Table 1).

3.1 Viral infections

Autoimmunity triggered by viral infections can play an important role in the etiology of T1D (Figure 1). Enteroviruses have been implicated at multiple levels in the etiopathogenesis of T1D, from infecting pancreatic β-cells to inducing autoimmunity against them (20). Cocksackie B viruses have been most frequently associated with the incidence of T1D (21, 22, 43). At the onset of disease in individuals with T1D, enterovirus proteins have been found in the pancreas (44). Since pancreatic β-cells also express several receptors used by enteroviruses to enter cells, various enterovirus species have been shown to infect and negatively impact the function of pancreatic β-cells (44). These viral infections trigger the production of interferons (IFN), which promote gene transcription; this IFN-stimulated gene expression has been shown in newly diagnosed T1D patients (44). This gene transcription has also been associated with the subsequent appearance of autoantibodies against pancreatic β-cells (44). As children with rapid onset T1D were found in the TEDDY study to be absent of viremia, this suggests that infections could induce autoimmunity progressively over time rather than acutely (45).

Furthermore, some viruses such as enteroviruses share structural similarities with pancreatic beta cell antigens. This resemblance may lead to a phenomenon known as molecular mimicry, where the immune system, activated to fight the virus, mistakenly attacks the body's own cells, including insulin-producing beta cells and initiation of T1D (17).

3.2 Pesticide exposure

Pesticide exposure has been implicated in the development of T1D. Pesticides are chemicals designed to control pests and are widely used in agriculture, but their potential impact on human health has raised concerns. While research in this area is ongoing and findings are not conclusive, studies have explored the association between pesticide exposure and T1D (46). Epidemiological studies have suggested a possible link between pesticide exposure and T1D. Pesticide exposure has been associated with the incidence of T1D and prediabetes, termed abnormal glucose regulation, even at low concentrations (47). The

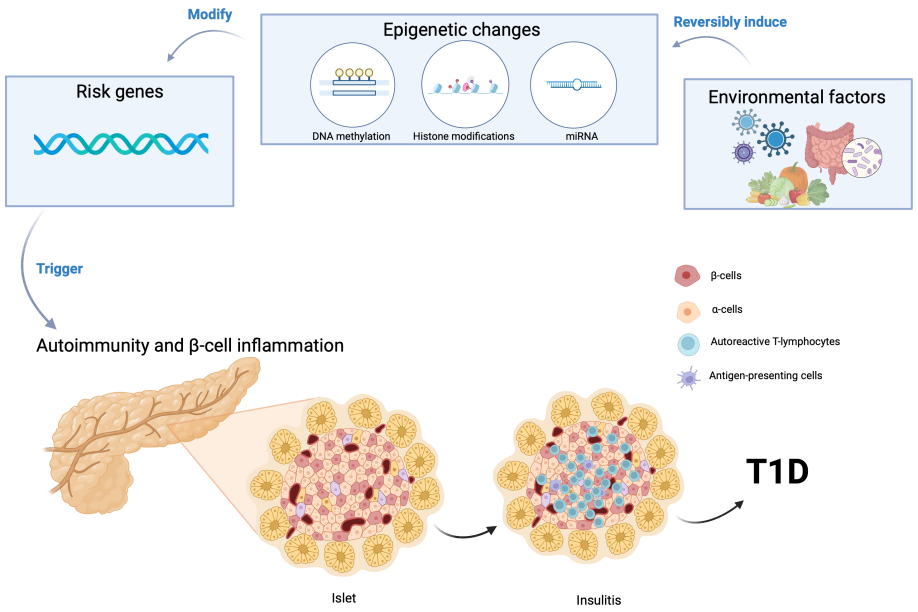

FIGURE 1
Gene-environment interaction can induce epigenetic modifications, initiating the autoimmune destruction of pancreatic β cells and consequently triggering the onset of Type 1 Diabetes (T1D). Created using BioRender.com.

causal relationship between pesticide exposure and abnormal glucose regulation differed between men and women, as a U-shaped dose-response relationship was more clearly demonstrated in men (47).

It has been hypothesized that pesticides may trigger or accelerate the autoimmune response that leads to beta-cell destruction in the pancreas. The mechanisms underlying this potential association are not fully understood but may involve the disruption of immune function or the induction of oxidative stress.

3.3 Other factors

3.3.1 Mode of delivery and antibiotic use

Studies have suggested a correlation between antibiotic use and increased predisposition to T1D (48–50). The use of broad-spectrum antibiotics during the first two years of life has been associated with an increased risk of developing T1D depending on the mode of delivery (51). Intriguingly, the association of broad-spectrum antibiotics with T1D was only observed in children delivered through cesarean section but not in vaginally delivered babies (51). However, other studies do not observe any correlation between antibiotic use and T1D (52, 53). Further studies are warranted to decipher the effect of mode of delivery and antibiotic use in the development of T1D.

3.3.2 Lifestyle and dietary factors

The influence of lifestyle and dietary factors on the development of Type 1 Diabetes (T1D) has been a subject of extensive research, revealing various associations and potential mechanisms (50). While the exact mechanisms are still being explored, it is evident that dietary habits leading to changes in gut microbiota composition may play a significant role in the development of T1D.

3.3.3 Vitamin D deficiency

Low levels of Vitamin D have been associated with the development of T1D (54–57). This association is thought to be due to Vitamin D's potential role in modulating the immune system, possibly impacting the autoimmune processes involved in T1D. However, other studies have observed no correlation between low levels of Vitamin D and a higher risk of T1D (58, 59). Additionally, the question of whether Vitamin D supplementation can reduce the risk of T1D remains under investigation, with mixed results from various studies (60). Further studies are warranted to elucidate the precise role of Vitamin D in T1D.

4 Gene-environment interaction and T1D

Despite the individual roles of genetic susceptibility and environmental risk factors, it is still unknown what triggers pancreatic β cell destruction and development of T1D in some patients. There is an emerging hypothesis that the interaction of environmental factors with genetic predisposition plays a crucial role in the pathophysiology of T1D (Figure 1). Environmental factors may exaggerate the effect of gene variants inducing autoimmunity and leading to the clinical manifestations of T1D.

Epigenetic modulators have emerged as pivotal regulators of gene expression and cellular phenotype, operating in conjunction with environmental factors (37, 61–66). Epigenetics is regarded as one of the primary molecular mechanisms by which gene-environment interactions may increase susceptibility to T1D

(61, 62, 67). Epigenetic mechanisms such as DNA methylation alterations have been a focus of investigation, with findings indicating anomalous patterns in genes linked to immune function and insulin regulation in T1D individuals (68–71). Furthermore, histone modifications have shown their influence on immune response gene dysregulation in the context of T1D (72). The role of microRNAs, another facet of epigenetics, has also been underscored, particularly in controlling immune and inflammatory responses in T1D (61, 73–76). Epigenetic alterations associated with T1D risk not only hold implications for biomarker discovery but also open doors to precision medicine strategies in T1D diagnosis, risk assessment, and therapeutic intervention.

The other possible mechanism through which the gene-environment interaction can influence the onset of T1D is through alterations in the gut microbiome, which can impact mucosal integrity and immune tolerance (Figure 1) (77–81). This has been shown to increase the risk of T1D by increasing gut permeability through molecular mimicry and modulation of the gut immune system (82). Individuals with T1D and those at risk to develop T1D have exhibited an increase of *Bacteroides* and *Bifidobacterium* spp and a decrease of *Lactococcus* spp in their gut microbiome compared to healthy controls (82). Recent advancements in genetic technologies and gut microbiome determination techniques such as multi-omics signatures have allowed us to determine differences in the gut microbiome between patients with T1D and healthy controls at the functional level (83). In addition to the differences in *Bacteroides*, *Bifidobacterium*, and *Lactococcus* spp found in the gut microbiome, taxonomic analyses of the gut microbiota identified 51 species that differed in absolute abundance between T1D and healthy controls, with T1D patients showing increases in 17 species and decreases in 34 species (83). HLA class II haplotypes with known effects on T1D incidence may directly correlate to changes in the gut microbiome, but exactly how the genes influence changes in the gut microbiome requires further investigations. Further studies are also warranted to decipher how changes in the gut microbiome leads to the development of autoimmunity and T1D.

5 Discussion

In this perspective article, we delve into the multifaceted relationship between genetic predispositions and environmental factors in the onset and progression of T1D. This exploration is crucial, as it provides insights into how specific genetic profiles interact with environmental triggers, leading to the development of T1D.

Although pathophysiology of T1D is complex, genetics has been strongly implicated in the development of disease. Gene variants in *INS*, *GLIS3*, *CCR5*, *BAD*, *GPX7*, *GSTT1*, and *SNX19* have been associated with T1D (9–16) (Table 1). However, not all the individuals harboring these gene variants develop T1D again highlighting the crucial role of gene-environment interplay in predisposition to T1D.

Besides genetics, environmental factors such as viral infections and pesticide exposure have been implicated with the development of T1D (18–22) (Supplementary Table 1). However, the causal relationship between viral infections and T1D remains complex and multifaceted. Not all individuals exposed to diabetogenic viruses develop T1D. Although compelling evidence supports the association between viral infections and T1D, further research is warranted regarding the specific viruses involved, timing of infection, the underlying molecular mechanisms of immune dysregulation, and the potential for preventive interventions. In a similar context, while pesticide exposure is being investigated as a potential environmental risk factor for T1D, there is a need to understand its interaction with genetic susceptibility, viral infections, and other environmental influences. This comprehensive understanding is vital for unraveling the complex etiology of the disease. A better knowledge about the causative relationship between pesticide exposure and T1D can contribute to preventive strategies and public health recommendations. Individuals, especially those in occupations with potential pesticide exposure and families residing in agricultural areas, should be aware of potential risks and take appropriate precautions to minimize exposure. Additionally, ongoing surveillance and research are crucial to further elucidate the impact of pesticide exposure on T1D.

Although the precise molecular mechanisms through which gene-environment dynamic interplay leads to the development of T1D are still not clear, epigenetics and changes in gut microbiome have been hypothesized to play a pivotal role. Epigenetic changes such as abnormal methylation patterns can occur in genes related to immune function or insulin production, altering their expression, and potentially triggering an autoimmune response against pancreatic beta cells (37, 61–66). While there has been significant progress in understanding how epigenetics contributes to T1D, several research gaps remain that need to be addressed for a more comprehensive understanding. There is a need to understand the causal relationship between epigenetic modifications and the initiation and progression of T1D. Deciphering the role of epigenetics will provide a deeper understanding of T1D etiology.

The changes in gut microbiome have been hypothesized to play a pivotal role in gene-environment interaction and development of T1D (77–81). Although some progress has been made in understanding the role of the gut microbiome in T1D, there are still many unanswered questions. The exact mechanisms by which alterations in the gut microbiota leads to autoimmune responses against pancreatic β-cells are not fully understood. Understanding these mechanisms is crucial for developing potential therapeutic interventions. Furthermore, there is a need to perform more longitudinal studies to understand how early-life exposures and changes in the gut microbiome contribute to the development of T1D, especially using emerging techniques such as omics technology. The information derived from these studies would provide insights into the temporal dynamics of microbiome changes and their association with T1D onset.

6 Conclusions and future directions

The availability of preclinical animal models and epidemiological studies using large cohorts can significantly increase our understanding regarding the role of gene-environment interplay in the molecular underpinnings of T1D. There is a need to discover novel therapeutic interventions that facilitate demethylating key DNA regions implicated in the pathogenesis of T1D. In addition, there is a need to understand the precise functional role of the gut microbiome in the pathophysiology of T1D using emerging omics technologies. Simultaneously, there is a critical emphasis on developing therapeutic strategies aimed at reducing gut dysbiosis observed in T1D individuals and restoring the normal gut microbiome. Considering the crucial role of epigenetics in the disease process, the other avenue of research should be focused on determining the efficacy of "epidrugs" already available in the market for prevention and treatment of T1D (84). Repurposing Food and Drug Administration (FDA) approved drugs significantly reduces the cost, time, labor, and high-risk process of drug development with greater rates of success. The development of novel interventions that focus on the interplay between genes and the environment offers significant hope for the efficient management of T1D in pursuit of improving the quality of life of affected individuals and their families/caregivers.

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

RM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. NC: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JL: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. KH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1335435/full#supplementary-material

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Advances in risk predictive performance of pre-symptomatic type 1 diabetes via the multiplex Antibody-Detection-by-Agglutination-PCR assay

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Introduction: Achieving early diagnosis of pre-symptomatic type 1 diabetes is critical to reduce potentially life-threatening diabetic ketoacidosis (DKA) at symptom onset, link patients to FDA approved therapeutics that can delay disease progression and support novel interventional drugs development. The presence of two or more islet autoantibodies in pre-symptomatic type 1 diabetes patients indicates high-risk of progression to clinical manifestation.

Method: Herein, we characterized the capability of multiplex ADAP assay to predict type 1 diabetes progression. We obtained retrospective coded sera from a cohort of 48 progressors and 44 non-progressors from the NIDDK DPT-1 study.

Result: The multiplex ADAP assay and radiobinding assays had positive predictive value (PPV)/negative predictive value (NPV) of 68%/92% and 67%/66% respectively. The improved NPV stemmed from 12 progressors tested positive for multiple islet autoantibodies by multiplex ADAP assay but not by RBA. Furthermore, 6 out of these 12 patients tested positive for multiple islet autoantibodies by RBA in subsequent sampling events with a median delay of 2.8 years compared to multiplex ADAP assay.

Discussion: In summary, multiplex ADAP assay could be an ideal tool for type 1 diabetes risk testing due to its sample-sparing nature (4μL), non-radioactiveness, compatibility with widely available real-time qPCR instruments and favorable risk prediction capability.

KEYWORDS

type 1 diabetes mellitus, immunology, islet autoantibodies, pediatrics, autoimmune diseases

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease that affects more than 1.6 million children and adults in the US (1). Early detection of T1D is critical because the initiation of the autoimmune process that leads to T1D clinical presentation begins well in advance of the symptoms. Indeed, the American Diabetes Association (ADA), JDRF, and the American Endocrine Society published a joint statement in 2015 to recognize T1D as a disease continuum and update the definition of T1D diagnosis into several distinct stages (2–4). Patients with stage 1 and stage 2 T1D are positive for multiple islet autoantibodies and are at high risk of progressing to stage 3 T1D with clinical symptoms (e.g., hyperglycemia) (3, 4). This classification system was later confirmed by a joint statement from the NIDDK TrialNet study group (4).

Early diagnosis of stage 1 or 2 T1D with regular monitoring and follow-up could improve the clinical outcomes of T1D (5–7). First, the rates of diabetic ketoacidosis (DKA) at stage 3 T1D onset could be reduced, leading to lower HbA1c levels and a reduced risk of complications such as retinopathy and nephropathy (5–7). Second, FDA-approved therapeutics such as teplizumab could delay the clinical diagnosis of stage 3 T1D by years (8). Third, new generations of interventional therapeutics (e.g., NCT01773707 and NCT03428945) would benefit from a pool of early stage T1D patients to support ongoing clinical trials (9). This creates a positive feedback loop for T1D patients in that early diagnosis not only improves the outcome for the individual patient but also creates an opportunity to develop more effective therapeutics to benefit future T1D patients.

Nevertheless, the identification of stage 1 or stage 2 T1D patients is challenging because they are asymptomatic, and over 85% of them do not have a family history (2–4). Therefore, large-scale testing by the general public remains the only effective means of systematically identifying them. There are several methods to detect islet autoantibodies for the identification of patients with stage 1 or stage 2 T1D. The radiobinding assay (RBA) remains the gold standard and the most used assay format in large-scale testing programs for early T1D. Newer non-radioactive assays, such as ELISA, ECL, and LIPS, have been used either solely or in combination with RBA in recent testing programs (4, 10–13).

The multiplex Antibody Detection by Agglutination-PCR (ADAP) islet autoantibody assay used in this study was based on a highly sensitive ADAP platform (14–17). The multiplex ADAP assay is valuable for early T1D diagnosis because it uses a small-sample volume for testing (e.g., 1 µL–4 µL). Considering that a significant portion of stage 1 or stage 2 T1D patients are pediatric, reduction of sample collection burden with small volumes is critical. Furthermore, ADAP multiplexed all relevant islet autoantibodies in a single assay, further minimizing the sample volume requirement and increasing laboratory throughput. In addition, ADAP does not rely on radioactive reagents and uses standard RT-qPCR as an assay readout, making the test readily adoptable in standard clinical laboratories. These technical attributes and the high sensitivity/specificity of ADAP make it an attractive option for early T1D diagnosis.

Previously, this assay was validated for islet autoantibody detection in several studies with favorable performance characteristics, including the islet autoantibody standardization program (IASP) (10, 15–17). Nevertheless, these validations were conducted primarily on stage 3 new-onset or stage 4 established T1D patients. Despite satisfactory sensitivity and specificity, it was unclear whether the ADAP assay could be used to identify stage 1 or stage 2 T1D patients who are at risk of progressing to stage 3 T1D. Herein, we report the results of a pilot validation with retrospective serum samples from subjects who had been tested by RBA for islet autoantibodies and were followed up for 8 years. This unique cohort enabled the analysis of positive and negative predictive values (PPV and NPV) for T1D risk prediction, providing data to support the use of multiplex ADAP for the early diagnosis of presymptomatic T1D.

Methods

Human specimen characteristics

The specimens used in this study were obtained from the DPT-1 trial cohort sponsored by the NIDDK between 1994 and 2003 (18). Detailed patient recruitment and study protocols have been reported previously (18). Briefly, all participants were first- or second-degree of relatives of a person with T1D and were tested for islet cell autoantibodies (ICAs). Written informed consent was obtained from all the subjects in the study group. Patients with ICA autoantibodies were offered additional testing for GAD, IA-2, and insulin autoantibodies. Islet autoantibody testing records, follow-up records, and clinical diagnosis of stage 3 T1D records were available from the NIDDK biorepository.

Sera collected within 6 months of study enrollment were obtained from a total of 48 subjects who progressed to stage 3 T1D and 44 subjects who did not progress to stage 3 T1D during the follow-up. The subjects were randomly selected by the NIDDK central repository staff. These subjects either developed stage 3 T1D during follow-up or were followed up for at least 5 years. The demographic characteristics of the study participants are presented in Table 1. Notably, the study participants were predominantly non-Hispanic white individuals. There were more male than female participants. The samples were transferred to Enable Biosciences for multiplex ADAP analysis as de-identified-coded specimens. The result was only unblinded by the NIDDK central repository after testing was completed. The study was approved by the Western IRB (IRB number #20180015) to Enable Biosciences.

Multiplex ADAP assay analysis

Previously, we reported a multiplex ADAP method for detecting three islet autoantibodies (15). In addition, we described an automated Hamilton MicroLab STAR system to carry out the 3-

TABLE 1 Demographic of study subjects.

Subjects	Progressors	Non-progressors
Number	48	44
Age at testing (median and IQR) (year old)	8.1 (5.5–11.3)	13 (8.6–29.6)
Ethnicity	46 non-Hispanic white	42 non-Hispanic white
Sex	33 Male, 15 Female	26 Male, 18 Female
Age at stage 3 T1D diagnosis (median) (year old)	12.0 (9.5–14.8)	N/A

A detailed description of the study subjects was provided below.

plex ADAP assay (16). Recently, we expanded the assay to 5-plex to test for IAA, GADA, IA2A, ZnT8A, and TGA on a modified version of Hamilton MicroLab STAR to achieve full automation (17). Herein, we restricted the ADAP assay to a 4-plex assay to test for all four islet autoantibodies (IAA, GADA, IA2A, and ZnT8A) on the Hamilton MicroLab STAR system. Briefly, 4 µL of serum was incubated with 8 µL of DNA-barcoded autoantigens at 37°C for 30 min. If present in the specimens, autoantibodies agglutinate autoantigens into a dense immune complex. Then, 4 µL of the mixture was aspired and mixed with 116 µL of ligation mixtures, where nearby DNA in the dense immune complex was ligated to form a full-length DNA amplicon. Next, 25 µL of the above mixture was further mixed with 25 µL of PCR amplification mixtures containing primers for all five autoantibodies for a total of 13 PCR cycles using an on-deck thermocycler (ODTC, Inheco, Martinsried, Germany). The amplified products were then aspired to 384 well plates in which each well contained the cognate primer pairs for each autoantibody to achieve specific quantification by real-time quantitative PCR (RT-qPCR). The qPCR-ready plates were transferred to Bio-Rad CFX384 to enable an automated sample-to-answer solution. The samples were analyzed in a coded and randomized manner. The results were unblinded after sample testing was completed. The assay cutoffs were determined by testing 80 healthy controls and set at the 99th percentile. The cut-offs for IAA, GADA, IA2A, and ZnT8 were 0.99, 3.1, 2.3, and 2.0, respectively.

Radiobinding assay analysis

The GAD, IA-2, and insulin autoantibody testing results were obtained from the NIDDK central repository database. Laboratory procedures for GAD, IA-2, and insulin autoantibody analyses have been extensively reported (18). Briefly, GAD and IA-2 autoantibodies were detetcted at the Barbara Davis Center (Denver, CO, USA). Insulin autoantibody levels were determined at the Barbara Davis Center or Joslin Diabetes Center (Boston, MA, USA). The cut-off values for the GAD and IA-2 assays were 0.032 and 0.049, respectively. For the insulin assays, the cut-off was 0.01 at the Barbara Davis Center and 0.02 at Joslin Diabetes Center. The cutoffs were determined using the 99th percentile of the healthy controls. A combined radiobinding assay was performed for GAD and IA-2 autoantibodies using radioactively labeled H3-GAD65 and S35-IA-2.

Data analysis

Positive predictive value (PPV) was defined as the probability that a subject with a positive test result actually progressed to clinical presentation of the disease. The negative predictive value (NPV) was defined as the probability that a subject with a negative test result truly did not progress to disease clinical presentation. For instance, in this study, a positive test result was defined as having two or more islet autoantibodies, unless otherwise noted. The overall PPV was calculated based on the number of individuals that progressed to stage 3 T1D during the entire follow-up period, while the overall NPV was calculated based on the number of individuals who did not progress to stage 3 T1D during the entire follow-up period. The 5-year risk PPV and NPV were calculated similarly, except that we restricted the analysis to progression within 5 years. It should be noted that all study subjects had either been followed for 5 years or progressed to stage 3 T1D within 5 years. Kaplan–Meier estimates were used to plot progression risk and to compare probabilities of stage 3 T1D progression in subjects stratified by the number of islet autoantibodies, sex, or age groups. For all analyses, a 2-tailed P-value of 0.05 was considered significant. All statistical analyses were performed using Graphpad Prism (version 9.3.1).

Data and resource availability

All data generated or analyzed during this study are included in the published article (and its online Supplementary Files). The reagents used in this study are available from the corresponding author upon request.

Results

Positive and negative predictive value of multiplex ADAP islet autoantibody assays

In this study, we obtained 92 sera samples from 48 progressors and 44 non-progressors in the NIDDK DPT-1 study (18). All individuals either developed T1D during the follow-up period (progressors) or were followed up for at least 5 years (non-progressors). The sera were analyzed using multiplex ADAP assays for autoantibodies against GAD, IA-2, insulin, and ZnT8 (Figure 1, Table 1). Among them, 68 individuals tested positive for two or more islet autoantibodies, and 46 developed stage 3 T1D

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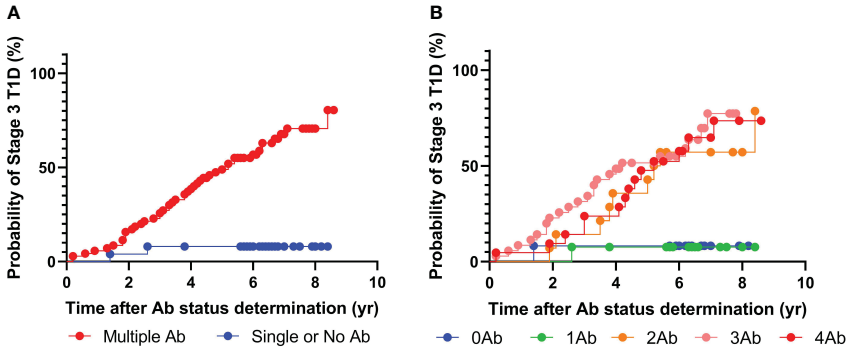

FIGURE 1
Progression to stage 3 T1D stratified based on islet autoantibody test results from 4-plex ADAP assay (GADA, IA2A, IAA, ZnT8). **(A)** Stratification based on harboring two or more islet autoantibodies. **(B)** Stratification based on number of islet autoantibodies.

during the follow-up period. The median time from positivity for two or more islet autoantibodies to stage 3 T1D diagnosis was 4.2 years (Range: 1.0–8.4 years). Among the 24 individuals with one or fewer islet autoantibodies, only two individuals progressed to stage 3 T1D. One of them, diagnosed at the age of 13.1 years old, had a high level of GAD autoantibody and IA2 autoantibody level immediately below the cut-off, while the other, diagnosed at age of 27.8 years old, was negative for all islet autoantibodies. The overall positive predictive value (PPV) and negative predictive value (NPV) of the multiplex ADAP islet autoantibody assay based on the presence of two or more islet autoantibodies were 68% (46/68) and 92% (22/24), respectively. Alternatively, the PPV and NPV for progression to stage 3 T1D within 5 years of testing were 49% and 92%, respectively. The 5-year PPV was lower than the overall PPV because some individuals developed stage 3 T1D after 5-years of initial testing. The 5-year PPV observed in this study is consistent with that of other longitudinal follow-up studies (19–21).

Next, we sought to further explore whether individuals with two, three, or four islet autoantibodies would have distinct progression risks to stage 3 T1D (Figure 1B). Progression rates ranged from 64% to 70% (Supplementary Table 1). The median time from positivity for two or more islet autoantibodies to clinical presentation was 3.9, 3.1, and 4.4 years for individuals with two, three, and four islet autoantibodies, respectively.

Furthermore, an analysis was conducted to evaluate whether the types of islet autoantibodies would influence the risks of progression to stage 3 T1D (Tables 2, 3). For individuals with two or more islet autoantibodies, if their autoantibody positivity included GAD, IA-2, insulin, or ZnT8 autoantibodies, the median time to diagnosis was 3.7, 3.8, 3.6, and 3.6 years, respectively, and the PPV were 68%, 68%, 65%, and 71%, respectively. If the autoantibody positivity included GAD/IA-2, GAD/insulin, GAD/ZnT8, IA-2/insulin, IA-2/ZnT8, Insulin/ZnT8 autoantibodies, the median time to diagnosis was 3.8, 3.6, 3.6, 3.8, 3.6, and 4.2 years, respectively and the PPV was 68%, 65%, 71%, 64%, 73%, and 67%, respectively.

Therefore, the above observation indicated that the presence of multiple islet autoantibodies was a critical risk factor for progression to stage 3 T1D.

Impact of age and sex on progression risk to stage 3 T1D

Patients positive for two or more islet autoantibodies might have distinct progression risks depending on their age and sex (22). To investigate this further, we first stratified the individuals into those under and above the age of 8 at the time of testing. For individuals under the age of 8 years, the multiplex ADAP assay had a PPV and NPV of 80% and 100%, respectively. For individuals over age of 8 years, the PPV and NPV were 58% and 90%, respectively. Therefore, the development of multiple islet autoantibodies at a young age appears to increase the risk of progression risk to stage 3 T1D. On the other hand, female patients had a slightly higher PPV than male patients (70% vs 67%), and the NPV was comparable (92% vs 91%).

TABLE 2 Multiplex ADAP islet autoantibody assay analysis results.

	Progressors (N = 48)	Non-progressors (N = 44)
Classification Scheme 1		
Two or more islet autoantibodies	46	22
One or less islet autoantibodies	2	22
Classification Scheme 2		
Four islet autoantibodies	14	7
Three islet autoantibodies	23	10
Two islet autoantibodies	9	5
One islet autoantibodies	1	11
Zero islet autoantibodies	1	11

In the classification scheme 1, subjects were classified based on whether they tested positive for two or more islet autoantibodies. In the classification scheme 2, subjects were classified based on the incremental number of islet autoantibody positivity.

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TABLE 3 Impact of islet autoantibody pattern of progression to Stage 3 T1D.

Stage 1 or stage 2 T1D autoantibody positivity pattern	Median time to diagnosis	PPV
GADA	3.7	0.68
IA2A	3.8	0.68
IAA	3.6	0.65
ZnT8	3.6	0.71
GAD/IA2	3.8	0.68
GAD/IAA	3.6	0.65
GAD/ZnT8	3.6	0.71
IA2/IAA	3.8	0.64
IA2/ZnT8	3.6	0.73
IAA/ZnT8	4.2	0.67

For the 48 subjects tested positive for two or more islet autoantibodies by ADAP assays, additional analysis was conducted to evaluate impact of islet autoantibody pattern of progression risk. For GADA, IA2A, and IAA, these indicated the subjects were positive for two or more islet autoantibodies, and one of the islet autoantibodies was the specified autoantibodies. For GADA/IA2A, GADA/IAA, GADA/ZnT8A, IA2A/IAA, IA2A/ZnT8, and IAA/ZnT8, these indicated the subjects were positive for two or more islet autoantibodies, and two of the islet autoantibodies were the specified autoantibodies.

Impact of ZnT8 autoantibodies in prediction of T1D risk prediction

Recently, ZnT8 autoantibodies were discovered. The value of ZnT8 autoantibodies in aiding the diagnosis of new-onset clinical diabetes and risk predictions has been widely reported (23). It is of great interest to investigate whether the exclusion of ZnT8 autoantibodies would substantially impact the prediction of stage 3 T1D progression risk.

To this end, the above analysis was performed again using only GAD, IA-2, and insulin autoantibodies (Figure 2). Intriguingly, 67 individuals tested positive for two or more islet autoantibodies, and 46 out of the 67 individuals eventually developed stage 3 T1D during follow-up. The median time from positivity for two or more islet autoantibodies to clinical presentations was 3.7 years. Similar to the previous analysis, only two out of 25 individuals with one or fewer islet autoantibodies progressed to stage 3 T1D. Accordingly, the positive predictive value (PPV) of the multiplex ADAP islet autoantibody assay with GAD, IA-2, and insulin autoantibodies was 68% (46/67) and the negative predictive value (NPV) was 92% (23/25). These predictive values were statistically indistinguishable from the predictive values when all four islet autoantibodies were included. The data thus support the use of three cardinal islet autoantibodies for the prediction of the risk of progression to stage 3 T1D.

Comparison of predictive value to radiobinding assays

The prediction of the risk of progression to stage 3 T1D has been extensively studied in several landmark studies using radiobinding assays to measure islet autoantibodies. Indeed, the underlying DPT-1 study was one of the earliest nationwide longitudinal studies to provide critical insight into the natural history of T1D development and inspired and shaped study designs for many other recent studies. Importantly, radiobinding assay data from the DPT-1 studies were available from the NIDDK biorepository. We sought to compare the risk prediction between the multiplex ADAP assays and radiobinding assays. It should be noted that DPT-1 study was conducted between 1994 and 2003 (18). The design and protocols for radiobinding assays have been improved in recent studies (10). Nevertheless, the data will provide a valuable context to help understand whether the observed multiplex ADAP assay performance is satisfactory.

Among the 92 patients with radiobinding assay data for GAD, IA-2, and insulin autoantibodies, 51 tested positive for two or more islet autoantibodies, and 34 progressed to stage 3 T1D during the follow-up period, with a median time to diagnosis of 3.4 years (Figure 3, Table 4). Fourteen of the 41 individuals with one or no islet autoantibodies progressed to stage 3 T1D, with a median time to diagnosis of 4.3 years. The overall PPV and NPV of the radiobinding assays were 67% and 66%, respectively. The 5-year PPV and NPV for the radiobinding assays were 51% and 78%, respectively.

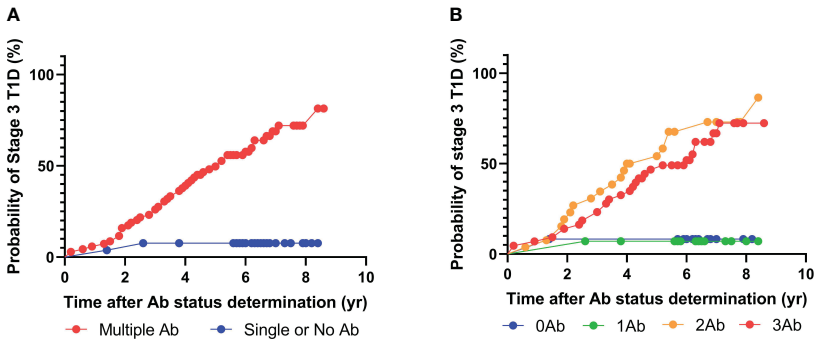

FIGURE 2
Progression to stage 3 T1D stratified based on islet autoantibody test results from 3-plex ADAP assay (GADA, IA2A, IAA). **(A)** Stratification based on harboring two or more islet autoantibodies. **(B)** Stratification based on number of islet autoantibodies.

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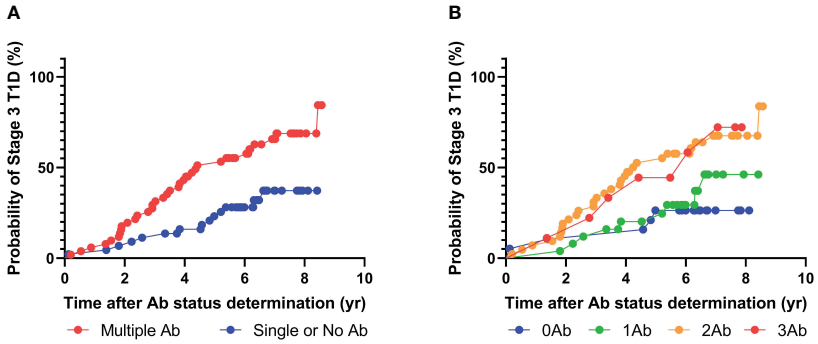

FIGURE 3
Progression to stage 3 T1D stratified based on islet autoantibody test results from radiobinding assays (GADA, IA2A, IAA). **(A)** Stratification based on harboring two or more islet autoantibodies. **(B)** Stratification based on number of islet autoantibodies.

To compare performance, we first restricted the multiplex ADAP assay analysis to GAD, IA-2, and insulin autoantibodies, given that ZnT8 autoantibodies were not yet discovered at the time of the DPT-1 study. The multiplex ADAP assay and radiobinding assays had similar PPV of 68% and 67%, respectively. Nevertheless, the NPV differences were statistically significant (92% vs 66%, respectively). To elucidate the potential sources of the NPV differences, it was noted that multiplex ADAP assays identified 46 out of 48 patients that progressed to stage 3 T1D as multiple islet autoantibody-positive. In contrast, radiobinding assays only identified 34 out of 48 progressors as multiple islet autoantibody-positive, leading to a lower NPV.

We further compared the pattern of islet autoantibodies for the 12 progressors that had discrepant assigned risk profiles using multiplex ADAP and radiobinding assays (Table 5). Five of the 12 progressors were positive for GAD/IA-2/insulin autoantibodies, and the remaining seven individuals were positive for GAD/IA-2 autoantibodies with multiplex ADAP assays. On the other hand, seven out of the 12 progressors were single positive for GAD autoantibodies, one out of 12 was single positive for IA-2 autoantibodies, one out of 12 was single positive for insulin autoantibodies, and three out of 12 were negative for all islet autoantibodies by radiobinding assays. Thus, it appears that the discrepant risk profiles were not a result of specific islet autoantibodies. Nevertheless, it was noted that for the seven individuals with single GAD autoantibodies by radiobinding assays, their GAD autoantobody signals measured by ADAP ranged from 7.59 to 11.63, whereas those five were missed by radiobinding assays, and their GAD autoantibody signals measured by ADAP ranged from 4.79 to 6.54. Similarly, for the one individual with insulin autoantibodies by radiobinding assays, the ADAP signal was 4.92, while rest of 4 ADAP insulin autoantibody-positive individuals had signals from 1.07 to 2.07. These observations suggested that ADAP had improved sensitivities over radiobinding assays for GAD and insulin autoantibodies, as the samples were only radiobinding assay-positive if their ADAP signals were higher in values. In contrast, for IA-2 autoantibodies, the only radiobinding assay-positive sample had an ADAP signal of 7.78, but the remaining 11 samples had ADAP signals from 2.42 to 11.64. Should sensitivities be the only factor, we would expect those samples with ADAP signals above 7.78 to be positive by radiobinding assays. The fact that several samples with strong ADAP signals were negative by radiobinding assays implied that the two assays might have additional differences for IA-2 autoantibody detection, such as autoantibody epitopes and isotypes.

Notably, the NIDDK biorepository had longitudinal radiobinding assay data for a portion of DPT-1 study samples. For these 12 progressors who were initially positive for one or fewer islet autoantibodies by radiobinding assays, five later developed two or more islet autoantibodies. The ADAP assay preceded the radiobinding assay by a median of 2.8 years for detecting two or more islet autoantibodies in these five samples. The remaining seven progressors did not develop two or more islet autoantibodies by radiobinding assays during the follow-up. While the sample size was limited, this is preliminary evidence that the ADAP assay could enable earlier diagnosis of stage 1 or stage 2 T1D.

The overall sensitivity of the multiplex ADAP sand radiobinding assay was 96% and 71%, respectively, whereas the overall specificity of the multiplex ADAP sand radiobinding assay was 50% and 61%, respectively.

TABLE 4 Radiobinding assay analysis results.

	Progressors (N = 48)	Non-progressors (N = 44)
Classification Scheme 1		
Two or more islet autoantibodies	34	17
One or less islet autoantibodies	14	27
Classification Scheme 2		
Three islet autoantibodies	10	3
Two islet autoantibodies	24	14
One islet autoantibodies	10	15
Zero islet autoantibodies	4	12

In the classification scheme 1, subjects were classified based on whether they tested positive for two or more islet autoantibodies. In the classification scheme 2, subjects were classified based on the incremental number of islet autoantibody positivity.

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TABLE 5 Discordant results from subjects that eventually progressed to Stage 3 T1D.

Subject	ADAP			Radiobinding assay		
	GADA	IA2A	IAA	GADA	IA2A	IAA
Progressor 1	11.63	3.55	2.07	0.86	−0.03	0.00
Progressor 2	5.58	7.78	1.07	−0.03	0.74	0.00
Progressor 3	6.01	3.19	4.92	−0.05	−0.01	0.12
Progressor 4	6.55	2.42	1.52	0.01	−0.02	0.00
Progressor 5	6.01	11.64	1.83	−0.01	0.02	0.00
Progressor 6	11.21	3.68	0.65	0.86	0.01	0.00
Progressor 7	9.24	3.06	0.81	0.36	0.01	0.00
Progressor 8	10.50	8.49	0.70	0.43	0.01	0.00
Progressor 9	9.38	5.46	0.62	0.12	0.00	0.00
Progressor 10	7.59	8.39	−0.03	0.04	−0.03	−0.02
Progressor 11	9.27	4.60	0.18	0.19	0.02	0.00
Progressor 12	4.79	2.70	0.92	−0.04	−0.02	0.00

A total of 12 subjects that eventually progressed to stage 3 T1D within the following up period had discordant results by the multiplex ADAP assays and radiobinding assays. Given that radiobinding assays only analyzed GADA, IA2A, and IAA during the DPT-1 study, the ADAP data shown here were restricted to the same three autoantibodies. Positive results were highlighted in red.

Discussion

Over the past two decades, our understanding of the risk factors, progression profiles, and prevention and intervention strategies for T1D has dramatically improved. Historically, T1D is a disease that can only be managed by insulin administration and glucose monitoring and cannot be prevented or cured. Teplizumab was recently approved by the FDA as the first drug to delay or prevent progression to stage 3 T1D (8). This has sparked widespread interest in building infrastructure to identify stage 1 or stage 2 T1D patients that may benefit from immunomodulatory drugs and create a pool of eligible patients to support the development of newer generations of interventional therapeutics (4). Considering that more than 85% of patients with stage 3 T1D have no family history, testing efforts have been increasingly directed toward the general population, including landmark Fr1da and ASK studies (24, 25).

The multiplex ADAP islet autoantibody assay may be a suitable tool for large-scale testing of stage 1 or stage 2 T1D in the general population. The ADAP assay features low sample volume consumption (as little as 1 μL–4 μL), is multiplex, and does not rely on hazardous radioactive reagents. These attributes are relevant in that most of the testing targets would be young children, where phlebotomy blood draw would create a substantial sample collection burden and decrease testing access. Extensive validation of the multiplex ADAP assay focused on evaluating assay performance in stage 3 or stage 4 T1D patients. While these validation data were promising in nature, they did not address the predictive value of T1D progression risk.

This study leveraged elegant retrospective samples from the DPT-1 study to fill this critical gap and provided valuable validation of risk prediction using the multiplex ADAP assay platform. The results showed satisfactory PPV and NPV values of 68% and 92%, respectively. Importantly, these data support the use of GAD, IA-2, and insulin autoantibodies to achieve effective risk prediction. In comparison, the radiobinding assays had PPV and NPV of 67% and 66%, respectively. The marked improvement in NPV was likely a combined result of the enhanced sensitivities of ADAP assays and intrinsic differences in assay epitope exposures. Notably, of the 48 individuals who eventually progressed to stage 3 T1D, the multiplex ADAP assay classified 46 as stage 1 or stage 2 T1D, whereas the radiobinding assay identified 34. These data complement previous validations using new onset/established T1D patient samples and demonstrate the robust performance of the multiplex ADAP assay.

Nevertheless, this study had some limitations. the DPT-1 study was conducted between 1994 and 2003. Therefore, the radiobinding assays used in the DPT-1 study improved over time. The observed lower performance of radiobinding assays in the DPT-1 study may not represent the performance of radiobinding assays (10). For instance, in a recent report in 2013 (21), radiobinding assays achieved an NPV of 87.3%–99.6% and a PPV of 61.6%–79.1%. These values were comparable to the multiplex ADAP assay performance reported in this study. Second, the sample size used in this study was limited. Third, the study was conducted using serum samples collected from phlebotomy blood samples. Finger-prick whole blood or dried blood spot should be used to fully realize the sample-sparing nature of the ADAP assay. Future studies should investigate risk prediction using ADAP assays with these easily collectable sample formats. Fourth, this study was primarily based on samples from relatives of T1D patients who tested positive by islet-cell antigen assays. It is desirable to conduct pilot testing with longitudinal follow-up in the general population setting to definitively evaluate the PPV and NPV. Finally, this study focused on clinical risk prediction accuracy and did not address the overall impact of improved prediction on patient outcomes and healthcare economics. Future studies should be

designed to evaluate whether improved predictions can lead to better patient outcomes and economic savings.

In addition to radiobinding assays, several new generations of islet autoantibody assays have been developed and reported, including ELISA, electrochemiluminescence (ECL), and luciferase immunoprecipitation (LIPS) (10–13). It is desirable to compare the ADAP assay performance beyond the radiobinding assay with these newer assay formats. Based on the comparison results, it might be possible to design a T1D risk-testing algorithm in which a highly sensitive assay is used as the first-line screening assay and the sample is reflected in a confirmatory assay with a high positive predictive value. These types of algorithms may achieve performance above and beyond what is possible with a single assay format. Additional considerations should be considered when designing these algorithms. For instance, the first-line and confirmatory assays should be compatible with the same sample type. Furthermore, the first-line assay should have minimal sample consumption, such that sufficient samples are available for confirmatory assays. Meeting these requirements would prevent the need for additional sample collection and increase participation in testing.

In conclusion, this study provides valuable evidence for establishing the predictive value of the multiplex ADAP assay for the risk to stage 3 T1D. The enhanced analytical sensitivities of ADAP translate to higher identification rates in stage 1 or stage 2 individuals who eventually progress to clinical T1D. The assay also achieved earlier identification of stage 1 or 2 T1D. These favorable clinical performances, together with the low sample consumption and multiplex capability, render the ADAP assay a potentially useful tool for large-scale testing of stage 1 or stage 2 T1D in the general population.

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 author.

Ethics statement

The studies involving humans were approved by the Western IRB at Enable Biosciences using de-identified specimens. The specimens were sourced from the NIDDK biorepository in the DPT-1 study. The DPT-1 study protocol was approved by the institutional review boards at all participating locations across the U.S. and Canada, including 91 sites in the study. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians or next of kin.

Author contributions

DT: Investigation, Methodology, Data curation, Validation, Writing – review & editing. BH: Writing – review & editing, Data curation, Investigation, Validation. FJC: Writing – review & editing, Data curation, Investigation, Methodology, Project administration. DS: Writing – review & editing. PR: Writing – review & editing. C-tT: Investigation, Methodology, Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – original draft.

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Conflict of interest

DT, BH, FJC, PR, DS, and C-tT were employed by Enable Biosciences. FJC, DT, PR, DS, and C-tT are shareholders of Enable Biosciences. PR and C-tT are inventors of the ADAP patent licensed from University of California, Berkeley to Enable Biosciences. The ADAP assay used in this study is a product in development. This does not alter our adherence to journal policies on sharing data and materials.

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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.2024.1340436/full#supplementary-material

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Preserved C-peptide is common and associated with higher time in range in Chinese type 1 diabetes

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Objective: The aim of this study is to determine the residual C-peptide level and to explore the clinical significance of preserved C-peptide secretion in glycemic control in Chinese individuals with type 1 diabetes (T1D).

Research design and methods: A total of 534 participants with T1D were enrolled and divided into two groups, low–C-peptide group (fasting C-peptide ≤10 pmol/L) and preserved–C-peptide group (fasting C-peptide >10 pmol/L), and clinical factors were compared between the two groups. In 174 participants who were followed, factors associated with C-peptide loss were also identified by Cox regression. In addition, glucose metrics derived from intermittently scanned continuous glucose monitoring were compared between individuals with low C-peptide and those with preserved C-peptide in 178 participants.

Results: The lack of preserved C-peptide was associated with longer diabetes duration, glutamic acid decarboxylase autoantibody, and higher daily insulin doses, after adjustment {OR, 1.10 [interquartile range (IQR), 1.06–1.14]; OR, 0.46 (IQR, 0.27–0.77); OR, 1.04 (IQR, 1.02–1.06)}. In the longitudinal analysis, the percentages of individuals with preserved C-peptide were 71.4%, 56.8%, 71.7%, 62.5%, and 22.2% over 5 years of follow-up. Preserved C-peptide was also associated with higher time in range after adjustment of diabetes duration [62.4 (IQR, 47.3–76.6) vs. 50.3 (IQR, 36.2–63.0) %, adjusted P = 0.003].

Conclusions: Our results indicate that a high proportion of Chinese patients with T1D had preserved C-peptide secretion. Meanwhile, residual C-peptide was associated with favorable glycemic control, suggesting the importance of research on adjunctive therapy to maintain β-cell function in T1D.

KEYWORDS

type 1 diabetes, preserved C-peptide, beta cell, CGM, glycemic control

Introduction

Type 1 diabetes (T1D) is characterized by progressive autoimmune destruction of β cells. The loss of β cells leading to the diagnosis of T1D is gradual and continues after clinical onset. Initially, a significant number of β cells remain, and relatively low doses of exogenous insulin are required to limit glucose variability and hypoglycemia. Although it has been assumed that β cells are irreversibly lost after diagnosis, recent studies have shown that not all β cells are destroyed and that many people with T1D continue to produce insulin even after long-term disease course (1, 2). The Diabetes Control and Complications Trial showed that the persistence of residual β cells, as measured by C-peptide secretion, is associated with better glycemic control, reduced glycemic variability, and a lower incidence of microvascular complications (3, 4). Understanding the presence and trends of residual β-cell function and its relationship to the heterogeneity of glycemic control may provide insights into the natural history of the disease and facilitate possible interventions to modify disease progression.

Previous studies have suggested heterogeneity in preserved β-cell function in T1D across cohorts and according to the definition of "preserved C-peptide secretion." In the Scottish Diabetes Research Network Type 1 Bioresource cohort, 37.7% of participants retain detectable non-fasting C-peptide (>5 pmol/L) (5). In addition, in the T1D Exchange Clinic Network, detectable non-fasting C-peptide (>17 pmol/L) was found in 29% of participants, and the frequency of non-fasting C-peptide ≥200 pmol/L was 10% (6). Meanwhile, even minimal levels of C-peptide have clinical significance in established T1D. Kuhtreiber et al. found that fasting C-peptide levels >10 pmol/L were associated with protection from complications (7), and Fraser et al. found that, under the same definition of preserved C-peptide, it was associated with fewer low glucose events and lower glucose variability on intermittently scanned continuous glucose monitoring (isCGM) (8). Although the maintenance of C-peptide secretion has been well studied in the Caucasian population, little is known about non-Caucasian populations, particularly East Asians. The aim of this study was to evaluate residual β-cell function, the underlying clinical factors contributing to the preservation of C-peptide secretion, and its impact on glycemic control in Chinese individuals with T1D.

Research design and methods

Study design and participants

A total of 631 individuals with T1D treated at Peking University People's Hospital from January 2017 to October 2022 were screened for eligibility. The diagnosis of T1D was made independently by two endocrinologists based on clinical manifestations: diabetes ketoacidosis at the onset of disease, initiation of insulin therapy within 6 months of diagnosis and continued thereafter, or positive diabetes autoantibody [islet cell autoantibody (ICA)/insulin autoantibody (IAA)/glutamic acid decarboxylase (GAD) autoantibody]. Moreover, individuals with fasting C-peptide >1,500 pmol/L were excluded to limit the possibility of including people with diagnoses other than T1D (N = 4). Sixty-six participants lacking the data of C-peptide and 27 participants lacking the information of diabetes duration were excluded. Cross-sectional analysis were performed in the remained 534 participants. Of the participants, 174 people who returned to the clinic and had regular β-cell function assessments were included in the longitudinal analysis to determine the change in C-peptide secretion over the course of the disease. Meanwhile, 178 participants who wore professional isCGM were also included for analysis of glucose control according to C-peptide levels (Figure 1).

The study was conducted in accordance with the ethical principles in the Declaration of Helsinki and was approved by the Peking University People's Hospital Ethics Committee (2022PHB407-001). Informed consent was obtained from all participants.

Physical and laboratory measurements

Blood samples were taken in the morning after an 8-h to 10-h fast, and a mixed meal tolerance test (MMTT) was performed (9). During the MMTT, participants consumed a standardized breakfast calculated on the basis of total caloric requirements (25%–30% of daily caloric intake; 50% of calories as carbohydrates, 33% of calories as lipids, and 17% of calories as proteins). Glucose, low-density lipoprotein cholesterol (LDL-C), triglycerides, and uric acid were measured using an automated biochemistry analyzer. HbA_{1c} was measured by high-performance liquid chromatography (Primus Ultra 2, Trinity Biotech, Bray, Co-Wicklow, Ireland). Insulin and C-peptide were assayed by electrochemiluminescence immunoassay on a Roche autoanalyzer (Cobas e601, Germany) using Elecsys C-Peptide (Roche Diagnostics GmbH, Mannheim, Germany). The inter-assay CVs for the low–, medium–, and high–C-peptide controls were 3.4%, 2.6%, and 1.8%, respectively.

Professional isCGM

isCGM was placed at clinic by care givers. A professional CGM (Freestyle Libre H, Abbott, US) was used to collect glucose data every 15 min for 14 days. The glucose metrics were calculated using data from 174 participants who had sensor activation over 90% during the 14 days period. Standard deviation (SD), mean glucose (MG), coefficient of variance (CV), interquartile range (IQR), mean amplitude of glucose excursions (MAGE), time below range (TBR), time above range (TAR), and time in range (TIR) were calculated according to isCGM data.

Statistical analyses

Unless explicitly stated otherwise, statistical analysis for this study was performed as follows. Continuous variables that followed a normal distribution were expressed as mean ± SD, whereas non-

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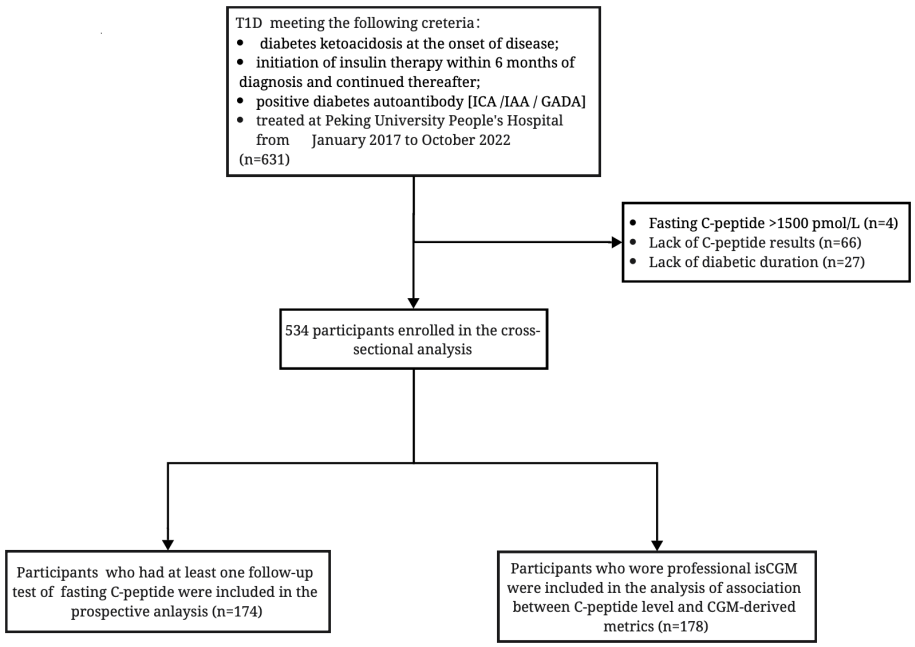

FIGURE 1
Inclusion flowchart of 534 participants with diabetic duration information and fasting C-peptide data; 174 participants had a at least one follow-up test of fasting C-peptide test; 178 participants had CGM-derived data. ICA, islet cell autoantibody; IAA, insulin autoantibody; GADA, glutamic acid decarboxylase autoantibody.

normally distributed variables were presented as median with IQR. Categorical variables were reported as proportions.

Participants were divided into a low–C-peptide group (fasting C-peptide ≤10 pmol/L) and a preserved–C-peptide group (fasting C-peptide >10 pmol/L). One-way ANOVA and Mann–Whitney U-tests were used to compare continuous variables between the two cohorts, depending on the distribution of the variables. Chi-squared tests were used for categorical variables.

Factors identified in the univariate analysis, including age at diagnosis, duration of diabetes, body mass index (BMI), positive GAD autoantibody, estimated glomerular filtration rate (eGFR), daily insulin dose, and HbA1c category were then examined using binary logistic regression analysis. In the longitudinal cohort, the change in C-peptide levels from baseline to last follow-up (ΔC-peptide $_{\text{last follow-up - baseline}}$) was used to define individuals with sustained and failed β-cell function. Cox regression analysis was also performed to determine the influence of age at diagnosis, duration of diabetes, HbA_{1c}, and positive GAD autoantibodies on β-cell function. Diabetes duration was adjusted in the logistic model to assess the association between C-peptide level and CGM metric.

Statistical analysis was performed using SPSS software (version 26), and a p-value<0.05 was considered statistically significant. R (version 4.3.2) and GraphPad Prism (version 9.3.1) were used to generate the figures.

Results

A total of 534 people were included in the study, 46.1% of whom were men. The average age of the participants was 50 years, and the average duration of diabetes was 9 years. The average HbA_{1c} level of the participants was 8.9%, and 21.9% of the participants were under euglycemic control (HbA_{1c} ≤7%).

Preserved C-peptide was common even with long duration of diabetes

Of the participants, 55.4% still had preserved C-peptide (fasting C-peptide >10 pmol/L). Among those who had diabetes for more than 20 years (n = 131), 38.9% still had detectable C-peptide levels (fasting C-peptide >3 pmol/L, Supplementary Figure 1). Fasting C-peptide levels decreased with diabetes duration, and the fitted curve suggested a non-linear association between C-peptide and disease duration (Supplementary Figure 2).

C-peptide levels independently associated with diabetes duration and positive GAD autoantibody

Participants in the preserved–C-peptide group were younger [48 (IQR, 34–61) vs. 54 (IQR, 38–64) years, P = 0.002], had a shorter diabetes duration [4.0 (IQR, 0.7–12.0) vs. 15.0 (IQR, 7.0–32.0) years, P <0.001], and had a lower insulin dose [29.4 (IQR, 20.0–40.0) vs. 36.0 (IQR, 28.3–46.0) U/d, P <0.001] compared with those in the low–C-peptide group. Meanwhile, BMI was lower in the preserved–C-peptide group than that in the low–C-peptide group (22.5 ± 3.4 vs. 23.1± 3.2 kg/m^2, P = 0.027). Positive GAD autoantibody was detected in 71.9% of participants in the preserved

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C-peptide and 42.9% in the low–C-peptide group (P <0.001). The eGFR was also higher in the preserved–C-peptide group [110.7 (IQR, 99.2–124.7) vs. 104.2 (IQR, 92.4–116.9) ml/min * 1.73 m^2, P< 0.001]. In addition, the rates of diabetic retinopathy and carotid plaque were lower in the preserved–C-peptide group (24.1% vs. 39.9%, P = 0.003; 49.3% vs. 62.9%, P = 0.010) Table 1.

After adjustment for age at diagnosis, duration of diabetes, BMI, GAD autoantibodies, eGFR, and HbA_{1c}, three factors including duration of diabetes, GAD autoantibodies, and daily insulin dosage were still associated with lack of preserved C-peptide [OR 1.10 (IQR, 1.06–1.14); OR, 0.46 (IQR, 0.27–0.77); OR, 1.04 (IQR, 1.02–1.06)] Table 2.

Sustained β-cell function associated with diabetes duration in the longitudinal cohort

The longitudinal analysis included 174 participants who had at least one follow-up visit with a fasting C-peptide test. The median follow-up was 2.0 years. Supplementary Figure 3A shown that β-cell function declined with increasing duration of diabetes. The proportions of participants with C-peptide >10 pmol/L were 71.4%, 56.8%, 71.7%, 62.5%, and 22.2% at baseline, < 1 year, 1 to 2 years, 2 to 3 years, 3 to 4 years, and 4 to 5 years follow-up, respectively (Supplementary Figure 3B). We divided these participants into two cohorts: those with failed β-cell function (ΔC-peptide $_{\text{the last follow-up - baseline}} \leq 0$) and those with sustained β-cell function (ΔC-peptide $_{\text{the last follow-up - baseline}} > 0$). Cox regression analysis showed that duration of diabetes was independently associated with sustained β-cell function (Supplementary Table 1).

Preserved C-peptide was associated with higher TIR

Mean glucose was lower in the preserved–C-peptide group compared with that in the low–C-peptide group [8.4 (IQR, 7.0–10.2) vs. 9.9 (IQR, 8.3–11.7) mmol/L, P <0.001]. In addition, TIR was

TABLE 1 Characteristics of the study participants according to the serum C-peptide level.

Characteristic	Total N = 534	Low C-peptide ≤ 10 pmol/L N = 238	Preserved C-peptide > 10 pmol/L N = 296	*P*
Age, years	50 (35, 62)	54 (38, 64)	48 (34, 61)	0.002
Male, n (%)	246 (46.1)	101 (42.4)	145 (49.0)	0.138
Smoking, n (%)	130 (30.2)	54 (28.0)	76 (31.9)	0.400
Age at diagnosis, years	36 (22, 50)	30 (17, 47)	38 (25, 52)	<0.001
Duration of diabetes, years	9.0 (2.0, 20.0)	15.0 (7.0, 32.0)	4.0 (0.7, 12.0)	<0.001
Weight, kg	59.5 (53.5, 67.6)	59.9 (54.1, 67.8)	59.3 (53.0, 67.5)	0.326
Body mass index, kg/m^2	22.8 (3.3)	23.1 (3.2)	22.5 (3.4)	0.027
Waist-to-hip ratio	0.88 (0.83, 0.92)	0.87 (0.82, 0.92)	0.89 (0.83, 0.93)	0.084
IAA antibody positivity, n (%)	67 (14.3)	35 (16.7)	32 (12.5)	0.232
ICA antibody positivity, n (%)	13 (2.8)	7 (3.4)	6 (2.3)	0.574
GAD antibody positivity, n (%)	294 (59.2)	94 (42.9)	200 (71.9)	<0.001
HbA_{1c}, n (%)				0.008
≤7%	115 (21.9)	63 (27.4)	52 (17.6)	
>7%	411 (78.1)	167 (72.6)	244 (82.4)	
SBP, mmHg	127 (116, 140)	128 (118, 140)	126 (113, 140)	0.264
DBP, mmHg	72 (66, 80)	72 (65, 80)	73 (66, 82)	0.120
TG, mmol/L	0.9 (0.7, 1.2)	0.9 (0.7, 1.2)	0.9 (0.7, 1.2)	0.951
LDL-C, mmol/L	2.5 (2.1, 3.1)	2.5 (2.1, 3.1)	2.5 (2.0, 3.1)	0.668
Urine microalbumin/creatinine ratio, mg/g	6.3 (3.1, 16.3)	7.6 (3.0, 32.3)	5.7 (3.2, 13.3)	0.065
eGFR, ml/min * 1.73m^2	107.7 (95.9, 122.2)	104.2 (92.4, 116.9)	110.7 (99.2, 124.7)	<0.001
Fasting plasma glucose, mmol/L	9.3 (6.3, 13.4)	10.1 (6.4, 14.8)	9.0 (6.3, 12.6)	0.026

(Continued)

TABLE 1 Continued

Characteristic	Total N = 534	Low C-peptide ≤ 10 pmol/L N = 238	Preserved C-peptide > 10 pmol/L N = 296	*P*
MMTT stimulated glucose, mmol/L	13.0 (5.2)	13.4 (5.7)	12.8 (4.8)	0.233
Fasting insulin, μIU/mL	2.9 (1.4, 6.7)	2.0 (0.7, 4.1)	3.6 (1.8, 8.0)	<0.001
Postprandial insulin, μIU/mL	3.9 (1.7, 15.0)	1.7 (0.7, 3.8)	8.1 (3.1, 21.1)	<0.001
MMTT stimulated C-peptide, pmol/L	70 (0, 350)	0 (0, 10)	25 (9, 540)	<0.001
Hypertension, n (%)	129 (29.9)	66 (34.2)	63 (26.5)	0.091
Diabetic retinopathy, n (%)	102 (30.6)	55 (39.9)	47 (24.1)	0.003
Carotid plaque, n (%)	213 (55.3)	110 (62.9)	103 (49.3)	0.010
Daily insulin dosage, U/d	33.0 (24.0, 42.0)	36.0 (28.3, 46.0)	29.4 (20.0, 40.0)	<0.001

Data are mean ± SD or median (IQR) unless otherwise indicated. IAA, insulin autoantibody; ICA, islet cell autoantibody; GAD autoantibody, glutamic acid decarboxylase autoantibody; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; MMTT, mixed meal tolerance test.

higher, and TAR was lower in the preserved–C-peptide group [62.4 (IQR, 47.3–76.6) vs. 50.3 (IQR, 36.2–63.0) %, P <0.001; 27.4 (IQR, 14.0–49.3) vs. 44.4 (IQR, 31.0–62.5), P <0.001). Glucose metrics indicating variability, including SD, IQR, and MAGE, were lower in the preserved–C-peptide group [3.1 ±.9 vs.3.8 ±.9 mmol/L, P <0.001; 4.2 ± 1.4 vs. 5.4 ± 1.5 mmol/L, P < 0.001; 7.1 (IQR, 4.2–13.3) vs. 10.8 (IQR, 4.9–16.3), P = 0.029]. After adjustment of diabetes duration, preserved C-peptide was still associated with higher TIR and lower TAR, SD, and IQR (P =0.003, P =0.003, P =0.03, P <0.001, and P <0.001) (Figure 2; Supplementary Table 2).

Conclusions

Our study showed that preserved C-peptide secretion was common in Chinese individuals with T1D and was associated with diabetes duration, positive GAD autoantibody, and insulin dosage. Meanwhile, preserved C-peptide was also associated with favorable glycemic control as represented by TIR.

Persistent C-peptide secretion, reflecting some degree of intrinsic β-cell function, is now recognized to be common in T1D (5, 8, 10). In the Joslin Medalist Study, residual C-peptide secretion was detected in a large proportion of Medalists, even after more than 50 years of follow-up (9). However, such studies were mainly conducted in Caucasian populations, and few studies have focused on Chinese, with the currently available studies having relatively short diabetes duration or small populations. In a cohort of 446 participants with T1D with a mean duration of 2.36 years, more than 80% of them had detectable C-peptide, but the percentage decreased rapidly with disease progression (11). In another study of 109 participants with T1D followed for at least 10 years, Cheng et al. showed that 38.5% of participants had detectable C-peptide secretion (random C-peptide ≥16.7 pmol/L) (12). Miao and colleagues reported that, in 443 participants with T1D for 2.38 years, stimulated C-peptide ≥200 pmol/L was detected in 64.3% of participants (13). To our knowledge, our study was the largest with a relatively long duration of diabetes in the Chinese population with T1D and suggested that more than half of them still had preserved insulin secretion.

TABLE 2 Variables independently associated with the preservation of C-peptide secretion.

	OR (95% CI)	*P*
Age at diagnosis	0.99 (0.97 to 1.01)	0.288
Duration of diabetes	1.10 (1.06 to 1.14)	<0.001
BMI	0.99 (0.92 to 1.07)	0.789
GAD autoantibody	0.46 (0.27 to 0.77)	0.003
eGFR	0.99 (0.98 to 1.00)	0.147
Daily insulin dosage	1.04 (1.02 to 1.06)	<0.001
HbA_{1c} > 7%	0.54 (0.26 to 1.14)	0.107

BMI, body mass index; GAD autoantibody, glutamic acid decarboxylase autoantibody; eGFR, estimated glomerular filtration rate.

Previous studies have suggested that age at diagnosis, duration of diabetes, autoantibody positivity, and Human Leukocyte antigen (HLA) genotype may influence serum C-peptide level (10, 14, 15). Our results were consistent with the previous studies that diabetes duration was negatively associated with residual β-cell function and that autoantibody positivity was correlated with sustained intrinsic insulin production (1, 13, 16). The relationship between longer disease duration and lower C-peptide is widely recognized according to previous studies, whereas the finding of a strong relationship between higher autoantibody levels and higher C-peptide levels is difficult to interpret. Autoantibodies are generally good predictors of disease onset but are not specific for disease outcome (17, 18). Our previous findings showed that 17.1% of Chinese patients with T1D with long duration of diabetes were with GAD autoantibody positive, and 14.7% had fasted serum C-peptide higher than 75 pmol/L (19). Further investigation of GAD autoantibody is clearly required. Meanwhile, our study suggested that residual β-cell function was associated with lower daily insulin dose, which was in line with previous studies (11, 20–23). Because C-peptide levels represent intrinsic β-cell function (24), a possible explanation is that participants with higher C-peptide levels had

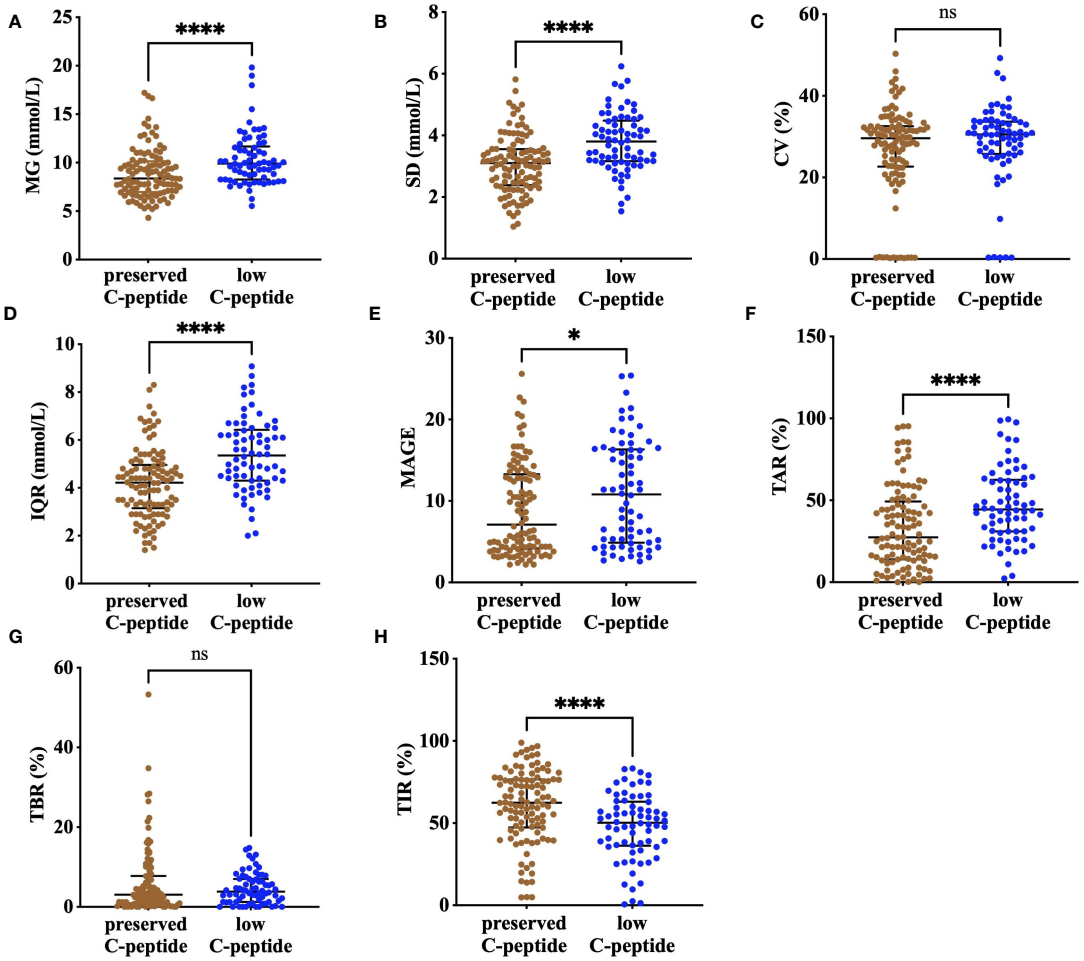

FIGURE 2
The comparison of CGM metrics, including MG **(A)**, SD **(B)**, CV **(C)**, IQR **(D)**, MAGE **(E)**, TAR **(F)**, TBR **(G)**, and TIR **(H)**, between the preserved–C-peptide group and the low–C-peptide group. **** means P<0.001, * means P<0.05, and ns means non-significant; MG, mean glucose; CV, coefficient of variance; IQR, interquartile range; SD, standard deviation; MAGE, mean amplitude of glycemia excursions; TBR, time below range (glucose concentrations below 3.9 mmol/L); TIR, time in range (glucose concentrations of 3.9–10.0 mmol/L); TAR, time above range (glucose concentrations over 10.0 mmol/L).

more endogenous insulin production and required lower doses of exogenous insulin. As accumulating evidence suggests that preserved C-peptide is associated with a lower likelihood of diabetes microvascular complications (5, 10, 25), the association between autoantibody positivity, residual β-cell function, and favorable diabetes outcomes should be further investigated and the underlying mechanisms explored.

Understanding how the residual β-cell function relates to the heterogeneity of glycemic control is important for people with diabetes and their clinicians. Moreover, a more personalized approach to diabetes care may be possible with a better understanding of the contribution of residual β-cell function to CGM-derived metrics such as TBR, TIR, TAR, and CV. Previous studies have investigated the impact of residual insulin secretion in T1D, as measured by the MMTT, on the maintenance of glycemic control, as measured by HbA_{1c} (20, 26, 27). Previous studies in Caucasian populations have investigated the association between residual β-cell function and TIR. Researchers found that, in the T1D Exchange participants, fasting C-peptide was correlated with higher TIR (28). In addition, in a recent study recruiting participants from The Netherlands, Coco et al. suggested that residual insulin secretion, as measured by urinary C-peptide to creatinine ratio, was associated with longer TIR, shorter TBR and TAR, and lower CV (23). Although a study conducted in Chinese patients with diabetes including T1D, type 2 diabetes, and latent autoimmune diabetes in adults showed a continuous spectrum of glycemic variability pattern (29), no large-scale study focusing on T1D population in Chinese has been reported. Our study provided a relatively large sample size covering the entire duration of T1D in Chinese and showed that residual β-cell function was associated with TIR after adjustment for potential confounders.

This study had several limitations. First, the cross-sectional design made it impossible to establish causality. However, it is most likely that preserved β-cell function has a positive effect on glycemic control and not vice versa, as recent studies have shown that tight glycemic control, even with an artificial pancreas, does not preserve β-cell function even in newly diagnosed T1D subjects (30, 31). Second, although fasted serum may be a good representation of β-

cell function, it is not considered the gold standard for measuring β-cell function. Therefore, we cannot exclude the possibility that our study underestimates the contribution of β-cell function to glycemic control. Third, our study was a single-center study, and the number of young patients with T1D was limited; we are planning on elaborate with some specialized children's hospitals in the future study. Finally, as the CGM data were not blinded to the participants, other important confounders related to glycemic control, such as diabetes management skills, emotional factors could also contribute to the individual's CGM metrics. However, as we used professional CGM in the study and all participants received standard T1D care from our specialists, the impact of individual procedures was minimized. Nevertheless, we point out that this observation further supports the concept that β-cell function contributes to better daily control, as we found strong and consistent associations with both TIR and TAR.

In conclusion, residual β-cell function was common in people with T1D, and preservation of C-peptide secretion was associated with shorter duration, positive GAD autoantibody, and lower insulin dosage. As glucose control measured by CGM is at least partly influenced by residual β-cell function, personalized glucose targets should be considered on the basis of individual C-peptide level. Furthermore, disease-modifying therapies aiming to preserve β-cell function should also be considered in the future.

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 humans were approved by Peking University People's Hospital Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by-product of routine care or industry. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

WL: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. YF: Data curation, Formal analysis, Visualization, Writing – original draft. XC: Conceptualization, Methodology, Writing – review & editing. YZ: Investigation, Writing – review & editing. MZ: Investigation, Writing – review & editing. XH: Investigation, Writing – review & editing. JL: Investigation, Writing – review & editing. SY: Investigation, Writing – review & editing. DC: Software, Writing – review & editing. JC: Software, Writing – review & editing. LW: Software, Writing – review & editing. DS: Software, Writing – review & editing. LJ: Conceptualization, Methodology, Supervision, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1335913/full#supplementary-material

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Prediction of personalised postprandial glycaemic response in type 1 diabetes mellitus

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Objectives: Patients with type 1 diabetes (T1D) face unique challenges in glycaemic control due to the complexity and uniqueness of the dietary structure in China, especially in terms of postprandial glycaemic response (PPGR). This study aimed to establish a personalized model for predicting PPGR in patients with T1D.

Materials and methods: Data provided by the First People's Hospital of Yunnan Province, 13 patients with T1D, were recruited and provided with an intervention for at least two weeks. All patients were asked to wear a continuous glucose monitoring (CGM) device under free-living conditions during the study period. To tackle the challenge of incomplete data from wearable devices for CGM measurements, the GAIN method was used in this paper to achieve a more rational interpolation process. In this study, patients' PPGRs were calculated, and a LightGBM prediction model was constructed based on a Bayesian hyperparameter optimisation algorithm and a random search algorithm, which integrated glucose measurement, insulin dose, dietary nutrient content, blood measurement and anthropometry as inputs.

Results: The experimental outcomes revealed that the PPGR prediction model presented in this paper demonstrated superior accuracy (R=0.63) compared to both the carbohydrate content only model (R=0.14) and the baseline model emulating the standard of care for insulin administration (R=0.43). In addition, the interpretation of the model using the SHAP method showed that blood glucose levels at meals and blood glucose trends 30 minutes before meals were the most important features of the model.

Conclusion: The proposed model offers a heightened precision in predicting PPGR in patients with T1D, so it can better guide the diet plan and insulin intake dose of patients with T1D.

KEYWORDS

type 1 diabetes, postprandial glycaemic response, personalized nutrition, continuous glucose monitors, dietary nutrients

1 Introduction

Diabetes is a metabolic disorder that causes abnormal regulation of blood glucose, if not managed properly, it can lead to short- and long-term health complications and even death (1). At the present time, there is no cure for diabetes. However, self-management of the disease, particularly keeping blood glucose levels within the recommended range, is central to treatment. This includes actively tracking blood glucose levels, managing physical activity, diet and insulin intake (2).

The postprandial glycaemic response (PPGR) has a very important impact on overall glycaemic control and is a difficult aspect of T1D glycaemic control (3). Optimally dosing insulin at each meal presents a significant challenge in disease management. Accurately determining the appropriate insulin dosage is critical for regulating blood glucose levels and avoiding both hyperglycaemia and hypoglycaemia (4). In previous studies, researchers have typically used carbohydrates and insulin doses to predict blood glucose concentrations. However, the predictive accuracy of these models varies from person to person (5, 6). In addition to the nutritional content characteristics of the food consumed, changes in blood glucose may also arise from preprandial blood glucose, the patient's lifestyle, and their clinical data. Mendes et al. (7) tested the efficacy of a prediction model for personalised postprandial glycaemic response developed using an Israeli cohort, which took into account characteristics such as food composition, blood, and lifestyle when applied to individuals in the Midwestern U.S. The results of the study demonstrated that the precision prediction method was more accurate in predicting blood glucose levels than the traditional method, which relied solely on the energy and carbohydrates in food. Thus, the most successful strategy for controlling blood glucose concentrations depends on the characteristics of each individual.

Eating habits are strongly influenced by ethnicity and region. For example, the Chinese have a very complex diet (8). A large number of current postprandial glucose prediction models for type 1 diabetes are based on Western dietary structures. Due to the complexity and uniqueness of the dietary structure, postprandial glycaemic control in Chinese patients with T1D faces unique challenges.

Therefore, the aim of this study was to construct a personalised model for predicting PPGR applicable to patients with T1D by collecting data on insulin dose, nutrient content of diet and additional clinical indicators from 13 patients with T1D in Kunming, Yunnan Province, in order to better guide the dietary plan as well as the dose of insulin intake in patients with T1D.

2 Materials and methods

2.1 Research object

This study used data provided by the First People's Hospital of Yunnan Province for the period from September 2023 to January 2024. Thirteen patients with T1D (10 females and 3 males) were recruited in Kunming, Yunnan Province, and an intervention lasting at least two weeks was provided to each patient. Following were the criteria for inclusion (1): aged 18 years or older. (2) Diagnosed with diabetes for more than 1 year. Participation in the study was excluded if the participant was suffering from active inflammatory, neoplastic disease, pregnancy or a history of antibiotic use in the three months before participation in the study, chemotherapy or radiotherapy in the past 2 months, chronic gastrointestinal disease, and chronic anaemia.

During the study period, all participating patients agreed to wear the SIBIONICS GS1 CGM continuous glucose monitoring device, which uses a subcutaneous sensor to measure blood glucose levels at five-minute intervals, under free-living conditions. The SIBIONICS GS1 Continuous Glucose Monitoring (CGM) System is a 14-day calibration-free RT-CGM that supports data sharing with caregivers and seamlessly integrates with the advanced ProView Remote Access Platform, enabling healthcare providers to monitor patients remotely. Clinical evaluations and user feedback have demonstrated excellent accuracy, with the GS1 CGM achieving a Mean Absolute Relative Difference (MARD) of 8.83%, a key measure of glucose monitor accuracy (lower MARD values indicate higher accuracy). In addition, the GS1 CGM has been tested in a variety of environments, including with over 1,600 hospitals, and has been used by over 600,000 users (9, 10).

Prior to wearing the continuous glucose monitoring device, medical staff collected comprehensive information from each patient, including anthropometric measurements (e.g., height, weight), a set of blood tests, and lifestyle and basic information questionnaires (gender, age, etc.). Patients were requested to adhere to their usual daily routines and dietary patterns, reporting their dietary intake for breakfast, lunch and dinner to physician on a daily basis in real-time. The weight of each meal was weighed by the patients themselves and then registered by the physician, and a mobile app - Sugar Sugar Circle's food bank of foods was used to measure carbohydrate, protein and fat content. Sugar Sugar Circle is a mobile app for blood sugar self-management and peer support for people with type 1 diabetes. It provides a food bank of up to more than 300,000 food items, which is very much in line with Chinese dietary habits, and allows for quick access to nutrient information for the food you want to find, as well as quick calculation of nutrient content using a weight scale. The physician must accurately document the specific nutritional components and timing details of patients' meals. Reported meal times were rounded to the nearest 5-minute interval. To improve compliance, patients were told that accurate recording was essential to obtain an accurate analysis of the PPGR of foods. Insulin was manually infused by the physicians before the patients' meals and the exact insulin dose was recorded.

The following filtering measures were applied to all meals recorded in this study: 1) To mitigate the potential impact of neighbouring meals and their antecedent insulin dosages, other meals recorded within 90 minutes were excluded from the analysis. Many studies have shown that the effects of mealtime insulin on insulin levels in subjects usually gradually return to basal levels within approximately 90-120 minutes after eating a meal. For example, the study by Hayashi et al. (11) details that in the oral glucose tolerance test (OGTT), insulin concentrations typically peak 30 to 60 minutes after glucose intake and approach basal levels 90 to 120 minutes after the meal. Shankar et al. (12) used the Mixed Meal Tolerance Test (MMTT) to study insulin levels and showed that insulin levels returned to basal levels within 90 to 120 minutes after a meal.

Therefore, to ensure that the effect observed was that of a single meal alone and not the result of multiple meals superimposed on each other, we chose 90 minutes as a threshold that would allow for a better separation of the effects of taking insulin between meals. 2) Incomplete meal records were deleted. 3) Records of meals with a carbohydrate content of greater than 200 grams were deleted. According to the recommendations of the Institute of Medicine of the National Academy of Sciences (13), carbohydrates should account for 45-65 per cent of total daily calories, so an intake of 200 grams of carbohydrates per meal is considered abnormally high, and these outliers can have an asymmetric effect on the overall analysis, leading to distorted results.

2.2 Data pre-processing

Dealing with missing data presents a significant obstacle in analysing information gathered from wearable devices, frequently stemming from incorrect or delayed usage. Statistical imputation, matrix decomposition, and machine learning algorithms are among the frequently used computational techniques for addressing the challenge of incomplete data. However, these approaches often fail to adequately capture the temporal fluctuations inherent in time series data, leading to occasional interpolation outcomes that may appear unreasonable (14).

GAIN (Generative Adversarial Imputation Networks) is a generative adversarial network (GAN) approach for processing missing data (15).The GAIN framework consists of a generator and a discriminator. In GAIN, the generator fills the data and the discriminator distinguishes between real and generated data. The discriminator aims to minimize classification errors, while the generator seeks to maximize the discriminator's error. Consequently, both networks undergo training through an adversarial process. To ensure that the adversarial training achieves the desired goal, GAIN assists the discriminator with a hint mechanism that ensures that the generator generates samples according to the distribution of the real data (Figure 1).

Generator: The generator G receives input consisting of a data matrix, a random matrix, and a mask matrix. The data matrix contains known data, but may also have missing values. The random matrix exclusively contains missing data and is populated with random values at the missing positions. The mask matrix is used to mark the positions of the missing values in the data matrix. Then, the generation process can be represented as follows:

$$\bar{X} = G(\tilde{X}, M, (1 - M) \odot Z)$$

where $\odot$ represents the multiplication at the element level, $\bar{X}$ represents the output matrix, M represents the mask matrix, $\tilde{X}$ represents the data matrix, and Z represents the random matrix. This configuration closely resembles a typical GAN, with Z resembling the noise variables introduced in that structure.

Discriminator: In the GAIN framework, a discriminator D is introduced to continually counter the generator G. Nevertheless, in contrast to conventional GANs, the generator's output comprises both genuine and spurious elements. The goal of the discriminator is not to identify the truth of the whole vector, but to identify which components of the vector are real and which are fake (i.e. interpolated).

Hint: The hint mechanism is intended to specify the positions of both the true and generated values, enhancing control over the direction of interpolation adjustments.

The Mean Absolute Error (MAE) criterion in this paper is adopted to assess the accuracy of the estimated values in relation to the actual values:

$$MAE = \min_{f} \sum_{(i,j)\epsilon M} \frac{\left|\hat{x}_{ij} - \bar{x}_{ij}\right|}{\|M\|}$$

2.3 Prediction of postprandial glycaemic response

In order to measure the effect of the meal on blood glucose, two metrics (PPGR and Glu_{max}) were calculated in this study (16).

Firstly, according to the method of Zeevi et al. (17), the PPGR for every meal was computed by integrating meal times using CGM data and determining the incremental area under the curve (iAUC)

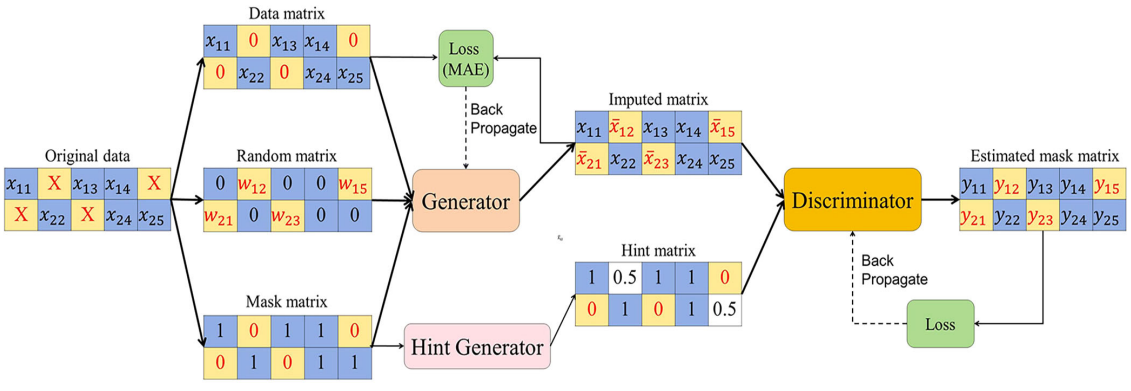

FIGURE 1
Process of missing data imputation using GAIN.

of the blood glucose curve within a 2-hour postprandial window. The median of the blood glucose values during the first 30 minutes of the meal was taken as the initial blood glucose concentration. This initial concentration will be used as the reference value for calculating the incremental area under the curve. The final result of this procedure is the PPGR per meal based on the calculated incremental area under the curve and the initial blood glucose concentration:

$$PPGR = \frac{\sum_{i=1}^{n} \frac{h_i}{2} \cdot (y_{i-1} + y_i - 2y_0)}{y_0}$$

where n represents the number of time points, h_i represents the time interval between two adjacent time points, y_{i-1} and y_i are the blood glucose measurements at two adjacent time points, and y_0 represents the initial blood glucose concentration.

Second, the variance in blood glucose levels at mealtime and the maximum blood glucose level within 2 h after the meal (Glu_{max}) was calculated. This metric was selected due to its reduced sensitivity to inaccuracies in patients' logging times:

$$Glu_{\max} = \max_{i=1}^{n} y_i - y_0$$

In order to predict these two metrics (PPGR and Glu_{max}), a LightGBM prediction model based on Bayesian hyperparameter optimisation algorithm combined with stochastic search algorithm was constructed in this paper. Model inputs consisted of 38 features in total, encompassing features such as meal composition and blood test outcomes, blood glucose measurements and insulin doses. 60% of the meals were utilized for training the model, while the remaining 40% were reserved for validation purposes.

The experiment was conducted on a computer with Windows 11 operating system. The simulation platform is Pycharm and is programmed using Python with sklearn, pandas and numpy libraries.

2.3.1 LightGBM model

The primary concept behind GBDT (Gradient Boosting Decision Tree) is to iteratively train using a weak classifier (decision tree) to obtain an optimal model, while LightGBM optimises the traditional GBDT algorithm as follows (18): histogram algorithm, gradient-based one-sided gradient sampling (GOSS), exclusive feature bundling (EFB), and leaf-wise growth strategy with depth constraints.

The basic idea of the histogram algorithm is to first discretise the continuous floating-point eigenvalues intointegers, and at the same time construct a histogram with a width of. When traversing the data, statistics are accumulated in the histogram based on the discrete values as indexes, when traversing the data once, the histogram accumulates the required statistics and then traverses to find the optimal segmentation point based on the discrete values of the histogram.

The basic idea of GOSS (gradient-based one-side sampling) is to calculate the gradient of the samples and then keep only the samples with larger gradient. This reduces the number of trainings samples and improves the training efficiency while maintaining similar information. The set of samples for GOSS sampling is N, and the threshold of gradient is α, then the sampling process is as follows:

$$N = \{i||n_i| > \alpha\}$$

where n_i is the gradient of the sample i.

The basic idea of the EFB (exclusive feature bundling) algorithm is to reduce the number of features and improve the generalisation ability of the model by bundling the features and merging the highly correlated features into one feature group.

The leaf-wise algorithm with depth constraints aims to reduce the complexity of the model and improve the training efficiency by controlling the depth of the tree and the number of leaf nodes (Figure 2). LightGBM firstly divides the dataset into different histograms according to the range of values of the features. Such a division can speed up the training process because the histograms can replace the original data in decision tree learning, reducing memory and computation. During each tree growth, instead of splitting based on nodes, the tree is split based on leaf nodes to find the leaf node with the maximum splitting gain among all current leaf nodes. Such a leaf splitting strategy reduces the risk of overfitting and improves model generalisation.

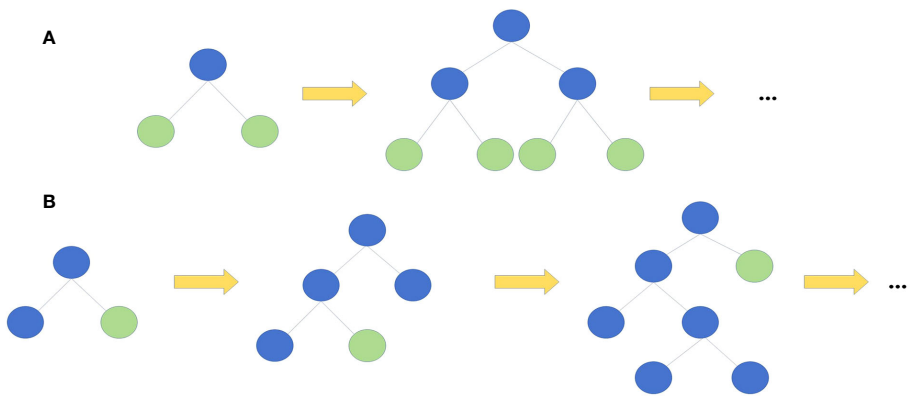

FIGURE 2
Two kinds of tree growth strategy. **(A)** Level-wise growth strategy **(B)** Leaf-wise growth strategy. ..., and so forth.

In LightGBM, the objective function consists of two parts, one is a measure of the fit to the training data and the other is a measure of the model complexity to avoid overfitting. The objective function can be represented like this:

$$Obj^{(k)} = \sum_{t=1}^{n} l(y_t, y_t^{(k)}) + \sum_{t=1}^{T} \Omega(f_t)$$

$$= \sum_{t=1}^{n} l(y_t, y_t^{(k-1)} + f_k(x_t)) + \sum_{t=1}^{T} \Omega(f_t)$$

where, k indicates the overall count of iterations, n represents the quantity of training samples, y_t is the true value of the t th training sample, $y_t^{(k)}$ is the predicted value of the t th training sample, $l(y_t, y_t^{(k)})$ is the loss function, $f_k(x_t)$ is the anticipated impact of the decision tree to the t th training sample x_t in the k th iteration, and $\Omega(f_t)$ is the regularisation term.

In each iteration, the goal of the model is to minimise the aggregate loss across all training samples by finding a new decision tree, while also considering the model's complexity aimed at mitigating overfitting. When adding a new decision tree, the model considers a combination of loss functions and regularisation terms to minimise the loss of training data while maintaining the model's ability to fit.

2.3.2 Bayesian hyperparametric optimisation algorithm

In order to improve the accuracy of the LightGBM prediction model, in this paper, a Bayesian hyperparameter optimisation algorithm combined with a stochastic search algorithm is used to automatically search for the optimal parameter configurations of the model. Hyperopt is one of the Bayesian optimisation libraries in Python, which uses an optimisation algorithm called Tree Parzen Estimation (TPE) (19). The core idea of TPE is to use the information about the parameter combinations that have been explored to dynamically adjust the parameter search space for the next iteration, so that better hyperparameter combinations can be found within a limited number of iterations. By transforming the generative process that describes the configuration space X, the TPE model $p(x|y)$ replaces the distribution of *a priori* configurations with non-parametric densities. Each iteration of TPE not only scales linearly according to the number of samples, but also optimises the number of dimensions in the parameter space by maintaining the ordering of the observed variables.

$$p(x|y) = \begin{cases} \ell(x) \text{ if } y < y^* \\ g(x) \text{ if } y \geq y^* \end{cases}$$

where $\ell(x)$ and $g(x)$ denote the observations and the rest of the observations.

When using Hyperopt for hyperparameter optimisation, a new approach is used where the data is first randomly sampled. The core idea of this approach is that since the sample is representative of the entire population, a sample can be used instead of the entire training dataset, and then Hyperopt is used to generate the optimal hyperparameters for LightGBM, an approach that greatly reduces the execution time required to generate the optimal hyperparameters (20).

Bayesian hyperparameter optimisation using Hyperopt is performed by combining a random search algorithm with a set of hyperparameters randomly selected from the search space to try in each iteration. By randomly sampling a set of hyperparameters in the hyperparameter space, their performance is evaluated and then the best performing set of hyperparameters is selected. This helps to avoid falling into a local optimal solution, thus enabling a more global search.

The set of hyperparameters for this study includes the following: learning_rate is used to control the magnitude of the update at each step, a smaller learning rate makes the model converge more slowly but may result in better generalisation performance; n_estimators specifies the number of weak learners, i.e. the number of decision trees to be trained; max_depth is the maximum depth of each tree, which controls the tree's complexity, a larger depth may lead to model overfitting; colsample_bytree is the proportion of features used in each tree, which controls the proportion of features randomly selected in constructing each tree and prevents overfitting; min_child_samples is the minimum number of samples required for each leaf node, which prevents overfitting; num_leaves is the number of maximum number of leaf nodes per tree; subsample is the proportion of samples used per tree, which controls the proportion of samples randomly selected during training of each tree and prevents overfitting.

2.3.3 LightGBM prediction model based on Bayesian hyperparameter optimisation algorithm combined with stochastic search algorithm

In this paper, a Bayesian hyperparameter optimisation algorithm combined with a stochastic search algorithm is used to optimise the LightGBM model and develop a model to predict PPGR in patients with T1D. The specific experimental procedure is shown in Figure 3.

Taken together, as described in Sections 2.3.2 and 2.3.2, the LightGBM model combining Bayesian hyperparameter optimisation algorithm and stochastic search algorithm has the advantages of high efficiency, adaptive and global optimisation, which can effectively improve the performance and generalisation of the model, and it can predict the patients' PPGRs more efficiently and accurately.

2.4 Feature attributions

To further understand the factors that influence model predictions, in this study, Shapley Additive exPlanations (SHAP) is employed to achieve model interpretability (21–23).

Shapley Additive exPlanations (SHAP) is a method for interpreting machine learning model predictions based on the concept of Shapley values from cooperative game theory. In machine learning, the SHAP method provides each feature with its contribution to the model prediction by applying this concept to the interaction between features. This method of interpretation not only provides interpretability for model predictions, but also can help understand the logic behind the model and the interactions between features. The ability to correctly interpret the predictive

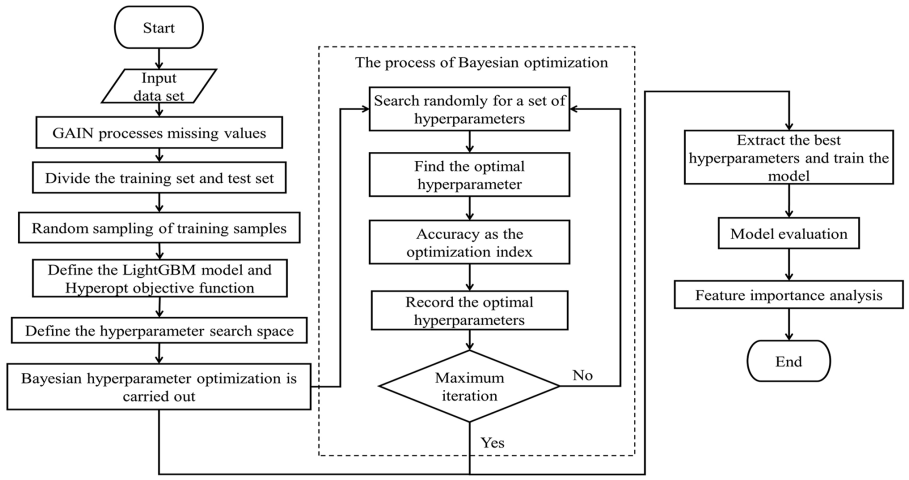

FIGURE 3
Experiment flow chart.

model outputs is important in providing insights into how to improve the model, as well as an understanding of the process being modelled.

SHAP has a wide range of applications for interpreting various types of machine learning models, including decision trees, neural networks, and integrated models, etc. SHAP estimates the contribution of each feature by ranking and combining a subset of features, and this feature-interaction-based interpretation approach allows SHAP to reveal the complexity of model predictions and help users understand the model's decision-making process. In clinical applications, these interpretations provide important information to guide doctor-patient discussions when the model categorises patients as being at high risk of certain adverse outcomes (24). Therefore, the SHAP method is important in interpreting machine learning models and has been widely used and recognized in practical applications.

In this paper, SHAP was used to interpret the PPGR prediction model in order to reveal important features affecting postprandial glucose elevation in patients with T1D, with the aim of providing more tailored guidance to healthcare professionals to help patients with T1D to improve their lifestyle habits and optimize the dose of insulin intake.

3 Experiments and results

3.1 Study population

A total of 13 patients with T1D (10 females and 3 males) were recruited into this study between September 2023 and January 2024, and a total of 867 meals were recorded during the study period, with a final sample of 826 usable meals selected for modelling. Of these, the mean age was 3810 years (median 35 years, interquartile range [IQR] 32-46 years), the mean BMI was 212.1 kg/m^2 (median 21.3 years, interquartile range [IQR] 20-22 kg/m^2), and the mean HbA1c level was 8.08%2.26% (see Table 1 for an analysis of all the blood test results).

In order to be able to visualize the patients' dietary habits more closely, the distribution of macronutrient intake in total energy intake was analysed in this study (Figure 4). The average carbohydrate, fat and protein consumption was 53.611.5g, 19.15.9g and 20.74.4g, respectively.

3.2 GAIN processing results

In this paper, the GAIN algorithm was applied to the processing of missing values in patients' continuous glucose data (CGM). In order to validate the effectiveness of the GAIN model used to process missing data, in this study, all instances of missing data in the original dataset were removed to obtain a intact validation set. For the validation set, ten percent of the data were randomly chosen to serve as missing values. The MAE between the generated interpolated values and the true dataset values was employed as the metric. In this paper we compared the results of three data interpolation methods for the blood sugar data processing, including GAIN, K-Nearest Neighbour (KNN) interpolation and Linear interpolation.

The experimental results demonstrate that the GAIN model achieved the optimal imputation performance for CGM data, with a mean absolute error (MAE) of 16.11 mg/dL, significantly lower than the KNN interpolation algorithm's 20.16 mg/dL and the linear interpolation algorithm's 19.8 mg/dL. This indicates that the GAIN model outperforms the other two traditional methods in imputing time-series blood glucose data.

3.3 Predicting the glycaemic response to a realistic diet

In this paper, a LightGBM prediction model was constructed based on a Bayesian hyperparameter optimization algorithm combined with a stochastic search algorithm. Additionally, the

TABLE 1 Blood test results.

Blood test result	Mean	Standard Deviation
HbA1c (%)	8.08	2.26
Creatinine (umol/l)	60.53	12.64
Sodium (mmol/l)	137.33	0.55
Potassium (mmol/l)	4.19	0.24
Serum chloride (mmol/l)	105.57	1.52
Calcium (mmol/l)	2.27	0.08
Total bilirubin (umol/l)	12.71	3.16
Uric acid (umol/l)	296.31	56.32
ALT (u/l)	16.86	6.55
AST (u/l)	19.03	5.74
ALP (u/l)	74.16	21.05
Total protein(g/l)	71.12	6.54
ALB (g/l)	43.05	2.57
Cholesterol (mmol/L)	9.11	10.79
Triglycerides (mmol/L)	1.05	0.54
HDL (mmol/L)	1.55	0.31
LDL (mmol/L)	2.74	1.24
TSH (mIU/L)	3.69	1.89
Fasting C peptide levels (nmol/L)	0.02	0.02

ability of the model to predict PPGR and Glu_{max} was assessed by calculating the Pearson correlation coefficients between the predicted and observed values:

$$p = \frac{\mathrm{cov}(X, \bar{X})}{\sigma_X \sigma_{\bar{X}}}$$

where $\bar{X}$ represents the actual observed values, $\bar{X}$ represents the predicted values, $\mathrm{cov}(X, \bar{X})$ is the covariance of X and $\bar{X}$, and σ_x and $\sigma_{\bar{x}}$ are the standard deviations of X and $\bar{X}$, respectively.

In order to validate the accuracy rate of the model, compared to other models using only carbohydrates as well as insulin dose to predict PPGR, in this study, a LightGBM prediction model that included the following three Bayesian hyperparametric optimisation algorithms combined with stochastic search algorithms was constructed: 1) a model based on carbohydrate content only: only one feature of carbohydrates in the food was used as an input. 2) An insulin administration baseline model: carbohydrate content, pre-meal insulin dose, and blood glucose level at the time of the meal were used as input features. 3) A full model: incorporating as inputs all of the information gathered from the patients over the duration of the study, encompassing features such as meal composition and blood test outcomes, blood glucose measurements, and insulin dosage (for features included in the model see Table 2).

In the prediction of PPGR, the model relying solely on carbohydrate content demonstrates a relatively low correlation

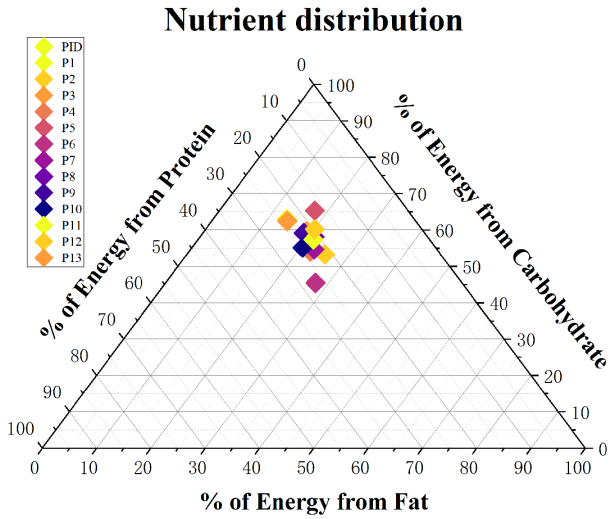

FIGURE 4
Distribution of the mean daily macronutrient proportions derived from the total energy intake of participants in the cohort (each participant represented by a single point).

(R=0.14, Figure 5A) of its predictions with observed PPGRs and explains only about 2% of the variance in glycaemic response. The insulin administration baseline model performs better (Figure 5B), with a correlation of its predictions with the observed PPGRs is 0.43 (R=0.43, $P<10^{-10}$) and explains 15% of the variance in glycaemic response. The full model integrating glucose measurements, insulin dose, meal content, and blood characteristics achieves a significantly higher correlation (R=0.63, $P<10^{-10}$) and the explained variance increases to 39% (Figure 5C).

After parameter optimisation of the LightGBM model using a Bayesian optimisation algorithm combined with a stochastic search algorithm, the optimal hyperparameter settings for the complete model are obtained as follows: learning_rate is 0.009, n_estimators is 345, max_depth is 5, colsample_bytree is 0.75, min_child_samples is 2, num_leaves is 36, and subsample is 0.69.

Similarly, for Glu_{max} predictions, the model relying solely on carbohydrate content has a correlation that is relatively low (R=0.15) (Figure 5D), the baseline model performs better (R=0.38, $P<10^{-10}$) (Figure 5E), and the full model has a significantly higher correlation (R=0.58, $P<10^{-10}$) (Figure 5F).

3.4 Characteristic attribution results

In order to clearly observe the relationship between the various parameters, a heat map was used. The heatmap provided a clearer visualisation of the linear relationship between the various features and PPGRs (Figure 6). From the heat map, it can be seen that the correlation coefficients of ALT, AST and TSH with PPGR seem to be close to 0, i.e., there is almost no linear relationship, whereas 4 hour base amount, and high dose of insulin have a strong positive correlation with blood glucose level at meal time.

To further understand the factors affecting the model predictions, Shapley Additive exPlanations (SHAP) was used in

TABLE 2 Features included in the model.

Category	Features
Meal content	Calorie, proteins, fats, carbohydrates, carbohydrate/fat ratio, carbohydrate/proteins ratio
Blood tests results	HbA1c,creatinine,sodium,potassium,serum chloride,calcium, total bilirubin,uric acid,alanine transaminase(ALT),aspartate transaminase(AST),total protein,alkaline phosphatase(ALP), cholesterol,triglycerides,HDL,LDL,thyrotropin,fasting C-peptide levels,glucose),alkaline phosphatase(ALP),total protein,albumin,cholesterol,triglycerides,HDL,LDL, thyrotropin,fasting C-peptide levels,glucose
Anthropometric measurements	Weight, height, waist and hips circumference, BMI
Survey-derived features	Age, gender
CGM-derived features	Glucose value at meal initiation,glucose trends calculated by subtraction of glucose value at meal initiation from The glucose values at 30, 60 and 120 minutes before the commencement of the meal.
Insulin	High dose of insulin before meal,4 hours basal insulin

this paper to achieve model interpretability. The results of using SHAP to assess feature importance are shown in Figure 7. The illustration portrays the influence of the top 20 most substantial features (arranged in descending order from top to bottom) on predicting PPGR for a particular data point within the test set. Each feature's effect on the prediction (SHAP value) is displayed on the scale. The distance from zero (indicated by a gray vertical line) indicates the magnitude of the feature's influence on the model. The colours represent the feature's value at each point, spanning from below-average (blue) through average (purple) to above average (red). For the most important feature blood glucose level at meals, it is clear that lower blood glucose levels at meals lead to significantly lower PPGR values.

It follows that the model's most impactful features with the highest mean absolute SHAP values include blood glucose level at meal time, blood glucose trend 30 minutes before the meal, gender, serum chloride, blood glucose trend 120 minutes before the meal, carbohydrate-to-protein ratio, protein content, calorie content, carbohydrate content, and fat content (Figure 7).

4 Discussions

In this paper, a personalised postprandial glycaemic response prediction model for patients with T1D is proposed, using LightGBM based on a Bayesian hyperparametric optimisation algorithm combined with a stochastic search algorithm. The input features of the model include features such as meal composition and blood test outcomes, glycaemic measurements and insulin dose.

Postprandial glycaemic response (PPGR) is an important indicator of the effectiveness of glycaemic control and glucose metabolism in all types of diabetic patients. Clinical trials have shown the importance of keeping postprandial glucose within the normal range (25, 26). In recent years, the increased use of continuous glucose monitors (CGMs) among diabetic patients (27) has radically improved the application of predicting postprandial glucose responses. However, modelling different individuals remains a challenge. For example, Kezhi Li et al. (28)

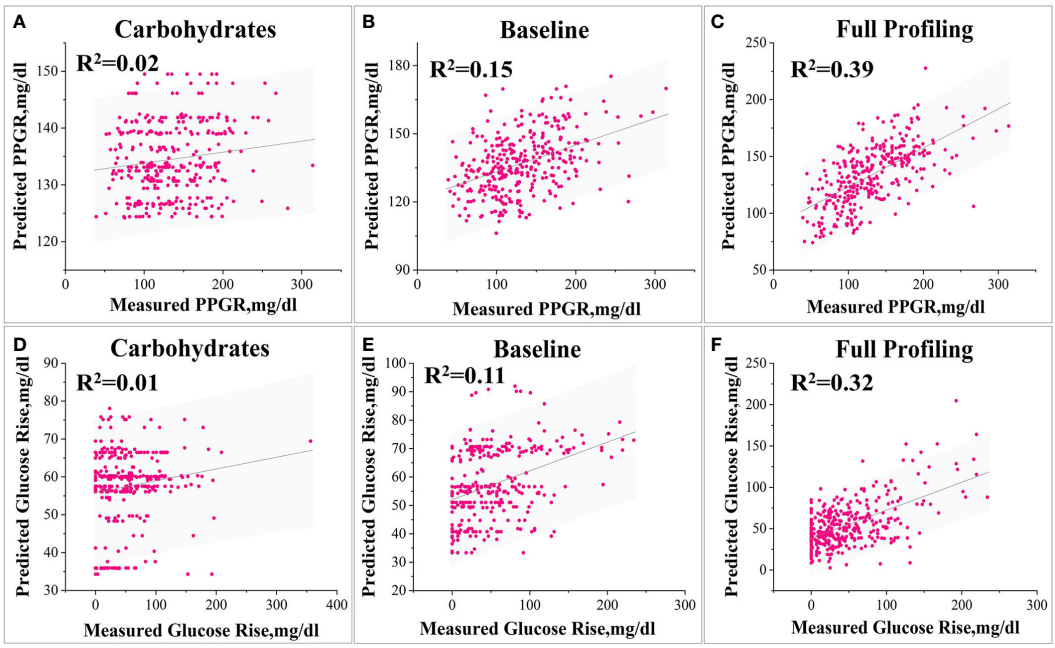

FIGURE 5
Various model predictions of PPGR and Glu_{max}. 1) Models for Predicting PPGR: Model **(A)** based solely on postprandial carbohydrate content, Baseline model simulating insulin administration **(B)**, and Model **(C)** utilizing all features. 2) Models for Predicting Glu_{max}: Model **(A)** based solely on postprandial carbohydrate content, Baseline model simulating insulin administration **(B)**, and Model **(C)** utilizing all features.

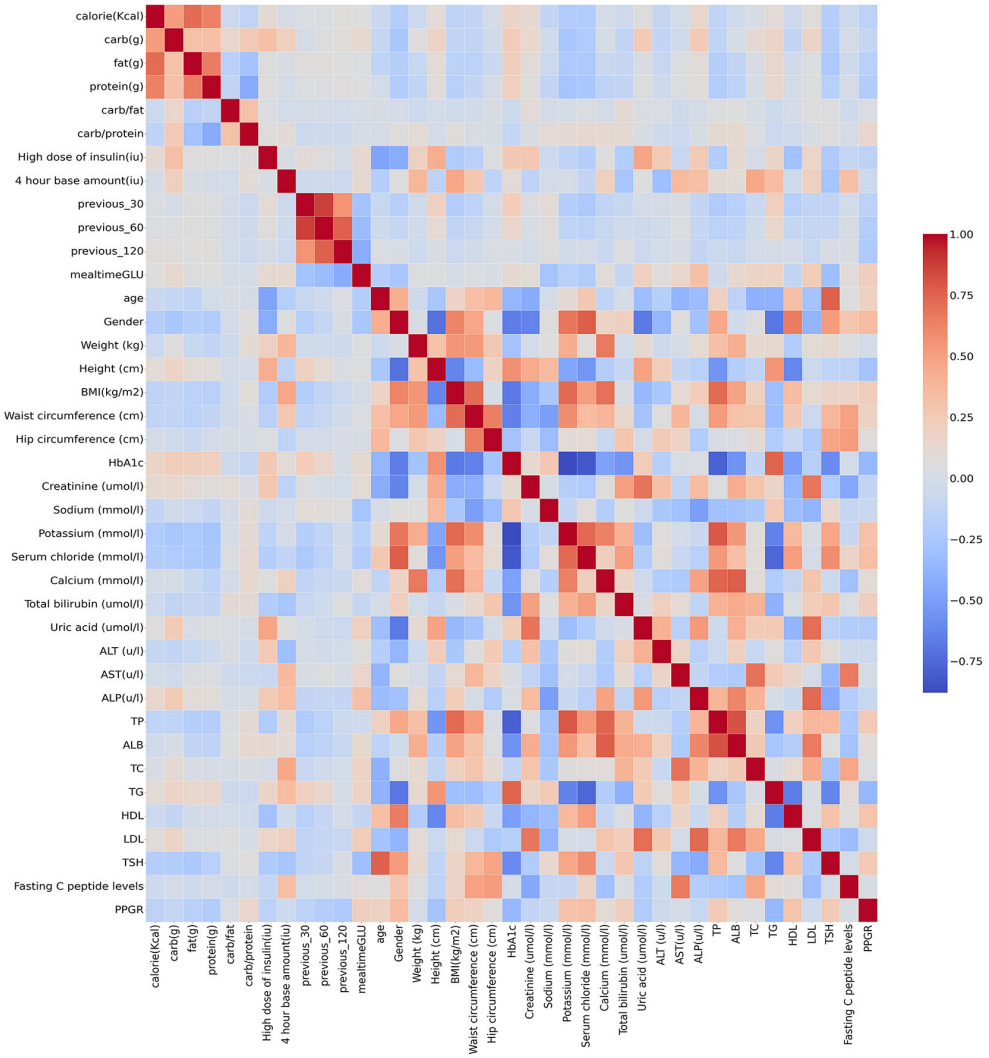

FIGURE 6
Correlation of clinical parameters (red for positive correlation, blue for negative correlation).

utilized GluNet, a personalized deep neural network framework, to predict the short-term (30-60 minutes) probability distribution of future CGM values in T1D subjects using historical data, including glucose measurements, dietary information, insulin dosage, and other factors. In 2017, KOREM et al. (29) conducted a randomised crossover trial in which 20 healthy subjects consumed two types of bread to compare their PPGRs and other clinical metrics. After careful examination of individual responses, it was found that there were significant differences in PPGRs between individuals after bread consumption. Models that incorporate individual-specific factors have been shown to be more effective in predicting an individual's PPGR than traditional methods. These personalised models rely on key variables, including anthropometric measurements, dietary intake, etc., to accurately predict PPGR.

There are also several studies of PPGR prediction models that include CGM-related characteristics, gut microbiome characteristics of individuals, anthropometrics, and dietary macronutrients, as shown in Table 3.

In the above study, Shilo et al. (16) developed a model for predicting PPGR in patients with T1D using a cohort of Israeli T1D patients and the inputs to the model also included microbiome profiles. Pustozerov et al. (30) used data from patients with gestational diabetes to construct a PPGR model. Whereas Zeevi et al. (17), Mendes-Soares et al. (31) and Tily et al. (32) all used healthy cohorts. Thus, the generally higher correlation between PPGRs obtained from CGM extracted from healthy individuals and PPGRs obtained from blood tests also suggests that predicting glycaemic response to diet is more challenging in patients with T1D than in healthy individuals, as patients with T1D have higher glycaemic variability.

These studies are all based on Western dietary structures, however, Chinese dietary habits are very complex, so more accurate postprandial glycaemic response (PPGR) prediction models are needed to guide postprandial glycaemic control in Chinese patients with T1D. In this paper, data from 13 patients with T1D in Kunming City, Yunnan Province are collected,

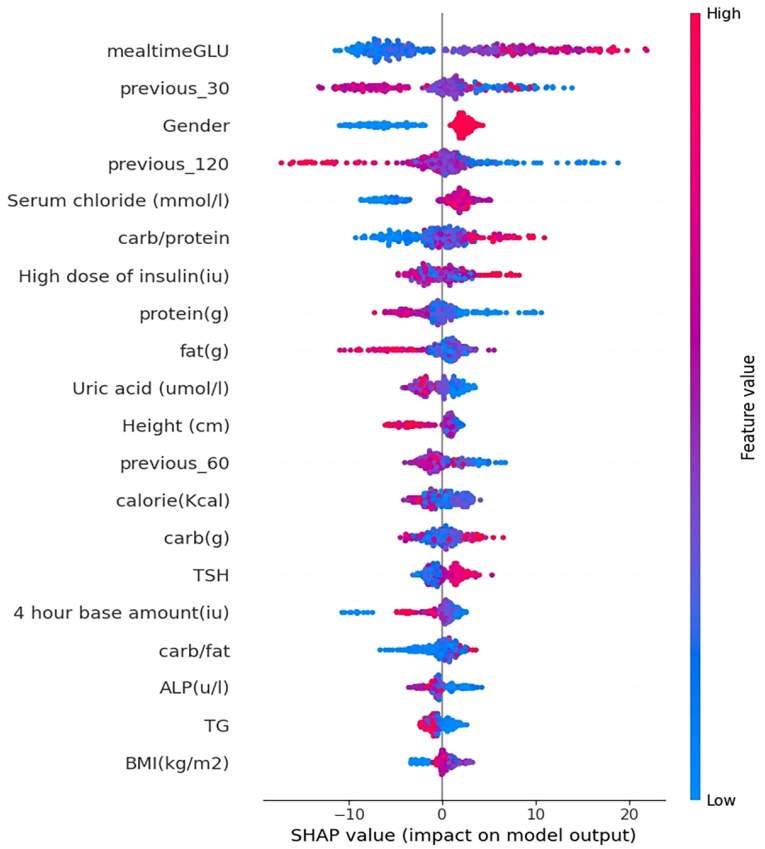

FIGURE 7
Interpretation of the prediction model.

provided by the First People's Hospital of Yunnan Province, and we developed a personalised PPGR prediction model for patients with T1D, using LightGBM based on Bayesian hyperparameter optimisation algorithm combined with a stochastic search algorithm to construct the model. The input features of the model include features such as meal composition and blood test outcomes, blood glucose measurements and insulin doses. The experimental results show that the correlation (R=0.63) between the predictions of the model in this paper and the observed PPGR is better than that of the Shilo et al. (16) 's model (R=0.59), and that the model developed here does not necessitate microbiome data as input, enhancing its accessibility for clinical application. In the prediction of both PPGR and Glumax, the proposed model also significantly outperforms the traditional model relying solely on carbohydrate content in food and the baseline model simulating the current standard of care for insulin administration.

TABLE 3 Summary statistics from previous studies (correlation coefficient R).

Reference	Statistics R	Cohort
Shilo et al., 2022 (16)	R=0.59, Full model	A cohort of Type 1 diabetes patients from Israel
Pustozerov et al., 2020 (30)	R=0.53, Full model	A cohort of patients with gestational diabetes
Zeevi et al., 2015 (17)	R=0.7, Full model	A cohort of non-diabetic adults from Israel
Mendes-Soares et al., 2019b (31)	R=0.62, Full model	A non-diabetic cohort from the Midwest
Tily et al., 2022 (32)	R=0.77, Full model	The U.S. Health Cohort, of which 73 per cent were Caucasian

In addition, although the advent of continuous glucose monitors (CGMs) in recent years has significantly enhanced the application of CGMs in glucose prediction by providing a large amount of time-series data through real-time monitoring of blood glucose levels, incomplete monitoring data may occur due to factors such as inappropriate or untimely wearing patterns and sensor malfunctions. These missing data may affect the accuracy and stability of the prediction model. In this study, in order to fill the missing values in the blood glucose data more rationally, the GAIN algorithm was used, which has a great advantage in capturing the temporal variations of time series data. And from the results of the study, GAIN does have higher accuracy than traditional methods in the processing of blood glucose data.

In this study, it reveals the drivers of postprandial glucose elevation in patients with T1D by analysing the factors influencing the prediction model using SHAP. From the results of

the study, the most influential features include blood glucose levels at the time of the meal, blood glucose trends 30 minutes before the meal, and carbohydrate to protein ratio. These results show that features related to CGM data have the greatest impact on the model, for example, the blood glucose level at mealtime, the blood glucose trend 30 minutes before meal and other features rank highly, followed by features related to dietary nutrient content. In this cohort, the lower the blood glucose level at mealtime and the lower the carbohydrate intake, the better the blood glucose control. These results are in good agreement with the results reported in the study by Shilo et al. (16).

Gender is also an important influencing factor in this study. In 2019, González-Rodríguez et al. (33) have demonstrated that the effects of dietary nutrients on postprandial glycaemic responses were different in women compared to men in a non-diabetic population. From the results in this study, it appears that gender characteristics also have an effect on postprandial glycaemic response in patients with T1D, but this conclusion may also be affected by the small sample size of the data and the uneven ratio of male to female patients.

In this study there has several limitations. Firstly, inaccuracies in patient self-reporting of dietary intake may affect the ability to predict postprandial glycaemic response. Secondly, because accurate dietary intake data are difficult to collect, the sample size of data in this paper is small and not representative of a broader population, and better predictions could have been obtained with more high-quality clinical data to train the model. Finally, although the PPGR prediction model proposed in this paper has a high level of accuracy, there is still potential for enhancement. For example, the inclusion of microbiome data and a detailed assessment of physical activity does increase costs, but may also improve the accuracy of the predictions.

5 Conclusions

In this study, a personalised PPGR prediction model for patients with T1D is proposed. For the model, glucose measurements, insulin dose, dietary content, blood measurements, and anthropometrics are integrated, and it is substantially superior to traditional models that rely solely on the amount of carbohydrates in food and baseline models that simulate the current standard of care for insulin administration. The proposed model could accurately predict postprandial glycaemic response in patients with T1D, and it maybe better guide patient dietary planning as well as insulin intake dosage. Furthermore, the proposed model can be further implemented within closed-loop systems, personalized decision support systems, and alert systems to mitigate anticipated hyperglycaemic and hypoglycaemic events in patients with Type 1 Diabetes (T1D). Additionally, the model can tailor dietary nutritional plans for T1D patients based on anticipated hypoglycaemic responses. In summary, the model represents a meaningful step forward in improving postprandial glycaemic control in T1D patients, providing direction for future research and development in personalized diabetes care.

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.

Ethics statement

The studies involving humans were approved by the Medical Ethics Committee of the First People's Hospital of Yunnan Province. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XX: Conceptualization, Formal analysis, Project administration, Validation, Supervision, Writing – review & editing. YX: Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. YC: Conceptualization, Data curation, Investigation, Project administration, Validation, Writing – review & editing. JH: Funding acquisition, Supervision, Writing – review & editing. HS: Funding acquisition, Resources, Validation, Writing – review & editing.

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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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Abnormal late postprandial glucagon response in type 1 diabetes is a function of differences in stimulated C-peptide concentrations

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Background: The functional changes in alpha cells in patients with type 1 diabetes (T1D) with different residual beta cell functions remain poorly elucidated. The study aimed to investigate the relationship between glucagon secretion and C-peptide levels and to explore the relationship between glucagon response and glucose increment in respond to a secretagogue in a steamed bread meal tolerance test (BMTT) in T1D.

Methods: The study enrolled 43 adult patients with T1D and 24 healthy control subjects. Patients with T1D who underwent BMTT were divided into two groups based on peak C-peptide levels: C peptide low (CPL; C-peptide < 200 pmol/L; n=14) and high (CPH; C peptide ≥ 200 pmol/L; n=29). Plasma glucose, C-peptide, glucagon levels at 0, 30, 60, 120, and 180 min were measured. The glucagon response to the BMTT was defined by areas under the curve (AUC) as early (AUC_{0-30}), late (AUC_{30-180}), or total (AUC_{0-180}) glucagon.

Results: Compared to healthy individuals, fasting plasma glucagon was lower and postprandial plasma glucagon level was increased in patients with T1D. Glucagon levels after BMTT between the CPL and CPH group showed significant group by time interaction. Peak glucagon and glucagon at 60-180 min, total and late glucagon response were higher in CPL than CPH group, while fasting glucagon and early glucagon response adjusted for glucose were comparable between CPL and CPH group. The higher late glucagon response and late glucagon response adjusted for glucose were associated with lower peak C-peptide in T1D. The higher late glucagon response and lower peak C-peptide were associated with the higher value of ▵glucose at 180 min.

Conclusion: Stimulated C-peptide levels affect the paradoxical increase in postprandial glucagon secretion in patients with T1D, especially late glucagon response. The exaggerated postprandial glucagon secretion further stimulates the elevation of postprandial glucose in patients with T1D.

KEYWORDS

type 1 diabetes, alpha cell regulation, glucagon, stimulated C-peptide, late glucagon response

1 Introduction

Blood glucose homeostasis is mainly regulated by pancreatic islet hormones, primarily insulin and glucagon. Insulin secretion by pancreatic beta cells has been intensively studied for its impact on glucagon secretion by pancreatic alpha cells under physiological conditions. The insulin receptor and its downstream signaling proteins are abundantly expressed in alpha cells, allowing insulin to suppress glucagon secretion (1). However, in pathological conditions, this negative feedback balance is disrupted due to impaired beta cell function. Patients with diabetes have been shown to exhibit insufficient suppression of glucagon secretion following oral ingestion of glucose intake or a meal (2, 3). Consequently, abnormalities in glucagon physiology may contribute to the development of fasting and postprandial hyperglycemia in the pathogenesis of type 1 diabetes (T1D) and its therapy.

Many studies have investigated the beta cell heterogeneity in T1D (4). However, clinical and immunologic characteristics of T1D vary significantly between different populations (5). For example, Chinese adults with newly diagnosed T1D have been reported to display high C-peptide levels (6). Our recent study further confirmed that Chinese patients with T1D exhibited substantial residuals beta cell mass despite ongoing autoimmune attacks (7). Despite this, the understanding of the stimulus-secretion coupling of alpha cell function and the residual beta cell function in response to an oral glucose challenge in T1D remains limited, with conflicting data emerging (8, 9). While previous studies had reported a significant exacerbation of postprandial hyperglucagonemia during the first one and five years after T1D diagnosis (9–11), two studies suggested that residual dysregulated glucagon secretion is not affected by beta cell function in T1D (12, 13). Thus, the primary question that arises is whether stimulated C-peptide levels could result in differential glucagon responses.

The phase of glucagon secretion in diabetes after oral ingestion of glucose intake or a meal remains unclear, with only a few studies focusing on type 2 diabetes (T2D). Early glucagon response, rather than late glucagon response, at baseline in non-diabetic individuals was significant associated with increased fasting glucose levels over 7 years (14). Additionally, the loss of early glucagon response suppression after oral glucose intake is only observed in T2D patients compared to healthy and pre-diabetes individuals, supporting the hypothesis that hyperglycemia in T2D is mainly related to impairment of the early glucagon response (15). These findings suggest that the levels of glucagon secretion following glucose load is crucial for the maintenance of normoglycemia. However, there are currently no studies that have looked in depth at the glucagon response in the early and late postprandial glucagon response in patients with T1D. Therefore, the second question of concern is whether the phases of postprandial glucagon secretion is associated with stimulated C-peptide and glucose increment in patients with T1D.

The aim of this study is to improve our understanding of the relationship between different phases of glucagon secretion and C-peptide concentrations and to investigate the relationship between the phases of glucagon response and glucose increments in response to a steamed bread meal tolerance test (BMTT) in Chinese patients with T1D.

2 Materials and methods

2.1 Study population

The present observational study was carried out in the Department of Endocrinology of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. Two types of subjects were enrolled in the study: 43 patients with T1D and 24 healthy control subjects. Major eligibility criteria for the patients with T1D included 1) age ≥ 18 years at the time of screening, 2) clinical diagnosis of T1D, 3) positivity for at least one islet antibody (IAA, ICA, GADA, or IA-2A) (16), and 4) able to provide written informed content. Exclusion criteria included 1) diabetic ketoacidosis or severe hypoglycemia within study preceding one month; 2) severe chronic diabetic complications (including proliferative retinopathy, autonomic neuropathy, macrovascular or central nervous system disease); 3) pregnancy or lactation, and 4) history of gastrointestinal surgery or pancreatectomy. The healthy controls were recruited from hospital and university staff,

and the inclusion criteria for the healthy controls included 1) age≥ 18 years at the time of screening; 2) normal glucose tolerance, and 3) no family history of diabetes.

Patients with T1D were divided into two groups according to the peak serum C-peptide level after BMTT. The BMTT has been used most often in China as a measurement tool to evaluate beta cell function during follow-up after individuals have been diagnosed with diabetes (6, 17). Patients with T1D with a peak serum C-peptide level below 200 pmol/L were defined as the C-peptide low (CPL) group. Patients with T1D and peak serum C-peptide values above 200 pmol/L were divided into a C-peptide high (CPH) group.

The study was approved by the local ethics committee of the First Affiliated Hospital of Nanjing Medical University (approval no. 2019-SR-121.A1). The study was carried out in accord with the principles expressed in the Declaration of Helsinki.

2.2 Experimental procedures

The BMTT was performed after an overnight fast, with no food or drink other than water from midnight. Prior to the study, the patients achieved satisfactory glycemic control for three consecutive days. Fasting glucose was measured using a glucometer before the initiation of the BMTT, ensuring that the glucose level was targeted within the range of 4-10 mmol/L (72-180 mg/dL). If the fasting glucose level is not within the target range, the BMTT will be rescheduled. Patients with multiple daily insulin injections (MDIs) were instructed to take usual long-acting insulin dose the night before the study, while patients with continuous subcutaneous insulin infusion (CSII) maintained their basal insulin infusion. The delivery of continuous subcutaneous insulin was halted 2 hours prior to the initiation of the BMTT. Participants were instructed not to administer premeal bolus insulin and any correction dose of rapid-acting insulin during the BMTT. The BMTT, provided by the First Affiliated Hospital of Nanjing Medical University, containing 75 g of glucose, approximately 7 g of protein, and 1 g of fat, amounting to a total of 337 kcal. Blood samples were obtained at 0, 30, 60, 120, and 180 min during the BMTT. Throughout the entire procedure, and continuous monitoring of glucose levels was conducted. After the 180-minute BMTT, the determination of the necessary dosage of rapid-acting insulin required to maintain glucose levels within the desired target range was promptly performed by a physician until the target range was achieved.

2.3 Laboratory methods

Serum glucose was measured with an automatic enzymatic analyzer (Beckman Coulter, USA). Serum C-peptide levels were measured by a chemiluminescence assay (Roche Diagnostics, Switzerland) with a detection limit of 3·33 pmol/L. Islet autoantibodies IAA, IA-2A, and GADA were measured by ELISA (Euroimmun Medizinische Labordiagnostika AG, Germany; Biomerica, USA). An indirect immunofluorescence technique was used to measure ICA autoantibodies (18).

EDTA tubes containing aprotinin (0.6 TIU/ml of blood) were used to collect blood samples for plasma glucagon measurements. Blood samples were centrifuged for 15 min immediately after collection and stored at -80°C. Plasma glucagon was analyzed with a solid phase two-site enzyme immunoassay (Mercodia, Sweden), which has a detection limit of 1 pmol/L. The coefficient of variation (CV) for intra-assay variation was 3·3–5·1%, and the CV for inter-assay variation was 7·3–9·4% (19).

2.4 Calculations

The change in glucagon levels at 30, 60, 120, and 180 min during the BMTT (△glucagon 30, 60, 120, and 180 min) was determined by comparing the glucagon levels at these time points to the baseline (0 min) glucagon level, as previously described (20). The change in glucose levels during the BMTT was calculated using the same method as △glucose 30, 60, 120, and 180 min. The glucagon response following the BMTT was expressed as the incremental area under the curve (iAUC), which were calculated using GraphPad Prism software (version 7.0). The iAUC from 0-180 and 0-30 min were calculated using the fasting value as the baseline, while the iAUC from 30-180 min was calculated using the 30min value as the baseline. Areas above the baseline were recorded as positive and areas below the baseline as negative. The iAUC from 0-180, 0-30 and 30-180 min was defined as the total, early and late glucagon response, respectively. In order to evaluate the glucagon response adjusted for glucose increment during the BMTT, we also calculated the ratio of iAUC glucagon to iAUC glucose from 0-180, 0-30 and 30-180min as previously described (21).

2.5 Statistical analysis

All statistical analysis were performed using IBM SPSS Statistics 22 and GraphPad Prism (version 7.0) software. Statistical significance for the parameter estimate was established with an alpha of 0.05.

2.5.1 Analytical approach

The normal distribution of continuous variables was assessed using the Shapiro-Wilk test or Kolmogorov-Smirnov test. Unpaired t test or Mann-Whitney U test were used to compare the difference in clinical characteristics, iAUC of glucagon, the ratio of iAUC glucagon to iAUC glucose, and fasting variables between two groups, where appropriate. The differences between the two groups in the repeated measured variables following the BMTT were compared using the generalized estimation equation (GEE) approach to indicate the effect of time, group, and group by time interaction with baseline measurement (0 min) as covariates. An exchangeable working correlation matrix was applied in the GEE approach to assess change over time. The group-by-time

interaction, which indicates the difference for given variables between two groups following the BMTT, was tested first. If significant, between-group differences at each timepoint were tested.

Relationships between variables were evaluated by spearman's rank correlations. Multiple linear regression analyses with backward elimination were performed to determine the association of glucagon response and peak C-peptide. Model 1 was applied with iAUC $_{30\text{-}180}$ glucagon as the dependent variable and including the following independent variables: peak C-peptide, sex, age, BMI, HbA1c, duration of diabetes, daily insulin dose, glucose 0, and peak glucose. Model 2 was applied with iAUC $_{30\text{-}180}$ glucagon/iAUC $_{30\text{-}180}$ glucose as the dependent variable and including the peak C-peptide, sex, age, BMI, HbA1c, duration of diabetes, and daily insulin dose as independent variables. Multiple linear regression analysis was also used to further explore the relationship between different phases of glucagon response and ΔGlucose 180 min, with sex, age at diagnosis, and daily insulin dose as the covariates.

2.5.2 Sensitivity analyses

To assess the robustness of glucagon levels analyses during the BMTT, two analytical approaches involving multiple covariates were performed (Table 1; Supplementary Table 2). The first approach included baseline measurement (0 min) as covariates in the GEE model; The second approach included clinical characteristics that differed between T1D and HC groups (age) or CPL and CPH groups (age and duration of diabetes) as additional covariates in the GEE model.

3 Results

3.1 Elevated glucagon response after the BMTT in T1D

A total of 43 patients with T1D (24 male and 19 female) and 24 healthy control subjects (10 male and 14 female) were enrolled in the study (Supplementary Figure 1). The sex and BMI of participant did not differ substantively between the T1D and healthy control groups, while patients with T1D showed higher median age compared with healthy control group (Table 2).

Compared with healthy control group, patients with T1D showed higher glucose and lower C-peptide levels during the BMTT (Figures 1A, B) (Supplementary Table 1). We found that the fasting glucagon concentration in the healthy control was higher than T1D group ($P < 0{\cdot}001$). A significant interaction between time and group was observed for glucagon after the BMTT ($P_{\text{group×time}} < 0{\cdot}001$), suggesting the different patterns of glucagon secretion after the BMTT between patients with T1D and healthy control group. After the BMTT, the healthy control group exhibited suppression of glucagon secretion, whereas patients with T1D showed elevated glucagon levels. The value of glucagon at each time point in T1D group were all higher than those in the healthy control group (Figure 1C) (Supplementary Table 1). The robustness of the glucagon level analyses between the T1D and HC groups was demonstrated in sensitivity analyses (Supplementary Table 2).

Meanwhile, the index of total glucagon response (iAUC$_{0\text{-}180}$ glucagon) in the T1D group were higher than that in the healthy

TABLE 1 Sensitivity analyses of the glucagon levels during the BMTT in participants with type 1 diabetes divided by peak C-peptide levels.

	Glucagon, pmol/L		Group×time interaction effect	CPL group vs. CPH group	
	CPL group (n=14)	CPH group (n=29)		Adjusted mean difference (95% CI)	*P* value
Multiple imputation					
0 min	4·20 ± 2·24	4·47 ± 2·28	0·003		
30 min	11·05 ± 5·66	8·32 ± 4·03		2·93 (-0·01 to 5·87)	0·051
60 min	11·07 ± 6·31	6·72 ± 3·41		4·55 (1·53 to 7·57)	0·003
120 min	10·31 ± 6·31	4·72 ± 2·37		5·79 (2·68 to 8·90)	<0·001
180 min	8·95 ± 5·03	4·31 ± 2·09		4·85 (2·41 to 7·29)	<0·001
Multiple imputation with adjustment for age, duration of diabetes and baseline measurement					
0 min	4·20 ± 2·24	4·47 ± 2·28	0·003		
30 min	11·05 ± 5·66	8·32 ± 4·03		2·11 (-0·61 to 4·84)	0·128
60 min	11·07 ± 6·31	6·72 ± 3·41		3·73 (1·12 to 6·34)	0·005
120 min	10·31 ± 6·31	4·72 ± 2·37		4·97 (2·26 to 7·68)	<0·001
180 min	8·95 ± 5·03	4·31 ± 2·09		4·03 (1·86 to 6·21)	<0·001

In the sensitivity analyses, the robustness of the results was assessed using 2 different analytical approaches. The repeated measured glucagon levels following the BMTT between two groups were investigated by generalized estimating equations. The first approach included baseline measurement (0 min) as covariates; The second approach included age, duration of diabetes and baseline measurement (0 min) as covariates. A significant group×time interaction indicated a significant difference for glucagon levels between two groups during the BMTT in all 2 approaches. BMTT, steamed bread meal tolerance test; CPL, C-peptide low; CPH, C-peptide high.

TABLE 2 Key clinical characteristics of type 1 diabetes and healthy control.

Characteristic	T1D group	HC group	*P* value
Subjects, n	43	24	NA
Male/Female, n	24/19	10/14	0·267[a]
Age, years	31·23 (23·81; 46·25)	23·95 (23·40; 27·75)	0·020
Duration of diabetes, years	3·00 (0·58; 7·00)	NA	NA
BMI, kg/m^2	20·33 ± 2·59	21·68 ± 3·41	0·073

Results were expressed as mean ± SD or median (25th; 75th). Variables were compared using the unpaired t test or Mann-Whitney U test; [a]Table was analyzed using Chi-squared test. NA, not applicable; T1D, type 1 diabetes; HC, healthy control.

control group [398·00 (37·10; 689·00) vs. -960·00 (-1449·75; -493·25) pmol/L*min, $P < 0·001$]. Compared with the HC group, the early glucagon response (iAUC$_{0-30}$ glucagon) was higher in the T1D group [62·80 (22·90; 87·10) vs. -78·05 (-119·50; -43·75) pmol/L*min, $P < 0·001$], while the late glucagon response (iAUC$_{30-180}$ glucagon) was lower in the T1D group [-319·00 (-485·00; -100·00) vs. -69·00 (-185·50; 53·68) pmol/L*min, $P = 0·002$] (Figures 1D–F).

3.2 Different glucagon responses between T1D divided by stimulated C-peptide levels

To investigate the association between beta-cell function and glucagon response in patients with T1D, the participant was divided into the CPL and CPH group according to peak C-peptide level. The CPL group showed higher age and longer diabetes duration compared with the CPH group. No significant differences were observed in sex, age at diagnosis, BMI, HbA1c, and daily insulin dose between two groups (Table 3).

Compared with the CPH group, the CPL group showed lower C-peptide and higher glucose levels during the BMTT (Figures 2A, B) (Supplementary Table 3). The fasting glucagon level was comparable between the CPL and CPH group ($P = 0·708$). There was a significant group by time interaction on glucagon during the BMTT between the CPL and CPH group ($P_{group×time} = 0·003$), suggesting a difference in glucagon secretion after the BMTT between the CPL and CPH group. The value of glucagon at 60, 120 and 180 min in the CPL group were all higher than CPH group in response to the BMTT (Figure 2C) (Supplementary Table 3). Sensitivity analyses showed the robustness of the glucagon level analyses between the CPL and CPH groups (Table 1). In addition, peak glucagon was also higher in the CPL group compared with the CPH group [11·01 (7·80; 18·82) vs. 8·24 (5·68; 10·62) pmol/L, $P = 0·018$].

We also found that the glucagon response and glucagon response adjusted for glucose increment during the BMTT was different between the CPL and CPH group. Firstly, the index of total (iAUC$_{0-180}$ glucagon), early (iAUC$_{0-30}$ glucagon), and late (iAUC$_{30-180}$ glucagon) glucagon response were all higher in the CPL group than the CPH group (Figures 2D–F). Secondly, the total and late glucagon response adjusted for glucose increment were both higher in the CPL group than in the CPH group (Figures 2G, I), while the early glucagon response adjusted for glucose increment was comparable between the two groups (Figure 2H) (Supplementary Table 4).

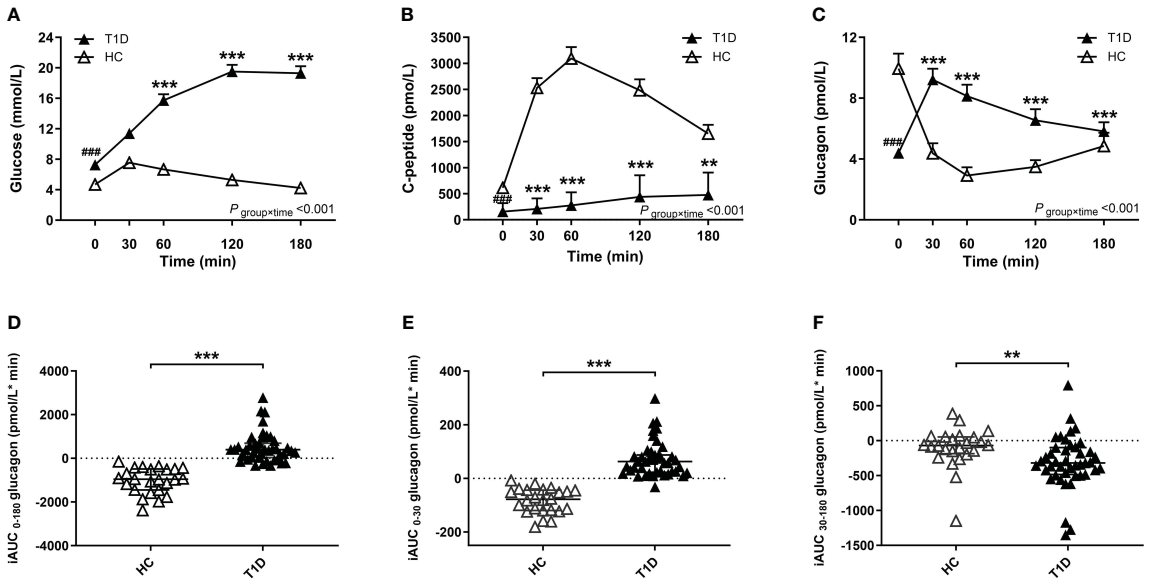

FIGURE 1
Results of the BMTT in the patients with T1D (n=43, filled triangles) and healthy control (n=24, open triangles). **(A–C)** The curve of plasma glucose, C-peptide and glucagon during the BMTT. * The repeated measured variables following the BMTT between two groups were investigated by generalized estimating equations with baseline measurement (0 min) as the covariates. * The simple effect of group was analyzed using the generalized estimation equation, ***P<0·001; **P<0·01. # The difference of baseline measurement was analyzed using the Mann-Whitney U test, ###P<0·001. **(D–F)** The incremental area under the curve of glucagon from 0 to 180 min, 0 to 30 min and 30 to 180 min during the BMTT, ***P<0·001; **P<0·01. BMTT, steamed bread meal tolerance test; HC, healthy control; T1D, type 1 diabetes; iAUC, incremental area under the curve.

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TABLE 3 Key clinical characteristics of patients with type 1 diabetes divided by peak C-peptide levels.

Characteristic	CPL group	CPH group	*P* value
Subjects, n	14	29	NA
Sex, male/female	6/8	18/11	0·235 [a]
Age, years	49·67 (31·89; 63·18)	29·84 (21·42; 33·19)	0·003
Age at diagnosis, years	29·35 (20·34; 45·14)	25·48 (19·98; 31·85)	0·238
Duration of diabetes, years	7·00 (4·13; 21·25)	1·00 (0·25; 3·50)	<0·001
BMI, kg/m^2	20·67 ± 2·51	20·17 ± 2·66	0·565
HbA1c, %	8·34 ± 1·63	9·73 ± 2·95	0·053
Daily insulin dose, U/day	31·84 ± 9·81	26·62 ± 7·09	0·053

Results were expressed as mean ± SD or median (25th; 75th). Variables were compared using the unpaired t test or Mann-Whitney U test; [a] Table was analyzed using Chi-squared test. NA, not applicable; CPL, C-peptide low; CPH, C-peptide high.

3.3 Relationship between peak C-peptide and glucagon secretion in response to the BMTT in individuals with T1D

Residual beta-cell function, defined as peak C-peptide concentration after the BMTT, was inversely related to the value of ∆glucagon at 120 and 180 min in patients with T1D (r = -0·476, P = 0·001; r = -0·530, P < 0·001, respectively). The peak C-peptide was inversely correlated with the iAUC $_{30\text{-}180}$ glucagon (r= -0·450, P = 0·002), but not with the iAUC $_{0\text{-}30}$ glucagon (r = -0·171, P = 0·274). Similarly, the peak C-peptide was also inversely correlated with the late glucagon response adjusted for glucose (iAUC $_{30\text{-}180}$ glucagon/iAUC $_{30\text{-}180}$ glucose) (r = -0·581, P < 0·001), but not with the early glucagon response adjusted for glucose (iAUC $_{0\text{-}30}$ glucagon/iAUC $_{0\text{-}30}$ glucose) (r= 0·044, P = 0·781).

Multiple linear regression analysis further showed that the peak C-peptide along with peak glucose affected the iAUC $_{30\text{-}180}$ glucagon after adjusted for sex, age, BMI, duration of diabetes, and daily insulin dose (R^2 = 0·335, P = 0·033). Moreover, the peak C-peptide, but not age and daily insulin dose, was also inversely related to the late glucagon response adjusted for glucose (iAUC $_{30\text{-}180}$ glucagon/ iAUC $_{30\text{-}180}$ glucose) (R^2 = 0·204, P = 0·029) (Table 4).

3.4 Relationship between glucagon secretion and glucose excursion in response to the BMTT in individuals with T1D

In order to determine the effect of glucagon response on the glucose increment in patients with T1D, we calculated the association between the paired ∆Glucagon and ∆Glucose during the BMTT. We found that the value of ∆Glucagon at 30, 60, 120, and 180 min were all positively correlated with the value of ∆Glucose at the same time point (Supplementary Figure 2).

We also investigated the effect of glucagon response on glucose increment in patients with T1D after the BMTT. The index of late glucagon response (iAUC $_{30\text{-}180}$ glucagon) were positive related to the value of ∆Glucose 180 min (r = 0·370, P = 0·014). From the multiple linear regression analysis, we found that the peak C-peptide along with iAUC $_{30\text{-}180}$ glucagon affected the value of ∆Glucose 180 min after adjusted for sex, age at diagnosis, and daily insulin dose (R^2 = 0·720, P < 0·001) (Table 5).

4 Discussion

Islet beta cells in patient with T1D exhibit ethnic heterogeneity, with better beta cell function observed in Chinese patients with T1D. The functional changes in alpha cells in patients with T1D with different stimulated C-peptide levels remain poorly elucidated. Our research aimed to investigate the association between insulin and the phase of glucagon secretion in patients with T1D exhibiting different stimulated C-peptide levels. Our findings can be summarized as follows: 1) stimulated C-peptide levels affected the paradoxical increase in postprandial glucagon secretion in patients with T1D, especially the elevated extent of the late glucagon response; 2) the late glucagon response affects the glucose increment in 180 min.

Glucose homeostasis is primarily regulated primarily by the two key regulatory hormones insulin and glucagon. Many studies have shown that the metabolic expression of uncontrolled diabetes is the consequence of abnormalities in these two hormones (22). During fasting, the balance between insulin and glucagon is crucial in preventing hypoglycemia. In individuals without diabetes, the basal glucagon concentration maintains approximately half of the basal hepatic glucose production, which regulates fasting plasma glucose levels (23). Brown et al. reported normal fasting glucagon concentrations for up to 12 months following the diagnosis of T1D (9). However, other studies have indicated slightly lower fasting glucagon levels in patients with T1D compared to healthy subjects (10, 20). In contrast, our findings revealed a significantly reduction in fasting glucagon concentration among patients with T1D compared to healthy controls. This insufficient fasting glucagon levels partly supported the susceptibility to hypoglycemia in patients with T1D. The persistence of C-peptide secretion exhibits considerable variability widely in individuals diagnosed as T1D (24, 25). Several studies have confirmed inadequate glucagon secretion during hypoglycemia in patients with T1D, with elevated glucagon correlating with beta cell function (26, 27). Our study did not identify in fasting glucagon levels in patients with T1D exhibiting different C-peptide levels, probably due to the fact that fasting is not accurately reflect the hypoglycemic status.

In addition to the abnormal fasting glucagon levels, the increasing postprandial glucagon in T1D after oral glucose and mixed-meal intake has been demonstrated in several studies (9, 28). Our results support these previous reports by revealing elevated glucagon levels at 30-180min and total glucagon response during the BMTT in patients with T1D. Local insulin secretion in intra-islets plays a critical role in suppressing glucagon secretion during

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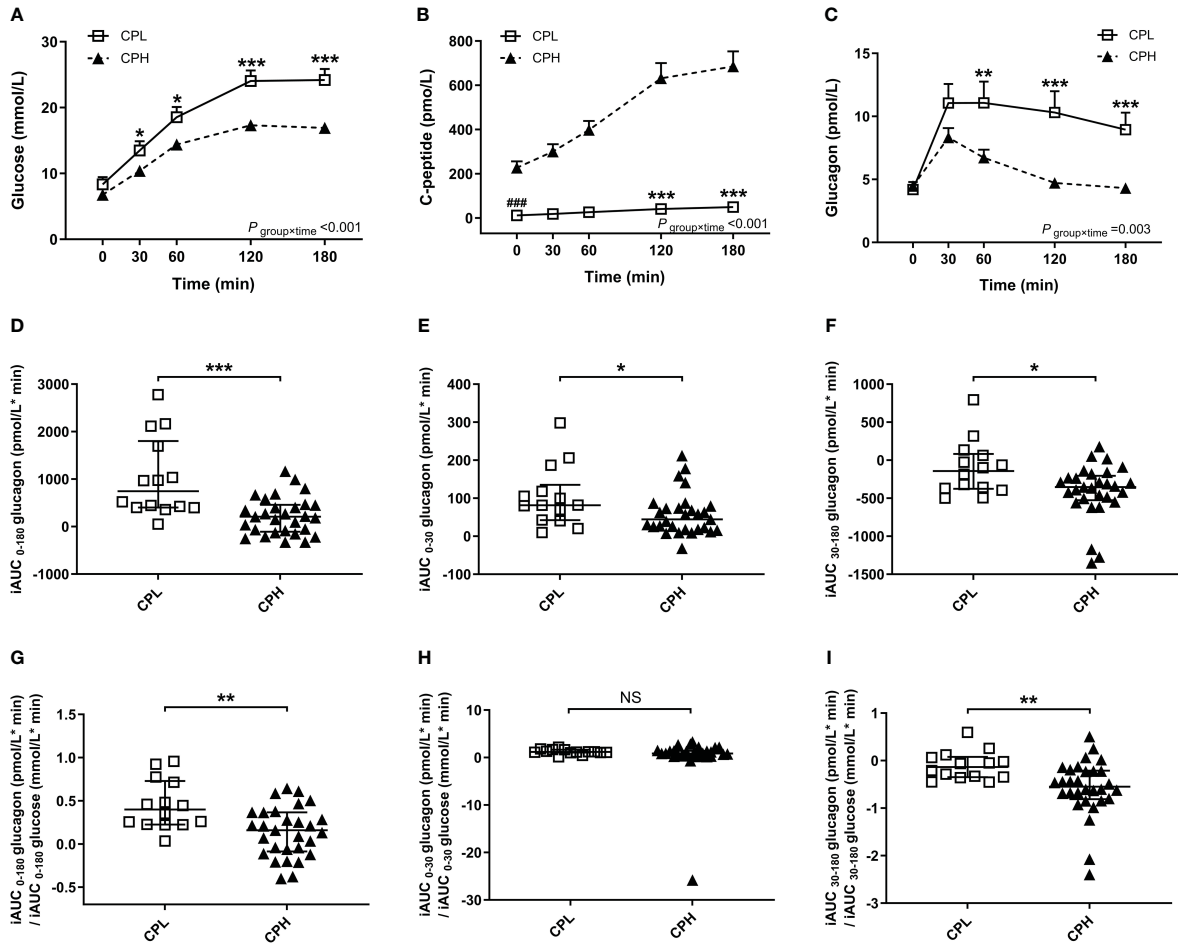

FIGURE 2
Results of the BMTT in the patients with T1D divide into subgroups according to peak C-peptide level: CPL group (peak C-peptide < 200pmol/L, n=14, open squares), CPH group (peak C-peptide ≥ 200pmol/L, n=29, filled triangles). **(A–C)** The curve of plasma glucose, C-peptide and glucagon during the BMTT. * The repeated measured variables following the BMTT between two groups were investigated by generalized estimating equations with baseline measurement (0 min) as the covariates. * The simple effect of group was analyzed using the generalized estimation equation; *P<0·05; **P<0·01; ***P<0·001. # The difference of baseline measurement was analyzed using the Mann-Whitney U test; ###P<0·001. **(D–F)** The incremental area under the curve of glucagon from 0 to 180min, 0 to 30 min and 30 to 180 min during the BMTT. **(G–I)** The ratio of iAUC glucagon to iAUC glucose from 0 to 180 min, 0 to 30 min and 30 to 180 min during the BMTT. *P<0·05; **P<0·01; ***P<0·001. BMTT, steamed bread meal tolerance test; T1D, type 1 diabetes; CPL, C-peptide low; CPH, C-peptide high; iAUC, incremental area under the curve; NS, nonsignificant.

hyperglycemia (29). The Diabetes Control and Complications Trial (DCCT) demonstrated that a stimulated C-peptide value ≥ 200 pmol/L showed benefits for glycemic control and a minimal number of complications (30). Therefore, we set the threshold of 200 pmol/L for C-peptide to compare the differences in glucagon secretion in patients with T1D exhibiting different residual beta cell function. Our results showed that stimulated C-peptide levels in T1D did affect the paradoxical increase in glucagon secretion after the BMTT. Patients with peak C-peptide below 200 pmol/L exhibited higher peak glucagon and glucagon at 60-180 min. Furthermore, the value of ▵glucagon at 120 and 180 min was negatively correlated with peak C-peptide in response to the BMTT.

Our findings above are consistent with previous research indicating a decrease C-peptide levels and an increase in postprandial glucagon levels as T1D progresses (9, 11, 31). However, our results contrast with the previous studies that T1D individuals with different residual C-peptide had comparable glucagon response after oral glucose challenge (12, 13, 32). The observed discrepancy may be attributed to the difference in stimulation components. The previous study used a mixed meal tolerance test (MMTT) consisted of 50-72% carbohydrate, 18-37% protein and 10-14% fat, while our participant underwent the BMTT, which consisted of a high amount of 90·3% carbohydrate and a low proportion of 8·4% protein and 1·2% fat. It has been reported that alpha cells exhibit varying degrees of sensitivity to different stimuli. In healthy individuals, glucose administration inhibits glucagon secretion, while protein intake activates glucagon secretion (33). The inclusion of protein in the diet of patients with T1D has a significant impact on the total concentration and peak levels of glucagon (34). In fact, several amino acids, such as alanine, arginine, cystine, and proline, have been reported to stimulate glucagon secretion in rodents. Although

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TABLE 4 Multiple linear regression coefficients for the association of the late glucagon response with peak C-peptide in type 1 diabetes after the BMTT.

	Dependent variables		Beta	Regression coefficient (95% CI) [a]	*P* value
Model 1	iAUC 30-180 glucagon	Peak C-peptide	-0·47	-186·08 (-354·22 to -17·94)	0·031
		Sex	0·28	207·61 (-42·10 to 457·31)	0·100
		Age	-0·45	-176·07 (-358·45 to 6·31)	0·058
		BMI	0·08	33·13 (-121·14 to 187·40)	0·666
		Duration of diabetes	0·09	26·56 (-94·52 to 147·65)	0·659
		Daily insulin dose	-0·22	-119·07 (-308·35 to 70·21)	0·210
		Peak glucose	0·51	186·13 (34·75 to 337·50)	0·017
Model 2	iAUC 30-180 glucagon /iAUC 30-180 glucose	Peak C-peptide	-0·51	-0·29 (-0·50 to -0·08)	0·008
		Age	-0·08	-0·04 (-0·24 to 0·16)	0·672
		Daily insulin dose	-0·12	-0·09 (-0·34 to 0·16)	0·460

Multiple linear regression analysis was performed. In the model 1 where the dependent variable is iAUC 30-180 glucagon, F test = 2·515; R^2 = 0·335; P = 0·033. In the model 2 where the dependent variable is iAUC 30-180 glucagon/iAUC 30-180 glucose, F test = 3·330; R^2 = 0·204; P = 0·029. [a] Regression coefficient represent change in the iAUC 30-180 glucagon (pmol/L*min) or iAUC 30-180 glucagon/iAUC 30-180 glucose for per SD increase in the value of independent variables shown. BMTT, steamed bread meal tolerance test; Beta, standardized regression coefficient; CI, confidence interval; iAUC, incremental area under the curve; iAUC 30-180 glucagon/iAUC 30-180 glucose, the ratio of the glucagon iAUC from 30-180 min to the glucose iAUC from 30-180 min.

the glucagon-stimulating potency of individual amino acids is not yet known in humans (29), it is plausible that amino acids present in the diet may stimulate glucagon secretion.

Glucose-stimulated insulin secretion is characterized by a transient first phase followed by a sustained second phase (35). Given the inter-regulatory role of islet beta and alpha cells (29), it is reasonable to hypothesize that glucose-stimulated glucagon secretion may be a biphasic pattern. Two studies have utilized a 30-min time points after glucose intake to differentiate between early and late glucagon response in diabetic patients (14, 15), and highlighted the importance of early glucagon response in T2D, and had suggested that individuals with newly diagnosed T2D exhibited impairment only in early glucose-stimulated glucagon suppression (15). Similarly, our study revealed the iAUC of glucagon from 0-30 was higher in T1D than healthy control, suggesting that T1D also has an impairment in early suppression of glucagon stimulated by glucose compared with subjects with normal glucose tolerance. Our study found that the iAUC of glucagon from 30-180 min was higher in patients with peak C-peptide below 200 pmol/L, and this increase was negatively correlated with peak C-peptide. In contrast, there was no linear correlation between the iAUC of glucagon from 0-30 min and peak C-peptide levels, although the iAUC of glucagon from 0-30 min was also higher in patients with peak C-peptide below 200 pmol/L. Therefore, we identified the importance of the late glucagon response in T1D, and proposed, for the first time, that C-peptide levels influence the late glucagon response in T1D individuals following the BMTT in our present study.

TABLE 5 Multiple linear regression coefficients for the association of the glucagon response with △glucose 180 in type 1 diabetes after the BMTT.

	Beta	Regression coefficient (95% CI) [a]	*P* value
Peak C-peptide	-0·27	-1·29 (-2·40 to -0·18)	0·024
iAUC 0-30 glucagon	0·46	2·01 (1·01 to 3·02)	<0·001
iAUC 30-180 glucagon	0·39	1·73 (0·71 to 2·76)	0·002
Sex	-0·16	-1·44 (-3·23 to 0·35)	0·111
Age at diagnosis	0·27	1·30 (0·28 to 2·32)	0·014
Daily insulin dose	-0·07	-0·48 (-1·81 to 0·86)	0·472

Multiple regression analysis was performed, F test = 15·442; R^2 = 0·720; P < 0·001; [a] Regression coefficient represent change in the △glucose (mmol/L) for per SD increase in the value of independent variables shown. BMTT, steamed bread meal tolerance test; Beta, standardized regression coefficient; CI, confidence interval; iAUC, incremental area under the curve.

Glucose has a direct effect on glucagon secretion, with glucose administration inhibiting glucagon secretion in healthy individuals (33). However, the effect of glucose on glucagon secretion and the underlying mechanisms are complex and disputed. Salehi et al. found that elevated blood glucose levels elicited a dose-dependent stimulation of glucagon release (36). Vieira et al. found an inhibition of glucagon secretion from isolated mouse islets within the glucose range of 4 to 20 mmol/L, while glucagon secretion increased when glucose levels exceeded 20 mmol/L (37). Wang et al. reported an increase in glucagon secretion from the pancreases of insulin deficient T1D rats when perfused glucose concentration was raised from 5 to 25 mmol/L (38). Glucose elevations that are not accompanied by a parallel increase in insulin levels will result in hyperglycemia, which in turn stimulates glucagon secretion in a counter-regulatory manner. In our present study, we found higher glucose trend following the BMTT in patients with peak C-peptide below 200 pmol/L, and the mean value of glucose at 120-180 min were above 20 mmol/L. In addition, the peak C-peptide along with peak glucose were correlate with the $iAUC_{30\text{-}180}$ glucagon in the multiple linear regression. These finding suggest that excessive postprandial glucose levels following the BMTT in the patients

with peak C-peptide below 200 pmol/L may further promote postprandial glucagon secretion. However, the interaction between glucose and glucagon is complex, and as our study is a cross-sectional study, it is difficult to establish a causal association. Further investigations are warranted to validate these findings.

One remaining question is whether there is a differential glucagon response adjusted for glucose increment after oral glucose intake, and whether this response is dependent on the stimulated C-peptide levels in individuals with T1D. Kramer et al. found that glycemic normalization prior to oral glucose ingestion did not change the suppression of glucagon per glucose increment in long-duration T1D during an oral glucose tolerance test (21). In our present study, we utilized the ratio of iAUC glucagon to iAUC glucose to estimate the time course of glucose-induced glucagon secretion adjusted for glucose increments in patients with T1D exhibiting different stimulated C-peptide levels. The glucagon level adjusted for glucose increments was almost identical to those prior to correction. We found that the late glucagon response adjusted for glucose increment were higher in patients with T1D who had lower peak C-peptide levels, and these responses was negatively correlated with peak C-peptide levels. These results further highlighted the significant of the late glucagon response in individuals with T1D with different stimulated C-peptide levels.

In our present study, we found that glucose at 30-180 min following the BMTT was higher in patients with peak C-peptide < 200 pmol/L. Additionally, the pair of parameter between Δglucose and Δglucagon at 30-180 min was positively correlated. Importantly, the late glucagon response and peak C-peptide levels were correlated with the postprandial glucose increment after adjusting for confounding factors. These results suggested that patients with T1D exhibiting low stimulated C-peptide levels had greater paradoxical increase in postprandial glucagon secretion, especially in the late glucagon response. The lack of adequate insulin secretion along with excessive glucagon secretion usually lead to hyperglycemic state.

To our knowledge, this study is one of the few investigations examining the effect of stimulated C-peptide levels on glucagon secretion in Chinse patients with T1D. Moreover, it is the first study to report a significant influence of C-peptide levels on the glucagon response during the late postprandial phase in individuals with T1D. Notably, exogenously insulin, unlike endogenously insulin, is insufficient to provide high concentrations of insulin within the islets of Langerhans, resulting to elevated glucagon levels and further complicating glycemic control in T1D (39). Recent years have witnessed the development of various classes of glucose-lowering medications. Targeting excessive postprandial glucagon secretion represents a potential strategy to mitigate hyperglycemia in individuals with T1D. A phase I trial showed that a single dose of a glucagon receptor antibody (volagidemab) decreased insulin requirements and improved glycemic control in patients with T1D (40). Furthermore, in a subsequent phase II clinical trial, 12-week adjunctive therapy with volagidemab was associated with decreased HbA1c and stable insulin dose (41). Therefore, this study has important clinical and public health implications as it provides insight into the role of postprandial hyperglycemia in T1D. Furthermore, it may provide a theoretical basis for the development of glucagon-based therapeutic approaches for T1D.

Our study has some limitations which include a small sample size and an open-label, cross-sectional design. Secondly, glucagon-like peptide 1 (GLP-1) could inhibit glucagon release in a glucose-dependent manner. The absence of evaluation of GLP-1 in our study hinders our understanding of the precise role of stimulated C-peptide levels in glucagon secretion under the influence of other potential factors in patients with T1D. Thirdly, several previous studies found that sex influences postprandial glucagon secretion in healthy individuals (42) and patients with T1D (11). However, our study did not find a significant impact of sex on the conclusion that stimulated C-peptide affects postprandial glucagon secretion in patients with T1D. Further research is required to confirm this conclusion and explore the underlying mechanisms. Recent evidence has demonstrated a 'pancreatic' 29-amino-acid glucagon in patients who had undergone totally pancreatectomy, indicating the existence of extrapancreatic glucagon (43); Unfortunately, due to the inclusion of participants without any history of pancreatic and intestinal surgeries, we were unable to distinguish whether the glucagon detected in our study contained exocrine pancreatic secretion. The physiology of exocrine glucagon in T1D remains unclear.

5 Conclusion

In conclusion, this study investigated the regulation of glucagon secretion in individuals with T1D and demonstrated that stimulated C-peptide levels play a role in the paradoxical increase of postprandial glucagon secretion, particularly in the late glucagon response. This exaggerated postprandial glucagon secretion contributes to the elevation of blood glucose after oral glucose intake. Our findings have significant implications for understanding the pathophysiology of postprandial hyperglycemia in T1D and may provide the development of glucagon-based therapies for this condition.

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 author.

Ethics statement

The studies involving humans were approved by The local ethics committee of the First Affiliated Hospital of Nanjing Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LZ: Data curation, Formal analysis, Methodology, Writing – original draft. YQ: Data curation, Methodology, Project administration, Writing – review & editing. YH: Data curation, Writing – review & editing. QH: Data curation, Writing – review & editing. QW: Data curation, Writing – review & editing. XW: Data curation, Writing – review & editing. MZ: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1419329/full#supplementary-material.

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Engineered IRES-mediated promoter-free insulin-producing cells reverse hyperglycemia

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Background: Endogenous insulin supplementation is essential for individuals with type 1 diabetes (T1D). However, current treatments, including pancreas transplantation, insulin injections, and oral medications, have significant limitations. The development of engineered cells that can secrete endogenous insulin offers a promising new therapeutic strategy for type 1 diabetes (T1D). This approach could potentially circumvent autoimmune responses associated with the transplantation of differentiated β-cells or systemic delivery of viral vectors.

Methods: We utilized CRISPR/Cas9 gene editing coupled with homology-directed repair (HDR) to precisely integrate a promoter-free EMCVIRES-insulin cassette into the 3' untranslated region (UTR) of the GAPDH gene in human HEK-293T cells. Subsequently quantified insulin expression levels in these engineered cells, the viability and functionality of the engineered cells when seeded on different cell vectors (GelMA and Cytopore I) were also assessed. Finally, we investigated the therapeutic potential of EMCVIRES-based insulin secretion circuits in reversing Hyperglycaemia in T1D mice.

Result: Our results demonstrate that HDR-mediated gene editing successfully integrated the IRES-insulin loop into the genome of HEK-293T cells, a non-endocrine cell line, enabling the expression of human-derived insulin. Furthermore, Cytopore I microcarriers facilitated cell attachment and proliferation during *in vitro* culture and enhanced cell survival post-transplantation. Transplantation of these cell-laden microcarriers into mice led to the development of a stable, fat-encapsulated structure. This structure exhibited the expression of the platelet-endothelial cell adhesion molecule CD31, and no significant immune rejection was observed throughout the experiment. Diabetic mice that received the cell carriers reversed hyperglycemia, and blood glucose fluctuations under simulated feeding stimuli were very similar to those of healthy mice.

Conclusion: In summary, our study demonstrates that Cytopore I microcarriers are biocompatible and promote long-term cell survival *in vivo*. The promoter-free EMCVIRES-insulin loop enables non-endocrine cells to secrete mature

insulin, leading to a rapid reduction in glucose levels. We have presented a novel promoter-free genetic engineering strategy for insulin secretion and proposed an efficient cell transplantation method. Our findings suggest the potential to expand the range of cell sources available for the treatment of diabetes, offering new avenues for therapeutic interventions.

KEYWORDS

IRES, CRISPR/Cas9, promoter-free, insulin-producing cells, diabetes

Introduction

Diabetes mellitus (DM) is a complex and heterogeneous disease with an increasing prevalence worldwide. Projections indicate that the number of diabetic patients will reach 693 million by 2045 (1), continuing to rise at an alarming rate and becoming a significant global health burden (2, 3). The dysfunction of islet β-cells is a critical factor in the pathogenesis of diabetes. In type 1 diabetes (T1D), these cells are targeted by auto-reactive T cells, leading to a loss of 70–90% of β-cell mass and a consequent reduction or cessation of insulin secretion (4). In type 2 diabetes (T2D), environmental factors such as malnutrition or obesity impair insulin function, accelerating the depletion of islet β-cells (5). Despite ongoing research and advances in medication and treatment, DM remains a critical health issue due to its associated complications. Prolonged hyperglycemia can lead to severe pathological conditions, including renal failure, cardiovascular diseases, metabolic syndrome, and hormone dysfunctions (6, 7). The limitations of organ transplantation, including donor shortages and the requirement for immunosuppressive drugs (8, 9), make regular insulin administration the primary treatment for diabetes (10).

However, the burden of frequent insulin injections underscores the need for less invasive methods of exogenous insulin delivery or the restoration of β-cell function. These approaches offer great promise for achieving long-term glycemic control in the treatment of diabetes. Insulin-secreting cells generated from stem cells (11, 12) or through genetic engineering of various cell types have emerged as advanced alternative therapeutic strategies for diabetes (13–17). However, it is challenging that not all stem cell lines differentiate with equal efficiency (11). Due to the pluripotent nature of stem cells and the complexity of the differentiation process, there is a risk that unintended or potentially dangerous non-target cell types may persist within the final population of differentiated cells. Of particular concern is the possibility of highly proliferative undefined progenitor cells or residual human pluripotent stem cells, which could pose a tumorigenic risk (18, 19).

Gene-edited engineered cells represent a potentially more convenient and stable alternative to insulin-producing cells that require complex differentiation steps. The implantation of glucose-responsive insulin-expressing elements into extra-pancreatic mammalian cell types could offer protection against DM (20, 21). Previous studies have demonstrated that human embryonic kidney 293T (HEK-293T) cells are capable of producing high levels of anti-diabetic proteins (22–24).

However, current approaches to engineer insulin-secreting cells often rely on viral vectors, where insulin transcription and translation are driven by strong promoters (25, 26). A significant concern with this method is the potential for insertional mutagenesis, which can result from enhancer-mediated dysregulation of adjacent genes or abnormal splicing processes (27). To mitigate these risks, we have designed a promoter-free insulin secretion system using HEK-293T cells.

CRISPR-Cas9 is undoubtedly a powerful gene editing tool for our purposes. This technique allows for precise insertions or deletions within genomic DNA sequences, correcting even genetically mutated cells and tissues. Cells possess several mechanisms for repairing double-strand breaks (DSBs), including non-homologous end-joining (NHEJ), which typically introduces unpredictable mutations, and homology-directed repair (HDR), which involves copying donor DNA strands into DSB regions (28). Genome editing based on HDR is increasingly being studied for its ability to precisely insert DNA fragments, and it has become a well-established and precise gene editing method (29–34).

To achieve promoter-free insulin secretion, we selected the Internal ribosome entry site (IRES) as a key component. This natural translational enhancer, found in various mRNAs, has garnered increased attention due to its ability to initiate cap-independent translation (35–37). The encephalomyocarditis virus (EMCV) IRES, in particular, has been shown to be active in most tissues and organs (37). Consequently, EMCV-IRES-based vectors are frequently employed to co-express multiple therapeutic genes within the same transcription unit, playing a significant role in combined gene therapy (38–43).

In the current study, we successfully integrated a promoter-free IRES-human furin-cleavable human insulin (IRES-hINS) fragment into the GAPDH locus using a CRISPR-Cas9-mediated HDR-based knock-in strategy. This approach resulted in an increase in insulin secretion without altering gene transcription in the cell itself, successfully reversing STZ-induced diabetes in mice over a prolonged period. These findings suggest a highly promising approach in the field of diabetic therapeutics.

Materials and methods

Generation of insulin-producing cell lines

Cell culture

HEK-293T cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified eagle's medium (DMEM, D-glucose content 4.5g/L), supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin) and maintained in a humidified chamber at 37 °C and 5% CO_2. All cultured medium were obtained from Hyclone Laboratories Inc (Logan, UT, USA).

Plasmid construction and generation of EMCVIns

The donor plasmid, ires-eGFP (+HAs) donor-1 (Cat # 87865) with human GAPDH left and right homologous arms was purchased from Addgene. Codon-optimized furin-cleavable human-derived insulin (hIns) was synthesized according to previous reports (44). To be brief, modifications have been made to replace the 62nd arginine to leucine and lysine (29th and 31st) to arginine respectively, which could favor the furin-mediated cleavage at B chain junctions of pro-insulin to obtain mature insulin and C-peptide. Then, the green fluorescence protein (GFP) (next to the EMCV-IRES) was replaced with the above-mentioned codon-optimized mCherry-P2A-hInsulin (mchP2AIns) sequences by inserting EcoR I restriction site using the site-directed mutagenesis kit (Vazyme, China) and henceforth called ires-mchP2AIns (+HAs) donor plasmid. Later, the mcherry sequence in the ires-mchP2AIns (+HAs) donor plasmid was replaced with a puromycin DNA sequence to create ires-puroP2AIns (+HAs) donor plasmid. The sgRNA plasmids were constructed by inserting sg1 and sg4 sequences into pCas-Guide-GFP (Origene Cat # GE100012) as per the manufacturer's instructions and are referred to as Cas-sg1 and Cas-sg4, respectively. The primers used in this study are given in Supplementary Table 1.

Cas-sg1 and ires-puroP2AIns (+HAs) donor plasmids were co-transfected into HEK-293T cells using jetPRIME polyplus transfection reagent (Polyplus Transfection, France) following the manufacturer's protocol. Later, the cells were screened with 10 µg/mL of puromycin for five passages to get pure lines of insulin-producing HEK-293T cells and henceforth named as EMCVIns cells.

Transfection and integration verification

HEK-293T cells were seeded into 12-well plates at a density of 5 × 10^5 cells/well and allowed to attach overnight. Then, 1.5 ug DNA (1 ug donor plasmid + 0.5 ug Cas-sgRNA plasmid) and 3 µl jetPRIME polyplus transfection reagent (Polyplus Transfection, France) were used for transfection in each well following the manufacturer's protocol. After 48h, the successfully integrated cells showed red fluorescence and were imaged using an inverted fluorescence microscope (Nikon, Japan). The efficiency of the successful genomic integration of donor DNA (mchP2AIns) was calculated using Flow cytometer (BD Accuri C6, USA).

The genomic DNA was isolated from both transfected (donor and sgRNA plasmid transfection as mentioned above) and control (without transfection) cells using Multisource Genomic DNA Miniprep Kit (Axygen, USA) following the manufacturer's instructions. The target site was PCR amplified with different specific primers (Supplementary Table 2) and the PCR amplicons were analyzed with Tanaon-4200 Chemiluminescent Imaging System. The successful integration of the donor DNA (mchP2AIns/puroP2AIns) sequences into the precise GAPDH genomic locus was verified using DNA sequencing.

Immunofluorescence staining

EMCVIns cells were seeded into 12-well plates at a density of 5 × 10^5 cells/well and allowed to attach overnight. Next, cells were fixed with 4% paraformaldehyde for 15 min, permeabilizated with 0.1% Triton X-100 for 5 min and blocked for 1 h in 3% BSA (Sigma-Aldrich, SRE0096). Subsequently, cells were incubated with an anti-insulin primary antibody (1:100 dilution, Abcam, EPR17359) overnight at 4°C. After 3 times PBST washing, the cells were incubated with Alexa Fluor 488-conjugated secondary antibodies (1:500 dilution, Thermo Fisher Scientific, A32731) for 1 h at 37°C. Next, the cells were washed three times with PBST and counterstained with DAPI (0.5g/ml) for 5 mins at RT. Then the cells were imaged using confocal microscopy (Leica, Germany).

Insulin secretion assays

Cellular Insulin Secretion Assay

HEK-293T cells were seeded into 12–well plates at a density of 5 × 10^5 cells/well and incubated at 37°C overnight (DMEM, D-glucose content 4.5g/L). About 24h later, the cells were placed with fresh complete medium. 1.5 ug DNA (1 ug mchP2AIns/puroP2AIns donor plasmid+ 0.5 ug Cas-sgRNA plasmid) was transfected using polyplus transfection reagent as mentioned above. About 4 h later, the transfected cells were replaced with fresh complete medium. Then the supernatant was collected after 24h and 48h respectively post-transfection.

Glucose-stimulated insulin secretion

Screened pore EMCVIns cells were starved in Krebs-Ringer buffer supplemented with 2mM glucose for 2 h in a 37°C, 5% CO_2 incubator and were stimulated by 1000µL Krebs-Ringer buffer with low (5mM), middle (11mM) or high (25mM) glucose concentration. Supernatants were collected after 2 h post stimulation. To extract the insulin component from the cells, we treated the cells with 1000 µL of acid-ethanol solution (containing 74% [v/v] ethanol, 1.4% hydrochloric acid, and 24.6% ultrapure water) at 4°C overnight. All the secreted insulin level was measured using a sandwich ELISA kit (ABclonal, Wuhan, China) according to the manufacturer's instructions. Additionally,

the total DNA content of each sample was determined using a DNA Quantification Kit (TIANGEN, China) to standardize insulin secretion.

Microencapsulation of EMCVIns cells

Cytopore I (GE, USA) and GelMA (Engineering for Life, China) are two common commercial biomaterials to encapsulate cells. Before encapsulation, EMCVIns cells cultured in the 2D system were harvested, labeled with lipophilic tracer DiO (Yeasen, Shanghai, China) for 20 mins at 37°C and washed with D-PBS three times. The Cytopore I biomaterials were soaked in D-PBS, sterilized in high-pressure steam, and followed by washing with D-Hanks and stored in DMEM with 10% FBS before use. An adequate number of primed microcarriers were added to the non-treated tissue culture plate to cover the bottom, to which the DiO-stained EMCVIns cells were then added. Crystal violet staining and CCK-8 (Yeasen, Shanghai, China) kit was used to monitor cell proliferation. At the same time, EMCVIns cells were mixed with GelMA-60 by following the manufacturer's instructions. GelMA-60 inclusions were labeled with Calcein-AM (Yeasen, Shanghai, China) to identify living cells.

To measure the secreted human insulin in cell culture, the culture supernatants of Cytopore I and GelMA-60 encapsulated cells were collected after 24 h, centrifuged to remove the cell debris, and evaluated by ELISA kit. The empty microcarriers and GelMA-60 were used as controls.

Mouse studies

8-week-old male C57BL/6 mice were purchased from Beijing Vital River Laboratories and were randomly divided into four groups (n=6 in each group). STZ (Sigma Aldrich, USA) was dissolved in sterile citrate buffer (0.05 M sodium citrate, pH4.5) and injected intraperitoneally into mice (40 mg/kg) for five consecutive days. Control age-matched mice received the same volume of citrate buffer. Fourteen days after the initial STZ injection, serum glucose level was measured every 3 days from tail vein blood using a One-touch glucometer (Roche) in 6 h fasted mice. Mice with serum glucose levels ranging between 12 to 20 mmol/L for continuous 3 days were considered diabetic.

Transplantation of EMCVIns cells

About 5×10^6 EMCVIns cells were resuspended in DMEM, taken in a 2 mL syringe, and allowed to sink for a while before being seeded on Cytopore I. The extra medium was then expelled, and the encapsulated microcarriers were subsequently injected into the inguinal fat pad of the mice. Another set of mice received the same number of encapsulated control HEK-293T cells, whereas the control group received empty microcarriers without cells. Serum glucose level was monitored every 3 days after implantation.

The intraperitoneal glucose tolerance test

An intraperitoneal glucose tolerance test (IPGTT) was performed on mice on day 14 of post-implantation. Mice were given an intraperitoneal injection of glucose (2 g/kg body weight) after overnight fasting. The glucose levels were measured after the injection at regular intervals of 0, 15, 30, 60, 90, and 120 min, post-glucose injection. Healthy mice served as the control. To measure the human insulin in the mice plasma, mice were anesthetized and the blood was collected from the abdominal aorta followed by a centrifugation at 3000rpm for 10 min to get the serum. The obtained serum was subsequently analyzed with an ELISA kit to determine the quantities of human insulin.

Statistical analysis

Data were represented as means ± standard deviation (SD). Statistical comparisons were made using Student's t-test or one-way analysis of variance (ANOVA) and Tukey post-test. Statistical significance was considered if $P < 0.05$.

Results

In vitro expression of insulin by mchP2AIns-293 cells

We successfully engineered insulin-producing cells by integrating a modified insulin gene into the GAPDH locus of HEK-293T cells using CRISPR/Cas9-mediated HDR (Figure 1A). A reporter system coupled with the modified insulin gene allowed direct quantification of CRISPR/Cas9-induced HDR-mediated insulin gene integration. We selected two different sgRNA sequences targeting the human GAPDH locus from a previously published report (45). Flow cytometry analysis indicated that integration frequency was slightly higher with Cas9sg1 (4.8–5.1%) compared to Cas9sg4 (4.1–4.3%), with a transfection efficiency of 84.2% (Figure 1C). No mCherry-positive cells were detected in the absence of either sgRNA or donor plasmid. Subsequent genomic DNA PCR and sequencing of mCherry-positive cells confirmed the integration of mchP2AIns at the GAPDH 3'UTR, demonstrating HDR-mediated targeting (Figure 1D).

To assess whether the integrated modified insulin gene could secrete mature insulin into the culture medium, both mchP2AIns cells and their supernatant were analyzed by ELISA. A fresh culture medium served as a control. The culture supernatant of mchP2AIns cells and the intracellular level showed mature insulin production of 0.45 ± 0.061 μIU·10^5 cells^{-1}·mL^{-1}·24 h^{-1} and 0.38 ± 0.06 μIU·10^5 cells^{-1}·mL^{-1}·24 h^{-1}, respectively, indicating successful synthesis and secretion of mature insulin via CRISPR/Cas9-induced HDR-mediated gene integration (Figure 1B).

We then investigated the effect of various glucose concentrations and formulations on EMCV IRES-mediated insulin synthesis. mchP2AIns cells were subjected to glucose stimulation tests with both L- and D-glucose. Notably, the cells

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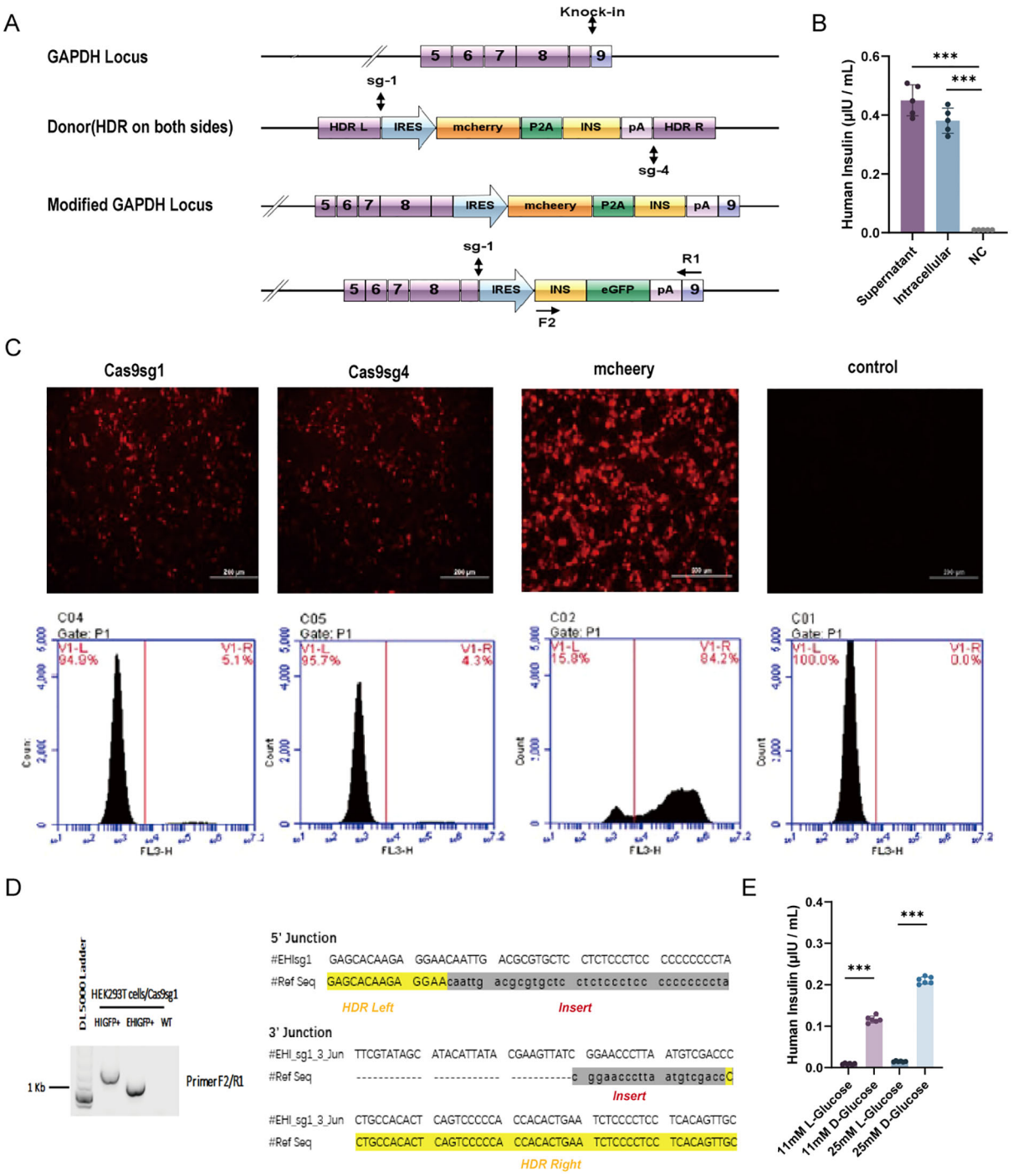

FIGURE 1
HDR-mediated modified human insulin gene knock-in in HEK293T cells. **(A)** Schematics of the donor plasmid and targeting strategy for HDR-mediated knock-in of the modified human insulin at GAPDH 3′-UTR. Dashed lines indicate sections of homology between the GAPDH genomic locus and donor plasmid DNA. Arrows indicate the positions of PCR primers for insulin integration examination. **(B)** The quantity of insulin in culture media (supernatant) and cell lysis(intracellular) were assessed using ELISA. Fresh culture medium was used as negative control (NC). **(C)** HDR-mediated integration efficiency of Cas9sg1 and Cas9sg4 using fluorescence images and Flow cytometer analysis. Cas9 plasmid without sgRNA was used as a control. **(D)** Genome PCR analysis of mcherry^{+} cells produced with Cas9sg1 in sequencing results of the PCR amplicons with expected modifications (human insulin gene) were integrated precisely at both 5′- and 3′-junctions. **(E)** Insulin secretion from mchP2AIns in response to different types of glucose stimulation. Data are expressed as mean ± SD. n = 5; ***p < 0.001 by student's t-test.

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responded to D-glucose, which is metabolically active in the human body (Figure 1E).

EMCVIns cells give stable insulin secretion *in vitro*

To obtain pure lines of engineered insulin-producing cells, we replaced the mCherry sequence in the ires-mchP2AIns (+HAs) donor plasmid with a puromycin DNA sequence, creating the EMCVP2AIns (+HAs) donor plasmid. We then co-transfected Cas-sg1 with the EMCVP2AIns (+HAs) donor plasmid into HEK-293T cells, as previously described. The cells were subsequently screened with 10 μg/mL of puromycin for five passages to obtain pure lines of insulin-secreting HEK-293T cells, designated as EMCVIns (Figure 2A). Immunofluorescent staining confirmed insulin expression in EMCVIns cells (Figure 2B).

Then, to evaluate whether the CRISPR/Cas9-induced HDR-mediated modified insulin gene integration in EMCVIns cells could successfully secrete mature insulin into the culture medium, both EMCVIns cells and their supernatant were analyzed by ELISA. The mature insulin production detected in the EMCVIns culture medium after stable transfection was 1.95 ± 0.26 $\mu IU \cdot 10^5 cells^{-1} \cdot mL^{-1} \cdot 24\ h^{-1}$ (Figure 2C). EMCVIns cells were then stimulated with different concentrations of D-glucose (5mM, 11mM, 25mM), and ELISA was used to assess changes in insulin levels. The results showed no significant changes in insulin levels with varying glucose concentrations (Figure 2D). Furthermore, we measured the total insulin synthesis versus secretion levels of engineered EMCVIns cells at 24 h, 48 h, and 72 h after cell seeding. The data indicated that the total insulin synthesis level did not increase over time (Figure 2E). However, the insulin secretion level increased with time and peaked at 48 hours (Figure 2F). These results suggest that mature insulin can be successfully synthesized and secreted into the supernatant using the CRISPR/Cas9-induced HDR-mediated gene integration method.

Cytopore I is favorable for EMCVIns microencapsulation

The triggered immune response is an unavoidable and crucial factor that must be considered during implantation (46, 47). To minimize immune responses potentially triggered by EMCVIns cell engraftments, the cells were encapsulated using Cytopore I or GelMA-60 at a density of 5 x 10^5 cells/mL. To assess the survival

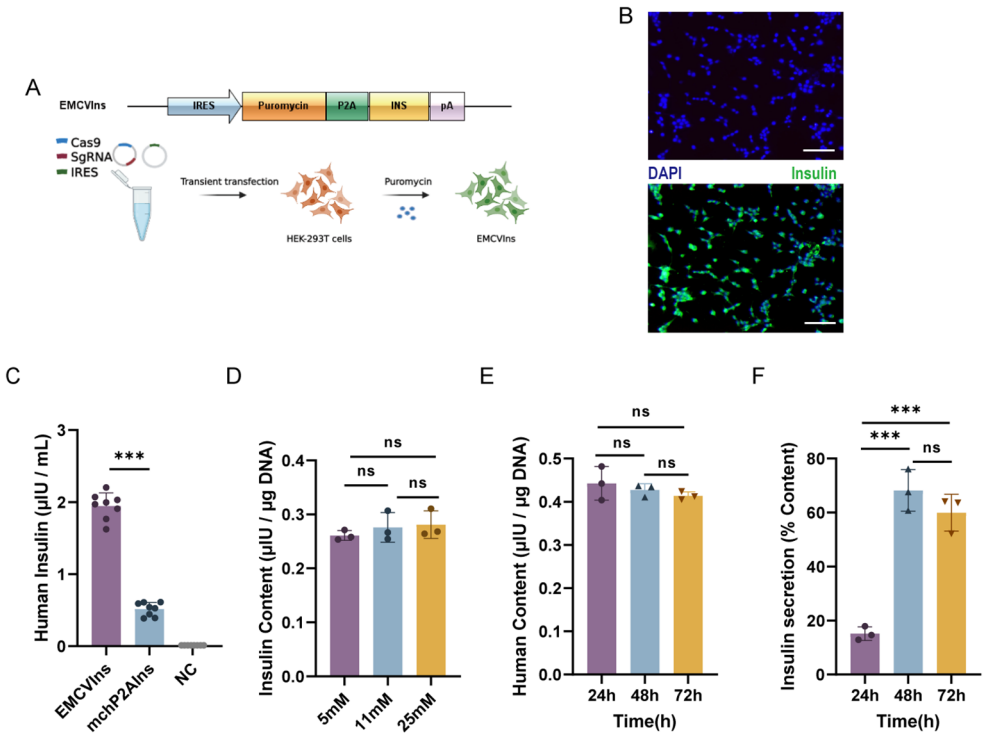

FIGURE 2
Engineering of stable EMCVIns cells for exogenous insulin. **(A)** Schematic of modified donor plasmids and progression to obtain stable insulin expressing EMCVIns cells. **(B)** Insulin expression was analyzed with immunofluorescence. Green fluorescence indicated human insulin. Scale bar: 50μm **(C)** Supernatant Insulin level produced by EMCVIns cells and mchP2AIns cells were assessed using ELISA. Total insulin content by EMCVIns cells at different concentrations of **(D)** glucose and **(E)** incubation times. **(F)** Insulin secretion from engineered cells at different times. Fresh culture medium was used as negative control (NC). Data are expressed as mean ± SD. n = 5; **p < 0.01, ***p < 0.001 by student's t-test, one-way ANOVA and Tukey post-test.

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of EMCVIns cells post-encapsulation, Calcein-AM staining was used to label living cells within the capsules. Both materials were found to carry living cells (indicated by green fluorescence), with Cytopore I microcarriers encapsulating a greater number of living cells (Figure 3A). The relative cell proliferation of EMCVIns cells encapsulated in both materials was determined using a CCK-8 assay by measuring absorbance at 450 nm. Cytopore I-encapsulated cells exhibited greater proliferative ability compared to GelMA-60 (Figures 3B, C), and viability was also confirmed by crystal violet staining (Figure 3D). To ensure continuous insulin secretion and to evaluate whether the encapsulation materials could hinder insulin secretion *in vitro*, the culture supernatant was collected and analyzed by ELISA. As shown in Figure 3E, the mature insulin production in the conditioned medium was 0.22 ± 0.02 μIU·10^5 $cells^{-1}$·mL^{-1}24 h^{-1} and 0.66 ± 0.08 μIU·10^5 $cells^{-1}$·mL^{-1}24 h^{-1} for Cytopore I-encapsulated EMCVIns cells. Overall, compared to GelMA-60, Cytopore I was superior for cell survival, proliferation, and did not interfere with insulin secretion, making it the preferred choice for further *in vivo* studies.

Implantation of insulin-secreting EMCVIns into streptozotocin-induced diabetic mice ameliorated hyperglycemia

Diabetic mice were generated by administering streptozotocin (STZ) at a dose of 40 mg/kg to C57BL/6 mice for five consecutive days, as described in the methods (Figure 4A). Hepatic glycogen depletion was confirmed by periodic acid-Schiff reactions in the livers of STZ-treated mice compared to controls (Supplementary Figure 2B). Additionally, H&E staining revealed clear pathological and morphological alterations in the pancreas of STZ-treated mice compared to controls (Supplementary Figure 2A).

To verify the ability of EMCVIns cells to ameliorate hyperglycemia in the diabetic mouse model, Cytopore I-encapsulated EMCVIns cells were implanted into STZ-induced diabetic mice. Fasting blood glucose levels were significantly reduced after EMCVIns + Cytopore transplantation in a time- and dose-dependent manner (Figures 4B, D). Compared to untreated diabetic mice, EMCVIns cells encapsulated in Cytopore

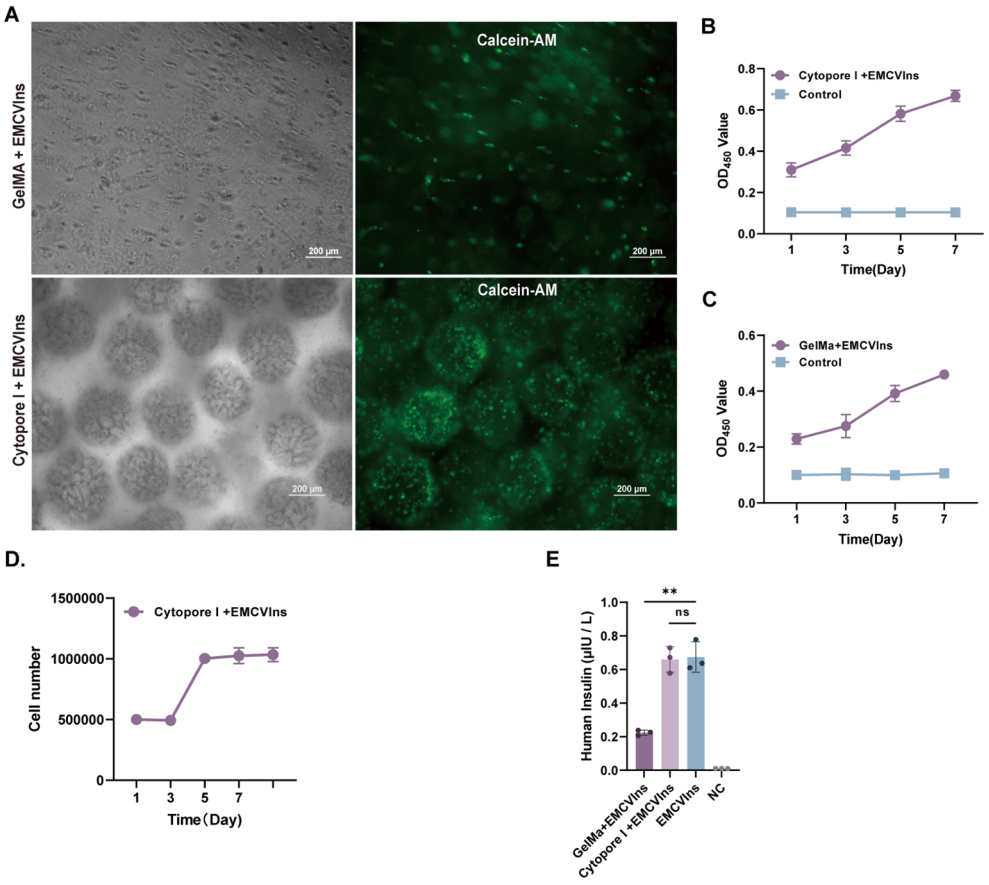

FIGURE 3
Microencapsulation supports the proliferation of EMCVIns cells. **(A)** Morphology of GelMA and Cytopore I encapsulated EMCVIns cells were imaged under bright field and fluorescence by inverted microscopy. Green fluorescence indicated living cells by Calcein-AM. Scale bar: 200 μm. The relative cell proliferation of **(B)** Cytopore I and **(C)** GelMA encapsulated EMCVIns cells were determined by CCK8 assay, respectively. **(D)** The absolute cell viability of Cytopre I encapsulated EMCVIns cells were tested using Crystal violet staining. Unencapsulated cells cultured in a 2D environment served as control. **(E)** Insulin level in the different microencapsulation group was checked by ELISA. An equal number of EMCVIns cells cultured in normal 2D-culture conditions was used as positive control, while fresh culture medium was used as negative control (NC). Data are expressed as mean ± SD. n = 3; **p < 0.01 by student's t-test and one-way ANOVA.

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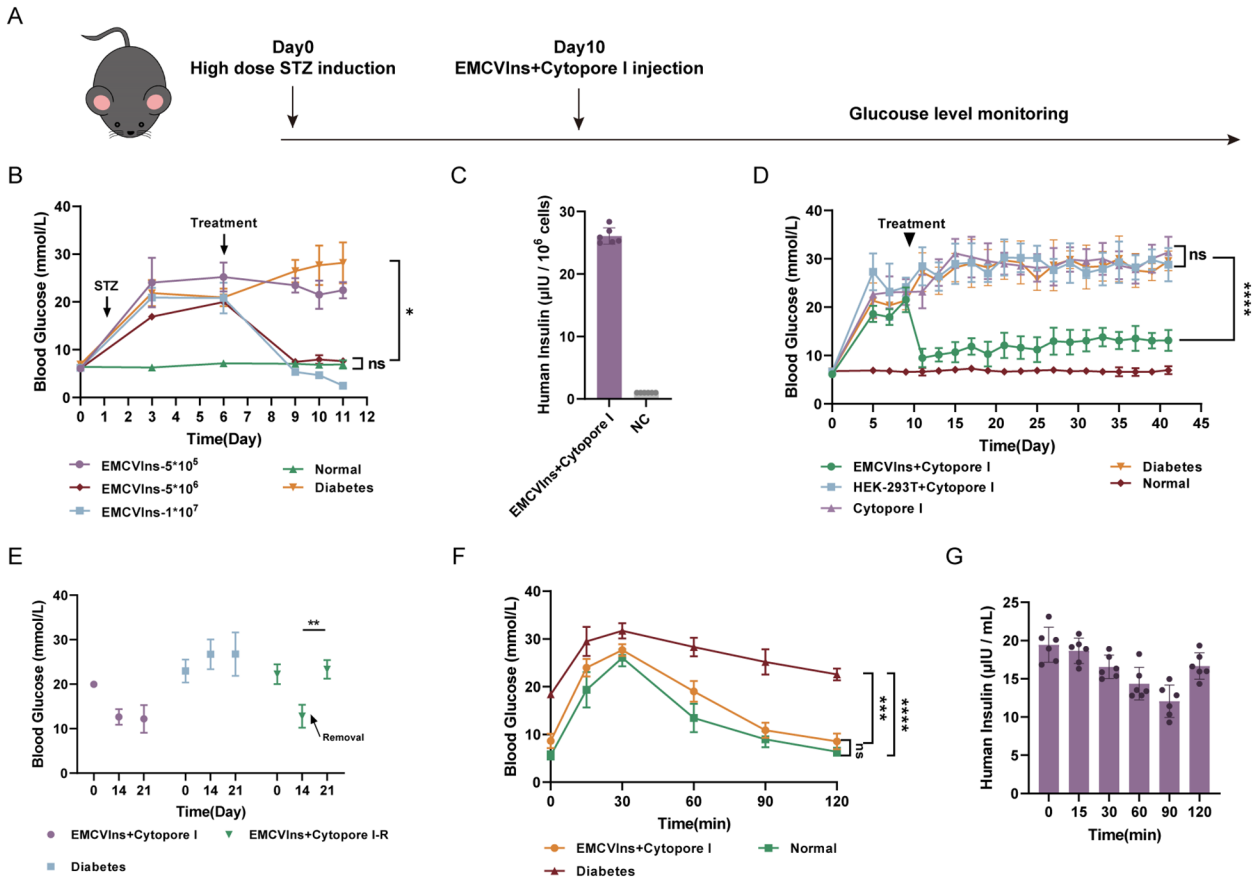

FIGURE 4
Implantation of Cytopore I encapsulated EMCVIns cells ameliorated hyperglycemia in diabetic mouse models. **(A)** Schematic timeline of diabetic mice model induction and implantation treatment. **(B)** Various numbers of EMCVIns cells were encapsulated into Cytopore I microcarriers and given to STZ-induced diabetic mice. Blood glucose was monitored at indicated time points. **(C)** The quantity of insulin produced by $5x10^6$ EMCVIns cells encapsulated in Cytopore I was determined by ELISA. A fresh culture medium was used as negative control (NC). **(D)** An equal number ($5x10^6$) of EMCVIns and HEK-293T cells were encapsulated by Cytopore I and implanted into an inguinal fat pad in STZ-induced diabetic mice, respectively. Blood glucose was monitored at indicated time points. **(E)** In the group of EMCVIns+ Cytopore-R, the implanted EMCVIns cells were removed from treated mice on day 14 as indicated by the arrows. Blood glucose was monitored at indicated time points. Blood **(F)** glucose and **(G)** human insulin levels were monitored at the indicated time point after intraperitoneal glucose stimulation. Data are expressed as mean ± SD.; *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001 by student's t-test, one-way ANOVA and Tukey post-test.

I (1×10^7) effectively reversed blood glucose levels within 72 hours post-implantation. However, this group developed persistent hypoglycemic symptoms and eventually died. In contrast, the group receiving 5×10^5 EMCVIns cells encapsulated in Cytopore I maintained the current glucose level without further increases. Notably, in the group implanted with 5×10^6 Cytopore I-encapsulated EMCVIns cells, fasting blood glucose levels remained close to the normal range throughout the experiment without inducing hypoglycemia (Figure 4B). Therefore, 5×10^6 EMCVIns cells were selected for encapsulation and transplantation in further studies. The insulin production by Cytopore+5×10^6 EMCVIns cells reached 26.09 6.0.13 μIU·10^6cells-1·mL-1 in the culture media, as tested by ELISA (Figure 4C).

Remarkably, EMCVIns + Cytopore implantation therapy reversed high blood glucose concentrations in diabetic mice over six weeks, maintaining fasting blood glucose levels at 11.6 ± 2.15 mmol/L (Figure 4D). Excision of grafts two weeks after transplantation resulted in a spike in fasting blood glucose levels, reverting to hyperglycemia (Figure 4E), confirming the effectiveness of the grafts.

An intraperitoneal glucose tolerance test was conducted on different groups of mice (Cytopore I + EMCVIns-treated STZ-induced mice, STZ-induced diabetic mice, and normal mice) to confirm the ability of EMCVIns cells encapsulated in Cytopore I to maintain blood glucose levels. On day 14, C57BL/6 mice were injected with a 20% glucose solution (2g/kg), and their blood glucose and insulin levels were monitored. Blood glucose levels increased in both the EMCVIns-implanted group and the normal mice group 30 min after glucose stimulation and then recovered, reaching normal levels approximately 2 hours after stimulation (Figure 4F). In the diabetic group, blood glucose levels decreased

slowly after the initial spike and remained hyperglycemic. Human insulin produced by EMCVIns cells, detected in the serum of the mice, declined within 90 min after glucose stimulation and then gradually rebounded (Figure 4G), suggesting its involvement in reducing glucose levels.

The tissue compatibility of microcarriers was examined two weeks post-implantation by retrieving the grafts for further analysis. As shown in Figure 5A, the implanted grafts were encapsulated by the host's adipose tissue, forming a solid, tissue-like structure with its own blood supply. The implants were then paraffin-embedded and sectioned for immunohistochemical (IHC) staining using CD31, a marker for endothelial cells, to detect the presence of endothelial cells in the invaded blood vessels of the excised implants. A strong CD31 signal was observed in the excised implants (Figure 5B), indicating successful blood vessel formation within the grafts. H&E staining was performed on the implants to visualize the morphology of encapsulated cells within the tissue-like structure (Figure 5C). Additionally, immunofluorescent staining for human insulin in the implant revealed significant insulin expression within the EMCVIns-encapsulated grafts (Figure 5C).

To evaluate the biocompatibility of the grafts, immunofluorescence staining was conducted, including the apoptosis factor TUNEL and immune cell markers CD3, CD4, and CD8, at the conclusion of the experiment. Compared to normal adipose tissue (Figure 6B), there was no substantial infiltration of immunological factors in the grafts, indicating that the grafted microspheres provided effective immune isolation. A minimal presence of TUNEL-positive cells suggested a low level of apoptosis within the grafts (Figure 6A). In summary, the study results indicate that Cytopore I is biocompatible and exhibits immune isolation effects. The absence of significant immune factor infiltration suggests that this transplantation method has a low risk of inflammation. Moreover, the grafts enable the formation of host blood vessels, which facilitates the exchange of nutrients, including oxygen, between the graft and the host. This environment supports the prolonged survival of engineered cells *in vivo* while preserving the normal insulin secretion function of EMCVIns.

Collectively, our results demonstrate that Cytopore I-encapsulated EMCVIns implants are capable of uninterrupted insulin secretion, which may contribute to the reversal of hyperglycemia and the potential achievement of long-term blood glucose homeostasis.

Discussion

Despite medical advancements, diabetic patients continue to rely on the invasive infusion of exogenous insulin, primarily insulin analogs, which remain a burden due to the need for multiple dosages. These structurally altered synthetic agonists interact differently with insulin receptors compared to endogenous insulin. Notably, unlike endogenous insulin, synthetic insulin analogs can act at nearly all ligand concentrations under abnormal physiological conditions, leading to shorter or longer receptor stimulation and potentially significant alterations in subsequent signaling and biological effects (48, 49). The use of

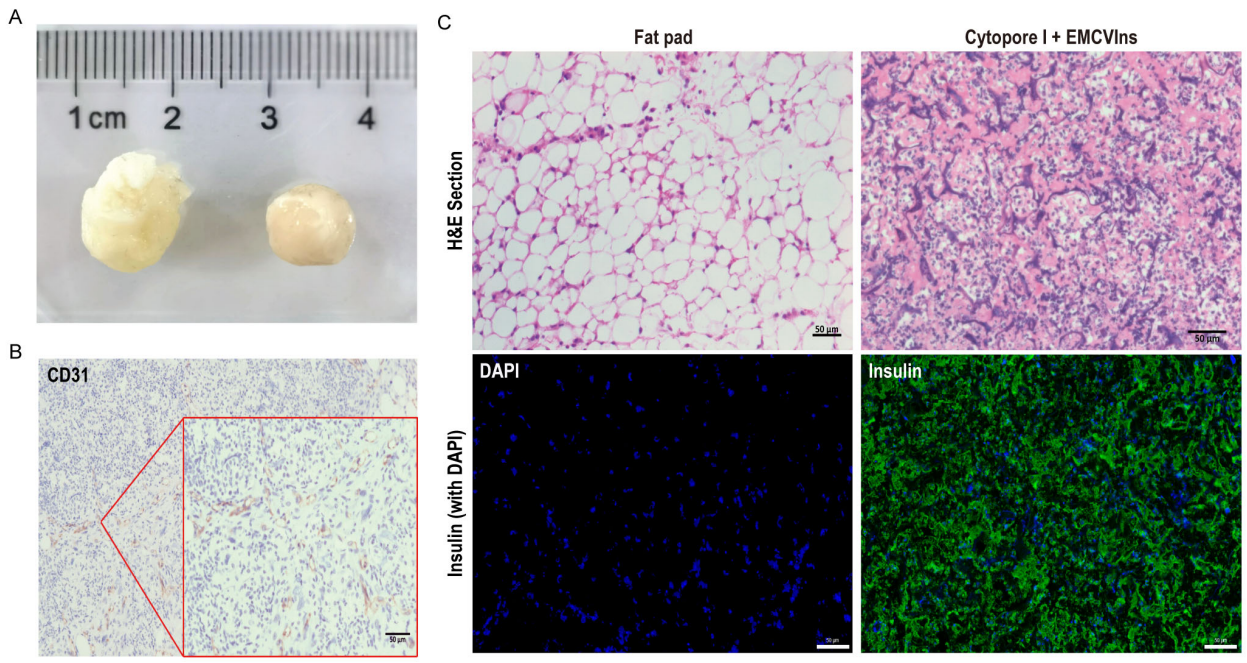

FIGURE 5
Tissue compatibility of Cytopore I microcarriers. 200 µL of Cytopore I encapsulated EMCVIns cells were injected into the inguinal fat pad of C57BL/6 mice. **(A)** Gross view of Cytopore I microcarriers formed structures 14 days after injection. **(B)** IHC staining to check the expression of CD31(platelet endothelial cell adhesion molecule 1) on sectioned Cytopore I microcarriers formed structures. **(C)** H&E staining on sections of the normal fat pad and Cytopore I+ EMCVIns cells- formed structures. Immunofluorescence staining to confirm human insulin expression (Green) on sections of the normal fat pad and Cytopore I+ EMCVIns cells- formed structures. Scale bar: 50 µm.

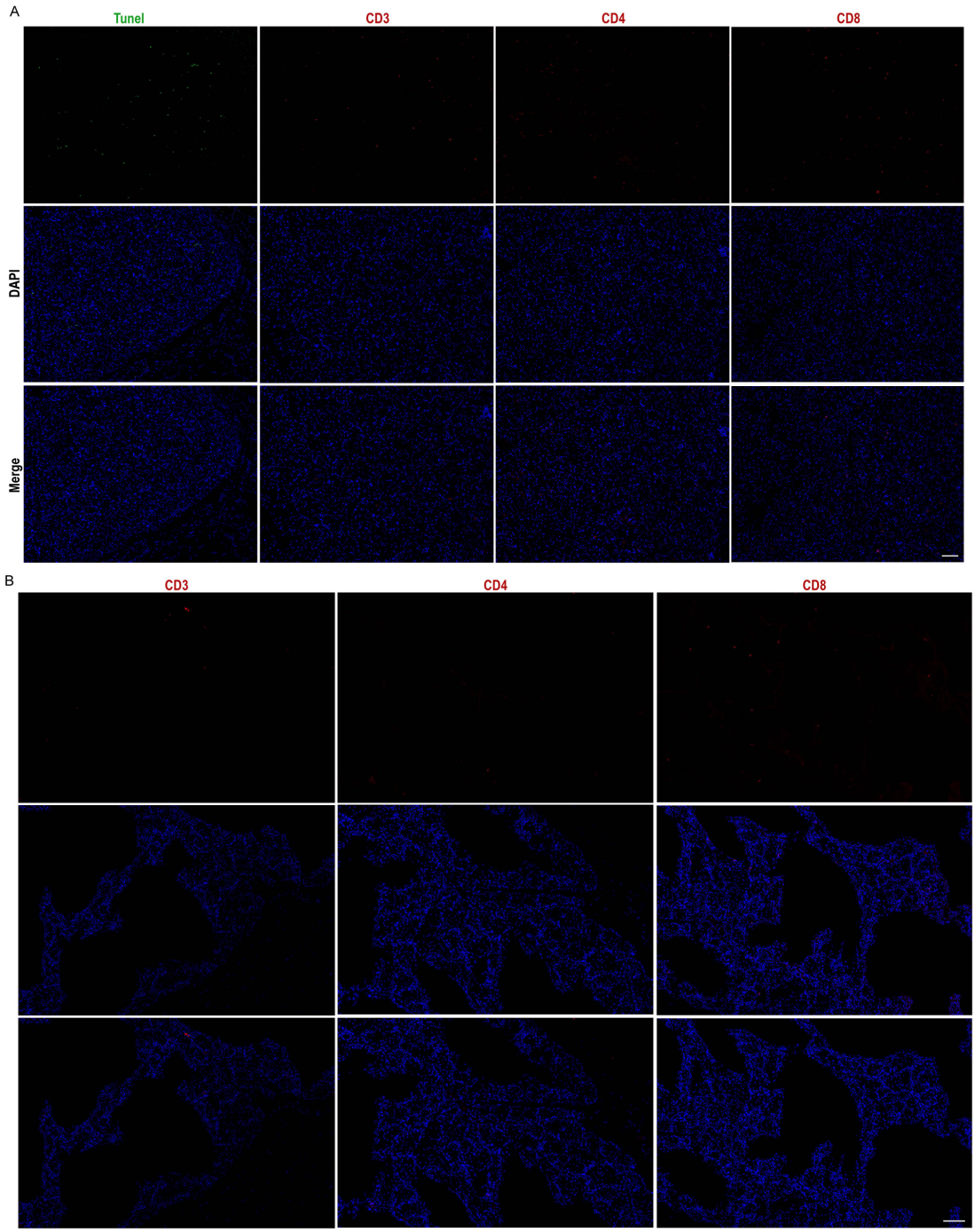

FIGURE 6
Grafts exhibit good *in vivo* biocompatibility. Cytopore I+ EMCVIns cells Graft **(A)** and normal mouse adipose tissue **(B)** apoptosis factor Tunel (green), immunity factors CD3, CD4, CD8 (red) characterization, and DAPI (blue). Scale bar: 100 μm.

gene editing to create insulin-secreting engineered cells typically involves introducing strong promoter-mediated insulin-secreting constructs into the genome or cells using lentiviruses or adenoviruses (50–52). However, it has been shown that the intervention of a strong promoter may trigger the silencing or aberrant expression of nearby genes. Additionally, studies have revealed that a vector-borne promoter, intended to drive the expression of the transgene, can be randomly integrated, potentially leading to the unexpected activation of nearby genes, including oncogenes (53, 54).

Site-specific gene integration enables stable expression of exogenous genes, heralding a new era in gene therapy. With the aid of CRISPR/Cas9 technology, targeted DNA breaks can be introduced at specific genomic sites using pre-designed sgRNAs, facilitating precise HDR-based integration that reduces the risk of off-target integration (55, 56). Leveraging this approach, we have

constructed a promoter-free, IRES-based expression system that couples insulin expression with the robust expression of GAPDH without disrupting its own expression.

To create a promoter-free endogenous insulin expression system, we synthesized modified insulin genes featuring furin-excisable sites (PC1/3 and PC2 recognition sites modified accordingly) based on previous research (44) These genes were integrated into a specific site of the GAPDH locus in HEK-293T cells using CRISPR-Cas9 tools (Figures 1A, C, D). The successful integration and secretion of mature insulin, facilitated by the modified furin-cleavable sites, were confirmed via ELISA (Figures 1B, E, 2B). However, the engineered cells based on this IRES were not glucose-responsive (Figures 2D, E). Stimulation with varying glucose concentrations (5mM, 11mM, 25mM) did not result in significant changes in total insulin content, likely because the EMCV-IRES is not inherently sensitive to glucose. To address this, replacing it with a different type of IRES or integrating glucose-sensitive components could be promising, and such work is ongoing. Additionally, the percentage of insulin secretion peaked at 48 hours (Figure 2F) and then decreased at 72 hours, possibly due to limited cell proliferation in the culture system.

Microcarriers have been effectively utilized for the culture of anchorage-dependent cells, facilitating easy scale-up and benefiting cell therapy applications (57, 58). Adequate oxygen supply and favorable substance exchange are crucial for cell survival post-transplantation (59). We observed that EMCVIns cells could proliferate and grow on both GelMA and Cytopore I microcarriers (Figures 3A–D), with microcarriers being more conducive to cell survival and insulin secretion. Encapsulation in GelMA resulted in insulin secretion levels in the medium supernatant that were less than one-third of those detected under normal conditions, possibly due to the electrostatic interaction between the negatively charged GelMA hydrogel and the positively charged insulin protein (Figure 3E).

Cytopore I microcarriers demonstrated an exceptional ability to form tissue-like structures that support encapsulated transplanted cells with an appropriate blood supply (Figure 5B). After extended *in vivo* transplantation, the cell-carrying microspheres were securely enveloped by the host's inguinal fat pad, creating a stable and robust fat inclusion body, free from vacuolar structures caused by apoptosis (Figures 5A,C). These inclusions simplified the localization of grafts in mice and could be removed as needed, potentially reducing immune risks associated with transplantation (57, 60). The cells within the grafts exhibited healthy growth, with a substantial amount of insulin detected (Figure 5C), and minimal apoptotic factors were observed at the end of the experiment, indicating active cell proliferation (Figures 6A, B). Throughout the six-week study, fasting blood glucose levels in the transplanted mice were maintained at 11.6 ± 2.15 mmol/L, representing a significant decrease compared to diabetic mice (25.16 ± 4.8 mmol/L) and reversing hyperglycemia [fasting blood glucose ≥ 16 mmol/L is considered to be diabetic (61)]. Upon graft removal, fasting blood glucose levels in the de-transplanted group rebounded to over 16 mmol/L (Figure 4E), strongly illustrating the hypoglycemic effect of the engineered cells.

To simulate the changes in blood glucose profile after feeding in mice, we conducted an IPGTT. The results were encouraging, as the blood glucose fluctuations in the EMCVIns-implanted group were comparable to those in the normal group. Following an intraperitoneal injection of glucose, the blood glucose levels in the mice increased rapidly, peaking at 30 min, and then declined until they stabilized at 2 hours, eventually returning to normal (Figure 4F). This indicates that the implanted EMCVIns cells have a beneficial hypoglycemic effect *in vivo*. However, it is disappointing that the current engineered EMCVIns cells did not exhibit glucose-sensing mediated regulation of insulin secretion, mirroring the *in vitro* experimental results. The observed decrease and subsequent increase in human insulin levels in the mice may be attributed to the continuous secretory nature of EMCV-IRES. Initially, insulin is used to equilibrate with the additional high glucose load, leading to depletion and then gradual recovery. Additionally, this may be due to the absence of insulin vesicle structures in the engineered cells, unlike β-cells (Supplementary Figure 3), which prevents the cells from releasing large amounts of stored insulin to address spikes in blood glucose. Further in-depth studies are required to address this limitation.

At the conclusion of the experiment, we assessed the grafts for immune factors, including CD3, CD4, and CD8, and detected only a minimal level of positive expression (Figures 6A, B). The mice in the transplantation group exhibited no signs of inflammation, such as skin ulceration or swelling, and maintained smooth hair and normal body condition. These findings imply that the transplantation of cell-carrying microspheres into the groin is a relatively safe approach. Both the fat pads and the microspheres may provide a degree of immune isolation for the engineered cells, which is beneficial for their long-term survival and the maintenance of their normal function within the host body.

In conclusion, this study—the first to demonstrate that pre-inoculation of IRES-mediated insulin-secreting cells on microcarriers lowers blood glucose in T1D diabetic mice—presents several significant findings: (i) It introduces a promoter-free protein expression system that does not interfere with the host's gene expression. (ii) It proposes a convenient and effective method of cell transplantation that has not triggered significant immune rejection, suggesting the potential for long-term *in vivo* functionality. (iii) It establishes a correlation between insulin production and the number of cells, indicating that the degree of blood glucose regulation can be modulated by adjusting the number of transplanted cells. (iv) It shows post-feeding glycemic kinetics comparable to those of a healthy group, suggesting that this approach may offer greater therapeutic potential for diabetes than long-acting or fast-acting insulin.

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.

Ethics statement

The animal study was approved by the Ethics Committee of the Laboratory Animal Center of Southeast University (No. 20230212028). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Conceptualization, Data curation, Formal analysis,Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft. CH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – review & editing, Funding acquisition. CN: Data curation, Methodology, Validation, Visualization, Writing – review & editing. RL: Data curation, Software, Visualization, Writing – review & editing. YZ: Software, Validation, Writing – review & editing. ZS: Conceptualization, Methodology, Writing – review & editing. HL: Investigation, Resources, Software, Writing – review & editing. ZX: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – review & editing. BS: Conceptualization, Formal analysis, Investigation, Project administration, Resources, Writing – review & editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1439351/full#supplementary-material

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A novel class of oral, non-immunosuppressive, beta cell-targeting, TXNIP-inhibiting T1D drugs is emerging

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Diabetes treatment options have improved dramatically over the last 100 years, however, close to 2 million individuals in the U.S. alone live with type 1 diabetes (T1D) and are still dependent on multiple daily insulin injections and/or continuous insulin infusion with a pump to stay alive and no oral medications are available. After decades of focusing on immunosuppressive/immunomodulatory approaches for T1D, it has now become apparent that at least after disease onset, this by itself may not be sufficient, and in order to be effective, therapies need to also address beta cell health. This Perspective article discusses the emergence of such a beta cell-targeting, novel class of oral T1D drugs targeting thioredoxin-interacting protein (TXNIP) and some very recent advances in this field that start to address this unmet medical need. It thereby focuses on repurposing of the antihypertensive drug, verapamil found to non-specifically inhibit TXNIP and on TIX100, a new chemical entity specifically developed as an oral anti-diabetic drug to inhibit TXNIP. Both have shown striking anti-diabetic effects in preclinical studies. Verapamil has also proven to be beneficial in adults and children with recent onset T1D, while TIX100 has just been cleared by the U.S. Food and Drug Administration (FDA) to proceed to clinical trials. Taken together, we propose that such non-immunosuppressive, adjunctive therapies to insulin, alone or in combination with immune modulatory approaches, are critical in order to achieve effective and durable disease-modifying treatments for T1D.

KEYWORDS

TXNIP, TIX100, verapamil, islets, diabetes, oral medication

Introduction

Since the discovery of insulin over 100 years ago, there have been a lot of advances in the treatment of diabetes. However, the overwhelming majority of novel medications is aimed at Type 2 Diabetes (T2D). In contrast, insulin has remained the main approved treatment for Type 1 Diabetes (T1D). While insulin therapy has come a long way and there have been a lot

of advances in the formulation of insulin and the technology of its delivery, including automated (closed-loop) insulin delivery systems, people with T1D still depend on multiple daily insulin injection or insulin infusions and there is still a lack of effective pharmacological approaches for T1D. Also, for decades the focus has almost exclusively been on identifying immunosuppressive and/or immunomodulatory approaches and this has indeed led to the FDA approval of teplizumab, an infusion regimen of humanized anti-CD3 monoclonal antibodies to delay progression from stage 2 (≥2 auto-antibodies, no symptoms) to stage 3 T1D (≥2 auto-antibodies, with symptoms) (1, 2). However, accumulating evidence from islet biology reveals that beta cells are not just 'victims' and rather play an active part in their own destruction and the pathogenesis of T1D (3). Since beta cells need to produce insulin, their level of protein synthesis is very high and as such they are more prone to endoplasmic reticulum (ER) stress. In addition, their relative lack of anti-oxidative enzymes such as superoxide dismutase, makes them more susceptible to oxidative stress. Thus, various factors such as metabolic stress or viral infection can initiate beta cell dysfunction, senescence, and death. This in turn leads to the release and formation of signals (e.g., chemokines, antigens) that can stimulate immune cells and trigger an autoimmune response. In fact, it has been suggested that such beta cell signals may precede T1D associated insulitis (4) and elevations in blood glucose have been demonstrated prior to the appearance of T1D auto-antibodies (5). It is therefore not surprising that purely immunosuppressive approaches have failed to yield the expected success. This has resulted in a major paradigm shift over the last several years that now recognizes beta cell pathology as an important factor that contributes to the pathogenesis of T1D and that needs to be addressed therapeutically (3, 5, 6). However, doing so has, until a short time ago, also been hampered by the lack of known, actionable targets. Nonetheless, some existing, orally available compounds have been studied in the context of T1D including among others the neurotransmitter, gamma aminobutyric acid (GABA) and the antihypertensive drug, verapamil as recently reviewed (7). However, while GABA has also been shown to act outside of the central nervous system and to exert beta cell protective and regenerative effect in preclinical mouse studies (8), a well-designed, randomized, placebo controlled trial failed to reach its primary endpoint of maintained C-peptide or beta cell function in recent onset T1D (9). On the other hand, verapamil has proven highly promising, demonstrating strong anti-diabetic effects in preclinical models as well as improvements in remaining C-peptide in independent human phase 2 and phase 3 trials in adults (10, 11) and children (12) with recent onset T1D. This is consistent with the fact that verapamil has been shown to downregulate the expression of TXNIP (13) and TXNIP in turn has been demonstrated to represent a promising target to preserve beta cells in T1D (14). In addition, this has led to the development of a new chemical entity, TIX100 (aka SRI-37330) (15), now specifically targeting the TXNIP signaling pathway believed to confer the beneficial verapamil effects (10, 13). TIX100 has just received clearance from the United States Food and Drug Administration (FDA) to start clinical trials and this Perspective therefore focuses on the identification, rationale, development, distinct properties and future implications of this novel class of TXNIP-inhibiting T1D drugs.

Target identification

TXNIP was originally identified as the top glucose-induced gene in a human islet gene expression profiling study (16). TXNIP is a 50kD cellular protein that binds and inhibits thioredoxin and thereby increases oxidative stress and impairs cell function and survival (17). However, the effects of TXNIP go beyond just inhibition of thioredoxin as it has also been demonstrated to play a major role in inflammasome activation especially in the context of ER stress (18, 19) and to modulate microRNAs involved in beta cell apoptosis and the regulation of insulin transcription (20, 21). In fact, TXNIP overexpression promotes beta cell apoptosis (14, 22, 23) and inhibits insulin production (20) (Figure 1**).** More recently, TXNIP (which is also expressed in non-beta cells) has been shown to promote diabetes-associated hyperglucagonemia and alpha cell glucagon secretion (24). TXNIP is well conserved across species and its expression is regulated primarily at the transcriptional level via an E-box motif in the TXNIP promoter (22). Importantly, TXNIP is not only induced by glucose *in vitro*, but pancreatic islet TXNIP expression is also elevated *in vivo* in various diabetes mouse models as well as in islets and beta cells of subjects with T1D and T2D (22, 23, 25). As such TXNIP is thought to contribute to a vicious cycle by further impairing islet function and in turn resulting in worsening of the hyperglycemia.

Genetic proof-of-concept

The role of TXNIP as a detrimental factor in islet biology and contributor to the pathogenesis of diabetes has further been demonstrated by multiple groups and by genetic TXNIP deletions (14, 18, 19, 22). Whole body TXNIP deficiency and beta cell specific TXNIP deletion have been shown to protect mice against diabetes in models of T1D and T2D including streptozotocin (STZ)-induced beta cell destruction and genetic obesity and insulin resistance (14). Moreover, TXNIP was found to represent a critical link between glucose toxicity and beta cell death (23). TXNIP deletion also has beneficial effects in the context other tissues affected by diabetes complications including diabetic cardiomyopathy (26, 27), nephropathy (28, 29), retinopathy (30, 31), and neuropathy (32, 33) further supporting the notion of TXNIP representing an attractive target for systemic inhibition in the treatment of diabetes (17, 34).

Pharmacological proof-of-concept

The elevated TXNIP expression found in human islets from individuals with T1D and T2D and the detrimental effects of increased TXNIP on beta cell survival and islet function provided a strong rationale for attempting to therapeutically inhibit islet TXNIP expression. In fact, the non–dihydropyridine L-type calcium channel blocker and approved antihypertensive drug, verapamil was found to non-specifically inhibit TXNIP expression (13). This effect is based on the verapamil-induced decrease in

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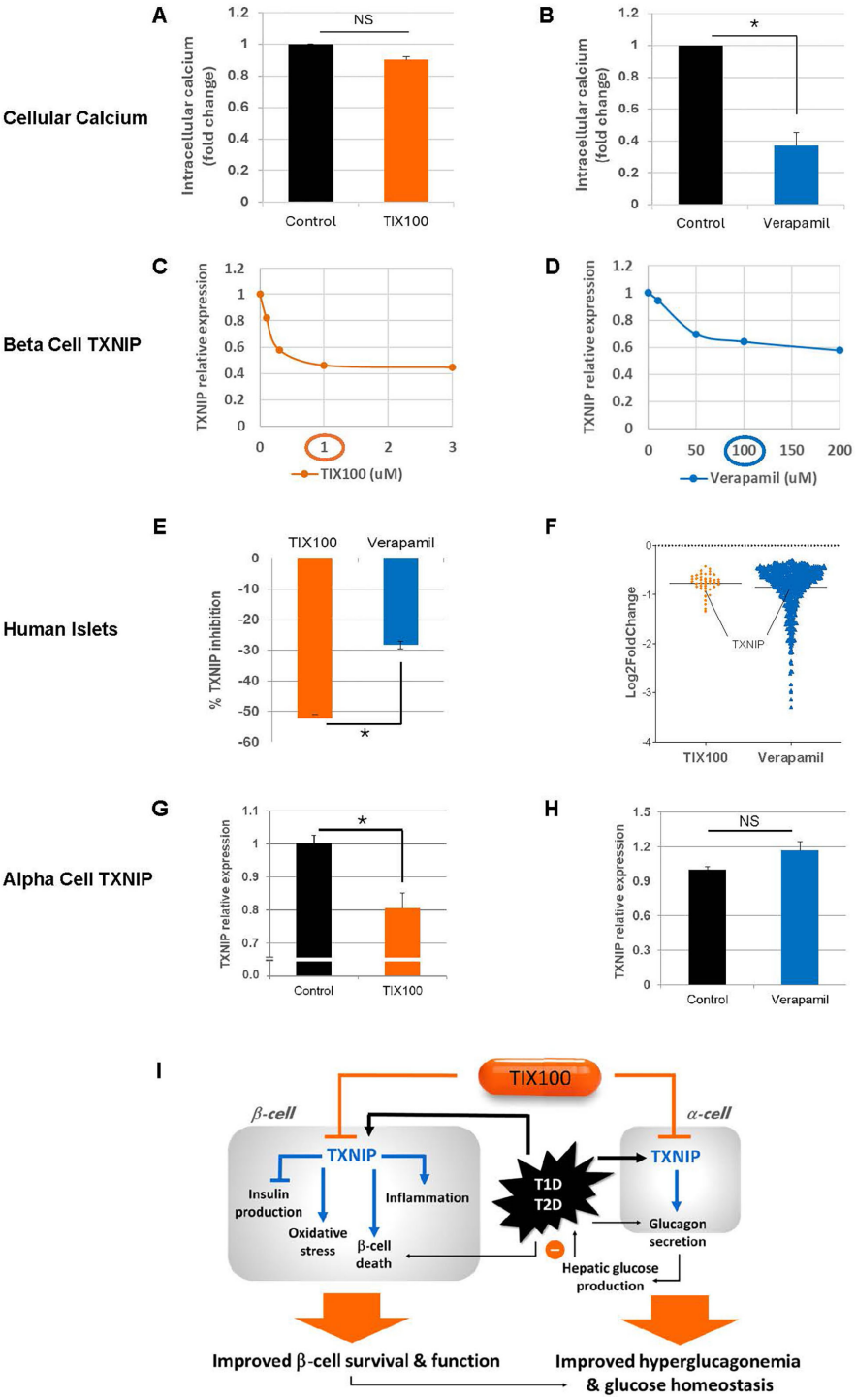

FIGURE 1
Additional insight into the effects of TIX100 and verapamil. Effects of **(A)** TIX100 (1μM) and **(B)** verapamil (100μM) on intracellular calcium of INS-1 cells as assessed by fluorometric calcium assay. Dose-response effects of **(C)** TIX100 and **(D)** verapamil on TXNIP expression as assessed by qPCR in INS-1 cells incubated for 24h at 11.1mM glucose. **(E)** Human islets were obtained from the Integrated Islet Distribution Program (IIDP) and were incubated for 24h at 25mM glucose and treated with TIX100 (1μM) or verapamil (100μM) and the % TXNIP inhibition was assessed by qPCR in the same 3 individual islets donors each serving as its own control. **(F)** Comparison of gene numbers found to be significantly downregulated (adjusted DESeq2 p-value < 0.05) by TIX100 (1μM) or verapamil (100μM) as assessed by RNA sequencing in the same 3 individual islets donors, each dot represents a gene and TXNIP is marked. **(G-H)** Alpha TC1-6 cells were incubated at 25 mM glucose and treated for 24h with TIX100 (1μM) or verapamil (100μM) prior to assessment of TXNIP by qPCR. Means ± SEM, n=3, *p<0.05, two-sided Student's t-test, NS (not significant). **(I)** Schematic of the effects of TIX100 on alpha and beta cells and the implications for beta cell biology and glucose control.

cellular calcium, also found with other calcium channel blockers or calcium chelators and is mediated by the inhibition of calcineurin signaling (13). Interestingly, verapamil was able to mimic the anti-diabetic effects of genetic TXNIP deletion observed in mouse models of T1D and T2D again using STZ-induced and obesity-induced diabetic mice treated with or without oral verapamil (13). Moreover, even when started after the onset of overt diabetes, verapamil was able to rescue the mice from diabetes due to STZ-induced beta cell destruction (13).

Clinical supportive evidence

These pre-clinical findings have now been translated into humans as a phase 2 randomized, double-blind, placebo-controlled trial in adult subjects with recent onset T1D found that individuals receiving once daily, oral, slow-release verapamil to inhibit TXNIP for 1 year had improved beta cell function as assessed by mixed-meal stimulated C-peptide area under the curve (AUC), required less insulin, spent more time within the blood glucose target range, and had significantly fewer hypoglycemic events (10). Importantly, these beneficial effects seem to persist for at least 2 years with continuous medication (11). In addition, an independent phase 3 trial has now further validated the beneficial effects of verapamil in children with recent onset T1D (12). This provides supportive clinical evidence that targeting and inhibiting TXNIP has also anti-diabetic effects in humans with T1D and that (at least in the case of this target) the mouse models used were predictive of the translatability to humans. While verapamil was overall well tolerated in these smaller U.S. studies (10–12, 35), additional larger trials are still ongoing in Europe (NCT04545151) to prove its safety, tolerability and efficacy in this special population of subjects with T1D. In fact, as a calcium channel blocker, verapamil can cause arrythmias and potentially life-threatening atrioventricular heart blocks as well as hypotension limiting its use in some individuals. While no adverse cardiovascular events were observed in the adult studies (10, 11), the pediatric trial reported that in the verapamil group, 6% of participants with one or more nonserious adverse events of special interest, showed electrocardiogram abnormalities including prolonged PR interval, second-degree heart block, and first-degree heart block, and 2% developed hypotension as compared to 0% in the placebo group (12).

New chemical entity for targeted therapy

Even though TXNIP has been validated as a promising therapeutic target for T1D, significant limitations are expected for the off-label use of verapamil to inhibit TXNIP for a T1D indication. Thus, a new chemical entity, specifically designed to inhibit glucose-induced TXNIP expression was developed using high throughput screening of 300,000 small molecules and extensive medicinal chemistry optimization resulting in TIX100, a substituted quinazoline sulfonamide (15). In contrast to verapamil, TIX100 does not function as an L-type calcium channel blocker (15) and as such does not pose a risk for the associated cardiovascular side effects. In addition, we have now confirmed that unlike verapamil, TIX100 does not alter cellular calcium concentrations (Figures 1A, B). TIX100 lowers TXNIP expression by specifically inhibiting the transcriptional activity from a conserved E-box motif of the TXNIP promoter (15). This inhibition is lost with mutation of just the first 7bp of this motif and thus seems to require the intact E-box repeat (15). Indeed, TIX100 was found to be highly effective in downregulating TXNIP expression in rodent and human islets (15).

Interestingly, our dose-response experiments now reveal that while TIX100 reaches its maximal TXNIP inhibitory effect at around 1μM, maximal TXNIP inhibition with verapamil occurs at around 100μM and a more than 100-fold lower concentration of TIX100 was sufficient to achieve comparable TXNIP inhibition (Figures 1C, D). With the molecular weight of both, TIX100 and verapamil being ~450 g/mol, this indicates a much higher potency of TIX100. Moreover, TIX100 is not only more potent, but also more effective than verapamil as suggested by its stronger maximal TXNIP inhibition observed in INS-1 cells (Figures 1C, D). We have now further confirmed this finding in human islets revealing a significantly bigger inhibitory effect in response to TIX100 as compared to verapamil in islets from the same donors (Figure 1E). Furthermore, RNA sequencing of human islets treated with/without TIX100 (15) or verapamil (11) revealed successful downregulation of TXNIP and its signaling pathway, however, while TXNIP ranked 7th of a total of 42 downregulated genes in response to TIX100, it was number 192 of 619 decreased genes in response to verapamil (Figure 1F). Also, while in the case of TIX100 pathway analysis suggested regulation of energy, glucose and apoptosis in line with the known roles of TXNIP (15), verapamil seemed to affect a variety of pathways (11). This large number of off-target effects in the case of verapamil is consistent with its non-specific TXNIP inhibition and its role as a calcium channel blocker. It also highlights the contrast to the much higher specificity of the TIX100 effects on human islets.

As a small molecule, TIX100 is orally available and oral administration protected and even rescued mice from overt diabetes as shown in models of T1D and T2D including again STZ-induced and obesity-induced diabetes (15). In fact, TIX100 mimicked the anti-diabetic effects of genetic TXNIP deletion, whereas it had no additional beneficial effects in the absence of TXNIP, confirming its mode of action via TXNIP targeting (15).

Diabetes, including T1D and T2D, has long been recognized as a bi-hormonal disease characterized not only by absolute or relative insulin deficiency, but also by inappropriately high levels of its counter-regulatory hormone glucagon (36, 37). This hyperglucagonemia leads to excessive hepatic glucose production in the face of already elevated blood glucose levels and results in worsening of the hyperglycemia. In fact, inhibition of glucagon action has previously been shown to ameliorate glucose control in diabetes (38, 39). However, the applicability of such glucagon receptor antagonism approaches has been limited as they have also been reported to cause hepatic steatosis, liver enzyme abnormalities, alpha cell hyperplasia and hyperglucagonemia (39). In contrast, TIX100 decreases alpha cell glucagon secretion and

serum glucagon levels and protects against hepatic steatosis without an increase in alpha cells or elevation in liver transaminases (15). Of note, TIX100 had no effect on glucagon secretion in the context of low glucose, which may help limit the hypoglycemic risk of TIX100. Indeed, even in the context of *in vivo* insulin-induced hypoglycemia, mice treated with TIX100 were able to defend their blood glucose levels equally well to untreated controls (15). This effect of TIX100 on glucagon secretion was also mediated by TXNIP inhibition (15) and mimicked by alpha cell-specific TXNIP deletion (24), but not observed in response to verapamil (10). In fact, we now have found that unlike TIX100, verapamil does not lower alpha cell TXNIP expression (Figures 1G, H), which helps explain why it does not have the glucagon-lowering effects. On the other hand, by controlling TXNIP in beta and alpha cells, TIX100 can improve beta cell health and function and also counteract hyperglucagonemia and excessive hepatic glucose production as summarized in our schematic (Figure 1I). These combined effects may explain the dramatic improvement in glucose homeostasis observed with TIX100 (15). Most recently, TIX100 completed all Investigational New Drug (IND) enabling safety and pharmacokinetic studies as well as chemistry, manufacturing, and control and has received clearance from the FDA to proceed to clinical trials. As such, there are now two TXNIP-inhibiting drugs available for clinical trials and we therefore provide a comparison of their currently known key features (Table 1).

Discussion

In summary, advances in the pharmacological treatment of T1D have been lagging behind those for T2D. Likely contributing factors include the predominant focus of industry on the larger market of T2D and, until recently, the over reliance of the field on technological advances and immunosuppressive approaches combined with the lack of good beta cell targets. Interestingly, the CLVer trial with its factorial design of participants receiving either intensive diabetes management with an automated insulin delivery system or standard diabetes care in addition to verapamil or placebo, nicely demonstrated that while advanced technology can yield optimal glucose control, this is not sufficient to impact beta cell pathology or delay disease progression (40) underlining the need for better pharmacological interventions. Indeed, the more recent realization that any disease-modifying T1D approach also needs to tackle beta cell pathology may lead to some novel breakthroughs. In this regard, targeting TXNIP inhibition seems to provide a promising approach. This is based on the fact that this approach targets an underlying disease pathology providing a strong rationale and that it has been validated in *in vitro* experiments, genetic mouse models, human islets studies and most importantly in adults and children with T1D (10–15, 18, 19, 22, 23, 41). Of note, based on the available preclinical data with verapamil and TIX100, TXNIP inhibitors are also expected to be useful in the treatment of T2D. In fact, several retrospective and a recent prospective clinical study with verapamil support this notion (42–44).

It is also worth noting that neither non-specific downregulation with verapamil, nor specific inhibition with TIX100 completely suppresses TXNIP expression (Figures 1C, D), yet effectively protected against diabetes in different preclinical models (13, 15). This is consistent with the stated therapeutic goal of just normalizing TXNIP to non-diabetic values and provides an additional safety margin (although even complete lack of TXNIP did not seem to cause any relevant detrimental effects in whole body TXNIP deficient mice (14).

While verapamil is immediately available for off-label use due to its FDA approval for hypertension and provides some control of beta cell TXNIP and improvement in beta cell health, it is associated with limitations due to its inherent risk for arrhythmias, heart blocks and hypotension and lacks other TIX100-associated benefits. Conversely, by reducing cellular calcium, verapamil has pleiotropic actions that go beyond TXNIP inhibition, and it remains to be seen whether these effects might provide additional benefits in the context of T1D or cause more side effects (Table 1). TIX100 has the advantage of higher specificity, potency, and effectiveness, and also improves hyperglucagonemia and excessive hepatic glucose production (Table 1), which obviously would also be beneficial in T2D. On the other hand, it still has to pass through the lengthy process of clinical trials to prove its safety, tolerability and efficacy in humans with T1D before becoming freely available in the clinic. Thus, verapamil provides a proof-of-principle for the translatability of the approach and may be helpful as an interim option as long as patients are carefully selected and monitored for any potential cardiovascular side effects. However, ultimately a more specific and targeted approach (such as with TIX100) could help avoid potential off-target effects while promoting the patient's proper endogenous islet cell function. We propose that such an adjunctive oral therapy to insulin alone or in combination with immune modulatory approaches, holds high promise as an effective and durable disease-modifying treatment for T1D.

TABLE 1 Comparison of key features of TIX100 and verapamil.

	TIX100	Verapamil	*References*
Controls beta cell TXNIP & improves beta cell health	✓✓	✓	(10–13, 15)
Controls alpha cell TXNIP & protects against hyperglucagonemia	✓	No	(15, 24)
Controls excessive hepatic glucose production	✓	No	(15)
Provides increased potency, effectiveness & specificity in TXNIP downregulation	✓	No	(11, 15), Figure 1
Maintains cellular calcium & avoids arrhythmia, heart block, or hypotension side effects	✓	No	(15), Figure 1
Reduces cellular calcium resulting in pleiotropic effects that might be beneficial in T1D	No	✓✓	(11), Figure 1

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 author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

GJ: Investigation, Methodology, Writing – review & editing. SJ: Investigation, Methodology, Writing – review & editing. AS: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

AS is also the co-founder and CSO of TIXiMED, Inc.

The remaining 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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Shifting the paradigm of type 1 diabetes: a narrative review of disease-modifying therapies

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A new diagnosis of type 1 diabetes (T1D) may be accompanied by numerous lifelong financial, emotional, and physical challenges, thus advancements in therapies that can delay the onset of clinical disease are crucial. T1D is an autoimmune condition involving destruction of pancreatic beta cells leading to insulin deficiency, hyperglycemia, and long-term insulin dependence. The pathogenesis of T1D is classified into stages, with the first signal being the detection of autoantibodies without any glycemic changes. In the second stage, dysglycemia develops without symptoms, and in stage 3, symptoms of hyperglycemia become apparent, and at this time a clinical diagnosis of T1D is made. As a greater understanding of these stages of T1D have evolved, research efforts have been devoted to delaying the onset of clinical disease. To date, only one medication, teplizumab, has been approved by the Food and Drug Administration (FDA) for the treatment of stage 2 T1D. This narrative review present published trials and ongoing research on disease modifying therapies (DMT) in T1D, the mechanisms of action for each therapy, and the stages of T1D that these interventions are being studied.

KEYWORDS

type 1 diabetes, stage 1 type 1 diabetes, stage 2 type 1 diabetes, stage 3 type 1 diabetes, teplizumab, disease-modifying therapies

Introduction

Type 1 diabetes (T1D) is an autoimmune condition that results in the destruction of insulin producing beta cells in the pancreas by $CD4^+$ and $CD8^+$ T cells and macrophages infiltrating the islets of Langerhans (1). Children with T1D commonly present with symptoms of polyuria, polydipsia, and weight loss, with about one-third of children presenting with diabetic ketoacidosis (2). Diagnostic criteria for diabetes include a fasting blood glucose concentration greater than or equal to 126 mg/dL, a random blood glucose concentration greater than or equal to 200 mg/dL with symptoms of hyperglycemia, a 2-hour glucose level of >200 mg/dL during an oral glucose tolerance test, or a glycated hemoglobin (HbA1c) greater than 6.5%. The exact trigger for the development of T1D is not well understood, however, a growing consensus suggests a convergence of genetic

predisposition and environmental triggers in its pathogenesis. T1D accounts for about 10% of all cases of diabetes worldwide, and it occurs most commonly in people of European descent (1). A study by Gregory et al. found that in 2021, there were about 8.4 million individuals worldwide with T1D, and that by 2040, this number was expected to reach 13.5-17.4 million (3). The predicted rapid rise in cases of T1D coincides with the belief that the environmental effect on susceptibility genes plays a role in its epidemiology (1).

Autoantibodies and screening for T1D

Regardless of the extent environmental and genetic causes are instigating a higher prevalence of T1D worldwide, an autoimmune response eventually occurs. The characterization of this autoimmune response has been known since the identification of autoantibodies in patient serum binding to islet cells dating back to 1974 (4). Identification of islet cell antibodies (ICA) sparked new research using advanced techniques, such as molecular cloning, gel electrophoresis, polymerase chain reaction, and DNA microarray analysis to discover more than ten target antigens related to the immune reaction (5). In 1983, the insulin autoantibody (IAA) was discovered in patients with newly diagnosed T1D (6). Following this, three additional autoantibodies were discovered to aid in screening, analysis, and prediction of T1D: GAD autoantibodies (GADA), discovered in 1990 (7), tyrosine phosphatase-like protein IA-2 autoantibodies (IA-2A), discovered in 1994 (8), and zinc transporter 8 autoantibodies (ZnT8A), discovered in 2007 (9). The type, number, and timing of developing autoantibodies improve predictions about timing of the onset of clinical disease and how the combination of autoantibodies predicts who may or may not respond to preventative therapies (10).

Most screening programs to identify individuals at risk for T1D, such as TrialNet and INNODIA, target relatives of people already diagnosed with T1D in an effort to improve yield and feasibility of using these autoantibodies as the screening tool. This, however, contradicts the fact that over 90% of those who go onto develop T1D do not have a family history (11). These programs have started to include monitoring or screening at risk individuals in the general population, now opting for online consent and optional at-home test kits. In total, the number of individuals without a relative with T1D who have been screened is greater than the number of relatives (11).

Stages of T1D

Multiple prospective, longitudinal studies have identified T1D pathogenesis as a continuum of disease that occurs sequentially at different rates through three separate stages prior to the onset of symptoms (12). While diabetes has historically been diagnosed secondary to symptoms associated with the onset of hyperglycemia, the screening of autoantibodies can now be used to predict risk of developing T1D. The presence of known T1D-associated antibodies and presence of dysglycemia can place screened individuals in one of the three stages. Stage 1 occurs with the presence of two or more T1D-associated autoantibodies with otherwise normal glucose levels. The transition from Stage 1 to Stage 2 occurs when they develop dysglycemia. Stage 2 T1D is notable for loss of beta cell function, leading to elevated fasting plasma glucose levels, impaired glucose tolerance, or mildly elevated HbA1c (12). Stage 3 involves developing clinical symptoms of T1D, including polyuria, polydipsia, or weight loss with hyperglycemia, but still have insulin secretion (12).

Methodology

A comprehensive literature review was conducted within PubMed utilizing the search terms "type 1 diabetes" and "disease modifying therapies." To identify specific medications currently under investigation, additional searches were conducted on ClinicalTrials.gov using the condition filter "type 1 diabetes" and the search terms "beta cell preservation" and "disease modifying." Breakthrough T1D (formerly the Juvenile Diabetes Research Foundation) and TrialNet websites were also reviewed to explore discussions on upcoming clinical trials. Identified medications were then searched in PubMed for publications. Given the relative paucity of literature in this field, exclusion criteria were fairly limited. However, a preference was given to medications demonstrating successful treatment outcomes. There were no limitations based on region of study or population studied. Information for the background studies was located through PubMed by employing the search terms "staging AND type 1 diabetes," and "antibodies AND type 1 diabetes." In total, 14 studies were included (Table 1).

To complement the initial literature search conducted within PubMed, a comprehensive exploration of ongoing and future clinical trials for disease-modifying therapies in early-stage (Table 2) and recent-onset (Table 3) T1D was undertaken. ClinicalTrials.gov was utilized as the primary platform for this investigation. The search strategy employed two filters: "condition" set to "diabetes mellitus, type 1" OR "type 1 diabetes" and a combination of search terms including "stage 1," "stage 2," "stage 3," "disease modifying," and "recent onset." Exclusion criteria were applied to filter out withdrawn or terminated studies. Conversely, inclusion encompassed any study matching the aforementioned search terms with a trial status listed as "recruiting," "active, not recruiting," or "completed" but lacking posted results. In sum, 16 studies relevant to early-stage and recent-onset T1D, summarized in Tables 2, 3, were identified through this search strategy. This approach aimed to provide a comprehensive overview of the current and emerging clinical trial landscape for T1D disease-modifying therapies.

Disease modifying therapies

With the classification of T1D into stages, therapies to intervene at each stage are becoming widely studied. Interventions that have the potential to preserve beta cell function may improve the metabolic and glycemic outcomes in new onset T1D (Figure 1). A majority of trials studying DMTs use C-peptide preservation to quantify responses (Table 1) (13).

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TABLE 1 Published clinical trials of disease modifying therapies in type 1 diabetes.

Medication Class	Studied Medications	Stage of T1D Studied	Longest Follow-Up	Results of Clinical Trial*
Anti-CD3 Monoclonal Antibodies	*Teplizumab*	2	2 years	At 2 years, patients in the treatment group had less reduction in MMTT-stimulated C-peptide AUC when compared to the control group (17).
		3	2.5 years	At a median follow up of 2.5 years, 50% of the teplizumab-treated population remained in stage 2 T1D compared to 22% of the placebo group (21).
Anti-CD20 Monoclonal Antibodies	*Rituximab*	3	1 year	At 1 year, the mean MMTT-stimulated C-peptide AUC was significantly higher in the rituximab group than in the placebo group (23).
Anti-IL-21 & GLP-1 agonists	*Anti-IL-21 & Liraglutide*	3	54 weeks	At 54 weeks, those receiving Anti-IL-21 and liraglutide had 48% higher MMTT-stimulated C-peptide levels when compared to placebo, representing only a 10% decrease from baseline (72)
Anti IL-12 & IL-23 Monoclonal Antibody	*Ustekinumab*	3	1 year	At 1 year, those receiving the intervention had 49% higher MMTT-stimulated C-peptide levels (27)
Dimeric Fusion Protein	*Alefacept*	3	2 years	At 2 years, the alefacept group had lower insulin requirements, fewer hypoglycemic episodes and higher MMTT-stimulated C-peptide levels when compared to the control group (30)
Anti-Thymocyte Globulins (ATG)	*Thymoglobulin*	3	2 years	A 2-year MMTT-stimulated C-peptide AUC was significantly elevated in ATG versus placebo, but not in ATG+GCSF versus placebo. Both ATG and ATG+GCSF were associated with reduced HbA1c at 2 years (32).
Calcium Channel Blockers	*Verapamil*	3	1 year	The treatment group had a 30% higher MMTT-stimulated C-peptide AUC (45).
CTLA-4 Analogs	*Abatacept*	3	2 years	At the 2 year follow up, MMTT-stimulated C-peptide AUC was found to be 59% higher in the treatment vs placebo group (40).
JAK Inhibitors	*Baricitinib*	3	48 weeks	Daily treatment over 48 weeks was associated with an increased meal-stimulated mean C-peptide level (36).
Tumor Necrosis Factor Alpha (TNF-α) Blockers	*Golimumab*	3	1 year	At 1 year, the MMTT-stimulated C-peptide AUC remained higher in the treatment versus placebo group (37). C-peptide AUC decreased 12% with golimumab compared to 56% with placebo.
Tyrosine Kinase Inhibitors	*Imatinib*	3	2 years	The treatment group had a higher MMTT-stimulated C-peptide AUC at 1 year, but this effect was not sustained at 2 years (41).
Neurotransmitter and antigen-based therapy	*GABA and GAD-alum*	3	1 year	No change in glycemia, fasting or meal-stimulated C-peptide AUC at 1 year. Mean fasting glucagon levels did not increase in the GABA or GABA/GAD-alum groups and meal-stimulated glucagon levels were lower in the intervention groups (50)
Autologous Dendritic Cell Therapy	*AVT001*	3	1 year	At 1 year, there were no differences in HbA1c or insulin dose, but there was less decline in C-peptide production (52)
Autologous Mesenchymal Stem Cells (MSC)	*Autologous bone marrow derived MSCs*	3	1 year	Those receiving MSCs had reductions in level 1 and level 2 hypoglycemia, and fewer hypoglycemia events. Earlier treatment (within the first year) was associated with lower HbA1c levels at 1 year when compared to later treatment (at least 1 year after T1D diagnosis) (53)

*AUC, Area Under the Curve; MMTT, mixed meal tolerance test; HbA1c, hemoglobin A1c; GCSF, granulocyte colony-stimulating factor.

Anti-CD3 monoclonal antibodies (*Teplizumab*)

In November 2022, Teplizumab was the first drug approved by the FDA to delay the progression from stage 2 to stage 3 T1D. Teplizumab is a humanized anti-CD3 monoclonal antibody that can reduce T-cell activation, proliferation, and cytokine release *in vitro*. Early studies of the drug's mechanism suggested that it could minimize the effects of $CD8^+$ T cells involved in the autoimmune-related destruction of pancreatic beta cells (14). The initial studies using teplizumab were conducted in those with stage 3 T1D, where participants received either a randomized, placebo, or standard of care design (14).

The first phase 1/2 randomized controlled trial (*Study 1*) tested a single 14-day course of teplizumab in those with recently diagnosed T1D (15). Compared to standard of care, the teplizumab group had preserved beta cell function when comparing C-peptide levels during mixed-meal tolerance tests

TABLE 2 Ongoing, future, and completed clinical trials investigating disease modifying therapies in stages 1 and 2 type 1 diabetes.

Medication Class	Studied Medications	Stage of T1D Studied	Trial Details*
Oral Insulin	*Recombinant Human Insulin (rH-insulin crystals)*	1	Randomized, triple-blind, phase 2 trial (*NCT02620072*) evaluating oral insulin to prevent T1D progression in stage 1, high-risk children aged 2-12 years. The study will assess prevention of dysglycemia or diabetes, as measured by oral glucose tolerance test. Participants are followed for at least 24 months (55).
Anti-CD3 Monoclonal Antibodies	*Teplizumab*	2	A single-arm, open-label, multicenter, phase 4 trial (*NCT05757713*) evaluating the safety and pharmacokinetics of teplizumab in young children (aged 0-8 years) with stage 2 T1D. The study will also assess the development of anti-drug antibodies and neutralizing antibodies. Each participant's involvement may extend up to approximately 26 months (56).
Glucagon-like Peptide-1 (GLP-1) Receptor Agonists	*GLP-1Ra with Teplizumab*	2	Early-phase 1, randomized, quadruple-masked, crossover trial (*NCT06338553*) assessing the safety and efficacy of a single GLP-1Ra dose in combination with teplizumab in participants with stage 2 T1D. Primary outcomes include changes in blood glucose levels, insulin function, and vascular health, as measured by multiple MMTTs conducted pre- and 3-5 months post-teplizumab treatment (57).
Polyclonal antibody	*Anti-Thymocyte Globulins (ATG)*	2	Phase 2, randomized, double-blind, placebo-controlled trial (*NCT04291703*) investigating low-dose ATG to prevent progression from stage 2 to stage 3 T1D in high-risk individuals. Participants are followed for up to 5 years (58).
Antigen-specific Immuno-modulators	*Diamyd*	1 & 2	Phase 2, randomized, parallel assignment, open-label trial (*NCT05683990*) evaluating Diamyd in children and adolescents aged 8-18 years with stage 1 or 2 T1D. Primary outcomes include safety and tolerability, assessed by hematology, clinical chemistry, metabolic status parameters (fasting C-peptide, HbA1c, fasting glucose) and urine analysis. Participants will be followed for 12 months (59).

*T1D, type 1 diabetes; MMTT, mixed meal tolerance test; HbA1c, hemoglobin A1c.

TABLE 3 Ongoing, future, and completed clinical trials investigating disease modifying therapies in recent-onset type 1 diabetes.

Class	Medication	Duration of T1D	Trial Details*
Supercoiled plasmid vector	NNC0361-0041	<48 months	Phase 1, placebo-controlled, double-blind, randomized, dose-escalation, sequential assignment trial (*NCT04279613*) evaluating the safety and tolerability of NNC0361-0041 plasmid in patients with T1D. Primary outcome measures include safety, as assessed by adverse event incidence, and efficacy, as determined by changes in C-peptide levels during MMTT from baseline to 12 months (60).
Interleukin Inhibitors	*Ustekinumab (Anti-Interleukin (IL)-12 and IL-23 Antibody)*	≤100 days	Phase 2/3, randomized, parallel assignment, double-blind, placebo-controlled trial (*NCT03941132*) assessing efficacy and safety of Ustekinumab in T1D. Primary outcome measures include baseline changes in 2-hour MMTT-stimulated C-peptide AUC, HbA1C, insulin use, and incidence of all adverse events. The follow up period is 12 and 18 months from the first dose (61).
Interferons	*Human Recombinant Interferon-Alpha (IFN-a)*	≤6 weeks	Phase 2, randomized, double-blind trial (*NCT00024518*) evaluating interferon-alpha in preserving residual endogenous insulin production. Primary outcomes include C-peptide levels and hemoglobin HbA1c. Participants are followed up at 3-month intervals over the course of 12 monthsr (62).
Interleukin Agonists	*Recombinant human IL-2*	≤ 3 months	Phase 2, randomized, quadruple-blind, parallel-assignment trial (*NCT02411253*) evaluating the efficacy and safety of low-dose IL-2 in preserving beta cell function in recent-onset T1D. Primary outcomes include change in C-peptide AUC, determined after a MMTT at month 12. The study duration is 24 months (63).
Imotopes	*IMCY-0098*	≤6 months	Phase 1, randomized, double-blind, sequential assignment trial (*NCT03272269*) assessing safety and immunogenicity of IMCY-0098 in participants with recent-onset T1D. Primary outcome measures include adverse events, changes in C-peptide production, HbA1c, and changes in IMCY-0098 specific T lymphocyte responses. Participants will be followed for 24 weeks post-enrollment (64).
Stem Cell Therapy	*Adipose Tissue-Derived Stem/Stromal Cells with Cholecalciferol*	≤4 months	Randomized, parallel assignment, open-label, prospective Phase 2 trial (*NCT03920397*) comparing adipose-derived stromal/stem cells plus cholecalciferol to cholecalciferol alone in patients with recent-onset T1D. Adverse effects will be recorded. In addition, glycated hemoglobin, insulin dose, frequency of hypoglycemia, glycemic variability, % of time in hyper and hypoglycemia and peak response of the C-peptide after the MMTT will be measured at three-month intervals for a 24-month period (65).

(Continued)

TABLE 3 Continued

Class	Medication	Duration of T1D	Trial Details*
Perinatal Tissue Derived Cells (PTDCs)	*CELZ-201*	≤6 months	Phase 1/2a, randomized controlled trial (*NCT05626712*) evaluating CELZ-201 therapy in recent-onset T1D. Primary outcome measures include safety and efficacy, assessed by changes in C-peptide during a 4-hour MMTT, HbA1c, exogenous insulin requirements, and autoantibody levels. Study duration is 24 months (66).
Vitamin D Analogs	*Calcitriol*	≤3 months	Phase 2, randomized, double-blind trial (*NCT01120119*) evaluating calcitriol in preserving beta cell function in recent-onset T1D. Primary outcome measures include changes in fasting and stimulated C-peptide, insulin requirements and HbA1c. Participants are followed up for 24 months (67).
JAK Inhibitors	*Abrocitnib; Ritlecitinib*	≤100 days	Phase 2, multi-center, randomized, double-blind, parallel assignment, placebo-controlled trial (*NCT05743244*) comparing two JAK inhibitors in individuals with recent-onset T1D. The primary outcome of interest is the change in stimulated C-peptide production during the 12-month follow-up period (68).
Polyclonal Regulatory T Cells	*$CD4^+CD127lo/-CD25^+$ & Interleukin-2 (IL-2)*	>3 and <24 months	Phase 1, single-arm, open-label (*NCT02772679*) trial evaluating safety and preliminary efficacy of polyclonal regulatory T cells (Tregs) plus IL-2 in patients with T1D. Primary outcome measures include safety, changes in beta cell function (C-peptide in response to serial MMTT), glycemia (HbA1c), and Treg survival (69).
Serine Protease Inhibitors (SERPINS)	*Alpha-1 Antitrypsin; Glassia*	≤6 months	Phase 1/2, randomized, parallel assignment trial (*NCT01304537*) evaluating safety and efficacy of alpha-1 antitrypsin (AAT) in T1D. Primary outcomes include incidence of adverse events, beta cell function, exogenous insulin requirements, and HbA1c. Participants will be followed for 12 months post-enrollment (70).

*T1D, type 1 diabetes; AUC, area under the curve; MMTT, mixed meal tolerance test; HbA1c, hemoglobin A1c.

(MMTT). Following this initial study, the Autoimmunity-Blocking Antibody for Tolerance (AbATE trial), a randomized phase 2 trial (with a 14-day course of teplizumab administered 12 months after diagnosis of T1D also successfully reduced the decline in C-peptide response to a MMTT at 24 months from initial treatment when compared to the control group (15–18). Two phase 3 clinical trials for teplizumab (*Protégé* and *Encore*) tested three different dosing regimens of the drug over two courses that were 6 months apart with the end points of exogenous insulin use and HbA1c (19). However, the *Protégé* study was terminated for not meeting its

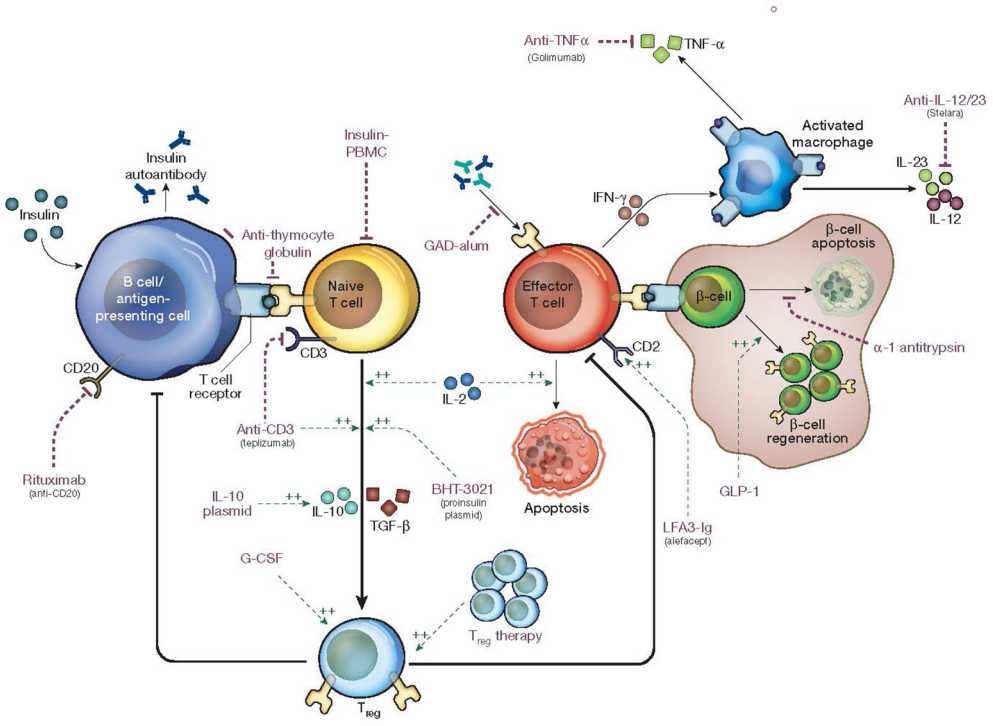

FIGURE 1
Mechanism of action of disease modifying therapies in type 1 diabetes. Edited with permission from Springer Nature (71).

primary endpoint. Finally, a fifth study (*Delay*) was a phase 2 trial that tested teplizumab in a cohort of patients recruited 4 to 12 months after T1D diagnosis who still had clinically significant levels of C-peptide (20). The onset of T1D was comparable to past studies that enrolled within 12 weeks of diagnosis. Endogenous insulin secretion was detectable in all interventions, consistent with preserved beta cell function (14).

Due to its success in stage 3 T1D, teplizumab was also studied in earlier stages of T1D. In *TN-10*, a randomized placed-controlled study including 76 participants with stage 2 disease and dysglycemia, suggested that teplizumab may delay beta cell degradation, although the change in magnitude was overall less than would be seen in Stage 3 T1D (20). A follow-up study completed at a median of 923 days after the initial study found that 50% of the teplizumab-treated population remained in Stage 2 T1D compared to only 22% of the control group. This change was attributed to partially exhausted memory T cells with reduced secretion in IFNγ and TNFα. This implies that a single course of teplizumab has a lasting affected in delaying stage 3 T1D in higher risk individuals (21). In a meta-analysis of 8 randomized, controlled trials including 866 patients with a clinical diagnosis of T1D who had received teplizumab, teplizumab use was found to be associated with decreased insulin use at 6, 12, and 18 months after diagnosis, and stimulated C-peptide AUC was higher at 12, 18, and 24 months (22). Thus, teplizumab has consistently showed improved endogenous insulin production when given during stage 2 or stage 3 diabetes.

Anti-CD20 monoclonal antibodies (*Rituximab*)

B cells are involved in a wide array of T lymphocyte diseases and play an important role as antigen presenting cells, expressing high levels of MHC class II which influence escape of auto-reactive T-cells thought to trigger autoimmune conditions (23). CD20 is a protein expressed on B cells and is involved in the proliferation and differentiation of B cells into plasma cells. A TrialNet study (*TN05*) researched the effects of rituximab, a monoclonal antibody against CD20 that has been used in both oncologic and rheumatologic presentations in the past. This study was a randomized, double-blind study in participants between ages 8 and 40 with stage 3 T1D who had at least one type of detectable diabetes autoantibody. At 12 months, the mean C-peptide area under the curve (AUC) was significantly higher in the rituximab group than in the placebo group. The rate of decline of C-peptide levels was also significantly slower in the treatment group (23).

Anti-IL-21 and GLP-1 Agonists (*Liraglutide*)

Interleukin-21 (IL-21), a cytokine produced by T cells, plays an important role in the trafficking and activation of autoreactive CD8+ T cells in the beta cell (72, 73), thus making it a potential therapy target in the prevention of T1D. In this study, Anti-IL-21, considered a milder, well-tolerated immunomodulatory agent, was tested alone and in combination with a GLP-1 agonist, liraglutide, which has been associated with decreased beta cell stress and preservation of insulin secretion. To test the isolated and synergistic effects on beta cell preservation, a randomized 4-arm placebo-controlled, doubledummy, double-blind phase 2 clinical trial evaluated the impact of IL-21 and liraglutide on C-peptide secretion over 54 weeks. Adults with T1D diagnosed within 20 weeks with at least two known T1D autoantibodies and residual beta cell function were included. Participants were randomly assigned equally to liraglutide, anti-IL-21, both, or placebo, receiving treatment over 54 weeks and monitored for another 26 weeks after the cessation of treatment. During the treatment period, C-peptide secretion decreased by 10% in the group receiving anti-IL-21 and liraglutide, compared to a 39% decrease with placebo. Further, Cpeptide secretion was 48% higher in the combination group when compared to the placebo group. No difference in C-peptide secretion was found when comparing single therapy with liraglutide or anti-IL-21 to placebo. During the 26-week observation period after cessation of therapy, no significant differences in C-peptide secretion, HbA1c, or total daily insulin dose were noted (72).

Ustekinumab (*Stelara*)

Ustekinumab (Stelara), most commonly used in plaque psoriasis, psoriatic arthritis, and inflammatory bowel disease (24), is a monoclonal antibody that binds to the p40 receptor and inhibits IL-12 and IL-23 cytokines, preventing the differentiation of $CD4^+$ cells into Th1 cells that produce IFN-gamma and Th17 cells that produce IL-17 (25). In a phase 1b open-label dose-finding study, it was found to reduce the percentage of circulating Th17, Th1, and Th17.1 cells and proinsulin-specific T cells that secreted IFN-γ and IL-17A and be safe for use in adults with T1D (26). Following this finding, a randomized, placebo-controlled, double-blinded, multi-center phase 2 study of ustekinumab (USTEKID Study) was conducted in adolescents who were diagnosed with T1D within 100 days and had at least 1 T1D autoantibody. Participants received 6 doses of ustekinumab over 48 weeks and were followed for 78 weeks following the first dose (27). At 12 months, those receiving the intervention had 49% higher meal-stimulated C-peptide levels and was also associated with lower levels of Th17.1 cells producing IL-17A, IFN-gamma, as well as B-cell stimulated Th17.1 cells (27).

Alefacept

In order to closely target effector T cells involved in autoimmune beta cell destruction, investigators studied Alefacept, a fusion protein on IgG1 that binds to CD2 on $CD4^+$ and $CD8^+$ effector T cells (28). Alefacept targets memory-effector T cells, preventing T cell activation and proliferation while also inducing T cell apoptosis in select cells (29). The T1Dal study, a multicenter, randomized, double blind placebo-controlled trial, was conducted to compare two 12-week courses of alefacept with placebo in 49 individuals with recently diagnosed T1D (30). At 24 months, the

group receiving alefacept had lower insulin requirements and 50% fewer episodes of hypoglycemia, however no meaningful differences in glycemia emerged. Not surprisingly, endogenous insulin secretion, measured by meal-stimulated 2- and 4-hour C-peptide AUC, was higher in the alefacept group when compared to the control group (30).

Anti-thymocyte globulins (*Thymoglobulin*)

Anti-Thymocyte Globulins (ATG) have historically been used in the cases of bone marrow transplant, solid organ transplant, and aplastic anemia for over four decades, and these cases are associated with a nearly complete immune suppression. Initial studies of high dose ATG in T1D were unsuccessful in demonstrating clinical significance, which may be related to the dose-dependent depletion of $CD4^+$ effector and regulatory cells (31). Later studies were completed using a low dose of ATG (Thymoglobulin) and ATG plus granulocyte colony-stimulating factor (GCSF). Following this study, Haller et al. tested low dose ATG in adolescents and young adults ages 12-45 with at least 1 autoantibody and were within 100 days of T1D diagnosis. They found that the 24-month MMTT stimulated C-peptide AUC was significantly higher in ATG versus placebo, but not in ATG+GCSF versus placebo. Both ATG and ATG+GCSF were associated with reduced HbA1c at 24 months (32).

Initial studies in non-obese diabetic (NOD) mouse models found that ATG plus GCSF demonstrated synergy and significant reversal of diabetes, likely due to the idea that ATG depletes pathogenic T cells while GCSF promotes regulatory T cells (32). The success of low-dose ATG is at least partially attributed to the fact that it was able to avoid long-term immunosuppression and maintain the beneficial regulatory functions of components like regulatory $CD4^+$ T cells that are essential to immune tolerance. Low-dose ATG led to a decrease in the number of $CD4^+$ T effector cells, an increase in the number in memory $CD4^+$ T cells, and overall preservation of the more naïve $CD8^+$ T cells (31).

JAK inhibitors (*Baricitinib*)

The hyperexpression of HLA-I molecules on pancreatic beta cells has been accepted as one of the leading components in the pathogenesis of T1D. This increased expression draws the attention of autoreactive $CD8^+$ T cells, which can accelerate autoimmune destruction. Interferons released by residual beta cells and autoreactive immune cells activate the JAK/STAT pathway, leading to more expression of genes involved in the autoimmune pathway (33).

In animal models, cytotoxic T cells that were deficient of *Tyk2*, a member of the JAK-STAT family, displayed overall reduced cytotoxicity. Treatment with a selective *Tyk2* inhibitor was also found to inhibit the expansion of autoreactive cytotoxic T cells, inflammation of beta cells, and onset of autoimmune T1D in NOD mice (34). Baricitinib, a JAK Inhibitor (JAKi) used in the treatment of rheumatoid arthritis (35), is one of the JAK inhibitors being studied in T1D. A phase 2, double-blind, randomized, placebo-controlled trial from Waibel et al. in 2023 found that daily treatment with baricitinib in patients within 100 days of diagnosis with stage 3 T1D over 48 weeks had a statistically significant change in mixed-meal stimulated mean C-peptide level, thus preserving beta cell function (36).

Tumor necrosis factor alpha blockers (*Golimumab*)

The *TIGER* study was a randomized, double masked, multicenter interventional phase 2 clinical trial assessing the effects of golimumab in new onset T1D. Golimumab, a Tumor Necrosis Factor Alpha (TNF-α) blocker, or placebo was administered in participants within 100 days of diagnosis of stage 3 T1D and who had at least one diabetes-related autoantibody. At the end of 12 months, the C-peptide AUC remained significantly greater in the treatment versus control group. The mean percent decrease in mean 4-hour C-peptide AUC was 12% in the golimumab group and 56% in the placebo group, and this difference in C-peptide secretion was found as early as week 12 (37). There was no statistically significant difference between HbA1c and hypoglycemia between the two groups (37). A 24-month follow up study also found that there were trends in decreased insulin use, higher meal-stimulated peak C-peptide levels, and an increase in those in partial remission (insulin dose–adjusted HbA1C ≤ 9) in the golimumab treatment group (38).

CTLA-4 analogs (*Abatacept*)

In order for a T-cell dependent B-cell response to occur, both a primary and secondary signal must be achieved. The first signal consists of a T-cell receptor binding to antigens presented by MHC class II molecules. A secondary signal consists of interactions between receptor-ligand pairs on T cells and antigen presenting cells that are non-antigen specific. The CD28/CTLA-4:CD80/CD86 costimulatory pathway is one of these pairs. CD28 and CTLA-4 are present on T cells while CD80 and CD86 are present on B cells. When CTLA-4 binds to CD80 and CD86, T-cell activation and proliferation is inhibited (39).

As CTLA-4 is a negative modulator for T-cell immunity, it serves as a method in which medication can become utilized. Abatacept, a CTLA-4 Analog, has been used successfully in conditions like psoriasis and rheumatoid arthritis. With the success abatacept has had in other presentations, it served as a good candidate for use in T1D as well. TrialNet completed a multicenter, randomized, double-blind, placebo-controlled trial (*TN09*) with the primary outcome of mean AUC serum C-peptide at a 24-month follow-up. Patients were required to have stage 3 T1D less than 100 days and have at least one diabetes-related autoantibody. At the 24-month follow up, C-peptide AUC was found to be 59% higher in the treatment vs placebo group, showing slowed reduction in beta cell function (40).

Tyrosine kinase inhibitors (*Imatinib*)

Imatinib, a tyrosine kinase inhibitor most commonly used to treat chronic myeloid leukemia, was also examined in a multicenter, randomized, double-blind, placebo-controlled, phase 2 trial in participants within 100 days of diagnosis with stage 3 T1D, aged 18-45 years old, with at least one positive diabetes related autoantibody. Participants were given either imatinib or placebo daily for 26 weeks. The study achieved its primary endpoint, with a higher C-peptide AUC at 12 months in the treatment group versus placebo, however this effect was unfortunately not sustained at 24 months (41).

Calcium channel blockers (*Verapamil*)

Verapamil, an antihypertensive calcium channel blocker, demonstrated the survival of insulin-producing beta cells and reversal of diabetes in mouse models (42). As diabetes develops, beta-cell TXNIP becomes overexpressed, triggering apoptosis of the beta cell (43). In murine models, verapamil reduced TXNIP expression and beta cell death and improved endogenous insulin production (43). To test the effect in humans, a randomized double-blind placebo-controlled phase 2 clinical trial in adults with a diagnosis of T1D within 3 months were given verapamil or placebo for 12 months. Both groups had similar HbA1c levels at the end of 12 months but those receiving verapamil had higher c-peptide production in response to MMTT at 3 and 12 months (44). Following this study, a double-blind, randomized clinical trial including 88 children and adolescents aged 7 to 17 years with newly diagnosed T1D was completed in 2023 (CLVeR Trial). Participants were treated within 31 days of diagnosis of stage 3 T1D and were randomized to daily verapamil or placebo for 52 weeks. Those receiving Verapamil had a 30% higher C-peptide AUC in response to a MMTT (45), consistent with increased endogenous insulin production. Thus, verapamil use was associated with preserved beta cell function in both pediatric and adults with a recent diagnosis of T1D.

Gamma aminobutyric acid and glutamic acid decarboxylase

Gamma aminobutyric acid (GABA) is a neurotransmitter that serves an autocrine and paracrine role in islet cells, with *in vitro* studies in human islets suggesting that GABA increases insulin secretion from beta cells and may also have a regulatory role for alpha and delta cells (46). Likewise, some studies have suggested that glutamic acid decarboxylase (GAD-alum) therapy may slow the loss of insulin secretion in stage 3 T1D (47, 48). Combination therapy with GABA and GAD-alum has prolonged the lifespan in transplanted islet cells non-obese diabetic mice, signifying its potential as a therapeutic agent to prolong islet cell function in early T1D (49). In a randomized double blind randomized (2:1) trial, participants received either GABA alone, a combination of GABA and GAD, or placebo for 5 weeks. While there was no change in fasting or meal-stimulated C-peptide AUC at 12 months and no change in glycemia, mean fasting glucagon levels had increased by 16.8% in the control group and 0-0.4% in the intervention groups. Further, meal-stimulated glucagon levels were lower in the intervention groups (50). Thus, additional studies are needed to evaluate how these agents influence insulin and glucagon secretion.

Novel autologous dendritic cell therapy

Regulatory T cells (Treg) are integral for maintaining immune tolerance, and abnormalities in $CD8^+$ Treg pathway have been identified in those with T1D (51). In a combined phase 1/2 trial, a vaccine (AVT001) comprised of immature autologous dendritic cells that had been primed with an oligopeptide was designed to correct the defective $CD8^+$ Treg pathway (52). The phase 1 portion of the randomized, double-blinded placebo-controlled study, the vaccine was administered to youth at least 16 years old within 12 months of T1D diagnosis and there were no serious adverse events during the 360 days of follow up. In the phase 2 study, there were no differences in HbA1c or insulin dose, but there was less decline in C-peptide production during the 360 day follow up, though the difference was small (52).

Autologous mesenchymal stem cell transplantation

Autologous mesenchymal stem cells (MSCs) pose great promise as a therapeutic immunomodulatory and regenerative agent in the pathogenesis of T1D (53). MSCs are multipotent progenitor stem cells that have beneficial healing and anti-inflammatory properties without activating immune responses (54). In a triple-blinded parallel randomized placebo-controlled trial, children and young adults ages 8-14 with a diagnosis of T1D within the previous 6 weeks were randomized to receive 2 doses of autologous MSCs or placebo (0 and 3 weeks) (53). Safety criteria were met in the phase 1 portion for the study. There was a meaningful reduction in level 1 and level 2 hypoglycemia as well as fewer total hypoglycemia events in the MSC group. The intervention group also produced higher levels of anti-inflammatory cytokines that persisted over the 12-month study period and lower levels of the pro-inflammatory TNF-alpha (53). Likewise, earlier treatment (within the 12 months) when compared to later treatment (at least 12 months after T1D diagnosis) was shown to have a more pronounce impact on lower of A1c levels for 12 months (53).

Ongoing/Future studies

As of August 2024, there are some additional studies about investigating DMT that could be used in recently diagnosed T1D (Tables 2, 3). TrialNet is also conducting the TOPPLE T1D study, a placebo-controlled, double-blinded within cohorts, randomized,

multiple ascending dose trial in assessing 12 weeks of once weekly dosing of the NNC0361-0041 plasmid, assessing C-peptide responses to multiple mixed-meal tolerance tests over 12 months. The intervention will be a recombinant supercoiled plasmid that encodes for four proteins: pre-proinsulin, transforming growth factor β1, IL-10, and IL-2 (Table 3).

Discussion

The emergence of a diverse array of disease-modifying therapies, particularly within the biologics and immunotherapeutic domains, presents a promising landscape for T1D management. As detailed in Tables 2, 3, a growing number of clinical trials are investigating interventions across various stages of T1D. While this review highlights a slight preponderance of studies focused on recent-onset T1D, the importance of early intervention cannot be overstated. Delaying the onset of T1D at earlier stages is associated with substantial benefits, especially for children, who may lose over 14 years of life expectancy if diagnosed before the age of 10 (20). Collectively, these emerging therapies can significantly improve health outcomes by addressing T1D across its entire disease trajectory.

The incidence of T1D continues to increase and rapid advancements are being made with preventative efforts to delay the onset of T1D. While only one medication, teplizumab, has been approved by the FDA in earlier stages of T1D, there are many other areas in the immune response in T1D that are being studied. Targeted therapies aimed at delaying the onset of T1D and preserving endogenous insulin secretion are vital to reducing the risk of severe long-term complications and have the potential to dramatically improve quality of life.

Author contributions

AO'D: Writing – original draft. SG: Writing – review & editing. LN: Writing – review & editing, Conceptualization, Supervision, Validation.

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Conflict of interest

Yale University School of Medicine conducts studies on the mentioned therapies through TrialNet. LN is a consultant for Medtronic, WebMD, and Calm.

The remaining 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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The role of GABA in type 1 diabetes

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Gamma aminobutyric acid (GABA) is synthesized from glutamate by glutamic decarboxylase (GAD). The entero-pancreatic biology of GABA, which is produced by pancreatic islets, GAD-expressing microbiota, enteric immune cells, or ingested through diet, supports an essential physiologic role of GABA in the health and disease. Outside the central nervous system (CNS), GABA is uniquely concentrated in pancreatic β-cells. They express GAD65, which is a type 1 diabetes (T1D) autoantigen. Glutamate constitutes 10% of the amino acids in dietary protein and is preeminently concentrated in human milk. GABA is enriched in many foods, such as tomato and fermented cheese, and is an over-the-counter supplement. Selected microbiota in the midgut have the enzymatic capacity to produce GABA. Intestinal microbiota interact with gut-associated lymphoid tissue to maintain host defenses and immune tolerance, which are implicated in autoimmune disease. Although GABA is a widely known inhibitory neurotransmitter, oral GABA does not cross the blood brain barrier. Three diabetes-related therapeutic actions are ascribed to GABA, namely, increasing pancreatic β-cell content, attenuating excess glucagon and tamping down T-cell immune destruction. These salutary actions have been observed in numerous rodent diabetes models that usually employed high or near-continuous GABA doses. Clinical studies, to date, have identified positive effects of oral GABA on peripheral blood mononuclear cell cytokine release and plasma glucagon. Going forward, it is reassuring that oral GABA therapy has been well-tolerated and devoid of serious adverse effects.

KEYWORDS

gamma aminobutyric acid (GABA), Type 1 diabetes, GABA treatment/diabetes, β-cells/pancreatic islets, α-cells/glucagon, diabetes/new therapies, GABA-producing microbes, microbiome/GABA/glutamate

1 Introduction

The pathogenesis of autoimmune type 1 diabetes mellitus (T1D) involves infiltration of the pancreatic islet cells by T-lymphocytes, macrophages, and other immune cells with consequent loss of insulin producing β-cells (1–3). Both genetic susceptibility related to HLA and non-HLA genes as well as environmental factors (infectious, dietary, the microbiome) participate in this process (4, 5). Clinical staging of at-risk subjects

according to autoantibodies and dysglycemia has guided potential preventive and therapeutic interventions. At the onset of T1D, more than 70% of β-cells are eradicated (6), thus, residual β-cell replication, intra islet cell transformations and progenitor ductal neogenesis may represent pathways for restoration of β–cell mass. (7). Studies from organ donor pancreata demonstrate insulin-containing islets despite decades following T1D onset (8) indicating ongoing β-cell renewal despite lasting autoimmunity and other stressors. A myriad of immunological abnormalities have been reported in those with T1D including, but not limited to, the production of autoantibodies and cytokines as well as the inability of regulatory T cells (Treg) to curtail the action of effector T cells (Teff); the latter distinct cell population participate in the immune destructive processes. Therefore, a vast majority of clinical studies attempting to curtail this immune foray have focused on immune suppression (9, 10). Additionally, dysfunction in the exocrine pancreas, aberrant sympathetic innervation, oxidative stress, ER stress, and altered autocrine and paracrine signaling within the islet cell are potential therapeutic targets in T1D (11–17).

Outside of the CNS, GABA is highly concentrated in the pancreatic islet wherein it has autocrine and paracrine actions to regulate β-cell insulin secretion and inhibit α-cell glucagon release. Well-known communal microbiota also produce GABA (18, 19). Rodent models have demonstrated reversal or prevention of diabetes with oral and intraperitoneal GABA treatments (20). Combination therapies of GABA with β-cell antigens, antiapoptotic agents, and immunotherapies show additive actions (21–23). In diabetic NOD-scid-γ (NSG) mice, GABA promotes β-cell neogenesis in human islet cell implants and reverses diabetes (24). In children with new onset T1D, low-dose, twice daily oral GABA, with/or without GAD-alum antigen stimulation, inhibited glucagon and reduced Th1 inflammatory cytokine release. Taken together, these studies support a unique role for GABA as a naturally derived oral agent with multifarious anti-diabetic actions. Given its excellent safety and tolerability, higher GABA doses, longer-acting preparations or combination therapies may bear salutary actions in stage 1, 2 or 3 diabetes (Figure 1). In this review, the potential role of GABA as an endogenous and exogenous disease modifier in T1D is presented.

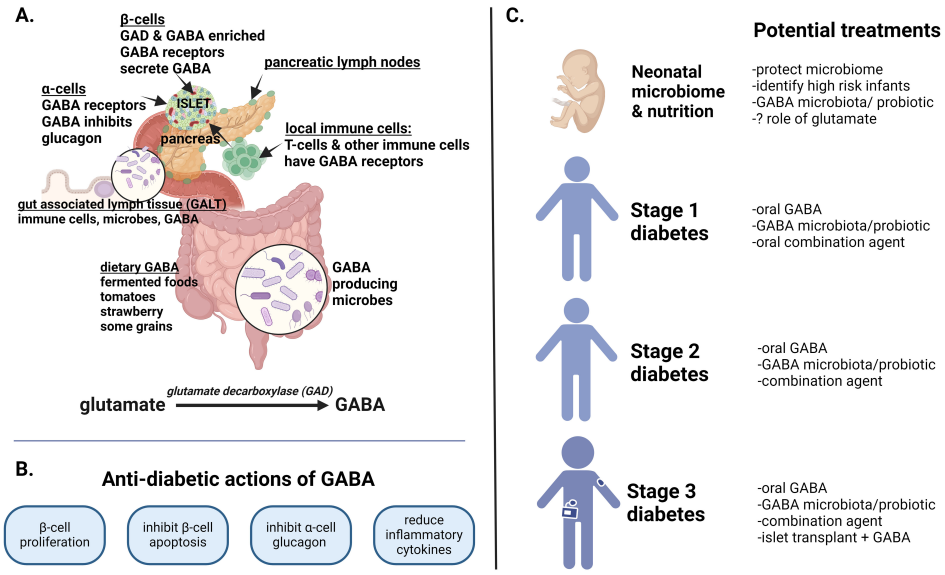

FIGURE 1
GABA in health and diabetes. This figure summarizes the role of GABA in the entero-pancreatic system, its anti-diabetic actions and potential as a therapeutic agent in type 1 diabetes (T1D). **(A)** GABA is synthesized from glutamate by glutamate decarboxylase (GAD65) which is also a T1D autoantigen. GABA is uniquely concentrated in β-cells but is also consumed in foods and produced by select GAD-containing microbiota in the upper and lower intestinal tract. From birth, gut associated lymph tissue (GALT) within the lamina propria are intricately involved in bodily defenses against autoimmunity and inflammation. **(B)** The key anti-diabetic actions of GABA are presented as validated in numerous preclinical rodent and human islet studies. In children with new onset T1D, oral GABA, with or without recombinant GAD, reduced serum glucagon as well as inflammatory cytokines. **(C)** The potential therapeutic role of GABA in T1D is shown from birth through stage 3 diabetes. The perinatal acquired microbiome is pivotal to lifetime immune defense. Whether GABA producing microbiota or glutamate have salutary immune actions is unexamined. In stage 1 diabetes (asymptomatic autoimmunity), GABA supplementation or precision probiotics might restrain the autoimmune process, particularly if GABA/GABA-producing microbiota are deficient. Combination therapy with a low risk oral therapy such as an islet antigen or anti-apoptosis agent might further preserve or expand β–cell mass. In stage 2 diabetes (autoimmunity with dysglycemia), GABA supplementation, with or without more potent combination therapies, might hamper autoimmune destruction. Possible co-therapies, noted to be effective in diabetic rodent studies, include oral T1D antigens, GLP-1 agonists, and positive allosteric modifiers that augment GABA action. Longer acting GABA formulations could also improve efficacy. In stage 3 diabetes (insulin dependent), higher dose GABA along with combination agents that preserve β-cell mass or induce β-cell proliferation are a consideration (anti-apoptotic agent, low dose immune therapies, GLP1 agonists, GABA receptor agonists). Finally, in humanized rodent diabetic models, GABA preserves implanted human islets while promoting β-cell proliferation - whether this has application for human islet transplant survival is intriguing.

2 Pancreatic GABA in diabetes

2.1 GABA in the pancreatic islet

GABA is present in assorted peripheral (non-CNS) tissues including pancreas, gonads, placenta, uterus, gastrointestinal tract, lymphatic and adrenal medulla (25, 26). Pancreatic β-cells have distinctly inordinate concentrations of GABA that are comparable to CNS tissue content (27). Several recent reviews underscore the role of GABA in the pancreatic islet (15, 28, 29). In human β-cells, GABA is synthesized from glutamate by the pyridoxal phosphate dependent enzyme GAD65, which is also a key diabetes autoantigen. GAD67 is an isoform of GAD65 found exclusively in mouse β-cells and brain, and both isoforms are present in rat β-cells (30).

Two GABA receptors are recognized. The GABA-A receptor (GABAAR) is a heteropentamer that functions as a fast-acting chloride channel, thereby altering membrane polarization. It is the primary GABA receptor in the human islet (31). Following GABA binding, there is efflux of chloride in β-cells (hypopolarization) but the opposite flow of chloride occurs in α-cells (hyperpolarization) (13). The GABA-B receptor (GABABR) is an inhibitory two-subunit G-protein receptor that reduces cAMP and modulates Ca^{+2} channels. Under basal conditions, human β-cells express only one of the two functionally necessary GABABR subunits. Modifiers that increase β-cell cAMP, such as forskolin, induce expression of the second subunit yielding functional activity of GABABR (decreasing insulin secretion) (32). Hence, while both GABA receptors are available in the human β-cell, GABAAR are functionally predominant. Regarding GABAA receptor affinity, human β-cells retain two main pentamer subunit subtypes; the stoiochiometry and arrangement of these subunits determines pharmacological selectivity regarding potential agonists and antagonist therapies (33, 34).

Autocrine and paracrine mechanisms account for the regulatory actions of ambient GABA on β- and α-cell function (15, 28, 29). By most accounts, α-cells are devoid of GAD, although this view has been disputed (35). Whether paracrine stimulation of δ-cell somatostatin secretion by GABA inhibits β-cell insulin release is unclear (35). Initial islet studies suggested the co-release of GABA and insulin by exocytosis and that the process was mediated via GABAA receptors. At 6 mM glucose, a GABAA receptor antagonist inhibited insulin secretion (31). Using patch clamp recording and PCR analysis of human islets, the authors demonstrated the presence of GABAA receptors on β-cell, δ-cells and α-cells implicating autocrine and paracrine roles for GABA. Using dynamic hormone secretion measurements in donor islets, GABA was later shown to regulate β-cell insulin release in an oscillatory pattern that was not glucose-dependent (35). GABA accumulates in the cytosol (rather than vesicles) and is secreted via volume regulatory channels. The autocrine action of GABA on β-cell insulin release was inhibitory. GABA attained local (interstitial) concentrations of 10 μM and patterned with the known oscillatory release of insulin. These investigations point to a stabilizing role of GABA in the dynamic regulation of β-cell insulin release. Menegaz and colleagues also demonstrated that T2D and T1D donor islets were 75% and 85% depleted of GABA, respectively, despite no difference in GAD65 content (35). The T2D islets lacked pulsatile insulin release until cellular GABA levels were restored by inhibiting GABA catabolism. Using single cell transcriptomics, islet cells with multiple hormone mRNA expression have been identified in human pancreatic islets (36). These mixed identity islet-cells most often express insulin/glucagon combinations but may also include somatostatin. In diabetic islets, glucagon predominant cell types are more frequent compared to controls. As to why the islet has mix-identity cells, the investigators underscore that the plasticity of the pancreatic islets (37), numerous regulatory factors, including GABA, and patterns of cellular neogenesis or dedifferentiation are all under investigation

Rodent and human islet studies demonstrate the complex autocrine and paracrine signaling that underpin nutrient-responsive crosstalk amongst α-, β- and δ- cells. Lesser-studied components include pancreatic polypeptide-secreting gamma cells and ghrelin-expressing epsilon cells which form <1% islet content. Each islet has a capillary and neural network that provides intimate connectivity with the immune system, gut, liver and CNS (17, 38–41).

Aside from receptor-mediated regulation by GABA, the metabolism of GABA via the intracellular GABA-shunt and TCA cycle further modulates β-cell GABA content and its energy metabolism. Beta-cells metabolize cytosolic GABA via the GABA shunt to meet cellular metabolic demands as the islet responds to the fluctuating energy shifts of the fasting and fed states (42).

2.2 Preclinical studies: GABA in diabetes

In numerous studies using diverse diabetic rodent models, GABA prevents and/or reverses hyperglycemia. Soltani et al. reported several gainful actions of GABA on β-cell mass, immune function, and clinical diabetes in two diabetic mouse models and also in INS-1 rat insulinoma cells (20). GABA increased $BrdU^{+}$ labelled β-cells 5-fold in CD1 mice following two i.p. injections of GABA (20 μmol/mouse over 48 hours). In the multiple dose STZ-diabetic (MDSD), daily i.p. injections of GABA(20 μmol/mouse, i.p.) for 7-days prior to STZ prevented hyperglycemia, increased serum insulin, decreased glucagon, restored β-cell mass and normalized α-cell mass. In the NOD mouse, a spontaneous immune-mediated diabetes model, treatment with GABA was preventative. GABA led to an abatement of insulinitis (lymphocyte infiltration), β-cell mass expansion and normalization of hyperglycemia (after *i.p.* glucose challenge). GABA treatment reduced MDSD- related inflammation by lowing cytokines (IL-1β, TNF-α, INF-γ and IL-10) and reducing LPS^{+}IFN-γ-stimulated splenic $CD4^{+}$ and $CD8^{+}$ cell numbers (20). Tian, et al. demonstrated that treatment of prediabetic NOD mice with GABA (delivered by subcutaneous pellet) from 6-34 weeks of age inhibited progression to overt diabetes by 70% and decreased GAD-specific INF-γ-secreting T-cells by 39% (43). Other rodent models also corroborate a salutary response to GABA in diabetes(median dose 1500 mg/kg/day, range 0.25-4500) (20, 22, 24, 44–52).

The anti-diabetic action of GABA has been studied in combination with other agents. Combination GABA with GAD immunization increased the duration of syngenic β-cell survival in diabetic NOD mice from 1 week in control-diabetic mice to 10 weeks

with maximal GABA doses (GABA 6 ml/ml in drinking water + 100 mg GAD immunization) (23). GABA with proinsulin immunization corrected hyperglycemia in newly diabetic NOD mice when compared to either agent alone (49). At the highest GABA doses (20 mg/ml in water) plus proinsulin immunization, diabetic mice achieved normoglycemia with 4/9 mice remaining normoglycemic for up to 50 weeks post onset of diabetes. Combined GABA plus proinsulin reduced insulinitis, increased β-cell replication and improved splenic Treg responses compared to monotherapy. In NOD mice prior to diabetes onset (4-6 weeks old), combination rapamycin (1 mg/kg daily) and GABA (~200 mg/kg/day divided twice daily) delayed the onset of diabetes for the entire 12 week experimental period, whereas with monotherapy 20% of the mice acquired diabetes (53). In overtly diabetic NOD mice, co-therapy with rapamycin and GABA was superior to monotherapy in reducing hyperglycemia and retaining β-cell function. In INS-1 cells and human pancreatic islets, combination therapy of GABA with a GLP-1 agonist (exendin-4) led to a reduction in cytokine-induced apoptosis and improved glucose-stimulated insulin release. Moreover, the anti-apoptotic actions of SIRT1 and α-Klotho expression were normalized with GABA plus exendin-4 (54).

Finally, in severely diabetic NOD mice, low-dose anti-CD3 (35 mcg) and lesgaberan, a GABA-B receptor agonist (0.08mg/ml in drinking water), rapidly lowered blood glucoses and preserved functional β-cells over a 25-week treatment period (21). After discontinuing treatment, mice were monitored for an additional 25 weeks. The co-therapy group was 83% relapse-free compared to 30% in the anti-CD3 monotherapy group. In a separate report, Tian et al. found that treatment of diabetic NOD mice for 25 weeks with low dose anti-CD3 treatment plus a GABA-A receptor agonist (homotaurine) reversed hyperglycemia and improved the percent of relapse free mice post treatment: 60% with combined therapy, 30% with anti-CD3 monotherapy and 10% with homotaurine alone (55).

Notably, GABA has shown anti-diabetic actions in diverse T1D rodent models, including NOD mice, multiple low dose STZ mice as well as humanized rodent models such as the NOD/Lt-SCID-IL2rg or NSG mouse (55–57). Concerning the NOD mouse, GABA not only forfends against diabetes onset but also reverses overt diabetes (20). As discussed in section 4 regarding GABA dosing and safety, to date, experimental rodent doses of GABA are comparatively higher and of longer duration than oral human dosing. Furthermore, conflicting or negative GABA effects were apparent when lower GABA doses were used in mice (44, 58). As concerns treatment of T!D, longer acting preparations of GABA, co-therapy with GABA receptor agonists, positive allosteric modifiers (59) or complimentary antidiabetic agents (discussed above) could potentially overcome a need for higher GABA doses to achieve efficacy.

2.3 GABA promotes β-cell proliferation and survival

Loss of β-cell mass due to a reduction in β-cell proliferation/regeneration and accelerated β-cell apoptosis are synergistic processes leading to the clinical manifestations of TID. Therapies that invigorate β-cell replication and reduce β-cell destruction may favorably improve the diabetogenic imbalance of cell growth/cell demise. These therapies are relevant to the survival of islet cell transplants as well as *in situ* β-cell function. GABA promotes β-cell growth and survival (24, 60–62). Mechanistically, via an autocrine route, GABA-mediated membrane depolarization (via GABAAR) in β-cells stimulates calcium influx via voltage-gated, calcium channels. The subsequent activation of the growth promoting Ca^{2+} dependent P13K/Akt pathways in INS-1 cells and isolated rodent and grafted human islets leads to increased β-cell proliferation and survival (20, 24). This GABAAR mediated process is potentiated by augmented expression of β3 receptor subunits as shown in the partial pancreatectomized mouse diabetes model (63). Humanized rodent models have advanced our understanding regarding the remarkable proliferative potential of human islets (57, 64).

Elevated TxNIP increases oxidative stress in β-cells and other tissues via thioredoxin (65, 66). In mouse islets from STZ-diabetic mice treated for 13 weeks with GABA (6 mg/ml in drinking water), the anti-apoptotic action of GABA was linked to TxNIP (67). They reported that both GABA and GLP-1 reduced hyperglycemia-associated increases in TxNIP through a common pathway (cAMP-β-cat) . If the effect of these agents is additive, then the combination of GLP-1 and GABA in T1D warrants investigation. Others have identified the role of SIRT-1 and α-Klotho in mediating the anti-apoptotic actions of GABA in the β-cells (47, 54).

Given the abundance of islet non-endocrine cells with pancreatic lineage such as exocrine cells from acinar or epithelial duct, the neogenesis of these cells into insulin-producing β-cells presents an enticing treatment for T1D. However, low-dose GABA over months failed to induce neogenesis of β-cells from ductal tissue based on lineage-labelled ductal tissue in Sox9CreER;R26R^{yfp} mice (68). Another experimental approach to β-cell insulin deficiency would be to induce transdifferentiation of α-cells to functional β-cells with GABA. This was accomplished by Ben-Othman, et al. (44). Experiments with wild-type mice showed a dose-dependent increase of insulin^{+} β-cell mass with 1-5 mg/kg GABA that persisted at a much lower dose of GABA (250μg/kg). In a related study, when C57BL/6J mice, rendered diabetic by STZ, were treated for 8 weeks with GABA (250μg/kg), blood glucose concentrations declined in concert with a ~3-fold increase in plasma insulin, yet plasma glucagon was unaltered. By histological staining, neither pancreatic β-cell nor α-cell mass was altered by GABA treatment alone. A nearly two-fold increase in α-to-β cell conversion was observed. The results of these studies could not be replicated (69). The discordant conclusions between labs could be consequent to heterogeneous experimental protocols to measure α-cell and β-cell transdifferentiation, and other such variables as mouse strains, diets, gut microbiota, and duration of GABA treatment. Worth considering, the three research groups aforementioned used GABA doses that were logarithmically lower than most *in vivo* GABA protocols.

In an attempt to resolve different experimental conclusions regarding GABA and β-cell regeneration, especially α- to β-transdifferentiation, von Herrath et al. independently conducted a series of experiments using similar GABA doses, additional delivery methods as well as assiduously reproducing identical experimental conditions (70). They were unable to demonstrate α- to β-

transdifferentiation by GABA, as well as, no effect on glucose homeostasis or α-cell/β-cell mass in normal or diabetic mice. However, there is an apparent dose-dependent trend that GABA decreased α-cell mass and the α-to β- ratio in the wild type mice.

2.4 GABA inhibits glucagon

Glucose control in diabetes is regulated in part by glucagon, not only through paracrine intra islet cell communication, but also through peripheral effects on hepatic, adipose and neural metabolism (17, 71, 72). Hyperglycemia triggers β-cell insulin release and suppression of α-cell glucagon secretion. Using rodent islets, Xu, et al. found that insulin secretion induces an Akt kinase dependent translocation of GABAA receptors to the membrane of pancreatic α-cells that augments the response to paracrine release of GABA from β-cells. The result is GABA-mediated membrane hyperpolarization and subsequent inhibition of glucagon secretion (73). GABA-deficient islets did not exhibit appropriate glucagon inhibition in response to increasing glucose concentrations *in vitro* (74), inferring that GABA is directly involved in the suppression of glucagon secretion in α-cells. Based on immunofluorescence studies in STZ-treated mice, daily intraperitoneal GABA (10 μg/kg) for 12 days thwarted the 7-fold rise in α-cell mass which transpired in the control-diabetic group and also preserved β-cell mass (75). The α-cell mass expansion in STZ mice likely develops in human T1D; for example, following the onset of T1D in humans, there is a progressive increase in serum glucagon for at least one year and sometimes 3-5 years thereafter (76–79). In diabetic animals, the effect of exogenous GABA on circulating glucagon and/or α-cell mass are conflicting. There was an approximate threefold reduction in serum glucagon in several studies (20, 75), but no change was noted by others (80, 81). As for the latter two studies, one involved rats and the other used a very low GABA dose (0.25mg/kg) - these experimental disparities could account for the conflicting findings. An excess of glucagon relative to insulin characterizes the metabolic dysregulation and hyperglycemia of diabetes. Treatment of children with T1D with low dose, twice-daily oral GABA, with and without GAD-alum, for 12 months reduced circulating glucagon without preserving serum c-peptide (82). In this trial, a secondary finding buttresses a role for glucagon in glycemic control: there was a significant relationship between fasting glucose and fasting glucagon. Moreover, at 12 months, there was an even more robust association between area under the curve (AUC) glucose and AUC glucagon following a mixed-meal challenge. Both of these glucagon-glucose relationships do not establish causation, yet provide intimations that compel further study.

2.5 GABA is anti-inflammatory

Type 1 diabetes is characterized by a multipronged inflammatory assault notable for infiltration of the pancreatic islet with autoreactive $CD4^+$ and $CD8^+$ T cells and macrophages begetting insulitis and β-cell demise. Antibodies to GAD65 and other β-cell antigens are present years prior to dysglycemia and overt symptomatic diabetes (4). Peripheral blood mononuclear cells (PBMC) release pro-inflammatory cytokines and chemokines that accelerate the process. Identifying safe immunomodulatory interventions to slow/abort the T1D autoimmune process or protect transplanted islets from immune destruction is imperative (14).

GABAARs are expressed in various immune cells, including T-cells and peripheral blood mononuclear cells, and are known to exert immune-inhibitory actions (43, 83, 84). Human T cells, dendritic cells, NK killer cells, and monocytes, also contain the enzymatic components for GABA production (including GAD) and catabolism (GABA transaminase) (85, 86). In NOD/*scid* mice, daily GABA (600 μg/day by subcutaneous pellet) inhibited the adoptive transfer of T1D indicating suppression of effector-T cells. In addition, continuous low-dose GABA for 30 weeks reduced the onset of diabetes in NOD mice: 90% of control mice developed diabetes compared to the 20% of those treated with GABA (43).

GABA suppresses the formation of IL-12 by macrophages, and IFN-γ by CD8 T-cells, underscoring its anti-inflammatory role of reducing cytokine production (20, 87). In rat INS-1 cells versus human β-cells, GABA attenuates cytokine-induced (IL-1β, TNF-α, INF-γ) apoptosis 75% and 30%, respectively; these actions were potentiated by a glucagon-like peptide-1 (GLP-1) (54). As recently reported, ambient glucose or insulin modulate the effect of GABA on inflammatory cytokine release in human CD4+T-cells (88). In children with new onset T1D, oral GABA, with or without GAD65-alum, curtailed the Th1 proinflammatory response relative to placebo (89). Following antigen stimulation of PBMC with GAD65, GABA/GAD treatment showed a blunted (absent) rise in INFγ and TNFα compared to the statistical increase in both proinflammatory cytokines in a placebo group from 0-12 months ($p<.05$).

3 GABA and the microbiome

3.1 GABA producing microbes

The intricate entero-pancreatic biology of GABA, ingested or synthesized by microbial glutamate decarboxylase(GAD), is conceivably germane to T1D pathophysiology. As aforementioned, GAD65 is concentrated in the β-cell (15) and found in discrete enteric bacteria (19). In microbiota, an intact GAD operon (includes both gadB or gadA plus the glutamate/GABA antiporter) is requisite for GABA metabolism (90) and acid/base tolerance (91). In the gastrointestinal tract, lactic acid bacteria such as L. brevis and L. reuteri (phyla Firmacutes), as well as bifidobacteria (phyla Actinobacteria) including B. adolescentis and B. dentium, are acclaimed GABA producers (92–94). Of 135 strains of Lactobacillus and Bifidobacterium from human donor enteric/salivary/vaginal specimens, 58 srains produced GABA from glutamate *in vitro* (94). The authors confirmed the presence of gadB/gadC genes in the bacteria and noted *in vitro* GABA production rates of 50-6000 mg/L in timed incubations. Standwitz, et al. identified a previously unculturable gram positive bacterium (KLE1738) that required a common GABA-producing gut microbe -Bacteroides fragilis- to grow *in vitro* (19). Genome-based metabolic

modelling uncovered genera of enteric bacteria capable of consuming or producing GABA. This work highlights the overlapping roles of GABA in microbiota as an energy source or pH modifier via the GABA shunt (95) versus its role in neuroendocrine signaling and immune regulation (96). The question whether GABA forming microbiota can alter plasma GABA is unresolved: two germ-free models employing metabolomics support this premise (97, 98) whereas another germ-free rodent study did not (99).

3.2 Microbial GABA in diabetes - preclinical studies

Several studies have examined the effect of GABA-producing microbes in streptozocin (STZ) diabetes. A single-dose streptozotocin (STZ) model was employed which causes abrupt chemically mediated β-cell destruction (100) and, hence, the results to not entirely translate to immune-mediated diabetes. Marques et al., treated STZ-diabetic rats with *Lactobacillus* GABBDPC6108 or GABA alone (2mg/kg/day or versus 200 mg/kg/day in drinking water) over 9 weeks (81). The investigators confirmed that the microbe-treated rats retained live, GABA-producing *L.brevis* in fecal samples at study end. Concerning diabetic parameters, there was a 26% decrease in blood glucose in the diabetic *L. brevis*-treated rats. GABA-treatment was associated with a 12-15% decrease in blood glucose. The serum GABA level was unchanged in the low-dose GABA group but increased 34% in the high dose GABA group. The investigators concluded that the nominal reduction in glucose by *L. brevis* or oral GABA was likely due to the massive β-cell destruction in their non-inflammatory STZ-dose rat model. Insofar as the effects of microbial-produced GABA is anti-inflammatory, perhaps a multiple dose STZ (MDSD) or autoimmune model, in which there is both inflammation and residual β-cells, would have revealed anti-diabetic actions in these experiments.

Using *specific pathogen-free* male C57BL/6 mice, Abdelazez et al. treated two groups of STZ-diabetic mice with different strains of *Lactobacillus brevis* (KLDS 1.0727 and KLDS 1.0373) and compared diabetes-related parameters relative to control mice and STZ-treated diabetic mice (no probiotic treatment) after 4 weeks. There was a 40% decrease in blood glucose in the *L. brevis*-treated STZ-mice compared to untreated STZ-controls (serum glucose 7mM versus 4 mM, respectively). The *L.brevis* strains were shown to contain a GAD gene and produce GABA. Proof of sustained enteric colonization with the Lactobacillus was not documented (101). In high fat-fed, insulin-resistant mice, *L. brevis* readily colonized the animals, increased insulin sensitivity, and, following an overnight fast, increased the GABA concentration in the small intestine 2.25-fold (102).

In aggregate, these STZ-diabetic rodent models showed modest metabolic actions on glucose and insulin with *L. brevis* treatment without reversal of diabetes. This supports that the primary salutary actions of microbial-GABA in T1D may be immunologic. Hence, long-term enhancement of GABA-producing microbiota, particularly in the entero-pancreatic region, may be requisite to mitigate autoimmune β-cell destruction. And, concerning the role of GUT health, many other factors, including nutrition, prebiotics, additional microbe-derived metabolites such as short chain fatty acids (SCFA), along with avoidance of unnecessary antibiotics, warrant study in T1D (16).

3.3 Crosstalk between gut microbiota and the pancreatic islet

The human gastrointestinal tract from the oral cavity to colon harbors distinct microbial ecosystems. Accordingly, microbes contained in a distal stool specimen, while experimentally convenient, differ considerably from proximal segments (103–105). In 21 healthy individuals age 59 ± 12.3 years who had endoscopy to obtain mucosal biopsies of the upper and lower GI tract (103) fecal microbiota by 16S ribosomal profiling did not mirror those in the upper intestinal mucosal microbiota. Noteworthy, lactobacilli (phylum Frimicutes), which includes many GABA-producing microbiota, were exclusive to the upper GI tract compared to fecal samples. Fecal GABA levels, however, correlate with Bifidobacterium abundance (phylum Actinobacteria) in healthy controls (106). In a catheterized rat model, serum GABA was measured in the venous effluent from small versus large intestine after selective ligation of abdominal arteries and veins. A two-fold increase in portal GABA concentration was found between the fasting and fed states, as well as a 50% diminishment in serum GABA in large versus small bowel effluent (99).

Concerning entero-pancreatic signaling, or crosstalk, between microbiota and the pancreas, local, as opposed to systemic, GABA levels are likely more relevant to autoimmune diabetes (107). The anatomical proximity and connections between microbiota in the nutrient-rich duodenum, gut-associated lymph tissue (GALT) and pancreatic lymph nodes (PLN) form a complex network that mediates immune tolerance (39, 108). For example, in control mice, pancreatic β-cells produce calthelicidin-related antimicrobial peptide (CRAMP) in response to microbial-derived SCFA; this response mechanism is deficient in both NOD mice and multiple dose STZ diabetes (MDSD) mice that are genetically CRAMP-negative (109). Replacing CRAMP forestalls diabetes in these rodents and is associated with reduced pancreatic immune cell infiltrates (B-cell, T-cell, and dendritic cells). This novel rodent study demonstrates that crosstalk between β-cells and the metabolites of intestinal microbiota may contribute to the immune backdrop that forfends against autoimmune diabetes. Studies in germ-free NOD,MyD88-deficientKO mice also highlight a protective interaction of commensal microbes with the immune system that reduces the incidence of diabetes (110). It is, therefore, reasonable to posit that within this enteric micro-environment, a healthy complement of GABA-producing microbes might favorably modulate T-cell immunity and islet cell function (81, 111, 112).

3.4 Microbial GABA and type 1 diabetes

Type 1 diabetes is associated with alterations in the composition of gastrointestinal microbiota (dysbiosis) and

breakdown of the gut barrier integrity (113–115). Longitudinal analysis from the TEDDY trial of fecal microbes and their metabolites from infancy to T1D-onset has uncovered bacterial imbalances notable for reduced ratios of Firmicutes to Bacteroidetes as well as deficient enteric SCFA (116–119). Serum metabolome analysis disclosed reduced GABA levels one year before seroconversion to insulin autoantibodies (IAA), but not before the appearance of GAD antibodies (120). This observation was corroborated in the Finnish Type 1 Prediction and prevention (DIPP) study wherein elevations in glutamate (precursor to GABA) were apparent prior to seroconversion. And, in a salient case study, an 8-fold spike in serum GABA and 13-fold increase in glutamate preceded the appearance of GAD antibodies by 2.5 years (121). The significance of these GABA/glutamate trends are unknown but may reflect compensatory immunomodulation, diet, microbiota, infection or unknown exogenous factors. Microbial dysbiosis has been implicated as a key element, in concert with genetic predisposition and environmental factors, which underpin the pathoetiology of T1D. It follows that a deficiency of GABA-producing microbiota, particularly in the duodenum, may be a component of diabetic dysbiosis. GABA receptors are abundant in the intestinal tract and on T-cells where anti-inflammatory actions are recognized (94, 113). T cells express functional GABAA receptors that are responsive to low dose GABA (43). As follows, GABA production by the microflora in the metabolically active small intestine could conceivably lessen pathogenic autoreactive T-cell responses in gut-associated lymphoid tissue (GALT) and/or pancreatic lymph nodes (117, 122).

A straightforward approach to dysbiosis in T1D is the introduction of probiotics (123, 124). Most human trials have tested the benefits of combinations of Lactobacillus (phyla Firmacutes) a bifidobacteria (phyla Actinobacteria) (124, 125). While GABA production was not the focus of these investigations, many lactobillus and bifidobacteria express GAD and, thereby, produce GABA (19, 90, 92, 94, 126, 127).

3.5 Microbial glutamate decarboxylase (GAD) and autoimmunity

GAD65 is a pyridoxal (B6)-dependent decarboxylase. The enzyme can alternate between an antigenic apoGAD65 format (no attached B6) versus its active and less antigenic holoGAD65 format (B6 bound). This contrasts with the non-antigenic holo-GAD67 that is only B6- bound (128). The hypothesis that GAD-containing microbiota might trigger an autoimmune attack against β-cell GAD65 was considered based on similarities in human versus bacterial GAD epitopes in the B6 binding region of GAD (129). The antigenic, pyridoxal linkage site of GAD65 in GABA-producing gut microbes aligns closely with human GAD65 such that microbial GAD could conceivably sensitize enteric T-cells to GAD65 leading to the pathogenic immune destruction of β-cells. In this model, B6 deficiency might enhance exposure of the antigenic catalytic site of GAD to autoimmune detection (130). Nevertheless, increased vitamin B6 intake was not protective against T1D progression in the TEDDY study (131).

3.6 Glutamate

Glutamate, the enzymatic precursor for GABA, constitutes about 10% of dietary amino acid content. Analogous to GABA, glutamate is a CNS neurotransmitter with additional actions outside the CNS. Glutamate receptors are widespread, found particularly in pancreas, adrenal gland, developing cartilage, gastrointestinal tract and lymphocytes (132). For unknown reasons, free glutamate is uniquely concentrated in human milk (133), several-fold higher than other amino acids . The free glutamate intake of breast-fed infants is 36 mg/kg compared to 0.7 mg/kg from dairy milk formulas; protein hydrolysate preparations provide 170 mg/kg. Enteral glutamate is rapidly oxidized for intestinal metabolic energy in piglets (134), preterm infants (as measured with stable isotopes) (135) and adults. Glutamate is, furthermore, the widely applied, unami food enhancer (mono-sodium glutamate or *MSG*). Inasmuch as oral glutamate is metabolized rapidly by enterocytes, there was no measurable rise in systemic GABA levels following an oral dose of glutamate (136, 137). Concerning glutamate metabolism and signaling in pancreatic islets, extracellular uptake of glutamate by AMPA receptors augments *a*-cell glucagon release (138). In β-cells, glutamate potentiates glucose and incretin-stimulated insulin signaling and islet survival via NMDA receptors (139). The intracellular metabolism of glutamate in β-cells involves mitochondrial glutamate dehydrogenase, glutamate decarboxylase (GAD), glutamine synthetase, and the synthesis of glutathione (140). The glutamate NMDA receptor is a proposed drug target for diabetes (140–142).

4 GABA dosing and safety

GABA is a water soluble, non-protein amino acid (C4H9NO2). It is considered a dietary supplement in the USA (143) and a pharmaceutical in Europe (144). The Dietary Supplement Label Database (https://dsld.od.nih.gov) records over 1500 GABA-containing supplements with daily doses ranging from 45 mg to 3000 mg/day, and most at 500-750mg/day (143). A toxicity study in rats administered oral GABA (500-2500 mg/kg/day) for 13 weeks and found no significant abnormalities in behavior, weight gain, or blood indices including general chemistries, glucose, renal function, hematology and liver function. Postmortem organ histopathology and weights were normal (145). The highest reported oral dose of GABA involved 14 adults (8 gram/kg/day divided into 4 doses) for up to 2 years and was well-tolerated (146). In healthy adults, single GABA doses of 5 gm, 10 gm or 18 gram/day for 4 days was without serious adverse side effects (147). Figure 2 presents a comparison of experimental daily GABA doses (mg/kg) in rodents and one human clinical study. Of importance, oral administered GABA does not cross the blood brain barrier (143), although this viewpoint may need further analysis in neonates (149).

Using immunoassay, adults with T1D had plasma GABA levels of 649 ± 42 nM compared to 501 ± 32 nM in controls (87). In T2DM, plasma GABA concentrations were 480 ± 28 nM in T2D compared to 516 ± 30nM in non-diabetic controls (33). In a clinical trial, baseline,

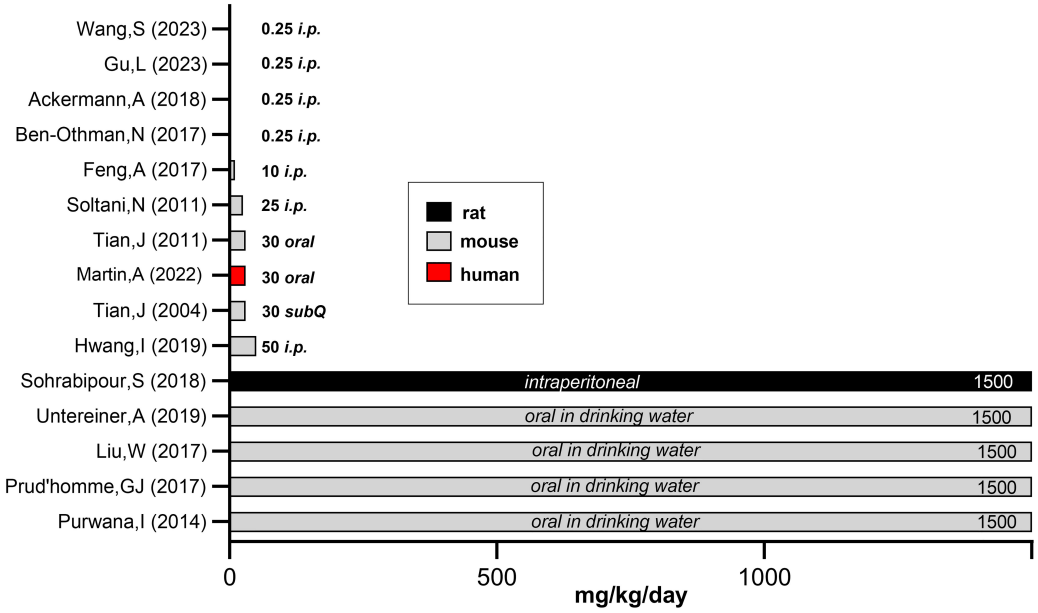

FIGURE 2
Comparison of experimental GABA doses used in rodent versus human studies. To compare the experimental GABA doses (mg/kg/day) used in rodent versus human studies, we estimated daily water intake and tabulated average adult rodent weights. When GABA was added to drinking water or given by injection, the daily intake approximated 1500 mg/kg/day based on estimated daily water consumption (148). This calculation does not take into account that diabetic animals have polydipsia, thus the actual GABA dose is vastly underestimated. Mouse body weights - unless noted by investigators in the methods section- were based on species and the average, non-diabetic weight in healthy animals. Figure 2 references (Y-axis): Wang, et al. (68), Gu, et al. (80), Ackermann et al. (69), Ben-Othman et al. (44), Feng et al. (75), Soltani et al. (20). Tian et al. (23), Martin et al. (82), Tian et al. (43), Hwang et al. (45), Sohrabipour et al. (48), Untereiner et al. (51), Liu et al. (22), Prud'homme et al. (47), Purwana et al. (24). Figure adapted from "A randomized trial of oral gamma aminobutyric acid (GABA) or the combination of GABA with glutamic acid decarboxylase (GAD) on pancreatic islet endocrine function in children with newly diagnosed type 1 diabetes," by Martin A, Mick GJ, Choat HM, Lunsford AA, Tse HM, McGwin GG Jr, and McCormick KL. Nat Commun. 2022 Dec 24;13(1):7928, Supplementary Data, Figure 6 (https://doi.org/10.1371/journal.pone.0197160).

fasting GABA levels were 248 ± 86 nM by LC-MS/MS in children with T1D (82). Peripheral blood GABA levels, as measured by HPLC, do not vary significantly by gender or exercise (150). Using LC-MS/MS, fasting GABA was 10 ng/ml (97 nM) in 12 healthy volunteers (151). Following a 2 gram oral dose of GABA, there was a rapid rise in plasma GABA (t_{max}: 0.5-1 hour, C_{max} 6.7 µM, $t_{1/2}$ = 5 hours). With repeated dosing of 2 grams GABA three times per day for 7 days (~85 mg/kg/day), GABA levels were at steady state. For comparison, in mice, GABA treatment (6 mg/ml in drinking water for ten weeks=1500mg/kg/day) raised plasma GABA five-fold over a baseline of 47.4± 4.8 ng/ml (460 nM) (51). In another report, fasting GABA levels were 16 ng/ml (155nM) in 11 male adult volunteers when measured by LC/MS/MS. Following ingestion of 888mg GABA in 1 liter of water, the pharmacokinetic variables were: t_{max} (h) = 0.5 and the C_{max} (ng/ml) = 75. Interestingly, ingestion of pureed tomatoes (innately high in GABA) that contain a comparable 888 mg dose of endogenous GABA, the GABA kinetics were: t_{max} (h) = 0.36 and the C_{max} (ng/ml) = 184.

In all pharmacologic interventions, a threshold concentration must be attained for efficacy. Hence, thrice-daily oral GABA, which is a practical outpatient regimen, and/or higher doses, is suggested given the short half-life of GABA. As emphasized by Kaufman's lab concerning the clinical utility of oral GABA, there is evidence that the GABAA receptor EC_{50} is of low affinity (50-400 µM) (55). By patch clamp technique, human islets attained maximum channel opening at 100-1000 nM GABA with desensitization occurring above this concentration range (33). The interstitial GABA concentration in the islet is unknown, yet reason dictates that continuous exposure or frequent and higher dose GABA may be required for efficacy. Alternative therapeutic options include longer acting receptor agonists such as lesogaberan, a GABA-B receptor agonist (21, 152), or homotaurine, a GABA-A receptor agonist (55). Other long- acting GABA formulations are in clinical trials (144) or early development (153, 154). Tian et al. demonstrated the role of positive allosteric modulators such as alprazolam to augment and/or prolong GABA actions (59).

5 Clinical studies using GABA in diabetes

5.1 GABA and GABA/GAD65-alum clinical trial in children with recent onset T1D

The GABA and GABA/GAD65 trial (82) was the first human, prospective, double blind, placebo-controlled and randomized clinical trial of oral GABA (with and without GAD65-alum) in new onset type 1 childhood diabetes. The investigators hypothesized that treatment

with oral GABA, or a combination of GABA/GAD65-alum, would halt or slow the progression of new onset type 1 diabetes (T1DM) by some/all of the following mechanisms: 1) increasing endogenous insulin secretion, 2) suppression of glucagon release, 3) dampening the T-cell mediated autoimmune process. This single center, one-year trial enrolled 97 children with T1D within 6 weeks of diagnosis (NCT02002130). Interventions included oral GABA (1 gram/M2/day up to a maximum of 1.5 gram/day or approximately 30 mg/kg/day, see Figure 2) divided into two daily doses with or without two GAD-alum injections (20 mcg/dose)-one at baseline and the other at one month. The FDA constrained the permissible

GABA treatment dose given that this was the first human trial, no less in children. While the primary outcome (preservation of fasting/meal-stimulated c-peptide) was not attained, the secondary outcome (reduction of glucagon) was demonstrated in the GABA/GAD group. Importantly, the safety and tolerability of oral GABA in children was confirmed. Considering the low oral GABA dose administered, it was not unforeseen that only glucagon inhibition was detected, corroborating the paracrine inhibitory effect of β-cell GABA on α-cells. Overnight fasting plasma GABA levels did not differ between T1D and controls in this pediatric trial, not unexpected with the short half-life of GABA. In contrast, adults with T1D had 13% higher fasting blood GABA levels compared to controls (87). Strengths of this T1D trial were the recruitment of young patients within 5 weeks of diagnosis and the inclusion of a combination antigen (GAD-alum) plus GABA study group (23). Limitations of this study were the low-dose of GABA and twice daily dosing to encourage study drug adherence. Compliance was measured by pill counts of returned study drug. The average compliance was 83% with 20% of patient visits recording <50% compliance over the study course. Based on a half-life of 5 hours after a two gram oral GABA dose (151), in combination with non-ideal study drug adherence, it is possible that islet GABA exposure was insufficient to achieve an anti-diabetic effect. Future human GABA trials could entail longer-acting preparations, higher doses, GABA agonists or precision GABA-producing probiotics. As previously discussed, preclinical studies support combination therapies (23, 54, 67, 155).

5.2 GABA and GABA/GAD65-alum alters Th-1 cytokine response in children with recent onset T1D

In the same cohort as the GABA/GAD-alum study (82), the potential immune effects of GABA treatment, with or without GAD65 immunization, were examined (89). B ased on cytokine responses in peripheral blood mononuclear cells following polyclonal and GAD65 antigen re-challenge, proinflammatory Th1 cytokine responses were attenuated in both the GABA and GABA/GAD65-alum groups over 12 months.

Peripheral blood mononuclear cell (PBMC) mRNA expression was measured following polyclonal stimulation with anti-CD3/CD28 Dynabeads. GABA treatment decreased IFNγ expression at 5 months compared to placebo and with GABA/GAD at 12 months ($p<0.05$). At 12 months, GABA increased expression of FOXP3, a transcriptional regulator of Treg differentiation ($p<.05$). Using an antigen-specific recall assay to GAD65, IFNγ mRNA decreased with GABA/GAD compared to GABA alone at 12 months ($p<.05$).

The cytokine/chemokine response of PBMC's was measured following antigen stimulation with GAD65 using a Milliplex MAP human cytokine/chemokine bead panel. GABA/GAD treatment showed a blunted (absent) rise in INFγ and TNFα compared to the statistical increase in both cytokines in the placebo group from 0-12 months ($p<.05$). GABA decreased the Th1 inflammatory chemokine CXCL10 response between 0 to 5 months but this diminishment reversed by 12 months. The placebo group, by contrast, had an increase in CXCL10 between 5-12 months ($p<0.05$) and 0-12 months ($p<0.01$).

In aggregate, by qPCR and cytokine/chemokine analysis, GABA and GABA/GAD reduced some but not all proinflammatory cytokines and chemokines consistent with an attenuated progression of the inflammatory phenotype. Subjects were next divided by high-risk haplotypes as either HLA-DR3-DQ2 and HLA-DR4-DQ8/other. The DR4 group manifested a Th1-skewed proinflammatory response in comparison to the DR3 group and responded differently to GABA alone versus GABA/GAD65-alum. Expression of IFNγ mRNA over 12 months was lower in the GABA/GAD group compared to placebo ($p<0.001$) or GABA alone ($p<0.01$) as well as compared to the same treatments in the HLA-DR4 cohort. At 12 month, GABA/GAD treatment led to decreased CXCL10 in the DR3 group compared to placebo ($p<0.05$) and the HLA-DR4/other GABA group. IL-2, which promotes expansion and maturation of naïve T-cell to T-eff, showed no differences with the placebo versus treatment groups.

These immune studies in PBMC from study subjects confirm the HLA-delineated immunomodulary actions of GABA and GABA/GAD65-alum in children with recent onset T1D. The results corroborate, in part, preclinical studies in MDSD mice showing that GABA decreased levels of circulating and CD4-released IFNγ, IL1β, TNFα, and IL-12 mice (20). The immunomodulary effect of GABA in NOD mice (600 mcg daily by subcut. pellet for 60 day) is also instructive (43). For example, in GAD-stimulated splenic T-cells from the NOD mice, GABA reduced INFγ formation 55%. The GABA dose used in the NOD mice (~30 mg/kg/day) (see Figure 2) is comparable to this clinical trial (82, 89).

Limitations of this study were the low dose of GABA and challenges with medication adherence as discussed previously. Concerning immunophenotyping of isolated PBMCs, it is evident that results do not perfectly mimic the localized immune response within the pancreatic islet. Corroborating the results in animal models of T1D treated with GABA and GAD65-alum could clarify whether the peripheral immune responses resemble the islet microenvironment.

In addition, both GABA alson and GABA with GAD65-alum treatment inhibited Th1 responses compared to placebo but showed no significant differences between the treatment cohorts. It is possible that multiple autoantigens are necessary to induce robust T cell proliferation as was shown in an analogous T1D study that used antigen recall assays and HLA delineation (5).

5.3 GABA trial in adults with prediabetes

In overweight adults with prediabetes, De Bie and colleagues examined the effect of oral GABA on glycemic control using a

double-blind, randomized and placebo-controlled study design (NCT04303468) (156). In this well-designed trial, 52 subjects, ages 50-70 years, were given 500 mg GABA orally thrice daily versus placebo for 95 days. Prediabetes was defined by abnormal oral glucose tolerance testing (OGTT). The primary outcome was the effect of GABA on OGTT, and the secondary exploratory outcomes included continuous glucose monitoring (CGM), cardiovascular indices and sleep quality. Blood sampling included glycated hemoglobin, insulin, glucagon, GABA, glutamate and lipids. Results did not establish the primary endpoint, although there was a 0.22 mmol/L decrease in fasting glucose in the GABA group after 95 days. Other secondary outcomes were not met. Given the role of excess hepatic glucose production and reduced glucose clearance in the pathophysiology of prediabetes (157) the inhibition of glucagon by GABA, in addition to β-cell replication, could favorably improve the insulin/glucagon ratio (158, 159).

5.4 Efficacy of combination therapy with GABA, a dipeptidyl peptidase-inhibitor and a proton pump inhibitor in adults with T1D.

This retrospective study examined the effect of GABA (500 mg orally 2-4 times/day) in combination with one of two DPP-4i (sitagliptin or saxagliptin) and a proton pump inhibitor (PPI) (omeprazole 20-40 mg/day) in 19 overweight adults (32± 13 years of age) with insulin dependent diabetes (160). The authors based this study on their preclinical report examining the effect of GABA, DPP-4i and PPI in NOD mice (161). T1D was characterized by low c-peptide (5/19 subjects) and GAD65 positivity (14/19 subjects). Patients were identified by chart review and were divided into two subgroups: early-therapy (begun within 12 months, mean 3 months, of starting insulin) and late therapy (begun more than 12 months, mean 168 months, after starting insulin). Treatment continued for 26-42 months. There were improvements in fasting blood glucose, HgA1C, IDAA1c, total daily dose of insulin, and c-peptide. Seventy percent of patients in the early- therapy subgroup no longer required insulin but none in the late-therapy group. Moreover, despite persistently low fasting c-peptide, the combination treatment led to improvements in glycemic control and reduced total daily insulin. The authors inferred that reduced glucagon secretion may have played a role. In T2D with insulitis, beta cell failure and glucagon excess would also likely benefit from this combination therapy. Preclinical studies support this possibility (22, 49, 54, 56, 67, 155).

5.5 GABA levels and GAD65 antibody titers in adults with T1D

Plasma GABA levels, GAD65 antibody titers, c-peptide, and serum cytokines were determined in 128 young adults: 45 healthy controls, 60 individuals with long standing T1D and 13 individuals with new onset T1D (162). Fasting morning blood was collected for analysis and GABA was measured by LC/MS/MS. Detectible serum c-peptide was found in 20% of patients with long-standing diabetes. Plasma GABA was similar in each group and correlated positively with fasting glucose and negatively with age. The authors posit that while circulating GABA levels were the same in all groups, GABA concentrations in the entero-pancreatic region and islets may be at variance. Moreover, both dietary intake and GABA- producing microbes are additional sources of GABA that may modify the islet milieu but not be reflected by circulating concentrations (19, 127, 163).

5.6 GABA induces a hormonal counterregulatory response in subjects with long-standing T1D

Six adult males enrolled in an open-label, 11 day study to test the safety, efficacy, pharmacokinetics and hormonal responses (including a hypoglycemic clamp) to a long-acting oral GABA preparation (Remygen, Diamyd Medical, Stockholm, Sweden) (144). Subjects were on average 25 years old and had long-standing diabetes. In 5 subjects the baseline c-peptide was <0.01 nmol/L. Results found that the long acting GABA preparation restored the counter-regulatory response (glucagon, cortisol, and adrenaline) to hypoglycemia (clamped at 2.5 mmol/L). The authors suggest a potential therapeutic action of their GABA preparation on hormonal counter-regulation during hypoglycemia. Note that with normal to high glucose, GABA inhibits alpha-cell glucagon release (15, 164).

6 GABA in hybrid diabetes

T1D and T2D have overlapping features such that both have relative or veritable insulin-deficiency (with or without autoimmunity) or insulin-resistance, both of which are identified in many patients who were previously classified to one or the other binary designation. Assorted recent classification schemes have been proposed to subdivide diabetes subjects as: double-diabetes, hybrid-diabetes, type 1.5-diabetes, early onset T2D or late-onset autoimmune diabetes (LADA) (165–167). The potential efficacy of GABA in hybrid diabetes is relevant given the purported capacity of GABA to increase beta cell mass and reduce glucagon (17, 80, 168). In the high fat fed/streptozocin type 2 diabetic rat model, GABA improved insulin sensitivity and reduced expression of lipogenic genes both in diabetic rat mothers and their offspring (169). Using the same model, Sohrabipour, et al. demonstrated that GABA (1.5 gr/kg/day, IP) normalized hyperglycemia, improved insulin sensitivity (measured by insulin clamp), reduced liver glucagon receptor mRNA (but not glucagon levels), and increased muscle GLUT4 translocation to plasma membrane as well as GLUT4 mRNA expression (48). Concerning the insulin-resistant phenotype, GABA treatment also reduced diabetic rat abdominal fat compared to an insulin-treated counterpart. In an olanzapine-induced insulin resistant model, GABA treatment (50mg/kg/day, *i.p.*) decreased insulin resistance through GABA-B receptor dependent mechanisms in adipose stromal vascular tissue (170). In pancreatic donor islets from non-diabetic versus T2D individuals (171), GABA-A receptor subunits in the T2D islets were downregulated compared to controls. The authors propound that deficient islet GABA signaling/content may contribute to the hyper-glucagonemia of T2D which again reinforces a role for GABA-based therapeutics.

7 Conclusion

The role of GABA as a safe and inexpensive therapeutic agent for diabetes is reviewed herein. Unlike exogenous interventions, GABA is a natural compound with a distinctive physiologic role in the pancreatic islet and nutrient gut. GABA is available in select foods and over-the-counter supplements. Pharmacokinetic and safety studies demonstrate that oral GABA has a short half-life, excellent tolerability and does not cross the blood brain barrier. Insofar as GAD autoantibodies are detected early in nearly all T1D, the logical segue was that islets would be depleted of the product of this enzyme, namely, GABA. Indeed, this has been confirmed in T1D and T2D donor islets. Preclinical studies demonstrated reversal of rodent diabetes (immune or chemically-mediated) using intermittent or continuous oral GABA dosing, as well as with subdermal implants. To date, experimental animal GABA doses (mg/kg/day) are generally many-fold higher than employed in clinical studies and, in general, higher doses (Figure 2) were more likely to elicit a favorable metabolic response. In adults, two small cohorts ingested 20-50 times the usual over-the-counter GABA dose (~1000 mg/day) for days to months without incident. There are no long-term safety data regarding GABA treatment. New approaches are emerging to prolong the half-life and efficacy of GABA using GABA receptor agonists (21, 55), long acting formulations (144), and positive allosteric modifiers (172). These agents may obviate the need for frequent and high oral doses of GABA.

slet studies in rodent and human islets, including single-cell transcriptomics, have unveiled a multitude of paracrine and autocrine GABA actions. There is more to learn regarding the GABA's role in modifying β-cell survival, regeneration and insulin secretory patterns. How GABA partakes in the regulatory crosstalk between the three major islet endocrine cells (β, α, and δ) is under study (15). Given its safety record and anti-inflammatory action, GABA may play a role in islet transplantation, either alone or in combination with other immunosuppressive or anti-apoptotic agents.

The endocrine and immunologic roles of GABA within the entero-pancreatic mid-gut as pertains to diet, the microbiome and the abundant gut-associated and pancreatic lymph tissue is likewise ripe for study (16, 163). A host of questions persists. Do GABA-enriched foods have health benefits? Do environmental toxins/antibiotics lead to GABA-deficient dysbiosis and reduced innate immunity? Human immune cells have GABA receptors including lymphocytes, CD4+. CD8+, PBMC, and monocytes. Do GABA-producing microbes have an immunosuppressive role concerning T1D autoimmunity? Could GABA-producing microbiota have analogous immune protective actions to SCFA-secreting microbes concerning β-cell immune protection and crosstalk in T1D? Do GABA-producing microbiota participate in primary TID prevention? To the point, disappearance of *bifidobacterium infantia* from the infant gut is implicated in the early dysbiosis of T1D (173). Of relevance, *b. infantia* is a recognized GABA-producer (111, 174). Probiotic trials frequently select Lactobacillus (phyla Firmacutes) as well as Bifidobacteria (phyla Actinobacteria) both of which contain recognized GABA-producing microbes via expressed glutamate decarboxylase (GAD). Whether microbial dysbiosis sensitizes the host immune system to GAD sequence dissimilarities between human and microbial is an alluring hypothesis (129) that deserves further consideration. Preventive management of gut dysbiosis might theoretically diminish this risk by correcting microbial imbalances and maintaining gut integrity.

GABA elicits an antidiabetic outcome by numerous routes. The fact that GABA can strikingly reverse hyperglycemia in diabetic mice, both STZ-induced and immune models, merits further clinical trials. Given the depletion of GABA in islets from patients with T1D and T2D (35), repletion of islet GABA may have pharmacologic application. Whether systemically administered GABA can replete this is unsettled, no less whether the experimentally measured islet cell deficit is indeed pathogenic. An alternative multipronged therapeutic approach would be GABA in conjunction with other immunomodulary or anti-diabetic compounds that have diverse mechanisms of action. Examples include GLP-1 agonists, DPP-4 inhibitors, TxNIP inhibitors, islet antigens, low-dose anti-CD3 antibody, and positive allosteric modifiers of GABA (13, 14, 21, 22, 59, 67).

The propitious safety profile of GABA renders early and longer-term GABA therapeutics particularly attractive, especially in stage 1 and 2 diabetes. The ongoing GPPAD-02 infant study (175) provides a paradigm for primary prevention with oral GABA. The underexplored role of endogenous GABA-producing microbiota in the immunoprotective enteropancreatic gut is apt for preclinical study and randomized controlled trials (RCT) with GABA producing probiotics in stage 1 diabetes (113). A lifetime of microbiome-protective nutritional and pharmacologic options for gut health may also defend against T1D. Combination therapy of GABA with a complimentary oral agents such as a TxNIP inhibitor or positive allosteric modifier in stage 2 T1D is an inexpensive intervention, and especially attractive insofar as the low toxicity. Based on residual β-cell function in stage 3 diabetes (176), β-cell preservation may also be feasible with longer acting or higher dose GABA formulations (82). Looking forward, GABA may have unique and previously underappreciated therapeutic benefits in TID to increase β-cell content, reduce excess glucagon and curtail the inflammatory T-cell dysfunction of type 1 diabetes.

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Glucagon-like peptide-1 receptor agonists and type 1 diabetes: a potential game changer?

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This mini review explores the increasing prevalence of obesity in type 1 diabetes (T1D) and the challenges patients face in achieving optimal glycemic control with current treatments. It discusses the evidence supporting the use of glucagon-like peptide-1 receptor agonists (GLP-1RA) as potential adjunctive therapy in T1D to reduce weight and improve insulin resistance. Potential benefits need to be weighed against the risk of hypoglycemia and lack of long-term data.

KEYWORDS

obesity, adiposity, insulin resistance, overweight, weight loss

1 Introduction

Since its introduction in 1921, insulin has changed the management and prognosis of patients with type 1 diabetes (T1D). New insulin formulations, together with advancements in insulin delivery and glucose monitoring technology, have changed the landscape for people with T1D. Notwithstanding these developments, only about 20% of patients with T1D achieve adequate glycemic control based on current targets (1). In addition, weight gain remains a significant concern for patients with T1D on intensive insulin therapy.

Based on recent data from National Health and Nutrition Examination Survey (NHANES) 2021-2023 (2), the prevalence of obesity in adults is 40.3% in the USA. Patients with T1D are equally affected by the obesity epidemic, and they have experienced a rapid increase in the prevalence of obesity in the last few years (3–6). Therefore, there is a strong need for new interventions to help manage obesity and hyperglycemia in T1D.

Glucagon-like peptide-1 receptor agonists (GLP-1RA) are effective to treat adult and pediatric populations with type 2 diabetes (T2D) and/or obesity, and they have an established safety profile. Their use in these populations has been associated with hemoglobin A1c reduction, significant weight loss, and a decrease in long-term microvascular and macrovascular complications (7–11). Even in patients without diabetes, semaglutide showed a decrease in cardiovascular events by ~20% (12). Recent American Diabetes Association (ADA) guidelines recommend GLP-1RA for weight

management in T2D, and also GLP-1RA as first-line therapy in patients with T2D and established cardiovascular disease (13). However, their use in T1D is not recommended by any current guidelines. This likely responds to the fact that patients with T1D were excluded from large randomized, controlled trials (RCTs) assessing cardiovascular and renal outcomes with the use of these drugs (7, 8, 11). Moreover, evidence from RCTs in T1D has not consistently shown benefits in A1c reduction, insulin dose reduction, or other outcomes. However, most of these studies were done with liraglutide or other daily GLP-1RA, which are not as effective for weight loss as the newer GLP-1RA (e.g., semaglutide or tirzepatide). In addition, many of the studies were not even designed to target patients with elevated BMI, who are the subjects likely to benefit the most from these compounds. One could hypothesize that the weight loss benefit of these drugs can be, at least in part, extrapolated to patients with T1D as it is mainly driven by appetite suppression (14).

This mini-review discusses clinical studies evaluating adjuvant treatment with GLP-1RA in patients with T1D as an opportunity to improve glycemic control, achieve weight loss, and decrease total daily insulin (TDI) requirements in these patients.

2 Obesity in type 1 diabetes

Obesity has become a significant global health burden (15), and patients with T1D, who were historically characterized as lean, are nowadays found to be overweight or obese with increasing frequency in clinics. The prevalence of overweight and obesity has increased in T1D in pediatric and adult populations, and this has occurred at a faster pace than in the general population (3–5). In a recent study in the USA, overweight and obesity were reported in 34% and 28%, respectively, of patients with T1D (6). Currently, patients with T1D have a similar prevalence of overweight and obesity compared to the general population (2).

Intensive insulin treatment improves glycemic control and reduces the risk of microvascular complications. However, it is considered an important risk factor for weight gain (16, 17) (Figure 1). In addition, prevention and treatment of hypoglycemia with excessive carbohydrates also contribute to weight gain. Fear of hypoglycemia can also lead to a reduction of physical activity and sedentarism, coupled with overeating. Other important contributing factors are eating disorders and depression, which are more common in patients with T1D compared to the overall population (18, 19). Weight gain causes insulin resistance (IR), which results in higher insulin requirements (20). Hyperinsulinemia is a key factor driving IR, thus leading to a positive feedback loop (i.e., hyperinsulinemia > IR > hyperinsulinemia).

Obesity increases the risk of obesity-related as well as diabetes-related complications in patients with T1D, including micro- and macrovascular complications, various types of cancer, and overall mortality (17, 20–22). For example, Wallace et al. showed that obesity in patients with T1D is associated with an increased risk of chronic kidney disease (CKD) compared with T2D (23). Obesity may also increase the risk of developing T1D (24, 25), and this could be one of the factors explaining the increasing incidence of T1D worldwide. Based on this hypothesis, obesity drives IR, which leads to hyperglycemia causing pancreatic β cell apoptosis. This process increases immunogenicity, which in turn leads to autoimmunity and the development of T1D in genetically predisposed patients (26, 27) (Figure 1).

3 Insulin resistance in type 1 diabetes

Insulin resistance is associated with micro- and macrovascular complications in patients with T1D (28). In these patients, IR is likely multifactorial and not fully understood (Supplementary Figure 1). While increased adiposity (i.e., overweight and obesity) is the usual driver of IR, other factors may play a role in the development of IR in T1D. For example, a family history of obesity and T2D may be an independent risk factor for obesity and IR in patients with T1D (29, 30). In a small study, Donge et al. compared IR measured by the gold-standard hyperinsulinemic euglycemic clamp in lean patients with T1D and healthy controls. They observed that patients with T1D were more insulin resistant than their BMI-matched counterparts at the levels of the liver, skeletal muscle, and adipose tissue (31). Similar results were found by larger studies (32). These findings suggest that there are additional factors contributing to IR other than obesity in T1D.

Insulin resistance in T1D could also be related to the insulin administration route. In subjects without type 1 diabetes, insulin is secreted from the pancreas into the portal vein, where 50-80% of insulin is metabolized in the first hepatic pass. On the contrary, exogenous insulin is absorbed from the subcutaneous tissue to the peripheral circulation, resulting in relative peripheral hyperinsulinemia and hepatic hypoinsulinemia (i.e., low portal-to-peripheral insulin ratio) (29). Indeed, peripheral insulin levels are ~2-fold higher in patients with T1D compared to patients matched for hyperglycemia (33). Compared to patients with MODY 2, who were well-matched for hyperglycemia and obesity, patients with T1D were significantly more insulin resistant, suggesting that IR is driven by peripheral hyperinsulinemia and not hyperglycemia (34). Indeed, peripheral insulin level was the strongest determinant of insulin resistance in this study. It is well-established that elevated peripheral insulin levels can modify insulin receptor expression and affect insulin sensitivity in skeletal muscle and adipose tissue (35). In support of this, when insulin is infused to healthy individuals without diabetes to levels observed in patients with T1D on insulin treatment, insulin sensitivity decreases (36). Moreover, when insulin administration route is changed from subcutaneous to intraperitoneal, glycemic control improves with lower insulin requirements, suggesting an improvement in insulin resistance (37). It has also been suggested that hepatic hypoinsulinemia may reduce insulin-like growth factor 1 (IGF-1)

Abbreviations: CSII, continuous subcutaneous insulin infusion; GLP-1, glucagon-like peptide-1; GLP-1RA, glucagon-like peptide-1 receptor agonists; IR, insulin resistance; MDI, multiple daily injections; RCT, randomized controlled trial; T1D, type 1 diabetes; T2D, type 2 diabetes; TDI- total daily insulin.

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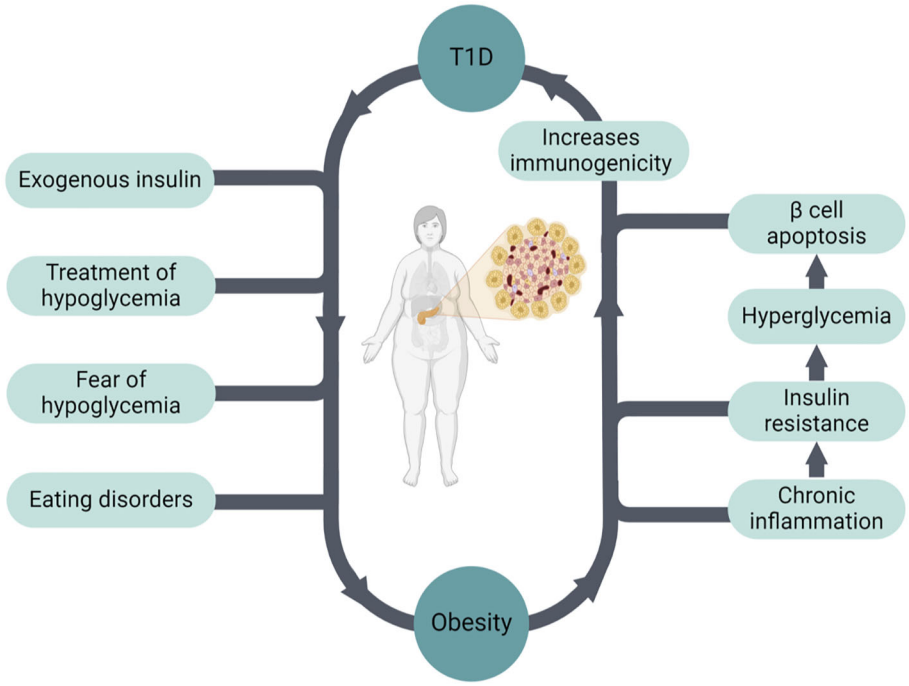

FIGURE 1
The bidirectional relationship between obesity and T1D.

levels, which may contribute to IR as well by increasing growth hormone and IGF binding globulins (29).

Defining IR in patients with T1D in the clinical setting is also challenging. While we can assume that increasing insulin requirements would be a surrogate marker, this will depend on the carbohydrate intake of the patients. The hyperinsulinemic euglycemic clamp technique is the gold standard for measuring insulin sensitivity. However, this technique is impractical in the clinical setting and it is mostly used for research purposes. Other methods to measure IR, such as homeostatic model assessment for insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) are based on the measurement of fasting insulin and glucose. Therefore, these equations cannot be used in patients with exogenous insulin use (38). Estimated glucose disposal rate (eGDR) is a validated tool to estimate insulin sensitivity in type 1 diabetes using HbA1c, waist circumference, and presence of hypertension (39). However, its clinical usefulness remains uncertain.

Despite important knowledge gaps in our understanding of IR in T1D, targeting IR in overweight/obese patients with T1D may decrease the risk of diabetes complications, contribute to weight loss, and improve glycemic control.

4 Glucagon-like peptide-1 receptor agonists in type 1 diabetes

Glucagon-like peptide-1 (GLP-1) is secreted in response to food consumption from intestinal L-cells. GLP-1 is a multifaceted hormone, and the use of its analogs is associated with broad metabolic effects: they increase insulin secretion, decrease glucagon release, increase glucose uptake in muscles, decrease glucose production in the liver, improve lipid profile, slow gastric emptying, and increase satiety leading to weight loss (40). GLP-1 regulates inflammatory response by lowering the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 6 and interleukin 1β, and stimulates activation of regulatory T cells. Specifically, GLP-1 reduces islet inflammation, inhibits apoptosis, and induces β cell proliferation in experimental models (41–43). This led to the consideration of GLP-1RA as medications that could change β cell function and survival in patients with T1D. Moreover, in patients with T2D, these compounds have shown cardiovascular, neurological, and renal protection (9, 12, 40, 44, 45), complications also frequently seen among patients with T1D.

GLP-1RA have been used to treat T2D for ~20 years, and they have transformed T2D management in pediatric and adult populations (9, 10, 44). One can hypothesize that their effects on glycemic control and reduction in long-term microvascular and macrovascular complications in patients with T2D might be equally beneficial to people with T1D, especially if overweight or obese. GLP-1RA can potentially improve quality of life through weight loss and reduction in insulin requirements. In addition, GLP-1RA reduce glucagon secretion, which can lessen postprandial hyperglycemia in patients with T1D (46, 47). However, there is concern about an increased risk of hypoglycemia and ketotic hyperglycemia. A recent study showed that, although not approved for T1D in the USA, GLP-1RA have been increasingly prescribed in patients with T1D (i.e., from 0.3% in 2010 to 6.6% in

2023) (48). As expected, the greatest increase in GLP-1RA prescriptions was among patients with T1D and obesity.

4.1 HbA1c reduction

4.1.1 Uncontrolled studies

In a small, uncontrolled study, weekly semaglutide in 10 patients with newly diagnosed T1D, mean HbA1c improved dramatically from 11.7 ± 2.1% to 5.7 ± 0.4% at 12 months (49). However, results are difficult to interpret as this study was uncontrolled and fasting baseline C-peptide levels were relatively high at 0.65 ± 0.33 ng/mL. It is possible that some of those patients were in honeymoon period, or had an alternative diagnosis, such as ketosis-prone diabetes or latent autoimmune diabetes in adults (LADA). Due to the retrospective nature of the data, other confounding factors may have affected the results as well. In 'real-world' studies evaluating the efficacy and safety of GLP-1RA in patients with T1D, HbA1c had significant reductions of 0.4-0.5% (50, 51). Because these studies allow for concomitant insulin adjustments, and they lack a controlled group (i.e., placebo) interpretation of the A1c reduction is very difficult.

4.1.2 RCTs

Several blinded, placebo-controlled RCTs have demonstrated a significant reduction in HbA1c when adding GLP-1RA to either multiple daily injections (MDI) or continuous subcutaneous infusion of insulin (CSII), ranging between 0.1-0.7% (52–61) (Table 1). The high heterogeneity of the results likely responds to different baseline A1c, variable rates of obesity, as well as different titration protocols to adjust insulin doses. Subgroup analysis in patients with positive baseline C-peptide levels showed a more robust reduction of HbA1c (i.e., 0.69-0.83%) on GLP-1RA in this group (56, 57). Overall, a meta-analysis of RCTs showed that A1c reductions were −0.28%, −0.21%, and −0.17% for liraglutide 1.8, 1.2, and 0.6 to 0.9 mg, respectively, and −0.17% for exenatide (62). Larger and longer studies are needed to evaluate this further.

4.2 Total daily insulin and C-peptide secretion

4.2.1 Uncontrolled studies

Using weekly semaglutide, prandial insulin was discontinued in all patients, and basal insulin was discontinued in 7 out of 10 patients. In addition, increased C-peptide levels and better glycemic control during the year of observation were noted (49). However, as aforementioned, due to the lack of a placebo group, high baseline C-peptide levels, and concomitant use of a restricted carbohydrate diet, these results are difficult to interpret or extrapolate to other populations. A small, short study had two patients on liraglutide with positive postprandial C-peptide at baseline completely discontinued insulin treatment with good glycemic control (63). In this study, insulin dose reduction was higher in patients who had a positive C-peptide at baseline, emphasizing that early initiation may be of benefit. Lower insulin requirements are also noted in 'real-world' studies (50, 51).

4.2.2 RCTs

In RCTs, insulin dose reductions were observed with the addition of GLP-1RA (52–61) (Table 1). Only dulaglutide treatment did not decrease TDI, however this was a small study (n=18) (52). Although β cell function improves in T2D patients, treatment with liraglutide in T1D did not significantly change mean C-peptide concentration (56–58). Similar results were seen in the study by Pozzilli et al., where the efficacy of once weekly albiglutide on preserving β cells was assessed in patients with newly diagnosed T1D. C-peptide levels were not significantly different between the intervention and placebo groups (53). However, all studies were performed on stage 3 type 1 diabetes, so whether initiation of these drugs at earlier stages of the disease can help prevent β cell function loss is unknown.

4.3 Weight change

4.3.1 Uncontrolled studies

While insulin monotherapy causes consistent weight gain, adding GLP-1RA leads to consistent weight loss. Weight loss appears to be more rapid in the first 10-12 weeks, but it continues to decrease at a slower rate thereafter (64). In 'real-world' studies weight loss was significant after 12 months and 3 years of treatment (50, 51).

4.3.2 RCTs

In RCTs (Table 1) (52–61), weight loss was observed in all trials, except with albiglutide. Weight loss was similar in patients on CSII, with a mean weight loss of 6.3 kg (55). Weight loss was also similar in studies with dulaglutide and exenatide (52, 54). Of note, not all studies focused on overweight or obese patients, with many allowing patients with BMI ≥20 kg/m^2. Even in these studies including patients without overweight or obesity, significant weight loss was observed. As expected, those studies limiting BMI to >25 kg/m^2 had a larger reduction in weight. In a meta-analysis with patients with T1D, weight loss with liraglutide 1.8 mg was estimated at ~5 kg compared to placebo (62), and there was a dose-response effect.

4.4 Safety

Hypoglycemia is common in patients with T1D. The average patient with T1D experiences two episodes of symptomatic hypoglycemia a week (65). In a survey of 436 participants with T1D, 72% of those who drive a vehicle reported having hypoglycemia events while driving, and 4.3% reported having a vehicular accident due to hypoglycemia in the previous 2 years (66).

In RCTs comparing GLP-1RA and placebo, insulin doses were reduced before GLP-1RA initiation to avoid hypoglycemia, and close monitoring and insulin adjustments were done periodically. As can be observed in Table 1, basal insulin was reduced by ~10-25% and

TABLE 1 Summary of randomized, controlled trials assessing GLP-1RA in patients with T1D.

Study	Treatment		n	Duration (weeks)	Δ HbA1c (%)	Δ Body weight (%)	Δ TDI (%)	Hypo-glycemia	Primary endpoint	DM stage	Insulin dose titration with GLP-1RA initiation	BMI Inclusion criteria (kg/m^2)
Frandsen et al., 2015 (59)	Liraglutide 1.2 mg		36	12	-0.1%	-5.7%	-7%	~	HbA1c	No β cell reserve	-25% bolus -10% basal	18-28
Lira-1 trial 2016 (58)	Liraglutide 1.8 mg		100	24	-0.2%	-7.3%	-15%	↓	HbA1c	> 1 year	-33% bolus -25% basal	>25
Kuhadiya et al., 2016 (60)	Liraglutide	1.2 mg	63	12	-0.48%	-4.9%	-14.3%	~	Weekly BG levels	> 1 year	-25% bolus -10% basal Only if HbA1c<7.5%	none
		1.8 mg			-0.12%	-5.4%	-16.8%	~				
ADJUNCT ONE trial 2016 (57)	Liraglutide	1.2 mg	1,398	52	-0.15%	-4.2%	-2%	↑	HbA1c, TDI, and weight	> 1 year	-25% TDI, plus -10% with dose escalation	≥20
		1.8 mg			-0.20%	-5.7%	-5%	↑				
ADJUNCT TWO trial 2016 (56)	Liraglutide	1.2 mg	835	26	-0.23%	-4.5%	-7%	↑	HbA1c	> 1 year	-25% TDI, plus -10% with dose escalation	≥20
		1.8 mg			-0.35%	-5.8%	-10%	~				
Lira Pump trial 2020 (55)	Liraglutide 1.8 mg		44	26	-0.7%	-7.4%	-15%	~	HbA1c	> 1 year	-15% bolus -10% basal	>25
MAG1C trial 2020 (54)	Exenatide 10μg TID		105	26	-0.1%	-5.0%	-15%	~	HbA1c	> 1 year	Adjusted individually	>22
Pozzilli et al., 2020 (53)	Albiglutide 50 mg		61	52	+0.1%	+0.2%	NR	~	Stimulated C-peptide	New *	Algorithm based on glucose levels	<32
DIAMOND-GLP-1 trial 2023 (52)	Dulaglutide 1.5 mg		18	24	-0.1%	-6.4%	NR	↓	HbA1c	With β cell reserve#	Not specified	16-30
NewLira trial 2024 (61)	Liraglutide 1.8 mg		68	52	-0.37%	-4.4%	-70%	↓	Stimulated C-peptide	New ^	-25% bolus -10% basal	>20

*4-8 weeks since insulin initiation.
#C-peptide levels above 15 pmol/l.
^up to 6 weeks from diagnosis.
All studies were blinded and placebo-controlled. Changes reflect the placebo-subtracted effect. n, number of subjects; TDI, total daily insulin; DM, diabetes; ↑, significantly elevated compared to placebo; ~, unchanged compared to placebo; ↓, decreased compared to placebo.

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bolus doses by ~15-33% in RCTs. Therefore, hypoglycemia rates may not reflect hypoglycemia rates observed in clinical practice. However, even in large 'real world' cohorts of patients with T1D on GLP-1RA, low rates of hypoglycemia were observed (50, 51).

ADJUNCT ONE and TWO trials, the 2 largest studies with GLP-1RA in T1D, reported increased rates of hypoglycemia. The ADJUNCT ONE trial found that symptomatic hypoglycemia had a dose-related effect with a rate ratio between 1.27 and 1.31 on doses of 1.2 and 1.8 mg of liraglutide, respectively. While the ADJUNCT TWO also reported an increased rate ratio of symptomatic hypoglycemia of 1.33 on 1.2 mg liraglutide, events were not significantly different with the 1.8 mg dose in that study. In other studies, GLP-1RA addition did not increase the risk of hypoglycemia. Overall, results are heterogeneous, but they point towards a small increase in the risk of hypoglycemia. However, adequate adjustment of insulin doses and close monitoring after GLP-1RA initiation may decrease this risk. Of note, a meta-analysis by Park et al. showed no differences in the frequency of symptomatic hypoglycemic events or severe hypoglycemia (62).

As insulin doses are reduced to prevent hypoglycemia, there is also a potential risk of hyperglycemia and eliciting diabetic ketoacidosis. The risk of ketotic hyperglycemia did not increase in the aforementioned meta-analysis (56). Among RCTs, increased ketotic hyperglycemia events were reported in the ADJUNCT trials in a dose-related manner (48, 49). However, it was not reported in smaller trials (53–55, 58–61), and the DIAMOND-GLP-1 trial reported a decrease in the frequency of events (52, 52).

In retrospective studies, acute pancreatitis was not reported in patients with T1D treated with GLP-1RA (45, 50, 51). In 2014, the FDA and the EMA found no causal association between GLP-1RA and pancreatic adverse events (67). Pancreatitis was not reported in the RCTs (52–61).

While some of the trials reported a decrease in appetite as a side effect, like the ADJUNCT trials (53–58, 60), it should be noted that this is one of the main mechanisms of action of these drugs to achieve weight loss. Nausea and gastrointestinal adverse effects were reported in about half of patients with T1D on liraglutide (56–58). However, a relatively low discontinuation rate was reported, 0-15%. For example, in the ADJUNCT ONE trial, the rate of nausea with the highest dose of liraglutide (1.8mg) was 49.6% compared to 12.1% in the placebo. Use of liraglutide 3mg daily in patients with obesity without diabetes was associated with nausea in 40.2% compared to 14.7% in the placebo (68). While it is difficult to compare these studies head-to-head, overall, it seems that gastrointestinal symptoms are relatively similar in patients with *vs.* without T1D. In a meta-analysis of GLP-1RA use in T1D, all adverse events were significantly higher in the GLP-1RA group, but there was no difference in serious adverse events (62).

4.5 Pediatrics

Only few small studies have assessed the use of GLP-1RA as adjuvant therapy in pediatric patients with T1D. A small trial including 8 patients with T1D showed improved postprandial hyperglycemia despite 20% insulin dose reduction (69).

5 Cardiovascular and renal outcomes with GLP-1RA in T1D

In patients with T2D, large RCT studies have shown that GLP-1RA treatment reduces the risk of cardiovascular and renal outcomes (7, 8, 11, 70). Moreover, the SELECT study showed that semaglutide significantly reduced the incidence of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke (hazard ratio 0.80) (12). Unfortunately, due to different FDA regulations and safety concerns, it would be hard for this type of RCTs to concomitantly include patients with T1D and T2D, and therefore patients with T1D were excluded from these trials. Therefore, results cannot be extrapolated to the T1D population. However, observational data has shown that obesity in T1D is associated with higher risk of microvascular and macrovascular complications (17, 20–22). Therefore, one can argue that targeting obesity in T1D may share some of the benefits observed in T2D or in obesity without diabetes.

6 Practical issues when prescribing GLP-1RA in T1D

GLP-1RA are not currently approved for treatment of T1D, but they may still be covered by insurance if prescribed to treat obesity in these patients (although insurance coverage for obesity remains limited). Based on data from RCTs in T1D and those with patients with T2D, initiation of a GLP-1RA should be followed by a decrease of total insulin dose to reduce events of hypoglycemia (71). The amount of reduction of insulin dose should be determined on an individual basis based on prior diabetes control, risk of hypoglycemia and ketosis, type of GLP-1RA started, among others. In RCT studies on T1D, at the initiation of GLP-1RA therapy, bolus insulin was reduced ~25-33% and basal insulin ~10-25% (56–61) as observed in Table 1. In some of the studies, an additional 10% dose reduction was done with drug escalation. In the study by Kuhadiya et al. insulin dose at GLP-1RA initiation was only decreased if HbA1c was below 7.5% (60). In the Lira Pump study, they decreased basal insulin by 10%, but increased bolus by 15% (55). Only in the ADJUNCT trials an increase in ketotic hyperglycemia events were reported with these dose adjustments (56, 57).

Our current practice is to consider a ~20% bolus insulin decrease and a ~10% basal insulin decrease before GLP-1RA initiation, although prior A1c, risk of hypoglycemia, potential risk of ketosis due to reduced insulin doses, as well as other patient-specific factors should guide the final insulin dose changes. Further adjustments may be needed as patients experience weight loss. Rapid weight loss or prolonged fasting periods due to appetite suppression is likely to be associated with a higher risk of hypoglycemia, and therefore, should be avoided in patients with T1D. Titration of GLP-1RA based on the individual's response in weight and glycemic control is recommended. Slow titration will also help decrease gastrointestinal symptoms that may arise. These symptoms improve with continuous use, as only 3% and 9% of patients reported nausea with the long- or short-acting GLP-1RA after 6 months of use, respectively (72). These medications, except exenatide, are safe for patients with mild to severe renal impairment without dose adjustments (73, 74). GLP-1RA can be used in patients

with hepatic impairment, without dose adjustments, although they have not been extensively studied in these circumstances (71).

7 Conclusions

Obesity increases the risk of microvascular and macrovascular complications, various types of cancer, and overall mortality in patients with T1D. New approaches are needed to address the rising rates of overweight and obesity in the T1D population. Despite new insulin formulations and improvements in the technology of insulin pumps and continuous glucose monitoring, glycemic control continues to be suboptimal in T1D, with about 80% of patients not reaching the recommended goals. Therefore, GLP-1RA is a promising group of medications to treat obesity, improve glycemic control, and decrease the risk of complications in these patients.

Overall, studies have shown that these medications have an established safety profile in T1D, with only a modest effect on HbA1c, but significant weight loss, and a reduction in TDI. These effects occur at the expense of a slight increase in hypoglycemia, but careful titration of insulin doses may mitigate this risk. Weight loss compared to placebo was ~5% in RCTs. However, there is lack of RCTs in T1D using the newest generation of GLP-1RA or dual GLP-1/GIP agonists, which are associated with significantly more weight loss. Moreover, because patients with T1D were excluded from studies looking at cardiovascular and renal outcomes with the use of GLP-1RA, it remains unknown whether those benefits observed in patients with T2D or obesity without T2D translate to the T1D population. In addition, whether early initiation of GLP-1RA in patients with newly diagnosed T1D can preserve β cell function remains to be determined in large RCTs. Until more research is available, the use of these drugs in T1D should be done carefully, with a thorough discussion with patients about potential risks and benefits of this approach.

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The partial clinical remission phase of type 1 diabetes: early-onset dyslipidemia, long-term complications, and disease-modifying therapies

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No therapy confers complete β-cell protection at any of the 3 stages of type 1 diabetes (T1D). Disease-modifying therapies in type 1 diabetes aim to prolong the preclinical (stages I and II) and the post-diagnostic partial clinical remission (PR) phases of T1D to reduce its short- and long-term complications. These therapies are focused on mitigating β-cell apoptosis by reducing autoimmune attacks on surviving β-cells through several pathways; as well as improving β-cell function to enable the production of functional endogenous insulin and C-peptide through the reduction of proinsulin to C-peptide ratios and other measures. These therapies target the 3 stages of T1D as monotherapy or combination therapy. Stage I of T1D is marked by the presence of at least one diabetes-associated autoantibody in an individual with normoglycemia; stage II is marked by the presence of diabetes-associated autoantibodies and dysglycemia; stage III is marked by the clinical diagnosis of T1D in an individual with antibodies, hyperglycemia, and symptoms. Conventional thinking suggests that the long-term complications of diabetes are principally rooted in early-stage hyperglycemia at the time of diagnosis of the disease, i.e., stage III of T1D. However, this theory of hyperglycemic memory is limited as it does not address the dichotomy in lipid-based atherosclerotic cardiovascular disease (ASCVD) risk in those with T1D. Given the current limitations to developing disease-modifying therapies in T1D because of the limited impact of current agents on β-cell preservation, we introduce the theory of hyperlipidemic memory of type 1 diabetes. This theory was developed by the author in 2022 using the same population as in this article to address the shortcomings of the theory of hyperglycemic memory and explain that the dichotomy in ASCVD risk is based on PR history. In this Review, the theory presents new pathways for disease-modifying therapies in T1D that focus on preventing early-phase dyslipidemia. It is hoped that including this theoretical framework in designing disease-modifying therapies in T1D will help move the field forward. This new theory supports the hypothesis that PR is an imprimatur rather than a process. It hypothesizes that pre-diagnostic interventions, at stages I or II of T1D, to ensure the occurrence of PR may be more effective in the long term than post-diagnostic interventions, at stage III, to prolong PR. This paradigm shift in approach to disease-modifying therapy in T1D is discussed in this review.

KEYWORDS

type 1 diabetes, type 2 diabetes, dyslipidemia, hyperglycemic memory, hyperlipidemic memory, honeymoon phase, partial clinical remission

1 Introduction

Diabetes mellitus affects 38.4 million Americans or 11.6% of the population (1). The leading cause of death in individuals with diabetes is atherosclerotic cardiovascular disease (ASCVD) (2). The total cost of diabetes care and management in the United States in 2022 was more than $412.9 billion (3). More than 50% of patients with type 2 diabetes (T2D) have pre-existing CVD at the time of diagnosis (4). However, there are no clear data on **early** CVD prevalence in patients with type 1 diabetes (T1D) (5), despite the high mortality from coronary artery disease of approximately 3- to 10-fold higher than in the general population (4).

1.1 The partial clinical remission phase of type 1 diabetes

T1D is marked by persistent hyperglycemia resulting from autoimmune destruction of the pancreatic β-cells (6). A period of partial clinical remission (PR) may follow the diagnosis of T1D, i.e., stage III of the disease, and this phase is marked by an increased functionality of the surviving β-cells with attendant endogenous insulin production (7, 8). Subjects who experienced PR are designated as remitters and those who did not are designated as non-remitters. PR typically lasts for 3-12 months (9), but could extend for decades into the established phase of T1D (10).

Despite the strong correlation between ASCVD and diabetes mellitus, the underlying mechanisms remain poorly understood (11), especially in T1D where 50% of the subjects undergo PR or honeymoon phase following the diagnosis of T1D (7, 12–14). However, the impact of PR on the earliest lipid phenotypes in individuals with T1D is not fully understood (15). Though PR has been reported to modulate the degree of early-phase dyslipidemia (16), mid-term microvascular disease risk (12), and long-term ASCVD risk (17), only one adult study (15) has directly compared the earliest phenotype of lipid-based ASCVD risk between subjects with T2D and T1D, after stratifying the subjects with T1D into remitters and non-remitters based on their PR history. Such stratifications are important to establish the nature and prevalence of dyslipidemia in T1D. PR-based stratified studies in patients with T1D will help to clarify unexamined contributors to diabetic dyslipidemia in children and adults with diabetes mellitus, such as the role of hyperlipidemic memory on post-diagnostic lipid phenotypes.

The Diabetes Control and Complications Trial demonstrated a protective role for C-peptide on the vasculature in remitters or patients with T1D who had residual β-cell function (17). The Medalist study (18) found that some adult patients with T1D for >50 years who were still producing endogenous insulin had better glycemic control and lipid profile compared to their peers. The T1D Exchange study (19) of 919 individuals reported that a great proportion of children and adult patients were still producing insulin several years after their diagnosis of T1D, i.e., at stage IV of T1D. This study reported the presence of residual C-peptide 3-5 years after the diagnosis of T1D in 78% of participants who were diagnosed at >18 years and 46% of those diagnosed at <18 years. They also found that 6% of subjects with childhood-onset-, and 16% of those with adult-onset T1D had residual C-peptide at 40 years or more following their diagnosis. This body of work and others form the basis for the current disease-modifying therapies in T1D to protect the β-cells, augment β-cells function, or expand the β-cell mass, Table 1.

Despite these landmark findings, there is a paucity of data on the characterization of early-onset, post-diagnostic lipid phenotypes in remitters and non-remitters (16) across the lifespan in both children and adults to enable the translation of crucial clinical data to PR-based ASCVD clinical guidelines. For example, there is currently no consensus on dyslipidemia in children and adolescents with T1D, as studies have reached differing conclusions, and it is believed that a lack of stratification of subjects by PR history may have confounded these results (32–35). Similarly, the literature in adults with diabetes mellitus showed that while the risk factors for ASCVD are well established in T2D (36), they are unclear in those with T1D (5, 11). In general, the lack of an understanding of the degree of PR-based dichotomy in early lipid phenotypes in subjects with T1D, i.e., remitters and non-remitters, and the assumption that subjects with T2D have worse lipid profiles than those with T1D have hindered a thorough assessment of the intrinsic differences in lipid phenotypes in patients with T1D (36) (5, 11),. As a result, the risk factors for ASCVD are well established in individuals with T2D (36), but not in those with T1D (5, 11).

Current knowledge indicates that several factors such as HbA1c concentration, diabetic nephropathy, hypertension, and dyslipidemia are important risk factors for ASCVD in adults with established T1D (37). However, the phenotype of the earliest ASCVD risk profile at the time of diagnosis of T1D, i.e., stage III of T1D, compared to T2D, and the cardinal role of PR on early lipid phenotype in those with T1D, which presages later ASCVD risk status, are not fully characterized.

This review article aims to address this important gap in knowledge with an emphasis on how this new lipid-based paradigm could be applied to pharmacologic interventions to augment the PR of T1D.

1.2 Prevalence of the partial clinical remission phase of type 1 diabetes

The introduction of the gold-standard clinical definition for PR, the insulin-dose adjusted hemoglobin A1c (IDAA1c) in 2009, has enabled a consensus on the estimation of PR in clinical practice (9). Recent studies show that the prevalence of PR in children and adolescents is approximately 50% (7, 12–14). This suggests that PR does not occur in a significant proportion of children and adolescents diagnosed with T1D (38–41). These individuals are referred to as non-remitters.

This high proportion of non-remitters reflects a key deficiency in the early management of children and adolescents T1D as there are no guidelines to prevent or address the early-onset dyslipidemia that occur in non-remitters (7, 12, 41, 42).

TABLE 1 Key disease-modifying therapies for type 1 diabetes that have been tested in clinical trials.

Disease Modifying Agent	Mechanisms	Effect on C-peptide_AUC and proinsulin-to-C-peptide (PI:C) ratio vs. placebo	Metabolic impact on glycemia and total daily dose of insulin (TDDI)	Safety concerns
Vitamin D Nwosu 2022 (20) Nwosu 2024 (21)	β-cell protection via reduction in β-cell stress through (a) reduced proinsulin to C-peptide ratio, and (b) tumor necrosis factor-alpha	• 20% higher C_AUC at 52 weeks, with an improved effect with time (N=36). • Reduction in PI:C	Vitamin D significantly reduced the temporal trends in A1c. No difference in the TDDI	Excellent safety profile.
Vitamin D and saxagliptin Yan et al. (22)	A combination of vitamin D's mechanism of action as noted above and a dipeptidyl peptidase 4 (DPP-4) inhibitor's role in preventing the degradation of glucagon-like peptide-1 which is associated with improved β-cell function	• The combination slowed the decrease in C_AUC at 24 months (N=301). No effect of saxaglipitin alone. • No data on PI:C	No difference in A1c. Significant reduction in the TDDI in the combination group.	No increased risk as monotherapy or combination therapy
Liraglutide (the New Lira Study) Dejgaard et al. (23)	Improved β-cell function by a GLP-1 receptor agonist	• Significantly higher AUC C-peptide in the Liraglutide vs placebo group: 176, 95 CI 142-208 nmol/L vs 120; 95 CI 97-143 nmol/L (N=68) • No data on PI:C	No significant difference in A1c. Significant reduction in the TDDI in the Liraglutide group	Gastrointestinal adverse effects. No increased risk for hypoglycemia
Vitamin D and Lansoprazole Reddy et al. (24)	A combination of vitamin D's mechanism of action as noted above and lansoprazole's proton pump inhibition which increases gastrin level and in turn increases β-cell neogenesis and survival	• No data on C_AUC • Slower reduction in fasting C-peptide at 6 months, 31% vs 48% (N=28). • No data on PI:C	No difference in A1c. Lower TDDI in the experimental group	No increased risk of adverse events.
Imatinib Gitelman 2021 (25)	Tyrosine kinase inhibitor that reduces β-cell endoplasmic reticulum stress and apoptosis	• 19% improvement at 12 months, no effect at 24 months (N=67). • No impact on PI:C	No difference in A1c or TDDI	Increased risk for side effects
Anti-IL-21 Liraglutide Trial Von Herrath 2021 (26)	A combination of the immunomodulatory activities of anti-IL21 antibody and the activities of Liraglutide, a GLP-1 receptor agonist to improve β-cell function	• Reduced C-peptide loss by combination therapy at 54 weeks, 10% vs 39% (N=308) • No impact by either anti-IL-2 or Liraglutide alone • No data on PI:C	Reduction in TDDI at 54 weeks in the combination arm versus placebo. No difference in A1c.	No increased risk.
Teplizumab Ramos 2023 (27)	Anti-CD 3 monoclonal antibody that protects the β cells by increasing the apoptosis of activated T cells while sparing regulatory T lymphocytes	• 59% higher C_AUC at 78 weeks (N=217). Effect stable over time. • No data on PI:C	No difference in A1c or TDDI	Headache, gastrointestinal symptoms, rash, lymphopenia, cytokine release syndrome
Bariticinib Waibel 2023 (28)	An oral Janus kinase (JAK) inhibitor that prevents the expression of cytokine-induced HLA-1 in islet cells and thus prevents CD8+ T cell activation	• 48% higher effect size for the median at 48 weeks (N=91). • No data on PI:C	No significant difference in A1c. Lower glucose variability and higher % time in range in the bariticinib group	No increased risk or acceptable side effect profile
Verapamil Forlenza 2023 (29)	Reduces thioredoxin-interacting protein expression that is linked to β-cell apoptosis	• 30% higher C-peptide level at 52 weeks (N=88). Effect stable over time. • No data on PI:C	No difference in A1c or TDDI	Increased risk associated with depression, nausea, vomiting, EKG abnormalities
REPAIR-T1D Trial: sitagliptin and lansoprazole Griffin et al. (30)	A combination of a dipeptidyl peptidase 4 (DPP-4) inhibitor's role in preventing the degradation of glucagon-like peptide-1 which is associated with improved β-cell function, and lansoprazole's proton pump inhibition which increases gastrin level and in turn increases β-cell neogenesis and survival	• No change (N=68). • No data on PI:C	No significant difference in metabolic outcomes between the groups	No increased risk

(Continued)

TABLE 1 Continued

Disease Modifying Agent	Mechanisms	Effect on C-peptide_AUC and proinsulin-to-C-peptide (PI:C) ratio vs. placebo	Metabolic impact on glycemia and total daily dose of insulin (TDDI)	Safety concerns
Low-dose anti-thymocyte globulin (ATG) and pegylated granulocyte colony-stimulating factor (GCSF) Haller et al. (31)	A combination of the immunomodulatory properties of ATG and GCSF to preserve residual β-cell function	• 40-50% effect size at 12 months (N=89) • Significantly higher AUC C-peptide in ATG cohort vs placebo, 0.646 nmol/L vs 0.406 nmol/L. • No difference in AUC C-peptide in those treated with combination ATG + GCSF • No data on PI:C	Lower A1c by ATG and ATG +GCSF versus placebo but no significant difference in the TDDI	Serum sickness, cytokine release syndrome, lymphopenia, musculoskeletal and connective tissue complaints

Despite increasing reports showing that remitters have a significant long-term prognostic advantage over non-remitters, this dichotomy in risk has not been considered in the early phase of diabetes management, as there is no clear guidance on strategies to prevent early-onset dyslipidemia in non-remitters, which represents a key drawback in early management T1D in children (7, 12, 41, 42). Additionally, the fact that approximately 50% of children with T1D will not experience PR suggests that post-diagnostic interventions at stage III of T1D might not be very useful in this subset of patients with T1D, i.e., the non-remitters.

1.3 The mechanism of the partial clinical remission phase of type 1 diabetes and associated theories of remission

1.3.1 The mechanisms of non-remission

The molecular mechanisms that determine the occurrence of remission or non-remission are not fully characterized (43). However, certain key factors have been identified. These include increased β-cell stress as marked by increased proinsulin-to-C-peptide (PI:C) ratio (44), increased glucagon concentration (45), unfavorable cytokine profile (46), and the role of immune mediators and genetic markers (43). Available data show that remitters possess a distinctive cytokine profile that protects the β-cells (46). Glucagon concentration is lower in remitters, which supports the premise that glucagon production is suppressed by intra-islet insulin production and release (45). Data supporting the key role for immune mechanisms in PR (47) show significantly lower concentrations of interferon-γ in remitters compared to non-remitters and controls, a higher frequency of $CD4^+$ $CD25^+$-$CD127^{hi}$ cells, and a non-Treg subset of memory T cell, which are all consistent with a slower rate of progression of T1D (48, 49). This supports the hypothesis that immune mediators could protect the β-cells and thus prolong PR. Moya et al. (49) suggested that the duration of PR could be predicted using a combination of the frequency of the $CD4^+$ $CD25^+$-$CD127^{hi}$ cells with glycemic markers at the time of diagnosis of T1D. In new-onset T1D, elevated islet antigen-specific interleukin-10-producing cells correlate with improved glycemia, while increased FoxP3 expression predicts a worse outcome (50). A genetic study (51) of patients with newly diagnosed T1D found that the level of circulating microRNA, has-miR-197-3p, at 3 months after T1D diagnosis strongly predicted the magnitude of residual β-cell function one year after the diagnosis of T1D. The marker for increased β-cell strain is poor proinsulin processing as data indicate that individuals who are more likely to undergo remission, such as overweight male children, have efficient proinsulin processing (44). Children with new-onset T1D generally have elevated PI:C ratio (52, 53). However, Nwosu et al. (21) recently demonstrated that high-dose vitamin D reduces PI:C ratio in this population. Clinical studies show that younger age at diagnosis, female sex, severe acidosis, and increased numbers of diabetes-associated autoantibodies are associated with non-remission (54). Thus, the occurrence and the duration of remission, or non-remission is determined by a constellation of genetic, immune, hormonal, and inflammatory factors.

2 Proposed theories to explain the partial clinical remission phase of type 1 diabetes

2.1 Impact of optimal glycemia on residual β-cell function

Data from the landmark Diabetes Control and Complications Trial suggested that intensive glycemic control following the diagnosis of T1D could preserve RBCF (17, 55). However, recent investigations in this area have shown mixed results, whereas some studies provide support for the DCCT findings (56), other studies (57–59) and systematic reviews (60) failed to show that improved glycemic control prolongs RBCF in subjects with new-onset T1D. A study by Enander et al. (59) is particularly interesting as it showed that RBCF at 2 years was associated with the initial A1c and C-peptide concentrations, but was independent of initial insulin regimens. This suggests that PR is rather a unique event or an imprint in the life history of T1D that occurs at the time of diagnosis of T1D and is determined by the constellation of prevailing factors at the time, i.e., stage III of T1D, such as the degrees of glucotoxicity and lipotoxicity (43). This suggests that interventions to prolong RBCF should focus on key pre-diagnostic pathways at stages I and II of T1D to either reduce β-cell stress through the use of agents such as high-dose vitamin D to reduce the PI:C ratio (21), or

immunomodulators such as teplizumab to reduce the impact of autoreactive T cells on β-cells (61), and not necessarily on interventions at stage III of T1D to alter the post-diagnostic glycemia. This new paradigm that RBCF is independent of post-diagnosis glycemia at stage III is supported by the theory of hyperlipidemic memory (62) and the concept of PR *imprimatur* where improved diabetes outcomes are largely independent of post-diagnosis glycemia, but rather on the dichotomy in lipid phenotypes that is determined by PR history (62).

We now examine the theories of hyperglycemic memory and hyperlipidemic memory in detail.

2.2 The theory of hyperglycemic memory of type 1 diabetes

Landmark studies in T1D show that intensive glycemic management preserves RBCF following the diagnosis of T1D (17, 55). The occurrence of residual endogenous insulin secretion in patients with T1D has been linked to reduced risk for severe hypoglycemia (63, 64), reduced development of diabetic retinopathy (65), promotion of statural growth in prepubertal children (66) and reduced risk for long-term complications of T1D (12, 17).

In contrast, the non-remitters experience chronic hyperglycemia from the time of diagnosis (12, 15). This initial phase of chronic hyperglycemia has been associated with long-term complications of diabetes mellitus, regardless of whether glycemia improved later in the history of the disease (43, 59). This phenomenology of diabetes complications arising from an initial chronic hyperglycemia has been christened the theory of hyperglycemic memory (67). Recent studies indicate that there are non-glycemic contributors to the phenomenon of hyperglycemic memory, and most of these factors are not fully characterized (43). As a result, some investigators now refer to this phenomenon as the glyco-metabolic theory (43). The researchers suggest that the mechanisms leading to glyco-metabolic memory are *interdependent* and act simultaneously. The 4 proposed mechanisms are oxidative stress, generation of advanced glycation end-products, chronic inflammation, and epigenetic changes (43). However, these studies did not examine the initial post-diagnostic lipid phenotypes in patients with newly diagnosed T1D to determine whether a dichotomy exists in the lipid parameters (between remitters and non-remitters) and whether non-remission is associated with both hyperglycemia and hyperlipidemia. Therefore, the theory of hyperglycemic memory has limited application as it does not explain the glycemia-independent dichotomy in early lipid phenotypes that presages subsequent divergence in ASCVD risks in patients with T1D.

2.3 The theory of hyperlipidemic memory of type 1 diabetes

As a result of the shortcomings of the theory of hyperglycemic memory, Nwosu (62) proposed the theory of *hyperlipidemic* memory of T1D which explains the divergence in lipid-based ASCVD risk and provides the necessary framework to understand the differences in lipid phenotypes between remitters and non-remitters on one hand, and between those with newly-diagnosed T1D or type 2 diabetes (T2D) on the other (Figure 1).

The theory of *hyperlipidemic* memory of T1D is premised on five years of research on the early post-diagnostic dichotomy in lipid phenotypes between remitters and non-remitters across the lifespan in children, adolescents, and adult patients with newly diagnosed T1D, T2D, and matched controls. This theory provides a rigorous explanation for the differences in lifelong ASCVD risk between remitters and non-remitters.

Nwosu and his colleagues (62) developed this theory by conducting 4 clinical studies involving children, adolescents, and adults to characterize the features of hyperlipidemic memory. In the first investigation (16), they explored the impact of the presence or absence of PR on lipid parameters in youth five years after their diagnosis of T1D.

In the second study (68), they investigated whether pubertal maturation influenced the dichotomy in lipid profiles in T1D; and whether pubertal lipid dichotomy occurred in age-matched healthy youth without a diagnosis of T1D.

In the third study (69), they used the findings from patients with T1D and control subjects to investigate early lipid changes in T2D by comparing the earliest lipid phenotypes of subjects with T2D to those of remitters, non-remitters, and controls. Finally, in the fourth study (15), they examined the impact of PR on the earliest lipid phenotypes in adult subjects with either T1D or T2D, and their matched controls.

In these 4 studies, the investigators found that remission was more robust in male than female subjects; and that remitters had significantly favorable lipid profiles compared to non-remitters (16) (68) (69) (15) as shown by significantly lower LDL-cholesterol, non-HDL cholesterol, total cholesterol, TC/HDL ratio, and mean composite scores for lipid-based ASCVD risks in the remitters. The early dyslipidemia in non-remitters was similar to that of the obese patients or those with T2D, while the favorable lipid profile in the remitters was similar to that of the normal-weight controls. These findings are similar to the results of a longitudinal study that reported a significantly reduced risk for chronic microvascular complications at 7-year follow-up in young adults who experienced PR (12).

This body of work across the lifespan in children, adolescents, and adults supports the theory of hyperlipidemic memory. This new theory clarifies why PR largely determines the risks for early-phase dyslipidemia, mid-term microvascular disease risk, and long-term ASCVD risk in subjects with T1D.

3 Support for PR-mediated hyperlipidemic memory as the primary determinant of early lipid phenotypes in both pediatric and adult type 1 diabetes

It is important to analyze the risk factors for dyslipidemia such as glycemia, BMI, and insulin resistance in patients with either T1D

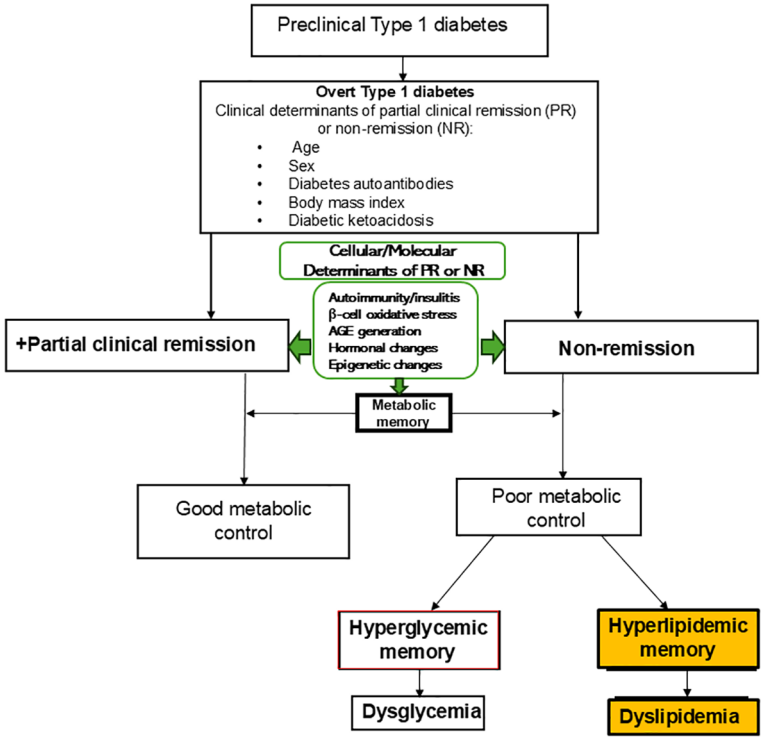

FIGURE 1
The theory of hyperlipidemic memory of type 1 diabetes explains the early dichotomy in lipid parameters in patients with type 1 diabetes (T1D) and the attendant divergence in dyslipidemic atherosclerotic cardiovascular disease risk in non-remitters compared to the remitters. The theory of hyperglycemic memory explains dysglycemia in non-remitters while the theory of hyperlipidemic memory explains dyslipidemia in non-remitters. This model shows the relationships between the overt clinical determinants of remission or non-remission and the less overt molecular and cellular factors that also predict the occurrence of remission or non-remission. These clinical, molecular, and cellular determinants impact metabolic memory and thus the long-term trajectory of the clinical course and complications of T1D. The concept of PR imprimatur calls for disease-modifying interventions in the preclinical stages of T1D to ensure robust metabolic memory and decreased long-term complications of T1D.

or T2D to understand the key role of hyperlipidemic memory on early lipid phenotypes in T1D. Nwosu et al. (16) examined the role of early glycemia in T1D. They found that both remitters and non-remitters have hyperglycemia at the time of diagnosis of T1D, but that glycemia improves markedly in the remitters and less so in the non-remitters, suggesting that hyperglycemia from poor glucose management could lead to dyslipidemia in these patients. However, they noted in their follow-up study that included patients with T2D (69), who had unfavorable lipid parameters at the time of the diagnosis, but with significantly lower mean A1c level of 6.7% compared to the mean A1c levels of the T1D cohort (8.8% for the non-remitters, and 8.6% for the remitters). These findings argue against hyperglycemia as the key determinant of early-phase dyslipidemia in children with either T1D or T2D. These data and previous reports (43, 59) show the limitations of the theory of *hyperglycemic* memory to explain the PR-mediated divergence in lipid phenotypes in patients with T1D.

Furthermore, though BMI predicts dyslipidemia, the occurrence of normal BMI z-scores in the non-remitters with a BMI z-score of 0.63 ± 0.9, despite having a similar lipid profile as the obese patients with T2D with a BMI z-score of 2.4 ± 0.4, suggests that increased BMI alone does not explain the increased dyslipidemia in the early phases of T1D in children. This is supported by an analysis of the proportion of subjects with dyslipidemia in that study (70) that showed that LDL-C of >130 mg/dL occurred in 7 (13.2%) of the subjects with T2D; 6 (7.6%) non-remitters; 2 (4.6%) remitters; and 4 (5.5%) controls. Additionally, TC of >200 mg/dL occurred in 15 (28.3%) of the subjects with T2D; 9 (11.4%) non-remitters; 3 (6.8%) remitters; and 4 (5.5%) controls. This analysis suggests that the non-remitters and the subjects with T2D, despite their differences in BMI z-scores, had a *higher frequency of dyslipidemia* compared to the remitters and controls.

Finally, the similarity of early lipid profiles in patients with T2D and the non-remitters, despite their significant differences in BMI z scores, also argues against IR as the primary determinant of dyslipidemia in non-remitters compared to the subjects with T2D. Taken together, these findings (15) establish PR as the principal determinant of early lipid phenotypes, and the divergence in ASCVD risks in both pediatric and adult patients with T1D.

4 Conclusions and future directions on disease-modifying therapies in type 1 diabetes to limit atherosclerotic cardiovascular disease risk

Partial clinical remission (PR) is a key event in the life history of T1D. When patients are stratified by PR status into remitters and

non-remitters, the non-remitters have less favorable lipid phenotypes than the remitters and controls. These findings support a dichotomy in ASCVD risk in subjects with T1D that favors the remitters. This divergence in ASCVD risk is explained by the theory of hyperlipidemic memory where the initial, early-onset hyperlipidemia in non-remitters persists across the lifespan leading to increased risk for ASCVD in this sub-population of subjects with T1D. In contrast, the imprimatur of PR in the remitters presages a lifetime of favorable lipid profile which has been confirmed in large studies (10, 17). The concept of PR imprimatur is fitting because the metabolic advantages of PR continue long after the end of partial remission (12, 18). This theory of hyperlipidemic memory explains the principal role of PR occurrence or *imprimatur* on the early dichotomy in lipid phenotypes in T1D, and the subsequent divergence in lipid-based ASCVD risks.

Therefore, we propose that the advantages conferred by PR occur at the time of the diagnosis of T1D, i.e., stage III of T1D, as an imprimatur, and not as a process that follows the diagnosis of the disease. Thus, the advantages of PR need not necessarily depend on the duration of PR, but on its singular occurrence, as it encompasses a constellation of factors that protects the β-cells from the initial shock of the diagnosis of T1D that resets the long-term trajectory of the complications of the disease in a favorable path.

This new paradigm provides a new structure for early and accurate quantification of ASCVD risk in subjects with T1D across the lifespan which may lead to the development of disease-modifying agents to address the risk for early-stage dyslipidemia at stages I and II before the diagnosis of T1D using high-risk population screening. This new model calls for the inclusion of lipid-based pathways in devising strategies and therapies to either augment PR following the diagnosis of T1D at stage III or develop agents or therapies to protect the β-cells prior to the diagnosis of T1D at stages I and II in individuals at high risk of the disease. For example, proinsulin to C-peptide ratio, a marker of β-cells endoplasmic reticulum stress, is increased in children and adolescents with new-onset T1D (53). Thus, agents that reduce PI:C ratio, such as high-dose vitamin D (21) could be used at stages I and II to protect the β-cells in individuals at high risk for developing T1D to ensure that these patients experience PR and the advantages of PR to reduce the short- and long-term complications of T1D.

Author contributions

BN: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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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Safety and efficacy of adjuvant Sotagliflozin therapy in patients with T1D - an update and systematic review and meta-analysis

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Objective: This meta-analysis aims to assess the safety and efficacy of Sotagliflozin in patients with type 1 diabetes (T1D).

Methods: Data on target organ protection, blood glucose levels, blood pressure, weight, insulin usage, and adverse events (AEs) associated with Sotagliflozin in the treatment of T1D were collected from databases including PubMed, Scopus, Web of Science, Embase, and the Cochrane Library. The search period extended until February 21, 2024, and included studies were restricted to randomized controlled trials (RCTs) investigating Sotagliflozin for T1D. The meta-analysis was performed using Stata 14 and RevMan 5.4.

Results: A total of 12 randomized controlled trials were included in the analysis, with treatment durations ranging from 14 to 52 weeks. Sotagliflozin, when used in combination with insulin therapy, resulted in significant reductions in cardiovascular disease (CVD) risk (–6.38%; *95% CI:* –7.63 to –5.1; $P < 0.05$) and end-stage kidney disease (ESKD) risk (–5.0%; *95% CI:* –7.62 to –2.3; $P < 0.05$). Additionally, Sotagliflozin significantly reduced blood glucose, blood pressure, and body weight, with these effects showing dose- and duration-dependent trends. Regarding adverse effects, the combination of insulin and Sotagliflozin was associated with an increased incidence of genital infections (Sotagliflozin group: 8% vs. control: 2%) but a reduced risk of fractures (Sotagliflozin group: 1% vs. control: 2%). No statistically significant differences were observed between the two groups for other outcomes, including diabetic ketoacidosis (DKA), hypoglycemia, mortality, cancer, nausea, diarrhea, urinary tract infections, or liver and kidney function impairment.

Conclusion: In T1D patients, Sotagliflozin adjunct therapy improves blood glycemia, stabilizes blood pressure, and reduces cardiovascular risk factors. It also shows potential in lowering fracture risk, but the risk of DKA requires further clinical validation.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/#joinuppage, identifier CRD42023467427.

KEYWORDS

Sotagliflozin, type 1 diabetes, SGLT1, SGLT2, meta-analysis

1 Introduction

Diabetes mellitus comprises two primary types: type 1 diabetes (T1D) and type 2 diabetes (T2D) (1). T1D, characterized by autoimmune-mediated pancreatic beta-cell destruction, leading to exclusive dependence on insulin therapy for management (2). However, this approach often leads to suboptimal glycemia with marked fluctuations, thereby increasing the risk of target-organ damage. Although sodium-glucose cotransporter 2 (SGLT2) inhibitors demonstrate partial efficacy in preserving target organs and improving glycemia, their use in T1D patients is associated with a higher incidence of adverse events (AEs) (3), particularly diabetic ketoacidosis (DKA), which contributes to elevated mortality rates in T1D (4, 5).

Sotagliflozin, a dual inhibitor of sodium-glucose cotransporters 1 and 2 (SGLT1/2), combines the advantages of SGLT2 inhibitors (6), such as improved glycemia, reduced cardiovascular adverse events, and enhanced survival benefits. Concurrently, SGLT1 inhibition mitigates side effects associated with SGLT2 inhibitors, including a lower incidence of DKA, urinary tract infections, and improved acid-base buffering capacity (7, 8). However, clinical randomized controlled trials (RCTs) have reported inconsistent findings, most notably an elevated risk of DKA, which remains contradictory and controversial (9, 10). Thus, this drug shows significant potential for T1D treatment if supported by robust evidence. To address this gap, we conducted an updated systematic review and meta-analysis with the following objectives: 1). Inclusion of high-quality RCTs to strengthen result reliability; 2). Comprehensive assessment of efficacy and safety, including target-organ protection, estimated glomerular filtration rate (eGFR), fracture rates, and major adverse cardiovascular events (MACE); 3). Subgroup analyses and meta-regression were conducted to explore correlations between outcomes, treatment duration, and drug dosage, thereby reinforcing the evidence for Sotagliflozin's safety and efficacy as an adjuvant therapy in T1D; 4. Comparative evaluation of current meta-analysis findings.

2 Materials and methods

2.1 Protocol

This systematic review and meta-analysis strictly adhered to the protocol registered with PROSPERO (CRD42023467427) and followed the guidelines outlined in the PRISMA statement.

2.2 Search crit

2.2.1 Inclusion criteria eria

The study design adhered to the PICOS framework: (1) Population (P): Patients diagnosed with T1D. (2) Intervention (I): Administration of Sotagliflozin. (3) Comparison (C): The control group comprising patients with T1D managed exclusively with insulin therapy. (4) Outcome Measures (O): Evaluation of cardiovascular disease (CVD) risk, end-stage kidney disease (ESKD), fasting plasma glucose (FPG), 2-hour postprandial plasma glucose (2H-PPG), glycosylated hemoglobin (HbA1c) levels, basal insulin usage, bolus insulin usage, total insulin consumption, systolic blood pressure (SBP), diastolic blood pressure (DBP), estimated eGFR, body weight (Bw), and monitoring of common adverse effects. (5) Study Type (S): Randomized Controlled Trials (RCTs).

2.2.2 Exclusion criteria

(1) Animal experiments,(2) Reviews and case reports,(3) Direct data from non-articles,(4) Duplicate published papers,(5) Patients with T1D treated with other medications.

2.3 Search databases

PubMed, Scopus, Web of Science, Embase, and Cochrane Library were searched from their establishment to February 21, 2024. The search strategy is shown in Supplementary Data Sheet 1.

2.4 Search strategy, data extraction, and quality assessments

Two independent researchers conducted literature screening and data extraction in accordance with established inclusion and exclusion criteria. Initially, titles and abstracts were reviewed, and any articles that did not meet the inclusion criteria were excluded. The remaining articles underwent full-text review to determine their final eligibility. In cases of disagreement, consensus was reached through discussion among all researchers. Two researchers evaluated the eligibility of RCTs using a bias assessment tool to assess the quality of the literature. This tool considered randomization, allocation concealment, blinding, completeness of outcome data, selective reporting, and other potential sources of bias. Disagreements in the assessment were resolved through group discussion. Subsequently, reorganization and classification of the limited number of included studies were performed to mitigate publication bias. Further details of this process are provided in Figure 1.

2.5 Statistical analysis

The meta-analysis was performed using Stata 14.0 and RevMan 5.4 software. For efficacy outcomes, the extracted data represent the change from baseline to post-treatment period. For safety outcomes, the total number of adverse events in both groups was recorded. A continuity correction (e.g., Bartlett's adjustment) was applied when AE incidence was 0% or 100%. Statistical heterogeneity among studies was evaluated using the Q-test and I^2 statistic, with heterogeneity defined as low ($I^2 < 50\%$) or high ($I^2 \geq 50\%$). A fixed-effects model was used in the absence of significant heterogeneity, whereas a random-effects model was applied when

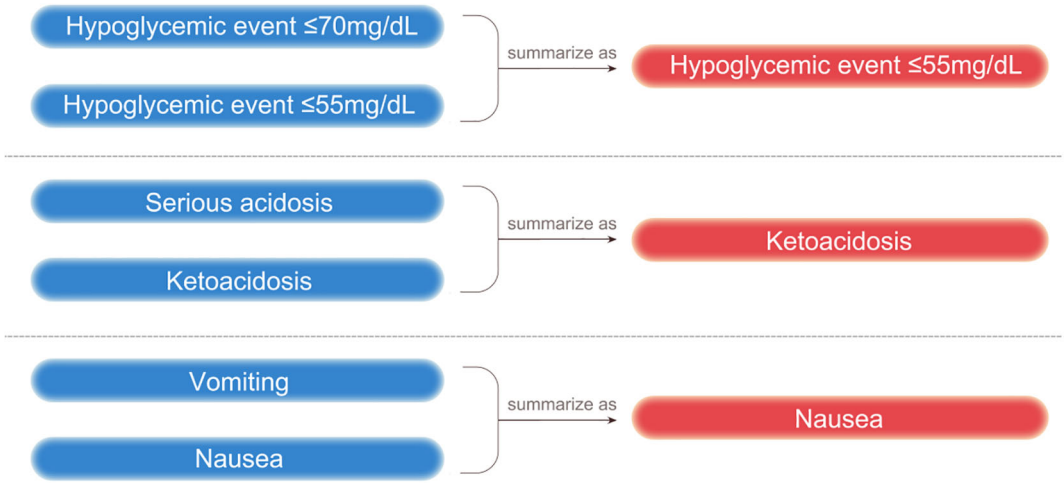

FIGURE 1
Infographic summarizing the categorization of indicators.

heterogeneity was detected. For outcomes with high heterogeneity, sensitivity analyses were conducted to identify potential sources. Meta-regression explored variable correlations, and publication bias was assessed via Egger's test, with $P < 0.05$ indicating potential bias. When bias was identified, the trim-and-fill method was used for adjustment. All outcomes were graded using the GRADE framework (Supplementary Data Sheet 2).

3 Results

3.1 Literature search results

In this study, an initial search retrieved 6,246 articles. After removing 2,055 duplicates, 37 unique articles remained for the first screening. Among them, 25 articles were excluded because they did not meet the inclusion criteria. As a result, 12 articles were retained for the final analysis. A visual representation of the literature screening process and its outcomes is presented in Figure 2.

3.2 Description of included trials

All of the studies were randomized controlled trials that examined a range of clinical parameters, including CVD, ESKD, FPG, 2H-PPG, HbA1c, basal insulin, bolus insulin, total insulin dosage, SBP, DBP, eGFR, body weight (Bw), DKA, adverse events, and serious adverse events. Detailed characteristics of these studies are provided in Supplementary Data Sheet 3.

3.3 Risk of bias assessments

All articles employed a randomized double-masked allocation method. However, it is important to note that the articles did not consistently clarify whether the statistical results underwent blinding procedures. To assess data reliability, the level of detail provided in the articles regarding patient follow-up and the recording of missed visits was crucial. The presence of selection bias depended on whether the articles explicitly defined specific population subgroups. Additionally, articles funded by public universities or charitable organizations were considered to have a low risk of other biases. The evaluations of treatment outcomes for each article are graphically depicted in Figure 3.

3.4 Target organ protection

A total of 1 study reported this outcome (11), with 1 arm, found that Sotagliflozin significantly reduced the likelihood of CVD [-6.38%, 95% CI: -7.63 to -5.1, P < 0.05] and ESKD [-5.0%, 95% CI: -7.62 to -2.3, P < 0.05] in T1D patients. However, a subgroup analysis of patients with a BMI ≥ 27 kg/m² revealed that the mitigating effect of Sotagliflozin on ESKD was significantly attenuated and no longer statistically significant. Nevertheless, it retained a substantial protective effect against CVD development (Figure 4).

3.5 Glucose regulation

A total of 4 studies reported on FPG (12–15), with a total of 12 arms. The results showed that adding Sotagliflozin to insulin therapy resulted in a significant reduction in FPG compared to insulin therapy alone [-15.86 mg/dL, *95% CI*: -19.43 to -23.30, $P < 0.05$] ($I^2 = 3.8$, $P>0.05$). A total of 3 studies reported on 2H-PPG (12, 13, 16), with a total of 6 arms. The results indicated that the combination of Sotagliflozin and insulin therapy led to a substantial reduction in 2H-PPG [-41.84 mg/dL, *95% CI*: -55.02 to -28.66, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). Subgroup analyses further revealed a positive correlation between drug concentration and the extent of 2H-PPG reduction within the 200 - 400 mg/day dose range. However, as the intervention duration increased, the reduction in

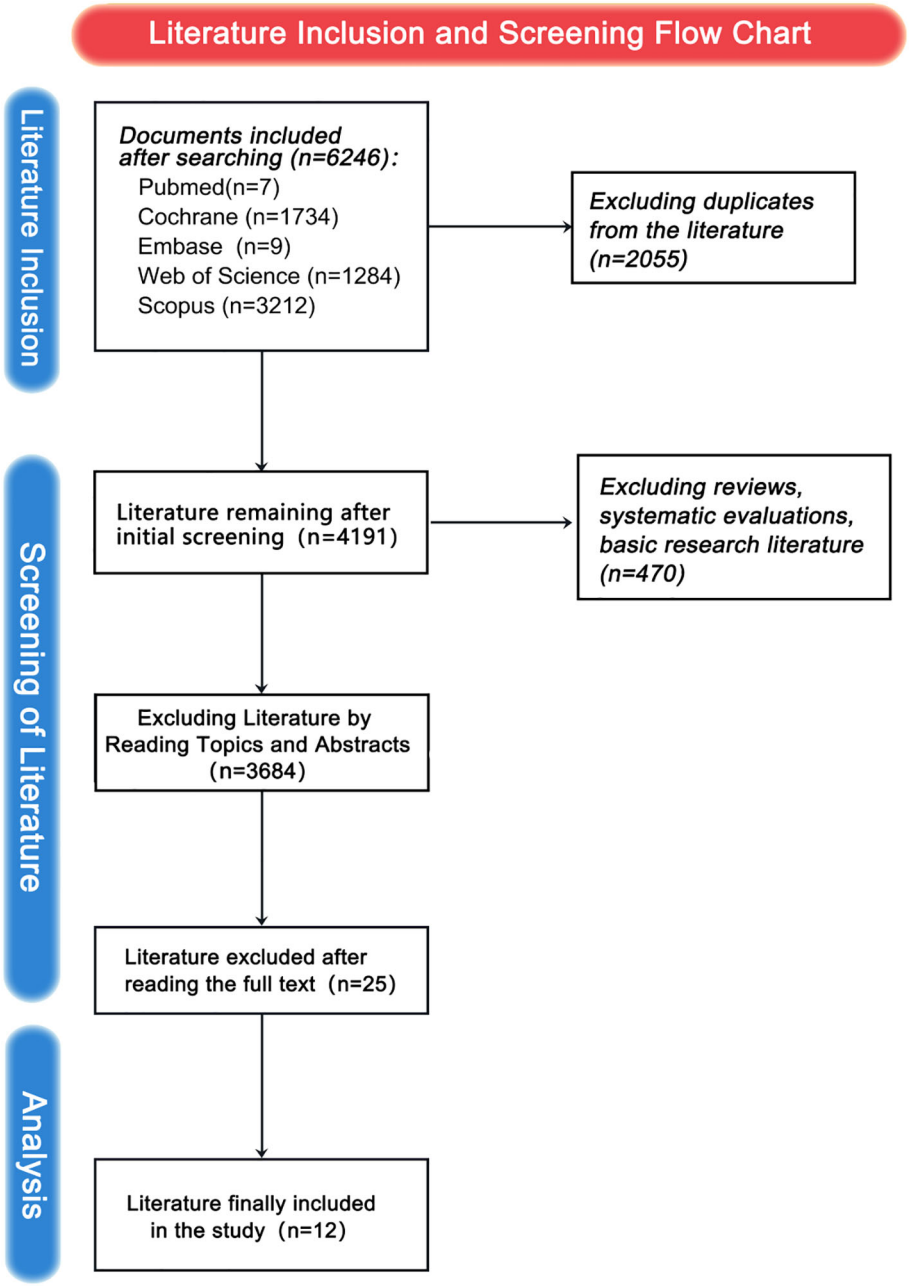

FIGURE 2
Process flowchart of article filtering.

blood glucose became more moderate, a trend consistent with the findings for HbA1c (12–15). All glycemic evaluation indices underwent Egger's test to assess publication bias, and Figure 5 visually presents the results.

3.6 Usage of insulin

A total of 2 studies reported this outcome (12, 15), with a total of 5 arms. The results demonstrated that the addition of Sotagliflozin to insulin therapy resulted in a significant reduction in basal insulin requirements compared to insulin therapy alone [-8.19%, *95% CI*: -9.90 to -6.48, $P < 0.01$] ($I^2 = 44.3$, $P>0.05$). Subgroup analyses revealed a consistent trend of reduced basal insulin dosage, a pattern also observed with bolus insulin as the duration of Sotagliflozin intervention increased and the drug concentration escalated. Additionally, A total of 3 studies reported total insulin requirements (12, 14, 15), with a total of 9 arms. The results showed that Sotagliflozin combined with insulin therapy led to a significant reduction in total insulin requirements [-8.6%, *95% CI*: -9.76 to -7.44, $P < 0.01$] ($I^2 = 28.2$, $P>0.05$). Subgroup analyses highlighted a consistent, significant decrease in

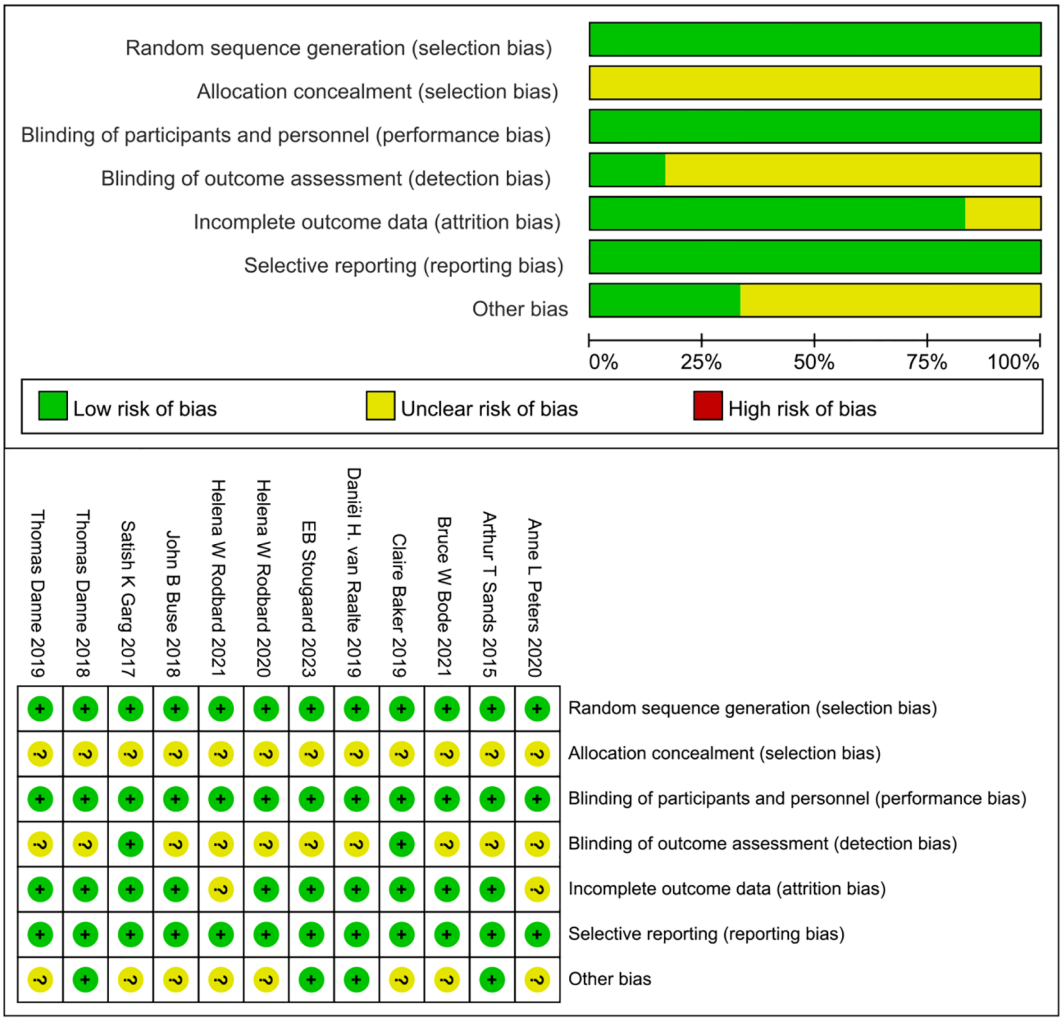

FIGURE 3
Quality evaluation chart of the literature.

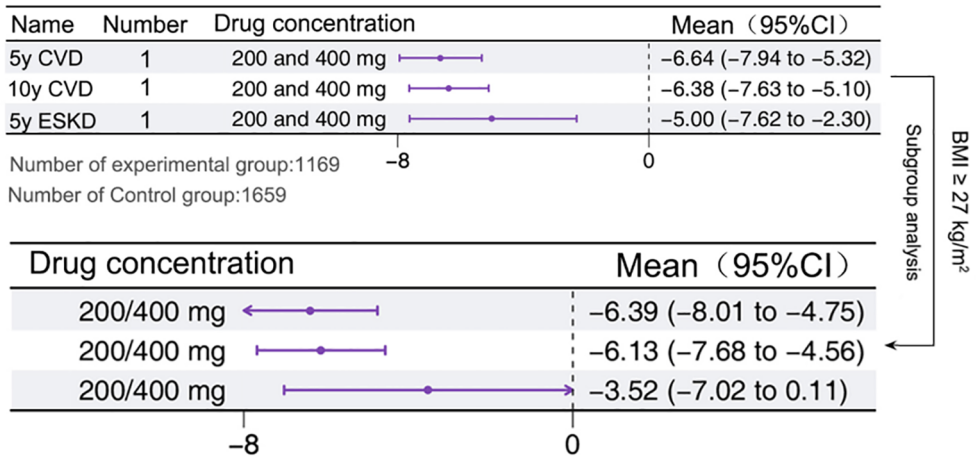

FIGURE 4
Protection of target organs in T1D patients treated with adjunctive therapy using Sotagliflozin.

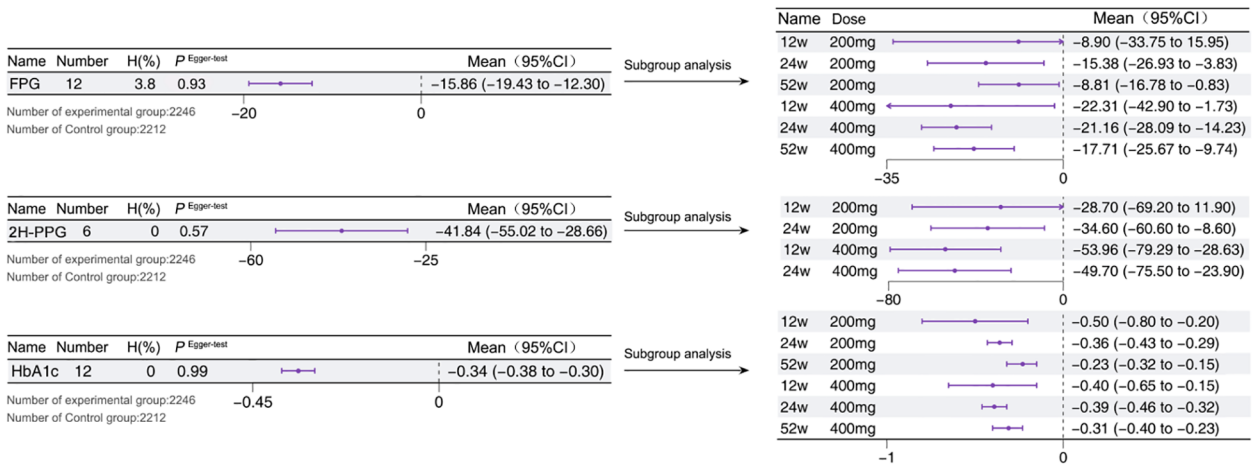

FIGURE 5
Glycemia in T1D patients treated with adjunctive therapy using Sotagliflozin.

total insulin usage with increasing drug concentration and longer intervention duration. All glycemic evaluation metrics underwent Egger's test to assess publication bias, and Figure 6 visually presents the results, confirming the absence of significant bias.

3.7 Continuous glucose monitoring time analysis

A total of 2 studies reported the outcome (12, 16), with a total of 2 arms. The studies found that patients using Sotagliflozin spent more time within the normal glucose range compared to the control group [8.53%, *95% CI*: 5.53 to 11.53, $P < 0.05$] ($I^2 = 45.1$, $P>0.05$). Among these, the group using 400 mg of Sotagliflozin showed a further trend of increased time within the normal glucose range [10.67%, *95% CI*: 6.78 to 14.55, $P < 0.05$]. In addition, A total of 2 studies reported the time spent with glucose levels <3.9 mmol/L (12, 16), with a total of 2 arms. The studies found no statistical difference between the two groups in the time spent with glucose levels <3.9 mmol/L [-0.06%, 95% CI: -0.33 to 0.22, P > 0.05] and <3.0 mmol/L [-0.07%, 95% CI: -0.21 to 0.07, P > 0.05]. A total of 1 study (16), with 1 arm, found that patients using Sotagliflozin had significantly less time with blood glucose >10.0 mmol/L [-8.44%, *95% CI*: -15.10 to -1.77, $P < 0.05$] and >13.9 mmol/L [-1.40%, *95% CI*: -2.38 to -0.42, $P < 0.05$] compared to the control group. These results indicate that patients using Sotagliflozin spent significantly more time in the normal glucose range, while the frequency of hyperglycemic events was lower compared to the control group.

3.8 Other results

The extent of blood pressure reduction showed a positive correlation with both the intervention duration and drug

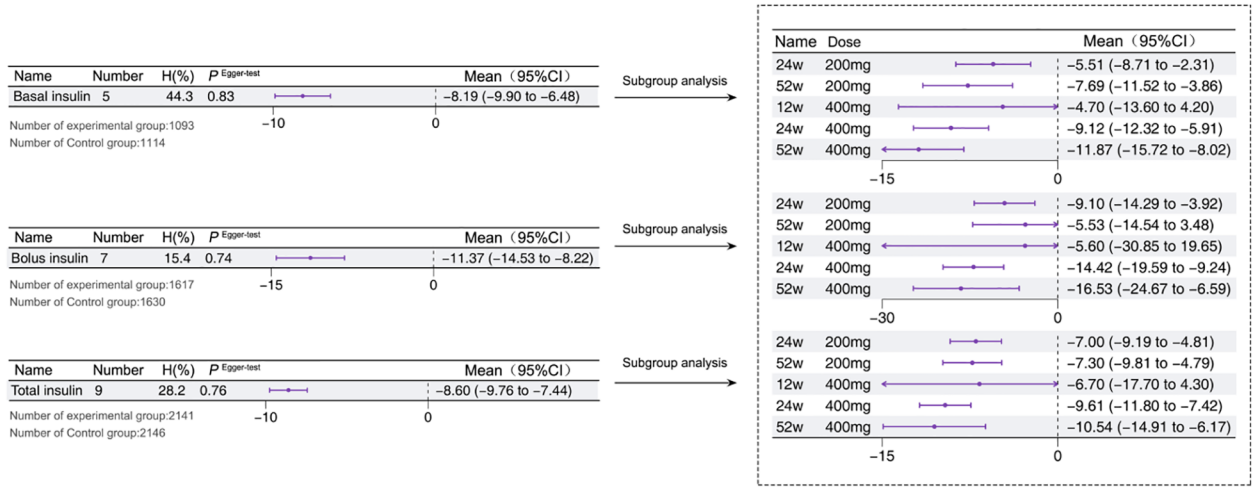

FIGURE 6
Insulin use in T1D patients treated with adjunctive therapy using Sotagliflozin.

concentration. A total of 5 studies reported on SBP (13–15, 18, 19), with a total of 10 arms. The studies indicated that combining Sotagliflozin with insulin therapy led to a significant reduction in SBP [-3.33 mmHg, *95% CI*: -3.13 to -2.63, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). However, potential publication bias was detected in the Egger's test, which was subsequently addressed using the cut-and-complement method, yielding revised results of [-3.00 mmHg, *95% CI*: -3.48 to -2.52, $P < 0.05$]. Additionally, a total of 3 studies reported the DBP (15, 18, 19), with a total of 8 arms. The studies indicated that combining Sotagliflozin with insulin therapy led to a significant reduction in DBP [-1.44 mmHg, *95% CI*: -1.78 to -1.11, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). Similarly, potential publication bias was suggested by the Egger's test, which was addressed using the cut-and-complement method, yielding revised results of [-1.24 mmHg, *95% CI*: -1.58 to -0.91, $P < 0.05$]. Furthermore, a total of 3 studies reported the eGFR, with a total of 7 arms. The analysis showed that the combination of Sotagliflozin and insulin therapy resulted in a reduction in eGFR [-1.51 mL/min/1.73m^2, *95% CI*: -2.19 to -0.82, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). Moreover, a total of 4 studies reported the body weight (Bw) (12–15), with a total of 12 arms. The analysis revealed a significant reduction in Bw [-2.69 kg, *95% CI*: -3.13 to -2.63, $P < 0.05$] ($I^2 = 77.4$, $P<0.05$). Notably, the difference in Bw between the experimental and control groups increased progressively with both the intervention duration and drug concentration, as shown in Figure 7.

3.9 Adverse effects

This meta-analysis explored the potential adverse effects of Sotagliflozin (12–15, 17, 20–22). A total of 7 studies reported adverse events (AE) (12–15, 17, 21, 22), with a total of 19 arms. The results indicated no statistically significant difference in the occurrence of AE[65%, *95% CI*: 59% to 72%, $P < 0.05$] ($I^2 = 92.4\%$, $P<0.05$) or SAE (12–15, 21, 22)[7%, *95% CI*: 5% to 9%, $P < 0.05$] ($I^2 = 89.4\%$, $P<0.05$) between the two groups. However, a total of 7 studies reported incidence of DKA (12–15, 17, 20, 22), with a total of 20 arms. The results indicated that the experimental group had a significantly higher incidence of DKA [3%, *95% CI*: 2% to 3%, $P < 0.05$] ($I^2 = 99.1\%$ $P<0.05$) compared to the control group [0%, *95% CI*: 0% to 0%, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). Notably, meta-regression analysis showed that the incidence of DKA was independent of both drug concentration and intervention duration, as depicted in Figure 8. The Egger's test suggested a potential publication bias ($P < 0.05$), prompting recalibration of the DKA incidence using the cut-and-patch method, which yielded an adjusted incidence of 0.00% [95% CI: -0.01 to 0.01]. This adjustment revealed no statistically significant difference in DKA incidence between the experimental and control groups. Additionally, a total of 6 studies reported incidence of genital infections (12–15, 21, 22), with a total of 20 arms. The results indicated a significant difference in the incidence of genital infections, with the experimental group [8%, *95% CI*: 7% to 10%, $P < 0.05$] ($I^2 = 85.0$, $P<0.05$) showing a higher rate than the control group [2%, *95% CI*: 1% to 2%, $P < 0.05$] ($I^2 = 0.0$, $P>0.05$). Meta-regression analysis indicated a positive correlation between the infection rate and both drug concentration and intervention duration. A total of 6 studies reported incidence of genital infections (12–15, 17, 21), with a total of 16 arms. The study also suggested a potential reduction in fracture incidence in the experimental group (1% compared to 2% in the control group). Furthermore, when the drug concentration ranged between 200-400 mg/day, a higher drug concentration was

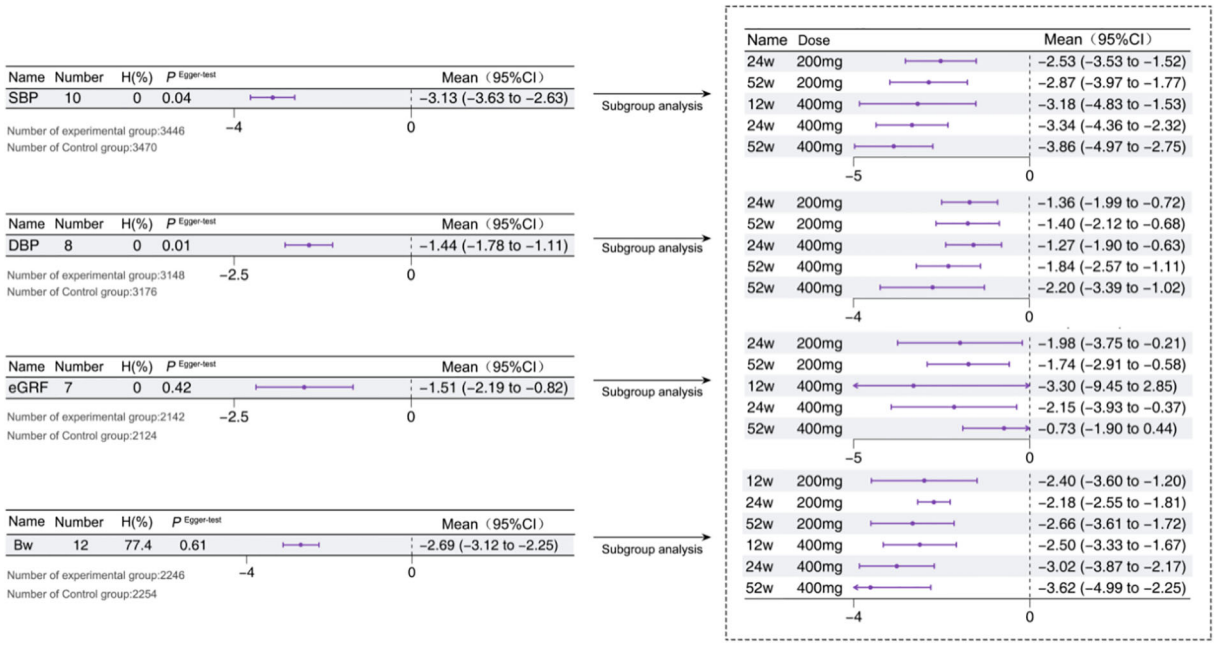

FIGURE 7
Other outcomes in T1D patients treated with adjunctive therapy using Sotagliflozin.

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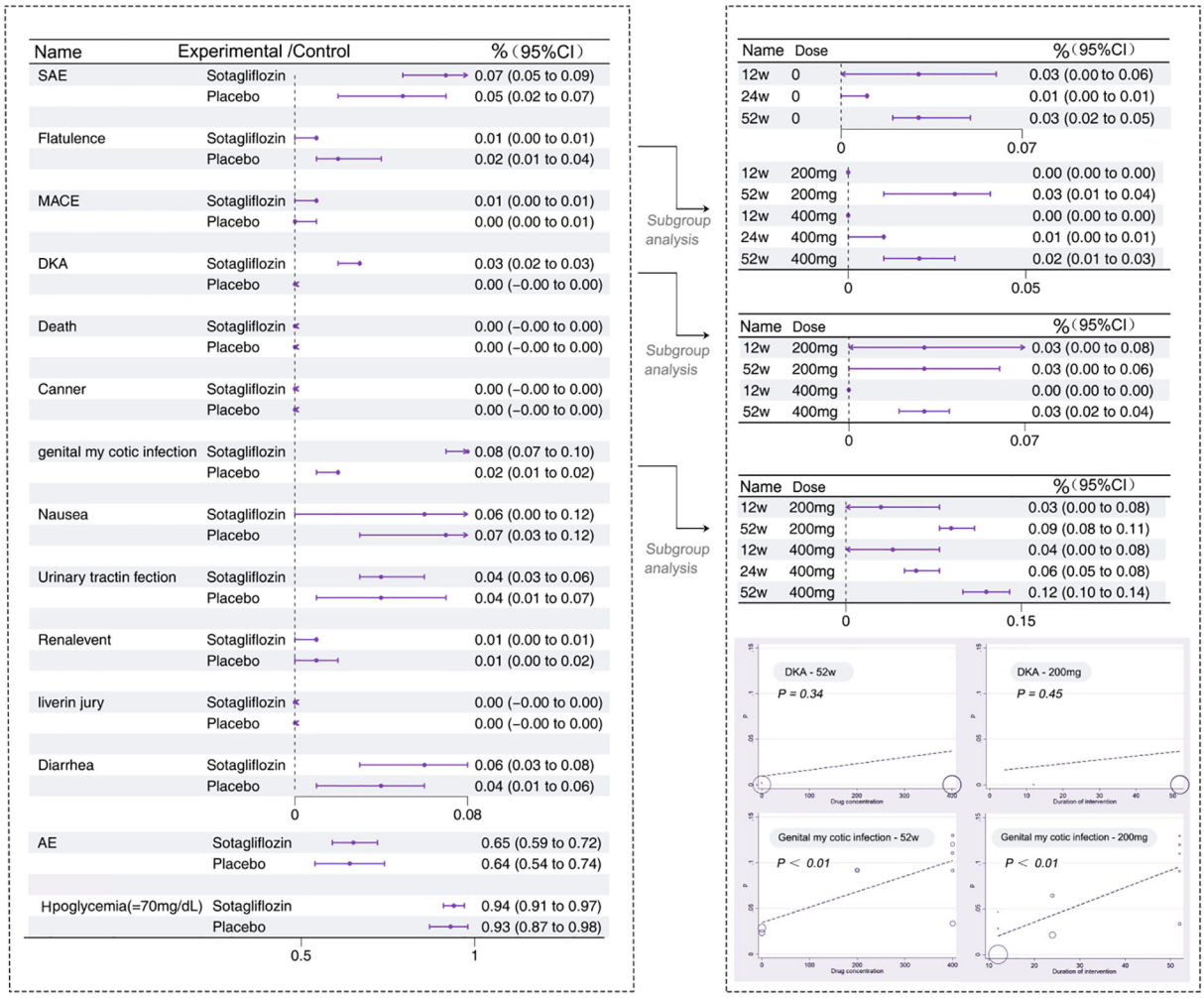

FIGURE 8
Summary graph of adverse effect analysis.

significantly associated with an enhanced fracture-delaying effect. Specific adverse effects are visually summarized in Figure 8.

4 Discussion

Diabetes mellitus is a significant global endocrine disorder, posing a considerable threat to human health. Sotagliflozin, a dual inhibitor of SGLT1 and SGLT2, offers a distinct profile compared to traditional SGLT2 inhibitors (23–25). It not only reduces hepatic β-oxidation but also enhances the buffering capacity of the acid-base homeostasis system (26–28). By inhibiting SGLT1, Sotagliflozin reduces glucose entry into the bloodstream, contributing to better long-term control of 2H-PPG, lower glucagon production, and a decreased risk of cardiovascular disease. These mechanisms suggest that Sotagliflozin holds significant potential in improving the safety and efficacy of treatments for individuals with T1D. In this study, Sotagliflozin treatment resulted in a reduction in the 10-year incidence of cardiovascular disease (CVD) [-6.38%, *95% CI*: -7.63 to -5.1, $P < 0.05$] and ESKD [-5.0%, *95% CI*: -7.62 to -2.3, $P < 0.05$]. These reductions are likely due to improved glycemic and blood pressure control, further mitigating the risk of complications. Notably, when Sotagliflozin was administered in doses ranging from 200 mg to 400 mg per day, there was a more substantial decrease in blood glucose with increasing drug concentration. However, as the intervention duration extended from 12 to 52 weeks, the effect on glycemia showed a declining trend, possibly due to a reduction in insulin dosage. Despite this, the experimental group continued to show superior blood glycemia compared to the control group, with no significant difference in the incidence of hypoglycemic events. Additionally, the study demonstrated a modest reduction in blood pressure due to Sotagliflozin use, although the change was not of substantial magnitude [SBP: -3.33 mmHg, *95% CI*: -3.13 to -2.63, $P < 0.05$; DBP: -1.44 mmHg, *95% CI*: -1.78 to -1.11, $P < 0.05$]. This reduction was potentially associated with weight loss [-2.69 kg, *95% CI*: -3.13 to -2.63, $P < 0.05$], suggesting the need for further exploration of the antihypertensive effect through subgroup analyses involving body

weight. In terms of adverse effects, Sotagliflozin did not increase the risk of urinary tract infections, consistent with previous studies. However, it did elevate the risk of genital infections, likely due to its pharmacological mechanism. Notably, genital mycotic infections were primarily observed in elderly patients within the first 30 days of treatment initiation. Interestingly, this study did not observe an increased risk of DKA, which contrasts with findings from previous meta-analyses. This discrepancy was investigated through Egger's test and meta-regression, which indicated potential publication bias. After adjusting for this bias using the cut-and-patch method, the results suggested that Sotagliflozin did not increase the risk of DKA, though additional confirmation via RCTs may be needed. The study also indicated a potential reduction in fracture incidence, though the 95% CI showed some overlap for this outcome. Subgroup analyses suggested a clear trend, particularly relevant for osteoporosis prevention in middle-aged patients. Furthermore, other adverse effects, including nausea, diarrhea, liver injury, renal impairments, and cancer, showed no statistically significant differences between the experimental and control groups. A comprehensive comparison with previously published meta-analyses is available in Figure 9 (29, 30).

This study found that Sotagliflozin can reduce the risk of CVD. On one hand, this may be attributed to its ability to more effectively control traditional risk factors such as weight, blood glucose, and blood pressure. The evidence from this study's evidence-based approach supports this view.

1) Weight Reduction: Sotagliflozin induces weight loss by inhibiting SGLT1 and SGLT2 in both the kidneys and the intestines, leading to increased renal glucose excretion. As glucose is excreted, water and sodium are also eliminated from the body. The significant excretion of glucose requires additional energy to process, which promotes fat loss and helps reduce body weight. Furthermore, Sotagliflozin improves insulin sensitivity, reduces insulin resistance, and stimulates fat metabolism. However, P.C. Lee et al. found that the effect of SGLT inhibition on weight reduction is moderate and diminishes over time, which may be partly due to compensatory mechanisms, such as an increase in energy intake, which attempt to maintain body weight (31).

2) Blood Glucose Reduction: As an SGLT1/2 inhibitor, Sotagliflozin can more effectively lower blood glucose levels in patients with T1D compared to a placebo when used as an adjunctive therapy (8). This meta-analysis found that Sotagliflozin helps stabilize blood glucose and increase time within the normal glucose range, primarily by reducing hyperglycemia. Its effect relies on renal and intestinal glucose excretion rather than insulin secretion, which reduces the risk of hypoglycemia, especially at lower glucose levels. Additionally, in T1D patients, Sotagliflozin reduces beta-cell stress, facilitating better blood glycemia, lowering hyperglycemia risk, and extending time spent within the normal glucose range (23). At the same time, Sotagliflozin may offer renal protection by reducing the activity of renal SGLT2, potentially slowing the progression of kidney damage induced by diabetes. This, in turn, could indirectly lower the cardiovascular risk associated with diabetes. However, some studies suggest that hyperglycemia is a relatively weak risk factor for cardiovascular diseases, and that merely controlling blood glucose may not be directly linked to the risk of cardiovascular events (32, 33).

3) Blood Pressure Reduction: The exact mechanism behind the antihypertensive effects of SGLT inhibitors is not fully understood, but it may be mediated by the osmotic and diuretic effects of SGLT2

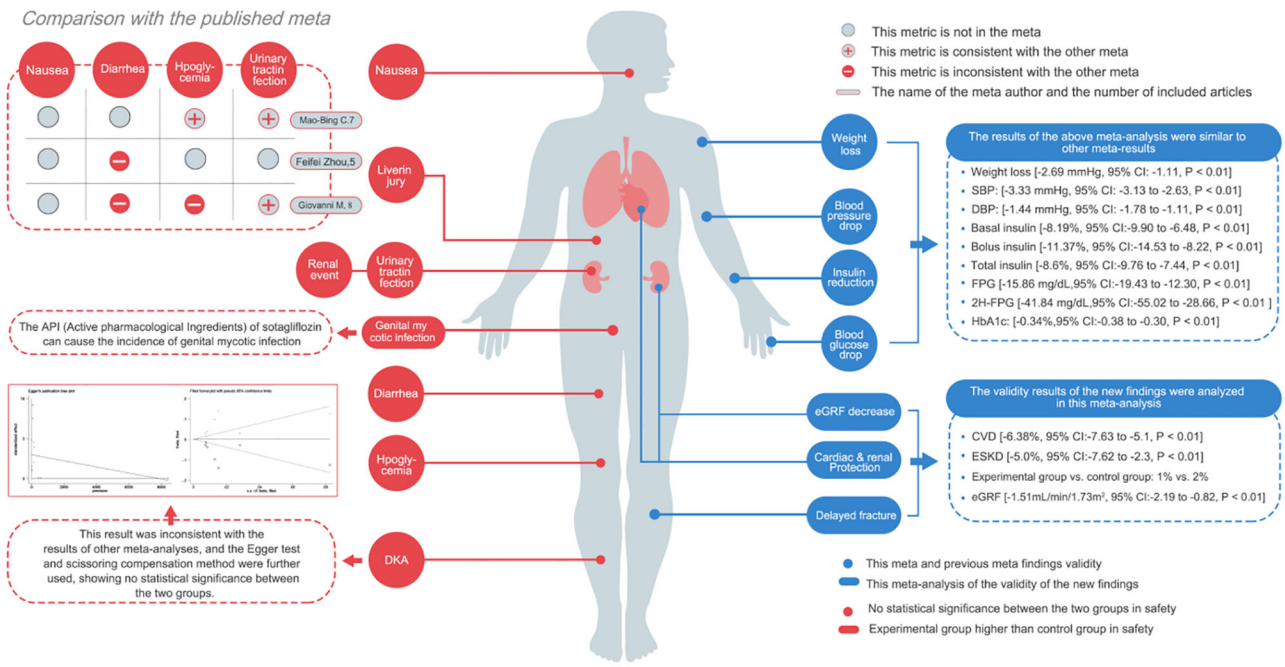

FIGURE 9
Comprehensive comparison with previously published meta-analyses.

inhibitors, which inhibit sodium reabsorption in the proximal renal tubules. Inhibition of SGLT2 can lead to an approximately 50% increase in urinary sodium excretion (34). Additionally, SGLT inhibition may reduce sympathetic nervous system activity, inhibit norepinephrine conversion in brown adipose tissue, and decrease the production of tyrosine hydroxylase (35). However, the blood pressure-lowering effect of SGLT2 inhibitors is moderate. Moreover, compared to other cardiovascular diseases, the impact of blood pressure reduction on stroke incidence is more pronounced. Therefore, the role of Sotagliflozin in reducing cardiovascular risk through blood pressure control remains limited (36). On the other hand, Sotagliflozin may reduce the risk of CVD through mechanisms such as improving cardiac energy metabolism, reducing oxidative stress, and protecting endothelial cells.

4) Improvement of cardiac energy metabolism: Sotagliflozin can increase circulating ketone levels, which results from the mobilization of fatty acids from adipose tissue. These fatty acids are then utilized by the liver for ketogenesis. The resulting ketone compounds provide an enhanced energy supply to the heart (37). Simultaneously, Sotagliflozin promotes autophagy and lysosomal degradation, which improves mitochondrial morphology and function. These mitochondrial changes are beneficial for the heart's energy supply. However, this enhanced energy supply does not necessarily correlate with improved efficiency of energy utilization by the heart (38). Additionally, SGLT1/2 inhibitors are associated with a reduction in the activity of calmodulin-dependent protein kinase II, which improves sarcoplasmic reticulum Ca^{2+} flux and increases cardiac contractility. This process may support cardiac energy conversion and help reduce the risk of CVD (39).

5) Reduction of oxidative stress and inflammatory response: Several studies have suggested that SGLT inhibitors can improve the inflammatory profile in patients with diabetes (40), potentially through extracellular matrix turnover and reduced fibrosis. Tsung-Ming Lee et al. found that Dapagliflozin exhibited significant antifibrotic effects by inhibiting collagen synthesis, thereby reducing the risk of cardiac remodeling. Moreover, the inhibition of SGLT1 in the heart may decrease myocardial sodium and glucose uptake, thereby reducing the generation of reactive oxygen species (ROS) induced by hyperglycemia (41, 42). However, some studies have indicated that dual SGLT1/2 inhibitors might exacerbate myocardial dysfunction in rats following myocardial infarction. Therefore, further investigation is required to assess the safety of Sotagliflozin in certain cardiovascular conditions (43).

6) Protection of endothelial cells: Studies have demonstrated that SGLT inhibition can improve vascular function by reducing endothelial cell activation, promoting direct vasodilation, alleviating endothelial dysfunction, and mitigating molecular changes associated with early atherosclerosis. These effects lead to decreased arterial stiffness and reduced total peripheral resistance (44). In this process, the inhibition of inflammatory pathways and the enhancement of mitochondrial function play crucial mediatory roles. Additionally, it has been proposed that SGLT2 inhibitors induce vasodilation through the activation of protein kinase G and voltage-gated potassium channels (45).

Regarding safety: This study found that Sotagliflozin does not increase the risk of fractures; rather, it appears to reduce the fracture risk, which may be linked to improved blood glycemia. As an adjunctive therapy, Sotagliflozin effectively lowers blood glucose levels, which helps alleviate diabetes-induced bone metabolism disorders, restore calcium and phosphate balance, and reduce skeletal damage caused by metabolic disturbances. The study also observed that, over the same follow-up period, the fracture risk associated with high-dose Sotagliflozin was lower than that associated with the low-dose regimen. Interestingly, while Sotaglifloxin did not increase the risk of urinary tract infections in our meta-analysis, we found that it elevated the risk of genital infections, particularly those caused by fungal pathogens, especially in elderly patients. Genital fungal infections predominantly occur within the first 30 days of treatment, and this phenomenon is also observed in patients with T2D (46). However, the exact mechanism remains unclear. It is likely related to the drug's unique pharmacological action. The active ingredients in Sotagliflozin may increase the incidence of genital fungal infections in diabetic patients, possibly due to altered glucose metabolism and changes in the local immune response in the genital area (47). Additionally, SGLT1/2 inhibitors reduce renal glucose reabsorption, leading to glucosuria (48), as the concentration of glucose in the urinary environment rises, *Candida albicans*, the primary pathogen in diabetic patients, proliferates rapidly, contributing to the development of genital fungal infections. Furthermore, the presence of *Candida albicans* is also associated with impaired immune function in patients. Since T1D is an autoimmune disease, studies suggest that genital microbiome dysbiosis induced by autoimmune imbalance, combined with environmental changes caused by Sotagliflozin, may further increase the risk of genital fungal infections during T1D treatment (44). Moreover, Nyirjesy et al. suggested that the use of antifungal creams as adjunctive therapy can effectively prevent genital fungal infections (45).

DKA, one of the most severe complications of diabetes, has long been a topic of concern. Current research findings suggest that Sotagliflozin may increase the risk of DKA. However, this result may be influenced by publication bias. After adjusting the data using the trim and fill method, we found that Sotaglifloxin did not significantly increase the risk for DKA. Therefore, the relationship between Sotaglifloxin and DKA remains uncertain and requires further high-quality clinical studies. Traditionally, it is believed that SGLT2 inhibitors induce DKA primarily through the following mechanisms: 1) SGLT2 inhibitors predominantly act on the kidneys, leading to substantial glucose excretion via urine, which increases the risk of urinary tract infections (UTIs) and may subsequently induce DKA. However, Sotagliflozin, acting on both the kidneys and the small intestine epithelium, reduces the glucose load entering the bloodstream, thus potentially lowering the risk of urinary tract infections. This viewpoint is supported by the current meta-analysis, which shows no significant difference in the incidence of urinary tract infections between patients using Sotagliflozin and those on placebo (46). 2) In patients with T1D,

insufficient insulin secretion, particularly after meals, leads to rapid exacerbation of hyperglycemia. This, in turn, increases the burden on the pancreas, promoting fatty acid oxidation and resulting in excessive ketone body production, potentially triggering DKA (47). Additionally, SGLT2 inhibitors cause continuous excretion of glucose by the kidneys, rapidly depleting endogenous glucose stores. In response, the body may break down fat to produce ketone bodies, thus maintaining energy supply (47). However, compared to SGLT2 inhibitors, Sotagliflozin has an advantage: its action on the intestinal epithelium reduces the rate at which glucose enters the bloodstream, thereby lowering insulin demand and preventing excessive fatty acid oxidation. Furthermore, as Sotagliflozin's effect on the kidneys is weaker than that of pure SGLT2 inhibitors, this allows more time for glucagon secretion, which reduces fat breakdown and ketone body production. 3) Traditional SGLT2 inhibitors increase hepatic β-oxidation, leading to a rise in ketone body production and a reduction in bicarbonate production. In contrast, Sotagliflozin may attenuate hepatic β-oxidation, thereby enhancing the buffering capacity of the acid-base system and reducing the risk of DKA (48).

Limitations of the Study: 1) This study is limited by the lack of follow-up data, which prevents the exploration of long-term patient outcomes. Additionally, the relatively small number of included articles may introduce potential bias, highlighting the need for more high-quality RCTs. 2) The study also failed to establish a clear dose-response relationship, limiting the ability to quantitatively assess the drug's safety and efficacy via response curves. Furthermore, the maximum observation period across the included studies was 52 weeks, which may not account for late-occurring adverse events such as major adverse cardiovascular events (MACE), mortality, or cancer. Future drug-targeted Mendelian randomized studies could be beneficial in further investigating and refining Sotagliflozin's safety and efficacy. 3) Although this study included 12 articles, some were *post-hoc* analyses, meaning the actual number of clinical studies is lower than the number of articles included. More clinical RCTs are needed in the future to strengthen the evidence level.

5 Conclusion

In T1D patients, Sotagliflozin adjunct therapy improves blood glycemia, stabilizes blood pressure, and reduces cardiovascular risk factors. It also shows potential in lowering fracture risk, but the risk of DKA requires further clinical validation.

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

YL: Conceptualization, Methodology, Software, Writing – original draft. SY: Writing – original draft, Data curation. ZW: Resources, Writing – original draft. QT: Funding acquisition, Writing – original draft. ZY: Project administration, Validation, Writing – review & editing. QJ: Writing – review & editing, Project administration.

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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 constructed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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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.2025.1506652/full#supplementary-material

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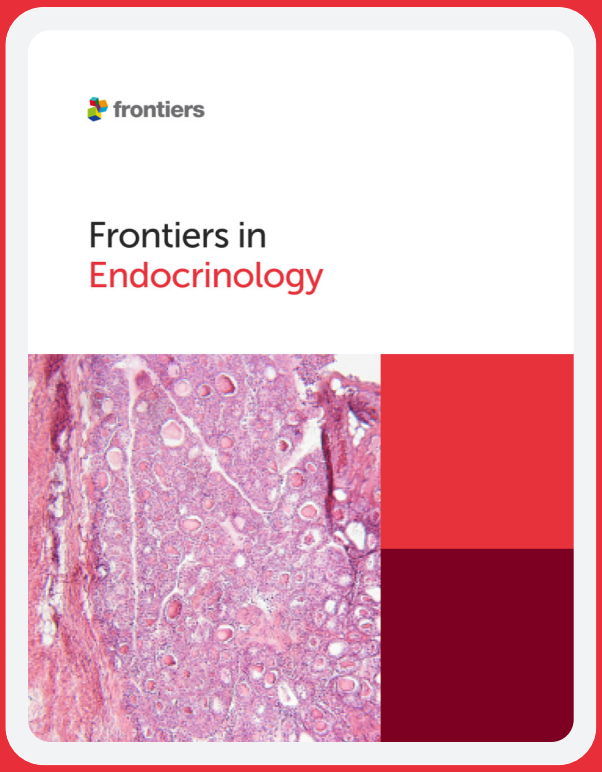

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