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ARTICLE

Increased plasma N-glycome complexity is associated with higher risk of type 2 diabetes

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Abstract
Aims/hypothesis Better understanding of type 2 diabetes and its prevention is a pressing need. Changes in human plasma N-glycome are associated with many diseases and represent promising diagnostic and prognostic biomarkers. Variations in glucose metabolism directly affect glycosylation through the hexosamine pathway but studies of plasma glycome in type 2 diabetes are scarce. The aim of this study was to determine whether plasma protein N-glycome is changed in individuals who are at greater risk of developing type 2 diabetes.
Methods Using a chromatographic approach, we analysed N-linked glycans from plasma proteins in two populations comprising individuals with registered hyperglycaemia during critical illness (increased risk for development of type 2 diabetes) and individuals who stayed normoglycaemic during the same condition: AcuteInflammation (59 cases vs 49 controls) and AcuteInflammation Replication (52 cases vs 14 controls) populations. N-glycome was also studied in individuals from FinRisk (37 incident cases of type 2 diabetes collected at baseline vs 37 controls), Orkney Complex Disease Study (ORCADES; 94 individuals with HbA_{1c} > 6.5% [47.5 mmol/mol] vs 658 controls) and Southall and Brent Revisited (SABRE) cohort studies (307 individuals with HbA_{1c} > 6.5% [47.5 mmol/mol] vs 307 controls).
Results Individuals with increased risk for diabetes type 2 development (AcuteInflammation and AcuteInflammation Replication populations), incident cases of type 2 diabetes collected at baseline (FinRisk population) and individuals with elevated HbA_{1c} (ORCADES and SABRE populations) all presented with increased branching, galactosylation and sialylation of plasma protein N-glycans and these changes were of similar magnitude.
Conclusions/interpretation Increased complexity of plasma N-glycan structures is associated with higher risk of developing type 2 diabetes and poorer regulation of blood glucose levels. Although further research is needed, this finding could offer a potential new approach for improvement in prevention of diabetes and its complications.

Keywords Diabetes predisposition · Hyperglycaemia · N-linked glycans · Plasma N-glycome · Type 2 diabetes

Abbreviations

2-AB	2-Aminobenzamide
ACN	Acetonitrile
BEH	Bridged ethylene hybrid

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HILIC Hydrophilic interaction liquid chromatography
ICU Intensive care unit
UPLC Ultra-performance liquid chromatography

Introduction

Type 2 diabetes represents one of the major public health challenges, with a constantly growing prevalence and a global projection of 7.3% for the year 2025 [1]. Intensive research during recent decades has resulted in the introduction of new oral and parenteral medications that may improve the management of the disease. However, recognising individuals at increased risk of developing type 2 diabetes is of equal importance, since many potential interventions are available that can postpone and maybe even prevent the disease onset.

It was shown recently that individuals without a history of diabetes but who developed hyperglycaemia (plasma glucose > 7.7 mmol/l) during critical illness, were at significantly increased risk of developing type 2 diabetes [2, 3]. The RR of type 2 diabetes development during 5 years after the acute illness was 5.6 (95% CI 3.1, 10.2). This phenomenon probably arises because people who develop hyperglycaemia in critical illness already have a latent disorder in glycaemic control. This disorder is not detected by the usual screening tests but manifests due to inflammatory and stress mediators generated during severe acute illness. Individuals without this latent disorder manage to overcome the hyperglycaemic effects of stress and inflammation and remain normoglycaemic. After the acute illness subsides, individuals who presented with hyperglycaemia become normoglycaemic again but the underlying disorder in glucose metabolism apparently makes them prone to type 2 diabetes development.

Glycosylation is the most frequent co- and post-translational modification of proteins, modulating their function in many cases [4]. Unlike glycation (a non-enzymatic chemical reaction), this highly complex enzymatic process is strictly regulated by a network of many enzymes, transcriptional factors, sugar nucleotides and other molecules [5]. Although proteins possess well-defined glycosylation sites, a great heterogeneity exists among their glycans. Human plasma N-glycome is quite stable within the individual [6] but is extremely sensitive to pathophysiological processes, reflecting the cell state from the time of protein secretion [7]. The main diagnostic deficit of glycans is in their low specificity; thus, they are more frequently studied as prognostic and stratification markers. Their great variability within populations and the significant heritability rate gives them great potential in risk assessments. Changes in the glycosylation of plasma proteins have been studied in many different diseases, including type 2 diabetes mellitus [8]. These changes have been confirmed in both individuals with diabetes and *db/db* mice [9, 10]. Although many studies identified biantennary glycans with core fucose as structures of main interest, differences between populations with and without diabetes were never pronounced enough to have a diagnostic potential, probably because of relatively good glycaemic control in those with diabetes, administration of medicaments or the existence of many different molecular mechanisms that lead to type 2 diabetes development.

The aim of this study was to determine whether plasma protein N-glycome is changed in individuals who are at greater risk of developing type 2 diabetes, using three different populations: two populations including individuals with registered hyperglycaemia in critical illness (increased risk for development of type 2 diabetes) and individuals with normoglycaemia in critical illness (AcuteInflammation and AcuteInflammation Replication populations); individuals who developed type 2 diabetes during the 10 years follow-up and those who remained normoglycaemic (FinRisk population).

To further confirm the connection of changes in N-glycome with glucose metabolism disruption, we used data from our previous plasma protein glycosylation analysis in two other cohorts (Orkney Complex Disease Study [ORCADES] and Southall and Brent Revisited [SABRE]) where we separated age- and sex-matched individuals into two subgroups differing in HbA_{1c} status (HbA_{1c} < 6.5% [47.5 mmol/mol] and HbA_{1c} > 6.5% [47.5 mmol/mol]).

Methods

Individuals with and without hyperglycaemia during critical illness

AcuteInflammation population We included individuals admitted to the medical intensive care unit (ICU) at the University Hospital Centre Zagreb during a period of 6 months (February to July 2013). Adults (aged > 18 years old) with negative history of diabetes who were admitted to the ICU and discharged from the hospital alive were eligible for inclusion. We excluded individuals diagnosed with diabetes or impaired glucose tolerance and/or impaired fasting glucose before or during hospitalisation, those with documented gestational diabetes, pregnant women and individuals taking glucocorticoids during or 3 months before the admission. Informed consent was obtained from participants by a member of the study team at the discharge from the ICU or the hospital. Consenting participants were asked to attend a follow-up appointment, 6–8 weeks after the hospital discharge. At this visit, inclusion/exclusion criteria were confirmed. Complete blood count and C-reactive protein levels were determined to exclude any persisting inflammatory process. Individuals with elevated markers of inflammation were retested after 2 weeks. All participants underwent an OGTT and measurement of HbA_{1c} to identify pre-existing diabetes. ADA criteria for diagnosis of diabetes were employed and any individual diagnosed with

existing diabetes or with impaired glucose tolerance was excluded. Height, weight and BMI were recorded and family history of diabetes was documented. For all participants, fasting blood samples for N-glycan profiling were collected in tubes containing anticoagulants (both EDTA and citrate for samples from each individual), plasma was separated immediately and stored at − 20°C until analysis. For each individual, samples were analysed in duplicate. In total, 108 participants were enrolled in the study.

AcuteInflammation Replication population All cardiac surgical patients operated on at the University Hospital Centre Zagreb (cardiac surgical procedures employing cardiopulmonary bypass) from October 2010 to February 2011 and discharged from the hospital alive were screened for the study. All individuals provided written informed consent. We excluded individuals diagnosed with diabetes or impaired glucose tolerance and/or impaired fasting glucose before or during hospitalisation. In total, 66 individuals were enrolled in the study and from all of them blood was drawn on the day of the surgery, before the surgical procedure. The blood samples were collected in tubes containing EDTA; plasma was separated and stored at − 20°C until analysis.

Relevant demographic data for both populations are summarised in Table 1.

Individuals who did or did not develop type 2 diabetes within 10 years from sampling (FinRisk study)

Plasma samples, stored at − 70°C, collected through a study designed to investigate risk factors in a population from Finland [11] were used. Population data were searched for individuals with incident type 2 diabetes after 10 years follow-up. Individuals who developed any autoimmune, malignant or other chronic disease were excluded. Thirty-seven individuals (aged 60 years [minimum–maximum; 34–72]), who developed type 2 diabetes and no other chronic disease, were identified and included in this study. Thirty-seven age- and sex-matched individuals (aged 61 years [35–73]) who remained healthy during the follow-up period were selected as a control group from the same population.

Individuals with different HbA_{1c} status (ORCADES and SABRE studies)

Plasma samples from the ORCADES, collected in the Scottish islands of Orkney, between 2005 and 2011 [12], as well as from the SABRE population were used [13]. Data on age, sex and HbA_{1c} levels were included in the study. Glycan data for these populations had been obtained for another purpose, but were used in this study to compare the N-glycome of individuals with $HbA_{1c} < 6.5\%$ (47.5 mmol/mol) and $HbA_{1c} > 6.5\%$ (47.5 mmol/mol). From the Orkney population, glycan and HbA_{1c} data from 752 individuals (aged 18–100 years) from a subgroup of ten islands were used for this analysis. Consecutively, 658 individuals (aged 63 years [18–97]) were identified as having $HbA_{1c} < 6.5\%$ (47.5 mmol/mol) and 94 (aged 63 years [19–88]) as having $HbA_{1c} > 6.5\%$ (47.5 mmol/mol). From the SABRE population, data from 307 individuals (aged 70 years [60–84]) with $HbA_{1c} < 6.5\%$ (47.5 mmol/mol) and 307 (aged 70 years [60–84]) with $HbA_{1c} > 6.5\%$ (47.5 mmol/mol) were included.

Ethics

All studies are supported by written informed consent from all individuals and approvals from eligible local Ethics Committees.

Glycan analysis

Glycan release and labelling Prior to analysis, all samples (cases and controls) were randomised throughout the multiwell plates. For each plate, four standards (pool of plasma samples) were added to minimise the experimental error. Each plasma sample (10 μl) was denatured by incubation with 20 μl SDS (2%wt/vol.; Invitrogen, Carlsbad, CA, USA) at 65°C for 10 min. Subsequently, 10 μl of 4% Igepal-CA630 (Sigma-Aldrich, St Louis, MO, USA) and 1.25 mU PNGase F

Table 1 Demographic characteristics of the AcuteInflammation and AcuteInflammation Replication populations

Characteristic	All participants		HG during acute illness		No HG during acute illness		*p* value (HG vs no HG)[a]	
Population	AI	AI_R	AI	AI_R	AI	AI_R	AI	AI_R
No. of participants (*N*)	108	66	59	52	49	14		
Age, median (min–max), years	55 (18–79)	67 (21–81)	58 (25–79)	68 (41–81)	54 (18–75)	62 (21–78)	0.388	0.121
Female sex, *n* (%)	32 (29.6)	19 (28.8)	18 (30.5)	15 (28.8)	14 (28.6)	4 (28.6)	0.826	0.984
BMI ± SD, kg/m^2	23.7 ± 4.0	28.2 ± 3.5	24.2 ± 4.1	28.5 ± 3.4	23.1 ± 3.9	27.2 ± 3.8	0.734	0.342
Family history of diabetes, *n* (%)	27 (25.0)	NA	19 (32.2)	NA	8 (16.3)	NA	0.039	NA

[a] *p* values were calculated using Mann–Whitney *U* test (significance level $\alpha = 0.05$) and for categorical variables χ^2 test was used

AI, AcuteInflammation; AI_R, AcuteInflammation Replication; HG, hyperglycaemia

(ProZyme, Hayward, CA, USA) in 10 μl 5× PBS were added. The samples were incubated overnight at 37°C to allow release of N-glycans. The released N-glycans were labelled with 2-aminobenzamide (2-AB). The labelling mixture was freshly prepared by dissolving 2-AB (19.2 mg/ml; Sigma-Aldrich) and 2-picoline borane (44.8 mg/ml; Sigma-Aldrich) in a mixture of DMSO (Sigma-Aldrich) and glacial acetic acid (Merck, Darmstadt, Germany) (70:30, vol./vol.). Labelling mixture (25 μl) was added to each N-glycan sample in the 96-well plate, which was then sealed using adhesive seal. Mixing was achieved by shaking for 10 min, followed by incubation at 65°C for 2 h. To each sample (75 μl), 300 μl of acetonitrile (ACN) (J. T. Baker, Phillipsburg, NJ, USA) was added to give a final concentration of 80% vol./vol. Free label and reducing agent were removed from the samples using hydrophilic interaction liquid chromatography (HILIC)–solid-phase extraction. To each well of a 0.45 μm GHP filter plate (Pall Corporation, Ann Arbor, MI, USA), 200 μl of 0.1 g/ml suspension of microcrystalline cellulose (Merck) in water was added. Solvent was removed by application of vacuum using a vacuum manifold (Millipore Corporation, Billerica, MA, USA). All wells were prewashed using 5 × 200 μl of water, followed by equilibration using 3 × 200 μl of ACN/water (80:20, vol./vol.). The samples were loaded into the wells, which were subsequently washed seven times using 200 μl of ACN/water (80:20, vol./vol.). Glycans were eluted with 2 × 100 μl of water and combined eluates were stored at − 20°C until usage.

HILIC–UPLC Fluorescently labelled N-glycans were separated by HILIC on Waters Acquity ultra-performance liquid chromatography (UPLC) instrument (Waters, Milford, MA, USA) consisting of a quaternary solvent manager, sample manager and a fluorescence detector set with excitation and emission wavelengths of 250 nm and 428 nm, respectively. The instrument was under the control of Empower 2 software, build 2145 (Waters). Labelled N-glycans were separated on a Waters bridged ethylene hybrid (BEH) Glycan chromatography column, 150 × 2.1 mm, 1.7 μm BEH particles, with 100 mmol/l ammonium formate, pH 4.4, as solvent A and ACN as solvent B. The separation method used a linear gradient of 70–53% acetonitrile (vol./vol.) at flow rate of 0.561 ml/min in a 25 min analytical run. Samples were maintained at 5°C before injection and the separation temperature was 25°C. The system was calibrated using an external standard of hydrolysed and 2-AB-labelled glucose oligomers from which the retention times for the individual glycans were converted to glucose units. Data was processed using an automated method with a traditional integration algorithm after which each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The chromatograms were all separated in the same manner into 46 peaks (GP1–GP46) and the content of glycans in each peak was expressed as a percentage of the total integrated area (Fig. 1).

Statistical analysis

To remove experimental variation from measurements, normalisation and batch correction were performed on UPLC glycan data. Also, normalisation by total area was performed in which the area of each glycan peak was divided by the total area of the corresponding chromatogram. Prior to batch correction, normalised glycan measurements were $\log_{10}$-transformed due to right skewness of their distributions and the multiplicative nature of batch effects. Batch correction was performed on $\log_{10}$-transformed measurements using the ComBat method, in which the technical source of variation (which sample was analysed on which plate) was modelled as a batch covariate. To obtain measurements corrected for experimental noise, estimated batch effects were subtracted from $\log_{10}$-transformed measurements.

From the 46 directly measured glycan traits, 12 derived traits were calculated (Table 2). These derived traits average glycosylation features such as branching, galactosylation and sialylation across different individual glycan structures and, consequently, they are more closely related to individual enzymatic activity and underlying genetic polymorphism. As derived traits represent sums of directly measured glycans, they were calculated using normalised and batch-corrected glycan measurements after transformation to the proportions (exponential transformation of batch-corrected measurements).

Analyses of associations between clinical trait of interest (hyperglycaemia in AcuteInflammation and AcuteInflammation Replication, future onset of diabetes in FinRisk cohort and HbA_{1c} > 6.5% [47.5 mmol/mol] in ORCADES and SABRE cohorts) and glycan measurements were performed using a regression model with age and sex included as additional covariates. Prior to analyses, for each cohort separately, glycan variables were transformed to a standard normal distribution by inverse transformation of ranks to normality. Using rank-transformed variables makes estimated effects of different glycans in different cohorts comparable, as transformed glycan variables have the same standardised variance. The false discovery rate was controlled using the Benjamini–Hochberg procedure.

Data were analysed and visualised using R programming language (version 3.0.1, https://cran.rstudio.com/bin/windows/base/old/).

Results

Analysis of plasma N-glycome composition in the AcuteInflammation and AcuteInflammation Replication populations

For each participant, N-glycans were released from plasma proteins, fluorescently labelled and analysed by HILIC–

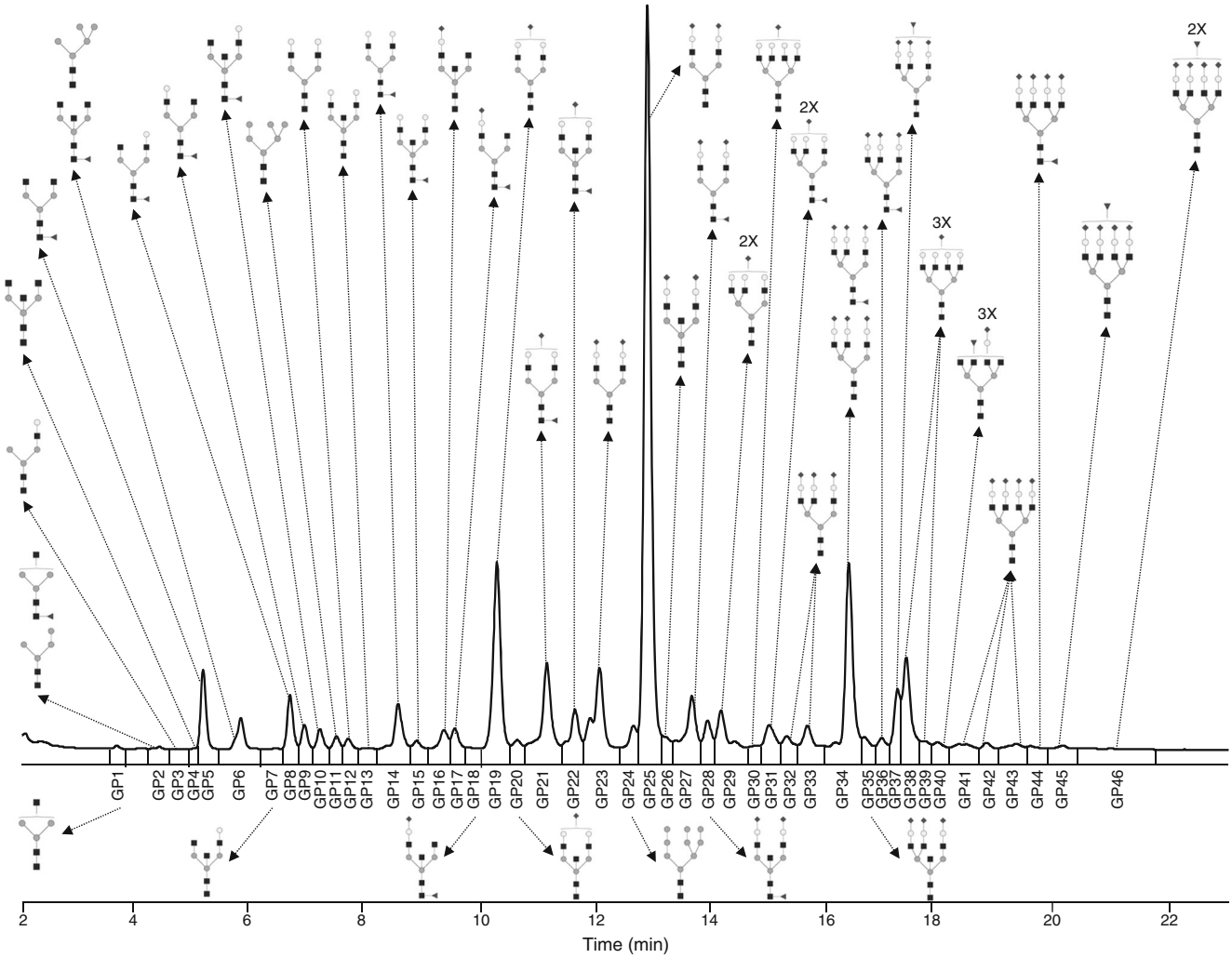

Fig. 1 HILIC–UPLC profile of N-linked glycans released from plasma proteins and fluorescently labelled with 2-AB. The glycans presented are the most abundant structures in each peak. Squares, *N*-acetylglucosamine; triangles, fucose; dark grey circles, mannose; light grey circles, galactose; diamonds, *N*-acetylneuraminic acid

UPLC, a method demonstrated to be the best approach for reliable and reproducible quantitative glycan analysis [14]. By this method, plasma glycome is separated into 46 chromatographic peaks, each containing a group of similar glycan structures. The individual glycan structures contained in each peak were characterised previously [15] and a representative chromatogram with the main structure present in each peak is shown in Fig. 1.

Next, we quantified each chromatographic peak (as a percentage of the total N-glycome) and calculated derived traits (Table 2). These derived traits represent common biologically meaningful features (glycan branching, galactosylation and sialylation) shared among several measured glycans. Then we compared these traits between two groups of individuals in the AcuteInflammation and AcuteInflammation Replication populations. Within the AcuteInflammation population, we compared those who developed hyperglycaemia during ICU admission due to acute condition and those who remained normoglycaemic during the same circumstances (Table 1). For the analysis, plasma samples taken 6–8 weeks after the hospital discharge were used and, meanwhile, the absence of inflammatory process was confirmed (acquired profiles thus represented the basal N-glycome for each person). Within the AcuteInflammation Replication population, we compared those who developed hyperglycaemia after cardiac surgery due to acute inflammation and those who remained normoglycaemic after the surgery (Table 1). For the analysis, plasma samples taken on the day of the surgery, before the surgical procedure were used.

The development of hyperglycaemia during acute inflammation represents significantly higher risk for type 2 diabetes development [2, 3].

Figure 2 and Table 3 show differences in the abundance of derived structural features of plasma protein N-glycans between individuals who developed hyperglycaemia during acute condition and their control groups. Differences are

Table 2 Twelve derived glycan traits calculated from 46 directly measured glycan traits

Structural feature	Formula
Low-branching	GP1+GP2+GP3+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15+GP16+GP17+GP18+GP19+GP20+GP21+GP22+GP23+GP24+GP25+GP26+GP27+GP28
High-branching	GP29+GP30+GP31+GP32+GP33+GP34+GP35+GP36+GP37+GP38+GP39+GP40+GP41+GP42+GP43+GP44+GP45+GP46
Agalactosylated	GP1+GP2+GP4+GP5+GP6
Monogalactosylated	GP3+GP7+GP8+GP9+GP10+GP16+GP17+GP18
Digalactosylated	GP12+GP13+GP14+GP15+GP19+GP20+GP21+GP22+GP23+GP25+GP26+GP27+GP28
Trigalactosylated	GP29+GP31+GP32+GP33+GP34+GP35+GP36+GP37+GP40
Tetragalactosylated	GP30+GP38+GP39+GP41+GP42+GP43+GP44+GP45+GP46
Neutral (not sialylated)	GP1+GP2+GP3+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15
Monosialylated	GP16+GP17+GP18+GP19+GP20+GP21+GP22+GP30
Disialylated	GP23+GP25+GP26+GP27+GP28+GP29+GP31
Trisialylated	GP32+GP33+GP34+GP35+GP36+GP37+GP38+GP39+GP40
Tetrasialylated	GP41+GP42+GP43+GP44+GP45+GP46

N-glycans were separated by HILIC and the peaks designated GP1–GP46

shown as the effect sizes estimated based on logistic regression (natural logarithm of OR). In the AcuteInflammation population, six derived traits were significantly different between the two groups: individuals who developed hyperglycaemia had decreased low-branching glycans, increased high-branching glycans, increased tri- and tetragalactosylated glycans, decreased neutral glycans and increased trisialylated glycans.

The differences found in the AcuteInflammation Replication population were in the same direction and were

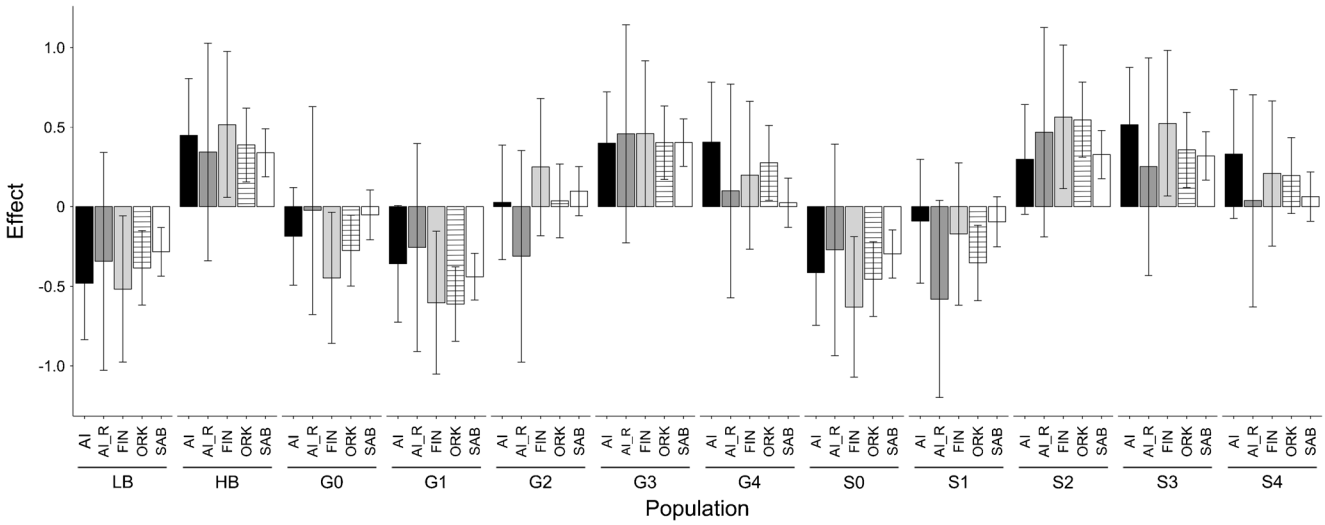

Fig. 2 Differences in abundance of structural features of plasma protein N-glycans in individuals who developed hyperglycaemia during acute condition (AcuteInflammation and AcuteInflammation Replication populations; black and dark grey bars, respectively), in individuals from the FinRisk population who developed type 2 diabetes during 10-year follow-up (light grey bars) and in individuals from the ORCADES (striped bars) and SABRE (white bars) cohorts divided according to HbA_{1c} level (< or > 6.5% [47.5 mmol/mol]). Pronounced differences were observed between cases and controls in all cohorts for several derived glycan structural features. Differences in derived glycan traits are shown as bar plots where height of bars represents the size of effects estimated based on logistic regression (natural logarithm of OR) and error bars represent 95% CIs of estimated effects. AI, AcuteInflammation; AI_R, AcuteInflammation Replication; FIN, FinRisk; ORK, ORCADES; SAB, SABRE; G0, sum of glycans with no galactose; G1, sum of glycans with one galactose; G2, sum of glycans with two galactoses; G3, sum of glycans with three galactoses; G4, sum of glycans with four galactoses; HB, high-branching, sum of triantennary and tetraantennary glycans (see Methods); LB, low-branching, sum of oligomannose, hybrid and biantennary glycans (see Methods); S0, sum of glycans with no sialic acid; S1, sum of glycans with one sialic acid; S2, sum of glycans with two sialic acids; S3, sum of glycans with three sialic acids; S4, sum of glycans with four sialic acids

Table 3 Differences in derived structural features of plasma protein N-glycans between the cases and the controls in all five populations

Derived glycan trait	AcuteInflammation				AcuteInflammation Replication				FinRisk				ORCADES				SABRE			
	Effect	SE	*p* value	Adj. *p* value[a]	Effect	SE	*p* value	Adj. *p* value[a]	Effect	SE	*p* value	Adj. *p* value[a]	Effect	SE	*p* value	Adj. *p* value[a]	Effect	SE	*p* value	Adj. *p* value[a]
LB	− 0.480	0.179	0.006	0.016*	− 0.344	0.342	0.281	0.393	− 0.517	0.230	0.022	0.040*	− 0.384	0.119	0.001	0.002**	− 0.284	0.077	0.000	0.001**
HB	0.448	0.180	0.010	0.024*	0.344	0.342	0.281	0.393	0.517	0.230	0.022	0.040*	0.388	0.119	0.001	0.002**	0.339	0.077	0.000	0.000***
G0	− 0.186	0.154	0.209	0.276	− 0.023	0.327	0.940	0.956	− 0.447	0.206	0.027	0.045*	− 0.276	0.113	0.014	0.019*	− 0.052	0.080	0.511	0.558
G1	− 0.359	0.184	0.044	0.068	− 0.257	0.326	0.399	0.498	− 0.603	0.225	0.007	0.017*	− 0.611	0.119	0.000	0.000***	− 0.440	0.074	0.000	0.000***
G2	0.027	0.182	0.878	0.878	− 0.312	0.332	0.314	0.428	0.250	0.217	0.236	0.290	0.036	0.118	0.759	0.759	0.097	0.079	0.213	0.276
G3	0.399	0.163	0.011	0.024*	0.458	0.343	0.153	0.236	0.459	0.230	0.041	0.066	0.403	0.118	0.001	0.002**	0.403	0.076	0.000	0.000***
G4	0.406	0.190	0.027	0.045*	0.099	0.335	0.750	0.818	0.198	0.234	0.381	0.446	0.276	0.120	0.021	0.025*	0.024	0.079	0.756	0.789
S0	− 0.413	0.167	0.011	0.024*	− 0.271	0.332	0.380	0.487	− 0.630	0.221	0.004	0.012*	− 0.455	0.119	0.000	0.001***	− 0.297	0.077	0.000	0.001***
S1	− 0.091	0.196	0.627	0.669	− 0.581	0.310	0.047	0.083	− 0.171	0.224	0.429	0.478	− 0.354	0.120	0.003	0.005**	− 0.096	0.080	0.230	0.290
S2	0.298	0.174	0.077	0.111	0.468	0.329	0.129	0.204	0.565	0.226	0.011	0.024*	0.547	0.120	0.000	0.000***	0.327	0.077	0.000	0.000***
S3	0.517	0.181	0.003	0.010*	0.252	0.341	0.428	0.514	0.525	0.230	0.020	0.039*	0.358	0.121	0.003	0.005**	0.319	0.077	0.000	0.000***
S4	0.331	0.204	0.093	0.128	0.038	0.333	0.902	0.933	0.209	0.229	0.347	0.416	0.195	0.122	0.106	0.115	0.063	0.079	0.424	0.478

Differences are shown as effects estimated based on logistic regression (natural logarithm of OR)

[a] *p* value corrected for multiple measures using Benjamini–Hochberg procedure

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

G0, sum of glycans with no galactose; G1, sum of glycans with one galactose; G2, sum of glycans with two galactoses; G3, sum of glycans with three galactoses; G4, sum of glycans with four galactoses; HB, high-branching, sum of triantennary and tetraantennary glycans (see Methods); LB, low-branching, sum of oligomannose, hybrid and biantennary glycans (see Methods); S0, sum of glycans with no sialic acid; S1, sum of glycans with one sialic acid; S2, sum of glycans with two sialic acids; S3, sum of glycans with three sialic acids; S4, sum of glycans with four sialic acids

of similar magnitude to those found in the AcuteInflammation population for almost all derived traits, except for digalactosylation. However, none of these differences reached statistical significance.

Analysis of plasma N-glycome composition in the FinRisk population

Differences in plasma protein N-glycome between individuals who would or would not develop type 2 diabetes was further valuated in the FinRisk study cohort, in which plasma samples were collected more than 10 years ago and biobanked. The 10-year follow-up was performed to record the incidence of type 2 diabetes. Thirty-seven individuals who developed type 2 diabetes and no other chronic disease and 37 individuals matched for age and sex (serving as a control group who remained healthy during follow-up period) were identified and included in the study. Plasma N-glycome was analysed in the same way as for the previous two populations.

Individuals who developed type 2 diabetes had significantly decreased low-branching glycans and increased high-branching glycans, decreased agalactosylated and monogalactosylated glycans, decreased neutral (no sialylation) glycans and increased di- and trisialylated glycans compared with individuals who remained normoglycaemic (Fig. 2 and Table 3). For all derived glycan traits, the differences found in FinRisk were in the same direction and of similar magnitude when compared with those found in AcuteInflammation population.

Analysis of plasma N-glycome composition in the ORCADES and SABRE cohorts

To further prove the connection of N-glycome with glucose metabolism disruption, we used data from our previous analysis of plasma protein glycosylation in two other cohorts (ORCADES and SABRE), where we separated age- and sex-matched individuals into two subgroups differing in HbA_{1c} status (HbA_{1c} < 6.5% [47.5 mmol/mol] and HbA_{1c} > 6.5% [47.5 mmol/mol]).

We compared the differences between the two subgroups in the same way as in the previous populations. Levels of most glycans were significantly different between the studied subgroups (ten for ORCADES and seven for SABRE) (Fig. 2 and Table 3) and followed the same direction as in the previous populations–higher levels of branching, galactosylation and sialylation.

Discussion

Our results indicate that plasma protein N-glycome is changed in individuals with increased risk for type 2 diabetes development (AcuteInflammation and AcuteInflammation Replication populations), incident cases of type 2 diabetes collected at baseline (FinRisk population) and people with increased HbA_{1c} (ORCADES and SABRE cohorts). Differences in plasma N-glycome were the same in all three populations and included higher levels of branching, galactosylation and sialylation. This suggests that the increased complexity of glycan structures represents a greater chance of developing type 2 diabetes and is also associated with poorer regulation of blood glucose levels.

One can only speculate whether these differences in plasma glycome are inborn and genetically conditioned or glycosylation is affected by the pathophysiological mechanisms that occur very early at diabetes onset and manifest themselves in the changes of the plasma glycome composition due to high susceptibility of glycans to changes in the cell metabolism. A possible explanation for this higher branching might be that higher glycaemia leads to altered flux through the hexosamine pathway, which produces uridine diphosphate–*N*-acetylglucosamine, the substrate for N-linked glycosylation [16]. Recently, it has also been shown that the hexosamine biosynthesis and *N*-acetylglucosamine salvage pathways contribute to glucose homeostasis through N-glycan branching on the glucagon receptor [17].

Previous studies showed that multi-branched and highly sialylated N-glycans were also elevated in response to inflammatory diseases, such as ulcerative colitis [18], chronic pancreatitis [19] and rheumatoid arthritis [20]. It is also well known that individuals with the metabolic syndrome and type 2 diabetes suffer from chronic low-grade inflammation [21]. Therefore, the changes in N-glycans observed in this study may also reflect the chronic inflammatory processes. Additional support for this hypothesis comes from recent studies of an NMR biomarker, GlycA. GlycA is a signal that mostly arises from *N*-acetylglucosamine residues attached to the plasma proteins and it has been shown to correlate with a wide spectrum of inflammatory diseases, including incident type 2 diabetes [22, 23]. Higher branching of N-glycans, which we found in our study, also implies a higher number of *N*-acetylglucosamine residues in glycans attached to the plasma proteins (similar to higher GlyA signal). Thus, the changes we saw in plasma protein N-glycome could indicate the start of chronic inflammation and the susceptibility for developing the metabolic syndrome. This could also explain why the differences in derived glycan traits between the cases and controls in the AcuteInflammation Replication population did not reach the threshold of significance–all individuals from this population had cardiovascular diseases and underwent cardiac surgery. Therefore, it is possible that most of them (both cases and controls) already had some degree of chronic inflammation. The other possible reason could be that the control group was too small (14 individuals).

We measured the whole plasma protein N-glycome, which is comprised of different glycans originating from many

different glycoproteins, yet we managed to find differences in glycan traits between individuals in the control groups and seemingly healthy individuals who had a higher chance of developing type 2 diabetes mellitus or who did develop the disease. The same changes were also associated with poorer regulation of blood glucose. Identifying the exact glycoproteins that contribute to these differences would probably help to develop stratification methods which could reliably distinguish individuals who are at risk of type 2 diabetes development and would improve the prevention of this widespread disease and its complications.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement OG, IGo, and GL conceived and designed the study. OG and GL supervised the study. TK, FV, NS, IGu, TP, EL, HG, BB, TT, AW, SM, VS, AH, JFW, NC, MP, HC and WW participated in data acquisition, collection, analysis or interpretation. OG, TK and FV drafted the manuscript. GL, NS, IGo, IGu, TP, EL, HG, BB, TT, AW, SM, VS, AH, JFW, NC, MP, HC and WW critically revised the manuscript for intellectual content. All authors approved the final version of the manuscript. OG is the guarantor of the study and is responsible for the integrity of the work as a whole.

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