Glycomic markers of inflammatory bowel disease and IgA nephropathy

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Chapter 3

Plasma *N*-Glycan Signatures Are Associated with Features of Inflammatory Bowel Diseases

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Abbreviations: MALDI-TOF-MS = matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, SA = sialic acid, TPNG = total plasma *N*-glycome.

Abstract

Background & aims

Biomarkers are needed for early detection of Crohn's disease (CD) and ulcerative colitis (UC) or to predict patient outcomes. Glycosylation is a common and complex posttranslational modification of proteins that affects their structure and activity. We compared plasma *N*-glycosylation profiles between patients with CD or UC and healthy individuals (controls).

Methods

We analyzed the total plasma *N*-glycomes of 2635 patients with inflammatory bowel diseases and 996 controls by mass spectrometry with a linkage-specific sialic acid derivatization technique. Plasma samples were acquired from 2 hospitals in Italy (discovery cohort, 1989 patients with inflammatory bowel disease [IBD] and 570 controls) and 1 medical center in the United States (validation cohort, 646 cases of IBD and 426 controls). Sixty-three glycoforms met our criteria for relative quantification and were extracted from the raw data with the software MassyTools. Common features shared by the glycan compositions were combined in 78 derived traits, including the number of antennae of complex-type glycans and levels of fucosylation, bisection, galactosylation, and sialylation. Associations of plasma *N*-glycomes with age, sex, CD, UC, and IBD-related parameters such as disease location, surgery and medication, level of C-reactive protein, and sedimentation rate were tested by linear and logistic regression.

Results

Plasma samples from patients with IBD had a higher abundance of large-size glycans compared with controls, a decreased relative abundance of hybrid and high-mannose structures, lower fucosylation, lower galactosylation, and higher sialylation (α2,3- and α2,6-linked). We could discriminate plasma from patients with CD from that of patients with UC based on higher bisection, lower galactosylation, and higher sialylation (α2,3-linked). Glycosylation patterns were associated with disease location and progression, the need for a more potent medication, and surgery. These results were replicated in a large independent cohort.

Conclusions

We performed high-throughput analysis to compare total plasma *N*-glycomes of individuals with vs without IBD and to identify patterns associated with disease features and the need for treatment. These profiles might be used in diagnosis and for predicting patients' responses to treatment.

Keywords: Crohn's Disease; ulcerative colitis; glycosylation; mass spectrometry

Background and context

The etiology of inflammatory bowel diseases is poorly understood and improved non-invasive diagnostic and prognostic tools are needed. Protein-linked carbohydrates (glycans) represent promising biomarker and therapeutic candidates.

New findings

This study found replicated associations of disease status and different clinical parameters with multiple glycosylation features of plasma proteins, including structures known to be crucial in immunological processes.

Limitations

Several dozens of different glycoproteins mainly derived from liver or immune cells contribute to the plasma glycan profile. Further studies assessing the glycosylation of specific proteins are needed to elucidate the mechanisms behind these glycosylation changes.

Impact

Multiple novel potential targets for diagnostic and prognostic approaches in inflammatory bowel disease management have been presented here.

Introduction

Inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory conditions of the digestive tract. Approximately 1% of the population in developed countries is affected, and the prevalence in developing countries is rising rapidly[1]. Genetic variation, environmental risk factors, and a dysregulation of the mucosal immune system are associated with an increased risk of IBD, although the exact etiology of the disease remains unknown[2-4]. The diagnosis of IBD is not always straightforward, lacks a

criterion standard test, and usually relies on a combination of medical history, laboratory investigations, and diagnostic procedures[3, 4]. Established markers such as C-reactive protein (CRP), erythrocyte sedimentation rate, or fecal calprotectin have neither the sensitivity nor specificity to reliably distinguish inflammation from functional symptoms and can reflect various autoimmune diseases, cancer, inflammation, or infections[5, 6]. Endoscopic investigations are the most reliable way to confirm the presence of IBD, differentiate CD from UC, and evaluate the efficacy of therapy at the mucosal level, but they are invasive and carry risks linked to the procedure and sedation of the patients[7]. Recent data suggest that serologic markers, such as anti-Saccharomyces cerevisiae antibodies (ASCA) or anti-neutrophilic cytoplasmic antibodies (ANCA), may be promising, but their use is controversial, and they have not been widely adopted in the management of IBD[8]. Consequently, there is an urgent need for noninvasive prognostic tests that will allow clinicians to predict the natural course of disease and target expensive therapies to those most likely to benefit[9, 10].

Glycosylation is recognized to be a complex and highly abundant posttranslational modification of proteins that can significantly affect the structural and functional properties of the conjugate[11, 12]. The modification is involved in the regulation of biological processes ranging from protein secretion and transport to receptor interaction and modulation of the immune response[12, 13]. Protein glycoforms found in healthy individuals are quite stable over time but can dramatically change because of a pathologic condition, especially upon inflammation[14, 15]. Furthermore, protein glycosylation is involved in the pathophysiology of major diseases such as cancer, diabetes, and rheumatoid arthritis[13, 16], and has previously been associated with IBD[17-20]. A particular glycosylation phenotype, that is, the linkage of sialic acids (SAs), which can be of α2,3- or α2,6-type, is reported to affect various biological processes, for example, protein-receptor interactions and the immune response in tumor cells[21, 22]. Sialyl-Lewis X structures are formed exclusively with α2,3-linked SA, and we envision that the evaluation of SA linkages in the context of IBD can provide insights into disease mechanisms and help the development of targeted treatment strategies[15, 22-24].

To this end, we evaluated the total plasma *N*-glycome (TPNG) of 2635 IBD patients and 996 healthy control individuals (HCs) distributed over 2 independent cohorts. The glycans were released from the plasma proteins, and their SAs were derivatized with our recently developed linkage-specific derivatization method and analyzed using high-throughput matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[25, 26].

Materials and methods

Clinical samples

Plasma samples were acquired from 3 repositories in Italy and the United States. The Italian sample set was combined from the University Hospital Careggi (Florence, Italy) and IRCCS-CSS Hospital S. Giovanni (Rotondo, Italy)[27]. The American sample set was collected at the Pediatric and Adult IBD Centers at Cedars-Sinai Medical Center (Los Angeles, CA). Informed written consent was obtained from the participants, and ethical approval was provided by the Ethics Review Board of the University Hospital Careggi and Casa Sollievo della Sofferenza. Hospital (Italy) and by the Cedars-Sinai Institutional Review Board (United States), respectively. The patients were categorized according to the Montreal classification of IBD[28]. Because of its larger number of patient samples, the Italian sample set (1989 IBD patients and 570 HCs) was chosen as the discovery cohort and the American cohort (646 IBD patients and 426 HCs) for replication. The demographics of the individuals retained for statistical analysis are presented in **Table 1.**

Table 1: Demographics of the individuals included for cross-sectional statistical analysis

Italian cohort (discovery)				
Sample numbers	HC	CD	UC	All
Total (males)	570 (355)	905 (524)	1084 (648)	2559 (1527)
Age, mean ± SD				
Males	50.5 ± 17.4	37.1 ± 14.0	43.4 ± 15.5	42.9 ± 16.2
Females	55.4 ± 18.2	38.5 ± 15.1	39.9 ± 15.5	42.6 ± 17.2
All	52.3 ± 17.9	37.7 ± 14.5	42.0 ± 15.6	42.8 ± 16.7
American cohort (replication)				
Sample numbers	HC	CD	UC	All
Total (males)	426 (180)	389 (220)	257 (124)	1072 (524)
Age, mean ± SD				
Males	46.3 ± 14.8	36.4 ± 14.5	42.3 ± 17.0	41.2 ± 15.8
Females	44.5 ± 13.7	39.9 ± 15.7	41.4 ± 15.1	42.3 ± 14.8
All	45.2 ± 14.2	37.9 ± 15.1	41.8 ± 16.0	41.8 ± 15.3

Patient information				
	Italian		**American**	
Disease group (counts)	CD	UC	CD	UC
Disease Location (CD)				
Ileum+UpperGI (L1+L4)	306+19		81+8	
Colon+UpperGI (L2+L4)	158+6		55+3	
Ileum+Colon+UpperGI (L3+L4)	333+22		197+35	
Upper GI (L4)	60		46	
Disease Location (UC)				
Rectum (E1)		124		7
Sigmoid + Left colon (E2)		507		64
Entire colon (E3)		447		183
Disease behaviour (CD)				
Inflammatory (B1)	519		139	
Stenosing (B2)	249		109	
Fistulizing (B3)	128		136	
Perianal disease modifier				
Yes	187	27	133	
No	667	982	216	
Surgery				
No	557	939	146	120
Before sample collection	250	39	119	85
After sample collection	33	7	44	14
Medication				
Mesalazine	186	329		
Steroids	242	371	all	all
Azathioprine/6-mercaptopurine	198	220	69	87
Anti-TNFα	186	89	310	148
Inflammation markers				
CRP levels			125	74
Sedimentation rate			140	77

Mass spectrometric glycomics and data analysis

The Italian plasma samples were prepared as previously described[26] and analyzed by MALDI-TOF-MS at the Max Planck Institute for Dynamics of Complex Technical Systems in Magdeburg, Germany. The American samples were prepared and measured on a robotized platform at the Leiden University Medical Center in Leiden, The Netherlands[25]. For this, the samples were randomly distributed in equal proportions of cases and controls on 96-well plates. The glycans were enzymatically released from the plasma proteins using peptide-*N*-glycosidase F, and the SAs were

derivatized by linkage-specific esterification. The derivatized glycans were then purified by hydrophilic interaction liquid chromatography solid-phase extraction and were analyzed by MALDI-TOF-MS in reflectron-positive mode on Bruker ultrafleXtreme instruments (Bruker Daltonics, Bremen, Germany)[25, 26]. A detailed description of the material and methods can be found in the **Supplementary Material**.

Data processing

In total, 63 glycoforms passed our quality criteria for relative quantification and were extracted from the raw data with the software MassyTools[29] (**Supplementary Table 1**). Common features shared by the glycan compositions were combined in 78 derived traits, including the number of antennae of complex-type glycans (CA), and the levels of fucosylation (F), bisection (B), galactosylation (G), and sialylation (S) (**Supplementary Table 2**)[30]. The relative glycan intensities and calculated derived traits were corrected for batch effects. Data points deviating more than 5 standard deviations (SDs) from their respective means were considered outliers and were excluded from further analysis.

Statistical analysis

The associations of the TPNG with age-, sex-, CD-, UC-, and IBD-related parameters such as disease location, surgery, medication, CRP levels, and sedimentation rate were tested by linear and logistic regression (for continuous and binary outcome variables, respectively) on standardized data (subtraction of the mean and division by the SD) using R, version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) and RStudio, version 1.0.136 (RStudio, Boston, MA) (see Supplementary Material). Age, sex, and their interaction were included as covariates in the models for the disease-related tests. The odds ratios (ORs) were calculated with their 95% confidence intervals (CIs) assuming a Student‘s t distribution and are referring to an increase of 1 SD in the tested traits. A meta-analysis of the associations with IBD found in the discovery and replication cohort was performed using a fixed effects model.

For the receiver operating characteristic analysis, 1 derived glycan trait was first selected from each of the derived trait classes (complex-type glycans, fucosylation, bisection, galactosylation, sialylation, E/L) based on having the strongest effect sizes in the meta-analysis and tested for significance in their prediction of diagnosis. Those found not to be significant were removed step-by-step from the equation until all remaining traits were deemed significant, thus achieving maximal prediction performance with a minimal set of traits. The model was trained on a random selection of 75% of the samples from the discovery cohort, and prediction was

validated on all available cases in the replication cohort. An internal prediction validation was also performed on the remaining cases of the discovery cohort (25%) to control for overfitting. The power of the prediction (area under the curve) was calculated on 10 repetitions of the random sampling in both cohorts.

In CD, we tested for association of the TPNG with localization of disease by comparing patients with ileal (L1) or colonic disease (L2) and associations with the disease severity by comparing concise CD (L1 or L2) to extended CD (ileocolon + upper gastrointestinal tract, L3 + L4), that is, L1 vs L2 vs L3 + L4 (see Supplementary Material). With respect to disease behavior in CD, we compared inflammatory (B1), stenosing (B2), and internal fistulizing behavior (B3) (B1 vs B2 vs B3), and furthermore compared patients with and without perianal disease. In UC, we compared distal disease (E1 + E2) and extensive disease (E3), that is, E1 + E2 vs E3, as indicated in **Table 1**. We also compared the TPNG profiles of colonic CD (L2) vs UC patients.

According to the clinical practices of the medical centers, patients were treated with mesalazine (mesalamine), steroids, azathioprine/6-mercaptopurine or anti–tumor necrosis factor (TNF)-α in the discovery (Italian) cohort, whereas all patients of the replication (US) cohort had been exposed to corticosteroid treatment accompanied by either azathioprine or anti– TNFα[31]. In the Italian cohort, patients were stratified into 4 groups according to the most potent drug class used at the time of sample collection. Patients exposed to both thiopurines and anti–TNF-α agents were classified as being exposed to anti–TNF-α agents for both cohorts. The TPNG profiles respective to each resulting drug class were compared with each other. Because the medication strategies differed between cohorts, we could not directly test for replication, but we looked for similarities instead.

Furthermore, we compared selected glycan traits of a small group of the replication cohort in paired samples (same individuals before and after surgery) using a Wilcoxon signed-rank test. Because cross-sectional surgery data were available in a larger patient group (Table 1), we tested for associations of the TPNG with the status of sample collection before (reference category) and after surgery in cross-sectional manner. We also tested the association of TPNG profiles with surgery by comparing patients requiring surgery with those who did not undergo surgery throughout the study period, thus testing for associations of the TPNG with the need of surgical intervention (see Supplementary Material).

Multiple testing correction was performed based on a false discovery rate of 5% (see **Supplementary Material**).

Results

The TPNG was analyzed by MALDI-TOF-MS for a total of 2635 IBD patients and 996 HCs from 2 independent cohorts. Sixty-three of the detected glycan compositions passed our quality criteria for relative quantification and were grouped into 78 derived traits based on their number of antennae, their bisection, galactosylation, fucosylation, and (linkage-specific) sialylation (Figure 1**, Supplementary Table 1, Supplementary Table 2**). Our primary objective was to find associations of the TPNG with UC or CD, compared with HCs, and CD vs UC. Additional secondary analyses were performed to evaluate the associations between the TPNG in patients and IBD-specific parameters, such as disease location, medication, and surgery. For the logistic models, we expressed the effect sizes as ORs, representing the odds of belonging to a group (eg, CD vs HC) in relation to an increase of 1 SD in the tested traits (eg, A2FS0G).

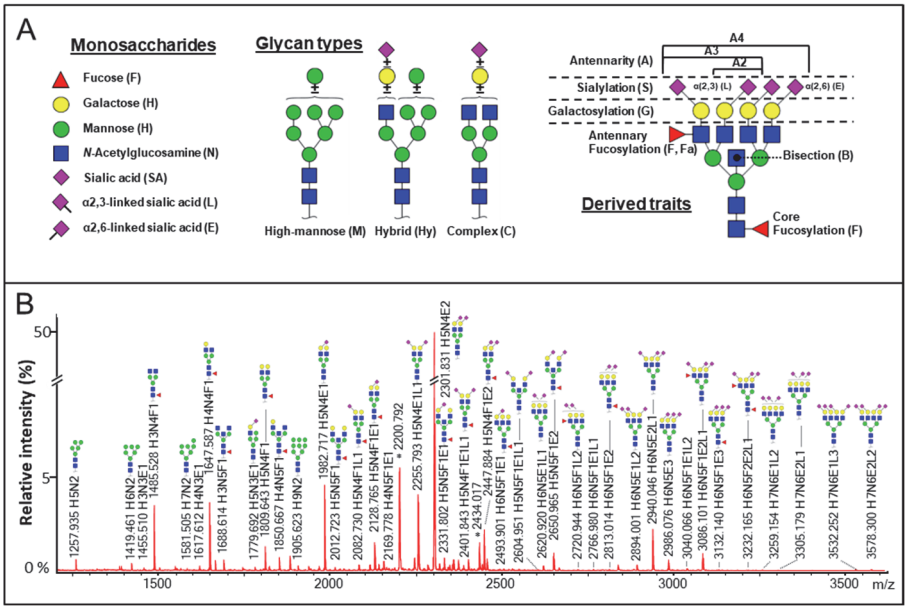


Figure 1. Glycan features and averaged spectrum of CD patients. *A) Schematic representation of glycan types and derived traits based on the common structural features of different individual glycans. B) Averaged MALDI-TOF-MS spectrum of CD patients from two 96-well plates of the discovery cohort (see Supporting information for details on the averaging of spectra). Major glycan peaks are assigned to compositions and structural schemes. The peaks marked with an asterisk (*) are satellites of the main peak.*

TPNG is associated with inflammatory bowel disease

The associations of the TPNG with IBD were tested by logistic regression on standardized data with age, sex, and their interaction as covariates in the model. In total, 45, 43, and 23 meta-analyzed associations were found in the 2 cohorts when comparing CD patients vs HCs, UC patients vs HCs, and UC vs CD patients, respectively (**Table 2, Supplementary Table 3**).

Table 2: Meta-analyzed associations of plasma *N*-glycans with CD and UC. *Red and blue labels indicate positive and negative replicated associations, respectively, with the second-called category being the reference. See Supplementary* ***Table 3*** *for the complete list of tests performed.*

Derived traits	Description of the derived traits	meta *p*-values			meta odds ratio (95% CI)		
		CD / Controls	UC / Controls	UC / CD	CD / Controls	UC / Controls	UC / CD
Glycan type							
CA2	Diantennary (A2) within complex-type	6.26E-28	5.60E-09	5.29E-16	0.53 (0.47-0.59)	0.75 (0.68-0.82)	1.34 (1.25-1.43)
CA3	Triantennary (A3) within complex-type	7.40E-28	2.06E-09	6.25E-15	1.88 (1.68-2.11)	1.35 (1.23-1.49)	0.76 (0.71-0.81)
CA4	Tetraantennary (A4) within complex-type	8.50E-08	2.43E-06	2.57E-01	1.32 (1.19-1.46)	1.27 (1.15-1.40)	1.04 (0.97-1.12)
TC	Complex-type within total	1.06E-28	8.14E-14	6.88E-08	1.83 (1.65-2.04)	1.45 (1.32-1.60)	0.82 (0.77-0.88)
TM	High-mannose (M) within total	3.63E-21	9.50E-12	4.85E-03	0.61 (0.55-0.67)	0.71 (0.65-0.78)	1.10 (1.03-1.18)
THy	Total hybrid within total	3.42E-32	2.51E-12	9.25E-25	0.52 (0.47-0.58)	0.71 (0.64-0.78)	1.48 (1.37-1.60)
TA2FS0	Total asialo fucosylated A2	1.25E-20	3.07E-07	1.00E-12	0.61 (0.55-0.67)	0.78 (0.71-0.86)	1.29 (1.20-1.38)
Fucosylation (F)							
CF	of complex-type	2.29E-13	1.70E-09	3.04E-06	0.68 (0.62-0.76)	0.74 (0.67-0.82)	1.18 (1.10-1.26)
CFa	Antennary F within complex-type	2.81E-01	4.24E-05	1.59E-07	1.05 (0.96-1.16)	0.82 (0.75-0.90)	0.83 (0.78-0.89)
A2Fa	Antennary F of A2	7.37E-23	5.85E-12	9.44E-05	0.59 (0.53-0.65)	0.70 (0.64-0.78)	1.15 (1.07-1.23)
A3Fa	Antennary F of A3	4.92E-04	4.69E-02	1.94E-17	1.19 (1.08-1.31)	0.91 (0.82-1.00)	0.73 (0.68-0.79)
A2F	of A2	2.97E-19	5.52E-10	1.42E-11	0.62 (0.56-0.69)	0.73 (0.67-0.81)	1.27 (1.19-1.36)
A3F	of A3	2.51E-05	2.02E-02	2.05E-25	1.24 (1.12-1.37)	0.89 (0.81-0.98)	0.68 (0.63-0.73)
A2SF	of A2 sialylated	1.30E-14	2.16E-10	2.24E-07	0.67 (0.61-0.74)	0.73 (0.66-0.80)	1.20 (1.12-1.29)
A2L0F	of A2 without α2,3 sialic acid (SA)	1.38E-17	2.78E-08	3.36E-12	0.64 (0.57-0.71)	0.76 (0.69-0.84)	1.28 (1.20-1.38)
A2LF	of A2 with α2,3 SA	1.79E-20	4.11E-29	5.73E-01	0.59 (0.52-0.66)	0.51 (0.45-0.57)	0.98 (0.91-1.05)
A3LF	of A3 with α2,3 SA	4.58E-04	3.90E-03	2.77E-25	1.19 (1.08-1.32)	0.87 (0.78-0.95)	0.68 (0.63-0.73)
A2EF	of A2 with α2,6 SA	1.85E-11	2.88E-08	2.34E-06	0.71 (0.64-0.79)	0.76 (0.69-0.84)	1.18 (1.10-1.27)
A3EF	of A3 with α2,6 SA	6.00E-06	3.80E-02	2.42E-26	1.26 (1.14-1.39)	0.90 (0.82-0.99)	0.67 (0.62-0.72)
Bisection (B)							
A2FB	of fucosylated A2	9.48E-13	2.16E-03	6.87E-06	1.49 (1.33-1.66)	1.17 (1.06-1.30)	0.85 (0.79-0.91)
A2FSB	of fucosylated sialylated A2	1.03E-14	1.15E-07	1.56E-02	1.51 (1.36-1.67)	1.31 (1.19-1.45)	0.92 (0.85-0.98)
A2F0S0B	of afucosylated nonsialylated A2	1.39E-10	4.60E-02	3.70E-20	1.41 (1.27-1.57)	0.90 (0.82-1.00)	0.71 (0.66-0.77)
Galactosylation (G)							
A2F0G	of afucosylated A2	1.26E-13	3.40E-05	5.55E-06	1.48 (1.33-1.64)	1.22 (1.11-1.34)	0.85 (0.79-0.91)
A2S0G	of asialo A2	1.12E-47	1.39E-13	2.11E-32	0.39 (0.35-0.45)	0.65 (0.58-0.73)	1.58 (1.47-1.70)

Derived traits	Description of the derived traits	meta *p*-values			meta odds ratio (95% CI)		
		CD / Controls	UC / Controls	UC / CD	CD / Controls	UC / Controls	UC / CD
A2FS0G	of fucosylated asialo A2	6.28E-51	1.34E-14	1.82E-36	0.37 (0.33-0.43)	0.64 (0.57-0.72)	1.63 (1.51-1.76)
A2F0SG	of afucosylated sialylated A2	2.03E-10	2.77E-07	5.49E-03	1.38 (1.25-1.53)	1.28 (1.17-1.41)	0.91 (0.85-0.97)
A2F0S0G	of afucosylated asialo A2	1.86E-10	1.15E-04	4.27E-04	0.72 (0.65-0.80)	0.82 (0.75-0.91)	1.14 (1.06-1.22)
Sialylation (S)							
CS	of complex	1.66E-22	3.89E-08	1.84E-12	1.70 (1.53-1.89)	1.31 (1.19-1.44)	0.78 (0.72-0.83)
A2S	of A2	1.12E-21	1.30E-08	1.27E-11	1.68 (1.51-1.87)	1.32 (1.20-1.45)	0.78 (0.73-0.84)
A2FS	of A2 fucosylated	1.47E-15	8.65E-03	1.38E-13	1.51 (1.37-1.67)	1.13 (1.03-1.24)	0.77 (0.72-0.82)
A2GS	per galactose in A2	8.27E-37	4.28E-12	9.34E-23	2.10 (1.87-2.35)	1.42 (1.28-1.56)	0.69 (0.64-0.74)
A3GS	per galactose in triantennary A3	2.06E-38	3.13E-20	5.75E-10	2.15 (1.92-2.41)	1.60 (1.45-1.77)	0.80 (0.75-0.86)
A2FGS	per galactose in fucosylated A2	2.24E-46	4.40E-09	7.40E-40	2.38 (2.12-2.68)	1.36 (1.22-1.50)	0.60 (0.55-0.65)
A3FGS	per galactose in fucosylated A3	1.59E-41	1.01E-15	5.94E-31	2.21 (1.97-2.48)	1.51 (1.37-1.67)	0.65 (0.60-0.70)
A3F0GS	per galactose in afucosylated A3	2.73E-15	1.39E-13	8.56E-01	1.51 (1.36-1.67)	1.45 (1.31-1.60)	0.99 (0.93-1.06)
A4F0GS	per galactose in afucosylated A4	1.12E-08	2.24E-12	9.32E-05	1.34 (1.21-1.48)	1.43 (1.30-1.59)	1.15 (1.07-1.23)
α2,3-linked sialylation (L)							
A2L	α2,3-linked (L) in A2	2.60E-13	5.88E-09	5.14E-03	1.46 (1.32-1.62)	1.34 (1.21-1.47)	0.91 (0.85-0.97)
A2F0L	L in afucosylated A2	1.89E-14	2.72E-16	5.73E-01	1.50 (1.36-1.67)	1.55 (1.40-1.72)	1.02 (0.95-1.09)
A2GL	L per galactose in A2	1.47E-12	2.24E-08	2.04E-02	1.44 (1.30-1.59)	1.32 (1.20-1.45)	0.92 (0.86-0.99)
A3GL	L per galactose in A3	4.95E-28	1.25E-24	3.87E-01	1.88 (1.68-2.10)	1.75 (1.57-1.95)	0.97 (0.91-1.04)
A2FGL	L per galactose in fucosylated A2	2.26E-15	3.33E-02	7.17E-24	1.53 (1.38-1.70)	1.11 (1.01-1.22)	0.69 (0.64-0.74)
A3FGL	L per galactose in fucosylated A3	2.38E-40	1.74E-22	1.12E-17	2.25 (1.99-2.53)	1.67 (1.51-1.85)	0.73 (0.68-0.78)
A2F0GL	L per galactose in afucosylated A2	3.51E-14	4.71E-16	5.36E-01	1.50 (1.35-1.66)	1.54 (1.39-1.71)	1.02 (0.95-1.09)
A3F0GL	L per galactose in afucosylated A3	6.98E-07	9.92E-17	3.18E-08	1.29 (1.17-1.42)	1.56 (1.41-1.74)	1.22 (1.14-1.31)
A4F0GL	L per galactose in afucosylated A4	1.27E-12	4.77E-14	6.02E-02	1.45 (1.31-1.61)	1.48 (1.34-1.64)	1.07 (1.00-1.14)
α2,6-linked sialylation (E)							
A2E	α2,6-linked (E) in A2	2.58E-17	2.57E-06	6.25E-10	1.56 (1.41-1.73)	1.25 (1.14-1.38)	0.80 (0.75-0.86)
A2FE	E in fucosylated A2	6.78E-12	1.98E-02	6.74E-09	1.42 (1.28-1.56)	1.12 (1.02-1.23)	0.82 (0.76-0.87)
A2GE	E per galactose in A2	7.00E-27	1.52E-07	5.89E-18	1.80 (1.61-2.00)	1.29 (1.18-1.43)	0.73 (0.68-0.78)
A2FGE	E per galactose in fucosylated A2	6.31E-35	3.72E-07	6.92E-25	1.99 (1.78-2.22)	1.29 (1.17-1.42)	0.68 (0.64-0.73)
A3FGE	E per galactose in fucosylated A3	1.47E-19	1.18E-04	1.74E-24	1.60 (1.45-1.78)	1.21 (1.10-1.33)	0.69 (0.64-0.74)
A3F0GE	E per galactose in afucosylated A3	8.01E-01	3.23E-07	5.02E-12	0.99 (0.90-1.09)	0.77 (0.70-0.85)	0.78 (0.73-0.84)
A4F0GE	E per galactose in afucosylated A4	2.14E-09	1.83E-06	6.21E-02	0.74 (0.67-0.82)	0.79 (0.72-0.87)	1.07 (1.00-1.14)

The TPNG-derived traits with the largest effect sizes between patients and HCs were found to be the lower levels of galactosylation in CD (metaOR [95% CI]$_{A2FS0G\ CD/HC}$, 0.37 [0.33–0.43]), the lower levels of fucosylation of diantennary species in UC (metaOR$_{A2LF\ UC/HC}$, [95% CI], 0.51 [0.45–0.57]), the higher levels of sialylation of diantennary fucosylated glycans in CD (metaOR$_{A2FGS\ CD/HC}$ [95% CI], 2.38 [2.12–2.68]), and specifically the α2,3-sialylation of fucosylated triantennary species in CD (metaOR$_{A3FGL\ CD/HC}$ [95% CI], 2.25 [1.99–2.53]). When comparing UC vs CD, the strongest associations were observed in the sialylation of diantennary fucosylated glycans (metaOR$_{A2FGS\ UC/CD}$ [95% CI], 0.60 [0.55–0.65]) and in fucosylation of triantennary glycans with α2,6-sialylation (metaOR$_{A3EF\ UC/CD}$ [95% CI] , 0.67 [0.62–0.72]), next to a higher level of galactosylation in UC (metaOR$_{A2FS0G\ UC/CD}$ [95% CI], 1.63 [1.51–1.76]) (Table 2).

Compared with HC, both CD and UC patients showed lower levels of diantennary glycans (CA2), higher levels of triantennary and tetra-antennary glycans (CA3 and CA4, respectively) and of complex glycans in general (TC), and lower levels of hybrid (THy) and high-mannose structures (TM). Compared with HCs, IBD patients had lower levels of diantennary fucosylated nonsialylated glycans (TA2FS0) that are mainly representative of IgG glycans[32] (Table 2**, Supplementary Table 3**).

IBD patients showed lower levels of fucosylation than HCs, especially for diantennary (sialylated) species (A2(S)F) with either α2,3- or α2,6-linked SAs (A2LF, A2EF) (Figure 2**,** Table 2). The fucosylation of triantennary glycans with α2,3- or α2,6-linked SAs (A3F, A3LF, A3EF) was lower in CD compared with UC patients.

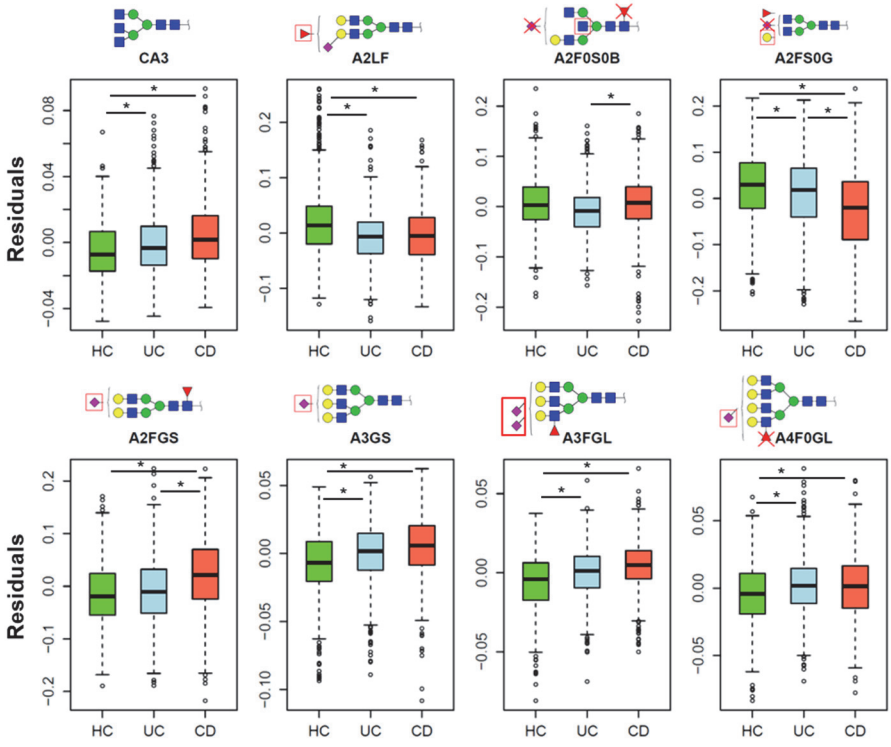


Figure 2: Main replicated associations between plasma *N*-glycan traits and IBD. *The 25th, 50th and 75th percentiles and whiskers at 1st quartile – 1.5 × IQR (Q1 – 1.5 × IQR) and Q3 + 1.5 × IQR based on the residuals after correction for age, sex, and age × sex are shown for data from the discovery cohort. P values, ORs and the 95% CIs are reported in Supplementary Table 3. Statistically significant and replicated associations are marked with an asterisk. IQR, interquartile range; Q, quartile.*

CD and UC patients displayed a higher bisection of diantennary fucosylated and sialylated glycans (A2FSB) compared with HCs, and CD patients had a higher bisection of nonfucosylated nonsialylated diantennary glycans (A2F0S0B) compared with UC patients (Table 2, Figure 2).

In IBD, the galactosylation of diantennary fucosylated glycans (A2FG) was lower than in HCs, whereas the galactosylation of nonfucosylated structures (A2F0G) was higher than in HCs. The galactosylation of diantennary structures without SA gradually decreased from HCs to UC patients to CD patients (A2(F)S0G and A2F0S0G), but in sialylated glycans (A2F0SG) it was higher in IBD patients compared with HCs (Table 2, Figure 2).

General sialylation was significantly increased in UC, and even more in CD, patients compared with HCs. CD and UC patients had higher relative levels of both α2,3- and α2,6-linked SA compared with HCs in all traits except for the α2,6-sialylation of tetra-antennary structures (A4F0GE), which was lower in IBD than HC. The strongest association distinguishing UC from CD was found in the sialylation per galactose in diantennary fucosylated glycans (A2FGS) (Table 2**,** Figure 2**)**

To explore the effect of known confounders of the TPNG[30, 33] also in IBD, we tested its associations with age and sex, stratified by disease group. Several traits of galactosylation, bisection, fucosylation, and sialylation showed associations with age (**Supplementary Table 4).** Some traits followed a nonlinear trend with age, for example A2FS0G in CD and UC patients but not in HCs (**Supplementary Figure 1**). We found previously unreported associations of SA linkage with sex. For instance, males had significantly higher fucosylation of diantennary glycans with α2,3-linked SA (A2LF) than the females in all 3 groups (**Supplementary Table 4**). For those patients in whom CRP levels and sedimentation rate were assessed (Table 1), we tested for associations with the TPNG. Multiple traits of sialylation, in particular α2,6-linked, as well as the fucosylation of triantennary glycans, were positively associated with both inflammation markers in CD, whereas IgG galactosylation showed a negative association. In UC patients, we observed similar associations, with additional negative associations with bisection (**Supplementary Table 4**).

Based on the associations found by logistic regression, we assessed receiver operating characteristic curves for the 3 groups. We found a good, fair, and poor discrimination for CD patients vs HCs, UC patients vs HCs, and UC vs CD patients, respectively, and this was replicated in the US cohort (**Figure 3**).

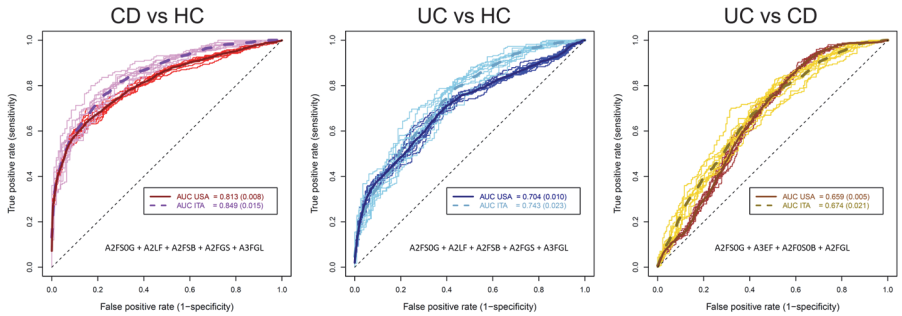
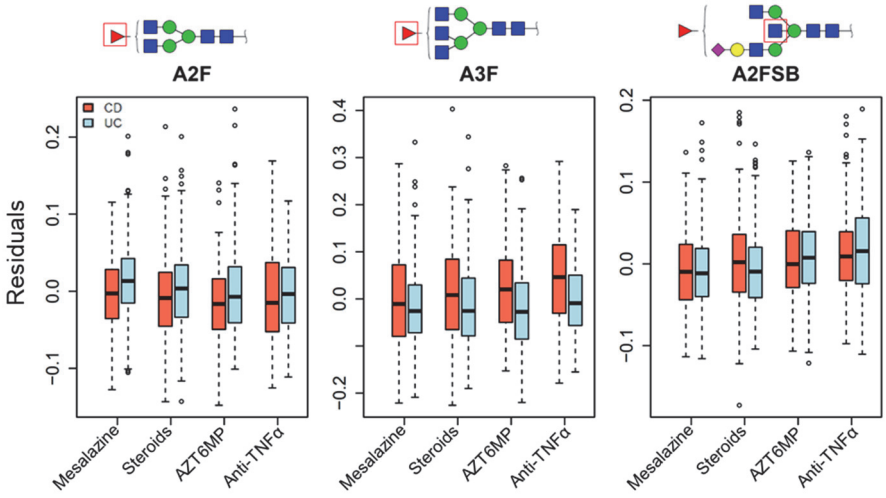


Figure 3: Receiver operating characteristic curves illustrating the power of selected glycan traits to predict CD and UC. *The means (and SDs) of 10 predictions are reported for the respective AUC for the 2 cohorts (Italy, discovery and United States, replication). In addition to the glycan traits listed in the respective panels, the interaction age × sex × glycan was included in the model. AUC, area under the curve*.

TPNG is associated with treatment

In CD patients, the need for increased drug potency was associated with elevated bisection (A2FSB) and sialylation (A2FGS, A2FGE, A3F(0)GE), as well as elevated fucosylation of triantennary structures with α2,3- and α2,6-linked SAs (A3F, A3LF, A3EF), whereas galactosylation (A2FSG, A2FS0G) was negatively associated with the need for more potent drugs (Figure 4**, Supplementary Table 5**). In UC patients of the discovery cohort, drug potency need was associated with higher antennarity, longer mannose branches (MM) and higher high-mannose–to–hybrid structures ratio (MHy), lower fucosylation (patients treated with mesalazine having higher levels than those taking other drugs), elevated bisection (especially anti–TNF-α users), lower galactosylation of diantennary glycans, and elevated sialylation (A2FGL, A3GL, A3FGL, A4F0GL), except for α2,3-linked sialylation in diantennary and afucosylated triantennary structures (A2L, A2F0L, A2GL, A2F0GL, A3F0GL) (Figure 4, **Supplementary Table 5**). In UC, only the associations with galactosylation of diantennary glycans were replicated.



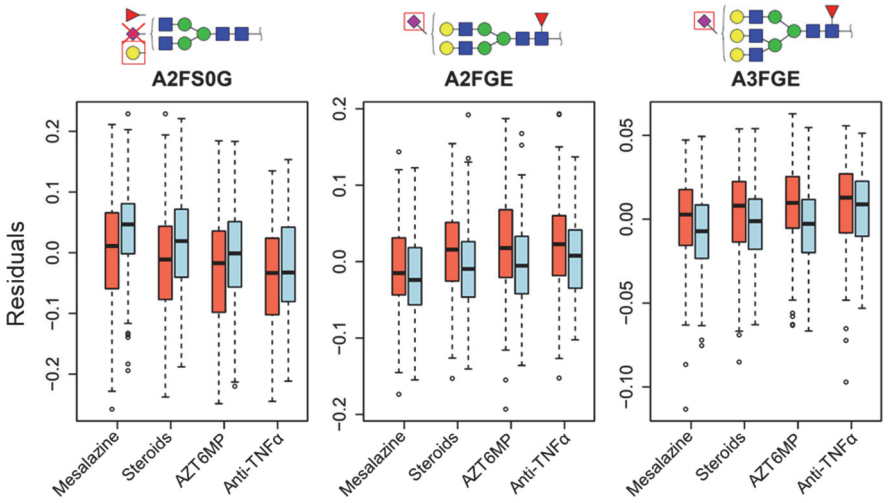


Figure 4: Main associations between plasma *N*-glycan traits and medication. *CD is colored red and UC is colored blue. The 25th, 50th, and 75th percentiles and whiskers at Q1 – 1.5 × IQR and Q3 + 1.5 × IQR based on the residuals after correction for age, sex, and the interaction thereof are shown for data from the discovery cohort. P values, ORs, and their 95% CIs are reported in Supplementary Table 5. IQR, interquartile range; Q, quartile* ***Supplementary Table 5****. IQR, interquartile range; Q, quartile.*

In the paired analysis of the TPNG from 9 UC patients of the replication cohort before and after surgery, we observed an increase in A2EF and IgG galactosylation and a decrease in A3FGL (**Figure 5**, and **Supplementary Table 6**). For CD patients (n = 19) we did not find any significant differences (**Supplementary Table 6**). Moreover, we performed a cross-sectional logistic regression analysis of the TPNG from patients whose plasma samples were collected before vs after surgery and did not find any replicated associations (**Supplementary Table 7**).

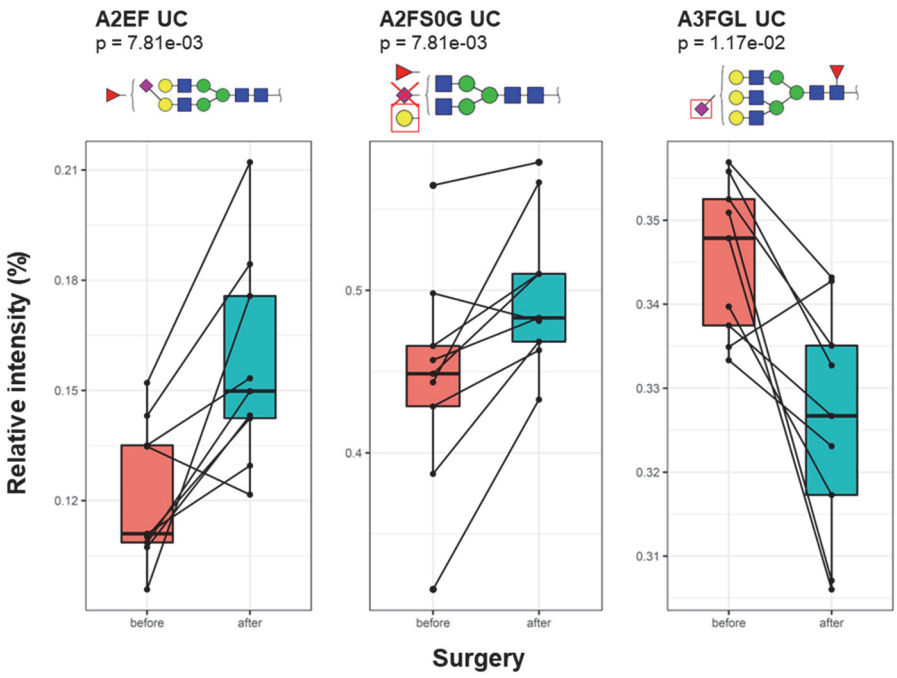


Figure 5: Postoperative *N*-glycosylation changes in UC patients. *P-values and relative intensities with 25th, 50th and 75th percentiles are shown with individual values for the 9 UC patients before and after surgery for the glycan traits significant after correction with false discovery rate of 10% (see **Supplementary Table 6** for the full list of tests performed, including CD data).*

Disease location and disease behavior

In both cohorts, UC patients in whom the colitis extended to the entire colon (E3) were found to have lower levels of galactosylation of the fucosylated and nonfucosylated diantennary nonsialylated glycans (A2(F)S0G) than distal colitis patients (E1 + E2) (**Supplementary Table 8**). Several fucosylation traits were higher in colonic CD than in ileal CD of the discovery cohort (L2 vs L1), but these associations were not replicated (**Supplementary Table 9).** Furthermore, no replicable associations of the TPNG were found with disease behavior, nor with the presence of perianal disease (**Supplementary Tables 10** and **11**). In the discovery cohort, the colonic CD subgroup (L2) vs UC showed similar associations as total CD vs UC, but those associations were not replicated (**Supplementary Table 12**).

Discussion

In this study we evaluated the differences in TPNG between 2635 IBD patients and 996 HCs by MALDI-TOF-MS. Glycosylation was found to differ between patients and HCs in all main glycosylation features, that is, complexity, fucosylation, bisection, galactosylation, and sialylation with discrimination of functionally distinct α2,3- and α2,6-linkage types. Novel associations of SA linkages with age and sex were found for both HCs and IBD patients. The availability of 2 distinct cohorts allowed the replication of the main findings and ensured the validity of the results.

One of the strongest and most robust associations with both CD and UC in our data appeared to be IgG galactosylation (A2FS0G)[32]. It was not only an important predictor for IBD in our receiver operating characteristic analyses but was also associated with the extent of disease and the need for surgery and more potent drugs. Moreover, it increased in UC patients after surgery. This is consistent with our recent findings on isolated IgG[20], and seems to mostly reflect the grade of inflammation, as is also evidenced by its strong negative association with CRP and sedimentation rate in our data and is in line with literature for IBD and other inflammatory conditions[34, 35]. In contrast, the galactosylation of afucosylated, sialylated diantennary glycans (A2F0(S)G) was higher in IBD. In healthy individuals, these glycans are mainly derived from hemopexin, serotransferrin, α1-antitrypsin, haptoglobin, fibrinogen, IgA, α1B-glycoprotein, and other acute-phase proteins from the liver[32]. Some of these proteins (e.g., α1-antitrypsin and haptoglobin) have been proposed as markers for IBD[36, 37]. At the same time, the per-galactose sialylation of related glycan traits (A2(F)GS) was also increased, contrary to IgG Fc sialylation, which was reported to decrease in CD, measured in the same cohort[20].

Although non-bisected A2FS-glycans are mostly attributable to a mixture of glycoproteins released by the liver, bisected A2FS-glycans are mostly derived from IgA, IgM, and the fragment antigen-binding portion of IgG.[32, 38]. Thus, the observed increase in diantennary sialylation might reflect acute-phase protein glycosylation and abundance changes, whereas the increased bisection of diantennary structures (A2FSB) in IBD may be attributed to increased bisection and/or levels of IgA or IgM[32, 39-41]. We reported on decreased IgG Fc bisection in UC in the same cohort as is analyzed here[20]. IgG bisection has been reported to enhance antibody-dependent cytotoxicity in vitro[42]. However, the role of bisection in IgA and IgM has not yet been established. Moreover, it needs to be taken into account that the applied analytical approach does not allow us to distinguish a bisecting GlcNAc from a nongalactosylated third antenna, because these result in exactly the same mass. Therefore, assignment is based on knowledge of the glycan biosynthetic pathways and literature on plasma protein glycosylation[12, 13, 32, 41].

Contrary to our results, no changes in fucosylation were found in the TPNG of UC patients in a previous study[43]. We found a decreased fucosylation of diantennary structures in IBD vs HCs, in colonic (L2) vs ileal (L1) or ileocolonic (L3) CD patients, and in UC with extensive (E3) compared with distal disease (E1 + E2). This likely reflects an increase of the mostly afucosylated liver-derived diantennary glycans compared with the mostly fucosylated IgG glycans (TA2FS0)[32, 34]. An increase of afucosylated IgG glycans as reported for UC[20], known to enhance antibody-dependent cellular cytotoxicity[44] may likewise contribute. Accordingly, we found a lower A2E fucosylation in UC before surgery compared with after surgery. Future studies are warranted to elucidate possible functional differences of IgG fucosylation between UC and CD. The fact that we found fewer or no replicated associations with disease location or disease behavior in CD may partly be due to the relatively small group sizes of the subcategories, which limited the power of our analyses.

The general relative increase of higher branched structures (CA3 and CA4) in IBD patients compared with HCs goes along with a general decrease of smaller structures (CA2 including IgG-derived TA2FS0; TM and THy). A decrease in TM could also be attributed to lower (relative) levels of proteins rich in high-mannose glycans (eg, α-2-macroglobulin, apolipoprotein B-100, and IgD, IgE, and IgM)[32, 43, 45, 46]. This is in line with our finding of a decreased A2F0S0G in IBD, which could possibly be linked to decreased levels of apolipoprotein B-100 in CD[45]. Higher branching, next to increased sialylation, has previously been reported for IBD, other autoimmune diseases, and acute inflammation[17, 43, 47, 48]. Similar *N*-glycome associations with disease and disease activity were found in a longitudinal study on rheumatoid arthritis, in which the serum *N*-glycome was measured with the same method as used here[49]. This was linked to an increase in the levels of acute phase proteins containing large, highly sialylated glycans, such as α1-acid glycoprotein and α1-antitrypsin[50, 51].

Plasma glycoproteins can contain SAs in 2 major linkage variants, that is, linked in the α2,3- or α2,6-position to an antenna galactose[13]. Because our novel method enabled us to discriminate between SA linkages, we were able to show some divergent, linkage-specific trends in our data. Although α2,3-sialylation was higher in IBD in various glycan traits, including both fucosylated and afucosylated structures, the α2,6-sialylation of afucosylated highly branched glycans (A3-4F0GE) was lower compared with HCs. These traits were strongly associated with inflammation markers in our data. Moreover, the expression of the enzyme generating α2,6-SAs was shown to be induced by inflammatory cytokines and to inhibit adhesion of B cells to endothelial cells[52]. Thus, although both α2,6- and α2,3-sialylation seem to increase during inflammation, our data suggest that in both CD and UC, the increased sialylation of highly branched structures is mainly driven by α2,3-sialyltransferases and, to a lesser extent, by α2,6-sialyltransferase. This may have consequences on the

immunologic pathways, because the ligands of SA-containing glycoproteins are mostly specific to the SA linkage type in humans. For instance, Siglecs on immune and epithelial cells recognize afucosylated α2,6-SA epitopes, whereas selectins specifically bind ligands carrying sialyl-Lewis X structures containing α2,3-SA and antennary fucose[21]. Correspondingly, the ORs of the α2,3-sialylation of fucosylated triantennary glycans (A3FGL) were considerably larger than those of the α2,6-sialylated variant (A3FGE). A3FGL decreased after surgery in UC patients only, which is in line with our meta-analysis finding of a lower A3FGL in HCs and indicates improvement in UC patients after surgical intervention. In a study of the serum glycome in rheumatoid arthritis, this trait did not differ between HCs and patients[49], which might point at a possible disease-specificity of A3FGL in IBD. The lack of significance in postoperative glycan changes in CD despite a higher sample size (n = 19 in CD vs n = 9 in UC) could be related to the fact that relapses occur more frequently in CD, whereas in UC surgery is able to cure the disease[37].

We observed a strongly reduced fucosylation of diantennary α2,3-sialylated (A2LF) glycans in IBD patients compared with HCs, which may be largely interpreted as an increase of nonfucosylated α2,3-sialylated diantennary structures (A2GL). These glycans are most likely liver derived, because immunoglobulins are known to mostly carry α2,6-linked SA and a core fucose[53, 54]. Moreover, as expected, the fucosylation of triantennary glycans (A3F) was strongly associated with inflammatory markers and, accordingly, was lower in UC compared with CD patients. This was furthermore true for both α2,6- and, α2,3-sialylated structures (A3EF, A3LF) and antennary fucosylation (A3Fa). The latter is most likely reflecting an overall increase of fucosylation of 2,3-sialylated antennae, resulting in increased sialyl-Lewis X structures on acute phase proteins known to occur during inflammation[55]. However, it was surprising that our findings of the various triantennary fucosylation traits were contradictory between the 2 cohorts when comparing UC and CD patients with HCs, which might be due to the different medication strategies between the cohorts, as further described in the following section.

We observed a decrease of IgG-related galactosylation (A2FS0G) with increasing drug potency in IBD. We assume that patients receiving more potent medication, such as anti–TNF-α, are likely to have a more severe disease[31, 56, 57] and, consequently, TPNG profiles, reflecting a stronger inflammation than, for example, mesalazine users. However, several other glycan traits showed rather unexpected association behavior, such as the fucosylation of triantennary glycans (A3F), which was higher in CD anti–TNF-α users compared with mesalazine-treated patients, whereas in Italian UC patients this was the case only for antennary fucosylation (A3Fa) (Supplementary Table 5). Surprisingly, A3Fa was lower in the Italian UC patients when compared with HCs (Supplementary Table 3). α2,3-sialylation in

diantennary, mainly afucosylated, structures was negatively associated with drug potency in Italian UC patients (eg, A2(F0)GL in anti-TNF vs mesalazine), whereas the fucosylated variant, A2FGL, showed a positive association (*i.e.*, increased in azathioprine/6-mercaptopurine compared with mesalazine). Moreover, this very same trait was distinguishing UC from CD in our meta-analysis and was not associated with inflammatory markers. When we compared UC patients with HCs, α2,3-sialylation in diantennary glycans including A2F0GL was positively associated with disease (Table 2). Thus, there seem to be underlying effects of interactions between CD or UC, medication, and various glycan traits, in particular, α2,3-sialylation and antennary fucosylation. This should be explored in more detail in future studies, preferably by taking into account disease scores.

Conclusion

To our knowledge, this study is the largest analysis of the TPNG in IBD patients to date and includes novel SA linkage-specific information. Here, we report on multiple associations of the TPNG with CD and UC in a case-control study, which were, in large part, replicated in an independent cohort. Glycan complexity, fucosylation, bisection, galactosylation, and sialylation were proven to differ in IBD patients compared with HCs. The novel information about SA linkages revealed yet unreported associations with age, sex, and IBD. Our high-throughput method allowed us to find differences within the groups of CD and UC patients regarding response to surgery and medication and disease localization. Established markers of inflammation, such as low galactosylation attributed to IgG and a higher sialylation and abundance of large structures typical of certain acute-phase proteins, were apparent in the TPNGs of CD and UC patients. Based on our data and knowledge of plasma protein glycosylation, we proposed protein scaffolds that might be affected in IBD. These specific proteins, for instance, IgA, haptoglobin, or α1-acid-glycoprotein, might be promising targets for future (glyco)proteomic investigations to gain insights into disease pathophysiology and to develop therapeutic targets. Future investigations, preferably in a longitudinal manner, are warranted to further elucidate the impact of the different treatments on the TPNG of IBD patients.

Overall, this study expands the knowledge about protein glycosylation and contributes to the further characterization of IBD by reporting multiple new associations and potential targets for further investigation.

Supplementary material

The supplementary material is available online at https://doi.org/10.1053/j.gastro.2018.05.030

https://www.gastrojournal.org/article/S0016-5085(18)34563-3/fulltext?referrer=https%3A%2F%2Fpubmed.ncbi.nlm.nih.gov%2F

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Conflict of interest

Dermot P.B. McGovern has consulted for Janssen, Cidara, Q Biologics, and Pfizer. The other authors declare that they have no conflict of interests.

Author Contributions

Florent Clerc and Mislav Novokmet; acquisition of data; statistical analysis, interpretation of the data, drafting of the manuscript. Viktoria Dotz and Karli R. Reiding; critical revision of the manuscript for important intellectual content and interpretation of the data; editing. Noortje de Haan, Guinevere S.M. Kammeijer, Hans Dalebout, Marco R. Bladergroen, Frano Vukovic and Erdmann Rapp; technical support. Stephan R. Targan, Gildardo Barron, Natalia Manetti, Anna Latiano, Dermot P.B. McGovern, Vito Annese; study design and patient recruitment. Gordan Lauc and Manfred Wuhrer; study supervision. All authors had access to the study data and approved the final manuscript.

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