Variation of IgG N-linked glycosylation profile in diabetic retinopathy

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Original investigation

Keywords: Diabetic retinopathy, IgG, Glycosylation, Biomarker

Posted Date: September 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-74884/v1

Full Title: Variation of IgG N-linked glycosylation profile in diabetic retinopathy

Running Head： IgG glycosylation and diabetic retinopathy

Word counts: 2424 words

Number of tables: 3

Number of figures: 3

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Abstract

Background: The relationship of IgG glycosylation with diabetes and diabetic nephropathy has been reported, while its role in diabetic retinopathy (DR) remained unclear. We aimed to investigate and validate the association of IgG glycosylation with DR.

Methods: We analyzed the IgG N-linked glycosylation profile and identified the specific panel in the discovery population using binary logistics model. Findings were validated in the replication population. The discriminative capacity of IgG glycosylation panel was explored by ROC analysis using cross validation and Brier score. Multiple sensitive analyses were performed on the whole population.

Results: 2 IgG glycans (GP15, GP20) and 2 derived traits (IGP32, IGP54) were identified and validated significantly associated with DR ($P<0.05$), and the adjusted OR were 0.676, 0.671, 1.770, 0.681 in combined population, respectively. The glycosylation panel achieved an average AUC of 0.67 and 0.60 in the discovery and replication population. The association was independent of blood pressure, glucose and lipids, thus improving the ROC and Brier score when the panel added. In addition, the results remained consistent when the controls were re-defined and 1:3 re-matched.

Conclusions: IgG glycosylation profile reflecting a pro-inflammatory status were associated with DR. The variation of IgG glycome deserves more attention in the aggravation of diabetes and the underlying mechanism warrants further research.

Keywords: Diabetic retinopathy; IgG; Glycosylation; Biomarker

Background

Type 2 diabetes, characterized by abnormal glycometabolism and impaired insulin function, has become a serious threat to global health. Type 2 diabetes accounts for the vast majority (around 90%) of diabetes worldwide and it is estimated that 171 million people have diabetes in 2000 and this number is projected to reach 366 million by 2030 [1]. People living with type 2 diabetes are at a higher risk of developing life-threatening complications, such as diabetic retinopathy (DR). About one third of individuals with diabetes have different degrees of DR, which is a common microvascular disease and the leading cause of blindness in the working-aged population [2,3]. In recent years, people with prediabetes, characterized by impaired glucose tolerance and/or impaired fasting glucose, are also increasingly, which signifies a potential risk of the future development of type 2 diabetes and DR [4]. However, the etiological mechanism of the aggravation of diabetes status remains unclear and the potential biological targets related with the onset of DR are urgently needed.

Glycometabolism is influenced by the interaction of genetic and environmental factors [5], among which glycosylation is one of the most common and posttranscriptional modifications. The glycans attached to proteins exert crucial biological effect including cellular recognition and molecular pathway regulation [6]. The variation of IgG glycans are mostly investigated and the covalently attached glycans are reported to be associated with the stability of IgG protein and its pro-inflammatory or anti-inflammatory effects [7]. Recently, the variation of IgG

glycans are emerging as potential biomarkers and biological pharmacological targets of various metabolic diseases, such as aging [8], dyslipidemia [9], immune disease [10] and type 2 diabetes [11-12]. In fact, type 2 diabetes is accompanied by glucose metabolic disorder and the impaired function of inflammation regulation. Moreover, the IgG glycosylation profiles have been linked with the risk factors of type 2 diabetes, such as obesity [13], blood pressure [14,15] and fasting blood glucose (FBG) [16]. Therefore, it is rational to infer that the specific IgG glycans or traits play an important role in the pathological process of DR. Lemmers et al. [11] and our team [12] have identified the differential IgG glycans between the diabetes population and health controls. However, the biological effect of the IgG glycosylation profile in the development of DR remains unclear.

In this study, we aim to investigate the association of the IgG glycosylation with the onset of DR, thus to identify the early glycome biomarkers related with DR.

Methods

Study design and population

In 2015, 54 subjects of new-onset DR and 108 matched controls (22 prediabetes and 86 diabetes), from the Beijing health management cohort, were enrolled in this study as the discovery population. Subsequently, 54 cases of DR and 108 matched controls (18 prediabetes and 90 diabetes) were recruited in 2016 as the replication population. The Beijing health management cohort is an ongoing population-based study of participants aged ≥18 years for metabolism-related diseases research, beginning from Jan 2008 [17]. All the participants in this cohort were asked to take in physical and

biochemical examinations, and the plasma samples were separated from the fasting blood for subsequent glycosylation experiment.

The following inclusion criteria were required: (1) signing informed consent prior to enrollment; (2) at least 18 years old; and (3) new onset of DR. The exclusion criteria were as follows: (1) history of type 1 diabetes; (2) history of mental illness, infectious disease, cardiovascular diseases, stroke, liver disease, renal failure, cancer or autoimmune diseases; (3) unable to collect the required data. This study was conducted following the Declaration of Helsinki and was approved by the Capital Medical University Ethics Committee.

IgG glycosylation experiment

The glycosylation experiment and analysis composed of four major processes: IgG protein isolation and purification from plasma, N-linked glycans release and fluorescence labeling, glycans quantitative detection, direct glycans and derived traits computation, as described previously [18, 19]. In brief, IgG protein was obtained from 2ml plasma using 96-well protein G monolithic plates using 1×phosphate buffer saline, 0.1 M formic acid and 1 M ammonium bicarbonate; the N-linked glycans were released from the purified IgG protein at 37℃ using 1.5 units of PNGase F and 5×phosphate buffer saline for 18 hours; subsequently, the released glycans were fluorescently labelled using 2-AB at 65℃ for 3 h and isolated with chromatography phase; the direct glycans were quantitatively detected using ultra-performance liquid chromatography platform (Waters, America) and the glycan traits were derived accordingly.

Finally, 24 direct glycan peaks (GP) were presented and quantitatively expressed with the percentage of the total integrated peak area. In addition, 54 glycan traits (IGP) were derived to reflect the relative abundance of the specific structure, such as galactosylation, sialylation, bisecting *N*-acetylglucosamine (GlcNAc), core fucosylation and mannose. The detailed information of each GP and IGP were shown in **Appendix Table A.1**. The amounts of GP and IGP were normalized by log-transformation and the batch size was considered and corrected for the subsequent analysis.

Covariates

The demographic characteristics like age and sex were obtained at baseline by questionnaires. The body mass index (BMI) was defined as weight (in kilograms)/height^2 (in meters squared), and was divided into <25 and ≥ 25. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were presented as the mean of twice measures on the right arm using sphygmomanometer after resting at least 10 min. High blood pressure (HBP) was defined as SBP ≥ 140 or DBP ≥ 90 accordingly. The fasting blood glucose (FBG) was measured after overnight fasting and the postprandial blood glucose (PBG) was measured after 2 hours from the beginning of meals using the glucose oxidase-peroxidase method (Mind Bioengineering Co. Ltd., Shanghai, China). Triglyceride, total cholesterol (TC), high density lipoprotein cholesterol (HDL-cholesterol, HDLC), low density lipoprotein cholesterol (LDL-cholesterol, LDLC) were measured with the Olympus Automatic Biochemical Analyzer (Hitachi 747; Tokyo, Japan).

Outcomes

Prediabetes, diabetes and DR were defined by physicians and ophthalmologists according to the American Diabetes Association standards [20] and the International Clinical Diabetic Retinopathy Disease Severity Scale [21] as follows: (1) prediabetes: 7.0>FBG>6.1 mmol/L, or 11.1>PBG≥7.8 mmol/L (2) diabetes: FBG≥7.0 mmol/L, or PBG≥11.1mmol/L, or regular use of anti-diabetes drugs, or history of diabetes; (3) DR: diagnosed by ophthalmologists among diabetes patients. The related signs include microarteoma, vein bead, intraretinal microvascular abnormalities, neovascularization, vitreous or retinal hemorrhage according to the mydriatic fundus examination by slit lamp and fundus photography.

Statistical analysis

Continuous variables adhering to the normal distribution were represented as the mean ± standard deviation (SD) and the differences between groups were tested by the independent student t tests; otherwise, the interquartile range (P_{25} - P_{75}) was used and the differences between groups were explored by Mann-Whitney U tests. Categorical variables were presented as n (%), and the differences were tested by the chi-square tests. The box plots were used to show the distribution of IgG glycans and traits between groups.

The controls were 1:2 matched based on age, sex and BMI. Binary logistics model was used to identify the IgG glycans and traits associated with the onset of DR in both discovery and replication population. Moreover, the association was explored after confounding covariates (age, sex, BMI, blood pressure, glucose and lipids) adjusted.

Further, the discriminative capacity of the differential IgG glycans and traits were explored by ROC analysis using 5-fold cross validation and calibration assessment using Brier score. In addition, the sensitive analyses were performed in the following situations: the ordinal logistics model was used to identify the substantially changed glycans and traits when the controls were re-defined as prediabetes and diabetes; the controls were 1:3 matched and re-analyzed.

All reported *P* values were two-tailed, and $P<0.05$ was considered statistically significant. All the analyses presented above were performed using the R software (version 3.6.3).

Results

Demographic and clinical characteristics

In the discovery population, the mean age of this population was 61.0 (range from 37.0 to 93), involving 131 males (80.9%). In the replication population, the mean age of this population was 60 (range from 27 to 88), involving 135 males (83.3%). The demographic characteristics were similar between the discovery and replication populations. Also, there were no significant difference in HBP, TG, LDLC, HDLC between DR group and the controls, while TC declined in DR group. The detailed information was shown in **Table 1**.

Associations of IgG glycosylation and DR

In the discovery population, 6 glycans and 9 traits were primarily identified. Then, GP15, GP20, IGP32, IGP54 were validated in the replication population as the glycosylation panel associated with DR. The distribution of the panel was shown in

Figure 1 and the adjusted OR in the combined population were 0.676, 0.671, 1.770, 0.681, respectively, as shown in **Table 2**. The detailed distribution of all these glycans and traits were shown in **Appendix Table A.2**.

Further, discriminative capacity of the glycosylation panel was shown in **Table 3** and the average AUC of 5 folds were 0.67 and 0.60 in the discovery and replication populations, respectively. In addition, the AUC and Brier score were slightly improved when the panel added to the simple model involving age, sex, BMI, HBP, FBG, DBG and blood lipids, as shown in **Figure 2**.

Sensitive analyses

The sensitive analyses were performed on the whole population due to the sample size. On one hand, the controls were separately defined as prediabetes and diabetes. And GP15, GP20, IGP54 were substantially decreased, while IGP32 increased. In the ordinal multivariable model, GP15 (OR: 0.64; 95%CI: 0.49-0.82), GP20 (OR: 0.60; 95%CI: 0.46-0.78), IGP32 (OR:1.94; 95%CI: 1.47 -2.61), IGP54 (OR:0.66; 95%CI: 0.51-0.85) still remained significantly associated with DR, as shown in **Figure 3(A)**. On the other hand, the control population was updated to 1:3 matching. Then, GP15 (OR: 0.74; 95%CI: 0.57-0.96), IGP32 (OR:1.58; 95%CI: 1.15-2.23), IGP54 (OR:0.74; 95%CI: 0.57-0.96) remained significantly associated with DR, while GP20 (OR: 0.81; 95%CI: 0.63-1.05) changed to indistinctive positive correlation in the multivariable model, as shown in **Figure 3(B)**.

Discussion

In this study, we investigated the relationship of IgG glycosylation profile and DR in

two matched populations. The panel of GP15, GP20, IGP32, IGP54 was validated to be strongly associated during the pathological process from prediabetes or diabetes to DR, which could also improve the discriminative capacity of the simple model. Moreover, the association remained consistent in the re-defined and re-matched populations. We proposed that the specific variation of IgG glycosylation profile, independent from the common clinical factors, plays an important role in the pathological process of DR. Meanwhile, the panel of GP15, GP20, IGP32, IGP54 could be potential biomarkers and drug targets, which could contribute to the early prevention and treatment of DR.

Both genetic and environmental factors affect the incidence and development of diabetes and its complications, and the glycosylation of IgG proteins is one of the most common post-translational modifications which is involved in almost all physiological processes of the body, such as signal pathways, cellular immunity, and the mutual recognition of proteins [22]. The variation of IgG glycosylation profiles, reflecting both the genetic and environmental characteristics [23], are reported to be associated with various diseases, especially the autoimmune diseases, and chronic metabolic and inflammatory diseases [24-26]. In fact, both the glycometabolism disorder and impaired immunologic function involve in the pathophysiological process of diabetes and DR. In this study, we found that GP15, GP20, IGP54 decreased and IGP32 increased, instead. The variation of IgG glycans and traits were in accordance with a decrease of digalactosylated biantennary glycan with bisecting GlcNAc and core fucose (GP15), digalactosylated monosialylated biantennary with

core and antennary fucose (GP20), digalactosylated biantennary glycan with core fucose structures in total neutral IgG glycans (IGP54) and an increase of disialylation of fucosylated digalactosylated structures with bisecting GlcNAc (IGP32).

The results above were largely in consistent with previous studies of IgG glycosylation profiles in type 2 diabetes and its related factors. Previous studies have reported that complex glycan structures with bisecting GlcNAc were highly associated with some inflammatory diseases, reflecting a body status of pro-inflammation [11,27]. IgG proteins were sensitive to biological inflammatory stress and the variation of IgG glycans could reverse its anti-inflammation function [28,29]. Therefore, the substantially increased proportion of the complex glycan structures such as disialylation of fucosylated digalactosylated structures with bisecting GlcNAc may be induced by the biological inflammation in the process of glucose aggravation and DR. In addition, the decreased proportion of galactosylation, accompanied by decreased percentage of sialylation as the sialic acids were attached to the galactose, was thought to strengthen the complement-dependent cytotoxicity (CDC) effect of IgG [30,31]. And the presence of bisecting GlcNAc and lack of core or antennary fucose were thought to strengthen the antibody-dependent cell-mediated cytotoxicity (ADCC) effect of IgG [29,32]. Both the CDC and ADCC effects of IgG were reported to switch its anti-inflammation role to pro-inflammation. Consistently, Lemmers et al. [11] found an glycosylation pattern of decreased galactosylation, sialylation, fucosylation structures and increased bisecting GlcNac structures associated with type 2 diabetes based on a European population. On a further step, we

found that the similar IgG glycosylation pattern was associated with the aggravation of diabetes from prediabetes or diabetes to DR in this study. The panel was related with an overall decrease digalactosylated fucosylated structures with and without GlcNAc, with monosialytion or without sialic acid. Moreover, the structures of bisecting GlcNac and disialylation seemed to exert synergetic effect in DR.

The strength of our study was that we analyzed the variation of IgG glycosylation profiles and identified the IgG glycans and traits associated with DR for the first time. As far, FBG, PBG and insulin resistance index have been applied in the diagnosis and intervention of diabetes and DR. It is of great importance of discover more metabolic biomarkers and potential drug targets for the prevention and treatment of DR [33]. However, the results should be interpreted in the context of some limitations. First, the sample size was relatively small and we could not claim a casual association due to lack of prospective. Second, the discriminative capacity of IgG glycosylation panel in differentiating DR was relatively poor, although it could improve the capacity of the generally monitored clinical factors. The biological mechanism of IgG glycosylation profiles in DR or other diabetic complications warrants further investigation in animal or cell level. Third, our study was based on the Chinese population, more collaborations are needed to validate the generalizability of results.

Conclusions

In general, IgG glycosylation profile, reflecting a pro-inflammatory status, was validated to be associated with DR. The variation of IgG glycans and traits could be novel biomarkers and potential drug targets for DR and other diabetic complications

which warrants further investigation.

List of abbreviations

DR: Diabetic retinopathy; **BMI**: Body mass index; **FBG**: Fasting blood glucose; **PBG**: Postprandial blood glucose; **SBP**: Systolic blood pressure; **DBP**: Diastolic blood pressure; **HBP**: High blood pressure; **TC**: Total cholesterol; **HDLC**: High density lipoprotein cholesterol; **LDLC**: Low density lipoprotein cholesterol; **GP**: Glycan peak; **ADCC**: antibody-dependent cell-mediated cytotoxicity; **CDC**: complement-dependent cytotoxicity.

Declarations

Ethics approval and consent to participate

The study followed the guidelines of the Helsinki Declaration, and was approved by the Ethics Committees of Capital Medical University.

Consent for publication

All participants have given the consent for publication.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

Our work was funded by the Program of Natural Science Fund of China (Serial Number: 81530087).The study funder was not involved in the design of the study; the collection, analysis, and interpretation of data; writing the report; and did not impose any restrictions regarding the publication.

Authors' contributions

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Acknowledgements

Not applicable

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Tables

Table1

The characteristics of participants in the discovery and replication populations.

	Discovery population			Validation population		
	Controls(n=108)	**DR (n=54)**	**P value**	**Controls(n=108)**	**DR (n=54)**	**P value**
Age†	60.41(12.22)	62.50(11.36)	0.295	59.26(11.85)	60.41(11.10)	0.554
Sex (male)‡	87(80.6)	44(81.5)	1	89(82.4)	46(85.2)	0.823
BMI (≥25)‡	70(64.8)	35(64.8)	1	80(74.1)	40(74.1)	1
FBG†	7.01[6.38,8.32]	8.00[6.52,10.05]	0.01	7.25[6.41,8.55]	8.00[6.46,9.68]	0.068
PBG†	11.00[9.40,13.17]	12.40[10.15,14.55]	0.08	10.80[9.70,13.31]	11.90[9.88,14.40]	0.134
HBP (yes)‡	35(32.4)	18(33.3)	1	37(34.3)	11(20.4)	0.1
TG §	1.43[1.06,1.88]	1.36[0.88,1.86]	0.423	1.43[0.97,2.06]	1.29[0.90,1.70]	0.123
LDLC §	2.69[2.15,3.47]	2.71[1.98,3.26]	0.243	2.71[2.15,3.45]	2.25[1.94,3.10]	0.041
HDLC §	1.35[1.05,1.58]	1.22[0.95,1.41]	0.049	1.25[1.06,1.45]	1.16[1.03,1.33]	0.101
TC §	4.55[3.91,5.40]	4.19[3.36,4.97]	0.018	4.41[3.87,5.25]	3.99[3.22,4.83]	0.004

† mean (SD), student t test; ‡ numbers of each category (%) are given, chi-square test;
§ median (P25 - P75), Mann-Whitney U test.

Table 2

Associations of IgG glycosylation and DR by binary logistics model.

	Discovery Population		Validation Population		Combined Population	
	OR	P value	OR	P value	OR	P value
GP15						
univariate	0.604	0.007	0.678	0.033	0.633	0.000
multivariate†	0.617	0.016	0.597	0.015	0.676	0.006
GP20						
univariate	0.654	0.016	0.640	0.011	0.608	0.000
multivariate†	0.643	0.022	0.585	0.025	0.671	0.008
IGP32						
univariate	1.898	0.009	1.861	0.010	1.995	0.000
multivariate†	2.123	0.005	1.813	0.023	1.770	0.002
IGP54						
univariate	0.587	0.005	0.677	0.033	0.635	0.000
multivariate†	0.609	0.013	0.610	0.018	0.681	0.007

† Age, sex, BMI, HBP, FBG, PBG, TG, TC, HDLC, LDLC were adjusted in the multivariate model.

Table 3

The AUC of IgG glycosylation panel using 5-fold cross validation.

	1-fold	2-fold	3-fold	4-fold	5-fold
Discovery Population	0.637	0.713	0.658	0.596	0.722
Validation Population	0.576	0.626	0.536	0.634	0.610
Combined Population	0.609	0.721	0.655	0.658	0.693

Figures:

Figure 1: The distribution boxplot of IgG glycosylation panel in the discovery and replication populations.

Figure 2: The discriminative capacity of IgG glycosylation panel.

Legend:

A: The ROC and Brier score with IgG glycosylation panel in the discovery population;

B: The ROC and Brier score with IgG glycosylation panel in the replication population;

C: The ROC and Brier score with IgG glycosylation panel in the combined population.

Simple model: involving age, sex, BMI, HBP, FBG, PBG, TG, TC, HDLC, LDLC;

Complex model: IgG glycosylation panel added in the simple model.

Figure 3: Results of sensitive analyses.

Legend:

A: Association of the IgG glycosylation and DR by ordinal logistics model given the controls defined as prediabetes and diabetes;

B: Association of the IgG glycosylation and DR in 1:3 matched population.

Supplementary materials:

Table A.1: The detailed descriptions of the IgG glycans and traits.

Table A.2: Distribution of all IgG glycans and traits in the discovery and replication populations.

Figures

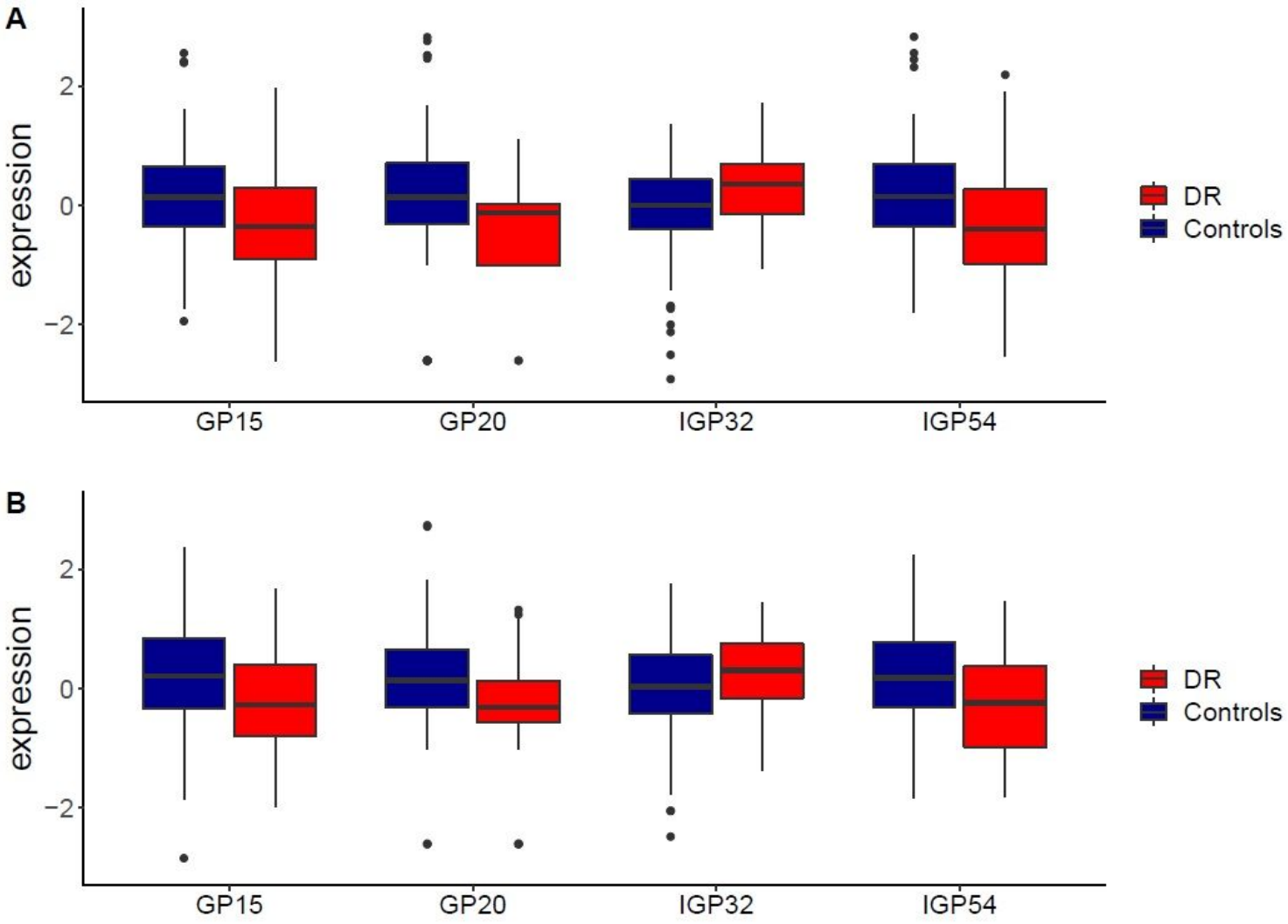

Figure 1

The distribution boxplot of IgG glycosylation panel in the discovery and replication populations.

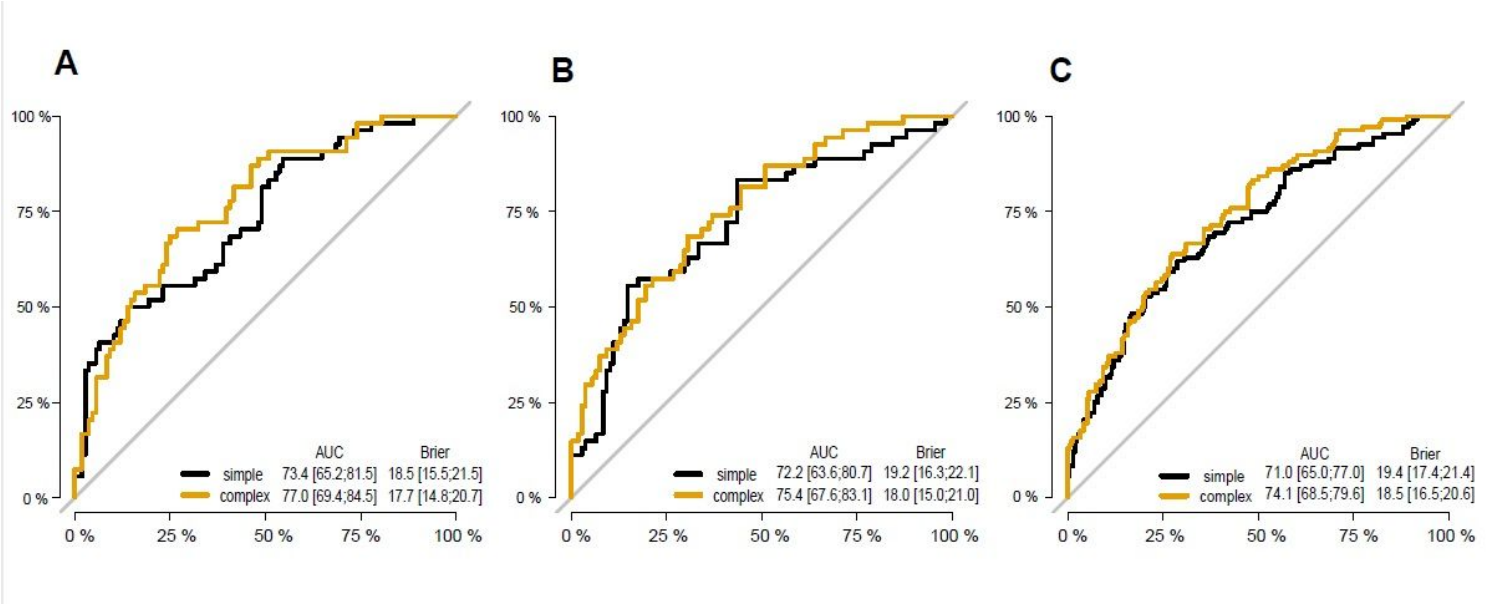

Figure 2

The discriminative capacity of IgG glycosylation panel. A: The ROC and Brier score with IgG glycosylation panel in the discovery population; B: The ROC and Brier score with IgG glycosylation panel in the replication population; C: The ROC and Brier score with IgG glycosylation panel in the combined population. Simple model: involving age, sex, BMI, HBP, FBG, PBG, TG, TC, HDLC, LDLC; Complex model: IgG glycosylation panel added in the simple model.

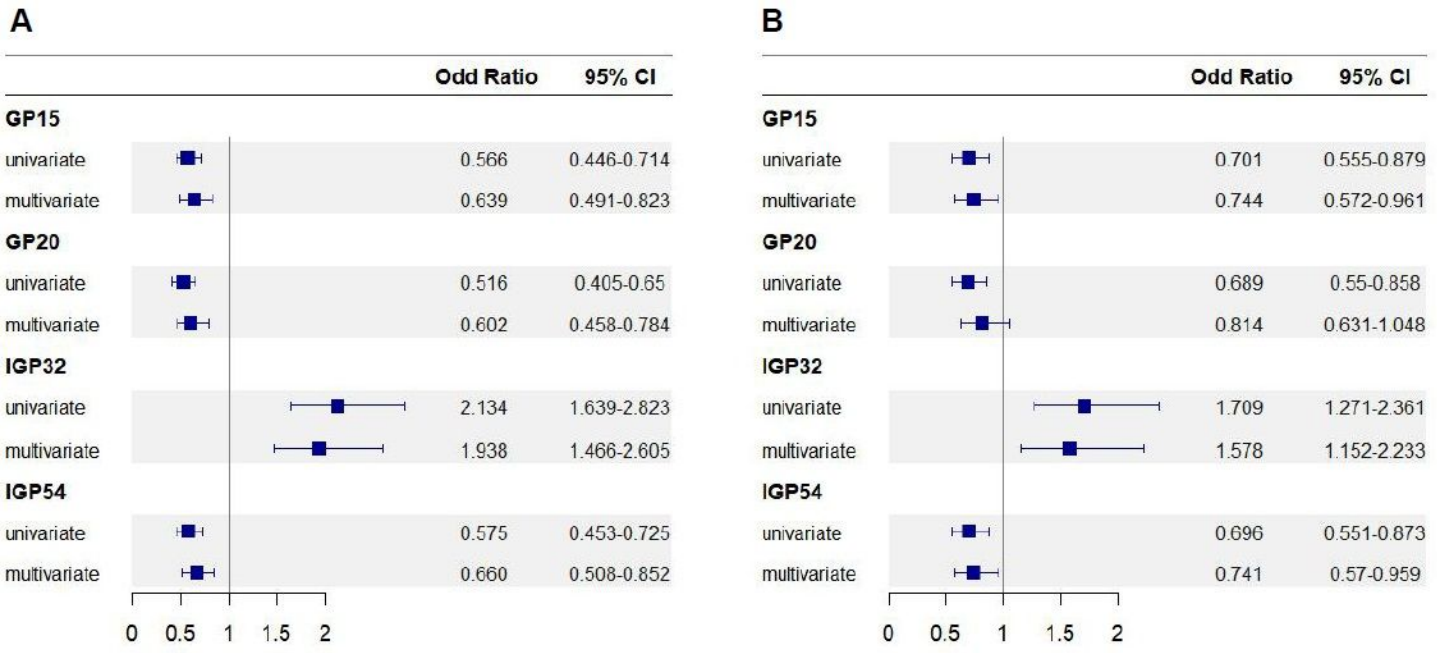

Figure 3

Results of sensitive analyses. A: Association of the IgG glycosylation and DR by ordinal logistics model given the controls defined as prediabetes and diabetes; B: Association of the IgG glycosylation and DR in

1:3 matched population.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableA.1.pdf
- TableA.2.pdf