Original Article

A Nested Case-Control Study to Explore the Association between Immunoglobulin G N-glycans and Ischemic Stroke*

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Abstract

Objective This study prospectively investigates the association between immunoglobulin G (IgG) N-glycan traits and ischemic stroke (IS) risk.

Methods A nested case-control study was conducted in the China suboptimal health cohort study, which recruited 4,313 individuals in 2013–2014. Cases were identified as patients diagnosed with IS, and controls were 1:1 matched by age and sex with cases. IgG N-glycans in baseline plasma samples were analyzed.

Results A total of 99 IS cases and 99 controls were included, and 24 directly measured glycan peaks (GPs) were separated from IgG N-glycans. In directly measured GPs, GP4, GP9, GP21, GP22, GP23, and GP24 were associated with the risk of IS in men after adjusting for age, waist and hip circumference, obesity, diabetes, hypertension, and dyslipidemia. Derived glycan traits representing decreased galactosylation and sialylation were associated with IS in men (FBG2S2/(FBG2 + FBG2S1 + FBG2S2): odds ratio (*OR*) = 0.92, 95% confidence interval (*CI*): 0.87–0.97; $G1^n$: *OR* = 0.74, 95% *CI*: 0.63–0.87; $G0^n$: *OR* = 1.12, 95% *CI*: 1.03–1.22). However, these associations were not found among women.

Conclusion This study validated that altered IgG N-glycan traits were associated with incident IS in men, suggesting that sex discrepancies might exist in these associations.

Key words: Ischemic stroke; Immunoglobulin G; N-glycans; Nested case-control study

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INTRODUCTION

According to the Global Burden of Diseases 2019 study, stroke has been a leading cause of disability-adjusted life-years (DALYs) lost among the elderly worldwide[1]. Approximately 80% of strokes are ischemic strokes (IS), constituting a major burden on the public health system[2]. In the past 30 years, the crude mortality rate of stroke in China has been rising rapidly, and the age-standardized incidence rate of IS increased by 34.7%[3]. IS is a multifactorial and complex

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syndrome triggered by a cerebral embolism resulting from the interaction of environmental and genetic factors[4].

N-glycosylation is a ubiquitous and complexly regulated posttranslational protein modification, with N-glycans covalently linked to asparagine residues in target proteins[5,6]. N-glycosylation participates in many key biological processes, ranging from protein folding, molecular trafficking and clearance, and cell adhesion to immune regulation[7-9]. Immunoglobulin G (IgG) is one of the most suitable candidates for glycomic research due to its unique structure with conserved N-glycosylation in the Fc domain of IgG[10]. Alterations of N-glycosylation in the Fc domain can affect the structure and function of IgG, which may regulate inflammation and trigger several inflammatory diseases[6,11,12]. In addition, N-glycosylation is remarkably stable in healthy individuals and can be modified under specific pathophysiological conditions[13,14].

Several studies have reported that IgG N-glycan profiles are associated with IS[15] and its risk factors, such as obesity[16,17], hypertension[18,19], type 2 diabetes[20], dyslipidemia[21], and inflammation-related diseases[14,22,23]. We recently reported proinflammatory alterations in IgG N-glycan profiles, including decreased sialylation and galactosylation, and increased levels of bisecting N-acetylglucosamine (GlcNAc) are linked to IS. They might be involved in the molecular mechanism of inflammation. However, most previous studies on N-glycans were case-control studies, and thus, the observed altered IgG N-glycosylation may be a consequence rather than a cause of IS. There is no prospective study linking IgG N-glycan profiles to IS.

In this study, we hypothesized that IgG N-glycan profiles are prospectively associated with IS and may serve as potential candidate biomarkers for incident IS. We examined the associations of IgG N-glycan profiles and IS using a nested case-control study in a prospective cohort. Previous studies have suggested sex-specific differences in IgG N-glycan traits[24,25], and sex hormones can modulate human IgG N-glycosylation[26]. Given the sex-specific differences in IgG N-glycosylation, we analyzed its stratification in men and women.

METHODS

Study Population

We conducted a nested case-control study in the China suboptimal health cohort study (COACS), an ongoing longitudinal study starting in 2013[27]. A detailed description of the COACS cohort has been published elsewhere[27]. In total, 4,313 participants from Tangshan city in northern China completed baseline questionnaires and physical examinations and provided peripheral venous blood samples. IS is a sudden onset focal neurological deficit caused by damage to an area in the central nervous system resulting from decreased or completely blocked blood flow. IS was diagnosed according to the International Classification of Disease (ICD-10) based on clinical symptoms, physical examinations, and evidence from brain X-ray computed tomography (CT) or magnetic resonance imaging (MRI)[28]. By the end of December 13, 2018, we identified 99 new incident IS cases in follow-up investigations. Controls were randomly selected from participants who were free of IS before or at the index date using risk-set sampling and were matched by age and sex with cases. Thus, the present study included 99 incident IS cases and 99 controls.

All voluntary participants provided written informed consent before participating in this study. The study was approved by the Ethics Committee of Capital Medical University, China, and abided by the principles of the Declaration of Helsinki.

Collection of Blood Samples and Covariates

After overnight fasting, venipuncture collected blood samples (5 mL) in the morning. Then, whole blood was centrifuged at 3,000 rpm for 10 min, and plasma was separated to detect IgG N-glycan profiles. Blood samples were stored at 4 °C and processed within 8 h. The separated plasma samples were stored at –80 °C until laboratory measurements.

During the baseline survey, trained interviewers collected information about the demographics (age, sex, ethnicity, and levels of education) and clinical history of all participants. Specialized nurses performed physical examinations to obtain information on height, weight, waist and hip circumference, and blood pressure. Blood lipids [total cholesterol (TC), total triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL)], and glucose [fasting blood glucose (FBG) concentrations] were measured immediately at a certified laboratory. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Obesity was defined as BMI > 28.0 kg/m^2[29]. Hypertension was defined as mean systolic blood pressure (SBP) ≥ 140 mmHg or mean diastolic blood pressure (DBP) ≥ 90 mmHg[30]. The participants were classified as having dyslipidemia

with TC ≥ 6.2 mmol/L, TGs ≥ 2.3 mmol/L, HDL < 1.0 mmol/L, and LDL ≥ 4.1 mmol/L, according to the Guidelines for the Prevention and Treatment of Dyslipidemia of adults in China[31]. The participants were diagnosed with T2D by physicians according to the 1999 WHO Criteria (FBG ≥ 7.0 mmol/L)[32].

Analysis of IgG N-glycan Traits

IgG N-glycan traits were separated into 24 glycan peaks (GP1–GP24) by Ultra-Performance Liquid Chromatography (UPLC, Water, USA) in our laboratory, according to standard operating procedures described in our previous publications[16,21,33,34]. First, 50 μL of plasma was diluted 10× with binding buffer (1× phosphate-buffered saline, pH = 7.4) and applied to washed and equilibrated 96-well protein G plates (Water, USA), followed by the immediate washing of protein G plates. Then, 0.1 mol/L formic acid (Sigma Aldrich, USA) and 1 mol/L ammonium bicarbonate (BBI Life Science, China) were used to elute and neutralize IgG, respectively. Samples of IgG were denatured with sodium dodecyl sulfate (SDS, Sigma Aldrich, USA) at 65 °C for 10 minutes. IgG N-glycans were then released with N-glycosidase F (Roche, Germany) and incubated overnight at 37 °C. Next, IgG N-glycans were labeled with mixtures of 2-aminobenzamide (Sigma Aldrich, USA), dimethylsulfoxide (Sigma Aldrich, USA), glacial acetic acid (Merck, Germany), and 2-picoline borane (Sigma Aldrich, USA) to make them visible by UPLC. Finally, 24 GPs were separated from IgG N-glycans by hydrophilic interaction chromatography-UPLC on a Waters BEH Glycan chromatography column. The glycan structures corresponding to each glycan peak are described in Supplementary Table S1 (available in www.besjournal.com)[35,36]. Each batch (96-well plates) included standard samples and blanks for quality control.

All chromatograms were divided similarly into 24 peaks, and the number of glycans in each peak was expressed as a percentage of the total integrated area. In addition, 54 derived glycan traits (DGs) were calculated from 24 directly measured glycan peaks to describe the relative abundances of galactosylation, sialylation, GlcNAc, and core fucosylation[37]. Detailed information on the derived glycan traits is presented in Supplementary Table S2 (available in www.besjournal.com). Normalization of UPLC data was performed as described previously[35].

Statistical Analysis

Baseline characteristics are presented as the median (25th–75th percentile) for continuous variables, whereas categorical variables are expressed as *n* (%). The differences in continuous variables among IS and controls were compared by a *t*-test or Mann-Whitney *U* test, and categorical variables were tested by a chi-square test or Fisher's exact test. Multiple logistic regression analyses were performed to identify the associations of 24 directly measured glycan peaks and 54 derived glycan traits with IS after adjusting for confounding factors, such as obesity, age, waist and hip circumference, diabetes, hypertension, and dyslipidemia. For multiple corrections, the false discovery rate (FDR) was controlled using the Benjamini–Hochberg procedure (*q*)[38]. The potential internal associations among IgG N-glycan traits could induce multicollinearity. Therefore, ridge, stepwise, and the least absolute shrinkage and selection operator (LASSO) based on logistic regression were performed to reduce the dimension of the feature set[39,40]. Fivefold cross-validation was used to evaluate the performance of the discriminant models. The false discrimination rates were used to compare the methods of dimension reduction. All analyses were performed with R (version 4.0.2, The R Foundation for Statistical Computing, Vienna, Austria) and SPSS Statistics (version 25.0, Chicago, IL, USA).

RESULTS

In total, 99 patients with incident IS and 99 matched controls were included in the nested case-control study. The baseline demographic and biochemical characteristics between IS patients and controls stratified by sex are shown in Table 1. Among male IS patients, waistline, BMI, SBP, DBP, TG, LDL, and TC were significantly higher than in the control group. In contrast, SBP and DBP were significantly higher than those in the control group among female IS patients.

When comparing the IgG N-glycome composition, including 24 directly measured GPs between the two groups, we observed significant differences in 7 GPs between IS patients and controls ($P < 0.05$, $q < 0.05$) (Supplementary Table S3, available in www.besjournal.com). The representative IgG N-glycan profiles of UPLC results for sex-specific IS patients and controls are shown in Figure 1. In the sex-stratified analysis, the comparisons of GPs and DGs at baseline between the IS and control groups are shown in Supplementary Tables S4–S5 (available in www.besjournal.com). Significant differences in 6 GPs and 5 DGs were observed in men after adjusting for

age, waist and hip circumference, obesity, diabetes, hypertension, and dyslipidemia (Figure 2, Supplementary Tables S6–S7 available in www.besjournal.com). However, the associations of IgG N-glycan profiles and IS were not found among women ($P < 0.05$, $q < 0.05$). For the initial GPs, higher GP4 and lower GP9, GP21, GP22, GP23, and GP24 were associated with a higher risk of IS in men (GP4: *OR* = 1.16, 95% *CI*: 1.05–1.29; GP9: *OR* = 0.70, 95% *CI*: 0.55–0.90; GP21: *OR* = 0.02, 95% *CI*: 0.01–0.27; GP22: *OR* = 0.01, 95% *CI*: 0.01–0.02; GP23: *OR* = 0.32, 95% *CI*: 0.13–0.78; GP24: *OR* = 0.21, 95% *CI*: 0.09–0.54) (Figure 2, Supplementary Table S6). Among the derived glycan traits, lower levels of sialylation [FBG2S2/(FBG2 + FBG2S1 + FBG2S2)] and galactosylation ($G1^n$) and higher levels of no galactosylation ($G0^n$) were found in the men IS group [FBG2S2/(FBG2 + FBG2S1 + FBG2S2]: *OR* = 0.92, 95% *CI*: 0.87–0.97; $G1^n$: *OR* = 0.74, 95% *CI*: 0.63–0.87; $G0^n$: *OR* = 1.12, 95% *CI*: 1.03–1.22) (Figure 2, Supplementary Table S7).

As shown in Supplementary Figure S1 (available in www.besjournal.com), the sex-specific correlation of IgG N-glycan traits indicated possible multicollinearity. GP4, GP9, and GP22 were selected by stepwise regression and ridge regression. In addition, GP4, GP9, GP22, and GP24 were selected by LASSO regression analysis in male participants. The false discrimination rates of the three methods were 0.267, 0.267, and 0.286, respectively (Supplementary Table S8, available in www.besjournal.com). In a confounder-adjusted and combined significant glycan peak logistic regression model, GP4, GP9, and GP22 were still significantly associated with IS in men (Supplementary Table S8).

DISCUSSION

In this study, the observational results showed higher levels of GP4 and lower levels of GP9, GP21, GP22, GP23, and GP24 in male patients with IS than in controls, although we did not find such associations in women. This is the first study to investigate the possible prospective link between IgG N-glycan traits and IS. Our findings indicated that decreased sialylation and galactosylation are associated with an increased risk of IS in men, which was also observed in our previous case-control study[15]. Several studies on the general population found that levels of N-glycosylation differed between men and women[24,25,41]. It was speculated that galactosylation and sialylation of IgG are influenced and regulated by different sex hormone levels[26]. Furthermore, a previous sex-stratified study indicated that N-glycan biomarkers could improve the prediction ability of type 2 diabetes and cardiovascular disease, with higher predictive efficacy in men[42]. A recent study also showed higher levels of galactosylation in female mice[11], which may explain the sex differences in our study.

Associations between N-glycan traits and cardiovascular diseases have been observed in

Table 1. Baseline characteristics of study subjects

Parameters	Men			Women		
	Incident IS (*n* = 58)	Control (*n* = 58)	*P*	Incident IS (*n* = 41)	Control (*n* = 41)	*P*
Age (years)	59 (47–61)	52 (40–62)	0.057	57 (48–61)	59 (54–62)	0.108
Waistline (cm)	92.00 (87.00–98.25)	88.00 (83.50–92.25)	**0.003**	84.00 (80.00–90.00)	85.00 (80.00–93.00)	0.700
Hipline (cm)	100.00 (96.75–105.00)	100.00 (97.00–102.00)	0.377	99.00 (95.00–103.50)	98.00 (96.00–103.00)	0.880
BMI (kg/m^2)	26.27 (24.51–27.68)	25.00 (23.44–26.45)	**0.021**	24.61 (23.00–26.49)	25.00 (22.83–26.76)	0.692
SBP (mmHg)	137.00 (125.00–144.00)	123.00 (115.00–130.25)	**< 0.001**	133.00 (120.00–147.50)	121.00 (111.00–134.00)	**0.004**
DBP (mmHg)	89.00 (83.00–95.25)	79.50 (72.75–87.25)	**< 0.001**	81.00 (74.50–90.00)	74.00 (67.50–82.00)	**0.007**
FBG (mmHg)	5.30 (4.90–5.90)	5.20 (4.90–5.81)	0.956	5.10 (4.90–5.50)	5.10 (4.80–5.70)	0.662
TG (mmol/L)	1.33 (1.02–1.97)	1.14 (0.87–1.55)	**0.031**	1.45 (0.95–1.78)	1.31 (0.86–1.85)	0.813
LDL (mmol/L)	2.58 (2.21–2.99)	2.25 (1.81–2.77)	**0.023**	2.63 (2.30–3.39)	2.65 (2.42–3.11)	0.289
HDL (mmol/L)	1.07 (0.93–1.25)	1.11 (0.94–1.33)	0.376	1.31 (1.15–1.43)	1.27 (1.17–1.47)	0.878
TC (mmol/L)	4.58 (3.99–5.16)	4.31 (3.30–4.82)	**0.047**	4.89 (4.34–5.78)	4.78 (4.33–5.18)	0.321

Note. IS, ischemic stroke; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TG, total triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol. Bold type indicates statistical significance.

previous studies[42,43]. Consistent with our results, a prospective study indicated that plasma N-glycan traits could improve cardiovascular event (including stroke) prediction with the established clinical risk score[42]. In this study, all glycan biomarkers of cardiovascular events stemmed from immunoglobulins, suggesting a particular role for the glycosylation-dependent immune response in

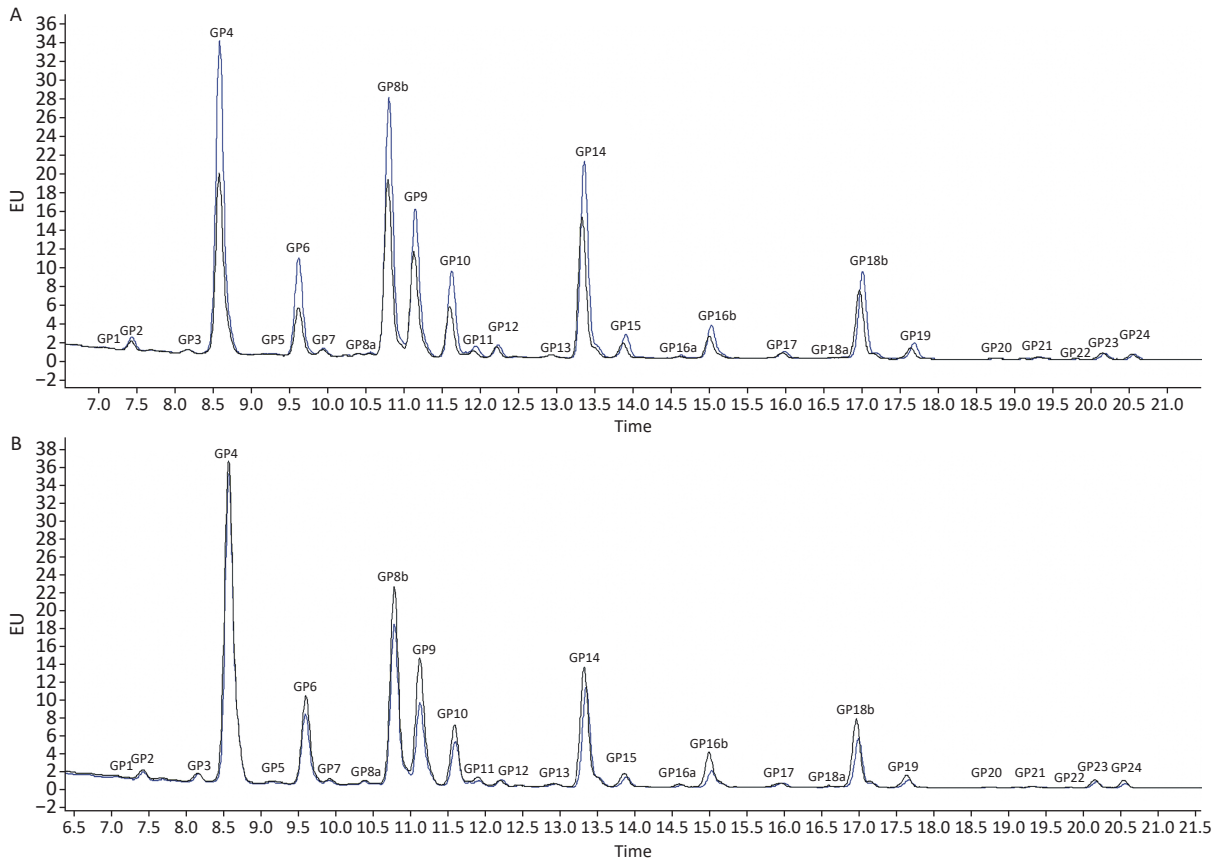

Figure 1. Ultra-Performance Liquid Chromatography of IS patients and controls in (A) men and (B) women. The profiles of glycan peaks were randomly selected from IS patients and matched controls. The 24 IgG glycan peaks are numbered. The blue line represents IS patients; the black line represents controls. IS, ischemic stroke.

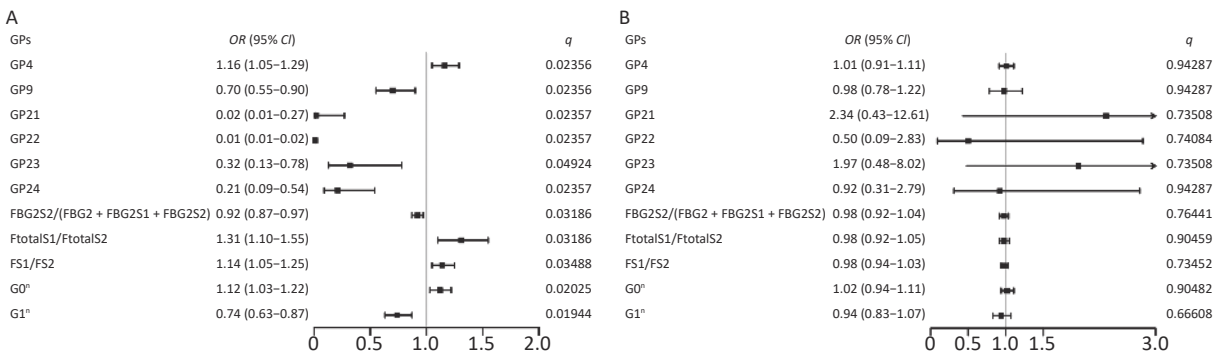

Figure 2. Odds ratios (*OR*) and 95% confidence intervals (95% *CI*) for the associations of IgG N-glycan traits with IS in (A) men and (B) women. Multiple logistic regression analysis was performed after adjusting for age, waist and hip circumference, obesity, diabetes, hypertension, and dyslipidemia. IS, ischemic stroke.

cardiovascular disease etiology. Similarly, a previous cohort study of stroke-free individuals also found that the N-glycome might be a potentially useful biomarker for silent brain infarcts. Individuals with silent brain infarcts have a 2- to 4-fold higher risk of stroke[44]. Menni et al. investigated the links between IgG N-glycan traits and the 10-year atherosclerotic cardiovascular disease risk score and subclinical atherosclerosis. They found that galactosylation and sialylation of IgG N-glycan traits were inversely associated with the 10-year atherosclerotic cardiovascular disease risk score[45].

Combined with previous research[15], our findings suggested that faintly aberrant IgG N-glycosylation might play a cascading role in the pathogenesis of IS. N-glycosylation compositions in the Fc segment of IgG can alter effector functions by modulating its affinity for distinct Fc receptors to mediate pro- and anti-inflammatory activities[46]. Abundant evidence has shown potential links between decreased galactosylation and BMI, measures of central adiposity, and hypertension[19,47,48], which are risk factors for IS. IgG N-glycosylation with decreased galactosylation mediates proinflammatory activity by recognizing mannose-binding lectin (MBL) and subsequently activating complement[49]. In addition, decreased galactosylation also enhances FcγRIII affinity, thus enhancing antibody-dependent cellular cytotoxicity (ADCC) activity[50]. Moreover, galactose deficiency also affects sialylation, as the addition of terminal sialylation requires galactose as the substrate for sialyltransferases[51].

A strength of this study was the use of incident cases from a prospective cohort study, which enables keeping the temporal association between IgG N-glycan traits and IS outcome in longitudinal studies. Several limitations should be noted. First, in a prospective setting, the relatively small sample size of the nested case-control study may result in an overfitted model. The results suggested that IgG N-glycan traits were prospectively associated with IS but did not provide a final assessment of predictive accuracy. Further validation of cohort studies in larger independent populations is necessary for our future work. Second, because medication information, such as antihypertensive treatment, was unavailable in the database, the potential for bias arising from medication information could not be assessed. Third, information on menopause time was not collected in the database, and more studies are needed to examine whether the changes in IgG N-glycosylation in women affected by menopausal status can influence the pathogenesis of IS in the future. Finally, this study did not include hemorrhagic stroke because of its low incidence in the cohort by the end of the follow-up. Further external validation of IgG N-glycan biomarkers is needed in large prospective studies.

CONCLUSION

In conclusion, the present study showed that IgG N-glycan traits with decreased galactosylation and sialylation were prospectively associated with incident IS in men, suggesting that sex discrepancy might exist in the association between IgG N-glycans and incident IS. Nevertheless, further research is needed to validate these biomarkers in cohort studies with larger sample sizes and multiethnic populations, which provide novel biomarkers to identify IS patients.

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AUTHOR CONTRIBUTIONS

WANG You Xin and XING Wei Jia conceptualized the study. WANG Bi Yan, SONG Man Shu, ZHANG Jie, and MENG Xiao Ni conducted the experiments on IgG N-glycome analysis, analyzed the data and drafted the manuscript. BW and WX recruited the participants and collected the demographic and clinical information. WANG You Xin, SONG Man Shu, and XING Wei Jia critically revised the manuscript.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared upon reasonable request to the corresponding author.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of Capital Medical University, China, and abided by the principles of the Declaration of Helsinki. All voluntary participants provided written informed consent before taking part in this study.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no financial or other conflicts of interest.

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