

## **Decreased Complexity of Serum *N*-glycan Structures Associates with Successful Fecal Microbiota Transplantation for Recurrent *Clostridioides difficile* Infection**

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## **Author contributions**

T.M.M., M. P-B, F.V. and D.K. designed the study, analysed the data and wrote the paper. M. P-B, I.W. and F.K. performed the experiments. F.V. and P.K. performed the statistical analyses. D.K., T.L., B.R., C.L., and P.K. developed the clinical sample cohort. All authors reviewed the manuscript, provided feedback, and approved the manuscript in its final form.

## **Competing Interests statement**

T.M.M. is a consultant for CHAIN Biotechnology. The remaining authors declare no competing interests.

## Introduction

*N*-glycosylation is a common and yet complex posttranslational process that covalently links glycans (complex oligosaccharides) to proteins and lipids, affecting cellular structure and function. Glycans have important biological functions in protein maturation and turnover, cell adhesion and trafficking, and receptor binding and activation.<sup>1</sup> In the immune system, glycosylation also modulates the function of immunoglobulin G (IgG). Differential *N*-glycosylation of its fragment crystallisable (Fc) affects IgG effector functions through modified binding affinity to the Fc-receptors (FcγRs), enabling its ability to act as a pro- or anti-inflammatory agent.<sup>1</sup>

Structural details of the attached glycans are of great physiological significance and many pathological conditions are associated with various types of glycan changes. Alterations in plasma protein glycosylation pathways with increased branching, galactosylation and sialylation are a hallmark of metabolic syndrome, cancers and inflammatory bowel diseases (IBD).<sup>1-2</sup> Therefore, glycans have the potential to help stratify patients according to disease predisposition, prognosis, and response to treatment.<sup>1</sup> For example, IBD patients with Crohn's disease or ulcerative colitis have lower plasma levels of IgG galactosylation than healthy controls<sup>3</sup> Furthermore, glycosylation patterns have been shown to be associated with IBD disease progression and need for surgery.<sup>3</sup>

Certain members of the gut microbiota, such as *Bacteroides* and *Bifidobacteria*, are coevolved to thrive on host- and diet-derived glycans,<sup>4</sup> where the former (*B. fragilis*) can efficiently deglycosylate complex *N*-linked glycans from the most abundant glycoproteins found in serum and serous fluid, thus conferring a competitive, nutritional advantage for extra-intestinal growth.<sup>5</sup> However, it is not known if modulation of the gut microbiota via faecal microbiota transplantation (FMT) can affect the host's glycosylation machinery, and if this may represent one mechanism by which FMT exerts its therapeutic efficacy against *Clostridioides difficile* infection (CDI), the leading infectious cause of antibiotic-associated diarrhea. Susceptibility to developing CDI typically occurs following disruption of the

intestinal microbiota through antibiotic usage. While current treatment options include standard antibiotics and emerging immunologics, microbiome restoration approaches such as FMT are highly effective for the treatment of recurrent CDI (rCDI). Nevertheless, the precise mechanisms that underlie the success of FMT remain largely unclear with current evidence suggesting that its effectiveness in part, may be related to reconstitution of the intestinal microbiota, restoration of bile acid and short chain fatty acid metabolism, activation of immune-mediated mechanisms.<sup>6</sup> Therefore, to address this gap in knowledge, we examined the composition of whole serum protein and subclass-specific IgG Fc *N*-glycome in subjects before and after FMT for rCDI.

## Methods

For *N*-glycome profiling, we retrospectively analysed a subset of archived sera from rCDI participants successfully treated in two independent trials comparing capsule versus colonoscopy delivered FMT [NCT02254811; discovery cohort] and fresh versus frozen enema delivered FMT [NCT01398969; replication cohort] for treatment of rCDI. Sera were profiled for total serum and IgG Fc *N*-glycome analysis by hydrophilic interaction ultra-performance liquid chromatography (HILIC-UPLC) and nano-liquid chromatography coupled with electrospray mass spectrometry (nanoLC-ESI-MS), respectively. For the discovery cohort, we evaluated 225 sera from 75 of 116 participants at screening, and compared with 4 and 12 weeks' post FMT. For the replication cohort, we assessed a total 110 sera from 55 of 178 participants before and at one time point after FMT [median 31 days, (range 7-277 days)] subject to sample availability. The baseline characteristics of both cohorts are illustrated in Supplementary Table 1. We analysed glycome changes for both cohorts individually and then aligned both discovery and replication data sets by comparing glycan signatures seen at the 4-week mark following FMT due to variability in sampling. Further details are described in the Supplementary Materials.

## Results

In the discovery cohort, HILIC-UPLC analysis of the total serum *N*-glycome identified 11 serum glycosylation structural features that changed significantly following FMT (Table 1). Specifically, we found a statistically significant increase in levels of low-branching, monosialylated, digalactosylated, oligomannose and bisecting *N*-acetylglucosamine glycans, while levels of high-branching, tri- and tetragalactosylated, tri- and tetrasialylated glycans and glycans with antennary fucosylation decreased following successful FMT. Meta-analysis confirmed that the effects of FMT were consistent across both the discovery and replication cohorts (Table 1). All 11 glycosylation traits that were significant in the discovery cohort remained significant in the meta-analysis of the combined studies.

For IgG Fc *N*-glycopeptide analysis, none of the glycosylation traits showed statistically significant changes in either discovery or replication cohorts (Supplementary Table 2). There were also no specific differences in the relative abundance of the different total serum and IgG *N*-glycome traits with age, sex, treatment modality, number of recurrent episodes prior to FMT, presence of IBD or immune status in either cohort.

In order to align the discovery and replication cohort sampling time points more evenly, we only selected sera that were collected around 4 weeks after FMT (n= 36 serum samples from 18 participants) in the replication cohort [median 31 days, (range 21-36)]. Here again, meta-analysis confirmed that 10 of 11 aforementioned serum glycan traits changed significantly and in the same direction to that seen for both cohorts.

## Discussion

This study represents the first exploratory analysis of whole serum and IgG *N*-glycosylation in participants undergoing FMT for rCDI. We demonstrate that successful FMT associates with a reduction in the complexity of serum *N*-glycosylation profiles, contrary to the complex

glycophenotypes typically encountered in many pathological states such as IBD, type 2 diabetes mellitus and cancer. Decreasing complexity of the serum *N*-glycome is mainly driven through a significant reduction in the relative abundance of high-branching, tetragalactosylated and trisialylated glycans and a corresponding increase in low-branching glycans. Although it is not known which specific cellular glycomic modifications occur following FMT, patients with autoimmune diseases and many inbred mouse strains display defective *N*-glycan branching on T cells, which may be restored by *N*-acetylglucosamine or vitamin D supplementation.<sup>7-8</sup> In conclusion, changes in the complexity of *N*-glycans in sera, may serve as an important molecular mechanism by which FMT exerts its beneficial effects in rCDI. Future studies will be required to assess *N*-glycome patterns in the context of treatment failure in order to assess their prognostic relevance in predicting FMT outcomes in rCDI.

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Glycan Structural Feature		Discovery Cohort - NCT02254811			Replication Cohort - NCT01398969			Meta-analysis			Direction of change
		Time effect	Time SE	Adj. p value	Time effect	Time SE	Adj. p value	Meta effect	SE	Adj. p value	
LB	Low-branching glycans	0.0333	0.0066	0	0.0257	0.0115	0.0893	0.0314	0.0057	0	↑
HB	High-branching glycans	-0.0282	0.0068	0.0002	-0.0247	0.0103	0.0717	-0.0272	0.0056	0	↓
S0	Neutral (not sialylated) glycans	0.0086	0.0071	0.2822	0.0097	0.0108	0.4972	0.0089	0.0059	0.1654	↔
S1	Monosialylated glycans	0.0286	0.0063	0.0001	0.016	0.0076	0.1004	0.0235	0.0049	0	↑
S2	Disialylated glycans	0.0029	0.0083	0.7241	-0.0015	0.0102	0.8814	0.0012	0.0064	0.8576	↔
S3	Trisialylated glycans	-0.0315	0.007	0.0001	-0.0258	0.0094	0.0392	-0.0295	0.0056	0	↓
S4	Tetrasialylated glycans	-0.0302	0.0081	0.0007	-0.0252	0.0139	0.1406	-0.0289	0.007	0.0001	↓
G0	Agalactosylated glycans	0.0023	0.0056	0.7218	0.0039	0.0091	0.7168	0.0028	0.0048	0.6004	↔
G1	Monogalactosylated glycans	0.0081	0.007	0.2887	0.0069	0.0103	0.5747	0.0077	0.0058	0.2116	↔
G2	Digalactosylated glycans	0.0222	0.0072	0.0035	0.0124	0.0078	0.1814	0.0177	0.0053	0.0012	↑
G3	Trigalactosylated glycans	-0.0265	0.0064	0.0002	-0.0246	0.009	0.0392	-0.0259	0.0052	0	↓
G4	Tetragalactosylated glycans	-0.0293	0.008	0.0008	-0.025	0.0141	0.1406	-0.0283	0.007	0.0001	↓
OM	Oligomannose glycans	0.0282	0.0075	0.0007	0.0215	0.0121	0.1406	0.0263	0.0064	0.0001	↑
B	Bisection (Glycans with bisecting GlcNAc)	0.02	0.0048	0.0002	0.0124	0.0098	0.3078	0.0186	0.0043	0	↑
CF	Core fucosylation	0.0087	0.0062	0.21	0.0083	0.0106	0.5353	0.0086	0.0053	0.1404	↔
AF	Antennary fucosylation	-0.014	0.0042	0.0019	-0.0242	0.0084	0.0392	-0.0161	0.0037	0	↓

Table 1. Changes in serum N-glycosylation traits across different time points by linear mixed modelling for individual cohorts and then combined using inverse-variance weighted meta-analysis (R package metaphor). SE = standard error. Upward arrow = increase, downward arrow = decrease, left/right arrow = no significant change

	Discovery Cohort - NCT02254811			Replication Cohort - NCT01398969		
	Capsule (n = 42)	Colonoscopy (n = 33)	P value	Fresh (n = 23)	Frozen (n = 32)	P value
Age, mean (SD)	59.3 (17.8)	55.6 (19.9)	0.874	75.7 (11.0)	71.9 (15.7)	0.2872
Females, n (%)	32 (76.2%)	18 (54.5%)	0.048	12 (52.2%)	17 (53.1%)	1
Charlson comorbidity index, median (Q1-Q3)	3 (2 - 5)	3 (0 - 4)	0.28			
Immunosuppressed patients, n (%)	6 (14.3%)	3 (9.1%)	0.492	6 (26.0%)	3 (9.4%)	0.1995
Use of immune modulator*, n (%)						
Corticosteroid	3 (7.1%)	2 (6.1%)	0.852			
Immunosuppressant	5 (11.9%)	1 (3.0%)	0.16	6 (26.0%)	6 (18.8%)	0.7498
Biologic	1 (2.4%)	2 (6.1%)	0.704	14 (60.9%)	14 (43.8)	0.3274
Body mass index (BMI), mean (SD)	25.4 (5.6)	25.9 (54.7)	0.566			
Inpatient status at screening, n (%)	4 (9.5%)	1 (3.0%)	0.263	16 (69.6%)	12 (37.5%)	0.03818
PPI use prior to FMT, n (%)	6 (14.3%)	3 (9.1%)	0.492	12 (52.2%)	12 (37.5%)	0.4198
Number of RCDI episodes prior to FMT, median	4 (3 - 4)	4 (3 - 5)	0.991	2 (2-3)	2 (2-3)	0.1077
Duration of RCDI prior to FMT (days), median	73 (49-97)	70 (52-122)	0.675			
Number of CDI related hospital admissions, median	0 (0 - 1)	0 (0 - 0)	0.053			
IBD, n (%)						
Ulcerative colitis	4 (9.5%)	3 (9.0%)	0.927	0 (0%)	1 (3%)	1
Crohn's disease	2 (4.8%)	1 (3.0%)		2 (8.7%)	2 (6.3%)	
Hemoglobin (g/dL), median	137 (129 - 145)	137 (128 - 145)	0.874			
WBC (10 <sup>9</sup> /L), median	7.7 (6.3 - 8.6)	6.5 (4.9 - 7.5)	0.007	12.4 (9.75-19.70)	9.90 (7.55-14.25)	0.09444
Albumin (g/L), median	40 (37 - 43)	39 (37 - 42)	0.312	31 (26.5-33.0)	32.50 (26.75-36.25)	0.194
CRP (mg/L), median	2.1 (1 - 4.4)	3.2 (1.1 - 11.2)	0.098			
Creatinine (mg/dL), median	71 (62 - 78)	71 (61 - 83)	0.951	73 (66.5-103.5)	75 (64.0-130.0)	0.7136

\*Some of these patients were on different immunomodulators.

Supplementary Table 1. Participant baseline characteristics at screening for discovery and replication cohorts.

IgG glycan traits	Description	Discovery Cohort - NCT02254811			Replication Cohort - NCT01398969			Meta-analysis			Direction of change
		Time effect	Time SE	Adj. p value	Time effect	Time SE	Adj. p value	Meta effect	SE	Adj. p value	
IgG1_H3N4F1	IgG1 glycopeptide with agalactosylated glycan	-0.008	0.004	0.2023	-0.0038	0.0079	0.6878	-0.0070	0.0032	0.1625	↔
IgG1_H4N4F1	IgG1 glycopeptide with monogalactosylated glycan	-0.001	0.003	0.7870	-0.0176	0.0100	0.5085	-0.0025	0.0027	0.6207	↔
IgG1_H5N4F1	IgG1 glycopeptide with digalactosylated glycan	0.001	0.003	0.7870	-0.0048	0.0063	0.6438	-0.0003	0.0028	0.9695	↔
IgG1_H5N4F1S1	IgG1 glycopeptide with digalactosylated and monosialylated glycan	0.003	0.004	0.6802	-0.0105	0.0068	0.5104	-0.0001	0.0033	0.9695	↔
IgG23_H3N4F1	IgG2&3 glycopeptides with agalactosylated glycan	-0.009	0.004	0.1729	-0.0064	0.0073	0.6438	-0.0085	0.0033	0.1100	↔
IgG23_H4N4F1	IgG2&3 glycopeptides with monogalactosylated glycan	-0.001	0.004	0.7870	-0.0082	0.0095	0.6438	-0.0024	0.0038	0.6357	↔
IgG23_H5N4F1	IgG2&3 glycopeptides with digalactosylated glycan	0.005	0.003	0.3723	0.0065	0.0050	0.5796	0.0052	0.0027	0.1677	↔
IgG23_H5N4F1S1	IgG2&3 glycopeptides with digalactosylated and monosialylated glycan	0.004	0.004	0.5708	-0.0074	0.0100	0.6438	0.0027	0.0036	0.6207	↔
IgG4_H3N4F1	IgG4 glycopeptide with agalactosylated glycan	-0.009	0.005	0.2155	-0.0063	0.0091	0.6438	-0.0087	0.0043	0.1625	↔
IgG4_H4N4F1	IgG4 glycopeptides with monogalactosylated glycan	-0.001	0.003	0.7870	-0.0145	0.0078	0.5085	-0.0022	0.0025	0.6207	↔
IgG4_H5N4F1	IgG4 glycopeptide with digalactosylated glycan	0.002	0.003	0.6802	0.0014	0.0083	0.8674	0.0022	0.0029	0.6207	↔
IgG4_H5N4F1S1	IgG4 glycopeptide with digalactosylated and monosialylated glycan	0.008	0.005	0.3723	-0.0068	0.0109	0.6438	0.0049	0.0047	0.6207	↔

Supplementary Table 2. Changes in the most abundant IgG Fc N-glycopeptides across different subclasses and time points (IgG1, IgG2/3 and IgG4) by linear mixed modelling for individual cohorts and then combined using inverse-variance weighted meta-analysis (R package metaphor). SE = standard error



## Supplementary Methods

### Patient clinical data, sample collection and storage

Participants with recurrent CDI in the capsule versus colonoscopy (NCT02254811; n = 75 of 116)<sup>1</sup> and fresh versus frozen enema-delivered (NCT01398969; n= 55 of 178)<sup>2</sup> FMT trials representing the discovery and replication cohorts respectively, were included in this study. Sera was separated from venous blood samples following centrifugation at 2200 g for 10 minutes at room temperature. Serum aliquots were stored at -80°C until ready for use. All archiving of sera was undertaken using standard operating protocols in the receiving centres which included labelling each sample with a study number and date of collection. Only serum samples with sufficient volume were selected for glycome profiling. For the derivation cohort, serum samples were collected over 26 months between October 2014 and December 2016 and stored at -80°C in the biobank at the University of Alberta. The mean storage time before testing was 820.99 days (SD 180.78). Of these 75 patients, 227 archived serum samples were available at screening, 4 and 12 weeks post-FMT and one case at two time points for capsule and colonoscopy for total serum and IgG Fc *N*-glycome profiling. For the validation cohort, serum samples were collected over 26 months between July 2012 and September 2014 at one time point following fresh or frozen FMT [median 31 days, (range 7-277 days)]. The mean storage time before sample testing was 1869 days (SD 233.57).

Immunosuppression was defined as those on prednisolone (>5 mg/d), immunomodulators (azathioprine, methotrexate, calcineurin inhibitor) or biologics.

Recurrent CDI cases were defined as having at least 2 episodes of CDI (NCT02254811) or at least 1 episode of CDI (NCT01398969) following an initial infection. Clinical and demographic information was collected from medical records. Participant baseline characteristics for both cohorts are shown in Supplementary Tables 1. Informed written consent was obtained from all participants, and ethical approval was provided by the Ethics

Review Boards of the University of Alberta (Pro 1994 and 49006), and St Joseph's Healthcare (#11-3622), Hamilton Health Sciences (#12-505).

## **Glycome analysis**

### **Experimental design**

Participant serum samples and in-house serum standards were thawed, vortexed and centrifuged for 3 minutes at 12 100 *g*. Each sample (100  $\mu$ L) was aliquoted to 2 mL 96-well collection plates (Waters, Milford, MA, USA) following a predetermined experimental design which included blocking of all known sources of variation (age, sex, time point, hospital) and sample randomization between the batches to reduce experimental error. In-house serum standards were aliquoted in seven to eight replicates per plate, to evaluate experimental error and integrity of generated data. An aliquot (10  $\mu$ L) of each sample was transferred to 1 mL 96-well collection plates (Waters, Milford, MA, USA) for *N*-glycome analysis and the rest was used for isolation of IgG followed by IgG Fc *N*-glycopeptide analysis.

### **Serum *N*-glycome analysis**

Serum *N*-glycans were enzymatically released from proteins by PNGase F, fluorescently labelled with 2-aminobenzamide and cleaned-up from the excess of reagents by hydrophilic interaction liquid chromatography solid phase extraction (HILIC-SPE), as previously described.<sup>3</sup> Fluorescently labelled and purified *N*-glycans were separated by HILIC on a Waters BEH Glycan chromatography column, 150  $\times$  2.1 mm i.d., 1.7  $\mu$ m BEH particles, installed on an Acquity ultra-performance liquid chromatography (UPLC) H-class system (Waters), consisting of a quaternary solvent manager, sample manager and a fluorescence detector set with excitation and emission wavelengths of 250 nm and 428 nm, respectively. Obtained chromatograms were separated into 39 peaks. The amount of *N*-glycans in each chromatographic peak was expressed as a percentage of total integrated area. From 39

directly measured glycan peaks, we calculated 12 derived traits which average particular glycosylation traits such as galactosylation, sialylation and branching across different individual glycan structures and are, consequently, more closely related to individual enzymatic activities and underlying genetic polymorphisms. Derived traits used were the proportion of low branching (LB) and high branching (HB) glycans, the proportion of a-, mono-, di-, tri- and tetra-galactosylated glycans (G0, G1, G2, G3 and G4, respectively), and a-, mono-, di-, tri- and tetra-sialylated glycans (S0, S1, S2, S3 and S4, respectively).

### **IgG Fc N-glycopeptides analysis**

Sample preparation and analysis of IgG *N*-glycopeptides was done using a previously described protocol with minor changes.<sup>4</sup> Briefly, IgG was isolated from 90 µL of serum samples by affinity chromatography using CIM<sup>®</sup> 96-well Protein G monolithic plate (BIA Separations, Ajdovščina, Slovenia). IgG *N*-glycopeptides were prepared by trypsin digestion of an aliquot of IgG isolates (25 µg on average per sample) followed by reverse-phase solid phase extraction (RP-SPE). Purified tryptic IgG *N*-glycopeptides were separated and measured on nanoAcquity chromatographic system (Waters) coupled to Compact Q-TOF mass spectrometer (Bruker, Bremen, Germany), equipped with Apollo II source and operated under HyStar software version 3.2.

The first four isotopic peaks of doubly and triply charged signals, belonging to the same glycopeptide species, were summed together, resulting in 20 Fc *N*-glycopeptides per IgG subclass. Predominant allotype variant of IgG3 tryptic peptide carrying *N*-glycans in Caucasian population has the same amino acid sequence as IgG2.<sup>5</sup> Therefore, IgG glycopeptides were separated into three chromatographic peaks labeled IgG1, IgG2/3 and IgG4. Signals of interest were normalised to the total area of each IgG subclass.

## Statistical analysis

All statistical analyses were performed in SPSS v.24 and R 3.5.1. Descriptive statistics for patient characteristics at baseline were reported using mean and standard deviation, median and interquartile ranges and percentages. Prior to analyses, glycan variables were all transformed to standard normal distribution (mean = 0, sd = 1) by inverse transformation of ranks to Normality (R package "GenABEL", function `rn transform`). Using rank transformed variables in analyses makes estimated effects of different glycans in different cohorts comparable as transformed glycan variables having the same standardized variance. Association analyses between *N*-glycome changes (through time) and clinical variables of interest were performed using a linear mixed model. Analyses were first performed for each cohort separately and then combined using inverse-variance weighted meta-analysis approach (R package `metafor`). False discovery rate was controlled using Benjamini-Hochberg procedure.

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