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## OPEN

# Inflammatory Bowel Disease Associates with Proinflammatory Potential of the Immunoglobulin G Glycome

Irena Trbojević Akmačić, MSc,\* Nicholas T. Ventham, MBBS,<sup>†</sup> Evropi Theodoratou, PhD,<sup>‡</sup> Frano Vučković, MSc,\* Nicholas A. Kennedy, MBBS,<sup>†</sup> Jasminka Krištić, MSc,\* Elaine R. Nimmo, PhD,<sup>†</sup> Rahul Kalla, MBChB,<sup>†</sup> Hazel Drummond, BSc,<sup>†</sup> Jerko Štambuk, MSc,\* Malcolm G. Dunlop, MD,<sup>§</sup> Mislav Novokmet, PhD,\* Yurii Aulchenko, PhD,<sup>||,¶</sup> Olga Gornik, PhD,\*\* IBD-BIOM Consortium, Harry Campbell, MD,<sup>‡</sup> Maja Pučić Baković,\* Jack Satsangi, DPhil,<sup>†</sup> and Gordan Lauc, PhD\*\*\*

**Background:** Glycobiology is an underexplored research area in inflammatory bowel disease (IBD), and glycans are relevant to many etiological mechanisms described in IBD. Alterations in *N*-glycans attached to the immunoglobulin G (IgG) Fc fragment can affect molecular structure and immunological function. Recent genome-wide association studies reveal pleiotropy between IBD and IgG glycosylation. This study aims to explore IgG glycan changes in ulcerative colitis (UC) and Crohn's disease (CD).

**Methods:** IgG glycome composition in patients with UC (n = 507), CD (n = 287), and controls (n = 320) was analyzed by ultra performance liquid chromatography.

**Results:** Statistically significant differences in IgG glycome composition between patients with UC or CD, compared with controls, were observed. Both UC and CD were associated with significantly decreased IgG galactosylation (digalactosylation, UC: odds ratio [OR] = 0.71; 95% confidence interval [CI], 0.5–0.9; *P* = 0.01; CD: OR = 0.41; CI, 0.3–0.6; *P* =  $1.4 \times 10^{-9}$ ) and significant decrease in the proportion of sialylated structures in CD (OR = 0.46, CI, 0.3–0.6, *P* =  $8.4 \times 10^{-8}$ ). Logistic regression models incorporating measured IgG glycan traits were able to distinguish UC and CD from controls (UC: *P* =  $2.13 \times 10^{-6}$  and CD: *P* =  $2.20 \times 10^{-16}$ ), with receiver–operator characteristic curves demonstrating better performance of the CD model (area under curve [AUC] = 0.77) over the UC model (AUC = 0.72) (*P* = 0.026). The ratio of the presence to absence of bisecting GlcNAc in monogalactosylated structures was increased in patients with UC undergoing colectomy compared with no colectomy (FDR-adjusted, *P* = 0.05).

**Conclusions:** The observed differences indicate significantly increased inflammatory potential of IgG in IBD. Changes in IgG glycosylation may contribute to IBD pathogenesis and could alter monoclonal antibody therapeutic efficacy. IgG glycan profiles have translational potential as IBD biomarkers.

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**Key Words:** inflammatory bowel disease, glycomics, IgG glycans, ulcerative colitis, Crohn's disease

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From the \*Genos Glycoscience Research Laboratory, Zagreb, Croatia; <sup>†</sup>Gastrointestinal Unit, Centre for Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom; <sup>‡</sup>Centre for Population Health Sciences, University of Edinburgh, Edinburgh, United Kingdom; <sup>§</sup>Colon Cancer Genetics Group, Institute of Genetics and Molecular Medicine, University of Edinburgh and Medical Research Council Human Genetics Unit, Edinburgh, United Kingdom; <sup>||</sup>Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia; <sup>¶</sup>Novosibirsk State University, Novosibirsk, Russia; and **\*\***Department of Biochemistry and Molecular Biology, University of Zagreb, Faculty of Pharmacy and Biochemistry, Zagreb, Croatia.

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G. Lauc is the founder and owner of Genos Ltd, a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field. D. Fernandes is the CEO of Ludger Ltd, a commercial company that specializes in the development and validation of glycoprofiling technology for biologic therapeutics and biological tissues. Some of the Ludger products include glycan purification kits and glycan release kits. There are no patents and products in development. I. K. Pemberton is the Research Director of IP Research Consulting SAS, a privately owned research-intensive SME under the commercial name of Photeomix Protein Discovery that specializes in the discovery and validation of biomarkers based on posttranslational protein modification activities. There are no patents, products in development, or marketed products to declare. Y. Aulchenko is the Director of PolyOmica—a privately owned research-intensive SME that specializes in Omics data analysis. The work of Y. Aulchenko was supported by grant from the Russian Science Foundation (Grant number 14-14-00313). E. Theodoratou is a recipient of the University of Edinburgh Chancellors Fellowship. The remaining authors have no conflicts of interest to disclose.

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I. Trbojević Akmačić, N. T. Ventham, E. Theodoratou, F. Vučković, J. Satsangi, and G. Lauc contributed equally.

Reprints: Gordan Lauc, PhD, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10000 Zagreb, Croatia (e-mail: [glauc@pharma.hr](mailto:glauc@pharma.hr)).

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The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are a cause of considerable morbidity among young people. The incidence of IBD is increasing,<sup>1</sup> and IBD has an estimated prevalence of 2.5 to 3 million in Europe.<sup>2,3</sup> IBD has a considerable economic burden with direct costs of €4.6 to 5.2 billion in Europe per year.<sup>2</sup>

IBD is believed to be caused by an aberrant immune response to gut microbiota in genetically susceptible individuals.<sup>4</sup> Although great progress has been made in understanding the genetic architecture of IBD,<sup>5–8</sup> only a limited proportion of heritability can be explained by genetics alone. Thus, determining the environmental priming and triggering factors of IBD is a research imperative. Glycobiology is an underexplored scientific area in the context of IBD. The 3 proposed pathogenic mechanisms of IBD: genetics, gut microbiota, and aberrant immune response may all be affected by or result in alterations in glycans.<sup>9</sup>

Glycans are complex carbohydrates conjugated to proteins (to form glycoproteins) by the activity of hundreds of enzymes organized into complex pathways.<sup>10,11</sup> Glycan moieties are not synthesized using a direct genetic template, and final glycan structure and glycome composition are determined by changes in concentrations, expression, and activity of the enzymes involved in glycan biosynthesis. Both inherited (genetic) and acquired (environmental) factors modulate glycosylation, and differential glycan structure and expression levels have been described in many diseases.<sup>12,13</sup> The complexity of glycans, given the nonlinear branched topology and differing interresidue linkages, potentially explains why this research field has lagged behind that of genomics and proteomics.<sup>10</sup>

In the colon, the dual mucus layer formed of *O*-linked glycosylated mucins, notably the glycan acceptor protein MUC2, has been extensively described, and a structurally and functionally deficient mucus layer is likely to contribute significantly to the pathogenesis of colitis.<sup>14–17</sup> The role of *N*-linked glycosylation of serum proteins in IBD has been less well described. A recent publication has reinvigorated interest in this field by profiling the whole serum *N*-glycome in patients with UC.<sup>18</sup> Miyahara et al<sup>18</sup> demonstrated a higher expression of *N*-glycans in patients with UC compared with controls, more specifically highly sialylated multibranched glycans and agalactosyl biantennary glycans, which have also been implicated in rheumatoid arthritis and diabetes.

A number of genetic studies compel studying the relationship between IBD and glycosylation of immunoglobulins. Immunoglobulin G (IgG) is the most abundant of circulating antibodies and acts against pathogens in the acquired immune system. A recent study by members of this consortium demonstrated large individual variability in IgG glycosylation.<sup>19</sup> The IgG glycome composition was up to 80% heritable, with the involvement of numerous genetic and epigenetic loci.<sup>20</sup> A recent genome-wide association study discovered 9 loci significantly associated with IgG glycosylation, several of which are also complex immune diseases susceptibility loci.<sup>21</sup> Particularly noticeable is the pleiotropy with IBD as 5 of 16 IgG glycome genome-wide

association study associations (attaining significance threshold of  $P = 5 \times 10^{-8}$ ) occurred in known IBD loci (*IKZF1*, *LAMB1*, *MGAT3*, *IL6ST*, and *BACH2*), indicating that IgG glycosylation may be an important factor in the development and course of IBD.<sup>5,8,21</sup>

IgG carries *N*-linked glycans at a single location on the C $\gamma$ 2 domain of its Fc fragment. Despite having a single *N*-glycan attachment site, more than 900 IgG glycoforms are theoretically possible.<sup>22</sup> Functionally, minute changes in glycan composition can significantly change the structural conformation of the Fc region with dramatic consequences for IgG effector functions.<sup>23,24</sup> IgG can demonstrate both pro- and anti-inflammatory activity depending on its glycosylation status. Sialylation of IgG converts IgG from a proinflammatory to an anti-inflammatory molecule.<sup>25</sup> Dynamic changes to the efficacy of IgG-mediated antibody-dependent cellular toxicity (ADCC) occur with changes in core fucosylation of IgG, with ADCC 100 times more likely to occur in IgG lacking a core fucose. Core fucosylation is present in 95% of circulating IgG molecules and may limit inappropriate and potentially destructive ADCC.<sup>19,26</sup>

To examine the potential role of individual variation in IgG glycosylation on IBD, we performed detailed characterization of IgG glycome composition in 507 patients with UC, 287 patients with CD, and 320 healthy controls.

## MATERIALS AND METHODS

### Clinical Samples and Ethical Considerations

#### IBD Cases

Patients with IBD were recruited in Edinburgh (Western General Hospital) and Dundee (Ninewells Hospital) in South East Scotland between 2001 and 2012. The appropriate ethical approvals were obtained before sample collection (Dundee Ethics [Tayside Ethics committee 226/02], Edinburgh Ethics [Lothian Ethics committee 2000/4/192, South East Scotland SAHSC Annotated BioResource 10/S1402/33]), and written informed consent was obtained from all participants. A clinical, radiological, endoscopic, and histopathological diagnosis of IBD was made according to Lennard-Jones<sup>27</sup> criteria. Disease location and behavior was classified according to the Montreal-classification.<sup>28</sup> The following outcome data were collected for UC: colectomy, colectomy reason (acute, chronic, and dysplasia), and time from diagnosis to colectomy; and for CD: biologic requirement, surgery, time from diagnosis to first surgery, and number of surgeries. Follow-up time was censored at the date of colectomy (or surgery) or on January 31, 2013, for participants who were not known to have had colectomy (or surgery).

#### Control Samples

Control samples used in the main analysis consisted of symptomatic patients with no discernable pathology after investigations (including colonoscopy) ( $n = 119$ ) and a set of healthy

volunteers recruited through the Study of Colorectal Cancer in Scotland (n = 201).<sup>29</sup> Both sets of controls were drawn from the same geographical area and were recruited during the same time period (Multicenter Ethics Committee for Scotland MREC/01/0/5).

## Analysis of IgG Glycans

### Isolation of IgG from Human Serum

The IgG was isolated using protein G monolithic plates (BIA Separations, Ajdovščina, Slovenia) as described previously.<sup>19</sup> Briefly, 50 to 90  $\mu$ L of serum was diluted 7 $\times$  with 1 $\times$  PBS, pH 7.4, applied to the protein G plate and instantly washed with 1 $\times$  PBS, pH 7.4, to remove unbound proteins. IgG was eluted with 1 mL of 0.1 M formic acid (Merck, Darmstadt, Germany) and neutralized with 1 M ammonium bicarbonate (Merck).

### Glycan Release and Labeling

Half of each IgG sample (350–400  $\mu$ g) was dried in a vacuum concentrator and denatured with addition of 30  $\mu$ L 1.33% SDS (wt/vol) (Invitrogen, Carlsbad, CA) and by incubation at 65°C for 10 minutes. Subsequently, 10  $\mu$ L of 4% Igepal-CA630 (vol/vol) (Sigma-Aldrich, St. Louis, MO) and 1.25 mU of PNGase F (ProZyme, Hayward, CA) in 10  $\mu$ L 5 $\times$  PBS were added. The samples were incubated overnight at 37°C for *N*-glycan release. The released *N*-glycans were labeled with 2-aminobenzamide (2-AB). The labeling mixture was freshly prepared by dissolving 2-AB (19.2 mg/mL; Sigma-Aldrich) and 2-picoline borane (44.8 mg/mL; Sigma-Aldrich) in DMSO (Sigma-Aldrich) and glacial acetic acid (Merck) mixture (70:30, vol/vol). A total of 25  $\mu$ L of labeling mixture was added to each *N*-glycan sample in the 96-well plate, and the plate was sealed using adhesive seal. Mixing was achieved by shaking for 10 minutes, followed by 2 hours incubation at 65°C. Samples (in a volume of 75  $\mu$ L) were brought to 80% ACN (vol/vol) by adding 300  $\mu$ L of ACN (J. T. Baker, Phillipsburg, NJ). Free label and reducing agent were removed from the samples using HILIC-SPE. A total of 200  $\mu$ L of 0.1 g/mL suspension of microcrystalline cellulose (Merck) in water was applied to each well of a 0.45- $\mu$ m GHP filter plate (Pall Corporation, Ann Arbor, MI). Solvent was removed by application of vacuum using a vacuum manifold (Millipore Corporation, Billerica, MA). All wells were prewashed using 5 $\times$  200  $\mu$ L water, followed by equilibration using 3 $\times$  200  $\mu$ L acetonitrile/water (80:20, vol/vol). The samples were loaded to the wells. The wells were subsequently washed 7 $\times$  using 200  $\mu$ L acetonitrile/water (80:20, vol/vol). Glycans were eluted 2 $\times$  with 100  $\mu$ L of water and combined eluates were stored at –20°C until usage.

### Hydrophilic Interaction Chromatography (HILIC)–Ultra Performance Liquid Chromatography

Fluorescently labeled *N*-glycans were separated by hydrophilic interaction chromatography on a Waters Acquity ultra performance liquid chromatography (UPLC) instrument (Milford, MA) consisting of a quaternary solvent manager, sample

manager, and an FLR fluorescence detector set with excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was under the control of Empower 2 software, build 2145 (Waters). Labeled *N*-glycans were separated on a Waters bridged ethylene hybrid, glycan chromatography column, 100  $\times$  2.1 mm internal diameter, 1.7- $\mu$ m bridged ethylene hybrid particles, with 100 mM ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B. The separation method used a linear gradient of 75% to 62% acetonitrile (vol/vol) at flow rate of 0.4 mL/min in a 25-minute analytical run. Samples were maintained at 5°C before injection, and the separation temperature was 60°C. The system was calibrated using an external standard of hydrolyzed and 2-AB labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units. Data processing was performed using an automatic processing method with a traditional integration algorithm after which each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The chromatograms were all separated in the same manner into 24 peaks, and the amount of glycans in each peak was expressed as % of total integrated area.

### Statistical Analysis

Clinical characteristics among the 3 groups (UC patients, CD patients, and healthy controls) were compared using Wilcoxon (for continuous outcomes) and exact tests (for categorical variables).

To remove experimental variation from measurements, normalization and batch correction were performed on UPLC glycan data. Total area normalization was applied, where peak area of each of the 24 glycan structures was divided by the total area of the corresponding chromatogram, resulting in normalized traits being expressed as proportions (in percentages) of specific glycan among total measured glycans. Before batch correction, normalized glycan measurements were log-transformed because of right-skewness of their distributions and the multiplicative nature of the batch effects. For each glycan group independently, batch correction was performed on log-transformed measurements using linear mixed models (R package lme4<sup>30</sup>). In the model, the batch membership (which sample was analyzed on which plate) was described as a random effect. To get measurements corrected for experimental noise, estimated batch effects were subtracted from log-transformed measurements.

High-throughput glycomics analysis techniques are currently relatively novel, thus additional care is needed to avoid false interpretation of results. Because of the chemical nature of glycans, glycan analysis is sensitive to slight variations in collection and analysis of samples. As patient and control samples originated from several centers, before comparing cases with controls, we first had to establish which glycans are robust regarding slight experimental variation in sample collection and time period of measurements. Two quality control metrics were used (on normalized, batch-corrected glycan measurements) for the selection, and both had to be satisfied for a glycan to be



selected for the further analysis. First, only the most abundant glycan structures (average abundance above 2% of the glycome) were considered for the analysis, as they generally show greater robustness compared with less abundant glycans.<sup>31</sup> Then, using Wilcoxon test, we compared 2 separately recruited parts of the control cohort and only glycans for which there were no statistically significant differences between the 2 subgroups of the control cohort (corrected for age) were used for comparison with IBD cases.

Eight glycan peaks passed both quality checks: GP4, GP6, GP8, GP9, GP10, GP14, GP18, and GP19. In addition to these 8 directly measured glycan structures, 15 derived traits were calculated from the directly measured glycans (see Table, Supplemental Digital Content 1, <http://links.lww.com/IBD/A791>). As derived traits represent ratios and sums of initial glycans, they were calculated using normalized and batch-corrected glycan measurements after transformation to the proportions (exponential of batch-corrected measurements).

Association analyses between disease status and glycotraits were performed using a logistic regression model with age, sex, and IBD cohort included as additional covariates. As a result of the large number of glycotraits investigated, the analysis was performed by including 1 glycotrait at a time in the model. We also performed an intracase analysis where we examined the associations between the percentage of each specific glycotrait and need for colectomy (patients with UC) or surgery (patients with CD) using a Cox proportional hazards model adjusted for age (when sample was taken), gender, and IBD cohort and accounting for follow-up time (time between diagnosis and colectomy or first surgery). Before logistic and Cox regression analysis, glycan variables were all transformed to standard Normal distribution by inverse transformation of ranks to Normality (R package “GenABEL,”<sup>32</sup> function `mtransform`). Using predictors, which are transformed to standard Normal, in logistic and Cox regression analysis makes estimated odds and hazard ratios of different glycans comparable, where the estimated odds or hazards ratio always corresponds to 1 SD change in the measured glycan trait. To evaluate the difference in changes in CD, compared with patients with UC, we defined the magnitude of change in a glycan as an absolute value of logarithm of odds ratio, which were then tested using Wilcoxon paired test. False discovery rate for both the logistic regression and Cox proportional hazards analyses was controlled using Benjamini–Hochberg procedure.<sup>33</sup>

To classify patients with UC and CD, a logistic regression model was applied. Only the selected measured glycans—GP4, GP6, GP8, GP9, GP10, GP14, GP18, and GP19—were used in the classification analysis. Before model training, measurements of initial glycans were adjusted for age (through residuals). To evaluate the performance of the discriminatory model, the leave-one-out cross-validation (LOOCV) procedure was used. In the LOOCV procedure, each partitioning of the data is done in a way that the validation set contains only 1 sample, whereas the predictive model is trained on all remaining samples. In each round of cross-validation, the full model (a model that contains all 8 initial glycans) was fitted into the training set. To remove from

the model those glycan variables that do not statistically significantly improve classification performance, variable selection was performed using the backward stepwise elimination procedure. The optimal model was determined using the Akaike information criterion,<sup>34</sup> where the best model is defined as one with the lowest Akaike information criterion score among all tested models (R package “stats”<sup>35</sup>; function `stepAIC`) with equivalent degrees of freedom = 2, which corresponds to the traditional Akaike information criterion). At the end of each LOOCV round, the optimal model was used for discrimination on a single sample validation set. Predictions made in each LOOCV round were pooled in 1 set, and the performance of the discriminatory model was evaluated on a pooled validation set based on accuracy criteria (threshold = 0.5) and area under the receiver operating characteristic curve criteria.<sup>36</sup> Differences in glycomes between patients and controls were visualized using principal component analysis. Principal component analysis was applied only on glycan variables that were selected by backward elimination procedure: GP4, GP8, GP9, GP14, and GP18 in HC/UC model and GP4, GP6, GP9, and GP14 in HC/CD model. The area under the receiver operating characteristic curve of the 2 classification models (HC/UC and HC/CD) were compared using the bootstrap test.

Data were analyzed using R (version 3.0.1) and STATA (version 12.0).

## RESULTS

Using the recently developed and thoroughly validated quantitative method for IgG glycosylation profiling, we have analyzed the composition of the IgG glycome in 507 patients with UC, 287 patients with CD, and 320 healthy controls. The distribution of current, non-, and ex-smokers was different between the patients with UC and controls and patients with CD (both  $P < 0.001$ ), with the difference mostly coming from the proportion of current smokers. In other parameters, there were no significant differences between the control and UC/CD groups (Table 1). Further detailed phenotypic information regarding IBD cases is listed in Table, Supplemental Digital Content 2, <http://links.lww.com/IBD/A792>. Chromatographic analysis separated the glycome in 24 chromatographic peaks (GP1–GP24), the majority of which represented a single glycan structure (Fig. 1). Eight of these structures, together with 15 additional derived traits were included in the current analysis.

Statistically significant differences were observed in a number of glycan traits as estimated in logistic regression analysis (Table 2, Fig. 2, and see Fig., Supplemental Digital Content 3, <http://links.lww.com/IBD/A793>). We have successfully replicated a significant decrease in IgG galactosylation reported in 2 previous studies (GP14 and G2N).<sup>18,37</sup> Thanks to a much larger cohort and improved analytical technology, we were also able to observe a decrease in monogalactosylated IgG glycans (GP9), which has not been reported previously. Another novel observation is the significant decrease in sialylation of bigalactosylated IgG glycans in CD (GP18 and S total). In CD, there was

**TABLE 1.** Demographics of Included Patients and Controls

	Healthy Controls n = 320	CD n = 287	UC n = 507	P (CD Versus HC)	P (UC Versus HC)	P (CD Versus UC)
Age, median [IQR], yr	46 (39–51)	42 (29–56)	45 (34–59)	0.05	0.54	0.006
Number of females (%)	175 (54.7)	183 (63.8)	292 (57.6)	0.03	0.47	0.01
Median age at diagnosis (IQR)	—	27 (21–49)	34 (25–47)	—	—	$1.11 \times 10^{-8}$
Smoking status at diagnosis (%)						
Current	53 (16.6)	87 (30.3)	48 (9.4)	0.01	$2.05 \times 10^{-5}$	$7.92 \times 10^{-14}$
Ex-smoker	73 (22.8)	67 (23.3)	200 (39.3)			
Never smoked	142 (44.4)	128 (44.6)	253 (49.9)			
Unknown	52 (16.3)	5 (1.7)	6 (1.2)			
Ethnicity (%)						
White	309 (96.6)	282 (98.3)	487 (96.1)	0.5	0.15	0.03
Non-white	6 (1.9)	3 (1)	19 (3.7)			
Unknown	5 (1.6)	2 (0.7)	1 (0.2)			
Family history of IBD (%)						
Yes	0	50 (17.4)	95 (18.7)	—	—	0.847
No	115 (35.9)	224 (78)	402 (79.3)			
Unknown	205 (64)	13 (4.5)	10 (2)			
Immunomodulator treatment (%)						
Yes	—	199 (69.3)	184 (36.2)	—	—	0.027
No		31 (10.8)	50 (9.9)			
Unknown		57 (19.9)	273 (53.8)			
Anti-TNF $\alpha$ or ciclosporin treatment (%)						
Yes	—	67 (23.3)	32 (6.3)	—	—	$8.61 \times 10^{-12}$
No		205 (71.4)	448 (88.4)			
Unknown		15 (5.2)	27 (5.3)			

also an increase in glycans with bisecting GlcNAc (B total) compared with controls. The pattern of glycosylation changes was very similar between UC and CD. For all glycans that were statistically significantly different between cases and controls, the intensity of change was consistently higher in CD than in UC ( $P = 5 \times 10^{-4}$ , Table 2).

### Correlation of Glycans with Clinical Phenotype

In total, 507 patients with UC included in the Cox regression analysis were followed up for 7090 person-years. Fifty two patients had a colectomy, and median follow-up was 11.1 years (interquartile range: 5.9–20.1 years). Glycan peak FA2BG2S1/FA2G2S1 was marginally significantly (false discovery rate  $p$  value = 0.05) increased in patients with UC undergoing colectomy compared with no colectomy (see Table, Supplemental Digital Content 4, <http://links.lww.com/IBD/A794>).

The 287 patients with CD included in the Cox regression analysis were followed up in total for 2628 person-years. Eighty-four patients had a surgery, and median follow-up was 6.9 years (interquartile range: 2.0–13.1 years). No glycan peaks were

statistically significantly different in those with CD undergoing surgery compared with no surgery (see Table, Supplemental Digital Content 4, <http://links.lww.com/IBD/A794>).

### Discrimination of Disease Status

Given the strong association of certain glycan traits with both UC and CD, we attempted to build discriminatory models for both diseases using logistic regression. To evaluate the discriminatory performance of the model based on glycan measurements, LOOCV procedure was used. Predictions from each LOOCV round were pooled in 1 set, and predictive model was evaluated on pooled set of predictions. For both models, a statistically significant discrimination power was observed (UC:  $P = 2 \times 10^{-6}$ ; CD:  $P < 2 \times 10^{-16}$ ; Table 3; Fig. 3A, B). A similar subset of glycan variables was selected as relevant features for disease discrimination for both the UC and CD models. In particular, GP4 (FA2), GP8 (FA2[6]G1), GP9 (FA2[3]G1), GP14 (FA2G2), and GP18 (FA2G2S1) were selected as informative variables for the UC discriminatory model, whereas GP4 (FA2), GP6 (FA2B), GP9 (FA2[3]G1), and GP14 (FA2G2) were chosen for the CD discriminatory model. Differences between patients and controls in the set

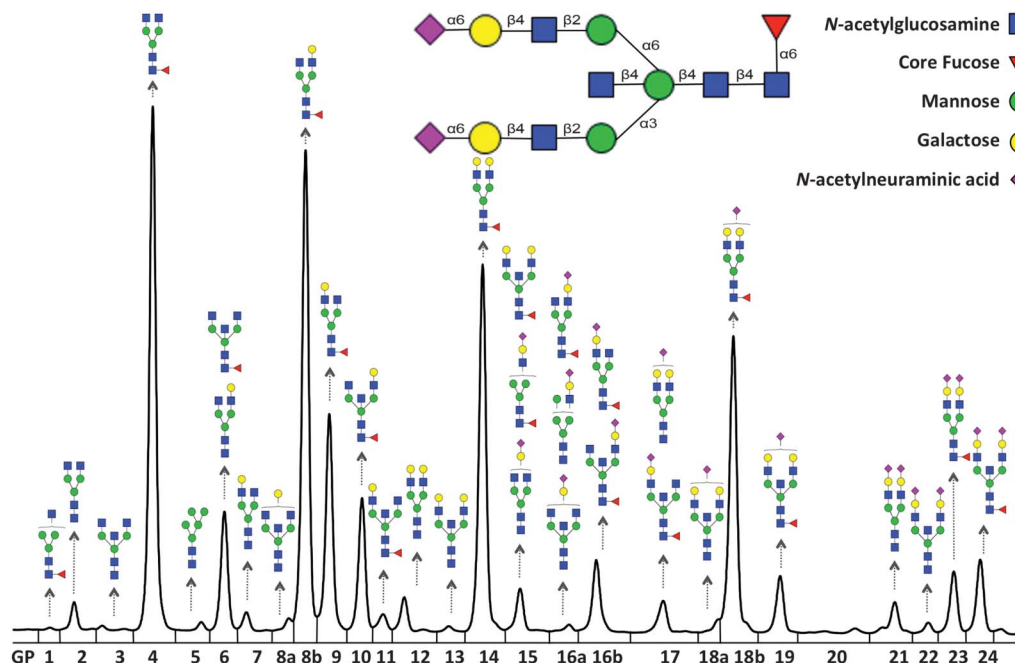


FIGURE 1. UPLC analysis of IgG glycosylation. Each IgG contains 1 conserved *N*-glycosylation site on Asn297 of its heavy chain. Different glycans can be attached to this site, and the process seems to be highly regulated. UPLC analysis can reveal composition of the glycome attached to a population of IgG molecules by separating total IgG *N*-glycome into 24 chromatographic glycan peaks (GP1–GP24), mostly corresponding to individual glycan structures.

of glycans that were used in the final discriminatory models were visualized using principal component analysis (Fig. 3C, D). Finally, analysis of ROC curves showed a superior performance of the CD discriminatory model over the UC model (CD: AUC = 0.77; UC: AUC = 0.72;  $P = 0.026$ ). This is in accordance with the results from the initial logistic regression analysis (Table 2), where the magnitude of change in glycan composition was larger for patients with CD compared with UC ( $P = 5 \times 10^{-4}$ ).

## DISCUSSION

This study used UPLC to determine differences in IgG glycome composition between patients with IBD and controls. There was significantly decreased galactosylation in patients with CD and UC compared with controls. CD was associated with significantly decreased sialylation and increased bisecting GlcNAc on digalactosylated IgG glycans. The observed differences may have some functional relevance for IBD because alterations of IgG have significant consequences on both IgG effector functions<sup>12,38</sup> and the immune system in general.<sup>39</sup>

IgG glycosylation was first associated with IBD in 2008 when a study on 58 patients with UC and 60 patients with CD reported significantly decreased galactosylation in both diseases.<sup>37</sup> A subsequent study performed in 2013, on 75 patients with UC also confirmed decreased galactosylation of IgG in UC.<sup>18</sup> Here, we successfully replicate the findings of the 2 aforementioned studies in a larger cohort with greater statistical power.

Furthermore, for the first time, we have demonstrated that monogalactosylated structures are decreased in both UC and CD.

Decreased IgG galactosylation can lead to a more proinflammatory antibody response.<sup>12</sup> This change in IgG galactosylation has been reported in a number of inflammatory diseases<sup>13</sup> but importantly also occurs with aging in the general population.<sup>20</sup> It can be postulated that the observed inflammatory IgG glycoforms are a consequence of inflammation rather than a cause of IBD. However, in rheumatoid arthritis, decreased galactosylation has been demonstrated to predate the onset of arthritis.<sup>40,41</sup> Thus, individual genetically predetermined differences in capacity to galactosylate IgG may be a predisposing factor for the development of IBD and other inflammatory diseases.<sup>42</sup>

*IL6ST* (IL-6 signal transducer or GP130) and *BACH2* have been identified as IBD susceptibility loci in genome-wide association study studies<sup>5,8</sup> and are both associated with IgG galactosylation.<sup>21</sup> This may provide a potential molecular mechanism linking *IL6ST* and *BACH2* polymorphisms with the aberrant IgG galactosylation with IBD demonstrated here. Additionally, IL-6 has been implicated in the pathogenesis of several immune-mediated diseases and tocilizumab, a monoclonal antibody directed against the IL-6 receptor has been developed to treat different inflammatory diseases.<sup>43</sup> A recent study on patients with rheumatoid arthritis treated with tocilizumab revealed significantly increased IgG galactosylation, providing further evidence that changes in galactosylation are functionally important.<sup>44</sup>

**TABLE 2.** Odds Ratios (OR), 95% Confidence Intervals (95% CI) and *P* Values for the Associations of the Normalized Glycan Variables (Adjusted for Age, Gender and IBD Cohort)

Glycan*	UC			CD			
	OR (95% CI)	<i>P</i>	q	OR (95% CI)	<i>P</i>	q	
Initial measurements	GP4	1.59 (1.25–2.04)	0.0001	0.0003	2.51 (1.85–3.5)	$2.0 \times 10^{-10}$	$2.8 \times 10^{-9}$
	GP6	1.44 (1.11–1.88)	0.01	0.01	2.95 (2.06–4.37)	$2.4 \times 10^{-10}$	$2.8 \times 10^{-9}$
	GP8	0.82 (0.66–1.02)	0.07	0.09	0.84 (0.65–1.08)	0.18	0.20
	GP9	0.58 (0.46–0.72)	$8.4 \times 10^{-7}$	$2.6 \times 10^{-6}$	0.69 (0.53–0.87)	0.002	0.005
	GP10	0.83 (0.66–1.04)	0.10	0.12	1.13 (0.87–1.47)	0.35	0.36
	GP14	0.71 (0.54–0.91)	0.01	0.01	0.41 (0.29–0.56)	$1.4 \times 10^{-9}$	$9.1 \times 10^{-9}$
	GP18	0.77 (0.6–0.99)	0.04	0.05	0.49 (0.36–0.66)	$7.3 \times 10^{-7}$	$2.6 \times 10^{-6}$
	GP19	1.12 (0.91–1.37)	0.30	0.32	0.62 (0.48–0.8)	0.0002	0.0004
	Neutral IgG glycans	GP4n	1.63 (1.28–2.09)	0.0001	0.0001	2.49 (1.84–3.49)	$2.4 \times 10^{-10}$
Neutral IgG glycans	GP6n	1.42 (1.09–1.85)	0.01	0.02	2.58 (1.82–3.75)	$1.6 \times 10^{-8}$	$9.3 \times 10^{-8}$
Neutral IgG glycans	GP8n	0.74 (0.59–0.93)	0.01	0.02	0.58 (0.44–0.76)	$5.7 \times 10^{-5}$	0.0001
Neutral IgG glycans	GP9n	0.54 (0.43–0.67)	$2.9 \times 10^{-8}$	$1.3 \times 10^{-7}$	0.54 (0.41–0.7)	$8.4 \times 10^{-7}$	$2.6 \times 10^{-6}$
Neutral IgG glycans	GP10n	0.81 (0.64–1.01)	0.06	0.08	0.96 (0.74–1.24)	0.74	0.74
Neutral IgG glycans	GP14n	0.72 (0.55–0.92)	0.01	0.02	0.39 (0.28–0.54)	$3.7 \times 10^{-10}$	$2.8 \times 10^{-9}$
Proportion of sialylated structures in total IgG glycans	S total	0.8 (0.63–1.02)	0.07	0.09	0.46 (0.34–0.62)	$8.4 \times 10^{-8}$	$3.2 \times 10^{-7}$
Proportion of structures with bisecting GlcNAc in total IgG glycans	B total	1.19 (0.93–1.53)	0.17	0.20	1.74 (1.28–2.39)	0.0003	0.0006
Proportion of agalactosylated structures in neutral glycans	G0n	1.67 (1.3–2.17)	$3.8 \times 10^{-5}$	0.0001	2.82 (2.03–4.05)	$8.9 \times 10^{-12}$	$4.1 \times 10^{-10}$
Proportion of monogalactosylated structures in neutral glycans	G1n	0.52 (0.4–0.66)	$2.8 \times 10^{-8}$	$1.3 \times 10^{-7}$	0.46 (0.34–0.62)	$4.4 \times 10^{-8}$	$1.8 \times 10^{-7}$
Proportion of digalactosylated structures in neutral glycans	G2n	0.72 (0.55–0.92)	0.01	0.02	0.39 (0.28–0.54)	$3.7 \times 10^{-10}$	$2.8 \times 10^{-9}$
Ratio of the presence to absence of bisecting GlcNAc in agalactosylated structures	FA2B/FA2	1.23 (0.99–1.52)	0.06	0.08	1.21 (0.94–1.56)	0.14	0.16
Ratio of the presence to absence of bisecting GlcNAc in monogalactosylated structures	FA2BG1/FA2G1	0.92 (0.73–1.17)	0.51	0.52	1.18 (0.9–1.56)	0.23	0.25
Ratio of the presence to absence of bisecting GlcNAc in monosialylated digalactosylated structures	FA2BG2S1/FA2G2S1	1.29 (1.02–1.62)	0.03	0.05	1.33 (1.02–1.74)	0.04	0.05
Ratio of monosialylated digalactosylated structures to digalactosylated structures without sialylation	FA2G2S1/FA2G2	1.21 (0.98–1.5)	0.07	0.09	1.33 (1.05–1.7)	0.02	0.03

Derived glycan structures are presented as the extension of the minimal core consisting of 2 GlcNAcs and 3 manoses with “G” representing glucose, “S” sialic acid, “F” core fucose, and “B” bisecting GlcNAc.

n, derived traits calculated from the subset of neutral glycans.

\*Directly Measured glycan peaks (GP1–GP24) are shown in Figure 1.

Because of technical limitations, previous studies of IgG glycosylation in IBD did not include other elements of IgG glycosylation such as sialylation, bisecting GlcNAc, and core fucose.<sup>18,37</sup> In this study, sialylation was significantly decreased in patients with CD (Figs. 2 and 3, Table 2). Given sialylation is

dependent, at least in part, on galactosylation, observed changes in sialylation may be driven by the known decrease in galactosylation seen in IBD.

Sialylation on IgG also results in an anti-inflammatory IgG phenotype.<sup>12</sup> Mechanisms include a reduction of the affinity of



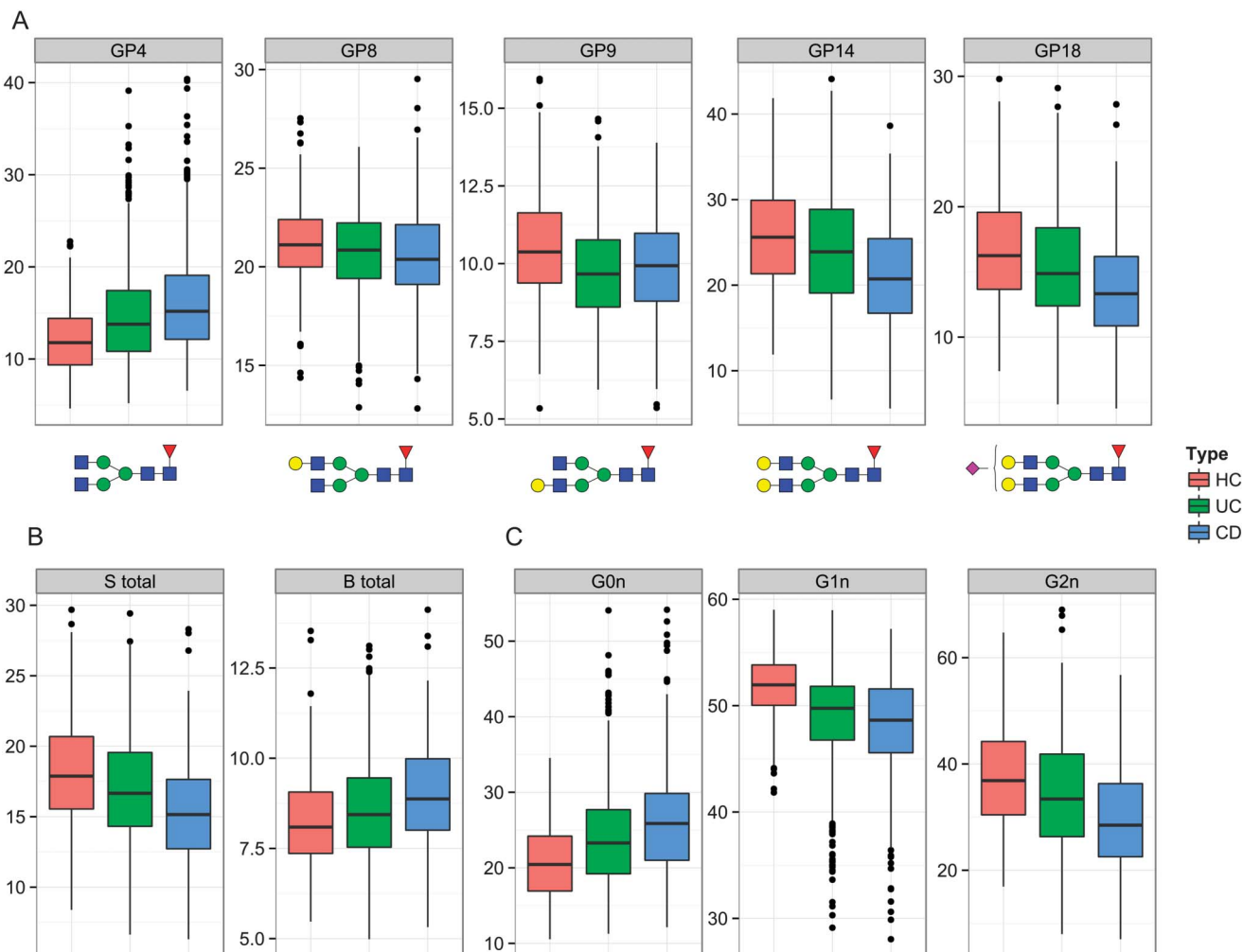


FIGURE 2. The distribution of IgG glycans in patients with UC and CD and healthy controls (HC). A, Directly measured glycan structures; B, Derived traits that measure sialylation and bisecting GlcNAc; C, Derived traits that measure galactosylation. Full set of glycans is available in Fig., Supplemental Digital Content 3, <http://links.lww.com/IBD/A793>.

IgG to bind to activated FcγRs<sup>45</sup> and promotion of recognition by DC-SIGN, which leads to increased expression of inhibitory FcγRIIB with consequent anti-inflammatory actions.<sup>45</sup> Sialylated IgG glycans are believed to be the active fraction that harbors the anti-inflammatory potential of intravenous immunoglobulins (IVIg). Therefore, through both decreased galactosylation and decreased sialylation, IgG in patients with IBD has significantly

greater proinflammatory properties than in healthy controls. In this study, we found this change to be more pronounced in CD compared with UC.

IVIg therapy is used to suppress inflammation in a number of diseases.<sup>39</sup> Although the therapeutic use of IVIg has been suggested in CD, its use in IBD is not routine.<sup>46</sup> The glycome composition we observed in patients with IBD (more pronounced in CD than in

**TABLE 3.** Performance Characteristics of the Logistic Regression Models Used to Discriminate Patients with UC and CD from Healthy Controls

Model	AUC	Number of Cases	Accuracy (95% CI)	Sensitivity	Specificity	P
UC	0.721	61	0.690 (0.657–0.722)	0.818	0.487	2.13 × 10 <sup>-6</sup>
CD	0.770	47	0.700 (0.662–0.736)	0.603	0.788	2.20 × 10 <sup>-16</sup>

Area under the ROC curve, accuracy, sensitivity, specificity, and significance were calculated for both UC and CD models.

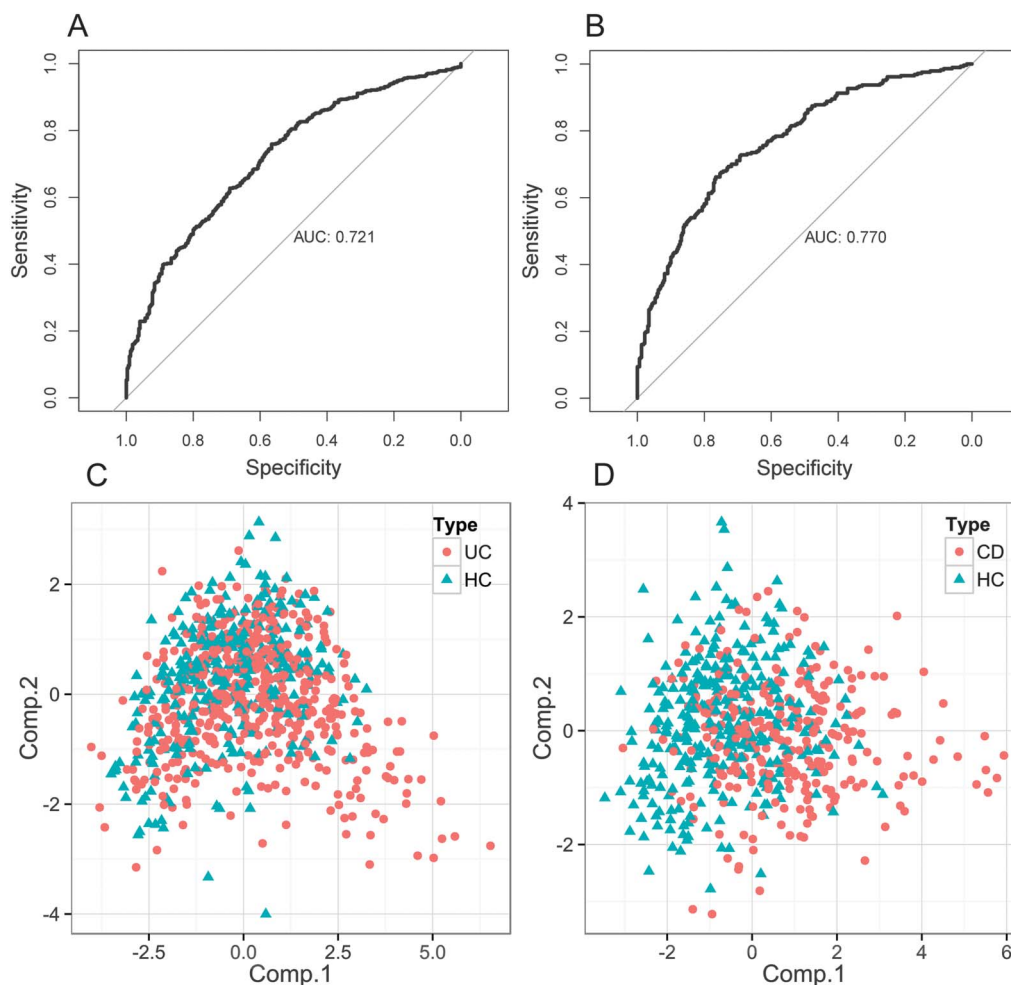


FIGURE 3. ROC curves illustrating the performance of logistic regression model in predicting disease status for patients with UC and healthy controls (A) and patients with CD and healthy controls (B). Principal component analysis plots for patients with UC and healthy controls (C) and patients with CD and healthy controls (D).

UC) is consistent with a decreased capacity of IgG to suppress inflammation, and thus could potentially be viewed as a decrease in “natural anti-inflammatory immunoglobulin therapy” in patients with IBD. The variation of IgG glycome composition in human population is very large,<sup>19</sup> and it is up to 80% heritable,<sup>20</sup> thus this more proinflammatory IgG glycome may also be a predisposition for the development of inflammatory diseases.

Approximately, 18% of IgG glycans contain bisecting GlcNAc, which significantly changes the structural properties of the glycan.<sup>19</sup> Although the decrease in bisecting GlcNAc on individual glycoforms did not reach statistical significance, the increase was consistently present on G0, G1, and G2 structures and the increase in total incidence of glycans with bisecting glycans was statistically significant in CD (B total, Table 2). The effects of bisecting GlcNAc on functional properties of IgG are not well understood,<sup>12</sup> but it is intriguing that an enzyme responsible for the addition of bisecting GlcNAc (GNT-III, coded by *MGAT3* gene) was also identified as the IBD susceptibility locus.<sup>5,47</sup>

The differential glycosylation described above may provide exciting insights into disease pathogenesis. However, causation is difficult to infer in data from those already diagnosed with the condition, and the observed changes may be consequence rather than cause of the disease. Nonetheless, glycobiology may be of immediate clinical relevance. Sialylation of IgG was found to be essential for the function of IVIg, and Fc fragments alone were found to be sufficient to suppress inflammation.<sup>48</sup> It seems that Fc with sialylated glycans suppresses inflammation through a novel  $T_H2$  pathway, which provides an intrinsic mechanism for maintaining immune homeostasis that could, in future, be manipulated to provide therapeutic benefit.<sup>49</sup> This is especially important given the widespread use of anti-TNF $\alpha$  monoclonal antibody therapy in IBD. Similarly, the mechanism of action of oncological monoclonal antibody therapies may derive from the therapeutic immunoglobulins lacking a core fucose, which in turn may lead to enhanced ADCC.<sup>50,51</sup> The analysis of individual variation of host immunoglobulin glycosylation also has great clinical potential

because it was recently demonstrated that variations in host IgG glycosylation may have an effect on the success of therapy.<sup>52</sup>

Glycan traits are also attractive candidates as biomarkers. Previous studies have attempted to define panels or ratios of *N*-linked serum glycans, which correlate with IBD diagnosis or disease course. Shinzaki et al<sup>37</sup> describe a ratio of IgG with no galactoses to 2 galactoses (G0:G2), which compares favorably as a diagnostic biomarker to the serological marker anti-*Saccharomyces cerevisiae* antibodies currently in clinical use. Miyahara et al demonstrate that a ratio of glycans of a specific molecular weight (*m/z* 2378/1914) and highly sialylated multibranched glycans correlates with UC disease activity (clinical activity index score >10), disease extent, and existing markers of disease activity such as C-reactive protein. On multi-variable analysis, this ratio of 2 specific glycan structures (*m/z* 2378/1914) was an independent prognostic factor for patients with UC requiring proctocolectomy.<sup>18</sup>

This is the largest study to date to compare IgG glycan structure in the context of IBD. This study both validates and expands upon the previous findings of serum glycan profiling in IBD,<sup>18</sup> using an alternative methodology (UPLC), which is also able to describe changes in sialylation, bisecting GlcNAc, and core fucosylation. Advancing technology, allowing high-throughput assessment of IgG glycan profiles, together with the ease of access to serum samples make glycan traits attractive and clinically feasible biomarkers.

The main limitation of this study was the retrospective nature of sample collection and limited phenotypic and outcome data available for included patients. Patients were sampled at various points in the disease course and after the administration of different therapeutics, thus providing only a cross sectional “snapshot” of glycosylation in IBD. It would be useful to observe longitudinal changes in glycan composition with differing disease courses and treatments. Ongoing prospective efforts by this consortium aim to validate the findings of this study while addressing some of the aforementioned limitations ([www.ibdbiom.eu](http://www.ibdbiom.eu)).

## CONCLUSIONS

We have described differences in glycosylation in IBD that are indicative of an increased inflammatory potential of IgG in IBD. Changes in IgG glycosylation may contribute to disease pathogenesis and could affect the efficacy of monoclonal antibody-based therapeutics commonly used in IBD. IgG glycan expression profiles may be developed as clinical useful biomarkers in the future.

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Chemistry, VU University Amsterdam, Amsterdam, the Netherlands), Dermot P. B. McGovern (F. Widjaja Family Foundation Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles), Vito Annese (Department of Medical and Surgical Sciences, Division of Gastroenterology, University Hospital Careggi, Florence, Italy), Iain K. Pemberton (IP Research Consulting SAS, Paris, France), Daniel IR Spencer (Ludger Ltd, Culham Science Centre, Oxford, United Kingdom), Vlatka Zoldoš (Department of Molecular Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia), Daryl L. Fernandes (Ludger Ltd, Culham Science Centre, Oxford, United Kingdom), Ray Boyapati (Centre for Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom), Ray Doran (Ludger Ltd, Culham Science Centre, Oxford, United Kingdom), Igor Rudan (Centre for Population Health Sciences, University of Edinburgh, Edinburgh, United Kingdom), Paolo Lionetti (Paediatric Gastroenterology Unit, AOU Meyer, Viale Pieraccini, Florence Italy), Natalia Manetti (Department of Medical and Surgical Sciences, Division of Gastroenterology, University Hospital Careggi, Florence, Italy), Anna Latiano (Department of Medical Sciences, Division of Gastroenterology, IRCCS-CSS Hospital, Viale Cappuccini, Rotondo, Italy), Anna Kohn (Division of Gastroenterology, S. Camillo Hospital, Rome, Italy), Renata D'Inca (Gastrointestinal Unit, University of Padua, Padua, Italy), Silvio Danese (IBD Unit, Humanitas Research Institute, Rozzano, Milan, Italy), Marieke Pierik (Maastricht University Medical Centre (MUMC), Maastricht, the Netherlands), and David C. Wilson (Centre for Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom).

Author contributions: *Study design*: G. Lauc, J. Satsangi, H. Campbell; *Sample provision*: N. T. Ventham, N. A. Kennedy, E. R. Nimmo, H. Drummond, J. Štambuk, R. Kalla; *Scientific analyses*: I. Trbojević Akmačić, J. Štambuk, J. Krišić, M. Pučić Baković, M. Novokmet, O. Gornik; *Statistical analyses*: F. Vučković, N. A. Kennedy, E. Theodoratou, N. T. Ventham, G. Lauc, Y. Aulchenko; *Drafting manuscript*: N. T. Ventham, I. T. Akmačić, E. Theodoratou, G. Lauc, J. Satsangi, Y. Aulchenko, N. A. Kennedy; *Critical review of manuscript*: H. Campbell, D. P. B. McGovern, V. Annese, D. Kolarich, M. Wührer, V. Zoldoš, I. K. Pemberton, D. L. Fernandes, E. R. Nimmo, Y. Aulchenko, D. R. I. Spencer, J. Satsangi, R. Kalla.

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