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Serum N-Glycomic Biomarkers Predict Treatment Escalation in Inflammatory Bowel Disease

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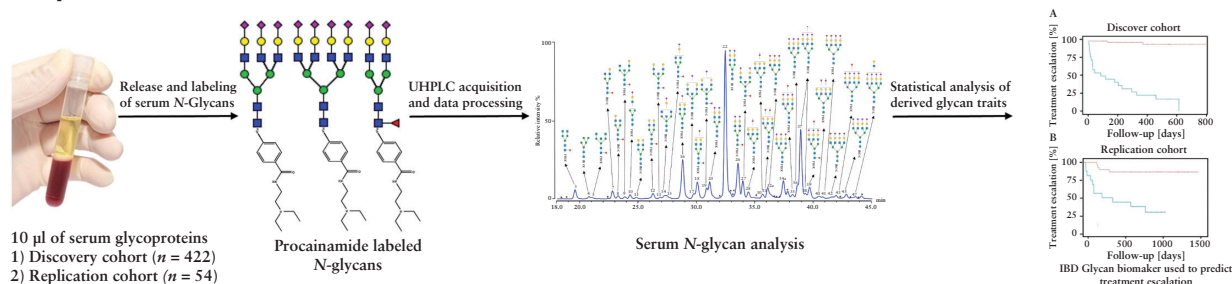
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Abstract

Biomarkers to guide clinical decision making at diagnosis of inflammatory bowel disease [IBD] are urgently needed. We investigated a composite serum N-glycomic biomarker to predict future disease course in a discovery cohort of 244 newly diagnosed IBD patients. In all, 47 individual glycan peaks were analysed using ultra-high performance liquid chromatography, identifying 105 glycoforms from which 24 derived glycan traits were calculated. Multivariable logistic regression was performed to determine associations of derived glycan traits with disease. Cox proportional hazard models were used to predict treatment escalation from first-line treatment to biologics or surgery (hazard ratio [HR] 25.9, $p = 1.1 \times 10^{-12}$; 95% confidence interval [CI], 8.52–78.78). Application to an independent replication cohort of 54 IBD patients yielded an HR of 5.1 [$p = 1.1 \times 10^{-5}$; 95% CI, 2.54–10.1]. These data demonstrate the prognostic capacity of serum N-glycan biomarkers and represent a step towards personalised medicine in IBD.

Key Words: N-Glycans; biomarker; Crohn's disease; ulcerative colitis; prognosis; personalised treatment

Graphical Abstract



1. Introduction

Inflammatory bowel disease [IBD] is an idiopathic chronic inflammatory disease of the gastrointestinal tract predominantly consisting of Crohn's disease [CD] and ulcerative colitis [UC], with some patients, especially early in the course of the disease, labelled as IBD unclassified [IBDU] where there is diagnostic uncertainty. The disease is characterised by an aberrant immune response to the intestinal

microbiota which results from a combination of genetic predisposition and environmental exposures.¹ In recent decades, an increasing incidence in newly industrialised countries has been suggested as the reason why IBD has become a global disease.² IBD has a considerable social, psychological, and financial impact on the patients' quality of life, and the disease accounts for significant costs to the health care system as well as to society.³

The clinical presentation of IBD is heterogeneous, both at diagnosis and during follow-up.⁴ Since many patients experience a progressive disease with frequent flares and various complications, much emphasis has been placed on the identifying prognostic markers of disease outcome. Several clinical characteristics that correlate with a severe disease course for IBD have been identified but their predictive power remains limited.⁵ Recent advances in molecular, serological, and proteomic-based technologies^{6–12} are expected to produce novel biomarkers for delivering personalised care in IBD. Identification of diagnostic and disease course-specific biomarkers would be immensely valuable, since they would allow stratification of patients for specific therapeutic interventions.^{6,13} In addition, such markers may provide insight into biological pathways of disease development and progression.

Most secreted proteins are glycosylated, largely as a result of post-translational modification, and these glycans play a vital role in regulation of key biological processes including protein folding, immune cell migration, cell adhesion, and recognition of pathogens.^{14,15} Recent glycomics studies have shown that aberrant glycosylation is associated with various cancer, neurodegenerative, and inflammatory diseases including IBD.^{16–20} Reflecting genetic, environmental, and pathological factors, the serum N-glycome is seen as an attractive source for biomarker discovery.^{21,22} Compared with healthy controls, a significant decrease in serum levels of galactosylation and sialylation and increased levels of bisection have been observed in patients with IBD.^{21,23,24} Clerc *et al.* reported differences in glycan associations such as glycan complexity, increase in relative abundance of higher antennary structures, and increase in α 2,3-linked sialylation in higher branched glycan structures in total plasma N-glycans in IBD patients from two different cohorts. The extent to which these previous results reflect causal associations or are explained by secondary effects of IBD treatment and disease course remains unknown, since all previous studies have examined patients with long-standing IBD. Furthermore, glycomics studies in IBD have hitherto never focused on the discovery of potential signatures of future disease course prediction or treatment escalation.

Here, we performed glycan analysis of total serum N-glycans [TSNG] by ultra-high performance liquid chromatography [UHPLC], followed by analysis of structurally related glycans called derived glycan traits. Statistical analysis involving these 24 derived glycan traits enabled profiling of glycosylation in IBD in newly diagnosed patients and controls, and compared the performance of the prognostic glycomics biomarker with standard inflammatory markers (eg, high-sensitivity C-reactive protein [hsCRP]). Finally, we identified glycomics signatures predictive of IBD treatment escalation prediction, and further validated this biomarker in an independent cohort of newly diagnosed IBD patients. These results provide an insight in understanding the translational aspects of glycomics in IBD. This could prove valuable in personalising treatment of IBD, wherein future longitudinal multi-omics studies should examine the utility of this novel glycomics signature for predicting and guiding treatment decisions for IBD patients at diagnosis.

2. Materials and Methods

2.1. Clinical samples and ethical statement

Adults over 18 presenting with de novo symptoms as well as patients presenting clinical, biochemical, or imaging data suggestive of IBD were part of the broad inclusion criteria

for patient recruitment. These patients were prospectively recruited from gastroenterology and endoscopy appointments at the Western General Hospital, Edinburgh, UK, as part of the IBD-BIOM study,²⁵ henceforth known as the discovery cohort. Patients were recruited close to diagnosis and were largely [69 %] treatment naive. Patients undergoing investigations for suspected IBD, but following endoscopic investigations found not to have IBD [eg, irritable bowel syndrome], were recruited as symptomatic controls. In addition, a previously described group of healthy volunteers with no self-reported gastrointestinal symptoms were included as healthy controls.²⁵ Exclusion criteria included children under 16 years, patients with established diagnosis of inflammatory bowel disease [ie, more than 3 months of IBD treatment], and/or patients diagnosed with other gastrointestinal pathology. A clinical, radiological, endoscopic, and histopathological diagnosis of CD and UC was made according to Lennard-Jones criteria and disease location and behaviour were classified according to the Montreal classification for these patients.^{26,27} Clinical activity scores were measured using the simple clinical colitis activity index [SCCAI] for UC²⁸ [Walmsley 1998] and Harvey-Bradshaw index [HBI] for CD²⁹ [Harvey-Bradshaw 1980; see [Supplementary Data File 1](#) for SCCAI/HBI and hsCRP scores for IBD patients from the discovery cohort, to assess the severity of clinical activity and/or inflammation). To validate the treatment escalation biomarker, an independent cohort of newly diagnosed IBD patients [76% treatment naïve], was recruited at the Department of Gastroenterology, Örebro University Hospital, Sweden, as part of the IBD-Character study, henceforth known as the replication cohort. All patients and controls provided written informed consent. Ethical approval was granted by the Tayside Committee on Medical Ethics B [UK, LREC 06/S1101/16, LREC 2000/4/192] and the Regional Ethics Committee [Sweden, 2006/245]. For study design, see [Supplementary Methods Section 1-Experimental](#).

2.2. Sample preparation and data acquisition

Single batches of reagents, buffers, laboratory consumables, reference standards, labelling kits, and clean-up plates were acquired from Ludger Ltd [UK]. The UHPLC column [BEH-Glycan 1.7 μ m, 2.1 x 150 mm column] was purchased from Waters [UK]. All samples and standards were prepared using the Hamilton Microlab STARlet liquid handling robot [Hamilton Robotics, Bonaduz, Switzerland].

Serum samples from discovery and replication cohorts were aliquoted into random well positions in 96-well plates irrespective of age, sex, and diagnosis, to avoid any positional bias. For the discovery cohort, 10 μ L of each serum sample and triplicate system suitability standards (human serum standard and human immunoglobulin G [IgG] glycoprotein standards) were distributed into six 96-well microtitre plates. The replication cohort was used to validate the treatment escalation biomarker along with system suitability standards, and was run on a single separate 96-well microtitre plate. N-Glycans were released using peptide-N-glycosidase F, fluorescently labelled with a procainamide tag, purified, and analysed using UHPLC [Supplementary Methods Section 1-Experimental](#). The serum N-glycan standards were used to determine the technical variation of the method and the discovery cohort data were used to determine the biological variation of the method. Thermo Dionex Ultimate 3000 UHPLC [ThermoFisher Scientific,

UK], equipped with a fluorescence detector [Supplementary Table 1], was used for data acquisition. Chromeleon [ThermoFisher] data software version 7.2 [Dionex, USA] was used for data analysis.

2.3. Data processing

All chromatograms were converted to an open text-based format using Chromeleon. Data processing was performed using HappyTools [Supplementary Data 1].³⁰ All chromatograms were retention-time calibrated [see Supplementary Table 2A and B for calibration parameters]. Peak alignment before and after HappyTools processing is shown in Supplementary Figure 1. Glycans were total area normalised to acquire relative areas, where 47 individual glycan peaks were integrated consisting of 105 glycoforms. Subsequently, all glycans were identified based on literature knowledge or mass spectrometry [MS]² fragmentation as depicted in Supplementary Table 3. Area under each peak was quantified, and the quantified areas were used to calculate quality criteria. A detailed description of HappyTools data processing can be found in the Supplementary Methods Section 2-Data Analysis.

Structurally related glycans were used to create 24 derived traits upon which all analyses were performed as previously described.^{31,32} These included fucosylation, bisection, sialylation, antennarity, and galactosylation, as well as galactose linkage [ie, the ratio between α 1,3—and α 1,6—mannose arm that contains mono-galactosylated species]. R [version 3.4.2] was used for calculating derived traits from raw data, and the full list and the calculations are presented in Supplementary Table 4A and B.

2.4. Statistical analysis

Generalised linear models [GLM], Cox proportional hazard [Cox PH] models [both limited to seven derived glycan traits] and receiver operating characteristic [ROC] analyses were performed using R [3.4.2]. Cox PH was used, as the follow-up time for the patients was not equal [between 1 and 797 days]. The model used a survival object of escalation status and time as the response variable, and the glycomics biomarker with age and sex correction as the terms. leave one out validation was performed to create receiver operating characteristic [ROC] curves, from which a threshold was determined by selecting the minimum distance from sensitivity and specificity of 1. The data were then stratified according to the determined threshold into a low-risk and high-risk group, which were used to determine the treatment escalation prediction using Kaplan-Meier curves. Cumulative hazard ratios were taken from these curves. Significance of the groups was assessed using a log-likelihood function. Furthermore, principal component analysis [PCA] was used to evaluate variation in datasets, and a Student's *t* test was used to test the significance of patient age with respect to the observed difference in PCA. Last, Bonferroni correction was used where relevant to correct for multiple testing.

2.4.1. Cross-sectional analyses

All associations between serum N-glycans, disease state, and clinical markers were assessed using age, sex, and age-sex interaction corrected logistic regression. Three separate comparisons were made for each derived glycan trait and disease state, namely IBD vs. controls, UC vs. controls, and CD vs. controls. Given no significant differences were found

between SC and HC, the two groups were merged for analysis. Statistical significance was defined as $P < 6.9 \times 10^{-4}$ for α of 0.05 after Bonferroni correction [24 derived traits $\times$ 3 disease states $n = 72$: $0.05/72 = 6.9 \times 10^{-4}$]. Associations of derived glycosylation traits with clinical markers were tested and the results are displayed in heatmaps [Supplementary Figure 6 and Supplementary Methods Section 2-Statistical Analysis].

2.4.2. Glycomics biomarkers for disease stratification

To assess the potential of glycomics biomarkers to distinguish between CD and UC, all 24 derived glycosylation traits were used in cross-validated [$n = 10$] ROC analyses using age, sex, and age-sex interaction corrected logistic regression. Extended models consisting of all combinations of up to a limit of seven derived traits were also tested and model performance was ranked using area under the curve [AUC].

2.5. Prediction of IBD treatment escalation

For the biomarker discovery study, data were available only for a sub-group of 91 patients who were recruited for on-going collection of clinical data which included their medication [54% escalated based on medication] and surgery [46% classed as escalators based on surgery]. Right-censored treatment escalation data were defined as the number of days until use of biologics, or ciclosporin ['second-line'] after any other initial medication ['first-line'], or surgery at any point. Changes between two first-line treatments or addition of a second first-line treatment was not considered an escalation. See Supplementary Table 5 for demographics of patients with disease escalation including disease location, disease activity scores, disease behaviour, and duration of follow-up. Furthermore, Supplementary Figure 2 shows a scatter plot depicting diagnosis time relative to sampling of blood from IBD patients from the discovery and replication cohorts. Supplementary Figure 3 illustrates association of derived glycan traits between individuals who were naïve to all therapies [treatment naïve] and individuals who had been exposed to IBD drugs [non-treatment naïve]. Three representative examples chosen from 24 derived glycan traits are depicted in this figure and this analysis showed that there were no significant differences between the two groups.

2.5.1. Glycomics markers

Kaplan-Meier analysis was performed using Cox PH models with age, sex, and age-sex interaction terms, with leave one out [LOO] validation to determine and estimate treatment escalation. Cox PH models for all single derived glycomics traits in predicting treatment escalation in IBD, and in UC and CD separately, were tested. Extended models comprising combinations of up to seven derived traits were also tested. Model performance was ranked using AUC. The best performing biomarker for predicting treatment escalation of IBD patients was tested in the validation cohort using age, sex, and age-sex interaction corrected Cox PH models with LOO validation.

2.5.2. Clinical markers

Cox PH models with LOO validation were used to evaluate standard clinical tests [hsCRP, $n = 91$ and Alb, $n = 91$] either alone or in combination for comparison with glycomics-derived models in predicting treatment escalation.

3. Results

Serum N-glycans of 244 IBD patients and 178 controls from a discovery cohort and 54 IBD patients from a replication cohort were analysed in this study. Demographics of the individuals from the discovery cohort and replication cohort are presented in Table 1. Using UHPLC with fluorescence detection, 47 glycan peaks corresponding to 105 procainamide-labelled glycans were quantified. Figure 1 depicts a typical chromatogram of human serum N-glycans. A detailed list

of glycan composition, glycan name, plausible glycan structure fragments, and relative abundance is reported in Supplementary Table 3. Figure 2 illustrates the total samples numbers used in this study for glycan association studies, as well as complete sample sets with follow-up data for IBD, CD, and UC, used in each biomarker analysis. Furthermore, derived traits were calculated to compare structurally related substrates, effectively describing single enzymatic steps across the glycoproteome.³³ These derived traits were calculated,

Table 1. Demographics of the individuals from discovery and replication cohorts

Discovery cohort									
Category		HC	SC	Total controls	CD	UC	IBDU	Total IBD	Total Individuals
Count	Female	35	65	100	40	48	8	96	196
	Male	16	62	78	63	76	9	148	226
	Total	51	127	178	103	124	17	244	422
Age [SD]	Female	42.9	32.4	35.9	39.3	39.3	42.4	39.4	37.7
		[15]	[10]	[14]	[15]	[16]	[12]	[15]	[14]
	Male	45.0	35.2	37.8	34.7	39.3	29.5	36.5	36.7
		[15]	[13]	[14]	[15]	[14]	[5]	[13]	[14]
	Total	43.6	33.8	36.7	36.5	39.3	35.5	37.7	37.3
		[15]	[12]	[14]	[15]	[15]	[11]	[15]	[14]
Replication cohort									
Category		CD			UC		IBDU	Total IBD	
Count	Female	10			12		3	25	
	Male	12			15		2	29	
	Total	22			27		5	54	
Age [SD]	Female	39.9			37.5		37.3	38.4	
		[13.52]			[11.22]		[3.51]	[11.35]	
	Male	28.9			40.9		36.5	35.6	
		[12.00]			[14.37]		[14.85]	[14.23]	
	Total	33.9			39.4		37.0	36.9	
		[13.62]			[12.94]		[7.84]	[12.93]	
Discovery cohort									
Category		CD			UC		IBDU	Total IBD	
E	Female	4			2		0	6	
	Male	7			11		0	18	
	Total	11			13		0	24	
NE	Female	13			14		0	27	
	Male	17			23		0	40	
	Total	30			37		0	67	
Replication cohort									
Category		CD			UC		IBDU	Total IBD	
E	Female	3			0		0	3	
	Male	9			3		0	12	
	Total	12			3		0	15	
NE	Female	7			12		3	22	
	Male	3			12		2	17	
	Total	10			24		5	39	

Escalators [E] were individuals whose medical therapy was altered from first-line therapeutics to biologics or who required surgical intervention due to disease severity. Non-escalators [NE] did not require treatment alteration or surgery.

HC, healthy controls; SC, symptomatic controls; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified.

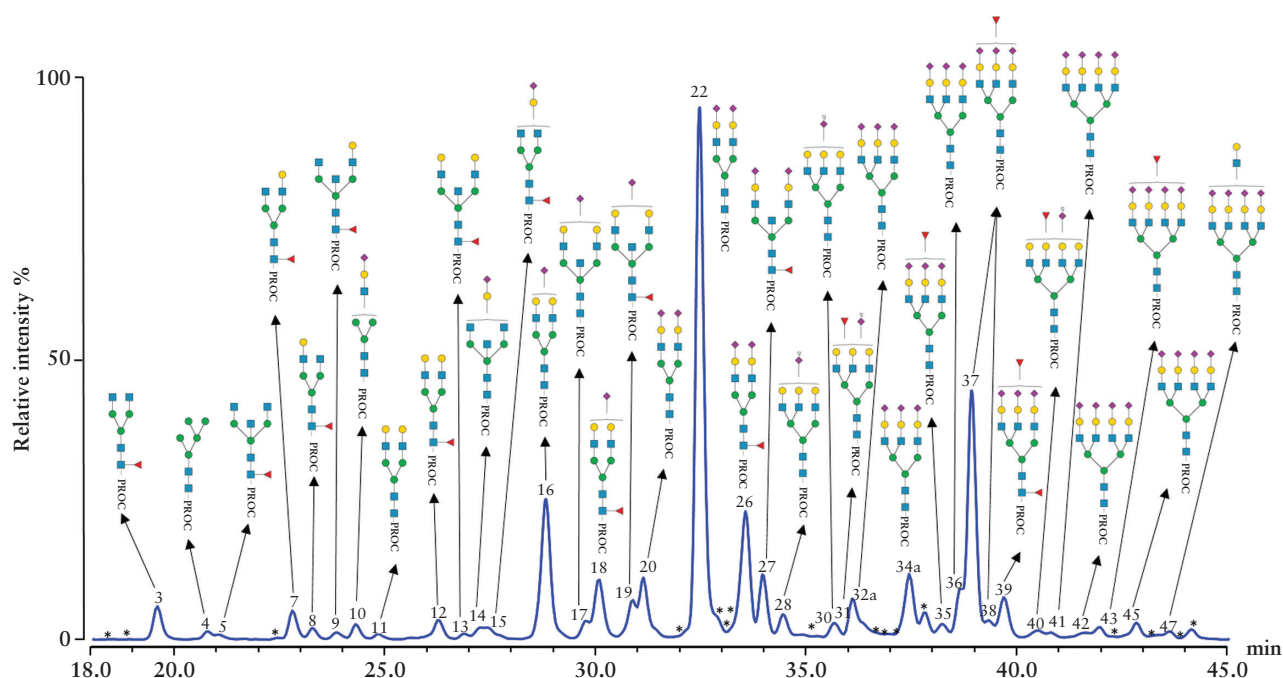


Figure 1. Typical chromatogram of procainamide-labelled serum N-glycans. Representative example taken from a female CD patient. For illustrative simplicity, most abundant N-glycan structures corresponding to each numbered peak are annotated [using Symbol Nomenclature for Glycans notation] and the additional glycans are illustrated in [Supplementary Table 2A](#). For a detailed list of procainamide-labelled glycans, glycan composition, plausible glycan structure assignment, glycan names, and glycan relative abundance see [Supplementary Table 3](#). Glycan monosaccharide key: N-acetylglucosamine [blue square], fucose [red triangle], mannose [green circle], galactose [yellow circle], and N-acetylneuraminic acid [purple diamond]. CD, Crohn's disease

altogether reporting on fucosylation, antennarity, bisection, galactosylation, sialylation, etc. Subsequently, all analyses were performed on 24 derived traits based on structural similarity of glycans [[Supplementary Table 4A and B](#)].

3.1. Technical and biological variation of serum N-glycan analysis

Technical variation was determined by measuring three serum standards per plate [total 18]. In total, 39 N-glycan peaks were quantified in each replicate and used to calculate the average relative intensities [RI], standard deviations [SD], and coefficients of variation [CV] after total area normalisation [[Supplementary Figure 4](#)]. The RI of the highest peak [A2G2S2] was 41.5% [SD $\pm$ 0.7%], with a CV of 1.6%. All glycan peaks with an RI above 2% gave CVs below 6.2%, with the exception of A2G2S1 that had an RI of 9.1% [SD $\pm$ 1.0%] and a CV of 11.1% [[Supplementary Table 6](#)]. The average RI of the highest peak [A2G2S2, peak 22] across all measurements was 36.3% [SD $\pm$ 2.1%, CV 5.8%] in healthy controls [HC] and 36.0% [SD $\pm$ 3.3%, CV 9.3%] in IBD—therefore biological variation was 3.6 times the technical variation in HC, 5.8 times in IBD.

3.2. Healthy controls vs. symptomatic controls

The HC and symptomatic controls [SC] were combined in all downstream analyses, since generalised linear models [GLMs] of derived glycan traits did not show any differences between the two groups after correcting for age, sex, and the interaction thereof [[Supplementary Table 7](#), [Supplementary Figure 5A](#)].²⁵

3.3. Symptomatic controls [IBS] vs. IBD [CD and UC]

The generalised linear model of derived glycan traits between those symptomatic controls with an IBS diagnosis, and IBD combining CD and UC individuals, showed significant

differences [13]. A representative example of three derived traits is shown in [Supplementary Figure 5B](#).

3.4. Associations of glycans with IBD

The association was tested between 24 derived glycosylation traits and the disease state or subtype [[Table 2](#)]. This showed that galactosylation of diantennary glycans was lower in both CD and UC vs. controls [[Figure 3A–C](#)], whereas bisection of diantennary glycans was generally higher in both CD and UC vs. controls [[Figure 3D and E](#)], except for bisection of diantennary fucosylated glycans [[Figure 3F](#)]. Fucosylation of triantennary glycans was higher in both CD and UC vs. controls [[Figure 3G](#)], whereas fucosylation of tetraantennary glycans was generally higher only for CD vs. controls [[Figure 3H](#)]. In addition, galactosylation was more abundant on the α 1,6—mannose arm as compared with the α 1,3—mannose arm for UC patients in comparison with CD patients [[Figure 3I](#)].

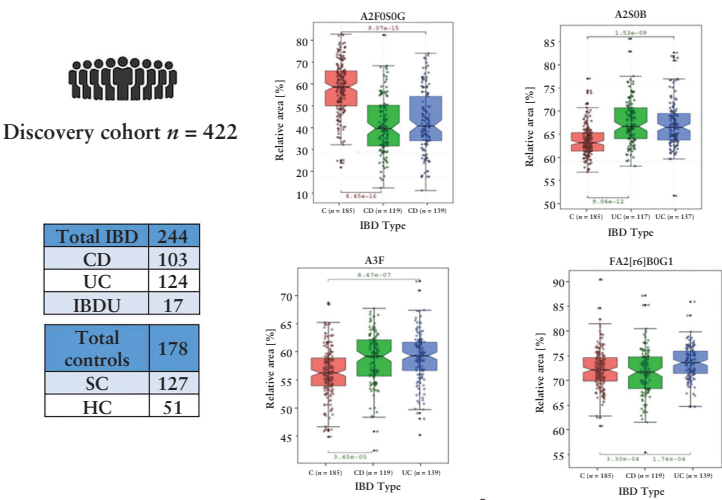
3.5. Association of glycans with clinical markers

HsCRP correlated with derived glycan traits for both controls and IBD patients, whereas platelets, haematocrit, haemoglobin, and albumin showed associations with glycans for IBD patients only. A detailed overview of derived glycan associations with clinical parameters is given in [Supplementary Results Section 3](#) and depicted in heatmaps in [Supplementary Figure 6](#).

3.6. Glycomics biomarkers for identification and classification of IBD

Cross-validated [$n=10$] analysis of the discovery cohort using GLMs consisting of up to seven derived glycans traits was performed in the discovery cohort. Individual traits including

A Glycan associations study in discovery cohort



B Escalation follow-up data in two cohorts

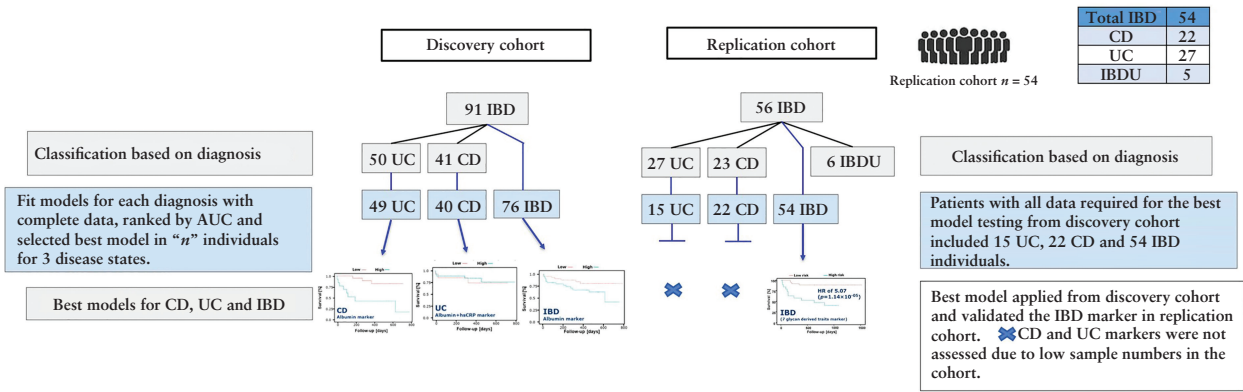


Figure 2. Infographic illustrating the breakdown of total number of samples used for different analysis in the discovery cohort and replication cohort. A) Depicts the sample numbers used to study the glycan associations of derived traits in the discovery cohort. B) Escalation follow-up data showing complete sample sets [ie, varying sample numbers used for the best model testing in ‘n’ individuals across three disease states] within the discovery cohort and replication cohort.

A2F0S0G, A2FS0G, A3F, and A3FS distinguished both CD and UC from controls alone (area under the curve [AUC] <0.85) with combinations of up to seven derived glycan traits [AUC <0.88] improving model performance. The best single derived trait for distinguishing between CD and UC was FA2[r6]G1 [AUC 0.62] and the best overall model [AUC 0.75] used a combination of five glycan markers; see [Supplementary Table 8](#).

3.7 Prediction of IBD treatment escalation using glycomic markers

Data on escalation of therapy, including biologics and surgery, during follow-up were available for 91 IBD patients in the discovery cohort [Figure 2]. Patients were recruited over a period of 4 years and followed up until escalation, loss to follow-up, or the end of the study period. All escalation events occurring within follow-up were considered with no horizon. For non-escalators, the median follow-up was 441 days with a range of follow-up days between 29 to 797 days. For escalators, the average number of follow-up days was 63, with a range of follow-up between 1 to 615 days. An endpoint

of ‘treatment escalation’ was defined as the requirement for either biologics or ciclosporin [$n=13$, median time to event 203 days], or surgery [$n=11$, median time to event 10 days]. Cox proportional hazard models including all combinations of up to seven derived glycan traits were created in this subset and ranked by the AUC from leave one out [LOO] cross-validation. A2F0S yielded the highest AUC [0.73] for a single derived trait, whereas A2F0S0G + A2FS for two traits and A2F0S0G + FA2[r6]B0G1 + FA2[r6]BG1 for three traits both had AUC of 0.84. All the other combination of markers that yielded the highest putative AUC for each length are presented in [Supplementary Table 9](#).

In the discovery cohort, the optimal biomarker for prediction of disease escalation in IBD produced an AUC of 0.94 [Figures 4A and 7] including the traits A4F, A2B, A2F0B, A2S0B, A2F0SB, A2S0G, and A2F0S. Addition of naivety to the IBD glycomics biomarker did not improve the results [AUC = 0.84]. Considering CD and UC diagnoses separately, the optimal biomarkers included A2F0B and A2S0G with an AUC of 0.96 [Figures 2 and 4C] and A2B, A2S0B, A2F0S0B, A2F0SB, A2FS, and FA2[r6]G1 with an AUC of 0.97 [Figures

Table 2. TSNG-derived trait associations with controls and IBD

Derived trait	C [RI %]	CD [RI %]	UC [RI %]	<i>p</i> -value [C-CD]	D	<i>p</i> -value [C-UC]	D	<i>p</i> -value [CD-UC]	D
A2F	32.8	32.7	32.1	8.7×10^{-1}	-	2.8×10^{-1}	-	3.2×10^{-1}	-
A3F	56.8	58.8	59.1	3.5×10^{-5}	↓	8.5×10^{-7}	↓	4.4×10^{-1}	-
A4F	15.9	18.1	17.0	1.5×10^{-5}	↓	6.2×10^{-2}	-	3.3×10^{-2}	-
A2B	16.3	16.4	16.2	4.9×10^{-1}	-	7.1×10^{-1}	-	4.6×10^{-1}	-
A2F0B	8.3	8.8	8.7	3.3×10^{-2}	-	1.1×10^{-1}	-	9.2×10^{-1}	-
A2FB	36.6	34.2	34.3	4.9×10^{-6}	↑	5.2×10^{-7}	↑	7.8×10^{-1}	-
A2S0B	64.1	67.8	67.3	9.0×10^{-12}	↓	1.5×10^{-9}	↓	2.7×10^{-1}	-
A2F0S0B	83.8	85.3	85.9	1.1×10^{-4}	↓	6.1×10^{-8}	↓	7.4×10^{-2}	-
A2FS0B	18.9	17.7	17.2	1.1×10^{-2}	-	3.3×10^{-5}	↑	1.1×10^{-1}	-
A2SB	11.1	11.0	10.5	8.8×10^{-1}	-	9.3×10^{-3}	↑	6.2×10^{-2}	-
A2F0SB	2.5	2.5	2.3	9.8×10^{-1}	-	1.4×10^{-5}	↑	1.1×10^{-3}	-
A2FSB	36.1	35.3	35.4	2.0×10^{-1}	-	9.5×10^{-2}	-	8.2×10^{-1}	-
A2S0G	56.2	46.6	47.5	5.0×10^{-15}	↑	3.1×10^{-14}	↑	3.5×10^{-1}	-
A2F0S0G	56.5	41.2	42.8	8.4×10^{-16}	↑	9.1×10^{-15}	↑	2.6×10^{-1}	-
A2FS0G	51.8	42.0	43.1	4.4×10^{-15}	↑	8.8×10^{-14}	↑	2.4×10^{-1}	-
A2S	80.7	81.2	81.1	2.9×10^{-1}	-	2.7×10^{-1}	-	9.8×10^{-1}	-
A2F0S	84.9	84.4	84.5	7.4×10^{-2}	-	3.9×10^{-1}	-	7.1×10^{-1}	-
A2FS	55.2	56.7	55.2	6.5×10^{-3}	-	8.1×10^{-1}	-	1.1×10^{-2}	-
A3S	100.5	99.5	101.2	5.7×10^{-1}	-	7.7×10^{-2}	-	3.2×10^{-2}	-
A3F0S	97.4	95.5	98.1	2.9×10^{-1}	-	1.2×10^{-1}	-	2.1×10^{-2}	-
A3FS	134.3	128.7	131.2	6.3×10^{-5}	↑	5.7×10^{-2}	-	4.0×10^{-2}	-
FA2[r6]G1	56.8	56.2	58.1	2.8×10^{-1}	-	6.3×10^{-3}	-	3.3×10^{-4}	↑
FA2[r6]B0G1	73.2	72.6	74.4	2.9×10^{-1}	-	1.5×10^{-3}	-	1.7×10^{-4}	↑
FA2[r6]BG1	33.8	33.4	33.9	7.3×10^{-1}	-	9.2×10^{-1}	-	7.5×10^{-1}	-

TSNG-derived trait comparison table displaying the average relative intensity [RI] for each sample type. grey background. The *p*-value of the age, sex, and age-sex interaction corrected generalised linear model and the direction [D] of change are shown for each comparison (controls [C] vs. CD, C vs. UC and CD vs. UC). Significant results are highlighted with bold text and a light grey background.

TSNG, total serum N-glycans; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

4E and 6] respectively. Kaplan-Meier plots for the optimal biomarkers for IBD, CD, and UC are depicted in [Figure 4B](#), [D](#), and [F](#) illustrating prediction of treatment escalation between high-risk and low-risk individuals. In the discovery cohort, the glycomics biomarkers for treatment escalation gave hazard ratios [HR] of 23.7 (*p*-value 6.8×10^{-6} ; 95% confidence interval [CI] 6.1–91.96) for CD, 30.8 [*p*-value 1.9×10^{-4} ; 95% CI, 7.9–120.4] for UC, and 25.9 [*p*-value 1.1×10^{-12} ; 95% CI, 8.52–78.78] in a combined IBD biomarker [See [Supplementary Table 10](#)]. Analysis of Schoenfeld residuals was evaluated and showed no significant time-dependent effects [see [Supplementary Figure 7](#)]. Addition of hsCRP to the best glycomics biomarker did not improve the results (IBD escalation HR = 9.0, *p*-value of 7.0×10^{-12} ; 95% CI, 6.84–11.85 [Supplementary Table 11](#))).

For comparison with glycomics markers, Cox proportional hazard models using clinical inflammatory markers were tested, none of which outperformed the glycomics markers [[Supplementary Table 12](#) and [Supplementary Figure 8](#)].

3.8. Validation of IBD treatment escalation using glycomics markers

The best performing glycomics biomarker for predicting treatment escalation in all IBD patients was reapplied to an independent cohort using an age, sex, and age-sex interaction corrected Cox model (AUC 0.76, *p*-value 1.1×10^{-5} , HR =

5.1; 95% CI, 2.54–10.1; [Figure 5](#))). Addition of naivety to the IBD glycomics biomarker in the validation cohort did not improve the results [AUC 0.74]. Individual analyses of CD and UC were not possible without a larger replication cohort. The impact of the collection centre was evaluated using principal component analysis [PCA], which showed a small effect. Patient age was the largest contributor to the observed effect. A Student's *t* test was used to evaluate the effect of patient age, which showed that it was not significant [*p*-value 0.987]. [[Supplementary Figure 9](#)].

4. Discussion

We performed glycosylation analysis of total serum N-glycans from 422 samples using an automated sample processing method including glycan release, enrichment, labelling with procainamide tag, and post-labelling clean-up followed by UHPLC analysis.³⁴ Our workflow did not involve time-consuming sample processing steps such as IgG purification and has the added advantage of providing an overview of glycosylation changes in the total serum N-glycome.

Using this robust analytical method,³⁵ we identified the differences in derived glycan traits between IBD and controls, as well as associations with clinical markers. The advantages of using derived traits are that they are calculated from enzymatically related glycans and are representative

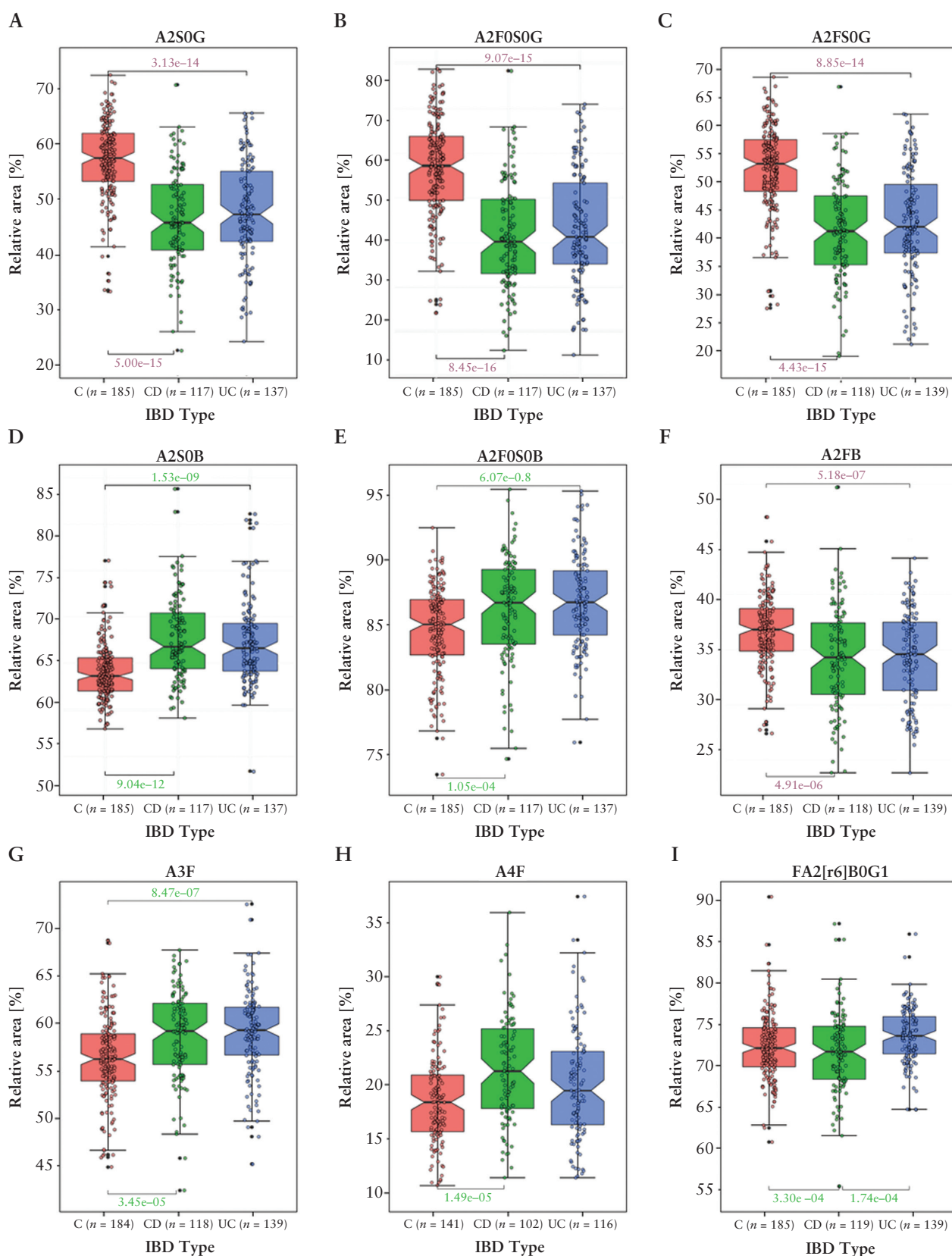


Figure 3. Association of derived glycan traits in controls, CD and UC patients. The notch represents 95% confidence interval for the median, the box represents the interquartile range, the dots within the upper and lower whiskers represent an individual's relative abundance for the trait, and dots outside the whiskers are possible outliers. The $n = x$ shown on the x-axis represents the number of measurements whose relative abundance was plotted for each group within the cohort. These age- and sex- corrected GLM boxplots with significant results are marked with a line, and coloured p -values [red indicates a negative slope and green indicates a positive slope] are shown inside each plot. [A] A2S0G = galactosylation of non-sialylated diantennary glycans, [B] A2F0S0G = galactosylation of non-sialylated non-fucosylated diantennary glycans, [C] A2FS0G = galactosylation of

of overall glycan characteristics [eg, galactosylation and/or fucosylation]. This does not only provide a clinically relevant metric but also improves the quality of the data by removing technical variation. Furthermore, we have reported novel predictions for IBD treatment escalation using derived glycomics traits and validated these in an independent replication cohort.

4.1. Association of glycans with clinical markers

We investigated the association of TSNG [derived traits] with most of the commonly measured clinical parameters such as hsCRP, albumin, differential white cell counts, and platelets. GLMs corrected for age, sex, and age-sex interaction terms were used to determine the potential correlation between each derived glycan trait and clinical markers, for controls and IBD patients. We assessed derived glycan trait associations with clinical markers and our results matched previously published data²³ with an exception of a few methodological differences. See [Supplementary Discussion-Section 4](#) and [Supplementary Figure 6](#) for full details.

4.2. Glycomics biomarkers for identification and classification of IBD

Many groups have studied the underlying differences and similarities between CD and UC, using multi-omics approaches to obtain insights into disease mechanisms and prognosis.^{6–10,25,36–38} We observed two glycomics traits differing between CD and UC, representing a shift towards a higher incidence of galactose on the α 1,6—mannose arm in UC patients as opposed to the α 1,3—mannose arm. This could be due to a shift in IgG sub-class concentration in serum of IBD patients. It is known that IgG1 and IgG4 sub-class serum concentrations are significantly higher in UC patients than in CD patients,^{39,40} and IgG1 and IgG4 sub-classes are known to have higher α 1,6-branch galactosylation than α 1,3-branch galactosylation.⁴¹ Although the subtle differences in glycan-derived traits differentiating CD and UC were identified in this study, we emphasise that the analysis performed for classification of IBD presented here is not proposed as a biomarker for stratification of IBD. A clinical test to distinguish CD versus UC would require much greater accuracy, but the glycomics markers identified in this study warrant further investigation.

4.3. Prediction of IBD treatment escalation

The need for and time to treatment escalation after diagnosis offer a useful insight into usual clinical practice as a result of clinical judgement in light of all the investigations and scores available to the treating physician and the tolerability of symptoms for each individual patient.⁴² As an endpoint they have been used in other studies, most notably in the derivation of the Predict-Immune molecular signature, now under extended investigation in the PROFILE trial⁴³ [ISRCTN 11808228] and under NICE assessment. In the IBD-CHARACTER cohort, this endpoint has allowed discovery of proteomic and molecular biomarkers. Mucosal healing may be better correlated

with disease activity; however, changes in medication are not always immediately pre-empted by endoscopy and as a measure this is subject to several biases [including inter-operator variability and differing rates of endoscopy].⁴⁴ Other robust endpoints have been recently suggested in the SPIRIT consensus, these at present being more suited to clinical trials than biomarker discovery.

4.3.1. Glycomics markers

Confidence in these findings was strengthened by the performance of the treatment escalation biomarker in a validation cohort of Swedish IBD patients [HR = 5.0, p -value 1.1×10^{-5} , 95% CI, 2.54–10.1] despite centre effects [[Supplementary Figure 9](#)]. Other variations between the cohorts could include patient and physician treatment preferences and baseline patient severity, as well as variations arising from different methods used for patient recruitment. As only few patients required treatment escalation, the IBD subtype-specific signatures could not be meaningfully assessed when stratifying patients in the validation cohort by subtype of IBD and analysing Crohn's disease and ulcerative colitis separately.

Decreased IgG galactosylation has been studied and reported in several publications with respect to ageing as well as inflammatory conditions such as rheumatoid arthritis and IBD.^{21,45} The genes *MGAT3* and *B4GALT1* encode glycosyltransferases known to glycosylate immunoglobulins. *MGAT3* codes for the enzyme *N*-acetylglucosaminyltransferase III that adds bisecting GlcNAc, and *B4GALT1* codes for galactosyltransferases that add galactose to immunoglobulin glycans.⁴⁶ It is also known that these enzymes that add galactose and bisecting GlcNAc compete for substrates such as G0 and G0F. For example, after the addition of a bisecting GlcNAc to the β 1,4-linked mannose in the three-mannose core of *N*-glycans, the bisecting GlcNAc prevents further branching, eliminates addition of core-fucose and diminishes addition of galactose residues.⁴⁷ Changes to levels of bisection have not been widely reported in IBD; however, associations of bisection and enzymes responsible for branching association with cancer have been discussed previously.⁴⁸ Genome-wide association studies have shown that *MGAT3* is one of the five glycosylation-associated genes [*IKZF1*, *LAMB1*, *MGAT3*, *IL6ST*, and *BACH2*] located within IBD susceptibility loci and it is also associated with CD pathogenesis.^{49,50} These findings may contribute to our result that altered bisection is seen in IBD patients requiring treatment escalation.⁴⁶

4.3.2. Clinical markers

To provide comparison with glycomics markers, we assessed the ability for widely available standard clinical inflammatory markers hsCRP and Alb to determine prediction of treatment escalation of IBD, CD, and UC. [[Supplementary Discussion Sections 4](#)] Among the two clinical blood markers for prediction of treatment escalation, Alb gave AUCs of 0.62, 0.77, and 0.57 for IBD, CD, and UC respectively. Although the model for Alb performed moderately, it is known from previously published data that serum albumin levels at the time of UC

non-sialylated fucosylated diantennary glycans, [D] A2S0B = bisection of non-sialylated diantennary glycans, [E] A2F0S0B = bisection of non-sialylated non-fucosylated diantennary glycans, [F] A2FB = bisection of fucosylated diantennary glycans, [G] A3F = fucosylation of triantennary glycans, [H] A4F = sialylation of fucosylated tetraantennary glycans, and [I] FA2[r6]B0G1 = the ratio of α 1,6 to α 1,6+ α 1,3 arm galactosylation of mono-galactosylated non-bisected core-fucosylated diantennary glycans. CD, Crohn's disease; UC, ulcerative colitis; GLM, generalised linear model.

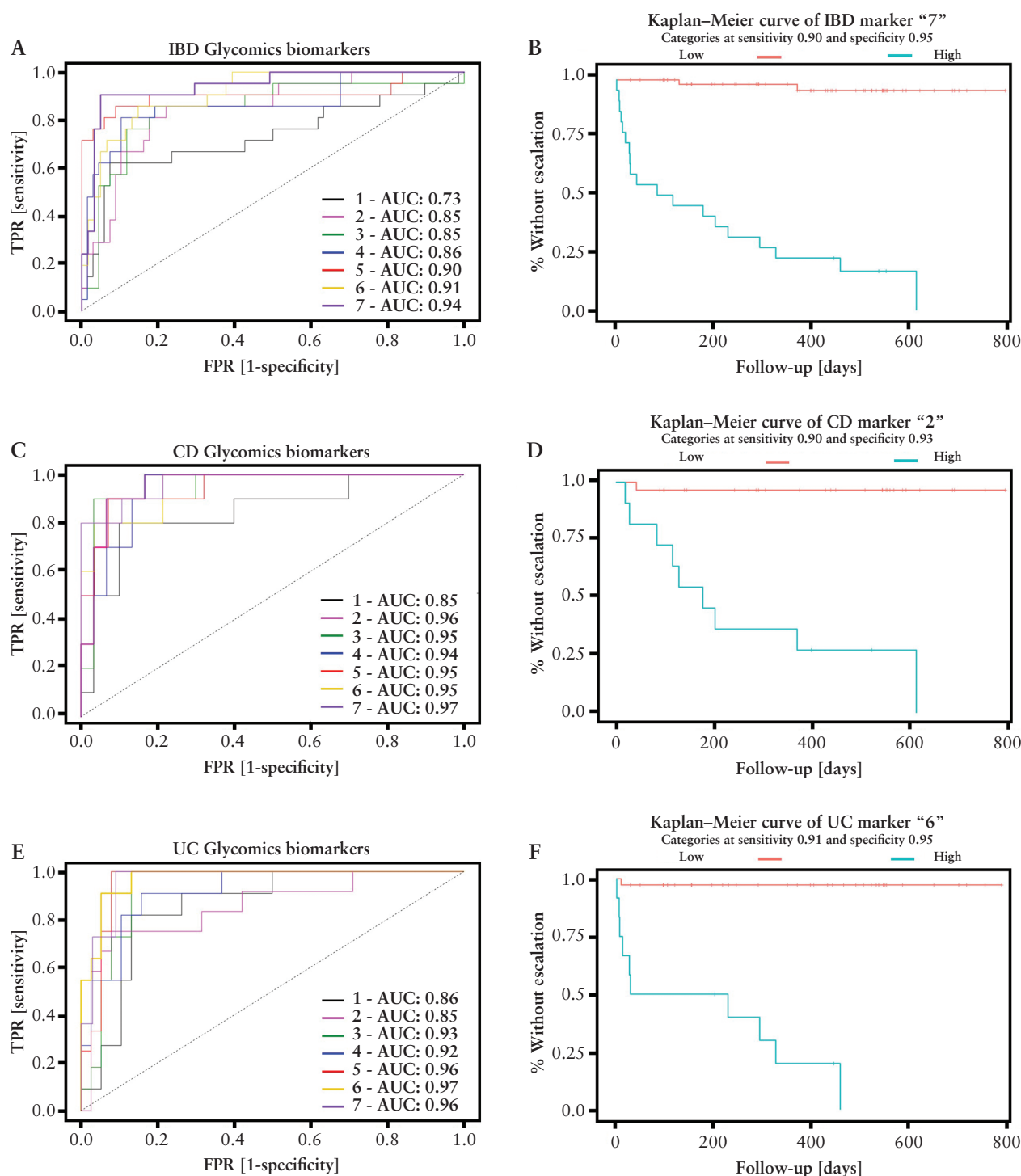


Figure 4. ROC curves and Kaplan-Meier plots for prediction of escalation of disease using glycomics biomarkers applied to IBD, CD, and UC patients. All possible combinations of between one and seven glycan-derived traits were used in leave one out validation [$n = 92$]. The combination that yielded the maximum area under the curve [AUC] for each length was stored to identify the optimal glycomics biomarker that can predict escalation of IBD [except in the case of CD, where only two derived traits gave an AUC of 0.96, therefore the combination of seven derived traits with AUC of 0.97 was disregarded]. The optimal biomarkers that were identified by this approach were used in a Kaplan-Meier test, where the samples were split into two groups based on the optimal sensitivity and specificity that were determined using the ROC curve [displayed under the title as categories with cut-offs for sensitivity and specificity]. [A] The optimal glycomics biomarker [red line] calculated by the model for prediction of IBD was A4F+A2B+A2F0B+A2S0B+A2F0SB+A2S0G+A2F0S, which yielded an AUC of 0.94. [B] Kaplan-Meier plot for the optimal marker for IBD. [C] The optimal glycomics biomarker for treatment escalation prediction for CD [purple line] was shown to be A2F0B+A2S0G, which yielded an AUC of 0.96. [D] Kaplan-Meier plot for the optimal marker for CD. [E] The optimal glycomics biomarker for treatment escalation prediction for UC [yellow line] was shown to be A2B+A2S0B+A2F0S0B+ A2F0SB+A2FS+FA2[r6]G1, which yielded an AUC of 0.97. [F] Kaplan-Meier plot for the optimal marker for UC. CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; ROC, receiver operating characteristic.

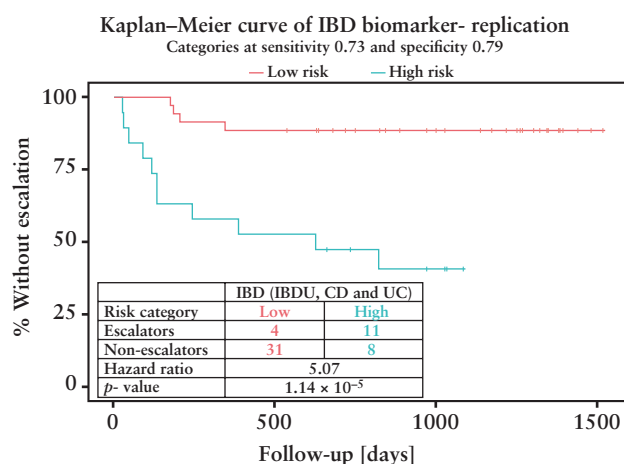


Figure 5. Kaplan–Meier plot from the validation cohort illustrating escalation of disease using the IBD glycomics biomarker. The glycomics biomarker for predicting treatment escalation of IBD was applied to a validation/replication cohort taken from Örebro, Sweden ($n = 54$). The model was applied using an LOO validated Cox PH model, which showed that the model was significant with a p -value = 1.14×10^{-5} , an HR of 5.0, and 95% confidence interval, 2.54–10.1. A table illustrating the number of patients escalated for each risk category is also included in the figure. IBD, inflammatory bowel disease; LOO, leave one out; PH, proportional hazard; HR, hazard ratio.

diagnosis could predict disease course.⁵¹ hsCRP gave AUCs of 0.57, 0.55, and 0.55 for IBD, CD, and UC, respectively, and although CRP and hsCRP are widely used as a blood-based biomarkers for prediction of systemic inflammation, they are not always sensitive or specific for IBD. Faecal calprotectin [FCP] is widely used as a dynamic indication of inflammatory activity in the gut; however, it is known that collecting stool samples from patients is problematic.⁵² The correlation between FCP and treatment escalation could not be assessed in this cohort, as stool samples were only available for five patients when restricted to those obtained from within 1 month of diagnosis. Patient reluctance to provide faecal samples and problems associated with sample delivery and processing of FCP limit the use of this marker in clinical practice. However in future multi-omics studies, we would like to increase the uptake of valuable clinical markers such as FCP and/or serum calprotectin to aid in prognosis of IBD.^{52,53}

4.4. IBD-treatment management

Studies have shown that a ‘top-down’ approach or early introduction of biologic therapy [second-line therapy, eg, anti-TNF] induces higher remission rates in CD^{54–56} and results in better clinical and endoscopic disease outcomes. However this approach may not be appropriate for all patients, given that up to 20% of IBD patients run a benign course with no requirement for drug escalation over 5 years. Therefore exposing these patients to potent therapies carries risks including infection and malignancy.^{57–59} Prognostic stratification at diagnosis are needed in IBD in order to personalise care and predict treatment outcomes.⁶⁰ Our data identify a unique glycan profile that associates with an aggressive disease course and the need for escalation over time. These data complement existing studies that are beginning to define the unique molecular profiles that associate with an aggressive disease course. Several studies have explored ‘omic’ technologies to identify prognostic profiles in IBD.⁶¹ A previous study by Lichtenstein *et al.* combined genetic markers

and quantitative serological clinical data to predict complications of Crohn’s disease behaviour.⁶² Biasci *et al.* have developed and validated a whole-blood quantitative polymerase chain reaction [qPCR] method that does not require cell separation, and this method can be used as a prognostic biomarker for prediction of disease course in newly diagnosed IBD patients.⁴³ A prospective transcriptomic-based, biomarker-stratified clinical trial [PROFILE study; ISRCTN 11808228] is currently recruiting to explore its clinical utility.⁶³ Using the escalation criteria, serum calprotectin, albumin, and CRP have also been shown to predict escalation, with HR of 2.1 to 2.4; a combined protein panel performed better than individual markers. There are also emerging data defining the methylome and proteome in patients who require intensification of therapy at diagnosis.^{64,65} In children, transcriptional risk scores have been generated that allow prediction of stricturing or penetrating CD over time.¹⁰ Previous ‘omics’ studies have examined patients with long-standing disease course, and therefore prediction of treatment escalation could reflect treatment effects instead of disease course. Because of this, an added advantage of our study is the presence of a large percentage of treatment-naïve patients in both discovery and replication cohorts.

The replication of a glycomics biomarker for treatment escalation in IBD [AUC: 0.76, HR = 5.0; 95% CI, 2.54–10.1] has shown very promising results that could potentially change IBD treatment management in the future. Based on our glycomics treatment escalation biomarker, clinicians would be able to provide personalised therapy and focus on patient-centred and individualised therapy management of IBD. Evidently, future clinical trials are needed to investigate the cost benefit and quality of life implications to adopting a ‘top-down’ approach in patients identified as being at risk using a glycomics-only biomarker.

In this study, various aspects of IBD treatment management and association with glycosylation remain unexplored: for example, association of glycosylation with response or non-response to specific therapeutic agents and the relationship to other multi-omic markers, particularly as multi-omic profiles that define the biology underpinning disease course may allow us to generate novel therapies, use markers to stratify patients in clinical drug trials, and achieve accurate selection of therapies early to improve outcomes in IBD. Advances in glycomics technology have allowed us to perform high-throughput, reliable, and reproducible glycosylation analysis using the gold standard UHPLC glycan analysis. However, this could be seen as a potential barrier to widespread adoption of this analytical method due to the complexity involved in the analysis. Developing and optimising a microtitre plate-based fluorometric assay for quantitative, high-throughput analyses of specific glycan biomarkers is a key objective for all our future glycomics-based research projects, as this method has a wider acceptance within the clinic. The current study focused on glycomics-based treatment escalation prediction strategy and the current technological advances in the glycomics field can easily translate it into the clinic.

Advantages of the glycomics biomarker include the non-invasive nature of the serum N-glycan test, low [10 μ L serum] sample volume needed for testing, and low cost per sample,⁶⁶ and automated high-throughput sample processing allows for scale-up of samples in the clinic. Additional advantages of glycomics markers include the high sensitivity and specificity for prediction of treatment escalation in IBD

[Figure 4]. These advantages make the glycomics marker method convenient, economical, and reproducible.

In this study, we demonstrated the robustness and repeatability of the analytical method used for total serum N-glycan measurement, and confirmed previous associations such as decreased galactosylation and increased bisection using a LC methodology. We were able to identify novel findings such as GalMan linkage, which shows significant differences between UC and CD patients.

Importantly, we were able to predict treatment escalation in IBD patients using glycomics analysis based on a small serum sample taken at diagnosis. To the best of our knowledge, we are the first group to identify glycomics biomarkers for prediction of treatment escalation in IBD. By using a single glycomics test [total serum N-glycan analysis] patients could potentially be selected for an individualised top-down approach to therapy in both CD and UC. As a result, the biomarker could be used to identify patients [either CD or UC] who are at a particularly high or low risk of severe disease. Once a risk category is identified for an individual, their treatment protocols could be a) adjusted to pre-emptively increase the treatment level and monitoring frequency in the case of high-risk patients, or b) to transfer management to primary care in the case of low-risk patients.⁶⁷ This may reduce cumulative intestinal damage, prolong periods of remission, and increase mucosal healing, possibly reducing the overall inflammatory burden and the concomitant risks and quality of life implications. Selecting individuals for expedited treatment based on TSNG, in contrast to a universal ‘bottom-up’ approach, would reduce exposure to treatment risks in those identified as at low risk and reduce unnecessary use of second-line treatments. Furthermore, the threshold of predicted risk at which action would be taken could be adjusted as required. Therefore, health care cost increases would be minimised by restricting biologics prescriptions to high-risk patients, and there is a potential for cost savings through reducing emergency hospitalisations and surgeries. Future perspectives include a clinical trial to assess real-life performance of the biomarkers. In conclusion, our validated glycomics biomarkers presented for prediction of treatment escalation of IBD patients could help bring about an era of personalised care in IBD.

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Conflict of Interest

Ludger Ltd is a commercial bioscience company that offers products and services for glycoproteomics technology. Some of Ludger's products are used in this study. RK: financial support for research EC IBD-Character, lecture fee[s]; Ferring, Nick Kennedy, financial support for research Wellcome Trust, conflict with Abbvie, MSD, Warner Chilcott, Ferring speaker fees, Shire Travel bursary. JS: financial support for research EC grant IBD-BIOM and IBD Character, Wellcome, CSO, MRC, conflict with consultant for Takeda, MSD speaker fees. JH: consultancy fees [advisory board/speaker] from AbbVie, Celgene, Ferring, Hospira, Janssen, Medivir, MSD, Pfizer, Prometheus, Renapharma-Vifor, Sandoz, Shire, Takeda,

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Author Contributions

Drafting of the manuscript: AS, BCJ, ATA. Sample preparation: AS, PAU, RAG. Data processing: BCJ, AS, ATA. Statistical analysis: BCJ, ATA, AS. Technical support: KRR, PAU, Patient recruitment: ATA, NTV, RK, DB, JH, JS. IBD-BIOM principal investigators: JS, DLF, MW, DIRS. All authors had access to the study data and approved the final manuscript.

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Data Availability Statement

The authors confirm that the source file data and code [R Script] supporting the findings of this study are available along with the Supplementary material.

Supplementary Data

Supplementary data are available at ECCO-JCC online.

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