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N-glycosylation of serum proteins in adult type 1 diabetes mellitus exposes further changes compared to children at the disease onset

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ABSTRACT

Objective: Previously we have shown that plasma protein N-glycosylation is changed in children at the onset of type 1 diabetes. In this study, we aim to identify N-glycan changes in adults with T1DM, compare them to those in children, and investigate their associations with disease duration, complications, glycaemic status, and smoking.
Methods: Serum protein N-glycans from 200 adults with type 1 diabetes and 298 healthy controls were analysed using ultra-high performance liquid chromatography and divided into 39 directly measured glycan groups from which 16 derived traits were calculated.
Results: Compared to healthy controls, subjects with type 1 diabetes showed differences in 19 glycan groups and a decrease in monogalactosylated, an increase in digalactosylated, monosialylated, and antennary fucosylated derived traits, from which changes in monogalactosylation and seven directly measured traits overlapped with previously reported in children. Changes in four directly measured and two derived traits previously seen in children were not detected in adults. HbA1c was positively associated with sialylated and highly branched structures, whereas N-glycome was not influenced by disease duration or diabetic complications.
Conclusions: Our results suggest potential N-glycome involvement in different stages of type 1 diabetes, including processes underlying its development, the disease itself, as well as those occurring after disease establishment.

1. Introduction

N-glycosylation is a highly conserved co– and post-translational modification [1] present in more than 7000 proteins of the human proteome [2]. Glycosylation plays a crucial role in development, neurogenesis, and immunity by regulating protein function. Glycosylation should not be confounded with glycation, with the latter representing a random reaction between glucose and proteins [3]. On the other hand, glycosylation is a strictly regulated enzymatic reaction under complex genetic regulation during which complex oligosaccharide structures, or glycans, are covalently attached to proteins, impacting their function in many instances [4,5]. Emphasizing the importance of this modification,

Abbreviations: T1DM, type 1 diabetes mellitus; IgG, immunoglobulin G; AGP, alpha-1-acid glycoprotein; AER, albumin excretion rate; GlcNAc, N-Acetylglucosamine; SDS, sodium dodecyl sulfate; 2-AB, 2-aminobenzamide; wwPTFE, water-wettable polytetrafluoroethylene; ACN, acetonitrile; UPLC, ultra-performance liquid chromatography; BEH, ethylene bridged hybrid; GP, glycan peak (directly measured N-glycan trait); ApoB-100, apolipoprotein B100; IgM, immunoglobulin M.

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it was found that changes in protein glycosylation are characteristic of numerous pathological conditions in humans, and in some cases, a change in glycosylation itself was identified as the main cause of the disease [4,6].

Aberrant N-glycosylation of serum and plasma proteins, as well as of immunoglobulin G (IgG) has been associated with various autoimmune diseases [7–10], including type 1 diabetes mellitus (T1DM) [11–14] while some N-glycan structures have been used to differentiate between different types of diabetes [15].

In the context of T1DM, changes in the N-glycome have been associated with microvascular complications in adults [11,12,14,16], while others have been associated with the onset of T1DM in children [13]. Poland and colleagues performed one of the first studies to explore the N-glycosylation in T1DM, in which they have shown that increased alpha-3 fucosylation of an acute phase protein, alpha-1-acid glycoprotein (AGP), was associated with T1DM and declining vascular function [14]. Moreover, recent studies on the N-glycosylation of total serum proteins in adults with T1DM have reported increased complexity of serum N-glycome with poor glycaemic control and declining renal function [11,12].

Thus far, we performed the only case-control study on children at the onset of T1DM, where it was shown that T1DM was associated with an increase in plasma and IgG high-mannose and bisecting GlcNAc structures, and a decrease in monogalactosylation [12]. Furthermore, the increase in the number of different auto-antibodies, a predictor of progression to overt diabetes, was followed by a decrease in some of the highly branched plasma N-glycans [13]. Following this, we recently also showed that allelic variants of N-glycosylation and complement genes contribute to altered plasma protein and IgG N-glycosylation in children at the onset of T1DM compared to their healthy siblings [17]. In this respect, these studies have identified glycans that may specifically accompany T1DM onset, i.e., may be important either in the disease process itself or be important indicators of that disease process and have identified novel genetic associations with such markers.

In this study, to address the association of glycan changes with processes that occur after T1DM establishment and to distinguish it from those that might be important for disease development, we investigated the persistence of glycan changes into adulthood in a case-control manner. Additionally, we examined the association between N-glycans of serum proteins and the presence or the severity of common diabetic complications, including albuminuria, retinopathy, and hypertension, as well as smoking habits that confer significant risk for the development of diabetic complications. We also assessed the correlation between N-glycome and indicators of glycaemic control such as haemoglobin A1c (HbA1c), postprandial and fasting glucose levels, and T1DM duration. These new insights will be important in understanding the autoimmunity process leading to T1DM and in generating tests that may monitor at-risk individuals.

2. Materials & methods

2.1. Subjects

The study cohort consisted of 200 individuals with T1DM recruited at Vuk Vrhovac Clinic for Diabetes, Endocrinology and Metabolic Diseases, Merkur University Hospital, Zagreb, Croatia and 298 matching healthy volunteers. All the participants were aged between 18 and 79 years (Table 1). Patient inclusion criteria were: T1DM diagnosed up to 35 years of age, positive relevant autoantibody status, active treatment with insulin for at least one year prior to sampling (minimal duration of the disease of 1 year), with or without microvascular complications (complication status being stationary for at least 3 months before recruitment).

The information on T1DM samples included laboratory data (such as fasting and postprandial glucose levels, HbA1c), smoking habits, and diabetic microvascular complication status. Complication status was based on the categorisation of the severity of common diabetic complications (albuminuria, hypertension, and retinopathy) into two or three distinct categories, depending on the complication. Albuminuria was categorised based on albumin excretion rate (AER) defined by the quantitative albuminuria during 24 h period: category (1) <30 mg/24 h (A1; no albuminuria or mild albuminuria); category (2) 30–300 mg/24 h (A2; moderate albuminuria); category (3) >300 mg/24 h (A3; severe albuminuria). Retinopathy status was classified based on ophthalmologist examination into three categories: category (1) no retinopathy; category, (2) non-proliferative retinopathy; category, (3) proliferative retinopathy. Hypertension was defined as current anti-hypertensive therapy or blood pressure ≥140/90 mmHg and categorized as absent (category 1) or present (category 2).

Table 1
Characteristics of the study participants.

Healthy volunteers	
Number of participants (male/female)	298 (125/173)
Age (years)	45 (18–79)
T1DM subjects	
Number of participants (male/female)	201 (86/115)
Age (years)	46 (18–70)
Smoking status	61 smokers, 140 non-smokers
T1DM duration median (years)	14 (1–47)
Laboratory data	
Fasting glucose (mmol/L)	7.7 (5.7–9.6)
Postprandial glucose (mmol/L)	11.4 (8.0–14.6)
HbA1c (%)	7.4 (6.6–8.3)

Diabetic complications

	Retinopathy	Albuminuria	Hypertension
Category 1	157	28	145
Category 2	31	162	56
Category 3	12	9	*NA*

Values for age and duration of the disease are medians (range) and values for biochemical parameters are medians (interquartile range). For diabetic complications, the number of participants pertaining to each category (N) is shown. *NA*: not applicable.

All subjects signed informed consent, whereafter ethical approvals from the local ethics committees were obtained. The study was conducted in accordance with the Declaration of Helsinki.

2.2. N-glycan release from total serum proteins

Serum samples (10 μl) were denatured by the addition of 20 μl of 2% (w/v) SDS (Sigma-Aldrich, St. Louis, MO, USA), incubated at 65 °C for 10 min, and consequently cooled down to room temperature. Following the denaturation, 10 μl of 4% Igepal-CA630 (Sigma-Aldrich, USA) were added to each sample and the samples were shaken on a plate shaker (GFL, Lauda-Königshofen, Germany) for 15 min. The release of N-glycans was achieved by the addition of 1,2 U of PNGase F (Promega, Madison, WI, USA) and overnight incubation at 37 °C.

2.3. Fluorescent labelling of released N-glycans and HILIC-SPE clean-up

The released N-glycans were fluorescently labelled using 2-aminobenzamide (2-AB). The labelling solution was prepared by dissolving 2-AB (19,2 mg/ml, Sigma-Aldrich, USA) and 2-picoline borane (44,8 mg/ml, Sigma-Aldrich, USA) in a mixture (70:30 v/v) of dimethyl sulfoxide (Sigma-Aldrich, USA) and glacial acetic acid (Sigma-Aldrich,

USA). After the addition of the labelling mixture (25 µl), the samples were incubated at 65 °C for 2 h and allowed to cool down to room temperature. Residual protein, enzyme, unbound dye, and the reducing agent were eliminated from the mixture utilizing hydrophilic interaction liquid chromatography solid-phase extraction (HILIC-SPE). Briefly, the 96-well 0,2 µm wwPTFE (Pall Corporation, New York, USA) filter plate was mounted on a vacuum manifold (Pall Corporation, USA), prewashed with 70% ethanol (J.T. Baker, Phillipsburg, NJ, USA) and ultra-pure water (Merck KGaA, Darmstadt, Germany), followed by equilibration with 96% acetonitrile (ACN). The samples were brought to 96% of ACN with 700 µl of ACN (VWR International, Radnor, PA, USA), and applied to the 0,2 µm wwPTFE (Pall Corporation, USA) filter plate. The wells were subsequently washed five times with 96% ACN and labelled N-glycans were eluted with a total of 180 µl of ultra-pure water and stored at – 20 °C until further use.

2.4. Separation of N-glycans by hydrophilic interaction liquid chromatography

Fluorescently labelled N-glycans were separated by an Acquity UPLC H-Class instrument (Waters, Milford, USA) composed of a quaternary solvent manager, sample manager and a fluorescence detector unit with excitation and emission wavelengths set to 330 nm and 420 nm, respectively. The instrument was managed by Empower 3 software, build 3471 (Waters, Milford, USA). The separation was based on hydrophilic interaction liquid chromatography and was performed on BEH Premier Waters 150 mm column (Waters, USA), with 100 mM ammonium formate (pH 4,4) and ACN (VWR International, USA) as solvents A and B, respectively. A linear gradient of 70–53% acetonitrile at flow rate 0.561 ml/min was applied in a 32.5 min analytical run.

The chromatography system was calibrated with an external standard of hydrolysed, 2-AB labelled glucose oligomers from which the retention times of the individual glycans were converted to glucose units (GU).

Glycan peaks were analysed based on their elution positions and measured in glucose units, followed by the comparison with the reference values in the "GlycoStore" database (https://glycostore.org/) for the structural assignment [18].

Data processing was performed manually, whereas each chromatogram was divided into 39 glycan peaks (GP1-GP39, Fig. 1). Each glycan peak was quantified as a percentage of the total integrated area. N-glycan structures pertaining to each GP are described in Appendix Table A.1. In addition to directly measured glycan traits, 16 derived traits that reveal changes in total activity of specific glycosidases and glycotransferases and represent glycans with a similar structural feature, such as galactosylation, fucosylation, bisecting GlcNAc, and sialylation,

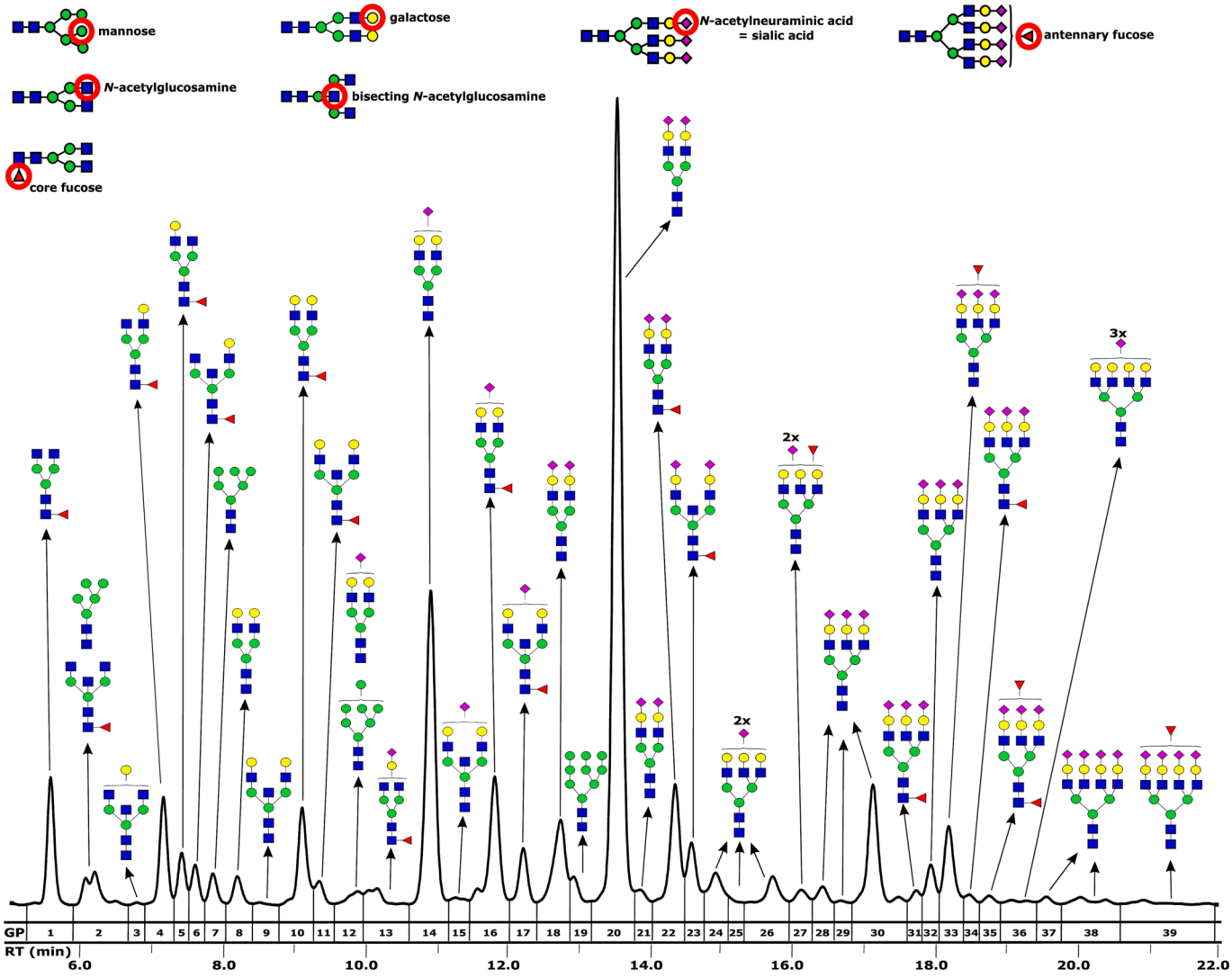

Fig. 1. HILIC-UPLC profile of the N-glycan structures released from the total serum glycoproteins. Only the most abundant glycoforms are shown for each glycan peak. GP: glycan peak; RT: retention time.

were also calculated for serum N-glycans (Appendix Table A.2).

2.5. Statistical analysis

Statistical analysis was performed in R programming language (build 4.1.3). UPLC N-glycan data were normalised by total chromatogram area and derived glycan traits were calculated as a sum of specific directly measured glycan traits. Analysis of the association between each N-glycan trait and T1DM was performed on the entire study cohort using general linear model. Log-transformed normalised glycan peak or derived trait abundance was defined as dependent variable while presence of T1DM was set as independent variable. Calculated coefficient represents natural logarithm of difference in glycan peak or derived trait abundancy in subjects with T1DM compared to controls. Correlations between N-glycan traits and glycaemic parameters, duration of the disease, complication status and smoking were also analysed by general linear model only for the subjects with T1DM. Log-transformed normalised glycan peak or derived trait abundance was defined as dependent variable. Independent variable was each additionally tested patient parameter along with age and sex to account for differences in their distribution among T1DM group. Calculated coefficient represents natural logarithm of change in glycan peak or derived trait abundancy for each unit or category used to describe the parameters. False discovery rate was controlled using the Benjamini–Hochberg method. P-value < 0.05 was considered significant.

Table 2
Associations of the directly measured and derived serum N-glycans with disease status, corrected for multiple testing. Significant N-glycan associations with T1DM are summarised in the column "In adults with T1DM" for comparison with significant N-glycan associations with T1DM observed in children at the onset reported by Rudman et al. [13].

Glycan trait	Description	β-coefficient	BH-adjusted p-value	In adults with T1DM	In children at T1DM onset [13]
GP1	FA2	−0.078	1.30×10^{-1}		
GP2	M5; FA2B	0.045	7.59×10^{-2}		Increased
GP3	A2BG1	0.042	4.13×10^{-1}		
GP4	FA2[6]G1	**−0.123**	$\mathbf{1.33 \times 10^{-3}}$	Decreased	Decreased
GP5	FA2[3]G1	**−0.191**	$\mathbf{2.53 \times 10^{-5}}$	Decreased	Decreased
GP6	FA2[6]BG1	−0.038	2.51×10^{-1}		
GP7	M6	**−0.183**	$\mathbf{1.69 \times 10^{-7}}$	Decreased	Increased
GP8	A2G2	**0.087**	$\mathbf{8.00 \times 10^{-7}}$	Increased	
GP9	A2BG2	0.056	5.04×10^{-2}		
GP10	FA2G2	−0.043	2.51×10^{-1}		Decreased
GP11	FA2BG2	−0.013	7.37×10^{-1}		
GP12	M7; A2G2S1	**0.131**	$\mathbf{1.14 \times 10^{-26}}$	Increased	Increased
GP13	FA2G1S1	**−0.139**	$\mathbf{2.69 \times 10^{-5}}$	Decreased	
GP14	A2G2S1	**0.042**	$\mathbf{7.77 \times 10^{-5}}$	Increased	
GP15	A2BG2S1	0.012	5.98×10^{-1}		
GP16	FA2G2S1	0.026	2.08×10^{-1}		
GP17	FA2BG2S1	0.007	8.25×10^{-1}		
GP18	A2G2S2	0.079	$\mathbf{1.50 \times 10^{-5}}$	Increased	
GP19	M9	−0.009	6.49×10^{-1}		
GP20	A2G2S2	−0.013	4.13×10^{-1}		
GP21	A2G2S2	**0.041**	$\mathbf{5.86 \times 10^{-3}}$	Increased	Increased
GP22	FA2G2S2	**0.080**	$\mathbf{3.62 \times 10^{-5}}$	Increased	Increased
GP23	FA2BG2S2	0.039	2.08×10^{-1}		Increased
GP24	A3G3S2	−0.043	5.87×10^{-2}		
GP25	A3G3S2	**0.137**	$\mathbf{1.21 \times 10^{-10}}$	Increased	Increased
GP26	A3G3S2	**−0.076**	$\mathbf{3.62 \times 10^{-5}}$	Decreased	
GP27	A3F1G3S2	**0.179**	$\mathbf{2.92 \times 10^{-7}}$	Increased	
GP28	A3G3S3	−0.010	7.35×10^{-1}		
GP29	A3G3S3	**0.117**	$\mathbf{4.49 \times 10^{-6}}$	Increased	Increased
GP30	A3G3S3	**−0.087**	$\mathbf{1.18 \times 10^{-4}}$	Decreased	
GP31	FA3G3S3	0.032	3.06×10^{-1}		
GP32	A3G3S3	**−0.130**	$\mathbf{1.78 \times 10^{-6}}$	Decreased	
GP33	A3F1G3S3	**0.137**	$\mathbf{6.02 \times 10^{-5}}$	Increased	
GP34	FA3G3S3	0.002	9.18×10^{-1}		
GP35	FA3F1G3S3	**0.194**	$\mathbf{7.72 \times 10^{-9}}$	Increased	
GP36	A4G4S3	0.008	7.35×10^{-1}		
GP37	A4G4S4	−0.009	7.69×10^{-1}		
GP38	A4G4S4	0.016	5.98×10^{-1}		
GP39	A4F1G4S4	**0.084**	$\mathbf{1.82 \times 10^{-2}}$	Increased	
LB	Low branching	0.002	5.70×10^{-1}		
HB	High branching	−0.006	6.60×10^{-1}		
G0	Agalactosylation	−0.032	4.98×10^{-1}		
G1	Monogalactosylation	**−0.123**	$\mathbf{1.32 \times 10^{-3}}$	Decreased	Decreased
G2	Digalactosylation	**0.020**	$\mathbf{2.26 \times 10^{-3}}$	Increased	
G3	Trigalactosylation	−0.011	4.98×10^{-1}		
G4	Tetragalactosylation	0.029	3.90×10^{-1}		
S0	Neutral glycans	−0.051	1.66×10^{-1}		
S1	Monosialylation	**0.034**	$\mathbf{4.43 \times 10^{-5}}$	Increased	
S2	Disialylation	0.008	4.98×10^{-1}		
S3	Trisialylation	−0.013	4.98×10^{-1}		
S4	Tetrasialylation	0.035	3.30×10^{-1}		
B	Bisecting GlcNAc	0.018	4.98×10^{-1}		Increased
AF	Antennary fucosylation	**0.140**	$\mathbf{4.15 \times 10^{-5}}$	Increased	
CF	Core fucosylation	−0.017	4.98×10^{-1}		
HM	Oligomannose	−0.020	1.66×10^{-1}		Increased

3. Results

3.1. N-glycosylation of total serum proteins in adults with type 1 diabetes mellitus

All N-glycan abundances for cases with T1DM and healthy controls are shown in Appendix Table A.3. We found that 19 directly measured N-glycan traits (GPs) and four derived glycosylation traits differed significantly between the studied groups as shown in Table 2.

Antennary fucosylation increased in adults with type 1 diabetes mellitus. The most prominent difference between the N-glycome of T1DM subjects and healthy controls was an increase in the derived trait of total antennary fucosylation with T1DM (coeff = 0.140, $P = 4.15 \times 10^{-5}$). This change was driven by an increase in the entirety of highly branched, complex antennary fucosylated structures found in serum N-glycome (GP27, GP33, GP35 and GP39). The single strongest association was found for GP35 (coeff = 0.194; $P = 7.72 \times 10^{-9}$), a triantennary and trisialylated structure with both core and antennary fucose (FA3F1G3S3).

Monogalactosylation decreased in adults with type 1 diabetes mellitus. Monogalactosylation trait (structures containing one galactose) was significantly lowered in adults with T1DM relative to healthy controls (coeff = −0.123; $P = 1.32 \times 10^{-3}$). This was mainly due to reduced levels of core-fucosylated, monogalactosylated glycans FA2[3]G1 (GP5) and FA2[6]G1 (GP4) as well as core-fucosylated, monogalactosylated and monosialylated glycan FA2G1S1 (GP13) in the group with T1DM.

Digalactosylation and monosialylation increased in adults with type 1 diabetes mellitus. Conversely, digalactosylation was increased in adults with T1DM (coeff = 0.020; $P = 2.26 \times 10^{-3}$) and was driven by an increase in asialylated digalactosylated GP8 (A2G2) as well as in mono- and disialylated, digalactosylated structures: GP12, GP14, GP18, GP21 and GP22. Monosialylation was also elevated (coeff = 0.034; $P = 4.43 \times 10^{-5}$) through an increase of A2G2S1 glycans (GP12 and GP14). Digalactosylated and disialylated GP21 (A2G2S2) and its core fucosylated variant GP22 (FA2G2S2) were increased as well in adults with T1DM.

Different isomeric behaviour in type 1 diabetes mellitus. Disialylated GP25 and GP26 (A3G3S2 isomers) were found to have inverse correlation with T1DM: GP25 was significantly increased while GP26 was decreased. Similarly, GP29 and GP30 (A3G3S3 isomers) had opposite correlation with T1DM; GP29 was elevated while GP30 decreased. Additionally, another A3G3S3 isomer, GP32, was decreased as well in T1DM.

3.2. Association of N-glycome with glycaemic regulation

Next, we assessed the connection between N-glycans and the following glycaemic parameters: HbA1c, fasting glucose levels and postprandial glucose levels. HbA1c was strongly correlated with 17 out of 39 directly measured glycan traits and with 6 out of 16 derived traits (Fig. 2). Conversely, postprandial and fasting glucose levels did not affect the N-glycoprofile of total serum proteins.

Higher HbA1c correlates with increased complexity of serum N-glycans. Higher HbA1c had negative correlation with simple, low-branched (coeff = −0.011; $P = 4.78 \times 10^{-9}$), monogalactosylated (coeff = −0.046; $P = 0.048$) and asialylated glycans (coeff = −0.034; $P = 0.048$). Conversely, positive correlation was observed with derived traits depicting total high-branched structures (coeff = 0.043; $P = 5.29 \times 10^{-8}$), trigalactosylated (coeff = 0.047; $P = 1.23 \times 10^{-8}$), and trisialylated structures (coeff = 0.047; $P = 1.69 \times 10^{-7}$). Association of all N-glycan traits with poor glycaemic control is shown in Appendix Table A.4.

Derived trait depicting total high mannose glycans did not reach statistical significance; however, positive association with HbA1c was observed for highly mannosylated GP19 (Man9).

Isomers of A3G3S2 structure (GP24, GP25 and GP26) did not have a uniform correlation with HbA1c. High HbA1c positively associated with GP24 and GP26 with, while negatively with GP25. Similar occurrence was observed for A3G3S3 isomers in which case GP28, GP30 and GP32

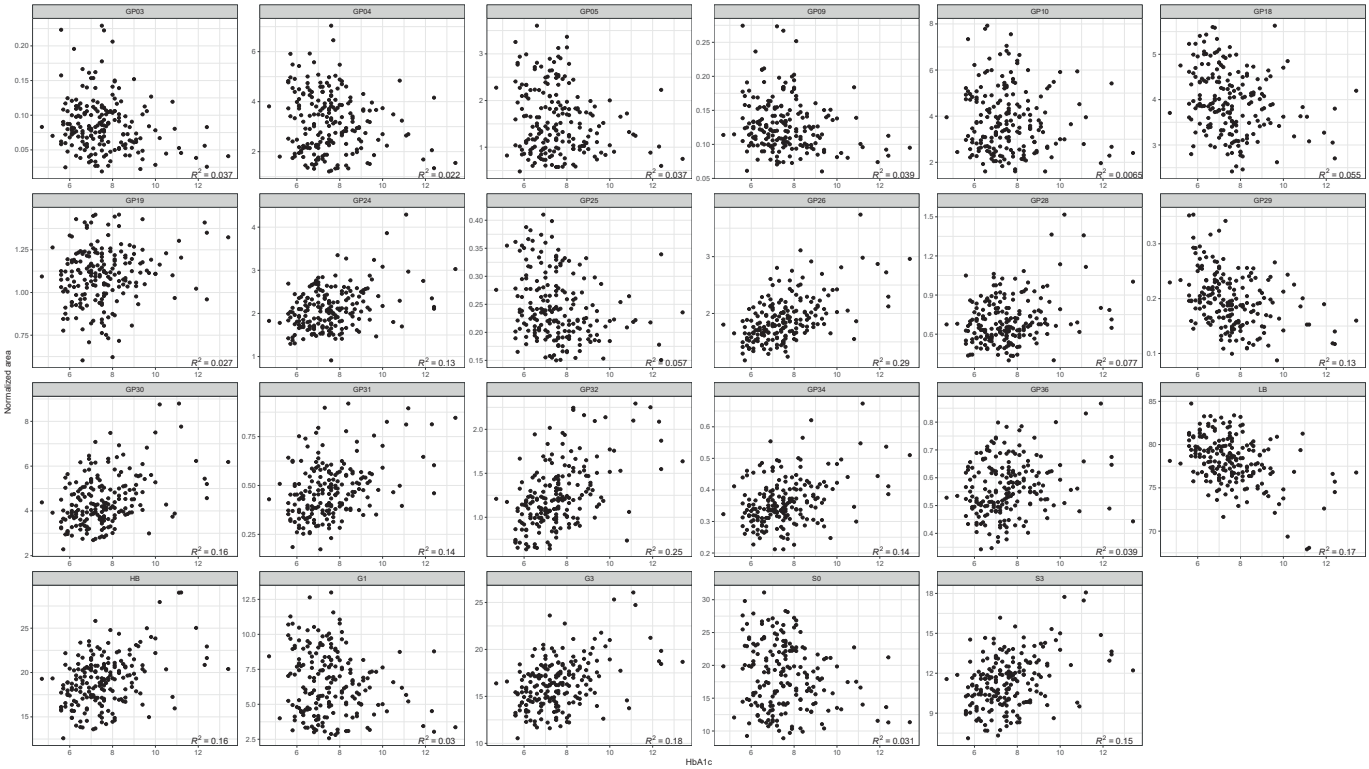

Fig. 2. Association of poor glycaemic control with relative abundance of serum N-glycans in adults with T1DM. Only significant results are shown ($p < 0.05$).

had positive correlation with high HbA1c, while GP29 had negative correlation.

GP29, on the other hand could be decreasing in people with high HbA1c on the account of its core-fucosylated form, GP31, which significantly increased with higher HbA1c values. Although no significant change was observed for derived trait depicting total core-fucosylation (adjusted P value = 0.066), some simple core-fucosylated glycans (GP4, GP5 and GP10) were negatively associated and conversely some complex trigalactosylated and trisialylated glycans (GP31 and GP34) bearing core fucose were positively associated with HbA1c.

3.3. *N-glycosylation of serum proteins in patients with different status of complications, effect of disease duration and smoking*

N-glycome in T1DM is not further influenced by disease complications. Further, we investigated the association of total serum protein N-glycosylation with the status of T1DM clinical complications. For association of N-glycans with diabetic complications, participants were divided into categories based on the severity of their complications (albuminuria, retinopathy, and hypertension; detailed in Section 2.1. Subjects). In our study, N-glycans did not correlate with diabetic complications. After correction for multiple testing, none of the directly measured, nor the derived glycan traits reached levels of significance (data shown in Appendix Tables A.5a–c).

N-glycosylation of serum proteins does not change with the

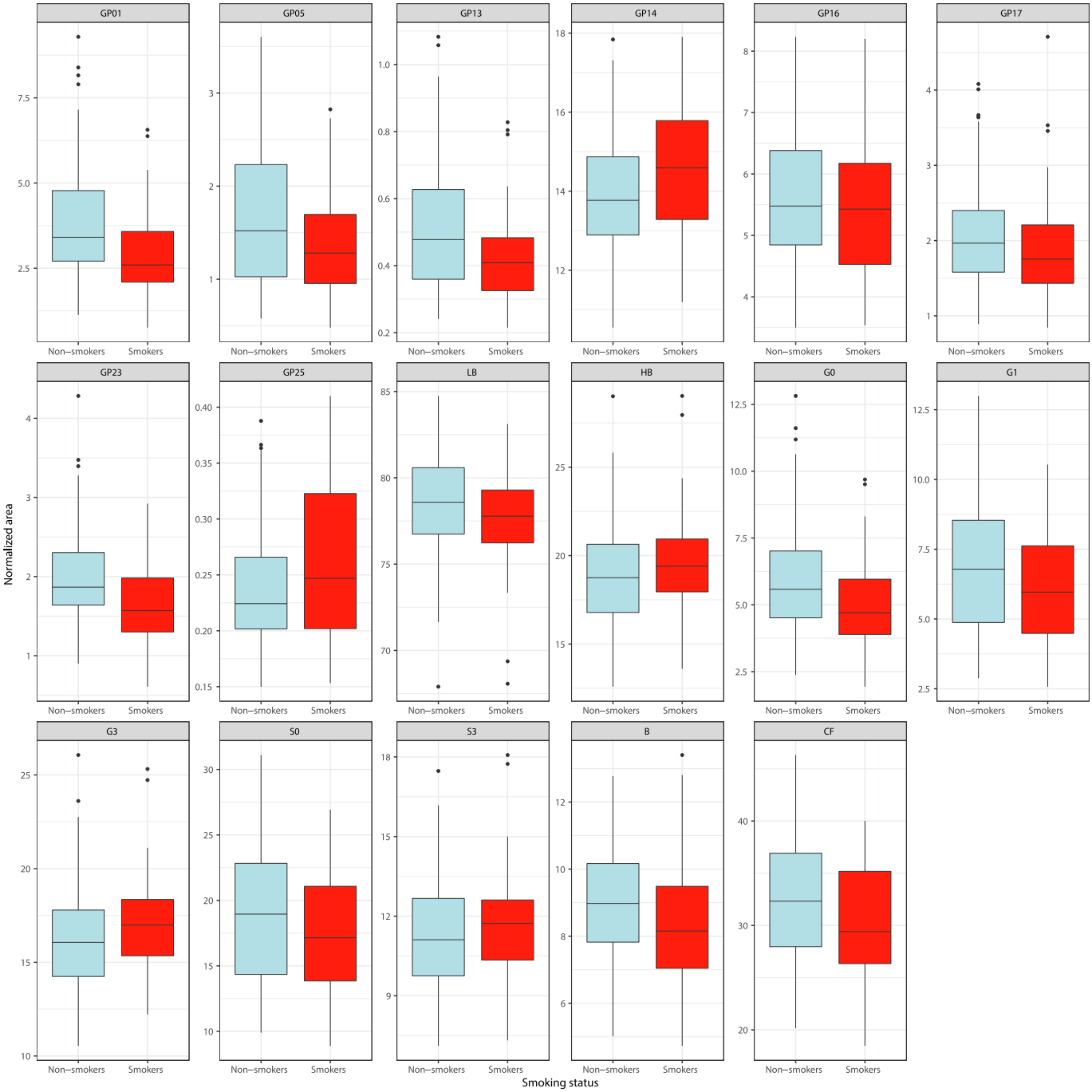

Fig. 3. Abundance of serum protein N-glycan traits in adults with T1DM divided into smokers and non-smokers. Only the significant changes are shown ($p < 0.05$).

duration of T1DM. Next, we examined the correlation of the disease duration with glycan traits. We did not identify any significant correlation between the duration of the disease and glycans, even though our study included individuals from 18 to 70 years of age with the duration of the disease between 1 and 47 years. Glycan traits that lost significance after p-value correction are shown in Appendix Table A.6.

Smoking affects serum N-glycome in subjects with type 1 diabetes mellitus. Herein, N-glycoprofiles of smokers had a significantly higher abundance of more complex, highly branched glycoforms, and, conversely, a lower abundance of simpler glycan structures in comparison to non-smokers. Significant associations of smoking habits with N-glycans are summarised in Fig. 3. Reduced levels of agalactosylated (coeff = −0.142; $P = 0.035$), monogalactosylated (coeff = −0.142; $P = 0.035$), asialylated (coeff = −0.106; $P = 0.036$), core fucosylated (coeff = −0.099; $P = 0.011$) structures and structures carrying bisecting GlcNAc (coeff = −0.093; $P = 0.024$) were observed. On the other hand, elevated levels of more complex trigalactosylated (coeff = 0.062; $P = 0.035$) and trisialylated (coeff = 0.061; $P = 0.043$) glycans were found.

4. Discussion

N-glycoprofiling was performed on total serum proteins from 200 adult T1DM subjects and 298 matching controls. Relative N-glycan levels were compared between subjects with diabetes and healthy controls and observed differences that reached the statistical significance were compared with the results of our previous study in children and adolescents at the onset of the disease [13] to identify similarities and differences in glycan behaviour (Table 2). Additionally, we examined the correlation between N-glycans and poor glycaemic control, complication status, disease duration, and smoking habits in T1DM.

4.1. Behaviour of N-glycan traits in adults and children with T1DM

When compared to the plasma protein N-glycome in children with T1DM, seven directly measured glycan traits and one derived trait (reduced monogalactosylation) remained significant from childhood to adulthood T1DM; twelve directly measured and three derived traits were observed to be new associations in adults; while four directly measured and two derived traits that were relevant in children, were not significant in adults.

The most prominent change associated with T1DM we observed in adults was an increase in the derived trait depicting total antennary fucosylation (or α1,3-fucosylation) of serum protein N-glycans. Increased rates of α1,3-fucosylation of tri-antennary and tetra-antennary glycans have previously been reported in sera of people with chronic inflammation [19], whereas the main carrier of α3-fucosylated structures in human serum was identified as AGP [20]. AGP is an acute phase protein which is produced and glycosylated in the liver and studies have shown that plasma concentrations of AGP do not differ significantly between adult subjects with diabetes and healthy individuals [14,21]. Although we did not measure serum AGP concentrations, these findings would suggest the increase in antennary fucosylation was indeed a consequence of changes in glycosylation and not caused by protein level disruption. Increased α3-fucosylation of AGP has previously been reported in individuals with T1DM relative to healthy controls, albeit in a lectin assay study [14]. Moreover, in a recent study we compared antennary fucosylation between diabetes types and showed that subjects with T1DM tend to have the highest levels of antennary fucosylation of AGP glycopeptides [15]. Even though this was the most prominent change observed in our study, none of these findings, including any of the directly measured glycan traits bearing antennary fucose, were reported in children at the onset of the disease [13] suggesting this change was not associated with islet autoimmunity at the onset of T1DM but could be a consequence of abnormal metabolic processes over the long term of T1DM.

Derived trait depicting monogalactosylated structures was negatively associated with T1DM in adult subjects. This change was mainly driven by reduced levels of the two structures (FA2[3]G1 and FA2[6]G1) originating entirely from serum IgG [20] and by FA2G1S1 glycan. We previously reported a similar finding of decreased monogalactosylation as well as both FA2[3]G1 and FA2[6]G1 structures in newly diagnosed T1DM children [13], however, a change in FA2G1S1 was not observed in their case. Interestingly, in our previous study of new-onset T1DM children two unaffected siblings with very low monogalactosylation (below Q1) were later diagnosed with T1DM. The persistence of decreased monogalactosylation may be mediated by genetic factors contributing to autoimmune disease susceptibility.

Conversely, digalactosylation was increased in adults with T1DM, driven by an increase in asialylated digalactosylated, and mono- and disialylated, digalactosylated structures, while monosialylation was also elevated through an increase of A2G2S1 glycans. A2G2S1 is a glycan found on several serum glycoproteins and most abundantly on alpha-1B-glycoprotein, haptoglobin, and IgA, respectively [20]. All of these proteins are reported to have a role in T1DM or are disrupted in people with the disease: levels of alpha-1B-glycoprotein are positively associated with poor glycaemic control and T1DM [22]; haptoglobin has a well-known role in susceptibility to diabetic cardiovascular diseases [23]; IgA has disrupted catabolism in T1DM [24]. Associations of these two derived traits are newly reported for T1DM and were not previously reported in children [13].

Digalactosylated and disialylated GP21 (A2G2S2) and its core fucosylated variant GP22 (FA2G2S2) were increased in both children and adults with T1DM. These structures are attributed to a range of glycoproteins, while by far most abundant are alpha-1-antitrypsin and hemopexin [20]. Alpha-1-antitrypsin has an established anti-inflammatory and immunomodulatory effects in chronic obstructive pulmonary disease [25] but increasing evidence is arising of its role in other inflammatory and immune-mediated conditions, such as diabetes [26] where efforts are made in discovering its therapeutic potential [27,28]. Hemopexin has also been shown to be up-regulated in plasma of T1DM subjects and its expression is linked to glucose-induced oxidative stress [29].

An increase in digalactosylated, monosialylated GP14 (A2G2S1) was not observed in children, but it should be noted that the N-glycome analysis on children was performed on plasma samples while the current study was done on serum samples. Although these two N-glycomes are comparable, some differences exist, mainly in structures originating from fibrinogen found in GP14 and GP20 [30]. These are A2G2S1 and A2G2S2, respectively, and they represent the most dominant structures attached to fibrinogen [30]; while these are also found on a number of different proteins in plasma, fibrinogen N-glycans account for a significant portion in plasma N-glycome, especially for GP14. The assessment of comparability of these glycan changes requires additional investigation.

GP12, a glycan trait which was increased in both children and adults with T1DM, implies two structures: oligomannose Man7 and monosialylated A2G2S1 glycan. Man7 in human serum is mostly derived from immunoglobulin E [20], but digalactosylated and monosialylated A2G2S1 structure stems from multiple proteins mentioned above. It is somewhat difficult to assess whether the increase in GP12 is driven by the same glycan both in the case of children and adults, since in the study on children, the derived trait depicting total mannosylation of plasma proteins was significantly increased, and was driven by an increase in GP2, GP7, and GP12 (Man5, Man6, and Man7, respectively) [13]. Herein, no significant change was observed for the derived trait describing highly mannosylated glycans. Recently, we also showed that highly mannosylated N-glycan structures on complement component C3 increase in children at the onset of T1DM [31]. In contrast with the finding observed in children, GP7 (Man6) was significantly decreased in adults with T1DM. Man6 glycan is mostly found on immunoglobulin M [20], which is a type of antibody that functions as a ligand for siglec-2 [32], an Ig-like lectin present on immune cells and insulin producing

pancreatic β-cells [33]. Although this result is in contrast to our findings in children, the opposite effect for this association could be explained by studies showing IgM concentration is increased at the onset of T1DM [34], and decreased in adults with the disease [35].

In our previous intra-family study of recent-onset T1DM children and adolescents, N-glycans with bisecting GlcNAc were significantly increased in the T1DM group in comparison with their healthy siblings and novel genetic variants that were not previously reported for the general European population contributing to these changes were identified [13,17]. The novel associations were found between genetic variants close to candidate gene *MGAT3* encoding *N*-acetylglucosaminyltransferase (bisecting GlcNAc transferase) and two IgG-bound glycans with bisecting GlcNAc – FA2BG2S2 and FA2BG2 [17]. These glycan changes were not shown herein to reflect differential glycaemic control (see section below); however, neither to persist into adulthood. In this respect, these glycans may be specifically important for processes at the onset or be important indicators of that disease process.

Di- and trisialylated N-glycan structures such as A3G3S2 and A3G3S3 are present in multiple glycan peaks of the chromatogram in different isomeric forms, differing in the type of sialic acid linkage. Herein sialic acid linkage preference toward particular glycans has been observed. Different isomers of disialylated A3G3S2 glycan were found to have inverse correlation with T1DM (GP25 increased and GP26 decreased). Alternatively, GP26 could be decreased due to the increase of its antennary fucosylated variant, GP27. While A3G3S2 could be attributed to various proteins, it was also reported to exist as an incomplete glycoform on AGP [20]. Similarly, A3G3S3 isomers had opposite correlation with T1DM (GP29 increased and GP30 decreased). Additionally, another A3G3S3 isomer, GP32, was decreased as well in T1DM; however, this could be due to increase in its antennary fucosylated variant (GP33). A3G3S3 is found on a number of different serum glycoproteins, most abundantly on AGP [20]. Interestingly, glycans GP25 and GP29 were also elevated in children with T1DM, but a decrease in their isomers (GP26 and GP30) was not observed [13]. These findings could indicate a sialic-acid linkage preference in adults with T1DM.

4.2. N-glycome and glycaemic regulation

In addition, we assessed associations between glycaemic parameters (HbA1c, fasting glucose levels and postprandial glucose levels) and N-glycoprofile of total serum proteins in adults with T1DM. The effect of HbA1c was strongly reflected in the N-glycome, while on the other hand no correlation was observed between postprandial and fasting glucose levels, and N-glycans of total serum proteins. Similar findings were also reported by Bermingham et al. [12].

The most prominent changes associated with high HbA1c can be summarized as significant increase in high-branched structures, bearing multiple galactose and sialic acid residues, meaning that poor glycaemic control is marked by increased complexity of the serum N-glycome. Consequently, low-branched glycans have a lower abundance in people with poor glycaemic control. This is partly congruent with previous studies where high HbA1c was associated with a lower relative abundance of simple biantennary N-glycans and a higher relative abundance of more complex structures with more branching, galactosylation, and sialylation [12,36]. Herein, a negative association of core-fucosylated monogalactosylated GP4 and GP5 (FA2G1) with high HbA1c was observed. These glycans negatively correlated with T1DM in both this study of adults with T1DM and in our previous study of children at the onset of T1DM [13], as well as with poor glycaemic control [12]; thus their real individual value in risk assessment should be evaluated after correcting for glycaemic differences.

Derived trait depicting total high mannose glycans did not reach statistical significance; however, positive association with HbA1c was observed for highly mannosylated GP19 (Man9). Clerc et al. reported that Man9 mostly derives from apolipoprotein B-100 (ApoB-100) and to a much lesser degree from immunoglobulin D [20]. It has also been reported that concentration of ApoB-100 tends to increase with poor glycemic control, hence this difference could be explained by an increase in relative concentrations of ApoB-100 [37,38].

Similarly as with T1DM, herein we observed different isomeric behaviour of A3G3S2 and A3G3S3 glycans with HbA1c. Interestingly, this linkage preference was inverse when associating glycans with T1DM: GP25 increased in T1DM and GP26 decreased, while their associations were opposite with HbA1c. Similar occurrence was observed for trigalactosylated and trisialylated A3G3S3 glycans: GP29 had negative correlation, while GP30 had positive correlation with HbA1c. In comparison with T1DM N-glycan associations, elevated levels of GP29 and reduced levels of GP30 were observed. This could indicate a preference for a sialic-acid linkage type in individuals with poor glycaemic control, suggesting an increased activity of specific sialyltransferases. Bias toward α2,6-linked sialylation was previously observed in type 2 diabetes [39] suggesting a common long-term response to elevated blood glucose levels.

Positive association of HbA1c with highly branched structures could also be explained by increased influx of glucose into the hexosamine biosynthesis pathway due to hyperglycaemia, thus impacting enzyme kinetics and the availability of the donor substrate for N-glycan branching, as already hypothesised by Bermingham et al. [12].

4.3. The effect of diabetic complications, disease duration and smoking on N-glycosylation in type 1 diabetes mellitus

While diabetic complications are often correlated with high HbA1c [40], the effect of which is shown to be strongly reflected in the N-glycome, N-glycans seemed not to correlate with the complication status in our study. In a separate study on diabetic retinopathy, an increase in the amount of glycans in vitreous fluid, particularly an increase in sialylated glycans was observed [16]. It has also been reported that *in vitro* high glucose stimulation of human retinal microvascular endothelial cells causes upregulation of sialyltransferases ST3GAL1 and ST3GAL4, indicating a local response to high systemic glucose levels [16]. Bermingham et al. reported association of N-glycome changes with diabetic kidney disease, specifically correlation of more complex N-glycans with a higher albumin-to-creatinine ratio and with a steeper decline in estimated glomerular filtration rate [12]. Poland et al. on the other hand observed an increase in AGP antennary fucosylation relative to increased urinary albumin excretion in T1DM [14]. None of the above-mentioned was corroborated in this study; however, this could be due to the study limitations: data categorisation and majority of cases having mild or moderate complications. In addition, we assessed the effect of the disease duration on serum N-glycoprofile, however, none of the glycosylation traits exhibited significant associations.

Together with poor glycaemic control, smoking contributes to a risk of developing diabetic complications. The fact that smoking induces changes in the glycosylation pattern of various glycoproteins is known from previous studies [41,42]. In our study, we observed a shift from simple towards complex, highly branched glycans, as well as changes in total core-fucosylation and glycans bearing bisecting GlcNAc in N-glycome of smokers. These changes in the serum N-glycome showed an inflammation-like pattern — the increase in high branched structures, highly galactosylated and highly sialylated glycans suggesting that smoking might lead to inflammation [43,44]. Our findings somewhat coincide with the previous studies [41,42,45], however, some differences appear to be present. An increase in antennary fucosylation was not observed, which was reported in other studies, and conversely, a decrease in structures bearing bisecting GlcNAc was observed, whereas the opposite association was reported previously, albeit for IgG glycans [45]. The observed differences could be a consequence of diabetes-smoking interaction, but this would require further investigation and replication. However, some novel associations were revealed herein,

which could help to elucidate the exact mechanisms of studied pathophysiological changes.

5. Conclusions

In summary, this study investigated the associations between N-glycans and T1DM in adults and evaluated the results in light of our previous findings in children at the onset of T1DM.

Our findings revealed that a decrease in monogalactosylation and changes in seven directly measured glycans were common to both children and adults, which may suggest a potential connection to genetic factors contributing to the autoimmune disease susceptibility. We also discovered changes in N-glycans that are specific to adults with T1DM, such as increased digalactosylation, monosialylation, and antennary fucosylation, which may reflect altered glucose metabolism and concomitant inflammation in adults with T1DM. Furthermore, certain changes in the N-glycome seem to be unique to children at T1DM onset, including an increase in oligomannose and glycans bearing bisecting GlcNAc. These changes were recently associated with newly reported genetic variants in T1DM genetic association studies, supporting their relevance in the disease onset and suggesting potential targets for intervention or monitoring of early disease progression.

We also found that N-glycome does not appear to be further influenced by the duration of the disease in adults, and that diabetic complications do not appear to contribute to changes in N-glycome. However, it is worth noting that this conclusion is based on our study design in which cases were categorised by the severity of the diabetic complication, and the majority had mild or moderate complications.

Our analysis of N-glycans in correlation with glycaemic control supports previous studies that have shown increased complexity in N-glycome. Additionally, we found that smoking in T1DM resulted in inflammation-related N-glycan changes, somewhat different from the findings reported in the general population.

To conclude, this study provides a more comprehensive portrayal of glycan changes that might be specific indicators or distinctively relevant for processes at the onset of T1DM and those occurring after T1DM establishment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon request.

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Contribution. OG, GM, and LD designed the research study. TB, MTo, BP, SVR, MVL and LD acquired the samples and the participants' data. MN performed the experiments, MT performed the statistical analysis and MN, OG, and NR analysed the data. MN and OG drafted the manuscript, and all authors edited the final version of the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary material

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