N-Glycan Profile and Kidney Disease in Type 1 Diabetes

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Mairead L. Bermingham,[1] Marco Colombo,[2] Stuart J. McGurnaghan,[1] Luke A.K. Blackbourn,[1] Frano Vučković,[3] Maja Pučić Baković,[3] Irena Trbojević-Akmačić,[3] Gordan Lauc,[3] Felix Agakov,[4] Anna S. Agakova,[4] Caroline Hayward,[5] Lucija Klarić,[2,5] Colin N.A. Palmer,[6] John R. Petrie,[7] John Chalmers,[8] Andrew Collier,[9] Fiona Green,[10] Robert S. Lindsay,[11] Sandra Macrury,[12] John A. McKnight,[13] Alan W. Patrick,[14] Sandeep Thekkepat,[15] Olga Gornik,[16] Paul M. McKeigue,[2,4] and Helen M. Colhoun,[1,17] on behalf of the SDRN Type 1 Bioresource Investigators

OBJECTIVE

Poorer glycemic control in type 1 diabetes may alter N-glycosylation patterns on circulating glycoproteins, and these alterations may be linked with diabetic kidney disease (DKD). We investigated associations between N-glycans and glycemic control and renal function in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Using serum samples from 818 adults who were considered to have extreme annual loss in estimated glomerular filtration rate (eGFR; i.e., slope) based on retrospective clinical records, from among 6,127 adults in the Scottish Diabetes Research Network Type 1 Bioresource Study, we measured total and IgG-specific N-glycan profiles. This yielded a relative abundance of 39 total (GP) and 24 IgG (IGP) N-glycans. Linear regression models were used to investigate associations between N-glycan structures and HbA_{1c}, albumin-to-creatinine ratio (ACR), and eGFR slope. Models were adjusted for age, sex, duration of type 1 diabetes, and total serum IgG.

RESULTS

Higher HbA_{1c} 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 (GP12, 26, 31, 32, and 34, and IGP19 and 23; all $P < 3.79 \times 10^{-4}$). Similar patterns were seen for ACR and greater mean annual loss of eGFR, which were also associated with fewer of the simpler N-glycans (all $P < 3.79 \times 10^{-4}$).

CONCLUSIONS

Higher HbA_{1c} in type 1 diabetes is associated with changes in the serum N-glycome that have elsewhere been shown to regulate the epidermal growth factor receptor and transforming growth factor-β pathways that are implicated in DKD. Furthermore, N-glycans are associated with ACR and eGFR slope. These data suggest that the role of altered N-glycans in DKD warrants further investigation.

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in diabetes (1). In people with type 1 diabetes, such CKD is usually due to specific diabetic kidney disease (DKD) in which a falling estimated glomerular filtration rate (eGFR) is usually accompanied by albuminuria (2). Diabetic nephropathy is the underlying pathologic process, the features of which include thickened basement membrane and disruption of the glycocalyx (3). Poorer glycemic control, as measured by the degree of nonenzymatic glycation of hemoglobin (HbA_{1c}), is associated with risk of CKD in diabetes and DKD. Such glucose-induced tissue damage is hypothesized to be mediated through a range of pathways, including increased flux of glucose through the hexosamine biosynthetic pathway (HBP) (4). However, considerable variation exists in individuals'

[1]MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, U.K.
[2]Centre for Population Health Sciences, Usher Institute, University of Edinburgh, Edinburgh, U.K.
[3]Genos Glycoscience Research Laboratory, Zagreb, Croatia
[4]Pharmatics Ltd, Edinburgh, UK
[5]MRC Human Genetics Unit, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, U.K.
[6]Cardiovascular and Diabetes Medicine, University of Dundee, Dundee, U.K.
[7]Institute of Cardiovascular & Medical Sciences, University of Glasgow, Glasgow, U.K.
[8]Diabetes Centre, Victoria Hospital, Kirkcaldy, U.K.
[9]Diabetic Day Centre University Hospital, Ayr, U.K.
[10]Research & Development Support Unit, Dumfries & Galloway Royal Infirmary, Dumfries, U.K.
[11]Institute for Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, U.K.
[12]Highland Diabetes Institute, Raigmore Hospital, NHS Highland, Inverness, U.K.
[13]Western General Hospital, NHS Lothian, Edinburgh, U.K.
[14]Royal Infirmary of Edinburgh, NHS Lothian, Edinburgh, U.K.
[15]David Matthews Diabetes Centre, Monklands Hospital, Airdrie, U.K.
[16]Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia
[17]Department of Public Health, NHS Fife, Kirkcaldy, U.K.

Corresponding author: Mairead L. Bermingham, mairead.bermingham@igmm.ed.ac.uk.

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M.L.B. and M.C. contributed equally to this work.

susceptibility to DKD for a given level of HbA_{1c}, with estimates of up to 35% for heritability of this complication (5).

Increased flux of glucose through the HBP in diabetes should lead to increased levels of uridine diphosphate-*N*-acetylglucosamine (6), the donor molecule for the enzymatic process of N-linked glycosylation of secreted proteins (7). The glycosylation process occurs when a block of 14 sugars (the dolichol phosphate precursor) is transferred cotranslationally to specific asparagine residues in newly synthesized polypeptides in the endoplasmic reticulum. The resulting N-glycans are subjected to extensive modification as the glycoproteins mature and move via the Golgi complex to their intracellular and extracellular destinations. The result is a myriad of modifications that can be described in terms of the amount of N-acetylglucosamine (GlcNAc), fucosylation, galactosylation, and sialylation (*N*-acetylneuraminic [sialic] acid), as well as branching (8). The degree of branching (biantennary, triantennary, and tetraantennary structures) has been shown to be determined by Golgi enzyme activity and by diphosphate-*N*-acetylglucosamine levels (7). We hypothesize that people with type 1 diabetes and poorer glycemic control would have increased complex branched N-glycan modifications in serum proteins. As detailed in the CONCLUSIONS, these complex branched structures have been shown to be determinants of many aspects of protein function with potential relevance to kidney disease; therefore we further hypothesize that N-glycan profiles may be associated with DKD. We examined the associations of N-glycans released from total serum protein and those released specifically from serum IgG with glycemia, albumin-to-creatinine ratio (ACR), and retrospective eGFR slope in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Study Population

The Scottish Diabetes Research Network Type 1 Bioresource Study (SDRNT1BIO) is a population-based cohort study of participants aged ≥16years who were enrolled between 1 December 2010 and 29 November 2013 and who had a clinical diagnosis of type 1 diabetes, maturity-onset diabetes of the young, or latent autoimmune diabetes in adults. They comprised consecutive attendees at annual routine hospital and primary care diabetes review visits in 10 of the 13 National Health Service Board areas in Scotland. Data collection from study participants comprised of a single study visit. Participants were asked to complete a self-report questionnaire that included information on demographic and behavioral attributes; details of the medical history of diabetes were collected during an interview on the day of the test. Clinical measures and nonfasting blood and urine samples were also taken. Participants are representative of the total adult population with type 1 diabetes in Scotland with regard to clinical history and clinical and demographic characteristics. The study data are linked retrospectively and prospectively to other routine data sources, including electronic health records for diabetes (9). For this study, type 1 diabetes was defined as a clinical diagnosis of type 1 diabetes, no evidence in the historical record of >1 year between diagnosis and insulin requirement, and no history of use of nonmetformin oral drug treatment. The study was approved by the Tayside Research Ethics Committee (reference 10/S1402/43) and conducted according to the principles of the Declaration of Helsinki. Written consent was obtained from the participants.

Acquisition of HbA_{1c}, ACR, and eGFR data

For all participants in the SDRNT1BIO, we linked HbA_{1c} measurements from the Scottish Care Information-Diabetes Collaboration database to participant data (9). Baseline HbA_{1c} was defined as the measurement closest to, and up to 1 year before, the date of consent.

Albuminuria status on the study day was based on measurement of albumin and creatinine in two spot urine samples taken several days apart. Albumin and creatinine concentrations were measured with an ADVIA 2400 Clinical Chemistry System (Siemens Healthcare, Camberley, U.K.) using immunoturbidimetric (antibody reaction) and modified Jaffé reaction methods, respectively, at the Ninewells Medical School & Hospital Biochemistry Laboratory, National Health Service Tayside, Ninewells Hospital & Medical School (Dundee, U.K.). We then modeled in the analyses the relationship of N-glycans to ACR, with ACR scaled to have a mean of 0 and an SD of 1. For the purposes of describing baseline characteristics, normoalbuminuria was defined as an ACR <30 mg/g (mean from the two readings), microalbuminuria as an ACR in the range of 30–300 mg/g, and macroalbuminuria as ACR >300 mg/g.

eGFR was estimated before the study day based on serum creatinine records from the Scottish Care Information-Diabetes Collaboration database linked to participant data. This database captures all serum creatinine values measured clinically in all participants since 2004. The mean number of creatinine readings per person over the period was 10 (interquartile range 7–17), excluding all creatinine measurements corresponding with a hospital stay. The CKD Epidemiology Collaboration formula was used to convert creatinine values to eGFR (10). eGFR was set at 10 mL/min/1.73 m^2 for the purpose of modeling continuous data distributions once renal replacement was started. The monthly median eGFR across the patient's retrospective record was then retained for inclusion in a later analysis. To validate the use of the eGFR from the electronic record, we confirmed that the health record–derived eGFR at time of sampling was highly correlated ($r >$ 0.97) with eGFR derived from directly measured serum creatinine.

Selection of Participants in This N-Glycan Study from the SDRNT1BIO Cohort

Funding did not permit measurement of N-glycans in the entire SDRNT1BIO cohort; therefore a subgroup of 818 participants were selected (Supplementary Fig. 1). We calculated for each person the retrospective eGFR slope. Only those with at least 3 years of longitudinal eGFR data and at least five eGFR observations over their history were considered for slope estimation. Two participants did not meet these inclusion criteria and were removed from subsequent analyses of eGFR slope. Slope was estimated by fitting a linear regression to the smoothed eGFR for each individual and estimating the effect of time on eGFR. To increase the power to detect associations, we then randomly selected participants across the range of slope but overweighted at the extremes of the distribution, such that 36% of all those included came from the top and bottom deciles of slope. The summary measure of slope was used as a single outcome variable in the subsequent analysis.

N-Glycan Analyses

IgG was isolated from serum samples using protein G monolithic plates (11), and N-glycans were released from samples using a previously described high-throughput method (12).

Hydrophilic Interaction Chromatography–Ultraperformance Liquid Chromatography

Fluorescently labeled N-glycans were separated by hydrophilic interaction chromatography on an ACQUITY ultraperformance liquid chromatography (UPLC) instrument (Waters, Milford, MA), as described previously (13). Glycan peaks were pooled based on similar structural or compositional features of the peak glycan members. Chromatograms for the total and IgG N-glycans were separated into 39 and 24 peaks using manual integration of chromatograms (12) and a semisupervised approach for the automatic chromatogram extraction (14), respectively. The amount of glycans in each peak was then expressed as a percentage of the total integrated area. The N-glycan traits are described in Supplementary Table 1.

Measurement of Within-Sample IgG and Glucose

We measured serum IgG and glucose levels in serum samples using an immunoturbidimetric assay (Tina-quant IgG Gen.2; Roche Diagnostics), and a GOD-PAP enzymatic colorimetric assay (Roche Diagnostics) at the Royal Devon and Exeter Hospital Blood Sciences Laboratory (Exeter, U.K.).

Statistical Analysis

To remove experimental variation, the UPLC N-glycan data were normalized for the total area, log transformed, and corrected for batch effects using the empirical Bayes method, implemented with the R package sva ComBat function (15). The exponent of batch-corrected measurements was then obtained to transform the UPLC N-glycan data to the original scale. An additional 15 and 54 derived traits were then calculated from the normalized and batch-corrected total and IgG N-glycan traits, respectively. These derived traits average particular glycosylation features (galactosylation, fucosylation, bisecting GlcNAc, and sialylation; Supplementary Table 1). All N-glycan and continuous clinical variables used in the association analyses were Gaussianized, centered, and scaled to have a mean of 0 and an SD of 1.

Before association analyses, we tested for associations between the N-glycan measurements and serum IgG and glucose levels in a linear regression model after adjusting for the effects of age, sex, and duration of type 1 diabetes. Serum glucose did not associate with total or IgG N-glycans. As such, N-glycosylation patterns are not confounded with within-sample variation in serum glucose levels. However, we observed that total and IgG N-glycan profiles varied with IgG levels. IgG levels were not associated with HbA_{1c}, ACR, or eGFR slope, but they showed some univariate association with eGFR. Therefore we adjusted all total and IgG N-glycan association analyses for IgG. However, adjusting for IgG in the analyses made no meaningful difference to any of the reported associations.

Linear regression models were used to test for associations between N-glycans and each of the following outcomes: glycemic control (HbA_{1c}), ACR, and the summary measure of historical eGFR slope. For HbA_{1c} associations, the first (minimal) model was adjusted for age, sex, duration of type 1 diabetes, and serum IgG levels. The second (full) model was additionally adjusted for baseline eGFR, ACR, BMI, smoking status, diastolic blood pressure, systolic blood pressure, total cholesterol, use of antihypertensive drugs (α-blockers, ACE inhibitors, angiotensin receptor blockers, β-blockers, calcium channel blockers, and diuretics), use of statins, use of anti-inflammatory drugs (δ-hydrocortisone, dexamethasone, hydrocortisone, Hydrocortone, Medrone, methylprednisolone, Plenadren, and triamcinolone), and use of oral contraceptives or hormone replacement therapy. For ACR and eGFR slope, the minimal model was adjusted for age, sex, duration of diabetes, and total IgG levels, and the full model was adjusted further for HbA_{1c} to examine to what extent N-glycans may capture an association of the underlying determinant (i.e., poor glycemic control) with DKD that is not already captured by the HbA_{1c} measure. We then added each of the N-glycans separately to the first model to test for association with the outcomes of interest and confirmed that associations were robust to the adjustments in the second model. In total, 132 N-glycan variables were tested, and we adopted a Bonferroni-corrected threshold of 3.79×10^{-4} (0.05/number of N-glycans tested) to indicate statistical significance. Reported *P* values are nominal. All analyses were conducted using the R software package version 3.3.1.

RESULTS

The study population comprised 818 persons with an interquartile range of age of 31.24–54.59 years, duration of diabetes 11.28–30.08 years, HbA_{1c} 63.00–86.00 mmol/mol, and eGFR 67.16–111.28 mL/min/1.73 m^2. Just 23.72% of participants had microalbuminuria or macroalbuminuria (Table 1). After adjusting for age, sex, and diabetes duration, higher HbA_{1c} was associated with ACR (β= 0.32; 95% CI 0.25, 0.38); $P = 2.00 \times 10^{-16}$) and a steeper eGFR slope (β = −0.09; 95% CI −0.16, −0.02; $P = 8.36 \times 10^{-03}$).

Table 1—Characteristics of the study population

Characteristic	Data set
Sample size (*N*)	818
Age (years)	43.07 (31.24, 54.59)
Male sex	50.98
Duration (years)	20.62 (11.28, 30.08)
HbA_{1c} (mmol/mol)	74.00 (63.00, 86.00)
ACR (mg/g)	5.56 (2.82, 26.44)
Normoalbuminuria (ACR <30 mg/g)	76.28
Microalbuminuria (ACR 30–300 mg/g)	13.45
Macroalbuminuria (ACR >300 mg/g)	10.27
eGFR (mL/min/1.73 m^2)	97.73 (67.16, 111.28)
eGFR slope* (mL/min/1.73 m^2/year)	−1.46 (−3.37, −0.21)
IgG (μmol/L)	9.90 (8.50, 11.30)
Glucose (mmol/L)	10.80 (6.90, 15.30)

Data are median (interquartile range) or percentage unless otherwise indicated. *Rate of change in 1 year.

The data are structured with groups of highly correlated N-glycans (Supplementary Fig. 2). We tested the association of HbA_{1c} level with the relative abundance of each total and IgG N-linked glycan using the Wald test for linear regression models and adjusted for age, sex, duration of type 1 diabetes, and total serum IgG. The lists of top and all associations are shown in Table 2 and Supplementary Table 2, respectively. The data are most easily visualized in Figs. 1 and 2, where annotated chromatograms of the total and IgG N-glycans show identified associations with HbA_{1c}, ACR, and eGFR slope. For total N-glycans, higher HbA_{1c} was associated with a shift toward a greater relative abundance of more

Table 2—Total and IgG N-glycan associations (ordered by regression coefficients) with HbA_{1c}, after adjusting for covariates in the minimal and full clinical models, and with ACR and eGFR slope after adjusting for covariates in the minimal and minimal plus HbA_{1c} clinical models

			Minimal model		Full model	
Associations	Code	Major structure	β (95% CI)	*P* value	β (95%CI)	*P* value
HbA_{1c}	N-glycans					
	Total					
	GP4	FA2[6]G1	−0.19 (−0.28, −0.11)	**2.63×10^{-6}**	−0.13 (−0.20, −0.05)	1.49×10^{-3}
	GP5	FA2[3]G1	−0.16 (−0.24, −0.09)	**3.67×10^{-5}**	−0.10 (−0.17, −0.02)	9.00×10^{-3}
	GP10	FA2G2	−0.16 (−0.24, −0.09)	**1.70×10^{-5}**	−0.10 (−0.17, −0.03)	6.26×10^{-3}
	GP6	FA2[6]BG1	−0.15 (−0.23, −0.07)	**2.59×10^{-4}**	−0.11 (−0.18, −0.03)	5.26×10^{-3}
	GP31	FA3G3S3	0.18 (0.11, 0.25)	**4.10×10^{-7}**	0.17 (0.10, 0.23)	**1.47×10^{-6}**
	GP32	A3F1G3S3	0.18 (0.11, 0.25)	**3.14×10^{-7}**	0.14 (0.07, 0.21)	**4.55×10^{-5}**
	GP34	A4G4S3	0.18 (0.11, 0.25)	**4.32×10^{-7}**	0.15 (0.08, 0.21)	**1.96×10^{-5}**
	GP12	A2[3]BG1S1	0.22 (0.15, 0.30)	**1.43×10^{-8}**	0.18 (0.10, 0.26)	**3.94×10^{-6}**
	GP26	A3G3S2	0.25 (0.18, 0.32)	**3.42×10^{-12}**	0.20 (0.13, 0.27)	**2.43×10^{-8}**
	IgG					
	IGP7	FA2[6]G1	−0.18 (−0.24, −0.11)	**4.79×10^{-7}**	−0.14 (−0.20, −0.07)	**4.28×10^{-5}**
	IGP23	FA2BG2S2	0.12 (0.05, 0.19)	**4.75×10^{-4}**	0.06 (−0.01, 0.12)	8.96×10^{-2}
	IGP19	FA2FG2S1	0.13 (0.06, 0.20)	**2.06×10^{-4}**	0.10 (0.04, 0.17)	3.08×10^{-3}
			Minimal model		Minimal model plus HbA_{1c}	
ACR	N-glycans					
	Total					
	GP10	FA2G2	−0.21 (−0.29, −0.14)	**1.31×10^{-8}**	−0.17 (−0.24, −0.10)	**4.61×10^{-6}**
	GP5	FA2[3]G1	−0.15 (−0.22, −0.07)	**2.48×10^{-4}**	−0.10 (−0.17, −0.02)	1.23×10^{-2}
	GP16	FA2G2S1	−0.15 (−0.22, −0.07)	**8.92×10^{-5}**	−0.14 (−0.21, −0.07)	**1.29×10^{-4}**
	GP29	FA3G3S3	−0.13 (−0.20, −0.06)	**3.15×10^{-4}**	−0.09 (−0.16, −0.03)	5.46×10^{-3}
	GP34	A4G4S3	0.15 (0.08, 0.22)	**1.74×10^{-5}**	0.10 (0.03, 0.17)	4.03×10^{-3}
	GP26	A3G3S2	0.17 (0.09, 0.24)	**6.22×10^{-6}**	0.09 (0.02, 0.16)	1.18×10^{-2}
	GP32	A3F1G3S3	0.19 (0.12, 0.26)	**8.30×10^{-8}**	0.14 (0.07, 0.20)	**6.75×10^{-5}**
	IgG					
	IGP13	*FA2G2*	−0.20 (−0.28, −0.12)	**7.38×10^{-7}**	−0.17 (−0.25, −0.10)	**1.15×10^{-5}**
	IGP11	*A2G2*	−0.16 (−0.23, −0.10)	**3.15×10^{-6}**	−0.13 (−0.20, −0.07)	**6.10×10^{-5}**
	IGP6	*A2G1*	−0.13 (−0.20, −0.06)	**4.41×10^{-4}**	−0.10 (−0.17, −0.03)	3.57×10^{-3}
	IGP12	*A2BG2*	−0.13 (−0.20, −0.06)	**2.61×10^{-4}**	−0.12 (−0.19, −0.06)	**3.37×10^{-4}**
	IGP23	*FA2BG2S2*	0.12 (0.06, 0.19)	**3.62×10^{-4}**	0.09 (0.02, 0.15)	9.21×10^{-3}
	IGP3	*FA2*	0.18 (0.10, 0.25)	**3.70×10^{-6}**	0.14 (0.07, 0.22)	**9.91×10^{-5}**
eGFR slope	N-glycans					
	Total					
	GP7	M6	0.12 (0.06, 0.19)	**4.16×10^{-4}**	0.12 (0.06, 0.19)	**3.54×10^{-4}**
	GP16	FA2G2S1	0.16 (0.08, 0.24)	**3.58×10^{-5}**	0.16 (0.08, 0.23)	**5.02×10^{-5}**
	GP10	FA2G2	0.19 (0.12, 0.27)	**6.57×10^{-7}**	0.18 (0.10, 0.26)	**3.64×10^{-6}**
	IgG					
	IGP3	*FA2*	−0.22 (−0.29, −0.14)	**3.94×10^{-8}**	−0.21 (−0.28, −0.13)	**1.45×10^{-7}**
	IGP_	—	−0.17 (−0.24, −0.10)	**3.58×10^{-6}**	−0.16 (−0.23, −0.09)	**9.89×10^{-6}**
	IGP1	*FA1*	−0.15 (−0.22, −0.08)	**5.90×10^{-5}**	−0.14 (−0.21, −0.07)	**7.43×10^{-5}**
	IGP12	*A2BG2*	0.13 (0.06, 0.20)	**4.17×10^{-4}**	0.13 (0.06, 0.20)	5.21×10^{-4}
	IGP14	*FA2BG2*	0.18 (0.10, 0.25)	**2.39×10^{-6}**	0.17 (0.10, 0.24)	**6.49×10^{-6}**
	IGP17	*FA2G2S1*	0.21 (0.12, 0.29)	**9.10×10^{-7}**	0.20 (0.12, 0.28)	**1.16×10^{-6}**
	IGP13	*FA2G2*	0.24 (0.15, 0.32)	**1.66×10^{-8}**	0.23 (0.15, 0.31)	**5.12×10^{-8}**

Covariates in the minimal clinical model were age, sex, duration of type 1 diabetes, and total serum IgG. Covariates in the full clinical model were age sex, duration of type 1 diabetes, total serum IgG, baseline eGFR, ACR, BMI, smoking status, diastolic blood pressure, systolic blood pressure, total cholesterol, use of antihypertensive drugs, use of statins, use of anti-inflammatory drugs, and use of oral contraceptives or hormone replacement therapy. Boldface *P* values indicate $P < 3.79 \times 10^{-4}$ (multiple testing correction).

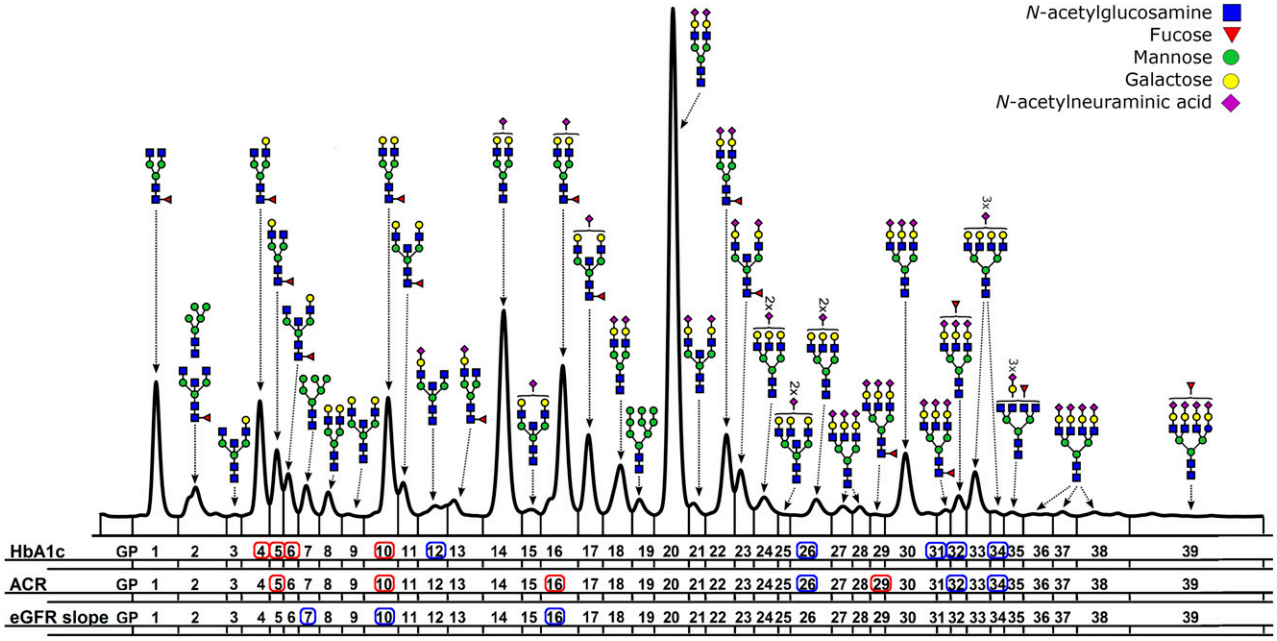

Figure 1—Chromatogram of total N-linked glycans released from serum glycoproteins and separated by UPLC–hydrophilic interaction chromatography. The integration areas, together with the structures present in each glycan group, are given. Groups of glycans are numbered from glycan peaks 1–39, mostly corresponding to individual glycan structures. Blue squares, red triangles, green circles, yellow circles, and purple diamonds represent GlcNAc, fucose, mannose, galactose, and *N*-acetylneuraminic (sialic) acid residues, respectively. The blue and red rounded rectangles below the chromatogram highlight the identified associations between N-glycan peaks and glycemic control (HbA_{1c}), ACR, and eGFR slope in this study. Positive associations are displayed in blue and negative associations, in red. Note that N-glycans with the same underlying direction with DKD show an opposite direction of association with ACR than with eGFR slope.

complex structures that have more GlcNAc and are more heavily galactosylated and sialylated (Table 2). Higher HbA_{1c} was associated with a greater relative abundance of GP12, a monogalactosylated and monosialylated biantennary glycan with a bisecting GlcNAc, and with greater relative abundance of some triantennary and tetra-antennary structures (GP26, 31, 32, and 34). Conversely, higher HbA_{1c} was associated with a lower abundance of simpler biantennary structures with less GlcNAc, less galactose, and less sialic acid (GP4, 5, 6, and 10).

For IgG N-glycans, which do not exist in more complex triantennary or tetra-antennary forms, higher HbA_{1c} was associated with a greater relative abundance of the more galactosylated and sialylated biantennary structures (IGP19 and 23) and less of the simpler monogalactosylated biantennary glycan IGP7 (Fig. 2). The same pattern of association was seen with further adjustment for drug treatments and blood pressure, as well as for baseline eGFR, ACR, and eGFR slope (Table 2).

No association was found between N-glycan levels and glucose level at the time N-glycans were sampled (data not shown). Thus the N-glycan associations with HbA_{1c} observed in this study do not reflect confounding by in-sample glucose levels.

We tested the association of ACR with each total and IgG N-glycan using the Wald test in linear regression models adjusted for age, sex, duration of type 1 diabetes, and total serum IgG. The top associations are shown in Table 2, and the full list of associations is shown in Supplementary Table 3. Similar to the pattern seen for higher HbA_{1c}, for total plasma N-glycans higher ACR was associated with a shift toward a greater relative abundance of more complex structures that have more GlcNAc, are more heavily galactosylated and sialylated, and are mostly triantennnary (GP26, 32, and 34) (Fig. 1). Conversely, higher ACR was associated with a smaller abundance of simpler biantennary structures with less GlcNAc, less galactose, and less sialic acid (GP5, 10, and 16), and with less of the core fucosylated GP29.

For IgG N-glycans, higher ACR was associated with a greater relative abundance of the more complex disialylated, digalactosylated structure with a bisecting GlcNAc (IGP23), as was found for higher HbA_{1c}. Higher ACR was also associated with relatively more of the simpler monogalactosylated IGP3 and less of the digalactosylated biantennary glycans (IGP6, 11, 12, and 13) (Fig. 2). The associations of the following N-glycans remained after adjusting for HbA_{1c}: GP10, 16, and 32; IGP3, 11, 12, and 13) (Table 2).

We tested for associations of N-glycans with eGFR slope (Table 2 and Supplementary Table 4), where a negative slope represents a greater mean annual decline in eGFR over the observed history of the patient. Some evidence indicated a pattern similar to that seen for HbA_{1c} and ACR. Those with a steeper decline in eGFR over time had relatively fewer of the simpler total N-glycans (GP7, 10, and 16) (Fig. 1). These associations were not restricted to those with an already reduced eGFR at entry, as would be expected if they reflected reverse causation. Indeed, they were strongest in those with eGFR $>$60 mL/min/1.73 m^2 on the study day.

For IgG N-glycans, steeper decline was also associated with relatively more very simple monoantennary and biantennary agalactosylated glycans that have core fucose (IGP1 and 3) and fewer of the biantennary galactosylated glycans (IGP12, 13, and 14) (Fig. 2). Similar to that seen with ACR, relatively less of the biantennary galactosylated sialylated glycan IGP17 was

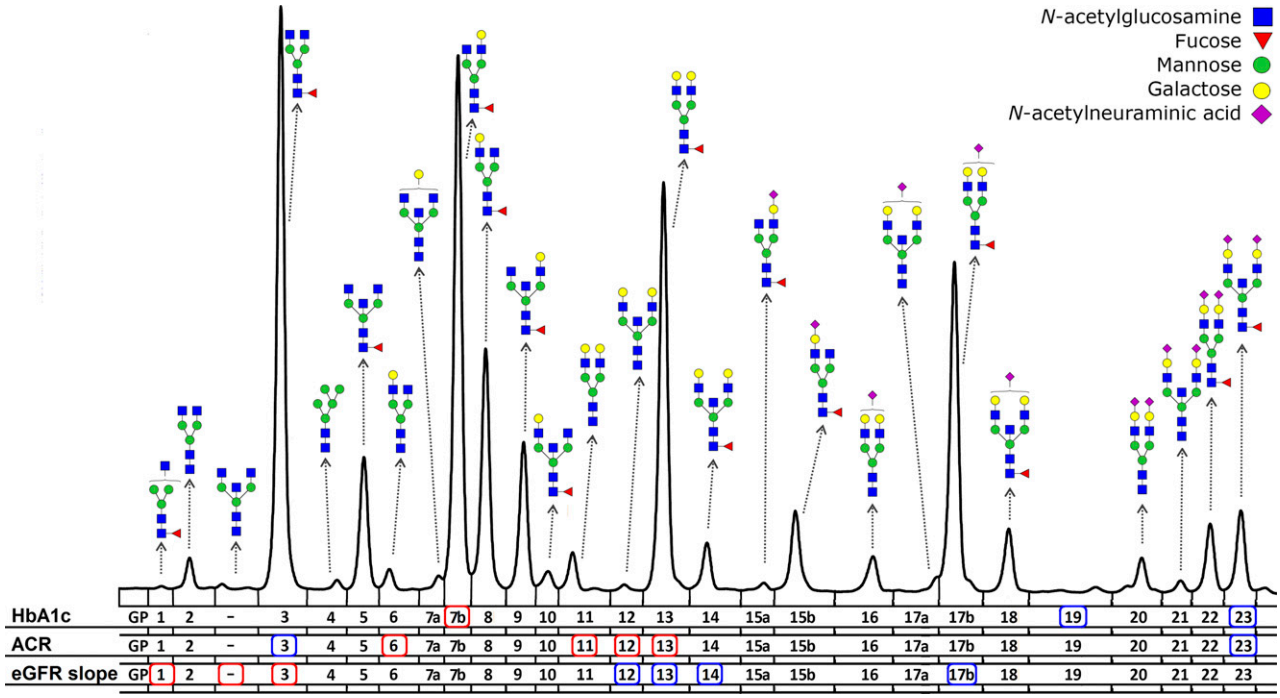

Figure 2—Chromatogram of IgG N-linked glycans released from serum glycoproteins and separated by UPLC–hydrophilic interaction chromatography. The integration areas, together with structures present in each glycan group, are given. Groups of glycans are numbered from glycan peaks 1–23, corresponding to individual glycan structures. Blue squares, red triangles, green circles, yellow circles, and purple diamonds represent GlcNAc, fucose, mannose, galactose, and *N*-acetylneuraminic (sialic) acid residues, respectively. The blue and red rounded rectangles below the chromatogram highlight the identified associations between glycemic control (HbA_{1c}), ACR, and eGFR slope and IgG N-glycosylation patterns in this study. Positive associations are displayed in blue and negative associations, in red. Note that N-glycans with the same underlying direction with DKD show an opposite direction of association with ACR than with eGFR slope.

also found. These associations mostly remained significant at $P < 3.79 \times 10^{-4}$ after adjusting for HbA_{1c} (Table 2), and many were also independent of ACR (GP7 and IGP1, 14, and 17) (Table 2). Although the angiotensin II receptor blocker and ACE inhibitors were significantly associated with higher ACR, the strength of the association of the reported N-glycans with ACR was reduced but still clearly present upon adjustment for these drugs. The associations of N-glycans with ACR and eGFR slope were attenuated when further adjusted for variables associated with these renal measures, including blood pressure and blood pressure treatment.

CONCLUSIONS

In this study we found evidence that the degree of glycemic control in type 1 diabetes was associated with substantial alterations in the relative abundance of released total and IgG N-glycans in serum. With higher prevailing glycemia, the N-glycan profile shifted toward more triantennary and tetra-antennary structures (which are also more galactosylated and sialylated). For IgG N-glycans, there were also highly significant alterations in the pattern with glycemic control. To our knowledge, this is the first report of N-glycan profiles in type 1 diabetes, despite the strong basis for expecting such alterations to occur in this condition. We further sought evidence of any potential impact of such N-glycans on renal complications of diabetes. The same shift toward more complex total N-glycans was observed in cross-sectional associations with higher ACR and with a steeper decline in eGFR, defined using retrospective data. These associations were independent of the association of HbA_{1c} with these renal measures, consistent with the idea that some aspects of glycemic exposure relevant to complications may not be captured by HbA_{1c}. Our data are congruent with increased HBP flux being manifest in altered N-glycan branching in diabetes. Previous studies have proposed that glycemia-related damage in type 1 diabetes is at least partly attributable to increased flux through the HBP (16). Under normal conditions, approximately 2–5% of total glucose is converted to diphosphate-*N*-acetylglucosamine via the HBP (7), but this would be expected to increase under conditions of high glycemia. However, such reports usually consider *O*-linked glycosylation as the main potential mediating mechanism for the role of increased HBP flux in diabetes complications (17). The degree of N-glycan branching has previously been reported to depend in part on the availability of the common donor substrate diphosphate-*N*-acetylglucosamine, the end product of HBP, and on branching enzyme kinetics (7). Thus, we hypothesized that we would see shifts in branching pattern in type 1 diabetes; this was confirmed by the data. Surprisingly, despite recent increasing interest in glycobiology, we are not aware of any large evaluations of N-glycan profiles in type 1 diabetes. However, previous studies of N-glycosylation in 562 people with type 2 diabetes reported reduced levels of certain monogalactosylated N-glycans (NG1[6]A2F, equivalent to GP6 in our study) in people with type 2 diabetes compared with controls (18); this is consistent with the association of lower GP6 with higher HbA_{1c} found in our study. Another small study of 17 people with diabetes and controls found increased sialylated

N-glycans in the vitreous fluid of people with proliferative retinopathy (19). A study of one specific glycoprotein, α1-acid glycoprotein, in type 1 diabetes reported altered fucosylation (20). The fundamental role of N-glycans is increasingly recognized in mediating a wide range of biological processes, including protein folding, cell adhesion, and receptor activity modulation (21). An important question, therefore, is whether the altered N-glycan profile in type 1 diabetes is part of the mechanism of glucose-induced kidney damage. We found supportive evidence, in that N-glycan profiles showed highly significant variation, with higher ACR and steeper eGFR decline in the 818 people studied. Certainly, there could plausibly be a causal role in DKD in particular with respect to the shift to triantennary and tetra-antennary branched structures that we observed in this study. It has been shown that triantennary and tetra-antennary N-glycans bind to galectins and form a molecular lattice on the cell surface that opposes glycoprotein endocytosis, thereby regulating surface levels of important glycoproteins in the glycocalyx (7). Termination of branches with galactose and/or sialic acid also increases and decreases galectin affinities, respectively (7). Of particular relevance to DKD is evidence that hexosamine/N-glycan branching may differentially regulate epidermal growth factor (EGF) receptor and transforming growth factor-β (TGF-β) signaling (7). Given the pivotal role of TGF-β in extracellular matrix deposition in DKD, and the increasing evidence that persistent EGF receptor activation is a pivotal process in diabetic nephropathy, a relationship of altered N-glycan branching to kidney damage in diabetes is highly plausible (22,23). Reverse causation is possible, with altered N-glycans resulting from reduced renal function. For example, progressive renal decline (as measured by eGFR) is associated with the presence of specific inflammatory cytokines (e.g., interleukin 6) in type 1 diabetes (24). Sustained inflammation is known to modify the glycosylation pattern of glycoproteins (25), and some glycosyltransferases have been shown to be regulated at the transcriptional level by proinflammatory cytokines such as interleukin 6 (26).

Increasing evidence indicates that alterations in branching, galactosylation, and sialylation of N-glycans on IgG dictate effector function (27–30). Glycosylation of IgG acts as a switch between proinflammatory and anti-inflammatory IgG functionality. Specifically, the decrease in galactosylated (IGP12, 13, and 14) and increase in agalactosylated (IGP1 and 3) glycoforms that we observed with more rapid eGFR decline would be expected to render a more proinflammatory IgG profile (29). A previous analysis of IgG N-glycans in relation to eGFR distribution in the TwinsUK cohort showed evidence similar to that reported here—that is, lower eGFR was associated with more IGP1 and IGP3 (called GP4 in that paper), and less IGP12, IGP13, and IGP14 (30).

We observed an increase in the digalactosylated and disialylated biantennary structure with a bisecting GlcNAc (IGP23) with glycemia and with higher ACR. We note that previous studies have shown elevation of total sialic acid in patients with diabetes and microvascular disease (31). However, unlike the proinflammatory changes seen with falling eGFR, this sialylation modification is expected to be anti-inflammatory (30,32), and we note that in the TwinsUK study this IGP23 was actually higher in those with a higher eGFR (30). A causal relationship of IgG glycan changes with DKD is plausible in that some evidence points to a role for the humoral immune system in DKD, with glomerular deposition of immune complexes (33). Also, IgG Fcγ receptor deficiency was protective in a mouse model of diabetic nephropathy (34–37).

Although the N-glycan profile patterns have some commonalities associated with higher HbA_{1c}, higher ACR, and lower eGFR, they do not perfectly overlap. ACR and eGFR represent aspects of renal function decline in diabetes, but even they do not overlap perfectly. Many people with declining renal function in diabetes are increasingly recognized as normoalbuminuric and, conversely, microalbuminuria may capture some aspects of widespread vascular damage other than only renal disease. However, the lack of perfect overlap highlights the importance of further proposed work to explore whether these relationships represent causal pathways.

Our study has several limitations. N-glycan profiling by examining released glycans is limited in that the proteins from which N-glycans have been released are not directly quantified. Thus any associations of HbA_{1c}, ACR, or eGFR slope with relative abundance of N-glycans may be confounded by associations of these variables with relative composition of the serum proteome. However, for the IgG N-glycans, we were able to quantify the relevant protein IgG, and adjusting for it made little difference to the associations found for IgG N-glycans.

Most importantly, we cannot demonstrate causality in the relationship of N-glycans with DKD in this study design, in which we have tested both cross-sectional and retrospective associations. Changes in N-glycans could be secondary to changes in renal function. However, the fact that such strong associations are shown with renal function and are independent of HbA_{1c} does suggest that further work is warranted. Furthermore, the associations of total N-glycans with eGFR slope were not restricted to those with an already substantially reduced eGFR (<60 mL/min/1.73 m^2). Prospective associations will be tested in the cohort as more data accrue. Also, because several of the N-glycans that we found to be associated with eGFR decline and ACR are partially controlled by genes (38,39), our next step is to test for causal associations of genetic predictors of risk for these traits with renal outcomes using Mendelian randomization. Development of N-glycans as clinical biomarkers for risk of DKD would also need to include the development of direct absolute quantification methods (40).

In summary, our results highlight that poorer glycemic control in type 1 diabetes is associated with substantial changes in the serum N-glycome that has elsewhere been shown to regulate EGF receptor and TGF-β signaling pathways that are generally considered important in mediating DKD. Furthermore, we found associations between N-glycans and both higher ACR and steeper eGFR slope in patients with type 1 diabetes. Our report serves to highlight the importance of further studies in understanding the pathogenic significance of these altered profiles in DKD.

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Author Contributions. M.L.B. interpreted the results and wrote the first draft of the manuscript. M.L.B., M.C., P.M.M., and H.M.C. designed the study. M.L.B and H.M.C. reviewed the literature. M.C. analyzed the baseline data. S.J.M. and L.A.K.B. undertook quality control of the clinical diabetes data sets. F.V., M.P.B., I.T.-A., G.L., and O.G. released the total and IgG N-linked glycans from serum samples. F.A. and A.S.A. undertook quality control of the N-glycan data. C.H. and L.K. provided expert advice on the interpretation of the results. C.N.A.P., J.R.P., J.C., A.C., F.G., R.S.L., S.J.M., J.A.M., A.W.P., and S.T were involved in the establishment of the SDRNT1BIO cohort and the collection of the clinical data used in this study. All authors contributed to the discussion and reviewed and edited the manuscript. H.M.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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