



Article

Longitudinal Profiling of Plasma N-Glycomic Alterations in an STZ-Induced Mouse Model of Hyperglycemia

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Abstract

The rising global incidence of Type 1 Diabetes Mellitus (T1DM) necessitates a deeper understanding of the molecular shifts underlying its metabolic complications, specifically the role of protein N-glycosylation. This study utilized a streptozotocin-induced C57Bl/6 mouse model to examine temporal changes in plasma N-glycan profiles at 2, 8, and 20 weeks post-induction using HILIC-UPLC-FLR-MS. Following the successful establishment of persistent hyperglycemia and weight loss, glycomic analysis revealed significant structural remodeling of 20 individual glycan species, with complex, multi-sialylated structures proving most sensitive to disease progression. Notably, bi-antennary structures such as A2G1S1, A2G2S1, and A2G2S2(2) exhibited a marked decrease in relative abundance that strongly correlated with elevated blood glucose levels. In contrast, highly sialylated and fucosylated glycans like FA2G2S3 and FA3G3S3 showed a progressive increase over the 20-week period, suggesting an adaptive response to chronic metabolic stress and altered hepatic processing. Our findings demonstrate that chronic hyperglycemia is accompanied by substantial remodeling of the plasma N-glycome, characterized by increased sialylation and fucosylation. These alterations closely track the progression of metabolic dysregulation, suggesting that while they parallel blood glucose trends, they provide a distinct molecular readout of the systemic glycosylation response to glucotoxicity. This study offers a detailed longitudinal characterization of these glycomic changes, highlighting their potential value as descriptive markers of cumulative metabolic stress in rodent models of type 1 diabetes.

Keywords: diabetes; glycosylation; biomarkers; diagnostic potential; mouse model; translational research; exploratory analysis



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1. Introduction

Diabetes mellitus represents a significant global public health and economic challenge, with its prevalence continuing to rise, particularly among children and adolescents. Type 1 diabetes mellitus (T1DM) is especially concerning, as its incidence increases by approximately 3–5% annually worldwide [1]. T1DM is characterized by an autoimmune response that targets and destroys insulin-producing β -cells in the pancreatic islets of

Langerhans [2–4], leading to a severe deficiency in insulin production and resulting in hyperglycemia and significant metabolic dysregulation. Acute complications of T1DM, such as hypoglycemia and diabetic ketoacidosis, pose immediate health risks [5], while chronic hyperglycemia can lead to long-term microvascular damage manifesting as diabetic retinopathy, nephropathy, and neuropathy, as well as macrovascular complications like coronary artery disease [6]. These complications significantly impact the quality of life and reduce life expectancy in individuals with T1DM. Recent research has highlighted the critical role of glycosylation in understanding diabetes and its complications [7]. N-glycans and O-glycans represent two distinct protein glycosylation pathways with different structural and functional characteristics. N-glycans are attached to asparagine residues and typically display complex branching patterns with up to three antennary structures and variable sialylation. In contrast, O-glycans attach to serine or threonine residues and generally exhibit simpler, linear structures. While both glycosylation types regulate protein function, our study focuses on N-glycosylation because plasma N-glycans substantially reflect hepatic glycosyltransferase activity and metabolic dysregulation in diabetes. Alterations in glycosylation patterns have been observed in diabetic patients and are thought to influence various physiological processes. For instance, increased levels of fucosylated glycans in serum glycans have been linked to altered immune responses and chronic inflammation, potentially exacerbating diabetes-related complications [8]. Additionally, reduced sialic acid residues on glycans can impair receptor interactions and disrupt signaling pathways involved in glucose metabolism, further contributing to insulin resistance [9]. Distinct N-glycan profiles, including changes in branching and the presence of specific glycan structures, have also been identified in T1DM, indicating a shift in glycan composition that may reflect disease state [10]. Sialic acid residues are terminal moieties on N-glycans that serve as critical communicators in immune regulation, viral pathogenesis, and metabolic homeostasis. These negatively charged molecules mask cells from immune attack by creating a ‘self’ identity, preventing inappropriate immune activation—a process particularly relevant to type 1 diabetes, where autoimmune responses target pancreatic β -cells. Additionally, sialic acid regulates cell–cell interactions through selectin and siglec binding, influences blood clotting cascades, and modulates neural development. In the diabetic context, altered sialylation may impair immune tolerance, exacerbate autoimmune β -cell destruction, and dysregulate inflammatory signaling pathways. Diabetes can also disrupt normal glycan processing, leading to the accumulation of immature or misfolded glycans that exacerbate metabolic dysregulation [11]. Finally, hyperglycemic conditions can lead to non-enzymatic glycation of proteins, resulting in advanced glycation end products (AGEs) that adversely affect protein function and contribute to diabetic complications [12].

In this study, we utilized a highly sensitive analytical technique to profile plasma N-glycans in a streptozotocin (STZ)-induced mouse model of T1DM, providing a comprehensive understanding of the glycomic changes associated with the disease. C57Bl/6 mice were treated with STZ to induce diabetes, while age-matched controls were used for comparison. Plasma samples were collected at 2, 8, and 20 weeks post-induction to observe the dynamics of N-glycan alterations over time. Advanced mass spectrometry techniques were employed to perform detailed analyses of glycan structures, enabling the detection of subtle changes that may indicate disease progression and potential therapeutic targets.

2. Results

The aim of this study was to investigate alterations in N-glycosylation profiles in the plasma of type 1 diabetes mellitus (T1DM) mouse model compared to healthy control. We hypothesized that T1DM induces distinct changes in N-glycan structures, which may contribute to impaired glucose homeostasis and disease complications.

To confirm the successful induction of diabetes following STZ administration, we evaluated the physiological parameters of the experimental groups. STZ-treated mice exhibited a pronounced and sustained elevation in fasting blood glucose levels compared with the control group, confirming the establishment of persistent hyperglycemia. Blood glucose levels increased significantly over time, reaching approximately 32 mmol/L at 20 weeks post-treatment. In parallel, a small but significant reduction in body weight was observed in diabetic mice, which is a frequent sign of T1DM. These physiological changes confirm that the STZ model effectively mimics the metabolic dysregulation associated with diabetes. After validating the establishment of the diabetic phenotype through physiological parameters, we have examined whether hyperglycemia and metabolic imbalance were reflected at the glycomic level. To this end, plasma N-glycan profiles were analyzed to identify structural changes in glycosylation potentially associated with the diabetic state.

Plasma N-glycans were analyzed by HILIC-UPLC-FLR-MS separating 20 individual glycan species, mainly bi-antennary structures with different level of sialylation as it shown in Supplementary Figure S2. A detailed list of identified N-glycans, including *m/z* ratios, retention times and mass errors (ppm), are provided in Supplementary Table S1. The use of fluorescence chromatographic data for quantitation was facilitated by the extraction of individual ion-chromatograms from the MS data as shown in Supplementary Figure S2. To identify the relative glycan distribution differences among experimental groups, representative chromatograms were generated for each cohort. Distinct profiles were observed between control and diabetic mice, reflecting alterations in the relative abundance of specific N-glycan species. The chromatographic patterns of diabetic samples exhibited noticeable shifts in relative peak area ratios, indicating structural and compositional remodeling of plasma glycans in response to hyperglycemia.

After establishing that many glycan variables deviated from normal distribution, non-parametric tests were employed to evaluate intergroup differences across the four experimental conditions: Control (C), Diabetes 2 weeks (D2), Diabetes 8 weeks (D8), and Diabetes 20 weeks (D20). Several glycan structures exhibited statistically significant differences between the experimental groups, indicating pronounced glycosylation remodeling over the course of diabetes (Supplementary Table S4). The results indicate that more complex, highly sialylated glycans were the most responsive to diabetic progression. The early-stage glycans remained statistically stable, suggesting that glycosylation shifts are a late, disease-driven phenomenon rather than an immediate metabolic response. To complement the inferential analysis, violin plots were used for each glycan structure to visualize both the data dispersion and the probability density. This dual visualization provided insight into how not only the central tendency but also the distribution shape changed during diabetes progression. Several glycan structures exhibited statistically significant differences between the experimental groups, indicating pronounced glycosylation remodeling over the course of diabetes.

As it shown in Figure 1, the violin plots effectively demonstrated the evolution of glycan distributions across time points. Increased distributional spread and elevated medians were observed for A2G2S1 (bi-antennary bi-galactosylated and mono-sialylated) and A2G2S2(2) (bi-antennary bi-galactosylated and mono-sialylated) in D8 and D20 groups, corresponding to hyperglycemia-associated over-sialylation. Conversely, A2G1S1 showed a reduction in relative abundance at intermediate stages, indicating selective pathway regulation in mono-sialylated structures. This visual analysis complements the statistical outcomes by revealing distributional heterogeneity a hallmark of biochemical adaptation under chronic disease conditions. The widening of violin plot shapes in diabetic groups suggests biological variability, possibly due to differential enzyme activity in glycosyl-transferases

and sialyl-transferases. The observed group differences reflect dynamic remodeling of the plasma glycome during diabetes progression.

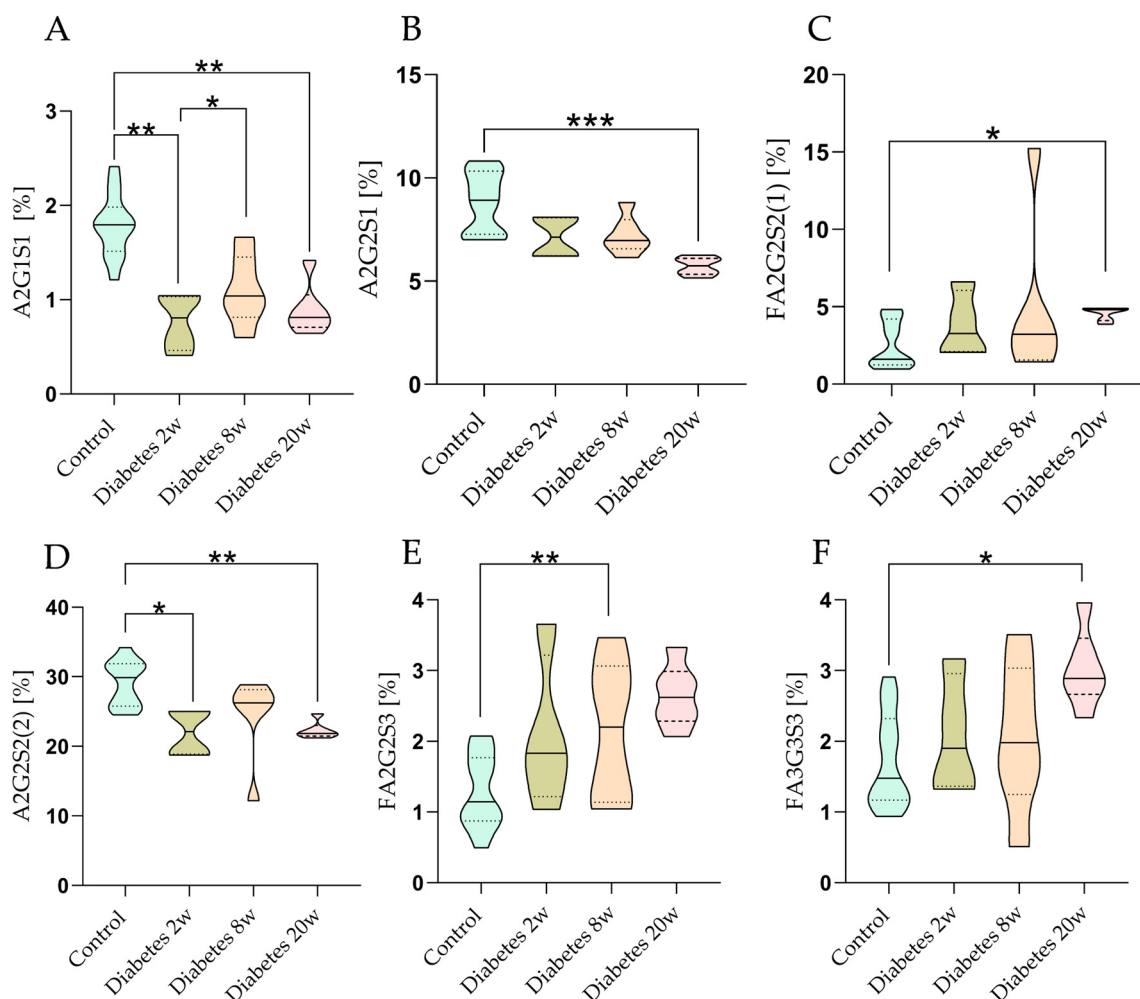


Figure 1. Significantly different glycan structure levels by Kruskal–Wallis Test. Relative abundance (%) of six specific glycan structures as a function of diabetes progression in 16 control and 16 diabetic mice (2, 8, and 20 weeks). The plots indicate that the levels of complex, sialylated glycans, such as (A): A2G1S1 (bi-antennary-monogalactosylated and monosialylated), (B): A2G2S1 (bi-antennary-bi-galactosylated and monosialylated), (C): FA2G2S2 (fucosylated, bi-antennary-bi-galactosylated, bi-sialylated), and (D): A2G2S2(2) (bi-antennary-bi-galactosylated and bi-sialylated), are significantly reduced in the diabetic group, with the most pronounced differences observed in the early phases of the disease (2 and 8 weeks) compared to controls. Conversely, the relative amounts of fucosylated structures, specifically (E): FA2G2S3 and (F): FA3G3S3, show a progressive increase in diabetic mice, reaching the highest and most significant deviations at 20 weeks. The violin plots display the data distribution and median values, revealing specific and time-dependent alterations in the glycosylation pattern associated with the diabetic phenotype (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

Correlation Analysis of Metabolic and Glycan Parameters

To identify the relationship between metabolic state and the plasma N-glycan profile, a Spearman correlation analysis was performed as an initial exploratory step. After applying Benjamini–Hochberg False Discovery Rate (FDR) correction, several robust associations remained statistically significant. Blood glucose levels showed a strong positive correlation with the relative abundance of FA2G2S3 and A2G2S2 (2), indicating that higher sialylation and fucosylation levels are linked to the diabetic state. Conversely, body weight and the A2G2S1 glycan structure exhibited a significant negative correlation with glucose levels, suggesting their association with the healthy metabolic state in this model (Figure 2).

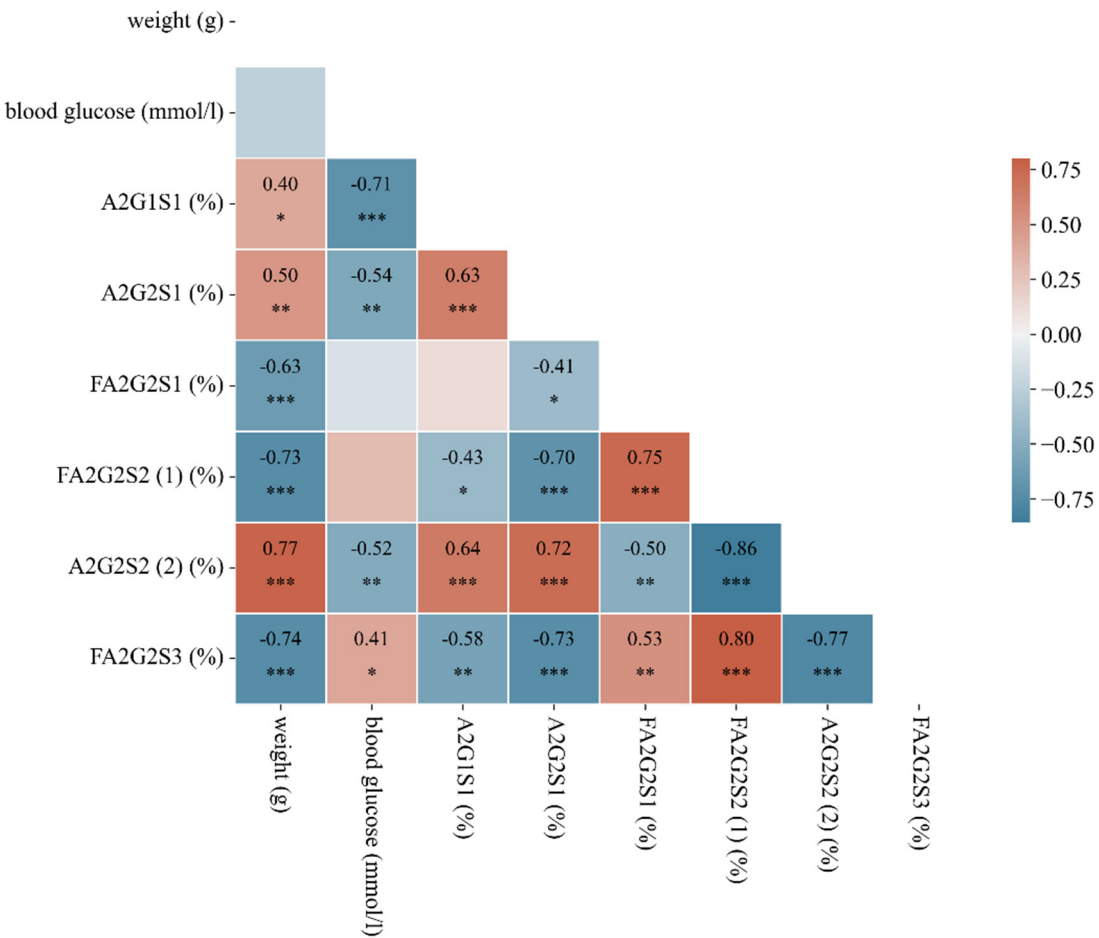


Figure 2. Exploratory correlation analysis of metabolic parameters and plasma N-glycans. Spearman’s rank correlation heatmap illustrating the relationships between key clinical markers (body weight, blood glucose) and selected plasma N-glycan structures. Correlation coefficients are color-coded (red: positive, blue: negative). Asterisks denote statistical significance after Benjamini–Hochberg False Discovery Rate (FDR) correction (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). The analysis reveals robust associations between hyperglycemia and increased levels of highly sialylated and fucosylated structures, while body weight and A2G2S1 show a significant inverse relationship with glucose levels.

Building upon the identified correlations, a logistic regression model was employed to evaluate the collective diagnostic potential of these parameters. Using stratified 5-fold cross-validation to ensure the generalizability of the results, the model achieved a mean Accuracy of 0.97 (± 0.07) and a perfect AUC of 1.00 (± 0.00). The classification yielded a Sensitivity of 1.00 (± 0.00) for the Diabetes class and a Specificity of 1.00 (± 0.00) for the Control class in this cohort. These metrics demonstrate that the plasma N-glycan profile provides distinct separation between the experimental groups, though we note that such clear demarcation is expected given the severity of the phenotype in the high-dose STZ model. These metrics demonstrate that the plasma N-glycan profile, combined with clinical markers, provides a robust basis for group separation.

The relative contribution of each parameter to the classification was assessed by analyzing the standardized regression coefficients across all validation folds. Blood glucose (mmol/L) was confirmed as the primary positive predictor for diabetes (Mean Coeff: $+0.90 \pm 0.10$). The most stable glycan-based indicators for the diabetic group were FA2G2S3 (%) (Mean Coeff: $+0.44 \pm 0.08$), FA3G3S3 (%) (Mean Coeff: $+0.38 \pm 0.03$), and A3G3S3 (%) (Mean Coeff: $+0.38 \pm 0.06$) (Figure 3).

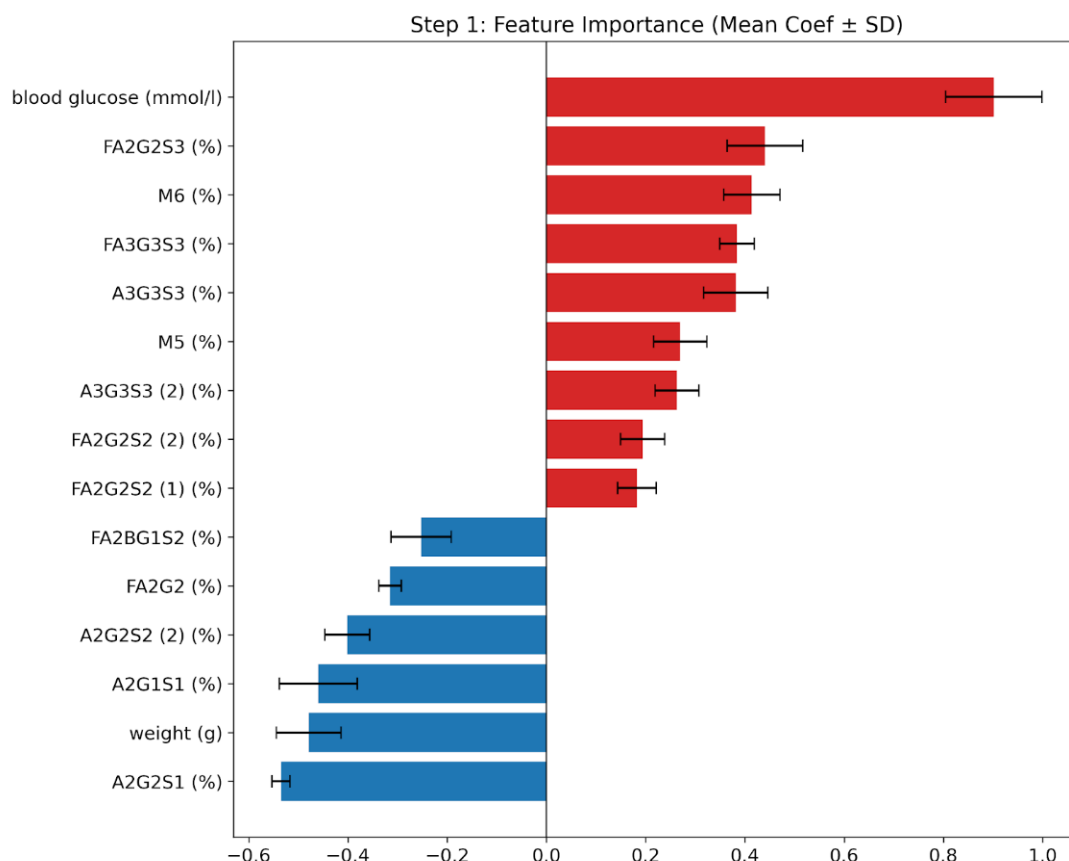


Figure 3. Feature Importance in Diabetes Classification. The bar chart displays the standardized regression coefficients (Mean Coef $\pm$ SD) for each parameter, indicating their relative contribution to distinguishing between diabetic and control states. Blood glucose (mmol/L) is the primary positive predictor for diabetes, with a mean coefficient of $+0.90 \pm 0.10$. Key glycan indicators for the diabetic group include FA2G2S3 (%), FA3G3S3 (%), and A3G3S3 (%). Conversely, prominent markers for the control group are A2G2S1 (%), weight (g), and A2G1S1 (%). The low standard deviations highlight the stability and systematic nature of these glycan shifts in the type 1 diabetes model.

On the other hand, the model identified several consistent markers of the healthy control state. The most prominent features were A2G2S1 (%) (Mean Coef: $+0.53 \pm 0.02$), weight (g) (Mean Coef: $+0.48 \pm 0.07$), and A2G1S1 (%) (Mean Coef: $+0.46 \pm 0.08$). The low standard deviations associated with these coefficients suggest that these glycan shifts are consistently associated with the diabetic phenotype in this dataset. However, given the high collinearity between blood glucose and glycan traits, these associations should be interpreted as correlative rather than independent predictive factors.

The scatter plots (Supplementary Figures S6–S8) reveal distinct structural trends. Highly sialylated and fucosylated glycans (e.g., FA2G2S3) exhibited a strong positive correlation with blood glucose, dynamically increasing in response to hyperglycemic conditions. In contrast, neutral and mono-sialylated bi-antennary structures (A2G2S1, A2G1S1) were negatively correlated with glucose and more closely associated with body weight, suggesting they may reflect the baseline metabolic state rather than the disease process itself.

3. Discussion

Glycosylation alterations play a pivotal role in the pathophysiology of diabetes, particularly in type 1 diabetes mellitus (T1DM). In our study, we observed significant variability in plasma N-glycan composition in the STZ-induced diabetic mouse model, indicating altered

regulation of glycosylation pathways under hyperglycemic conditions. This finding aligns with the work of [13], who reported significant changes in plasma protein N-glycosylation patterns among adults with T1DM, specifically highlighting increased antennary fucosylation and decreased monogalactosylation. These changes suggest that glycosylation profiles can serve as biomarkers for disease progression and metabolic dysregulation [13]. While some glycomic changes appeared by week 8, the most profound remodeling occurred at week 20. This suggests that these specific plasma N-glycan signatures serve less as early predictive markers of onset, but rather as robust indicators of cumulative glycemetic burden and long-term metabolic dysregulation, potentially useful for monitoring the efficacy of therapeutic interventions preventing diabetic complications.

Our results further support the notion that glycomic alterations are conserved across species, as we identified strong positive correlations between hyperglycemia and the abundance of sialylated glycans. These findings are consistent with previous research that has demonstrated similar associations in humans, indicating a mechanistic link between glucose dysregulation and glycosyltransferase activity [14,15]. Such correlations highlight the potential role of altered glycosylation in the progression of diabetic complications, including microvascular damage [16,17].

The observed structure-specific shifts in sialylation, characterized by the decrease in mono-sialylated species like A2G1S1 and the concurrent increase in tri-sialylated forms such as FA2G2S3, align with established literature suggesting that chronic hyperglycemia alters the activity of specific sialyltransferases. Plasma N-glycans represent a superposition of glycoproteins derived from hepatocytes—including major acute-phase proteins like fibrinogen, haptoglobin, and α 1-acid glycoprotein—and B-cells (immunoglobulins). The specific use of plasma in this study ensures that the glycomic profile captures the contribution of hepatic clotting factors (fibrinogen), which are absent in serum. The observed increase in highly sialylated and fucosylated structures (e.g., FA2G2S3) is characteristic of these hepatic acute-phase proteins, confirming that the identified T1DM signature strongly reflects hepatic metabolic adaptation.

Although we did not directly quantify glycosyltransferase expression, the observed structural shifts—specifically the increase in α 2,6-sialylation—are consistent with the hypothesis of ST6Gal1 upregulation, a phenomenon previously described in hyperglycemic states [18,19].

It is important to note that while the multiple low-dose streptozotocin (MLD-STZ) protocol is often preferred for studying the autoimmune etiology of T1DM, our study employed the single high-dose (SHD-STZ) protocol to rapidly induce a stable state of severe insulin-deficient hyperglycemia. This model was selected to specifically isolate the long-term metabolic consequences of chronic hyperglycemia on plasma protein glycosylation over a 20-week period, minimizing the confounding variables of fluctuating immune responses associated with the MLD model.

It is important to distinguish these T1DM-associated profiles from those reported in Type 2 Diabetes (T2DM). While T2DM is often characterized by hyperinsulinemia and low-grade inflammation leading to increased core fucosylation and branching, our T1DM model reflects a catabolic, hypoinsulinemic state with severe hyperglycemia (>30 mmol/L). The specific plasma signatures observed may be associated with increased hexosamine pathway flux driven by unmitigated hyperglycemia, distinct from the lipotoxicity-driven profiles often seen in T2DM.

It is essential to acknowledge several limitations of this study to provide a comprehensive understanding of its implications. Firstly, the study utilized a limited number of subjects, specifically 16 diabetic and 16 control mice. We acknowledge that the high classification accuracy (AUC > 0.99) is likely driven by the severity of the STZ-induced

phenotype and the small sample size, and should not be interpreted as validation of clinical diagnostic utility. These analyses are intended as exploratory demonstrations of the high dimensionality reduction and separability of the glycomic data, rather than as validated predictive models. The potential for variability in biological responses among individual animals should be considered, as this may influence the overall results. Secondly, the diagnostic potential of the identified glycan structures was assessed without independent validation in larger cohorts or human models. Additionally, the use of a single streptozotocin (STZ)-induced mouse model may not fully capture the complexity of type 1 diabetes mellitus (T1DM) in humans. Variations in glycosylation patterns may exist across different models or species, necessitating further research to establish the translational relevance of our findings.

4. Materials and Methods

4.1. Chemicals

For animal procedures, anesthesia and analgesia were achieved using a combination of Zoletil 50 (tiletamine/zolazepam, Virbac, Carros, France), Sedaxylan (xylazine, Dechra, Northwich, UK), and Torphadine (butorphanol 10 mg/mL, Dechra, Northwich, UK). Insuman Basal SoloStar (100 IU/mL, Sanofi-Aventis, Paris, France) was used for insulin supplementation when required. Phosphate-buffered saline (PBS) and sterile saline solutions were applied for injections, perfusion, and tissue rinsing throughout the experiments.

For the glycosylation analysis, formic acid, ammonium hydroxide, acetic acid, acetonitrile, picoline borane, procainamide-hydrochloride, dimethyl sulfoxide (DMSO), magnesium(II) nitrate hexahydrate, and ethanolamine were obtained from Sigma-Aldrich (St. Louis, MO, USA). PNGase F was purchased from New England Biolabs (Ipswich, MA, USA). Nickel(II) nitrate hexahydrate and sodium acetate were supplied by Thermo Fisher Scientific (Kandel, Germany), while manganese(II) nitrate tetrahydrate was obtained from Carl Roth (Karlsruhe, Germany). Cobalt(II) nitrate hexahydrate and iron(III) nitrate nonahydrate were purchased from VWR International (Radnor, PA, USA).

All aqueous solutions were prepared using ultrapure water (resistivity $\geq 18.2 \text{ M}\Omega\cdot\text{cm}$) produced by a Milli-Q purification system (Merck Millipore, Darmstadt, Germany), and all reagents were stored and handled according to the manufacturers' recommendations.

4.2. Study Design

Animal experiments were approved by the RCNS Institutional Animal Care and Use Committee (Permit number: PE/EA/748-2/2021) and conducted in accordance with national guidelines. Wild-type C57BL/6J mice were obtained from The Jackson Laboratories. All animals were maintained in accredited facilities at the HUN-REN Research Centre for Natural Sciences. At study initiation, mice were healthy and within the normal weight range. Animals were housed under standard laboratory conditions with a 12 h light/dark cycle and ad libitum access to food and water. Both male and female mice were used to minimize sex bias. Although female C57BL/6 mice are historically reported to be more resistant to STZ-induced diabetes, our high-dose protocol successfully induced sustained hyperglycemia ($>30 \text{ mmol/L}$) in both sexes. Preliminary stratification of the plasma glycomic data revealed no statistically significant interaction between sex and the identified glycan biomarkers, suggesting that the severity of the hyperglycemic insult in this model overrides subtle sex-specific metabolic differences.

Experimental groups included 16 streptozotocin-induced T1DM mice and 16 age-matched control mice. Streptozotocin-induced T1DM mice models typically show β -cell destruction and hyperglycemia, making them suitable for testing pharmacological interventions or therapies targeting hyperglycemia or autoimmune reactions. In the diabetic groups

blood samples were collected 2, 8 and 20 weeks after diabetes induction to obtain plasma for glycosylation analysis. The number of animals used in this study was determined in line with legal and ethical requirements, and represents the minimum number required to achieve statistically meaningful results. Details on sample sizes representing biological replicates are shown in the figure legends.

4.3. Diabetic Mouse Model: STZ Treatment

Streptozotocin (STZ), an antibiotic isolated in 1960, was discovered in 1963 to possess diabetogenic properties. Upon administration, STZ selectively induces β -cell destruction within the pancreatic islets, leading to insulin deficiency and hyperglycemia in experimental animals [20,21].

The STZ-induced diabetes model closely resembles human type 1 diabetes mellitus (T1DM), characterized by β -cell destruction and subsequent insulin deficiency. The mechanism of STZ-induced β -cell destruction involves the selective uptake of STZ into β -cells via the GLUT2 transporter, leading to DNA fragmentation and cell death [22]. Two primary protocols are utilized for inducing diabetes in rodents: multiple low-dose STZ administration and a single high-dose injection. The multiple low-dose regimen involves administering STZ at subdiabetogenic doses over several days, inducing a more gradual β -cell destruction and mimicking the autoimmune nature of human T1DM [23,24]. Conversely, a single high-dose STZ injection leads to rapid and extensive β -cell necrosis, resulting in acute hyperglycemia within 48 h [20]. Both models are widely used in diabetes research to study disease mechanisms and evaluate potential therapeutic interventions.

Mice received an intraperitoneal injection of streptozotocin (150 mg/kg; Tocris, 1621; Bristol, UK), as previously described [25]. 3.5-month-old mice were used for the experiments. Blood glucose levels were measured on the fifth day following STZ administration. Diabetes was defined as a blood glucose concentration exceeding 16 mmol/L. Animals with glucose levels below this threshold received an additional STZ injection at a dose of 150 mg/kg body weight. In cases where blood glucose exceeded 30 mmol/L, mice were administered insulin intraperitoneally once daily. Following the initial measurement, blood glucose levels were monitored weekly to track the progression of hyperglycemia. Blood glucose was measured from non-fasting animals.

4.4. Euthanasia and Blood Sampling

Prior to euthanasia, mice received a combined anesthetic and analgesic agent via intraperitoneal injection (Zoletil® 30 mg/kg (Virbac Corporate, Carros, France), Xylazine 12.5 mg/kg, Butorphanol 3 mg/kg). Once deep anesthesia was achieved, blood samples were collected. Heparin was added to the blood samples as an anticoagulant (final concentration: ~10–20 U/mL). The samples were kept on ice until further processing. To separate red blood cells, the samples were centrifuged (Eppendorf® Centrifuge 5804 G, 1000 g, 10 min, 4 °C (Horsholm, Denmark)). After centrifugation, the supernatant (plasma) was carefully collected and used for subsequent analyses. Plasma was selected for glycomic analysis to ensure the inclusion of all circulating glycoproteins, including clotting factors such as fibrinogen, which are typically removed during serum preparation. Since fibrinogen is a major acute-phase protein synthesized by the liver, its presence in plasma provides a more comprehensive representation of hepatic glycosylation status under inflammatory conditions compared to serum. Additionally, we respectfully clarify that all blood glucose measurements reported in this study were obtained from fasting animals to minimize postprandial variability.

4.5. N-Glycan Analysis

The N-glycans were released from plasma samples using the PNGase F protocol of New England Biolabs (Ipswich, MA, USA), with slight modifications. In this procedure, 9 μ L of plasma was transferred into a reaction tube and 1 μ L of denaturation buffer was added and incubated at 65 °C for 15 min. After this, 7 μ L of water and 2 μ L of Glycobuffer 2 were added with 1 μ L of enzyme and then incubated overnight at 37 °C. For detection, the released N-glycans were fluorescently labeled, as they lack intrinsic fluorescent properties. Glycans were labeled via reductive amination, a reaction that attaches a single fluorophore molecule to the specific reducing end of each free N-glycan. This ensures a strict 1:1 stoichiometric relationship between fluorescence intensity and molar abundance, thereby eliminating labeling bias and allowing for accurate relative quantification across different glycan structures. The labeling solution was freshly prepared by dissolving 100 mg of procainamide hydrochloride and 100 mg of 2-picoline borane complex in 700 μ L of dimethyl sulfoxide and 300 μ L of acetic acid. Finally, 10 μ L of the labeling reagent was added to each glycan sample, mixed thoroughly, and incubated at 65 °C for 5 h to ensure complete derivatization. The labeled glycans were purified by hydrophilic magnetic nanoparticles.

4.6. UPLC-FLR-MS Analysis

Prepared N-glycans were analyzed on a Waters Acquity UPLC system equipped with a fluorescence detector and a Xevo G2-S qTOF mass spectrometer (Waters, Milford, MA, USA). Data were acquired with MassLynx 4.2 software. Separation was carried out on a Waters BEH Glycan column (100 $\times$ 2.1 mm, 1.7 μ m) at 60 °C using a linear gradient of 75–55% acetonitrile (mobile phase B) against 50 mM ammonium formate, pH 4.4 (mobile phase A). The flow rate was 0.4 mL/min, and 5 μ L of each sample was injected in partial loop mode. The sample compartment was maintained at 15 °C. Fluorescence detection was performed at λ_{ex} = 308 nm and λ_{em} = 359 nm.

Mass spectrometry was operated in positive ionization mode with a capillary voltage of 2.2 kV. The desolvation temperature was set to 120 °C with a gas flow of 800 L/h. Mass spectra were collected in the range of 500–2000 m/z , and MS/MS data were obtained at a collision energy of 45 kV.

4.7. Data Analysis

All patient samples were analyzed in triplicate, and chromatograms were quantified using UNIFI chromatography software (Waters, Milford, MA, USA). Data analysis was performed with IBM SPSS Statistics 25, PAST 4.02, and GraphPad Prism 8. GlycoWork-Bench 2.1 was used for the mass calculation of individual glycan structures, and glycan nomenclature followed the conventions described by Harvey et al. [26]. Figures were prepared using ChemDraw 22.2.0 and BioRender. Created in BioRender. Kutás, A. (2026) <https://BioRender.com/fl988j4>.

To ensure reliable statistical analyses, the dataset was first characterized using descriptive statistics. This included calculating the mean and standard deviation (SD) for each glycan structure. Normality testing was conducted using the Shapiro–Wilk test and Q–Q plots to assess distribution (Supplementary Figure S4). These steps were crucial for determining the right statistical methods for hypothesis testing and identifying variability among experimental groups. Descriptive statistics were calculated for four groups: Control, Diabetes 2 weeks (D2), Diabetes 8 weeks (D8), and Diabetes 20 weeks (D20). Standard deviations were important for assessing variability within each group. Low SD values indicated stable glycan expression, while high SD values suggested greater variability due to biological fluctuations. Several trends were observed: Control samples showed low SD values, indicating a consistent glycan composition. Early diabetic groups (D2, D8) had

moderate increases in SD, suggesting early metabolic changes. In late-stage diabetes (D20), higher SD values reflected increased biological variability due to chronic stress. The rising SD during diabetes progression indicated that glycan regulation becomes less stable. This variability likely results from activated glycosylation pathways or tissue-specific stress responses. The Shapiro–Wilk test revealed that most glycan variables deviated significantly from normality.

4.8. Statistical Analysis and Machine Learning Validation

To investigate the associations between clinical parameters (body weight, blood glucose levels) and the relative abundance of plasma N-glycans, Spearman's rank correlation analysis was performed, as it is robust to non-linear relationships. To account for the risk of Type I errors (false positives) arising from multiple simultaneous comparisons, raw *p*-values were subjected to Benjamini–Hochberg False Discovery Rate (FDR) correction. Only correlations with an adjusted *p*-value were considered statistically significant.

To evaluate the diagnostic potential of the plasma N-glycan profile and to model the differentiation between control and diabetic cohorts, a logistic regression algorithm was employed. Prior to modeling, all numerical variables were standardized (Z-score normalization) to ensure that features with different scales (e.g., body weight in grams versus glycan area percentages) contributed equally to the model.

To prevent statistical overfitting and to demonstrate the generalizability of the identified biomarkers, a Stratified 5-fold Cross-Validation approach was implemented. In this procedure, the dataset was partitioned into five subsets; the model was iteratively trained on 80% of the data and validated on the remaining 20%—a subset previously unseen by the algorithm in each cycle. The overall diagnostic performance was determined based on the aggregated results of these independent validation folds, quantified by ROC-AUC (Area Under the Curve), sensitivity, and specificity metrics. Feature importance was derived by calculating the mean and standard deviation of the logistic regression coefficients across the cross-validation folds (Mean Coeff $\pm$ SD). This rigorous approach ensured that the identified biomarkers represent stable, systematic biological shifts rather than artifacts of sampling noise.

5. Conclusions

In conclusion, this study provides valuable insights into the glycomic alterations associated with type 1 diabetes mellitus (T1DM) using a streptozotocin (STZ)-induced mouse model. The observed changes in plasma N-glycan profiles highlight the potential of glycosylation patterns as indicators for monitoring disease progression and metabolic dysregulation. These findings contribute to our understanding of the molecular mechanisms underlying diabetes and suggest that specific glycan structures may be associated with the diabetic state. However, it is crucial to approach these results with caution. The limitations of our study, including the small sample size, lack of independent validation, and the exploratory nature of the analyses, necessitate further investigation to substantiate these findings. Future research should focus on replicating these results in larger and more diverse populations, as well as validating the clinical applicability of the identified glycan alterations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27042010/s1>.

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