Letter to the Editor

Association between IgG *N*-glycans and Nonalcoholic Fatty Liver Disease in Han Chinese*

ZHAO Zhong Yao[1], LIU Di[1], CAO Wei Jie[1], SUN Ming[1], SONG Man Shu[1], WANG Wei[1,2], and WANG You Xin[1,2,#]

Nonalcoholic fatty liver disease (NAFLD) is a major public health issue worldwide. Immunoglobulin G (IgG) *N*-glycans are associated with risk factors for NAFLD, such as obesity and diabetes. A cross-sectional study involving 500 Han Chinese adults recruited from a community in Beijing was carried out to explore the association between IgG *N*-glycans and NAFLD. IgG *N*-glycosylation was significantly associated with NAFLD, with the disease showing a negative correlation with galactosylation (GP14, GP14^n, and G2^n), positive correlation with fucosylation (FBG2^n/G2^n), and positive correlation with bisecting *N*-acetylglucosamine (GlcNAc) [FBG2^n/FG2^n and FBG2^n/(FG2^n+FBG2^n)], after controlling age, gender, and prevalence of obesity, type 2 diabetes mellitus, hypertension, and hyperlipidemia. In other words, the present study showed a possible association between NAFLD and the loss of galactose and elevations of fucose and bisecting GlcNAc. Aberrant IgG glycosylation might therefore be a potential biomarker for the primary or secondary prevention of NAFLD.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the world, with an increasing prevalence from 15% in 2005 to 25% within 5 years[1]. It is commonly associated with metabolic comorbidities, including obesity, type 2 diabetes, dyslipidemia, and the metabolic syndrome, and has the potential to progress to advanced fibrosis and hepatocellular carcinoma (HCC), making a major public health issue. The increased morbidity and mortality, healthcare costs, and declining quality of life associated with NAFLD renders its early screening or intervention a matter of urgency.

The pathogenesis of NAFLD is a multifactorial and multistep process, involving the combination and interaction of genetic, demographic, clinical, and environmental factors, with up to 27% of heritability identified[2]. Besides the genetics, obesity, lifestyle variation, and diabetes are the most prevalent risk factors leading to the development of NAFLD. Recently, glycosylation, the most important and abundant post-translational modification, was demonstrated to be associated with obesity, diabetes, and several inflammatory or autoimmune diseases[3]. Glycans are involved in virtually all normal physiological and disease mechanisms in humans, as exemplified by immunoglobulin G (IgG) *N*-glycans[3]. IgG can exert both anti-inflammatory and proinflammatory responses, where the ability depends on modulation of the two *N*-glycans that attach to the conserved asparagine-297 in the Cγ2 domain. The effector functions of IgG are mediated by the interaction of the Fc fragment with the IgG-specific Fcγ receptors (FcγRs). The glycosylation pattern of IgG essentially influences its conformation and affinity for the FcγRs. Mehta et al. discovered that the major glycoprotein altered in patients with cirrhosis is IgG, which is specifically reactive to the heterophilic alpha-Gal epitope[4]. The present study aimed to explore the association between the IgG *N*-glycan profile and NAFLD.

Subject recruitment was carried out as described previously[5]. In brief, 913 Han Chinese adults aged 18 years or older and without a history of somatic and psychiatric abnormalities were recruited from a community in Xicheng District, Beijing, from January to April of 2012. Subjects with a diagnosis of specific severe diseases of the cardiovascular, respiratory, genitourinary, digestive, and hematic systems were excluded from the study. Consequently, 500 subjects with clinical abdominal ultrasound findings were included in the final analysis. The study was approved by the Ethical

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1. Beijing Key Laboratory of Clinical Epidemiology, School of Public Health, Capital Medical University, Beijing 100069, China; 2. School of Medical and Health Sciences, Edith Cowan University, Perth WA 6027, Australia

Committee of Capital Medical University. All subjects gave informed consent for participation.

Two experienced radiologists who were blinded to the clinical presentations and laboratory findings of the participants performed the liver ultrasonography, using a high-resolution B-mode topographic ultrasound system with a 3.5 MHz probe (ACUSON ×300, Siemens, Germany). NAFLD was diagnosed according to the Asia-Pacific Working Party on NAFLD and Chinese Association for the Study of Liver Disease[6,7]. Fatty liver was diagnosed by the presence of at least two of three abnormal findings on abdominal ultrasonography: diffusely increased echogenicity ('bright') that is greater than that of the kidney or spleen, vascular blurring, and deep attenuation of the ultrasound signal. NAFLD was defined as that in patients with a fatty liver and with an alcohol intake of less than 140 g weekly for men or 70 g weekly for women.

Blood samples, collected from the subjects after at least 10 h of fasting, were separated by centrifugation at 1,500 rpm for 15 min to obtain the plasma samples that were then stored at -80 °C until the glycosylation analysis. IgG was isolated from the plasma after washing and equilibrating on protein G monolithic plates. In brief, 90 mL of plasma was diluted 10 times with binding buffer, applied to the protein G plate, and then washed. The released IgG *N*-glycans were then labeled with 2-aminobenzamide and analyzed by hydrophilic interaction chromatography (HILIC)- ultra performance liquid chromatography (UPLC) to reveal 24 IgG glycan peaks (GPs)[5]. From these 24 directly measured traits, an additional 54 derived traits describing the percentages of galactosylation, sialylation, and fucosylation were also calculated. The glycan peak GP3 was excluded from the calculations owing to contamination.

Other measurements, namely the body mass index, blood pressure, waist circumference, fasting plasma glucose, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, and triglycerides, were determined by standard methods.

Normal distributions of all analysis results were checked using the Kolmogorov-Smirnov test. Data of characteristics are presented as means ± standard deviations, medians (quartile interval range), or *n* (%), when appropriate. The between-group comparisons were tested using the independent sample *t*-test, Wilcoxon rank-sum test, or Chi-square test. Correlation, partial correlation, and logistic regression analysis were used to determine the association between the IgG *N*-glycan profiles and NAFLD. Considering that UPLC-measured glycans are subject to high experimental variability, raw glycan intensities were normalized by total area, batch-corrected by applying the empirical Bayes method ComBat with parametric priors, and standardized to a mean of 0 and standard deviation of 1[5]. Statistical analyses were carried out with SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA) and *R* (version 3.1.0). Two-sided $P < 0.05$ was considered to be statistically significant.

The main clinical and laboratory characteristics of the patients are described in Table 1. Five hundred individuals were included in the final analysis, among which 143 were diagnosed with NAFLD. The gender, age, and prevalence of obesity, type 2 diabetes mellitus, hypertension, and hyperlipidemia differed significantly between the NAFLD and healthy control groups.

Table 1. Demographic and Biochemical Characteristics of the NAFLD and Healthy Controls

Variables	Total (*n* = 500)	NAFLD (*n* = 143)	Controls (*n* = 357)	*t*	*P*
Female (%)	351 (70.2)	77 (53.8)	274 (76.8)	25.61	< 0.001
Age (years)	47.6 ± 5.5	49.2 ± 5.7	47.0 ± 5.3	4.15	< 0.001
BMI (kg/m^2)	22.7 ± 7.5	24.5 ± 8.6	22.0 ± 6.9	3.16	0.002
Obesity				8.082	< 0.001
Normal	238 (47.7)	32 (22.4)	206 (57.9)		
Overweight	195 (39.1)	70 (49.0)	125 (35.1)		
Obesity	66 (13.2)	41 (28.7)	25 (7.0)		
Diabetes, *n* (%)				4.138	< 0.001
Normal	436 (87.2)	111 (77.6)	325 (91.0)		
Prediabetes	39 (7.8)	17 (11.9)	22 (6.2)		
Diabetes	25 (5.0)	15 (10.5)	10 (2.8)		
Blood pressure, *n* (%)				3.99	< 0.001
Normal	291 (61.7)	66 (49.6)	225 (66.4)		
Prehypertension	65 (13.8)	15 (11.3)	50 (14.7)		
Hypertension	116 (24.6)	52 (39.1)	64 (18.9)		
Hyperlipidemia, *n* (%)	140 (28.0)	68 (47.6)	72 (20.2)	37.98	< 0.001

In the ultra performance liquid chromatography (UPLC) analysis of IgG glycosylation, 23 chromatographic peaks (GP3 was excluded) and 54 additional traits describing the calculated percentages of galactosylation, sialylation, bisecting *N*-acetylglucosamine (GlcNAc), and fucosylation were included in the further analysis[5]. In the univariate analysis, 41 glycan traits were significantly different between the NAFLD and control groups (Table S1 available in www.besjournal.com). After controlling for gender and age, 14 glycan traits remained significantly different; namely, GP12, GP13, GP14, GP17, GP12^{n}, GP13^{n}, GP14^{n}, G1^{n}, G2^{n}, F$^{n\ total}$, FBG2^{n}/G2^{n}, B^{n}/(F^{n}+FB^{n}), FBG2^{n}/FG2^{n}, and FBG2^{n}/(FG2^{n}+FBG2^{n}) (Table 2). When adjusted further for the prevalence of obesity, type 2 diabetes mellitus, hypertension, and hyperlipidemia, GP14, GP14^{n}, G2^{n}, FBG2^{n}/G2^{n}, FBG2^{n}/FG2^{n}, and FBG2^{n}/(FG2^{n}+FBG2^{n}) were significantly different between the two groups (Table 2). In the discrimination model, each of the 6 glycan traits yielded an area of 0.610-0.643 under the receiver operating characteristic (ROC) curve (Figure 1). Compared with the model that only included age and gender, the area under the ROC curve of the model that included age and gender together with the 6 glycans increased to 0.804 [95% confidence interval (*CI*): 0.761-0.847] (Figure 1).

Table 2. The Odds Ratios (*OR*) and 95% Confidence Intervals (*CI*) of NAFLD According to the 1 Standard Deviation (SD) of Each Glycan Trait

Glycans	Crude Model		Adjusted Model 1*		Adjusted Model 2#	
	OR (95% *CI*)	*P*	*OR* (95% *CI*)	*P*	*OR* (95% *CI*)	*P*
GP4	1.46 (1.20-1.79)	**1.86×10^{-4}**	1.19 (0.95-1.49)	0.120	1.21 (0.94-1.56)	0.143
GP6	1.50 (1.22-1.84)	**1.06×10^{-4}**	1.14 (0.89-1.46)	0.287	1.19 (0.89-1.59)	0.246
GP11	1.26 (1.03-1.55)	**2.54×10^{-2}**	1.10 (0.92-1.32)	0.312	1.19 (0.90-1.57)	0.229
GP12	0.68 (0.55-0.85)	**5.78×10^{-4}**	0.75 (0.60-0.94)	**0.013**	0.89 (0.69-1.14)	0.337
GP13	0.69 (0.56-0.86)	**7.08×10^{-4}**	0.77 (0.62-0.96)	**0.020**	0.84 (0.66-1.07)	0.157
GP14	0.59 (0.47-0.73)	**1.15×10^{-6}**	0.74 (0.57-0.95)	**0.017**	0.74 (0.56-0.99)	**0.041**
GP17	0.67 (0.54-0.84)	**3.88×10^{-4}**	0.73 (0.58-0.91)	**0.005**	0.85 (0.67-1.09)	0.208
GP18	0.67 (0.54-0.82)	**1.21×10^{-4}**	0.84 (0.67-1.07)	0.155	0.83 (0.64-1.08)	0.160
FGS/(FG+FGS)	0.75 (0.62-0.92)	**5.28×10^{-3}**	0.87 (0.70-1.07)	0.192	0.87 (0.69-1.10)	0.245
FGS/(F+FG+FGS)	0.70 (0.57-0.86)	**6.24×10^{-4}**	0.85 (0.68-1.06)	0.143	0.84 (0.66-1.07)	0.161
FBS^{total}/FS^{total}	1.37 (1.14-1.66)	**9.77×10^{-4}**	1.09 (0.88-1.35)	0.433	1.13 (0.88-1.44)	0.348
FBS1/FS1	1.37 (1.14-1.65)	**9.49×10^{-4}**	1.12 (0.91-1.38)	0.285	1.16 (0.91-1.48)	0.221
FBS1/(FS1+FBS1)	1.38 (1.14-1.66)	**9.12×10^{-4}**	1.12 (0.91-1.39)	0.284	1.16 (0.91-1.48)	0.227
GP1^{n}	0.70 (0.57-0.85)	**4.58×10^{-4}**	0.83 (0.67-1.03)	0.097	0.83 (0.65-1.06)	0.127
GP2^{n}	0.68 (0.55-0.83)	**1.97×10^{-4}**	0.84 (0.67-1.05)	0.119	0.83 (0.65-1.07)	0.143
GP4^{n}	1.46 (1.20-1.79)	**1.86×10^{-4}**	1.19 (0.95-1.49)	0.120	1.21 (0.94-1.56)	0.143
GP6^{n}	1.46 (1.19-1.80)	**3.18×10^{-4}**	1.10 (0.86-1.41)	0.461	1.15 (0.85-1.53)	0.364
GP12^{n}	0.66 (0.53-0.83)	**2.88×10^{-4}**	0.74 (0.59-0.93)	**0.011**	0.87 (0.68-1.12)	0.280
GP13^{n}	0.66 (0.53-0.82)	**1.69×10^{-4}**	0.76 (0.60-0.94)	**0.013**	0.81 (0.64-1.04)	0.101
GP14^{n}	0.59 (0.47-0.73)	**9.32×10^{-7}**	0.73 (0.57-0.94)	**0.015**	0.73 (0.55-0.97)	**0.030**
G0^{n}	1.51 (1.23-1.85)	**7.11×10^{-5}**	1.19 (0.94-1.51)	0.139	1.22 (0.93-1.61)	0.143
G1^{n}	1.34 (1.08-1.66)	**6.93×10^{-3}**	1.27 (1.02-1.58)	**0.031**	1.18 (0.91-1.52)	0.203
G2^{n}	0.60 (0.48-0.74)	**1.49×10^{-6}**	0.74 (0.58-0.94)	**0.015**	0.76 (0.58-1.00)	**0.048**
F$^{n\ total}$	1.33 (1.07-1.65)	**1.03×10^{-2}**	1.29 (1.03-1.61)	**0.027**	1.13 (0.88-1.44)	0.348
FBG2^{n}/G2^{n}	1.55 (1.26-1.90)	**2.44×10^{-5}**	1.33 (1.06-1.65)	**0.012**	1.35 (1.05-1.75)	**0.019**
B^{n}/(F^{n}+FB^{n})	0.66 (0.53-0.82)	**1.74×10^{-4}**	0.75 (0.60-0.94)	**0.013**	0.82 (0.64-1.04)	0.104
FBG2^{n}/FG2^{n}	1.50 (1.22-1.84)	**8.64×10^{-5}**	1.27 (1.02-1.58)	**0.031**	1.34 (1.04-1.72)	**0.024**
FBG2^{n}/(FG2^{n}+FBG2^{n})	1.50 (1.23-1.84)	**8.42×10^{-5}**	1.28 (1.02-1.59)	**0.030**	1.34 (1.04-1.73)	**0.025**
FG2^{n}/(BG2^{n}+FBG2^{n})	0.67 (0.54-0.84)	**4.24×10^{-4}**	0.80 (0.63-1.02)	0.069	0.77 (0.58-1.01)	0.063

Note. *Adjusted for age and sex; #Adjusted for age, sex, and occurrence of obesity, diabetes, hypertension and hyperlipidemia.

This is the first study to explore the relationship between changes of the IgG *N*-glycan profile and NAFLD. We first demonstrated an independent association between IgG *N*-glycosylation and NAFLD, where the disease was negatively correlated to galactosylation (GP14, $GP14^n$, and $G2^n$), positively correlated to fucosylation ($FBG2^n/G2^n$), and positively correlated to bisecting GlcNAc [$FBG2^n/FG2^n$ and $FBG2^n/(FG2^n+FBG2^n)$], after controlling for age, gender, and prevalence of obesity, type 2 diabetes mellitus, hypertension, and hyperlipidemia. Metabolic comorbidities, such as obesity, type 2 diabetes mellitus, dyslipidemia, and the metabolic syndrome, were associated with both NAFLD and IgG *N*-glycosylation[1,3]. In the present study, we showed that the association between NAFLD and IgG *N*-glycosylation is independent to obesity, type 2 diabetes mellitus, hypertension, and hyperlipidemia, which suggests that disorders in IgG *N*-glycosylation contribute to the accelerated progression of end-stage liver disease or HCC in patients suffering from NAFLD.

Previous studies have shown that changes of glycoprotein *N*-glycosylation were associated with liver disease. Chen et al. reported that *N*-glycans in serum protein were associated with nonalcoholic steatohepatitis (NASH)-related fibrosis, suggesting that *N*-glycans could be a potential biomarker for NAFLD[8]. Clarke et al. demonstrated a significant loss of glycosylation of key uptake and efflux transporters in liver samples following the progression to NASH[9]. The present study further demonstrated that the *N*-glycosylation of IgG is associated with NAFLD. IgG is one of the most well-studied glycoproteins and represents an excellent example of protein function modulation by alternative glycosylation. The Fab parts of IgG are responsible for the recognition of antigens, whereas the Fc part executes the removal or destruction of substrates through interaction with Fcγ and other receptors[10]. The elevated bisecting GlcNAc and the reduced galactosylation contribute to the proinflammatory pattern, as observed for the IgG from patients with NAFLD compared with that from healthy controls. GP14 (the percentage of FA2G2 glycan in total IgG glycans), $GP14^n$ (the percentage of FA2G2 glycan in total neutral IgG glycans), and $G2^n$ (the percentage of digalactosylated structures in total neutral IgG glycans) indicate digalactosylation. $FBG2^n/FG2^n$ (the ratio of fucosylated digalactosylated structures with and without bisecting GlcNAc) and $FBG2^n/(FG2^n+FBG2^n)$ (the incidence of bisecting GlcNAc in all fucosylated digalactosylated structures in total neutral IgG glycans) indicate bisecting GlcNAc. The decreased IgG galactosylation in NAFLD described in this study parallels a similar observation of reduced serum IgG galactosylation in autoimmune and inflammatory diseases[10]. There is not much data on IgG containing bisecting GlcNAc and autoimmune diseases, but bisecting GlcNAc was

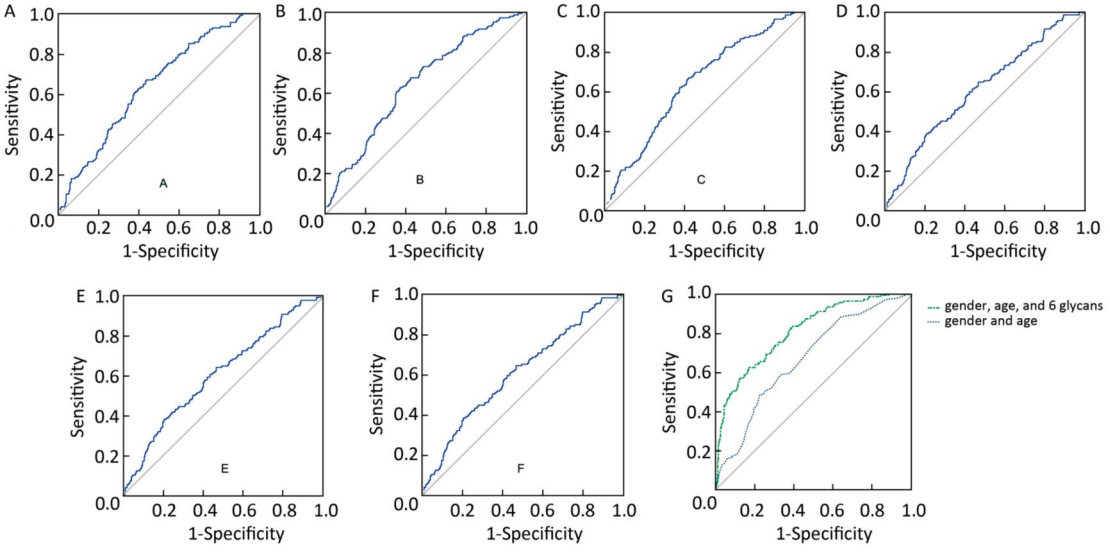

Figure 1. Receiver operating characteristic curves illustrating the performance of a regularized logistic regression model in predicting disease status of patients with nonalcoholic fatty liver disease (NAFLD) and healthy controls. (A) GP14; (B) $GP14^n$; (C) $G2^n$; (D) $FBG2^n/G2^n$; (E) $FBG2^n/FG2^n$; (F) $FBG2^n/(FG2^n+FBG2^n)$; (G) combination of 6 glycans, gender, and age compared to gender and age.

also elevated in the cerebrospinal fluid of patients with multiple sclerosis and in the serum of patients with Lambert-Eaton myasthenic syndrome[11,12]. Taken together, the alterations described here in patients with NAFLD (i.e., reduced galactosylation, increased bisecting GlcNAc) suggest that the IgG in these patients has a higher proinflammatory activity than that of controls.

Afucosylation was initially regarded as being proinflammatory owing to its enhancement of antibody-dependent cellular cytotoxicity (ADCC) *via* FcγRIIIa[10], but it was also found to have the capability to decrease complement activation[13]. In the present study, we showed that fucosylation ($FBG2^n/G2^n$, the percentage of fucosylation of digalactosylated structures) was increased in patients with NAFLD relative to the level in the controls, which may result in enhanced complement activation but less ADCC. Further study is urgently needed to elucidate these contradictory findings of IgG fucosylation or multiple functions in pathology.

Several limitations of this study should be noted when interpreting these findings. First, the design of the cross-sectional study prevented any inference of the causal effect between IgG and *N*-glycosylation in NAFLD. Second, lifestyle factors (e.g., smoking, drinking, etc.) were not controlled in this study, which may potentially confound the association. Third, the small sample size of this casecontrol study might result in less statistical power in establishing the association between glycan traits and NAFLD.

In conclusion, the present study showed a possible association between NAFLD and the loss of galactose and elevations of fucose and bisecting GlcNAc. The aberrant glycosylation of IgG might contribute to the incidence or progression of NAFLD via chronic inflammation, and thus serves as a potential biomarker for the primary or secondary prevention of this fatty liver disease.

No conflict of interest to declare.

WANG You Xin and WANG Wei designed the study and revised the manuscript. ZHAO Zhong Yao and LIU Di performed the analysis and interpretation of data, and drafted the manuscript. CAO Wei Jie and SUN Ming collected the samples. SONG Man Shu contributed analysis tools.

#Correspondence should be addressed to WANG You Xin, PhD, Tel: 86-10-83911779, E-mail: wangy@ccmu.edu.cn

Biographical note of the first author: ZHAO Zhong Yao, male, born in 1992, postgraduate, majoring in molecular epidemiology.

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