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Memarian, E.; Heijmans, R.; Slieker, R.C.; Sierra, A.; Gornik, O.; Beulens, J.W.J.; ... ; Hoek, M. van

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RESEARCH ARTICLE

IgG *N*-glycans are associated with prevalent and incident complications of type 2 diabetes

Elham Memarian[1,2] | Ralph Heijmans[3] | Roderick C. Slieker[4,5] | Adriana Sierra[6] | Olga Gornik[7] | Joline W. J. Beulens[5,8] | Maja Hanic[1] | Petra Elders[9] | Julio Pascual[6] | Eric Sijbrands[3] | Gordan Lauc[1,7] | Viktoria Dotz[2] | Clara Barrios[6] | Leen M. 't Hart[4,5,10] | Manfred Wuhrer[2] | Mandy van Hoek[3]

[1]Genos Glycoscience Research Laboratory, Zagreb, Croatia

[2]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

[3]Department of Internal Medicine, Erasmus MC - University Medical Center, Rotterdam, The Netherlands

[4]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands

[5]Department of Epidemiology and Data Science, Amsterdam University Medical Center, Location VUMC, Amsterdam Public Health Institute, Amsterdam, The Netherlands

[6]Department of Nephrology, Hospital del Mar, Institut Mar d´Investigacions Mediques, Barcelona, Spain

[7]Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

[8]Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands

[9]Department of General Practice, Amsterdam Public Health Institute, Amsterdam UMC, Location VUmc, Amsterdam, The Netherlands

[10]Department of Biomedical Data Sciences, Section Molecular Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

Correspondence

Mandy van Hoek, Department of Internal Medicine, Erasmus MC - University Medical Center Rotterdam, Doctor Molewaterplein 40, Rotterdam 3015 GD, The Netherlands.
Email: m.vanhoek@erasmusmc.nl

Clara Barrios, Department of Nephrology, Hospital del Mar, Institut Mar d'Investigacions Mediques, Passeig Marítim de la Barceloneta 25, Barcelona 08003, Spain.
Email: cbarrios@psmar.cat

Abstract

Aims/Hypothesis: Inflammation is important in the development of type 2 diabetes complications. The *N*-glycosylation of IgG influences its role in inflammation. To date, the association of plasma IgG *N*-glycosylation with type 2 diabetes complications has not been extensively investigated. We hypothesised that *N*-glycosylation of IgG may be related to the development of complications of type 2 diabetes.

Methods: In three independent type 2 diabetes cohorts, plasma IgG *N*-glycosylation was measured using ultra performance liquid chromatography (DiaGene $n = 1815$, GenodiabMar $n = 640$) and mass spectrometry (Hoorn Diabetes Care Study

Abbreviations: DCS, Hoorn Diabetes Care Study; GDM, GenoDiabMar; IBD, ischaemic brain disease; IHD, ischaemic heart disease; Non-HDL-c, cholesterol value minus HDL cholesterol; PAD, peripheral artery disease.

Mandy van Hoek and Clara Barrios shared corresponding authorship.

Elham Memarian and Ralph Heijmans shared first authorship.

Viktoria Dotz, Clara Barrios and Leen M. 't Hart shared penultimate authorship.

Manfred Wuhrer and Mandy van Hoek shared last authorship.

Present address
Viktoria Dotz, BioTherapeutics Analytical Development, Janssen Biologics BV, Einsteinweg 101, Leiden 2333 CB, The Netherlands.

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n = 1266). We investigated the associations of IgG *N*-glycosylation (fucosylation, galactosylation, sialylation and bisection) with incident and prevalent nephropathy, retinopathy and macrovascular disease using Cox- and logistic regression, followed by meta-analyses. The models were adjusted for age and sex and additionally for clinical risk factors.

Results: IgG galactosylation was negatively associated with prevalent and incident nephropathy and macrovascular disease after adjustment for clinical risk factors. Sialylation was negatively associated with incident diabetic nephropathy after adjustment for clinical risk factors. For incident retinopathy, similar associations were found for galactosylation, adjusted for age and sex.

Conclusions: We showed that IgG *N*-glycosylation, particularly galactosylation and to a lesser extent sialylation, is associated with a higher prevalence and future development of macro- and microvascular complications of diabetes. These findings indicate the predictive potential of IgG *N*-glycosylation in diabetes complications and should be analysed further in additional large cohorts to obtain the power to solidify these conclusions.

KEYWORDS
biomarkers, cox regression, glycobiology, ischaemic heart disease, nephropathy, peripheral artery disease

1 | INTRODUCTION

Type 2 diabetes is one of the most common chronic diseases worldwide. The long term microvascular and macrovascular complications cause an enormous burden of the disease.[1] It has been demonstrated that inflammation plays a key role in the pathophysiology of type 2 diabetes and its complications.[2] For instance, it has been shown that inflammatory markers have the most promise as warning signs of diabetic kidney disease (DKD).[3] Consequently, *N*-glycosylation of plasma IgG might contribute to pathophysiological processes of type 2 diabetes complications as glycosylation is known to influence IgG effector functions.[4] We therefore hypothesised that *N*-glycosylation of IgG may be involved in the development of complications of type 2 diabetes.

Posttranscriptional modifications of proteins are thought to play a role in almost every major disease. One of the most frequently occurring posttranscriptional protein modifications is glycosylation.[5] IgG *N*-glycosylation patterns vary between individuals and contain a genetic, heritable component.[6] However, IgG glycans show excellent temporal stability in a single individual and therefore have diagnostic potential.[7] Changes in IgG *N*-glycome composition are associated with a variety of diseases such as rheumatoid arthritis, inflammatory bowel disease, cancer, kidney disease and type 2 diabetes.[8–10] Recent retrospective studies have also shown associations between IgG glycosylation and diabetic retinopathy,[11] ischaemic stroke[12] and macrovascular risk score.[13] In addition, our team recently found relationships between *N*-glycosylation of total plasma proteins and type 2 diabetes complications.[14] However, to date, the effects of IgG glycosylation on clinical outcome in patients with type 2 diabetes have not been fully investigated. In the present study, we determined the relationships between IgG *N*-glycans and diabetic nephropathy, retinopathy and macrovascular complications in three large European prospective cohort studies. The outcomes can provide more insights into diabetes complication pathophysiology, contribute to biomarking development and identify patients with a higher risk profile for developing complications associated with type 2 diabetes.

2 | MATERIALS AND METHODS

2.1 | Patient cohorts

Three cohorts were included in this study. The DiaGene study has been described in more detail elsewhere.[15] In short, the DiaGene study is a multi-centre all-lines of care prospective cohort study. Between 2006 and 2011, individuals with and without type 2 diabetes were approached for participation in the region of Eindhoven, the Netherlands. Only cases with type 2 diabetes (*n* = 1886) were included in the current study.

The Hoorn Diabetes Care System (Hoorn DCS) is a cohort that comprises currently over 14,000 individuals with type 2 diabetes from the region of West Friesland in the Netherlands.[16] Annual examination data and biobanking materials for around 5500 persons, with the agreement to participate in DCS biobanks, have been collected. For this study, we randomly selected a subset of plasma samples from 1600 subjects who donated a sample in 2008/2009.

The GenoDiabMar study is a type 2 diabetes adult registry recruited between 2012 and 2015 from the healthcare area Litoral-Mar of Barcelona, Spain.[17,18] Patients older than 45 years with a medical history of type 2 diabetes for more than 10 years and treated with anti-diabetic drugs were included ($n = 640$ for the current study).

The medical ethics committees of the Erasmus Medical Center, VU University Medical Center Amsterdam, and Institut Mar d'Investigacions Mediques have approved DiaGene (DiaGene MEC 2004-230), Hoorn DCS (Hoorn DCS MEC 2007-57), and GenoDiabMar (MEC 2014/4323) studies, respectively. All individuals gave their written informed consent.

2.2 | Measurements and definitions of macro- and microvascular complications

In all three cohorts, data on micro- and macrovascular complications at inclusion and during follow-up were available. Definitions for vascular complications have been described in detail for DiaGene, GenoDiabMar and DCS cohorts.[15,16,18] In short, retinopathy for all studies was scored by an ophthalmologist based on fundus photography, and for the current study was defined as present or absent, where present includes all gradations and stages of diabetic retinopathy. Nephropathy was defined as microalbuminuria [albumin/creatinine-ratio (ACR) ≥2.5 mg/mmol for men or ≥3.5 mg/mmol for women] present at two of three consecutive measurements or when high micro-albuminuria or macro-albuminuria was present at one measurement (ACR ≥ 12.5 mg/mmol for men or ≥17.5 mg/mmol for women).[15,16,18] Cardiovascular disease derived from medical records and questionnaires was defined as myocardial infarction, percutaneous coronary intervention/coronary artery bypass graft (PCI/CABG), cerebrovascular accident, transient ischaemic attack, and peripheral arterial disease (PAD). Ischaemic brain disease (IBD) comprised both cerebrovascular incidents and transient ischaemic attacks combined. Our macrovascular endpoint consists of all the above macrovascular events combined.

2.3 | IgG N-glycosylation analysis and data quality control

In all three cohorts, IgG *N*-glycan profiles were determined at the moment of inclusion. IgG *N*-glycan isolation, release and labelling in the DiaGene and GenoDiabMar studies followed the same procedure and were described elsewhere.[10] Briefly, IgG was isolated from 100 μL of plasma per sample on a protein G monolithic plate (BIA Separations, Ajdovščina, Slovenia). Consequently, IgG *N*-glycans were released by PNGase F, fluorescently labelled with 2-aminobenzamide and cleaned up by hydrophilic interaction liquid chromatography solid phase extraction (HILIC-SPE) from the excess of reagents. IgG *N*-glycan profiles were obtained on an Acquity ultra performance liquid chromatography(UPLC) instrument (Waters, Milford, MA, USA) and separated into 24 glycan peaks. The relative number of glycans in each peak was expressed as a percentage (%) of the total integrated area. In Supplementary Table S1, a detailed description of the 24 glycan peaks is given. Of the 1886 participants with type 2 diabetes in the DiaGene study, plasma of 1837 participants was available. As 29 samples failed data quality control, a total of 1815 participants were included. In GenoDiabMar, data from 640 participants were used for statistical analysis after quality control.

Samples from the Hoorn DCS cohort were analysed using an ultrahigh resolution mass spectrometry method, which is described in detail in the Supplementary Method S1. In short, after capturing IgG from plasma samples, following enzymatic *N*-glycan release, sialic acids were stabilised by derivatisation. Subsequently, hydrophilic interaction liquid chromatography (HILIC) solid phase extraction and sample spotting were performed, and in line with previous literature, IgG *N*-glycans were measured by matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance (MALDI-FTICR) MS.[19] According to the mass spectrometry results, alpha 2–6 sialic acid linkages were found to be dominant, and only minor signals were observed that indicated the possible existence of trace amounts of alpha 2–3 sialylated species in the analysed samples. Consequently, alpha 2–3 sialylated species were excluded from the analysis. After performing quality control on mass spectra in the Hoorn DCS cohort, low intensity spectra were excluded, and 35 IgG glycan peaks were quantified. 19 glycan compositions were matched with the 24 UPLC glycan peaks and only the matching 19 peaks were used for further statistical analysis (Supplementary Table S1).

In all three cohorts, four IgG glycosylation derived traits were calculated for glycans that share structural similarities: fucosylation, galactosylation, sialylation, and bisecting GlcNAc as indicated in Supplementary Table S2. These features were used in the main analyses, with subsequent further detailed analyses in the 19 direct glycan peaks which were matched between the three cohorts.

2.4 | Statistical analyses

In baseline characteristics of the cohorts, continuous variables are expressed as mean and their standard deviations. Categorical variables are expressed as percentages.

Batch correction for glycan relative abundance was performed in all three cohorts. Initially, the normalisation of IgG glycans was performed using the total area under the expression peaks. After log-transformation of the relative abundance values, batch effects were corrected using the ComBat function in the R package *sva*, modified to correct for outliers.

Both cross-sectional and prospective analyses of the IgG glycome were adjusted for covariates known to influence vascular complications and/or IgG glycosylation. We considered three models: Model 1 adjusted for age, sex and their interaction. Age and sex are known to influence glycans, especially glycosylation profiles of females, with the biggest effect around menopausal age.[20] Model 2 was additionally adjusted for BMI, HDL-cholesterol, non-HDL-cholesterol, hypertension, HbA1c, duration of diabetes and smoking. To examine the direct correlation between IgG glycans and the

outcomes, all analyses were additionally performed without the addition of any covariables (model 0). The maximum proportion of imputation for clinical covariables in model 2 was 9.60% and 1.32% for DiaGene and DCS, respectively. In all cohorts, associations between prevalence (cross-sectional) and incidence (prospective) of macro- and microvascular complications and the 4 main IgG glycosylation features (fucosylation, galactosylation, sialylation and bisection) were evaluated using logistic and Cox regression models, respectively. Subsequently, a meta-analysis of these results was conducted. As a secondary analysis, associations between the 19 IgG glycan peaks and vascular complications were assessed in a similar manner. The presence of a vascular complication was entered as a dependent variable, and glycosylation features and adjusting covariates were entered as independent variables. For the prospective analyses, individuals with prevalent events at baseline were not included. Time-to-event was defined as the time between the date of inclusion and the date of the complication during follow-up, the date of death, or censoring at the end of follow-up. Across cohorts, results were random-effects meta-analysed using the metagen function from the R package meta. The Benjamini-Hochberg procedure was used to correct for multiple testing and an false discovery rate (FDR) adjusted p-value < 0.05 (FDR = 5%) was considered significant.

For statistical analyses, we used IBM SPSS Statistics version 25 in the DiaGene study, STATA version 15.1 in the GenoDiabMar study, and R (version R-3.6.3) and RStudio (version 1.4.1106) in the Hoorn DCS cohort.

3 | RESULTS

3.1 | Baseline characteristics

The clinical characteristics of the three study cohorts at baseline are shown in Table 1. All cohorts had a higher percentage of male participants, and the average age of patients with type 2 diabetes was approximately 66 years. The mean diabetes duration in the DiaGene, Hoorn DCS, and GenoDiabMar cohorts was 10.0 (SD = 8.4), 7.1 (±5.8), and 16.8 (±8.8) years, respectively. The mean duration of follow-up in these cohorts was 7.0 (SD = ±2.1), 7.5 (SD = ±2.3), and 4.7 (SD = ±1.3) years. In all cohorts, the most prevalent microvascular and macrovascular complications were nephropathy and ischaemic heart disease (IHD), respectively.

A graphical representation of all significant results and total results of IgG main glycan features can be found in Figure 1 and Supplementary Figure S1, respectively.

3.2 | Microvascular complications

IgG main glycan features that were significantly associated with vascular complications are shown in bold and highlighted in Tables 2 and 3. Several nominally significant results were found that did not remain significant after FDR correction; they are shown in Tables 2 and 3 highlighted only. Complete data on the individual 19 IgG *N*-glycan peaks outcomes can be found in Supplementary Table S3. Complete data on all IgG main glycan feature outcomes can be found in Supplementary Table S4. The level of heterogeneity as expressed by the I^2 is shown in Tables 2 and 3. For most significant findings the I^2 value was below 0.5, implicating low heterogeneity.

3.2.1 | Nephropathy

In the meta-analysis, galactosylation was negatively associated with prevalent (OR = 0.74, 95% CI = 0.65–0.84, $p_{FDR} = 3.23 \times 10^{-5}$) as well as incident diabetic nephropathy (HR = 0.81, 95% CI = 0.73–0.89, $p_{FDR} = 1.47 \times 10^{-4}$) in model 1. These associations remained significant after adjustment for clinical variables in model 2 (OR = 0.79, 95% CI = 0.68–0.93, $p_{FDR} = 1.21 \times 10^{-2}$ and HR = 0.86, 95% CI = 0.77–0.95, $p_{FDR} = 1.46 \times 10^{-2}$). The association of galactosylation with prevalent nephropathy in model 2 shows higher heterogeneity ($I^2 = 0.53$), indicating potential differences in effect between the meta-analysed cohorts. Sialylation was negatively associated with incident diabetic nephropathy in both models (model 1; HR = 0.83, 95% CI = 0.73–0.95, $p_{FDR} = 1.77 \times 10^{-2}$ and model 2; HR = 0.86, 95% CI = 0.78–0.96, $p_{FDR} = 1.46 \times 10^{-2}$). Figure 2 shows the effect sizes across all cohorts for galactosylation and sialylation regarding diabetic nephropathy. The GenoDiabMar cohort showed a larger effect size for nephropathy outcomes, which was most pronounced for galactosylation in model 2.

(Supplementary Figures S2–S4 show effect sizes across all cohorts for all vascular complications).

Considering the 19 IgG *N*-glycan peaks, the association with nephropathy appeared to be mainly driven by 2 glycan peaks. The strongest, statistically significant, negative associations were found for digalactosylated (GP14) and monosialylated digalactosylated (GP18) fucosylated biantennary glycans in model 1 in the prospective analysis (HR = 0.81, 95% CI = 0.72–0.91, $p_{FDR} = 2.57 \times 10^{-3}$ and HR = 0.81, 95% CI = 0.71–0.93, $p_{FDR} = 1.41 \times 10^{-2}$, respectively).

3.2.2 | Retinopathy

Galactosylation was negatively associated with diabetic retinopathy in model 1 in both cross-sectional (OR = 0.86, 95% CI = 0.78–0.95, $p_{FDR} = 5.96 \times 10^{-3}$) and prospective analyses (HR = 0.86, 95% CI = 0.77–0.97, $p_{FDR} = 4.18 \times 10^{-2}$). However, after adjustment for clinical confounders in model 2, these associations did not remain statistically significant. A negative association between bisection and diabetic retinopathy was found in model 1 in the cross-sectional analysis (OR = 0.86, 95% CI = 0.79–0.94, $p_{FDR} = 2.39 \times 10^{-3}$).

TABLE 1 DiaGene, Hoorn Diabetes Care Study (Hoorn DCS), and GenoDiabMar cohort characteristics.

	DiaGene	Hoorn DCS	GenoDiabMar
Number of participants, *n*	1815	1266	640
Sex, *n* (% male)	975 (53.7)	706 (55.8)	389 (60.8)
Age in years, mean (±SD)	65.2 (±10.5)	64.6 (±10.6)	69.65 (±9.3)
Duration of diabetes (years), mean (±SD)	10.0 (±8.4)	7.1 (±5.8)	16.82 (±8.8)
Duration of follow-up (years), mean (±SD)	7.0 (±2.1)	7.5 (±2.3)	4.7 (±1.3)
BMI, kg/m^2, mean (±SD)	30.5 (±5.5)	30.4 (±5.4)	30.31 (±5.1)
HbA1c, %, mean (±SD)	7.03 (±1.06)	6.78 (±1.0)	7.72 (±1.3)
HbA1c, mmol/mol, mean	53	51	61
Mean arterial pressure (mmHg), (±SD)	98.9 (±10.8)	99.0 (±11.3)	95.10 (±11.4)
HDL-c, mmol/L, mean (±SD)	1.17 (±0.3)	1.18 (±0.4)	1.22 (±0.3)
Non-HDL-c, mmol/L, mean (±SD)	3.12 (±0.9)	3.46 (±1.5)	3.27 (±0.9)
Hypertension *n* (%)	1042 (57.4)	910 (71.9)	585 (91.4)
Smoking			
Never, *n* (%)	602 (33.2)	783 (61.9)	259 (43.6)
Former, *n* (%)	921 (50.7)	269 (21.2)	222 (37.4)
Current, *n* (%)	292 (16.1)	214 (16.9)	113 (17.8)
Complications			
Prevalent IHD, *n* (%)	467 (25.7)	199 (15.7)	134 (20.9)
Incident IHD, *n* (%) (*n*-case/*n*-total)	45 (3.6)	71 (5.6)	74 (11.6)
Prevalent PAD, *n* (%) (*n*-case/*n*-total)	179 (9.9)	35 (2.8)	125 (19.5)
Incident PAD, *n* (%) (*n*-case/*n*-total)	93 (6.1)	17 (1.3)	54 (8.4)
Prevalent IBD, *n* (%) (*n*-case/*n*-total)	200 (11.0)	29 (2.3)	67 (10.5)
Incident IBD, *n* (%) (*n*-case/*n*-total)	63 (4.2)	14 (1.1)	28 (4.4)
Prevalent nephropathy, *n* (%) (*n*-case/*n*-total)	352 (19.4)	199 (15.7)	347 (54.2)
Incident nephropathy, *n* (%) (*n*-case/*n*-total)	243 (20.0)	131 (10.3)	129 (20.2)
Prevalent retinopathy, *n* (%) (*n*-case/*n*-total)	288 (15.9)	172 (13.7)	162 (25.3)
Incident retinopathy, *n* (%) (*n*-case/*n*-total)	220 (16.2)	104 (8.2)	134 (20.9)

Note: Details on the cohorts including definitions and biochemical measurements have been described elsewhere (15, 16, 18). For complications, numbers of participants are shown, and percentages are presented as *n*-case divided by *n*-total.

Abbreviations: BMI: Body mass index; HbA1c: Haemoglobin A1C; HDL-c: High-density lipoprotein (HDL) cholesterol; IBD: Ischaemic brain disease; IHD: Ischaemic heart disease; Non-HDL-c, cholesterol value minus HDL cholesterol; PAD: Peripheral artery disease; SD, standard deviation.

3.3 | Macrovascular complications

IgG main glycan features that were significantly associated with macrovascular complications are shown highlighted in Tables 2 and 3. Complete data on all IgG main glycan feature outcomes can be found in Supplementary Table S4. Complete data on the individual 19 IgG *N*-glycan peaks outcomes can be found in Supplementary Table S3. Complete data on the separate associations for the components of the macrovascular endpoint are shown in supplementary Table S4.

Regarding macrovascular complications, a negative association with galactosylation (OR = 0.86, 95% CI = 0.80–0.94, pFDR = 2.57×10^{-3}) and sialylation (OR = 0.88, 95% CI = 0.81–0.95, pFDR = 3.54×10^{-3}) was found in model 1 in the cross-sectional analysis. Galactosylation was also negatively associated with incident macrovascular complications (HR = 0.82, 95% CI = 0.73–0.93, pFDR = 6.35×10^{-3}) in model 1. This association remained significant after adjustment for clinical variables in model 2 (HR = 0.85, 95% CI = 0.74–0.96, pFDR = 4.16×10^{-2}). Crude models (model 0) for the macrovascular endpoints showed similar observations for galactosylation and sialylation albeit somewhat stronger effects that subside with the addition of age, sex (model 1) and additional covariables (model 2) (Supplementary Table S5).

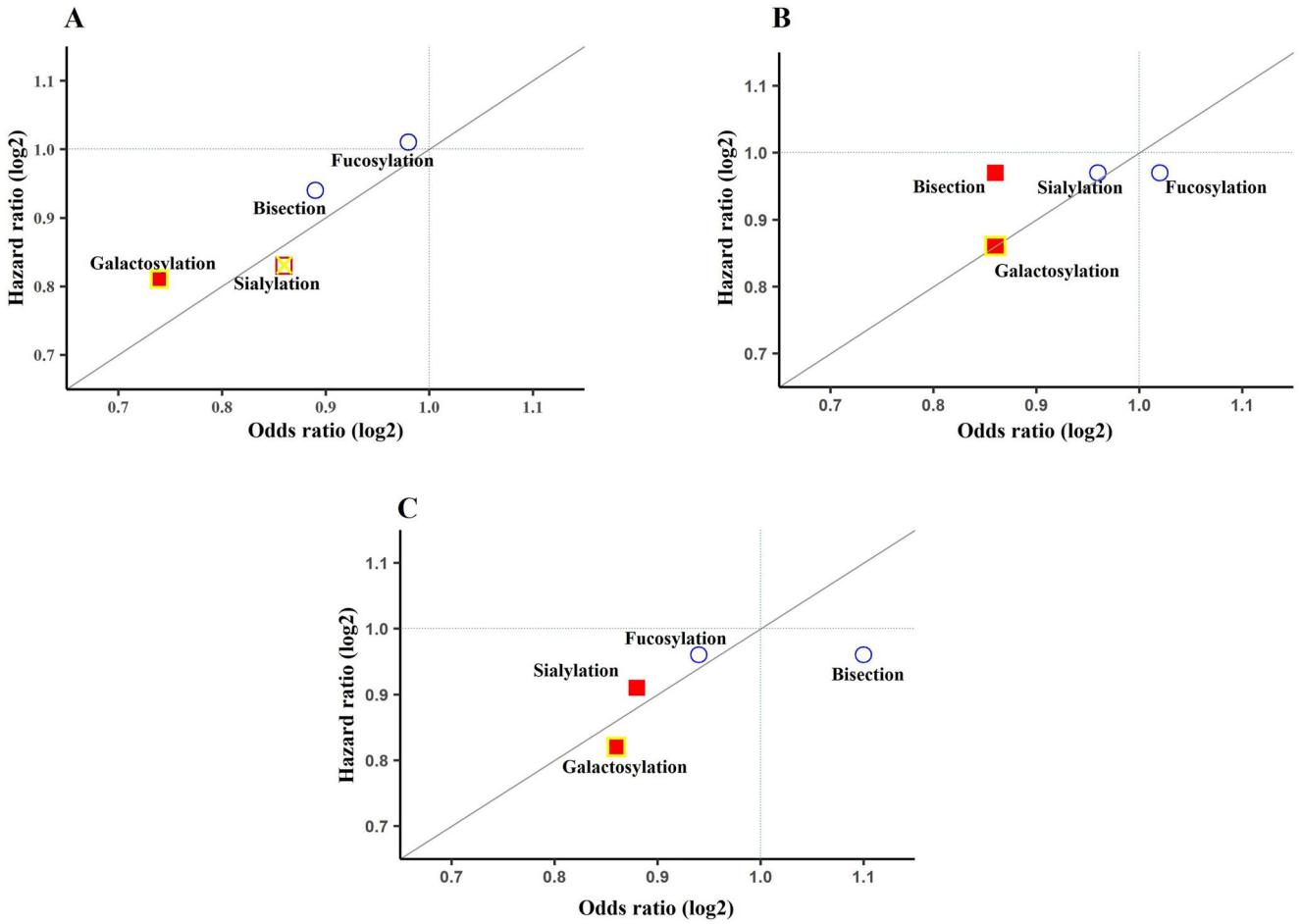

FIGURE 1 *N*-glycan-derived traits odds ratios (OR) and hazard ratios (HR) for meta-analysed data from DiaGene, Hoorn Diabetes Care Study, and GenoDiabMar studies in model 1 (adjusted for age, sex, and age × sex interaction). (A) Nephropathy, (B) Retinopathy, (C) Macrovascular. Red-filled yellow square: Significant in prevalent and incident complications after FDR correction. Red unfilled square with a yellow cross: Significant in prevalent complications before FDR correction and in incident complications after FDR correction. Red-filled square: Significant in prevalent complications after FDR correction. Yellow-filled circle: Significant in incident complications before FDR correction. Red-filled circle with a yellow cross: Significant in incident complications after FDR correction. Red unfilled circle: Significant in prevalent complications before FDR correction. Blue unfilled circle: Non-significant. FDR, false discovery rate.

4 | DISCUSSION

This study is the first to present associations between IgG *N*-glycosylation and prevalent as well as incident vascular complications of type 2 diabetes in three large cohort studies. In a meta-analysis, we found negative associations of galactosylation with prevalent and incident nephropathy and macrovascular disease, also after adjustment for clinical risk factors. Galactosylation was also negatively associated with prevalent as well as incident retinopathy in the age- and sex-corrected model. Furthermore, we found negative associations between sialylation and incident nephropathy that remained statistically significant after adjustment for clinical risk factors.

Type 2 diabetes and its complications result from complex interactions between environmental, metabolic and genetic factors and several of its risk factors are already known to influence IgG *N*-glycosylation patterns.[10] IgG *N*-glycan patterns are partly heritable and have temporal stability,[6,7,21] but can change in time by changes in cellular environment and disease, and have shown to influence IgG function.[4,22] Associations between inflammation and all microvascular and macrovascular complications have been demonstrated. Therefore, IgG glycans are potential biomarkers for complications of type 2 diabetes.

4.1 | Microvascular complications

The pathogenesis of nephropathy is complex, multifactorial and yet to be fully elucidated. We now demonstrate that galactosylation is negatively associated with both prevalent and incident diabetic nephropathy and that sialylation is negatively associated with incident diabetic nephropathy. It has been documented that

TABLE 2 Meta-analysed associations of main IgG glycosylation features with type 2 diabetes complications.

	Nephropathy						Retinopathy						Macrovascular (IHD, IBD, PAD)					
	Prevalent (logistic)			Incident (cox)			Prevalent (logistic)			Incident (cox)			Prevalent (logistic)			Incident (cox)		
Derived traits	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square
Galactosylation	**0.74**	**3.23×10^{-5}**	**0.46**	**0.81**	**1.47×10^{-4}**	**0**	**0.86**	**5.96×10^{-3}**	**0**	**0.86**	**4.18×10^{-2}**	**0**	**0.86**	**2.57×10^{-3}**	**0**	**0.82**	**6.35×10^{-3}**	**0**
Bisection	0.89	3.54×10^{-1}	0.74	0.94	3.43×10^{-1}	0	**0.86**	**2.39×10^{-3}**	**0**	0.97	5.60×10^{-1}	0	1.1	4.19×10^{-1}	0.84	0.96	5.42×10^{-1}	0
Sialylation	0.86	1.07×10^{-1}	0.51	**0.83**	**1.77×10^{-2}**	**0.36**	0.96	5.90×10^{-1}	0	0.97	5.60×10^{-1}	0	**0.88**	**3.54×10^{-3}**	**0**	0.91	5.05×10^{-1}	0.39
Fucosylation	0.98	6.67×10^{-1}	0	1.01	9.27×10^{-1}	0.71	1.02	5.90×10^{-1}	0	0.97	5.60×10^{-1}	0	0.94	1.42×10^{-1}	0	0.96	5.42×10^{-1}	0.22

Note: FDR-significant values are marked bold ($p < 0.05$), and values highlighted in yellow are nominal-significant (Model 1: Age, sex, age×sex interaction).

Abbreviations: FDR, false discovery rate; HR, hazard ratio; OR, odds ratio.

TABLE 3 Meta-analysed associations of main IgG glycosylation features with type 2 diabetes complications.

	Nephropathy						Retinopathy						Macrovascular (IHD, IBD, PAD)					
	Prevalent (logistic)			Incident (cox)			Prevalent (logistic)			Incident (cox)			Prevalent (logistic)			Incident (cox)		
Derived traits	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square	OR	*p*-value	*I*-square	HR	*p*-value	*I*-square
Galactosylation	**0.79**	**1.21×10^{-2}**	**0.53**	**0.86**	**1.46×10^{-2}**	**0**	0.88	5.38×10^{-2}	0	0.9	3.57×10^{-1}	0	0.91	9.54×10^{-2}	0	**0.85**	**4.16×10^{-2}**	**0**
Bisection	0.92	6.09×10^{-1}	0.67	0.95	5.07×10^{-1}	0	0.89	5.38×10^{-2}	0	1.01	8.84×10^{-1}	0	1.1	4.00×10^{-1}	0.82	0.96	5.14×10^{-1}	0
Sialylation	0.91	4.13×10^{-1}	0.39	**0.86**	**1.46×10^{-2}**	**0**	0.96	8.73×10^{-1}	0	0.98	8.84×10^{-1}	0	0.92	9.54×10^{-2}	0	0.93	5.14×10^{-1}	0.17
Fucosylation	0.98	6.54×10^{-1}	0.03	1.02	7.65×10^{-1}	0.61	1.01	8.73×10^{-1}	0	0.96	8.84×10^{-1}	0	0.93	9.54×10^{-2}	0	0.95	5.14×10^{-1}	0.14

Note: FDR-significant values are marked bold ($p < 0.05$), and values highlighted in yellow are nominal-significant (Model 2: Age, sex, age×sex interaction, BMI, HDL-cholesterol, non-HDL-cholesterol, hypertension, HbA1c, duration of diabetes and smoking).

Abbreviations: FDR, false discovery rate; HR, hazard ratio; OR, odds ratio.

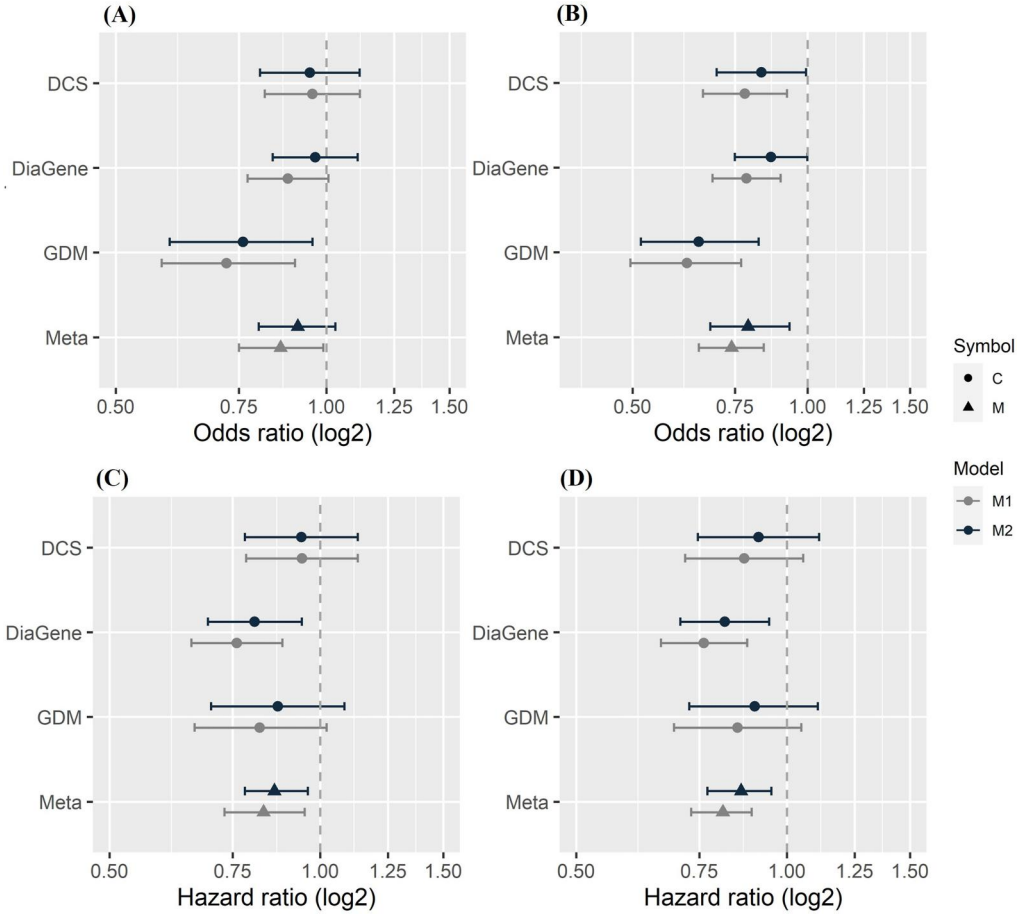

FIGURE 2 Forest plots of associations of *N*-glycan sialylation and galactosylation-derived traits with nephropathy. Data from DiaGene, Hoorn Diabetes Care Study, and GenoDiabMar (GDM) studies and meta-analysed data are from model 1 and 2 analyses. (A) IgG Sialylation in Nephropathy (prevalent), (B) IgG Galactosylation in Nephropathy (prevalent), (C) IgG Sialylation in Nephropathy (incident), (D) IgG Galactosylation in Nephropathy (incident).

hyperglycaemia in type 2 diabetes induces the activation of inflammatory pathways, causes advanced glycation end-products, reactive oxygen species and activation of RAAS, eventually leading to tissue damage and vascular complications.[23–25] Subsequent pro-inflammatory cascades lead to changes in kidney structure and function. The essential role of inflammation, and involvement of IgG was shown in vivo by Lopez-Parra et al.,[26] demonstrating that Fcγ receptor blockade is renoprotective, reducing renal hypertrophy, inflammation, and fibrosis in diabetic mice. There is evidence to suggest that galactosylation and sialylation can alter the effector function of IgG. Studies have shown the relationship between low galactosylation in IgG *N*-glycans and increased disease severity in various auto-immune diseases, indicating the ability of galactose to decrease inflammation.[22] Notably, nonclinical laboratory studies have found contradictory results with an increase in galactosylation induced inflammation through the alternative and lectin route of the complement system[9] or enhancement of the classical pathway of the complement cascade.[27] These nonclinical laboratory findings are, however, not in line with findings in most clinical studies so far. Additionally, IgG is thought to acquire anti-inflammatory properties upon glycan sialylation as it potentially decreases binding affinities for Fcγ receptors and total IgG sialylation decreases during an active pro-inflammatory immune response.[28] However, the exact biological mechanism remains incompletely understood to date. Thus, galactosylation and sialylation cannot be considered anti-inflammatory in all circumstances due to the complexity of biological pathways.

The associations found in this study are in line with our previous work in the DiaGene Study where monogalactosylated structures and GP14 (FA2G2) were associated with slower kidney function decline.[29] In a non-diabetic population, Barrios et al.[8] found that individuals with galactosylated (notably GP14) and sialylated IgG glycans had a lower risk of developing chronic kidney disease. Furthermore, another study showed that agalactosylated IgG is associated with a more rapid eGFR decline in type 1 diabetes.[30]

Similarly in retinopathy, hyperglycaemia activates inflammatory processes leading to oxidative stress, vascular leakage and eventually ganglion cell loss in the retina.[31] We found negative associations for galactosylation and bisection in the cross-sectional

analysis. Galactosylation was also negatively associated with retinopathy prospectively in model 1, which could indicate an indirect effect through clinical risk factors or merely lower power in model 2 as the HR was similar to model 1. As discussed above, galactosylation is thought to have an anti-inflammatory effect on IgG. Conversely, in vitro studies have shown that bisecting GlcNAc increases FcγRIII affinity and results in a pro-inflammatory effect.[32,33] The negative association with bisection therefore seems contradictory and cannot be fully explained. Recently, Wu et al.[34] found similar associations with diabetic retinopathy in a retrospective study. Whether this is an effect associated with an inflammatory response triggered in the presence of retinopathy is unknown. We did not see a similar association in the prospective analyses, making it difficult to determine whether the finding is cause or consequence, although our results suggest the latter.

4.2 | Macrovascular complications

For macrovascular complications, we found that galactosylation was inversely associated both cross-sectionally and prospectively, and remained so after correction for clinical risk factors. Sialylation was only cross-sectionally associated with macrovascular disease cross-sectionally in the basic model 1. The number of macrovascular events in these analyses is smaller than for our microvascular analyses and therefore some caution should be exerted. These findings indicate a possible pro-inflammatory state of IgG associated with macrovascular disease. Atherosclerosis is a chronic inflammatory condition of the artery walls. Also, risk factors of macrovascular disease are known to influence IgG *N*-glycosylation patterns.[10,35,36] Therefore, the associations found could (partly) be attributed to the influence of risk factors on IgG glycosylation. Even though we have corrected for many of those risk factors in model 2, residual effects cannot be excluded. The effect of risk factors on these associations is also illustrated when comparing the ORs and HRs of the crude model 0 to models 1 and 2. It shows that the effect size of the glycosylation trait becomes considerably smaller when adding more cardiovascular risk factors. Recently, Menni et al.[13] described associations between IgG *N*-glycan patterns and 10-year atherosclerotic cardiovascular disease risk score in individuals without diabetes. Among the glycan traits associated with a low macrovascular risk score found by Menni et al. were GP14 and GP18, the former containing galactose, the latter containing both galactose and sialic acid. Interestingly, these glycan peaks had a similar direction of effect as the corresponding glycan features in our prospective analyses. For comparison with the study of Menni et al, we should keep in mind that in diabetes, different mechanisms may contribute to atherogenesis compared to individuals without diabetes.

Overall the I^2 values for the meta-analyses were low, implicating comparable effects across cohorts for the associations. As depicted in Figure 2, the cross-sectional differences in glycosylation profiles in relation to nephropathy were more evident in the GDM cohort, a cohort with a high prevalence of nephropathy. For galactosylation and prevalent nephropathy this results in one slightly higher I^2. Overall, this reinforces our findings and supports the relationship between IgG *N*-glycan patterns and nephropathy, which is stronger in populations with a high burden of disease.

4.3 | Strengths and limitations

A major strength of this study is the use of three large independent populations that represent both primary and secondary type 2 diabetes care, which allowed us to meta-analyse our findings and optimise statistical power. Furthermore, extensive phenotyping in all cohorts allowed adjustment for known confounders of type 2 diabetes complications and known factors affecting IgG *N*-glycosylation. In addition, due to the prospective nature of our studies, we were able to indicate the temporal sequence between IgG *N*-glycan patterns and diabetic complications. For nephropathy, macrovascular disease and partly for retinopathy, the findings suggest that IgG *N*-glycosylation patterns precede the onset of the complication. This is important in light of biomarker potential and gives us a glance at the potential role of IgG *N*-glycosylation in the pathophysiology of diabetes complications. Future experimental studies and Mendelian randomisation should be conducted to further clarify this potential causation. However, considering causality, it cannot be excluded that preclinical aspects of nephropathy were present at baseline that have driven the association rather than IgG *N*-glycosylation having a causal effect on future complication development.

In our study, IgG *N*-glycosylation was measured at baseline in all cohorts. It is known that glycosylation can change in case of acute inflammation,[37] and that glycans remain fairly stable over time.[7] Nonetheless, multiple measurements during follow-up may have given a better insight into the impact of glycosylation on diabetic complications. Likewise, for the prospective analyses, one cannot exclude any changes in confounding covariables during the lag time of follow up that may impact the relationship between IgG glycan measurement and the outcome. In addition, the cohorts did not have serum markers of inflammation, such as CRP, available to investigate inflammation as an underlying mechanism more in depth.

Even though this is one of the largest studies to investigate the relationship between IgG *N*-glycans and vascular complications to date, macrovascular complications are still quite rare and thus underpowered. Of note, 27% of the participants in the three cohorts had more than one vascular complication at baseline. It is possible that the same IgG *N*-glycan patterns precede or succeed different vascular complications and may thereby confound each other's association. This would, however, have led to an underestimation rather than an overestimation of the results. Due to limited statistical power, it was not possible to adjust for the presence of multiple complications.

4.4 | Conclusion

To conclude, in three large type 2 diabetes cohorts, we show that IgG *N*-glycosylation is significantly associated with prevalent and incident nephropathy, retinopathy and macrovascular disease. The IgG associated glycosylation features are in line with previous reports on IgG glycosylation in other health conditions where inflammation plays a key role. This fuels genetic, experimental and epidemiological studies to dive further into the underlying mechanisms and predictive potential of IgG *N*-glycosylation in the context of diabetes complications.

AUTHOR CONTRIBUTIONS

Elham Memarian developed an automated method for glycomics study and performed glycomic analysis of the Hoorn DCS cohort, processed Hoorn DCS glycomics raw data, developed the R-scripts for data visualisation, and wrote and reviewed/edited the manuscript. Ralph Heijmans completed the DiaGene database, performed statistical analyses, and wrote and reviewed/edited the manuscript. Roderick C. Slieker performed statistical analyses and reviewed/edited the manuscript. Adriana Sierra contributed to the collection and design of the GenoDiabMar (GDM) study and reviewed/edited the manuscript. Olga Gornik reviewed/edited the manuscript. Joline W. J. Beulens coordinated the DCS study and reviewed/edited the manuscript. Maja Hanic performed glycomic analysis of the DiaGene cohort and reviewed/edited the manuscript. Petra Elders coordinated the DCS study and reviewed/edited the manuscript. Julio Pascual reviewed/edited the manuscript. Eric Sijbrands contributed to the collection, design, and coordination of the DiaGene study and reviewed/edited the manuscript. Gordan Lauc contributed to the conception of the research question, reviewed/edited the manuscript, and contributed to the discussion. Viktoria Dotz supervised automated-method development for glycomics study in DCS cohort, developed R-script for MS data processing, interpreted results, reviewed/edited the manuscript, and contributed to the discussion. Clara Barrios coordinated the GDM study, contributed to the conception of the research question, reviewed/edited the manuscript, and contributed to the discussion. Leen M. 't Hart contributed to the coordination of the Hoorn DCS Study, performed statistical analyses, and reviewed/edited the manuscript. Manfred Wuhrer supervised the glycomics study, contributed to the discussion, and reviewed/edited the manuscript. Mandy van Hoek contributed to the conception of the research question, collection, and coordination of the DiaGene study, reviewed/edited the manuscript, and contributed to the discussion.

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CONFLICT OF INTEREST STATEMENT

Manfred Wuhrer is the inventor on a patent application on sialic acid derivatisation by ethyl esterification, and Elham Memarian and Maja Hanic are employed by Genos Ltd; Viktoria Dotz is currently employed by Janssen Biologics BV. No other potential conflicts of interest relevant to this article were reported.

ETHICS STATEMENT

None.

DATA AVAILABILITY STATEMENT

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

STATEMENTS OF ASSISTANCE

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GUARANTOR

Mandy van Hoek 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.

ORCID

Elham Memarian https://orcid.org/0000-0001-5862-4375
Roderick C. Slieker https://orcid.org/0000-0003-0961-9152
Maja Hanic https://orcid.org/0000-0001-6152-2054
Eric Sijbrands https://orcid.org/0000-0001-8857-7389
Viktoria Dotz https://orcid.org/0000-0002-9888-7409
Leen M. 't Hart https://orcid.org/0000-0003-4401-2938
Mandy van Hoek https://orcid.org/0000-0002-2957-5436

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