U.PORTO

TESE DE DOUTORAMENTO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO EM
PATOLOGIA E GENÉTICA MOLECULAR

The role of protein glycosylation in the pathogenesis of IBD: clinical applications

Márcia Micaela Santos Pereira

D
2020

U.PORTO

Márcia Micaela Santos Pereira. The role of protein glycosylation in the pathogenesis of IBD: clinical applications.

D.ICBAS **2020**

The role of protein glycosylation in the pathogenesis of IBD: clinical applications.
Márcia Micaela Santos Pereira

SEDE ADMINISTRATIVA INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
FACULDADE DE MEDICINA

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

The role of protein glycosylation in the pathogenesis of IBD: clinical applications

Márcia Micaela Santos Pereira

TESE DE DOUTORAMENTO APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO EM PATOLOGIA E GENÉTICA MOLECULAR

Márcia Micaela Santos Pereira

The role of protein glycosylation in the pathogenesis of IBD: clinical applications

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular; Programa Doutoral da Universidade do Porto e submetida ao Instituto de Ciências Biomédicas de Abel Salazar e Faculdade de Farmácia

Orientador – Professora Doutora Salomé Soares de Pinho

Categoria – Professora Afiliada

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Professora Doutora Séverine Alice Robert Andrée Vermeire

Categoria – Full professor

Afiliação – Department of Internal Medicine, University of Leuven

Co-orientador – Professor Doutor Ricardo Jorge Marcos Pinto

Categoria – Professor Auxiliar Convidado

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Funding

The work presented within this thesis was financially supported by the PhD Fellowship (SFRH/BD/110148/2015) provided by the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education.

“It always seems impossible until it’s done.”

– Nelson Mandela

“How wonderful it is that nobody need wait a single moment before starting to improve the world.”

– Anne Frank

Declaration

The author of this thesis declares that, in accordance with number 2, article 31 of "Decreto-lei nº 178/2009", provided a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted articles and submitted or in preparation articles:

Scientific publications:

Original articles

- **Pereira MS**, Petralia F, Štambuk, Novokmet M, Vučković F, Trbojevic-Akmacic I, Maia L, Pinto V, Alves I, Marcos-Pinto R, Colombel JF, Lago P, Lauc G, Pinho SS**. Glycosylation of IgG as a blood biomarker to predict immunogenicity to anti-TNF in Crohn´s Disease patients.** (*Under submission*).

- **Pereira MS,** Durães C, Catarino TA, Costa JL, Cleynen I, Novokmet M, Krištić J, Štambuk J, Nádia Conceição-Neto, Machado JC, Marcos-Pinto R, Magro F, Vermeire S, Lauc G, Lago P, Pinho SS. **Genetic variants of the *MGAT5* gene are functionally implicated in the modulation of T cells glycosylation and serum IgG glycome composition in Ulcerative Colitis.** *Clinical and Translational Gastroenterology*, 2020 Apr 22; 11(4):e00166. 10.14309/ctg.0000000000000166.

- **Pereira MS,** Maia L, Azevedo LF, Campos S, Carvalho S, Dias AM, Albergaria A, Lima J, Marcos-Pinto R, Lago P, Pinho SS. **A (glyco)biomarker that predicts failure to standard therapy in Ulcerative Colitis patients.** *Journal of Crohn's and Colitis*, 2019 Jan 1; 13(1):39-49. doi: 10.1093/ecco-jcc/jjy139.

Reviews

- **Pereira MS*, Alves I*,** Vicente M, Campar A, Silva MC, Padrão NA, Pinto V, Fernandes Â, Dias AM, Pinho SS. **Glycans as key checkpoints of T cell activity and function.** *Frontiers Immunology*, 2018 Nov 27;9:2754. doi: 10.3389/fimmu.2018.02754. *These authors contributed equally to this work.

Acknowledgements

Uma bela e intensa etapa da minha vida que só foi possível porque tinha os melhores comigo. São tantas as pessoas a quem agradeço!

À minha orientadora, Professora Doutora Salomé Pinho, muito obrigada pela excelente orientação, por todo o apoio científico e emocional. A paixão e o entusiamo que tem pela ciência é sentida por todos os que a rodeiam. Obrigada por todas as oportunidades que me proporcionou, obrigada pela confiança, obrigada pelos incentivos sempre que precisava deles, obrigada por me fazer acreditar que tudo é possível e por me fazer acreditar em mim mesma. Sou hoje uma pessoa bem mais forte e confiante, e muito se deve a si. Obrigada por todos os momentos de lazer e por fazer com que o nosso grupo seja o grupo tão unido que é.

To my co-supervisor Professor Séverine Vermeire, I want to express my gratitude for all the clinical and scientific support. I am very grateful for the opportunity to be part of your team and for hosting me so well in KU Leuven.

Ao meu co-orientador, Professor Doutor Ricardo Marcos-Pinto, por toda a contribuição clínica, por todas as sugestões e por todo o interesse dados ao longo desta etapa.

À Professora Doutora Fátima Gärtner, pela incansável disponibilidade que sempre manifestou enquanto diretora do programa doutoral. Tornou todo o processo muito mais fácil. Obrigada.

Ao Professor Doutor Celso Reis pelo apoio científico ao longo deste percurso e pela sua amabilidade.

Obrigada a todos os colaboradores do Centro Hospitalar do Porto, especialmente à Dra. Paula Lago, Dr. Luís Maia, Dr. Ricardo Marcos-Pinto e Dra. Isabel Pedroto, muito obrigada pela oportunidade e por toda a disponibilidade, interesse e motivação demonstrados. Obrigada ao Prof. Dr. Fernando Magro pela colaboração e interesse prestado ao longo destes anos. A colaboração com a clínica não poderia ter sido mais enriquecedora, sem vocês este trabalho não seria possível. Obrigada pela cedência de amostras e por todo a contribuição clínica ao longo destes anos. Foi crucial para esta tese.

To all the co-authors of the publications for their important suggestions and contributions, namely, Prof. José Carlos Machado, Prof. José Luís Costa, Telmo Catarino, Prof. Jorge

Lima, Prof, André Albergaria, Prof. Luís Azevedo, Sara Campos, Nuno Padrão, Prof. Gordan Lauc, Prof. Harry Sokol, Aonghus Lavelle, Prof. Jean-Fred Colombel, Prof. Francesca Petralia.

Um especial agradecimento à Cecília por toda a ajuda e discussão ao longo destes anos e ao Luís Maia por toda a dedicação e boa disposição.

Ao grupo mais incrível que poderia ter! Tudo se torna mais fácil quando nos rodeamos de pessoas tão boas e tão cooperativas como aquelas que me acompanharam. Sou uma sortuda! Todos acrescentam algo de bom a cada um. Obrigada por todas as discussões científicas, pelos excelentes momentos tanto no trabalho como fora dele. Inês, as palavras não são suficientes para descrever toda a gratidão que tenho por ti. Levo em mim uma amiga para a vida. As noitadas no laboratório não custam quando se tem alguém como tu por perto. A minha Dudis, que tanta luz trouxe à minha vida! Já estava na hora de nos cruzarmos! A doce Mariana que está sempre pronta a ajudar, o Manu sempre com a sua boa energia e disposição, a Ângela sempre com aquelas palavras boas de se ouvir, a Vanda a melhor lab manager de sempre, o Nuno com a sua alegria contagiante, a Ana com a sua memória extraordinária para pormenores de experiências pessoais bons de recordar, a Ana Campar com as suas excelentes intervenções, a Joana que desde que entrou se fez sentir pela excelente boa disposição, as meninas Bia e Eduarda sempre bem dispostas e sempre super prestáveis. Obrigada do fundo do coração!

To all the people from Targid group, thank you for the nice atmosphere and for making me fell at home. You are really the best! Thank you, Wiebe, Prof. Marc, Prof. Isabelle, João, Maaike and Bram for all the suggestions and support. Brecht, Manuel, Wiebe, Kathleen and Magali thank you for all the nice conversations and funny/memorable moments.

Ao grupo de Glycobiology in Cancer, por todos os excelentes momentos e por todo o apoio. Filipe obrigada pela amizade e apoio incondicionais, Dani obrigada pelas incríveis conversas que tanto reconfortam, Henrique obrigada por estares comigo lado a lado desde o início, por todas as risadas, todas as aventuras, Joana, Diana e Catarina obrigada por todos os bons momentos e boa disposição. Stefan and Meritxell, thank you for all the friendship, for the amazing and funny moments. You will be always in my heart. Tornaram todos este caminho muito mais doce.

Aos meus amigos de sempre, que são o meu pilar em todas as fases da minha vida. Que sorte tenho em vos ter! Sem vocês e sem todo o vosso ânimo e apoio

incondicionais, nada disto seria possível. Ju, mesmo longe tive-te tão perto em tantos momentos deste percurso, Je o doutoramento não seria o mesmo se não estivesses mesmo ali no piso de cima. Fafenses, Happy Family, Meninas de mestrado, Framboesas e Fotogénicos, este percurso um pouquinho de cada um de vós! Obrigada!

À Catarina por ter sido uma excelente companheira de vida durante todo o doutoramento. Crescemos juntas! O 509 vai ser sempre especial.

A ti Leandro, por teres entrado na minha vida e por teres tornado esta reta final tão especial!

Pai, mãe e Rui, sem vocês não chegaria onde cheguei. Obrigada pelo apoio incondicional e por todos os valores que me transmitem e que me seguem onde quer que esteja.

Avô, obrigada por toda essa constante boa disposição e tranquilidade que transmites. Avó, és um exemplo para mim. Sei que estás orgulhosa!

Ficam recordações que me enchem o coração.

Resumo

A doença inflamatória intestinal (DII) engloba a colite ulcerativa (UC) e doença de Crohn (CD) e consiste num distúrbio imuno-mediado crónico e recorrente do trato gastrointestinal de etiologia desconhecida. A heterogeneidade da doença tendo em conta a severidade e a eficácia terapêutica reflete a complexa interação entre a genética do hospedeiro, a resposta imune e fatores externos, como microorganismos. A glicosilação é um processo fundamental na resposta imune e a sua desregulação já foi evidenciada na DII. Um défice na *N*-glicosilação ramificada (mediada pelo glico-gene *MGAT5*) nas células T intestinais e a sua associação com a hiper- ativação das células T são evidentes em alguns pacientes com UC. Se esta deficiência pode ser usada para predizer a resposta à terapia é desconhecido (1), bem como a causa da sua desregulação (2) e a sua influência na composição do microbiota intestinal (3). Para além destes três pontos principais, nesta tese nós também explorámos os glicanos das imunoglobulinas G (IgG) circulantes como potenciais biomarcadores na resposta a terapia (4).

1) Verificámos que a expressão ramificada de *N*-glicanos na lâmina própria do cólon, avaliada próximo do diagnóstico, é capaz de distinguir os pacientes com UC que irão responder à terapia convencional daqueles que serão refratários à terapia, com uma especificidade de 75%. Baixos níveis de *N*-glicanos ramificados e altos níveis de proteína C reativa foram mostrados como sendo preditores independentes da não resposta à terapia convencional e de beneficiarem um do outro no seu desempenho preditivo. 2) Identificámos alterações genéticas no *MGAT5* que estão associadas à desregulação da expressão do glico-gene em células T do cólon e circulantes de pacientes com UC, bem como a baixos níveis de galactosilação das IgGs circulantes, frequentemente associados a um fenótipo pró-inflamatório. Estas variantes genéticas funcionais do *MGAT5* e outras de regiões reguladoras do gene foram capazes de discriminar pacientes com UC de acordo com a severidade da doença. Para além disso, 3) análises *in vivo* mostraram que a deficiência de *MGAT5* altera a composição do microbiota, que parece estar associada a uma maior suscetibilidade em desenvolver uma colite severa induzida por DSS. Por último, 4) verificámos que a glicosilação da fração Fc das IgGs em pacientes com DC parece distinta em pacientes que perdem a resposta à terapia anti-TNF daqueles que sempre respondem. Um perfil de glicosilação específico das IgGs também parece estar associado ao desenvolvimento de imunogenicidade contra a terapia anti-TNF.

Em conclusão, a desregulação do *MGAT5* e dos *N*-glicanos ramificados que medeia observados em contexto de DII, são uma ferramenta importante a ser usada o mais

perto do diagnóstico possível para identificar pacientes que irão desenvolver uma doença mais severa, provavelmente devido à sua influência na resposta imune e na composição do microbiota. Nesta tese nós propomos potenciais [glico]biomarcadores *in situ* e no sangue associados a maus resultados clínicos, permitindo terapia personalizada através de estratégias preventivas e terapêuticas.

Abstract

Inflammatory bowel disease (IBD), which encompasses ulcerative colitis (UC) and Crohn's disease (CD), is a relapsing chronic immune-mediated disorder of the gastrointestinal tract of unknown etiology. The heterogeneity of the disease regarding severity and therapeutic outcomes mirror the complex interaction between host genetics, immune response and external factors, such as microorganisms. Glycosylation is instrumental in immune response and has been shown to be dysregulated in IBD. The deficiency of branched *N*-glycosylation (mediated by *MGAT5* glycogene) on intestinal T cells and its association with hyperactivation of T cells are evident in some UC patients. Whether this deficiency might predict therapy outcome is unknown (1), as well as, its dysregulation cause (2) and its influence on microbiota composition (3). In addition to these three main points, in this thesis we also explored glycans from circulating immunoglobulins G (IgG) as potential biomarkers for therapy response (4).

1) We verified that branched *N*-glycans expression in colonic lamina propria, evaluated close to diagnosis, were able to distinguish UC patients who will response to the conventional therapy from those that will be refractory to the therapy with a specificity of 75%. Low branched *N*-glycans and high C- reactive protein were shown to act as independent predictors of non-response to standard therapy and to benefit from each other for its predictive performance. 2) We identified genetic variants on *MGAT5* that were associated with altered expression of *MGAT5* mRNA levels in colonic and circulating T cells from UC patients, as well as with low galactosylation of plasma IgGs, often associated with a pro-inflammatory phenotype. These functional genetic variants of *MGAT5* together with other variants from the regulatory regions of the gene were able to discriminate UC patients according with the disease severity. Furthermore, 3) *in vivo* analysis showed that the deficiency on *MGAT5* alters microbiota composition which seems to be associated with the higher susceptibility to develop a severe DSS-induced colitis. Lastly, 4) we verified that the glycosylation of the Fc fraction of IgG in CD patients seems distinct in patients who lost response to anti-TNF therapy from those that always respond. A specific glycosylation profile from IgG also seems to be associated with the development of immunogenicity to anti-TNF drug.

Overall, the dysregulation of *MGAT5* and its mediated branched *N*-glycans observed in IBD context, are a valuable tool to be used early in diagnosis to identify patients that will display a severe disease probably due to its implications on immune response and microbiota composition. In this thesis we propose *in situ* and blood potential [glyco]biormarkers associated with poor clinical outcomes, enabling a personalized medicine through optimized preventive and therapeutic strategies.

Abbreviations

3’UTR – 3 prime untranslated region

5-ASA – 5-aminosalicylates

ADA – Anti-drug antibodies

ADCC – Antibody dependent cellular cytotoxicity

ADCP – Antibody dependent cellular phagocytosis

AMP – Antimicrobial peptides

APC – Antigen presenting cells

ASCA – Anti-Saccharomyces cerevisiae antibodies

Asn – Asparagine

ATG16L1 – Autophagy related 16 like 1

BCR – B cell receptor

C. difficile – Clostridium difficile

CARD9 – Caspase recruitment domain-containing protein 9

CD – cluster of differentiation

CD – Crohn’s disease

CDC – Complement dependent cytotoxicity

CDH1 – Cadherin-1

CLR – C-type lectin receptor

CRP – C reactive protein

CTLA-4 – Cytotoxic T-lymphocyte–associated antigen 4

DC – Dendritic cells

DC-SIGN – Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DNA – Deoxyribonucleic acid

Dol-P – Dolichol phosphate

DSS – Dextran sulfate sodium

E.Coli – Escherichia coli

ER – Endoplasmic reticulum

Fab – Antigen-binding fragment

Fc – Fragment crystallizable

FcγR – Fc gamma receptor

FMT – Fecal microbiota transplantation

FUT1 – α1,6 fucosyltransferase

FUT2 – α1,2 fucosyltransferase

FUT8 – α1,6 fucosyltransferase

Gal – Galectin

Gal-T – Galactosyltransferase

GBP – Glycan binding proteins

Glc – Glucose

GlcNAc – N-acetylglucosamine

GlycA – Glycoprotein acetylation

GnT – N-acetylglucosaminyltransferase

GWAS – Genome-wide association studies

HBI - Harvey-Bradshaw Index

HIV – Human immunodeficiency virus

HLA – Human leukocyte antigen

IBD - Inflammatory bowel diseases

IECs – Intestinal epithelial cells

IFN-γ – Interferon gamma

Ig – Immunoglobulin

IgG – Immunoglobulin G

IL – Interleukin

ILC – Innate lymphocyte cells

KO – Knock-out

LacNAc – N-acetyllactosamine

L-PHA – Phaseolus Vulgaris Leucoagglutinin

LPS – Lipopolysaccharides

mAb – Monoclonal antibody

Man – Mannose

MAN1A1 – alpha-mannosidase IA

MAN1B1 – alpha-mannosidase IB

MAN2A1 – alpha-mannosidase IIA

MAN2B1 – alpha-mannosidase IIB

MayoE – Mayo endoscopic subscore

MBP – Mannose-binding lectin

MGAT – Mannosidase acetylglucosaminyltransferase

MHC – Major histocompatibility complex

miRNA – micro ribonucleic acid

MLN – Mesenteric lymph nodes

mTNF – Transmembrane tumor necrosis factor alpha

MUC – mucin

NFκB – Nuclear factor κB

NK – Natural killer

NOD2 – Nucleotide-binding oligomerization domain containing 2

OST – Oligosaccharyltransferase

pANCA – perinuclear anti-neutrophil cytoplasmic antibodies

PD-1 – Programmed cell death protein 1

PD-L1 – Programmed cell death-ligand 1

pLacNAc - Poly-N-acetyllactosamine

PNR – Primary non-responders

PSA – Polysaccharides-A

RNA – Ribonucleic acid

SCFA – Short-chain fatty acids

Ser – Serine

SIGNR3 – Specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3

SNP – Single nucleotide polymorphisms

SNR – Secondary non-responders

STAT – Signal transducer and activator of transcription

TCR – T cell receptor

TDM – Therapy drug monitoring

Th – T helper cells

Thr – Threonine

TNF-α – Tumor necrosis factor alpha

TNFR – Tumor necrosis factor receptor

Treg – T regulatory cells

UC – Ulcerative colitis

UDP – Uridine diphosphate

VEO – Very early onset

WT – Wild type

Table of Contents

Chapter I

General Introduction

1. Inflammatory bowel disease

1.1. Epidemiology

Inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and Ulcerative colitis (UC), is a multifactorial disorder characterized by a chronic and relapsing inflammation of the gastrointestinal tract (7, 8). The prevalence of IBD is increasing worldwide, due to the accelerating incidence in newly industrialized countries (such as South America, eastern Europe, Asia, and Africa), which turns IBD a global disease in 21st century (Figure 1) (2, 9, 10). The incidence in adult life of IBD patients is stabilizing in western countries (9), but the incidence in the pediatric-onset IBD continue to increase in certain regions (11-13), indicating its emergence as a global disease. The highest peak of prevalence is observed in these countries, affecting approximately 2.5 million people in Europe and 1.5 million people in USA (9, 14). The highest prevalence for UC occurs in northern Europe (505 per 100,000 persons) and for CD in North America (412 per 100,000 persons) (9). Portugal presents an intermediate prevalence comparing with global scenario (2, 9), which increased from 86 to 146 patients per 100 000 in 2003 to 2007 (15).

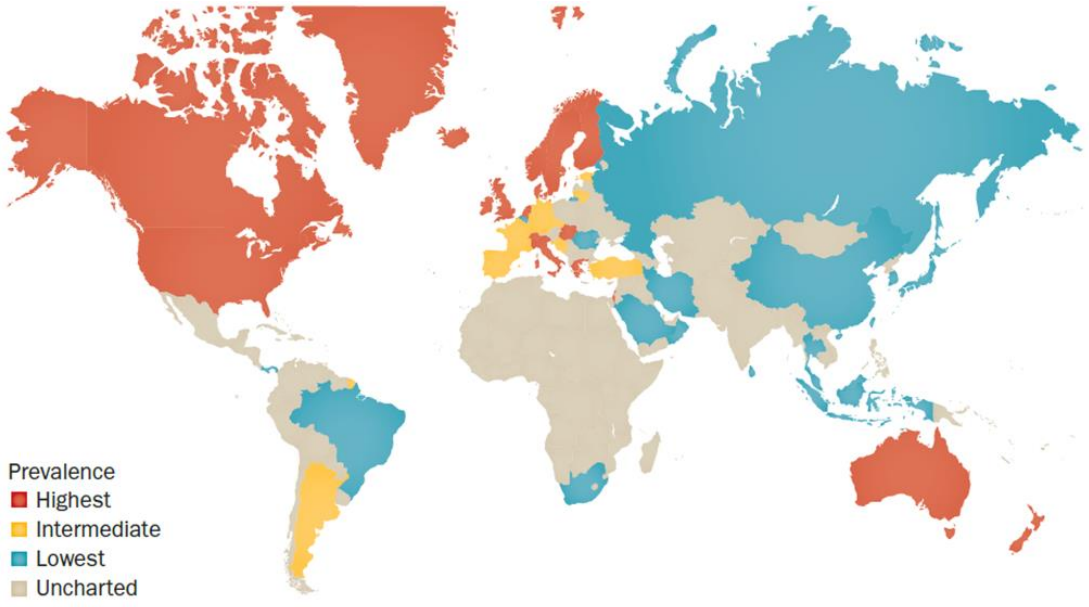


***Figure 1**_Global prevalence of Inflammatory bowel disease. Adapted from (2).*

The incidence of IBD varies with age and gender. IBD can affect individuals from infants to the elderly, but the peak of onset is between 15 and 30 years old (16, 17). A second but smaller peak is also observed between 50 and 70 years (16, 18). Overall, CD patients display an age at diagnosis of 5-10 years earlier than UC patients (19) and around

25% of all IBD are diagnosed before 18 years old (17, 20). Female display lower risk than male to develop CD during childhood, but higher risk later in life (25-29 years and older than 35 years). While in UC patients, the gender-ratio of UC incidence is mainly different after 45 years old, with higher male predominance (21).

1.2. Diagnosis and prognosis

The diagnosis of UC and CD is mainly based on clinical, endoscopic, histologic and radiologic features (16, 17), that in some cases are not enough for a distinct diagnosis. Clinical symptoms such as bloody diarrhea, chronical abdominal pain and weigh loss are suggestive of IBD. Moreover, first-degree relatives of patients with IBD increases 10-15-fold risk for IBD (16, 17). Generally, while UC display a continuous and mucosal confined inflammation, which can extend from the rectum (proctitis) to the left side of the colon (left-sided colitis) or to the entire colon (pancolitis), CD inflammation is transmural and might affect other regions of the intestine in addition to colon and ileum, often discontinuously (16, 17). Fistulas, fissures and abscesses are common complications of CD but not of UC.

The management of IBD is based on disease activity, in which the main goal is to improve patient's quality life through the elimination of symptoms. In UC, the Mayo clinical score is widely used to score disease activity. This index is based on the combination of clinical symptoms (stool frequency, rectal bleeding and physician's global assessment) and endoscopic observations (vascular pattern, friability, bleeding and ulcers). Each clinical parameter and also the Mayo endoscopic subscore (22) are rated from zero to three, classifying the disease as normal or inactive (Mayo 0), mild (Mayo 1), moderate (Mayo 2) or severe (Mayo 3) (23). In contrast to UC, the assessment of disease activity in CD is exclusively based on clinical parameters, the Harvey-Bradshaw Index (HBI) (24), which includes patients well-being, abdominal pain, stool frequency and complications. However, absence of clinical symptoms might not guarantee endoscopic and histological remission, and vice versa (25, 26). The persistence of a degree of subclinical inflammation in mucosa of the gut might shorten the periods between remission and relapse, thus increasing IBD burden. This issue is of major concern in the clinical management, once might be associated with the risk of IBD patients to develop colorectal carcinoma. Patients with higher disease extension and disease duration display the highest risk (23, 27).

Physicians are challenged daily to define different aspects of the IBD such as diagnosis, prognosis, determine disease activity, severity and the therapeutic outcome. Serological antibodies such as autoantibodies and anti-microbial glycans or microbial antigens are used to distinguish UC and CD. For instance, the anti-neutrophil cytoplasmic

antibodies (ANCAs; autoantibody), more specifically the atypical perinuclear ANCA (pANCA), despite the low sensitivity for IBD diagnosis, discriminates UC from CD with a relative high performance (52% sensitivity and 91% specificity). On the other hand, the anti-glycan antibodies against *Saccharomyces cerevisiae* cell wall mannan (ASCA) are used as a clinical diagnostic marker of CD (28, 29) and combined with pANCA distinguish both disease subtypes with a specificity of approximately 90% (30). Antibodies against microbial components were also shown to be helpful in the discrimination of the patients regarding their disease severity (31, 32).

C reactive protein (CRP), produced in response to acute and chronic inflammation by hepatocytes (33), is one of the most commonly non-invasive biomarker used in clinical practice, being helpful in IBD diagnosis (without distinguishing the two types) and in the determination of disease activity (34), severity (34) and therapy response (35, 36). The sensitivity of CRP is relatively high (approximately 80% for CD *vs* 50% for UC) (37, 38); however, the specificity is far from being the ideal since it detects general inflammation (in response to circulating IL6 and to a lesser extent to IL1β and TNF-α), which could be promoted by other diseases, thus not reflecting specifically the endoscopic inflammation (35). Despite the low sensitivity in UC, CRP levels at diagnosis are proportional to disease extension and elevated levels of CRP in patients with extensive colitis predicts the need of surgery as well (37). Fecal markers as calprotectin and lactoferrin overcome the specificity issue of CRP. The quantification of calprotectin (a calcium- and zinc- binding protein) and lactoferrin (iron binding protein) reflect the neutrophil migration in the colon, being thus highly sensitive (89-98%) and specific (81-91%) in the diagnosis of IBD. These fecal markers are also helpful in the prediction of IBD patient clinical relapse (39, 40), endoscopic activity (41) and therapy response (42, 43), displaying a better overall performance than CRP (44-46).

Clinicians have been taking into consideration some clinical/pathological parameters associated with disease progression as a guidance of therapy decision-making: younger age of onset (less than 40 years old) and extensive disease in both IBD subtypes; deep ulceration and perianal disease in CD; family history in UC (36). These factors are associated with the development of severe/complicated disease courses, in which are included hospitalizations, 12 months of disabling symptoms, IBD-related surgeries, multiple corticosteroid courses and need for immunosuppressive or escalation to biologic therapy (36). However, the early identification of the patients with high risk to develop a severe/complicated disease remains a challenge for clinicians.

1.3. Current therapeutic approaches

IBD remains an uncurable disease. The main goal of the current therapy is to increase patients' quality of life, which is affected by the chronic pain and fatigue associated with inflammatory symptoms, reduces their ability to work, increases social stigma and restricts career choices (47, 48). The development of new and more effective drugs allowed the achievement of long-term remissions, evolving from the clinical improvement to mucosal healing (the so-called "deep remission") (49, 50). This led to better outcomes by minimizing disease progression and complications as the need of surgery, hospitalizations and corticosteroid use (51-53).

Despite some patients only achieve or maintain remission with the more recent and potent drugs, others can control disease with the so-called conventional therapy, that are cheaper and display less side-effects. The lack of molecular markers able to discriminate patients accordingly to their therapeutic needs, lead to the use of step-up treatment approach (Figure 2).

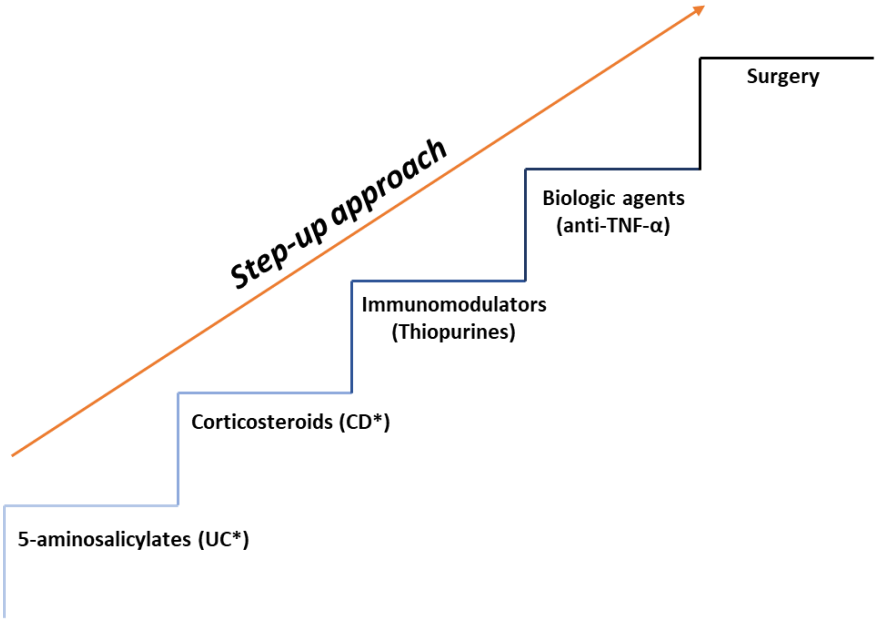


Figure 2_*Common step-up therapeutic approach used in ulcerative colitis and Crohn's patients. *first line therapy.*

5-aminosalicylates (5-ASA) are the first line therapy, mainly for mild-to-moderate left-sided UC patients, which was shown to induce and maintain remission (54), being however not effective in CD (44, 55). The molecular mechanism underlying its anti-inflammatory effect is not completely clear. Evidences suggest that 5-ASA act as scavenger of reactive oxygen species that might decrease the production of prostaglandin and leukotriene and inhibit leukocyte chemotaxis (56, 57), being also demonstrated to be a peroxisome proliferative activated receptor-γ agonist that might affect nuclear factor κB (NFκB) activity (57, 58).

Around 35% and 52% of UC patients fail to reach or maintain remission with 5-ASA, respectively (59). Corticosteroids and immunomodulators (thiopurine drugs such as the prodrug azathioprine that possess a cytotoxic effect through its active metabolite mercaptopurine) are used as alternative or first choice depending on disease subtype and severity (44, 54). Despite the proven efficacy of these drugs, some patients remain refractory (44, 54). It would be crucial to identify early at diagnosis the patients that will not response to conventional therapy to avoid this step-up approach and guide them early to the most appropriate therapy.

1.3.1. Biologics

Since the successful treatment of CD patients with the monoclonal antibody (mAb) against TNF-α Infliximab in 1993 (60), and the transversal efficacy to severe UC patients (61), TNF-α was considered a major pathological cytokine in the disease.

TNF-α is presented in a transmembrane form (mTNF) and in a soluble form (when cleaved) and exert its function through the interaction with its receptors (TNFRI and TNFRII). In addition to Infliximab, that is a mouse-human chimera mAb, other human anti-TNF mAb were developed, as Adalimumab and Golimumab (62). Infliximab and Adalimumab are the most used biologics in IBD treatment. The introduction of biologic agents as a new therapy choice significantly improved patients' quality life and decreased the number of surgeries. In fact, the use of biologic agents demonstrated a high efficacy in the induction and maintenance of clinical remission for IBD patients who failed to standard therapies (61, 63-66). However, despite the efficacy of anti-TNF, there are up to 30% of IBD patients that do not respond to this therapeutic class since the beginning (primary non-responders - PNR) and up to 50% display a transient efficacy of the drug, losing the response during time (secondary non-responders - SNR) (67, 68).

To overcome this problem, alternative therapies targeting other molecules have been developed and approved, including integrins mAb (natalizumab and vedolizumab) and anti- IL12/IL23 (ustekinumab), that were demonstrated to be effective in the control of IBD immune response (69-71). Thus, the therapeutic strategy for PNR is to swap therapy to other drug class (as anti-integrin e.g.), while the SNR usually combine therapy with immunosuppressants, alter dose and time interval of the drug or switch for a biologic drug within the same drug class (67, 72, 73).

The effectiveness of the biologics can be monitored not only based in clinical parameters, but also through the measurement of drug levels (which can involve clinical decision regarding dose intensification) and anti-drug antibodies (ADA – clinical decision for switching to another anti-TNF drug; Figure 3) (73, 74). Patients with therapeutic levels of the drug without the production of ADA displays mechanistic issues, thus need to switch or swap to other biologics. The development of immunogenicity against infliximab and adalimumab, ADA production and reduced through levels of the drug, underlies another part of the secondary failure of the drugs (73, 74). Therapy drug monitoring (TDM) by measuring drug and ADA levels has been shown to be useful in clinical decision (73), however its use in IBD is still debatable (75).

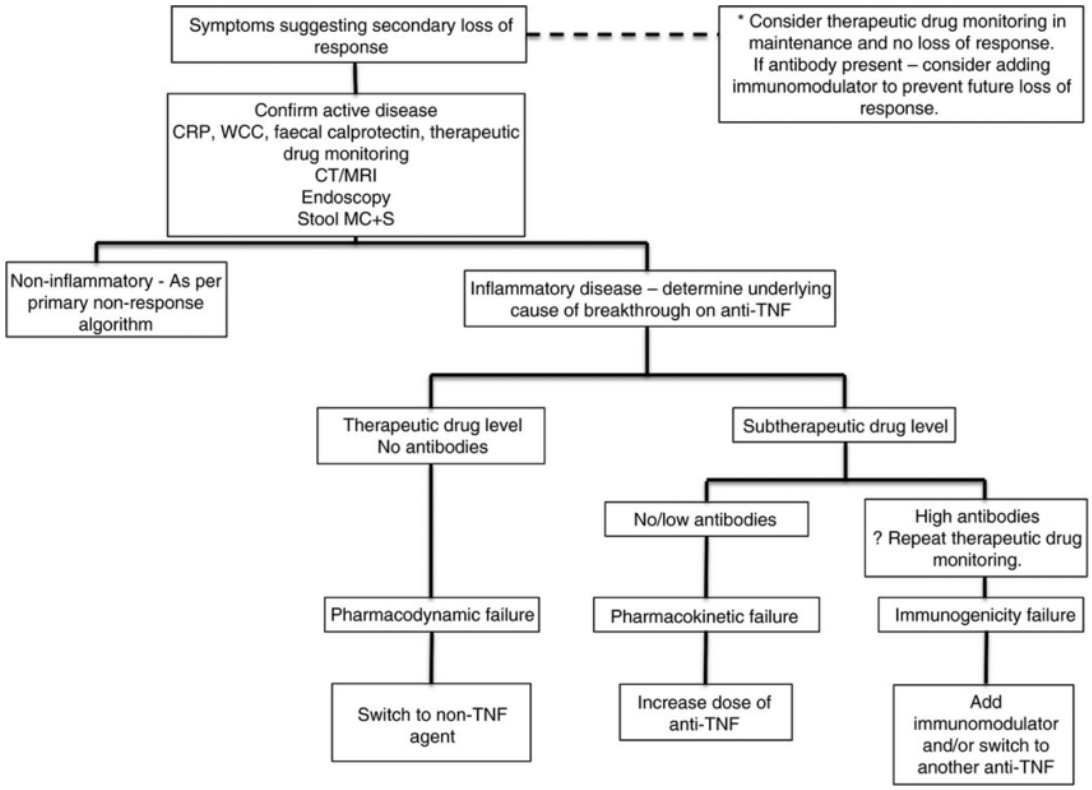


Figure 3_ *Proposed management algorithm for secondary loss of response to anti-TNF therapy in IBD. (6)*

There is a pressing need in the field to identify reliable and minimally invasive biomarkers to stratify those patients that will fail to respond to anti-TNF therapies, even for mechanistic issues or for the development of immunogenicity and elect them, early in disease course, to other alternative therapies. This tailored approach will improve clinical remission, avoiding risk of relapse and side effects as a cost-effective approach that will improve the long time course of the disease and patients‘ quality of life.

1.4. Genetics

The pathogenesis of IBD is unknown, but evidences suggest that IBD results from the genetic susceptibility of an individual to environmental exposures, which leads to the initiation and propagation of an abnormal immune response and microbiota unbalance (76, 77). In fact, the genetic burden in IBD is reported in familial and twin studies. Familial clustering of IBD has been demonstrated in 5% to 16% of the patients diagnosed with IBD (78) and studies in identical twins also show a significant concordance in the development of the disease, mainly CD (20-55% of CD *vs* 6.3-17% of UC) (79). This highlights the influence of environmental factors that includes tabaco, diet and use of antibiotics as well as microbiota (79-81) in the risk to develop IBD.

Meta-analysis of genome-wide association studies (GWAS) together with an ImmunoCHIP (a chip that includes around 200,000 single nucleotide polymorphisms (SNPs) relevant to multiple different immune-mediated diseases) identified SNPs in more than 200 loci associated to higher risk to develop IBD (82, 83). These risk loci are enriched in genes related with other immune-mediated disorders (84), implicated in epithelial barrier function and repair (*CDH1*), innate immunity (*NOD2*, *CARD9*), lymphocyte activation and regulation (*IL23R*, *IL10*, *STAT3*, *STAT5B*, *SP110*) and autophagy (*ATG16L1*) (82, 83). The impairment of immune system and the altered response to molecules of bacterial origin (*NOD2*) or their effective removal (ATG16L1), highlight the predisposition for host-microbe interactions as main triggers of IBD pathogenesis. However, this huge number of loci are far to explain the total disease variance in IBD risk (only 13.1% and 8.2% for CD and UC, respectively) (82). In fact, the risk loci to develop IBD may not explains the course that the disease takes over time, concerning disease relapsing, need of surgical intervention and also therapeutic outcomes. A recent study showed that loci associated with CD prognosis are independent on the loci that contributes to disease susceptibility, as in human leukocyte antigen (HLA) region (85), being this discrepancy also demonstrated in UC (86). Variants on HLA genes were also associated with therapy response, as predictors of the need of immunomodulatory therapy (87) and increased immunogenicity to the drug (88).

Each GWAS-identified risk loci are typically composed of ~250 kb that might comprise more than 50 genes or any gene (“gene desert”), depending on the loci. The large clusters of SNPs in linkage disequilibrium present in each loci hamper the identification of the causative variants and genes, making it difficult to distinguish between causal and neutral SNPs in linkage. Hundreds of variants are associated to each loci, and the majority are from non-coding regions (89). The non-coding variants are mainly regulatory variants that perturb the expression of one or more genes. A recent study showed that 63 of the 200 risk loci associated to IBD are driven by regulatory models that affect multiple genes in

multiple tissues, including causative genes (90). In fact, the power of regulatory variants is enormous, and might affect different layers of gene regulation like inception mode: SNPs on promoters, enhancers regions or in 3-prime untranslated region (3'UTR) might affect directly the transcription or post-transcription of the causal/phenotypic gene; other SNPs in the same regulatory regions might affect genes encoding proteins involved in other levels of regulation as in translational and post-translational processes, which highly increases the panel of causal/phenotypic genes. The "post-transcriptional regulome" of the 3'UTR has been shown to have implications in immune-mediated disorders through the control of immune gene expression (91). For instance, in IBD, SNP in 3'UTR from *IL23R* disrupt the microRNA (miRNA) recognition elements for specific miRNAs and leads to an increased IL23R expression (92).

1.5. Immune response

Gut homeostasis is a balance between host immunity and intestinal environmental factors (as microbiota and food antigens). Disruption of the control of this immunological balance results in abnormal stimulatory signals associated with the loss of immune tolerance, associated with IBD. Different layers of defense are stated in gut immunity, the so-called gut firewall: epithelial mucosal barrier, innate and adaptive immune response.

1.5.1. Mucosal barrier and innate immune response

The first line of defense of the gut is essentially physical and is promoted by intestinal epithelium, which prevents antigens reaching the lamina propria. To reach lamina propria, microorganisms need to pass through different layers including the outer mucus layer, composed by gel-forming mucins, and then the inner mucus layer composed by the glycocalyx (including membrane associated glycoproteins mucins) presented in the surface of intestinal epithelial cells (IECs). These layers are biochemically reinforced by the accumulation of secreted antimicrobial peptides (AMP) produced by Paneth cells (in small intestine) and other IECs (in colon) (93, 94), being also a net for IgA binding (discussed below). In IBD patients, a decreased mucus layer is reported due to decreased expression of mucins and AMPs (95). In fact, genetic variants of membrane mucins, such as MUC3*A*, are associated with higher susceptibility to IBD (89), and mice KO for the most abundant secretory mucin in the colon (MUCIN 2) spontaneously developed colitis (96). In the IEC layer, the paracellular permeability is controlled by intercellular tight junctions, as occludins and claudins (97). The expression of sealing claudins (claudin 3, 5 and 8) seems to be downregulated in IBD patients, promoting a barrier disruption (98). Dendritic cells (DC) and

macrophages are highly abundant in IBD and secret pro-inflammatory mediators as IL1, IL6, TNF-α and IFN-γ (99-102), which might induce epithelial cell apoptosis thus also weakening the IEC layer as a barrier to the intestinal mucosa (103).

Cytokines are the main drivers of inflammation and might possess pro- and anti-inflammatory properties. Gut mucosa displays more sources of cytokines, such as those produced by IECs, DC, macrophages, natural killer cells, innate lymphoid cells, T effector and regulatory (Treg) cells.

1.5.2. Adaptive response

In IBD, the disruption of mucosal barrier leads to the recruitment of immune cells in lamina propria, in order to eliminate microbes, toxin related microbes or food-derived antigens. In fact, IBD lamina propria is enriched of both innate and adaptive immune cells (104). Antigen presenting cells (APC) as DC and macrophages are the bridge from innate to adaptive immunity, sensing antigens that might be presented in epithelium and inducing differentiation of naïve T and B cells, indirectly through the secretion of cytokines and directly through antigen presentation.

1.5.2.1. T cells

Naïve T cells differentiate into effector T cells as helper T cells (Th, CD4+) or cytotoxic CD8+ T cells, depending on the inflammatory cytokine milieu (105). Mucosal CD4+ T cells are thought to play a major role in the chronic inflammation of IBD. CD is associated with a predominance of Th1 response, whose differentiation is mainly induced by IL12 (released from macrophages and DC) (106), that when blocked was demonstrated to control colitis in mice (107). On the other hand, UC exhibit a more Th2- related immune response, known to be induced by IL4. The distinction between Th1 and Th2 stands for the elevated production of IFN-γ, IL2 and TNF-α for Th1 response, while Th2 is associated with production of high amount of IL4, IL5, IL13 and IL10 (108, 109). However, the Th2 response in UC remains controversial, due to the low levels of IL13 observed in colonic mucosa from UC patients (110, 111) and the higher abundance of IL6 (112) comparing with CD.

Under inflammatory condition, Th17 cells (stimulated by TGF-β, IL6, IL21 and IL23), predominantly secrete IL17A, IL17F, IL22, IL21 and TNF-α, which are drastically increased in both UC and CD (113). The importance of Th17 in IBD was highlighted by the requirement of IL23 in the spontaneous development of colitis in IL10- deficient mice (114). The levels of colonic TNF-α, IFN-γ and IL6 are decreased in the absence of IL13 (115).

The pro-inflammatory function of Th17 might be driven by IL17 cytokines, as observed by the increased levels of IL17 cytokines in inflamed colons (116-118). IL17 might target immune and epithelial cells, inducing the recruitment of neutrophils for instance (tissue infiltration), inducing pro-inflammatory cytokines (as IL6 and TNF-α), chemokines and matrix metalloproteases (119-122). Despite the elevated homology between IL17A and IL17F (123, 124), the function of the cytokines are distinct in IBD mice models. While inhibition of IL17F had been shown to attenuate inflammatory response (125, 126) due to the increase of Treg in intestine (126), IL17A has a protective role in DSS- induced colitis (125, 127) and T cell-induced colitis (128). This protective role of IL17A collaborates in epithelial repair (129) and in the maintenance of intestinal epithelial tight junctions (130, 131).

In fact, Th17 is presented in normal intestinal lamina propria and associated with homeostasis (132). IL22 is another Th17 cytokine that also shows a protective role in colitis mice models (133-135). IL22 display a pleotropic effect on mucosa immunity, maintaining the integrity of mucosal barrier inducing pro-survival and anti-apoptotic signaling in IECs; as well as by increasing mucus and AMP production (134-136). IL22 is not exclusive from Th17 and natural killer (NK) cells. Innate lymphocyte cells (ILCs) and γδ T cells, which express the TH17-associated transcription factors retinoic-acid-receptor-related orphan receptor (ROR)γ, are also IL17 and IL22 producers (116). In fact, ILC and innate like T cells - γδ T cells (that express the heterodimer T cell receptor γδ instead of αβ) are mucosal resident cells that are shown to play a role in IBD pathogenesis (137-139). They respond to environmental stress signals and protect mucosa from pathogens. ILC protect against extracellular bacteria, helminths and virus, displaying different subsets characterized by the production of IFNγ (ILC1), IL5 and IL13 (ILC2) and IL17 and IL22 (ILC3) (137). γδ T cells are highly present in the gut and act as a pattern recognition receptor, recognizing a large variety of ligands, as bacterial phosphoantigens, nonclassical MHC-I molecules and unprocessed proteins (140). Although γδ T cells have been shown to be increased in IBD mucosa and to be considered a major source of the IFN-γ (138), it has also been shown to play an important role in gut homeostasis. This protective effect was supported by the increased susceptibility to DSS-induced colitis in γδ T-cell-deficient TCRδ(-/-) mice (141, 142). Depending on the cytokine they produce, they might be implicated either in IBD pathogenesis or function as immunoregulator, through production IFN-γ (TH1-like) or IL17 and IL22 (TH17-like), respectively.

The maintenance of homeostasis is possible through regulatory pathways that when disrupted might lead to the loss of tolerance and development of aberrant effector responses in the gut, as the case of Foxp3+ regulatory T (Treg) cells. Treg cells (induced

by TGF-β and IL2) are known to be fundamental for the maintenance of homeostasis mainly through IL10 secretion. Its differentiation from naïve T cells also occur in the gut, and studies suggest an importance of intestinal microbiota or dietary antigens in Treg activation (143, 144). In fact, IL10-deficient mice develop spontaneous colitis only in the presence of microbiota (145). Although the potent suppressive function of Tregs from mucosa of IBD patients (146, 147), the number of Treg cells in both inflamed or non-inflamed tissue is higher comparing with healthy individuals (146, 148). Since TGF-β might induce both Treg and Th17, the presence of theses T cell subsets highly depend on the cytokine milieu, which may favor one subset rather than the other.

1.5.2.2. B cells – focus on plasma cells and IgG

The host intestinal mucosa contains the largest population of plasma cells, displaying 80% of all antibody-secreting B cells (149). Plasma cells are effector B cells, known as factories of antibodies production. After the recognition of antigens through B cell receptor (BCR), B cells proliferate and became activated in a dependent or independent contact with T cells. Activated B cells undergo to class switching from the initial immunoglobulin (Ig)M and IgD of BCR to IgG, IgA and IgE, differentiating into plasma cells (150-152).

The elevated amounts of the daily secreted IgA are crucial for the gut homeostasis (153, 154), by targeting mostly microbes that drive IBD (155). The high affinity for pathogens (IgA^+) and low affinity to commensal microbes (IgA^-), highlight their importance as balancers of microbiota composition (156). The affinity of IgA depend on T cell activation in which T cell dependent induction leads to high-affinity IgA whether T cell independent manner induces low-affinity IgA (157).

Plasma cells are highly abundant in lamina propria from IBD patients (158, 159), as well as the serum IgG against gut microbiota and autoantigens, as mentioned in section of *"Diagnosis and Prognosis"*. IgG is the most abundant Ig in the serum and is responsible for pathogens and toxin antigen neutralization and elimination through the induction of an effector immune response (160). In fact, IgG-coated fecal bacteria have been shown to be elevated in IBD patients (161-163), being enriched of IgGs that bind mostly pathobionts (potentially pathogenic microbes) (162, 164). Both IgA and IgG-coated bacteria and the soluble form of the IgGs are increased in IBD and associated with disease activity (165). Some studies support that the pronounced humoral immune response in IBD is mainly driven by mucosal IgG+ plasma cells and in and in less extent IgA (166, 167), as confirmed in colitis-induced in mice (168). CD and UC patients are distinct concerning humoral

response, in which UC usually display higher mucosal IgG+ plasma cells and serum IgG (169) than CD, being in fact the plasmocytic infiltration a histological marker of UC (170, 171).

IgG accounts for 75% of total Igs in the serum and around 20% of all plasma protein (160). IgG are subdivided into four subclasses: IgG1, IgG2, IgG3 and IgG4. IgG1 is the most abundant displaying the highest affinity and being largely generated in a T cell dependent manner. This Ig is used in the generation of therapeutic drugs (160, 172). IgG2 is mainly involved in bacterial capsular polysaccharide antigens response (160). IgG3 has a short half-life being the most effective subclass in terms of their activating effector functions, acting as a potent pro-inflammatory antibody (160). Finally, IgG4 is mainly produced by allergens, and may be over-represented in immune responses to therapeutic proteins, as for instance adalimumab (160, 173).

IgG is a glycoprotein composed by four polypeptide chains, organized in two identical heavy chains and two light chains, linked by inter-chain disulfide bonds. Both heavy and light chains contain a *N*-terminal variable domain and constant domains that from the variable fraction (Fab) and constant fraction (Fc) of IgG, respectively (160). Fab is responsible for the recognition and binding of the antigens and Fc is involved in the effector function of the IgG. A highly conserved *N*-linked glycosylation site at position 297 is located in Fc fraction, being crucial for their function (this topic will be further discussed in the section of *"Glycosylation in IgG"*). The effector functions of IgG variates accordingly with the IgG subclass and includes: 1) direct neutralization of microbes and toxins; 2) lysis of cells through complement activation (complement dependent cytotoxicity – CDC; Figure 4); 3) interaction with Fc gamma receptors (FcγRs) expressed in a range of effector immune cells (included monocytes and macrophages) to engage antibody dependent cellular cytotoxicity (ADCC; Figure 4) or antibody dependent cellular phagocytosis (ADCP) (174). The binding of the Fc portion of IgG to FcγRs, triggers an activating or inhibiting signaling depending on the FcγRs (in humans FcγRIIA, IIIA, and IIIB or FcγRIIB, respectively) (174). The IgG-immune complex (IC)-FcγR signaling depends on the relative engagement of activating or inhibitory FcγRs (the A:I ratio) expressed in immune cells.

Mucosal IgGs from CD and UC are highly reactive to cytoplasmatic proteins of commensal bacteria (166). In UC, it was shown to have a higher IgG1 response against surface antigens, while in CD the response was preferentially by IgG2 (175). The same differential proportion of IgG subtypes were observed in the serum of both UC and CD (169). The functional effects of these IgGs were observed by increased respiratory burst in polymorphonuclear leukocytes (175). Also, the increased production of mucosal IgG against

commensal microorganisms in UC patients was shown to induce intestinal inflammation through the production of TNF-α, IL1-β and neutrophil-recruiting chemokines via activation of FcγR presented on resident mucosal macrophages (168, 176). The IgG-IC- FcγR engagement, and the consequent IL1-β production was also shown to induce IL17A and IL22 cytokine production by both Th17 cells and γδ T cells (168).

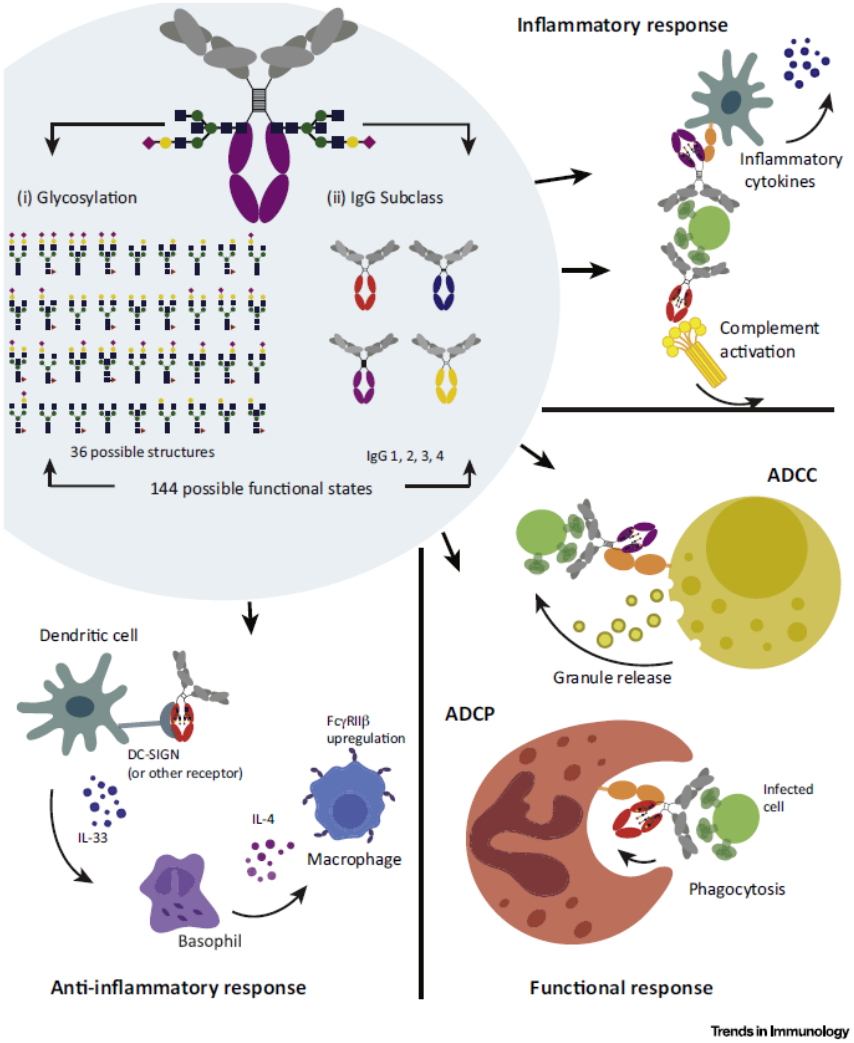


Figure 4_ *The diverse effector functions of IgG depend on the N-glycan attached to the Fc fraction of IgG (3).*

1.6. Microbiome

The gut mucosa is colonized by a complex and vast number of microorganisms that includes bacteria, fungi and viruses, collective called as microbiota (177, 178). The genome of microbiota is termed microbiome and gives information about the microbial composition and function, which varies along the gut being influenced by age and environmental factors as diet (179-181). The maintenance of homeostasis in the presence of such tremendous amount of microorganisms is driven by a tight immune control, in which immunogenicity against pathogens balance with tolerance to commensals (182). This explains why intestine

contain the biggest armament of T and B cells in the body. Also, the mutual benefits of both host and microbiome is provided by the symbiotic nature of microbiota that provides a variety of nutrients, metabolizes food and prevents colonization by pathogens (183).

The microbiota of an individual starts to be established through the birth canal and continue during breast feed. Breast milk contains microbes, IgA, metabolites, immune cells and cytokines, which is believed to be important in the shaping of immune system and microbiota colonization (182). In fact, studies in germ-free mice showed the relevance of microbiota in the development of Peyer's patches, CD4+ T cells and IgA-producing plasma cells (182). The composition of microbiota changes since birth until adulthood with increasing microbiota diversity along time (184). More than 1000 bacterial species are presented in gastrointestinal microbiome, in which the more prevalent phyla are *Firmicutes* (where *Clostridia* class are enriched) and *Bacteroidetes* (185-189), counting for 90% of gut microbiota. Other phyla as *Actinobacteria* and *Proteobacteria* are present but at low percentage (189). *Lachnospiraceae* and *Ruminococcaceae* (from *Clostridia* class) family as well as *Bacteroides and Prevotella* (from *Bacteroidia* class) genera are the most abundant (189).

Despite the permanent fluctuations of microbiome in the gut mucosa, the typical compositional pattern is maintained due to microbial resilience. IBD patients display a compositional and functional imbalance of the microbiota - termed as dysbiosis (190, 191). In a scenario of dysbiosis, the microbiota diversity is decreased and disturb the resistance that commensal provide against the outgrowth of commensals with potential pathogenic influence on the host (pathobionts) or opportunistic pathogens (192). Gut microbiota from IBD patients is commonly characterized by reduced abundance and diversity (193), where *Firmicutes* and *Bacteroidetes* are usually under-represented while *Actinobacteria* and *Proteobacteria* are over-represented (188). *Ruminococcaceae* and *Lachnospiraceae* families belonging to *Firmicutes* phylum are the most common decrease in IBD patients (194-197).

External factors such as diet and smoke habits are shown to be associated with alterations on microbial composition in IBD (198-200). Also, the disturbance of microbiome due to the exposure to antibiotics early in life is associated with higher risk to develop pediatric IBD (201, 202). These evidences suggest that the effect of external factors on microbial composition might be underlying the pathogenesis of IBD. In fact, many IBD susceptibility genes are related with bacterial recognition and process, mucosal barrier function (83), which might confer a predisposition to develop dysbiosis.

However, whether dysbiosis is a cause of IBD or a consequence of the inflammation remains to be elucidated. Inflammation provide extreme conditions that probably only the more resistant bacteria, as enteric pathogen, can survive and proliferate. In fact, facultative anaerobes (as *Enterobacteriaceae*) take advantage in an inflammatory condition in comparison to obligate anaerobes (commonly found in healthy conditions), which can perpetuate inflammation (203). Concordantly, treatment with 5-ASA decreases inflammation in IBD patients and also decreases the facultative anaerobe *Escherichia/Shigella* - an increased genus from *Enterobacteriaceae* family associated with IBD (200, 204, 205).

Pathobionts are considered colitogenic as they trigger intestinal inflammation, as observed in mice models of spontaneous colitis (206), where monocolonization of *E. coli* or *Enterococcus faecalis*, in germ free IL10- or IL2- deficient mice induces colitis. These bacteria are mainly associated with the ileal mucosa of patients with CD (207). The resistance to the colonization of harmful bacteria is driven by the competition between commensals and pathobionts, mainly for nutrients that include glycans (further discussed), and host metabolites (208). Commensals also impact pathogen colonization and expansion through the inhibition of virulence-related gene expression (209) and through the modulation of host immune response. *Clostridium* (belonging to *Firmicutes*) species (210, 211), as well as other organisms, as *Bifidobacterium infantis and Bacteroides fragilis* (212, 213), promote Treg response. *Clostridium butyricum* was also shown to suppress inflammatory response through stimulation of IL10-producing macrophages involved in prevention of DSS-induced acute colitis in mice (214). Furthermore, filamentous bacteria are able to induce Th17 response protecting the gut mucosa against *Citrobacter rodentium* infection, in mice (215). Another example of Treg regulation mediated by bacteria is through the production of short-chain fatty acids (SCFA) metabolites, resultant from digestion of fibers. The butyrate producing specie such as *Faecalibacterium prausnitzii* display an anti-inflammatory effect and its loss in ileal CD is associated with worse prognosis (216). Furthermore, *Faecalibacterium prausnitzii* and SCFA-producing bacteria *Roseburia* are also inversely correlated with UC disease activity (217). The anti-inflammatory properties might be through the downregulation of pro-inflammatory mediators of lamina propria macrophages (218) and through macrophages and DC - mediated Treg induction (219).

The colonization and overgrowth of the pathogen often occurs when there is low competition from commensal microorganisms. Infection *with Clostridium difficile* (*C. difficile*) is a classical example, which might occur after a long-term antibiotic treatment and cause pseudomembranous colitis which results in severe diarrhea, fever and abdominal pain (220, 221). The modification of the gut microbiota through of fecal microbiota transplantation

(FMT) from a donor to the gastrointestinal tract of individuals with recurrent *C. difficile* infection, leads to the resolution of symptoms within 2–3 days post-FMT (222, 223). Furthermore, FMT was demonstrated to be responsible for the clearance of the *C. difficile* infection (223, 224), highlighting the protective effect of commensal microorganisms as a community. Indeed, FMT increase microbial diversity and richness (225) and it was shown to induce disease remission, although not consistently (226, 227). The clinical improvement by re-shaping gut microbiota highlights the contribution of microbiota to IBD pathogenesis.

2. Protein glycosylation

The information that is transferred between DNA, RNA and protein in not enough to explain the inherent complexity of a cell. All cells in nature express carbohydrates, monosaccharides or polysaccharides structures (glycans), being the repertoire of glycans of an organism (glycome) more complex than the genome or proteome (228, 229). Post-translational modifications of proteins, through the addition of carbohydrates – termed glycosylation - , have been changed the paradigm of molecular biology by introducing a marked diversity in proteins and a novel layer of information. Glycosylation consists in the covalent attachment of a carbohydrate to proteins, lipids or other carbohydrates (Figure 5), through the action glycosyltransferases, producing different families of glycoconjugates (5). An enormous repertoire might be originated from glycosylation pathway, once glycosylation is cell and tissue specific and it depends on the availability of sugar substrates and on the expression and localization of the glycosyltransferases and glycosidases involved.

Cells are covered by a complex coat of glycans (glycocalyx) and almost all secreted proteins are glycosylated, which turn glycans a major tool in cellular interaction with the microenvironment. Glycans are important on cell-cell and cell-matrix adhesion, protein folding and stability, trafficking and clearance, regulation of receptor activation and signal transduction. The biological relevance of glycans is huge, in which glycans encode important biological information that regulate fundamental cellular and molecular mechanisms both in health and disease (230, 231). Alterations on glycans expression, caused by external or internal factors, might alter cellular phenotype and behavior with impact on both homeostatic and pathological conditions such as inflammation, autoimmunity and cancer (5, 232).

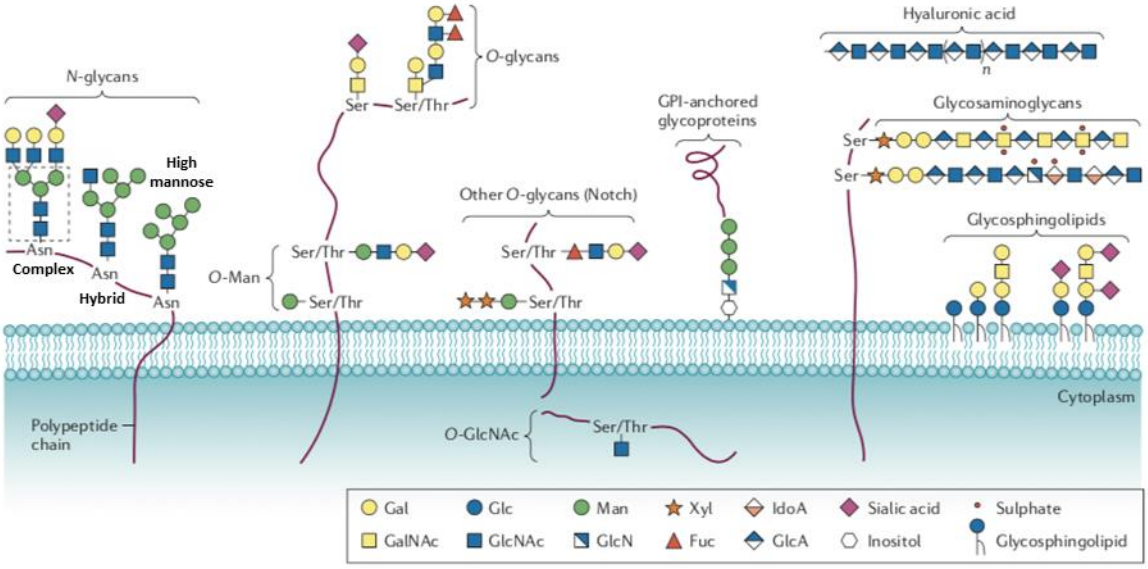


Figure 5_ *Major classes of glycoconjugates in mammalian cells (5).*

Protein glycosylation is a process that occurs in the lumen of the endoplasmic reticulum (ER) and in the Golgi apparatus (230) and encompasses two main types of glycans : *N*-glycans and *O*-glycans. Particularly, *N*-glycosylation (the focus of this thesis) consists in the attachment of *N*-acetylglucosamine (GlcNAc) to the nitrogen atom of an asparagine (Asn) side chain by a β-1N linkage, in a consensus sequence Asn-X-serine (Ser)/ threonine (Thr), where X represents any amino acid excluding proline (233). While in *O*-glycosylation, the attach of sugar residues occur in amino acids with functional hydroxyl groups, as Ser and Thr (233). The thesis will focus on *N*-glycosylation.

2.1. *N*-Glycosylation

About 90% of the glycoproteins in eukaryotic organisms are *N*-glycosylated, and *N*-glycans have a crucial role in regulating many intracellular and extracellular functions within a cell. The *N*-glycosylation process occurs firstly in endoplasmic reticulum (ER), in a highly conserved pathway, and secondly in Golgi apparatus, where the diversity of protein glycosylation arises.

N-glycans contain a common glycoconjugate core linked to Asn, composed by GlcNAc2 mannose (Man)3, which is further modified by the addition and removal of other monosaccharides. This results in three major final structures: high mannose *N*-glycan, only extended by Man residues; complex *N*-glycans, extended by GlcNAc; hybrid *N*-glycans, extended by Man in Manα1-6 arm and one or two GlcNAc in Manα1-3 arm (1). Both Hybrid and complex glycans might be further elongated and decorated thorough galactosylation,

GlcNAclyation, sialylation and fucosylation, which determine the final structure of the glycan.

These structures depend on the formation of a lipid-linked oligosaccharide precursor in ER cytoplasmic side, in which GlcNAc link to the lipid carrier dolichol phosphate (Dol-P) and is branched with other GlcNAc and five residues of Man. This lipid precursor is then translocated to ER luminal membrane site, where is decorated with four Man and three Glucose (Glc) residues, to form a 14-sugar structure — Glc3Man9GlcNAc2. The completed Dol-P–carbohydrate structure is then transferred *en bloc* to the side-chain Asn residue of the glycoprotein by oligosaccharyltransferase (OST). The nascent carbohydrate–protein conjugate suffers additional quality-control processes, involving removal of the glucose residues, determining whether the newly made membrane and secreted proteins continue to the Golgi apparatus or targeted for degradation (234-237).

The high-mannose structures move to *cis*-Golgi where mannosidases - α1-2 mannosidase IA and IB (*MAN1A1*, *MAN1B1*) – give rise to $Man_5GlcNAc_2$, which is a key intermediate in the pathway to hybrid and complex *N*-glycans (Figure 6). These *N*-glycan forms are further produced in *medial*- and *trans*- Golgi compartments, starting with the action of the *N*-acetylglucosaminyltransferase (GnT) I (GnT-I, encoded by Mannosidase acetylglucosaminyltransferase (*MGAT*)*1*), which adds a GlcNAc in β1–2 linkage to produce a hybrid *N*-glycan structure (1). GnT-I initiates the GlcNAc-bearing branches (referred as antenna), in which after the removal of terminal α1-3Man and α1-6Man residues through α-Mannosidase II enzymes (*MAN2A1*, *MAN2B1*), is continued by GnT II (encoded by *Mgat2). Additional antennas might be added by GnT-IV (encoded by MGAT4) and GnT-V (encoded by MGAT5), through the formation of* β1-4GlcNAc branch to the Manα1-3 core and β1,6GlcNAc arm to the Manα1-6, respectively. Both hybrid and complex *N*-glycans might also carry a bisecting GlcNAc residue to the β-linked mannose residue as a result of the action of GnT-III (encoded by *MGAT3*) (1). The presence of bisecting GlcNAc can block further branching by GnT-II, -IV and -V (238). The formation of branching *N*-glycans occurs sequentially (GnT-I, II, IV, V) , along the hexosamine pathway using the substrate UDP-GlcNAc, for which the affinity of enzymes also decrease in a sequential manner (from GntT-I to GntV) (239).

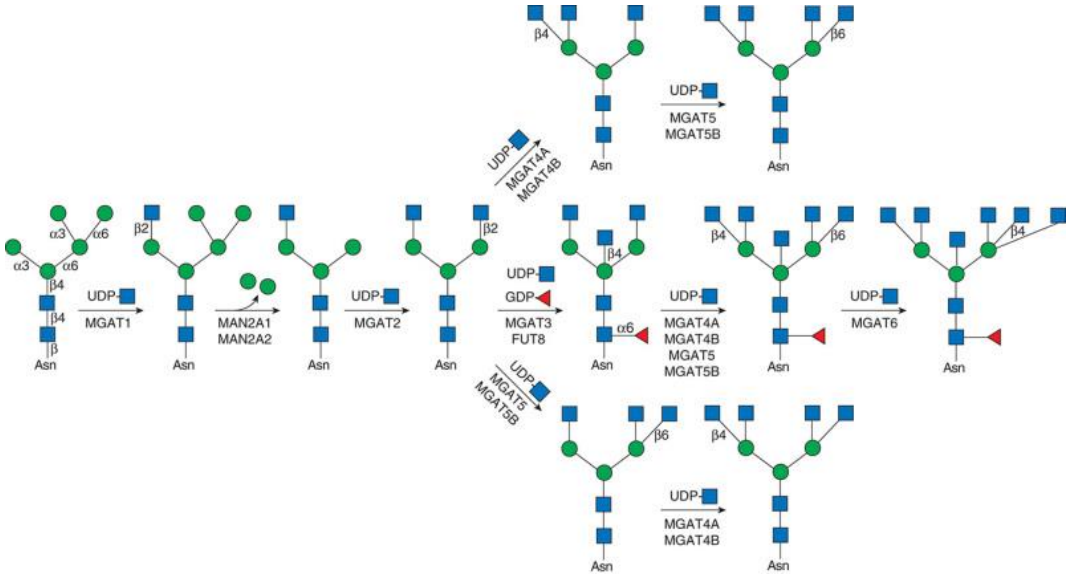


Figure 6_ *N-glycosylation pathway that determines the branching and core modification of complex N-glycans. Adapted from (1).*

The maturation of the glycoconjugate occurs in trans-Golgi, where all antenna, with the exception of bisecting GlcNAc, might be elongated and decorated with other glycan moieties (Figure 7) (1). Elongation starts with the attachment of galactose to the branch GlcNAc (catalyzed by β1,4 galactosyltransferase - β4Gal-T) generating *N*-acetyllactosamine (LacNAc) units, which by sequential addition of galactose and GlcNAc originates poly-*N*-acetyllactosamine (pLacNAc) (Figure 7) that are highly recognize by the glycan binding proteins (GBP) galectins, which might regulate immune response (240). The addition of a fucose to the core of *N*-glycans (catalyzed by α1-6 fucosyltransferase (FUT8)) consist in the major core modification in vertebrates (1). The decoration of the glycoconjugate is amplified by terminal glycan structures, as galactose, fucose and sialic acids, which per se, and in combination display diverse functions in the cell, through cell recognition for instance and stimulation of effector functions, as the case of sialic acids that are important immunoregulators.

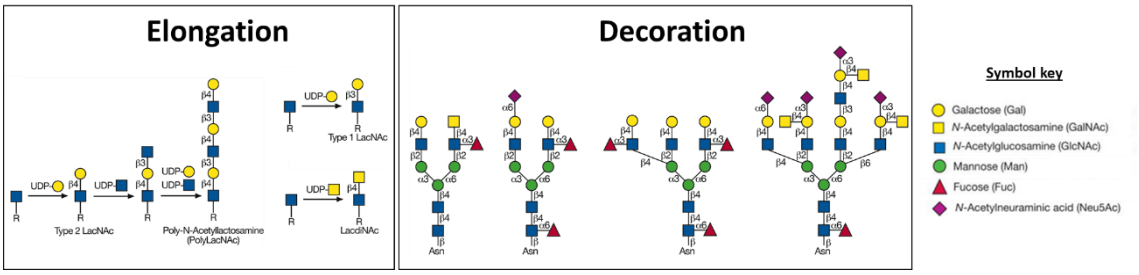


Figure 7_ *Maturation of the N-glycanconjugate, through elongation and decoration with different glycan moieties. Adapted from (1)*

3. Glycosylation in immune response – IBD related

Almost all of the key molecules involved in antigen recognition and the subsequent immune events are glycosylated, including pattern recognition receptors, the class I and class II major histocompatibility complex proteins (MHC class I and MHC class II) and T and B cells receptors and co-receptors, as well as cytokines, chemokines and antibodies (230, 240, 241). Alterations on glycosylation profile might thus interfere with the control of immune tolerance, autoimmunity, and chronic inflammation (as revised in the attached manuscripts (232, 242)). The impact of the glycans in both innate and adaptive immunity is dependent on the dynamic regulation of glycans attached to proteins and on its interplay with the GBP, including the C-type lectins, Siglecs, and galectins (240), that are expressed on DCs, macrophages, T and B lymphocytes (230, 240, 241, 243).

For instance, the C-type lectin receptor (CLR) dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) that is present in DC and macrophages, triggers different signaling pathways depending on the carbohydrate structure that recognizes (244). As example, pathogens expressing fucose (as *Helicobacter pylori*) instructs the suppression of proinflammatory response, whereas mannose expressing pathogens (as *Mycobacterium tuberculosis* and human immunodeficiency virus type 1 – HIV-1) enhances a proinflammatory response (245, 246). The murine related DC-SIGN – specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3, SIGNR3 – was demonstrated to recognize intestinal fungi and to display an immune regulatory role in colitis (247).

Antigen glycosylation has been implicated in autoimmune disorders (248, 249). The abrogation of complex *N*-glycans formation through the deficiency on alpha-mannosidase II (MAN2A1) in mice, promotes spontaneous autoimmunity that seem to be independent on T cell function, once lymphocytes retain high amounts of branching *N*-glycans due to compensatory mechanism of another mannosidase (250, 251). The exposition of cell surface mannose in this mice model hyper-activates innate immune response through binding to mannose-binding lectin receptors (251), and its specific deficiency on epithelial cells was shown to promote intestinal inflammation through neutrophil recruitment (252).

3.1. Glycosylation in T cells

Over the years it has been shown that the recognition mediated by T cells is not exclusive to proteins and peptides. Glycopeptides or only oligosaccharide products can be

presented on MHC molecules and induce T cell response. The polysaccharides-A (PSA) from *Bacteroides* capsule is endocytosed and processed into low molecular weight molecules in APC and presented by MHCII, mediating APC/T cell engagement through αβ T cell receptor (TCR) recognition and eliciting a clonal CD4+ T cell response (253, 254). This recognition is also dependent on the presence of branched *N*-glycosylation in MHCII, once loss of complex *N*-glycans in APC reduces the MHCII binding and presentation of this polysaccharide (255). Glycosylation of proteins also impact on cellular uptake and the proteolytic processing of the antigen or glycoantigen, as well as in the binding to MHC, interfering thus in T-cell priming (256, 257).

IBD patients display a dysregulation on mucosal-associated galectins (Gal-1, -3, -4, -9) (258). Specifically, Gal-3 is reduced (259-261) and displays a protective role in DSS-induced colitis and in the T-cell transfer colitis models, through suppression of IL6 or by induction of $Foxp3^+$ Treg cells (262, 263). The binding of galectins to transmembrane receptors regulate their mobility in the cell surface, something especially relevant for TCR clustering in T cells activation. Glycoproteins display affinity for galectins in proportion to the number of *N*-glycans and the *N*-glycan branching that it possesses (264). TCR contains seven sites of *N*-glycosylation, and branching *N*-glycans mediated by *MGAT5* were shown to increase the binding to Gal-3, which impedes TCR clustering and increases the threshold for activation by APC (265). In fact, *MAGT5* deficient mice was shown to develop spontaneous autoimmune disease and a severe DSS-induce colitis (265, 266). The loss of self-tolerance was found to be promoted by lowering T cell activation threshold through TCR clusters and impacting on the cytotoxic T-lymphocyte antigen (CTLA)-4 surface retention, an important autoimmune inhibitor (239, 265).

The activation of CD4+ and CD8+ T cells through TCR recognition of antigens bound to MHC involves alterations on the glycoprofile of the cell (267). Upon activation of naïve T cells, branched *N*-glycans increases together with the upregulation of *MGAT5* expression, while maintaining low levels of *Mgat1* and *Mgat2*, which compete for the same sugar substrates (268). This increase is important for immune response regulation, once the increase of branching *N*-glycans controls T cell proliferation and promotes T cell arrest (265, 266, 268). Accordingly, *MGAT5*-mediated branched *N*-glycans expression (detected by *Phaseolus Vulgaris Leucoagglutinin* (*L-PHA*)) was found to be increased in Treg and to correlate with enhanced ability to suppress $CD4^+$ and $CD8^+$ T-cell proliferation and conventional $CD4^+$ T-cells activation (269). This is explained by the cell surface stability of receptors imposed by branched *N*-glycans expression in Treg suppressive functions (CTLA-4, PD-1, PD-L1, e.g.) (269). Thus, a deficiency on *MGAT5* expression and consequent decrease of branching *N*-glycans will impair immune tolerance. Accordingly, IBD patients

display low levels of branching *N*-glycans on lamina propria CD3+ T cells, together with a reduction on *MGAT5* mRNA expression (270). This defect was recovered by the supplementation with the substrate of GnT-V (encoded by *MGAT5*) the UDP-GlcNAc, which reduced TCR signaling and T cell proinflammatory response (266). Thus the metabolic supplementation with glycans that are salvaged into the hexosamine pathway, can represent a promising immunomodulatory therapeutic strategy for IBD, as already observed DSS-colitis induce mice (266) and in children with IBD (271).

In opposition, the over-expression of *MGAT5* in mice was shown to exacerbate DSS-mediated colitis by inducing macrophage dysfunction (272), suggesting that complex branched *N*-glycans may have different roles in different immune cells. In addition, T cells from IBD intestinal mucosa also display methylation in the promoter of *MGAT3* (273) and an increase in core fucosylation (mediated by *FUT8*) (274). Core fucosylation of TCR was also shown to be required for induction of colitis in mice, due to its positive effect on T cell signaling and production of inflammatory cytokines (274).

3.2. Glycosylation in IgG

As mentioned previously, IgG is a glycoprotein in which both Fab and Fc are glycosylated. Fc region of IgG is covalently attached to a single bi-antennary *N*-glycan that has a key structural role in stabilizing the conformation of the Fc region (Figure 5) (275). Alterations on glycans composition alter dramatically the IgG effector function, as observed by the loss of function in non-glycosylated IgGs (276). Pharmaceutical companies have been taking into consideration for the quality of an antibody, the profile of glycosylation, once Fab glycosylation influences the binding to antigen and Fc glycosylation influences the clearance rate, pharmacokinetic, pharmacodynamic and the binding to Fc receptors (277, 278).

IgG comprises 10-20% of the total plasma (160) and its Fc linked *N*-glycan can comprises different glycoforms, generally resulting from the combination of the presence or absence of fucose, galactose, sialic acid and bisecting GlcNAc (Figure 8) (279). Although heterogenous, the profile of IgG glycosylation is quite constant, displaying high proportion of fucosylation (core fucosylation; 96%), low bisecting (8%) and sialylation (4%), and intermediate galactosylation (40%) (280). Galactosylation and sialylation decrease with age and showed significant gender dependence (280), and can be rapidly altered in situations of disturbed homeostasis (281).

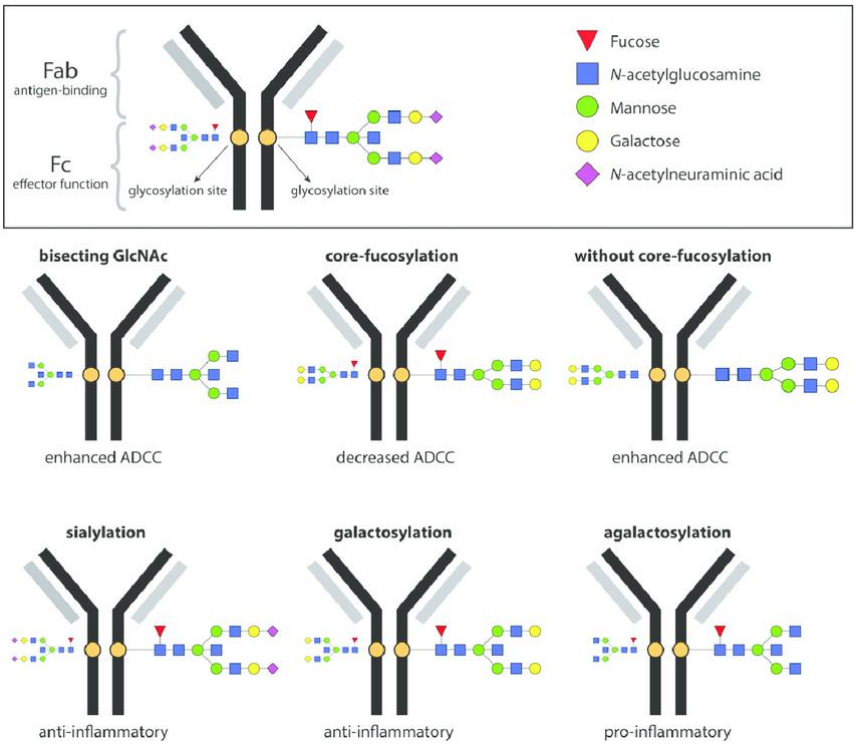


Figure 8_*IgG glycome and its effector function in IgG (4).*

Alterations in the glycosylation of IgG have shown to impact on biological functions and structure of IgG, switching the phenotype of IgG from anti-inflammatory to pro-inflammatory. Circulating IgG in IBD patients were shown to display alterations of IgG glycosylation, comparing with healthy individuals (282). Alterations on IgG glycosylation, such as the loss of sialylation and low levels of galactosylation (282, 283), are also associated with inflammatory markers (284) and other autoimmune disorders as rheumatoid arthritis and Systemic Lupus Erythematosus (285, 286).

Terminal sialic acids were shown to display an anti-inflammatory function on IgG, by increasing expression of inhibitory FcγRIIB and reducing affinity to activating FcγRIII/IV (287-289), suggesting that sialic acid in Fc promotes a conformational change in IgG and increases the I:A ration of FcγR in effector cells. Sialic acids were also shown to bind to DC-SIGN which was associated with up-regulation of FcγRIIB (290). In addition, terminal galactose was also shown to increase affinity of IgG to inhibitory FcγR and C-type lectin–like receptor dectin-1 (291), exerting an anti-inflammatory property. Contrary, other studies suggest that galactosylation may also affect binding to FcγRIIIa in a positive way (292-294). Agalactosyl IgG with terminal GlcNAc was shown to enhance antibody-dependent phagocytosis (295) and to bind to mannose-binding lectin (MBP), and thus selectively activate the lectin-arm of complement (296). However, other study suggest that the effector function of agalacosyl IgG is dependent on activating FcγR and not on MBP (297). The

exposition of *N*-acetylglucosamine residues in IgG was also shown to increase the uptake from mannose receptor in macrophages and DC, potentially generating epitopes recognized by T cells (298).

In contrast to other proteins from the plasma (299), IgG is typically core fucosylated, which is known to reduce IgG binding to the activating FcγRIIIA (300-302) and consequently their potential to induce ADCC. An Increased proportion afucosylated IgGs and bisecting GlcNAc, also demonstrated to enhance ADCC (303-306), were also observed in UC patients (282). Thus, IBD patients display glycosylation alterations on IgG that might participate in the pathogenesis of the disease.

4. Glycans in microbiome

The interplay between microbial organisms and the host is driven by the thick glycocalyx that both present at the cell surface. Host and microbes are phyloglycomic distinct, and host took advantage of this difference to discriminate self- from non-self and instruct immune responses based on glycan recognition through Toll like receptors and C-type lectins (307).

Focusing on bacteria, they display complex biosynthetic pathways that leads to a variety of glycans: polysaccharide capsules, peptidoglycans, lipopolysaccharides (LPS) and both *N*- and *O*- linked protein glycosylation, ranging from mono- and disaccharides to complex glycans with repeating carbohydrate units and extensive branching. The majority of the glycoproteins are surface-located, as it is the case of the adhesins, the surface hair-like extension (pili) and the flagella (308, 309). A coordinated interplay between host glycans degradation and the bacteria polysaccharide capsule synthesis occurs (310). In fact the interplay between host and bacteria is evident with the different abundances of glycosyltransferases that a germ-free mice and conventionally raised mice display (311), which alter Muc2 *O*-glycan biosynthesis. Thus, it is not surprising that alterations on host glycosylation might altered the pattern of the microflora.

In order to fulfill their energy needs, microbiota displays a complex mechanism that makes them capable of processing diet derived glycans that human is unable to process, as well as, host- produced glycans, as the case of mucins. This source of energy imposes a high level of competition between bacterial community, but also an impressive cooperation.

For instance, fucose is wildly used by symbiotic gut bacteria as a source of energy or to incorporate in their own glycans for the proper function of the glycoproteins or for its fitness in the gut (312, 313). To take advantage from diet- and host- derived fucose, bacteria must liberate fucose using α-fucosidases, which allows the availability of fucose to all bacteria community. Commensal bacteria as *Bacteroides thetaiotaomicron* also take advantage from the host glycosylation machinery, by increasing the expression of α1,2-fucosyltransferase mRNA in intestinal epithelial cells and using their own fucosidases and fucose permeases to cleave and internalize the terminal fucose residues incorporating into the capsular polysaccharides or bacterial glycoproteins (313, 314). This mechanism is not exhibited in all pathogens and, in fact, α1,2-fucosylation is increased upon an infection which seems to be a mechanism of protection from the host to supply symbiotic bacteria with fucose (315). This interplay between host and commensal bacteria maintains homeostasis and suppresses virulence genes as is the case of pathogenic *Escherichia coli* (*E.Coli*) (316).

Epithelial interleukin-22 receptor IL22RA1 signaling was also shown to promote intestinal fucosylation via induction of the fucosyltransferase *FUT2*, which protects host against the *Citrobacter Rodentium* pathogen infection and DSS – induced colitis through resistance to *Enterococcus faecalis* (317). The selective outgrowth of opportunistic pathogens *Enterococci* following intestinal dysbiosis (either due to antibiotic treatment, intestinal inflammation or infection) might result from the lack of competition for nutrients and space (317, 318). In fact, the administration of fucosylated oligoscharides restore dybiosis induced by *Citrobacter rodentium* infection in a IL22-deficient mice, reducing *Enterococcus faecalis (317)* potentially through the increase of *Bacteroides* and *Ruminococcaceae*, which benefit from host-derived fucosylated glycans (319, 320).

In addition to fucose, sialic acid is also found to be liberated by symbionts like *Bacteroides species,* which are important for *Salmonella enterica serovar Typhimurium* and *C. difficile* expansion. However, these pathogens are only able to catabolize microbiota-liberated mucosal carbohydrates when bacteria that normally consume it are depleted, as is the case of antibiotic-induced disruption of the resident microbiota (321). In fact, sialic acid catabolism might confer a growth advantage to *Enterobacteriaceae* in the inflamed gut. The increase of sialidase activity observed after DSS administration is critical for the infection of opportunist pathogens as *E.Coli*, once the oral administration of sialidase inhibitors in mice decreases the outgrowth of E.Coli and the severity of the disease (322).

Some bacteria encode sialidases, which cleave and release sialic acid, but lack the catabolic pathway, being thus unable to consume the sugar. On the other hand, other

bacteria are only able to catabolize sialic acid but do not express sialidases (323). The incorporation of sialic acids by the bacteria serve as a "self" molecular pattern and a process of camouflage, the glycan mimicry, that pathogens use to avoid the recognition as non-self from host lectin and hampering an immune response against the bacteria. Instead of synthesizing glycans de novo, there are bacteria that incorporate glycans from the host in order to persist and cause infection, as it is the case of *H. influenzae* and *E.Coli* that acquire sialic acid to decorate its capsule and lipopolysaccharide (314). Trans-sialidases are used by bacteria to obtain and incorporate sialic acid from the host. The modification of antigens with sialic acid alters their immunogenicity by inducing T regulatory cells and inhibiting T proliferation through the loaded of the glycoantigen by dendritic cells and their interaction with sialic acid binding immunoglobulin type lectins (Siglec E) (324, 325). Also, the mechanism that bacteria uses to mimic host glycosylation can induce the production of self-antibodies which may be associated with auto-immune reactions. This could be a problem for the development of vaccines with the glycoantigens (326).

All of these glycosylation strategies are important to maintain microbial symbiose, highlighting the importance of glycosylation in host- microbiome interactions associated with IBD pathogenesis.

5. Glycans as markers in IBD

5.1. Genetic glycobiomarkers

As mentioned above, glycosylation constitutes a key player in immunity. The contribution of glycans-encoding genes to IBD pathogenesis is far from being fully characterized. In fact, the emerging role of glycoconjugates in IBD pathogenesis gave rise to the creation of an online database called Glycosylation and Gut Associated Immune Tolerance (GlycoGAIT-https://apps.connexios.com/glycogait/), in which the expression patterns of glycome genomics can be analyzed under different inflammatory conditions (327). Genetic alterations in glycogenes have been associated with multiple primary immunodeficiency diseases, known as congenital disorders of glycosylation, often embryonically lethal and associated with poor survival rates (328, 329). Accordingly, mouse genetics in which selected glycogenes, as *MGAT1*, are mutated revealed a remarkable impact in T cell development and thymus positive and negative selection (330). Interestingly, transgenic mice with overexpression of α1,2-fucosyltransferase (*FUT1*) spontaneously develop colitis, by influencing T cell development and altering glycosylation

of mucosal barrier (331). The effect of glycosylation in T cell development was presented in the review in attach (242).

Genetic variants associated with loss-of-function of *FUT2* were demonstrated to increase susceptibility to CD by altering host-microbial interactions. Specifically, the non-secretor status of those patients, display an altered microbiome at both composition and functional levels potentially explaining the CD risk (332, 333). Additionally, *FUT3* polymorphisms were also associated with higher susceptibility of Southeast China individuals to UC (334). Other genetic variants on *MAN2A1* was highlighted in UC patients, which seems to play a role in promoting intestinal inflammation through neutrophil recruitment (252). Genetic alterations on specific glycogenes (*ST6GAL1, B4GALT1, FUT8, MGAT3 and FUT6-FUT3*) have been shown to interfere in IgG glycosylation (335, 336). Furthermore, genetic variants on *MGAT5*, *FUT8*, *FUT6*/*FUT3*, *B3GAT1* were also shown to affect the total plasma *N*-glycome composition in healthy European individuals (337), which as well in IgG, the glycan profile is also alter in IBD patients (338). Genetic and epigenetic alterations in *MGAT3* were also found to be associated with both IgG glycosylation and IBD, suggesting a role of GnT-III in IBD pathogenesis (273, 336).

Furthermore, genetic alterations in immuno-genes, associated with IBD, can indirectly lead to alterations on glycogenes/glycosylation, with a synergistic impact in the dysregulation of the immune system. The very early onset (VEO) – IBD can be caused by loss-of-function mutations of *IL10* and *IL10R*. A point mutation in the intronic region of *IL10R1*, observed in three VEO-IBD patients, impaired the mRNA splicing and the *N*-linked glycosylation, interfering with the trafficking of IL10R to the plasma membrane and thus with the anti-inflammatory signal mediated by IL10 (339). Also, protective genetic variants of *IL23Rα* for IBD was shown to display different receptor maturation in glycosylation, being associated with lower cell surface expression and with an impairment in ER to Golgi trafficking (340). Interestingly, IL22/IL22RA1 signaling in intestinal organoids was shown to up-regulate glycosylation genes such as *FUT2*, *FUT8*, *SEC1* and *B4GALT1*, in which IL22RA1/FUT2 axis was demonstrated to be involved in the maintenance of healthy microbiota (317). Mice experiments also revealed a protective role of *B4GALT1*, which encodes for b-1,4-galactosyltransferase I and it is involved in the addition of galactose moieties to glycoproteins. Higher galactosylation of *N*-glycans of mucus proteins were associated with an increase *Firmicutes*/*Bacteroidetes* ratio, induced protection against TNF-induced systemic inflammation and DSS-induced colitis (341). Taken together, glycogenes constitute important genetic determinants with functional impact in IBD pathogenesis and prognosis.

5.2. Glycans as non-invasive biomarkers

Non-invasive biomarkers are attracting tools for monitoring disease activity and for predicting therapy response, and much of them are glycosylated, as the case of fecal Lactoferrin and the antibodies against specific bacterial glycans (29, 342, 343). Alterations on plasma proteins glycome were observed in IBD patients (338, 344). The glycan profile were composed by decreased proportions of galactosylation and fucosylation and increased sialylation and higher proportion of hybrid and high mannose and it was associated with the extension of disease, the need for surgery and need of more potent drugs (338).

Comparative analysis of IgG glycome between healthy individuals and IBD patients showed a distinct pattern of IgG glycosylation, usually with less complexity in IBD scenario, as observed by decreased galactosylation and sialylation and increased bisecting GlcNAc (282, 283). Fucosylation of IgG was also shown to be alters but differentially in UC and CD, being decreased and increased, respectively. The combination of agalactosyl IgG with ASCA further showed higher specificity in IBD diagnosis (345). Alterations on IgG glycosylation were also associated with IBD clinical features, as decreased galactosylation with the need of surgery and with patients that were under more aggressive therapy (282). Moreover, the analysis of plasma glycoprofile of 75 UC patients (346) demonstrated that high sialylation in IgG was associated with higher disease activity. A ratio between the presence of bisecting GlcNAc in monogalactosylated structures with the absence of bisecting GlcNAc in these structures were associated with a poor prognosis of UC patients (283). These evidences suggest a potential role of plasma IgG glycosylation as a non-invasive diagnostic and prognostic biomarkers in IBD.

Another proposed biomarker in serum and plasma for IBD is the glycoprotein acetylation (GlycA) measured by nuclear magnetic resonance. This biomarker reflects the glycan groups of certain acute-phase glycoproteins, and was shown to be increased in active IBD patients, when compared to healthy individuals. Also, GlycA was correlated with CRP and fecal calprotectin levels, discriminating however CRP-negative patients that display an active disease (347).

Overall, the determination of the glycosylation signature of human biological fluids, such as serum constitute a non-invasive tool with promising prognostic value that is worth explored.

Aims

Rational and novelty

IBD is influenced by multiple factors as genetics, environment, gut microbiota and host immune response, which drives the disease to diverse directions concerning severity and response to therapy. Glycosylation has gained more attention over the years in immune-mediated disorders, mainly due to its important role in T cells and IgG functions. Particularly, alterations on *MGAT5*-mediated branched *N*-glycans of colonic lymphocytes have been identified in UC patients, but the underlying cause remain unknown. This dysregulation is associated with an hyperimmune response, however its implication on microbiota composition and in patients' clinical and therapeutic outcomes are still unexplored. Also, the glycan profile of the IgGs has been demonstrated to be altered in IBD, but the influence of this alteration on therapy response is also poorly explored.

Main goals

The main goal of this thesis was to identify novel glycan based-biomarkers with prognostic value in IBD and with potential to be included in therapy decision-making process, further exploring the influence of branched *N*-glycan alterations in gut microbiota composition associated with IBD.

Hypothesis

I. Different levels of branched *N*-glycans at diagnosis may predict disease course regarding therapeutic outcomes.

II. Genetic variants of *MGAT5* might be associated with alterations in T cells and IgG glycosylation with impact on therapeutic outcomes.

III. Glycoengineering of *Mgat5* increases susceptibility to develop colitis that might be due to microbiome composition.

IV. The profile of IgG glycosylation might be different in CD patients who respond to anti-TNF from those that lose response.

Specific aims

I. To determine the branched *N*-glycan expression in colonic lamina propria early at diagnosis and correlate with the need of UC patients to step-up to anti-TNF during disease course.

II. To determine the functional impact of specific *MGAT5* genetic variants on mRNA expression levels of the gene in colonic and circulating T cells, as well as, in IgG

glycosylation from UC patients, further exploring these and other *MGAT5* genetic variants in relation to UC prognosis.

III. To evaluate the impact of changes in branched *N*-glycans expression on gut microbial composition and on colitis susceptibility and immune response.

IV. To determine the predictive capacity of circulating IgG glycosylation to discriminate responders *versus* non-responders to anti-TNF therapy (Infliximab or Adalimumab), based on immunogenicity issues to the drug.

References

1. Stanley P, Taniguchi N, Aebi M. 2015. N-Glycans. In *Essentials of Glycobiology*, ed. rd, A Varki, RD Cummings, JD Esko, P Stanley, GW Hart, M Aebi, AG Darvill, T Kinoshita, NH Packer, JH Prestegard, RL Schnaar, PH Seeberger, pp. 99-111. Cold Spring Harbor (NY)
2. Kaplan GG. 2015. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 12: 720-7
3. Jennewein MF, Alter G. 2017. The Immunoregulatory Roles of Antibody Glycosylation. *Trends Immunol* 38: 358-72
4. Lauc G, Pezer M, Rudan I, Campbell H. 2016. Mechanisms of disease: The human N-glycome. *Biochim Biophys Acta* 1860: 1574-82
5. Pinho SS, Reis CA. 2015. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* 15: 540-55
6. Ding NS, Hart A, De Cruz P. 2016. Systematic review: predicting and optimising response to anti-TNF therapy in Crohn's disease - algorithm for practical management. *Aliment Pharmacol Ther* 43: 30-51
7. Ordas I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. 2012. Ulcerative colitis. *Lancet* 380: 1606-19
8. Baumgart DC, Sandborn WJ. 2012. Crohn's disease. *Lancet* 380: 1590-605
9. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. 2018. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 390: 2769-78
10. Mak WY, Zhao M, Ng SC, Burisch J. 2019. The epidemiology of inflammatory bowel disease: East meets West. *J Gastroenterol Hepatol*
11. Benchimol EI, Guttmann A, Griffiths AM, Rabeneck L, Mack DR, Brill H, Howard J, Guan J, To T. 2009. Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data. *Gut* 58: 1490-7
12. Benchimol EI, Manuel DG, Guttmann A, Nguyen GC, Mojaverian N, Quach P, Mack DR. 2014. Changing age demographics of inflammatory bowel disease in Ontario, Canada: a population-based cohort study of epidemiology trends. *Inflamm Bowel Dis* 20: 1761-9
13. Sykora J, Pomahacova R, Kreslova M, Cvalinova D, Stych P, Schwarz J. 2018. Current global trends in the incidence of pediatric-onset inflammatory bowel disease. *World J Gastroenterol* 24: 2741-63

14. Burisch J, Jess T, Martinato M, Lakatos PL, EpiCom E. 2013. The burden of inflammatory bowel disease in Europe. *J Crohns Colitis* 7: 322-37
15. Azevedo LF, Magro F, Portela F, Lago P, Deus J, Cotter J, Cremers I, Vieira A, Peixe P, Caldeira P, Lopes H, Goncalves R, Reis J, Cravo M, Barros L, Ministro P, Lurdes M, Duarte A, Campos M, Carvalho L, Costa-Pereira A. 2010. Estimating the prevalence of inflammatory bowel disease in Portugal using a pharmaco-epidemiological approach. *Pharmacoepidemiol Drug Saf* 19: 499-510
16. Conrad K, Roggenbuck D, Laass MW. 2014. Diagnosis and classification of ulcerative colitis. *Autoimmun Rev* 13: 463-6
17. Laass MW, Roggenbuck D, Conrad K. 2014. Diagnosis and classification of Crohn's disease. *Autoimmun Rev* 13: 467-71
18. Hanauer SB. 2006. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 12 Suppl 1: S3-9
19. Duricova D, Burisch J, Jess T, Gower-Rousseau C, Lakatos PL, EpiCom E. 2014. Age-related differences in presentation and course of inflammatory bowel disease: an update on the population-based literature. *J Crohns Colitis* 8: 1351-61
20. Sairenji T, Collins KL, Evans DV. 2017. An Update on Inflammatory Bowel Disease. *Prim Care* 44: 673-92
21. Shah SC, Khalili H, Gower-Rousseau C, Olen O, Benchimol EI, Lynge E, Nielsen KR, Brassard P, Vutcovici M, Bitton A, Bernstein CN, Leddin D, Tamim H, Stefansson T, Loftus EV, Jr., Moum B, Tang W, Ng SC, Gearry R, Sincic B, Bell S, Sands BE, Lakatos PL, Vegh Z, Ott C, Kaplan GG, Burisch J, Colombel JF. 2018. Sex-Based Differences in Incidence of Inflammatory Bowel Diseases-Pooled Analysis of Population-Based Studies From Western Countries. *Gastroenterology* 155: 1079-89 e3
22. Schroeder KW, Tremaine WJ, Ilstrup DM. 1987. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 317: 1625-9
23. Magro F, Gionchetti P, Eliakim R, Ardizzone S, Armuzzi A, Barreiro-de Acosta M, Burisch J, Gecse KB, Hart AL, Hindryckx P, Langner C, Limdi JK, Pellino G, Zagorowicz E, Raine T, Harbord M, Rieder F, European Cs, Colitis O. 2017. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 1: Definitions, Diagnosis, Extra-intestinal Manifestations, Pregnancy, Cancer Surveillance, Surgery, and Ileo-anal Pouch Disorders. *J Crohns Colitis* 11: 649-70
24. Harvey RF, Bradshaw JM. 1980. A simple index of Crohn's-disease activity. *Lancet* 1: 514

25. af Bjorkesten CG, Nieminen U, Turunen U, Arkkila P, Sipponen T, Farkkila M. 2012. Surrogate markers and clinical indices, alone or combined, as indicators for endoscopic remission in anti-TNF-treated luminal Crohn's disease. *Scand J Gastroenterol* 47: 528-37
26. Peyrin-Biroulet L, Reinisch W, Colombel JF, Mantzaris GJ, Kornbluth A, Diamond R, Rutgeerts P, Tang LK, Cornillie FJ, Sandborn WJ. 2014. Clinical disease activity, C-reactive protein normalisation and mucosal healing in Crohn's disease in the SONIC trial. *Gut* 63: 88-95
27. Chang M, Chang L, Chang HM, Chang F. 2018. Intestinal and Extraintestinal Cancers Associated With Inflammatory Bowel Disease. *Clin Colorectal Cancer* 17: e29-e37
28. Lichtenstein GR, McGovern DPB. 2016. Using Markers in IBD to Predict Disease and Treatment Outcomes: Rationale and a Review of Current Status. *The American Journal Of Gastroenterology Supplements* 3: 17
29. Lakatos PL, Papp M, Rieder F. 2011. Serologic antiglycan antibodies in inflammatory bowel disease. *Am J Gastroenterol* 106: 406-12
30. Reese GE, Constantinides VA, Simillis C, Darzi AW, Orchard TR, Fazio VW, Tekkis PP. 2006. Diagnostic precision of anti-Saccharomyces cerevisiae antibodies and perinuclear antineutrophil cytoplasmic antibodies in inflammatory bowel disease. *Am J Gastroenterol* 101: 2410-22
31. Paul S, Boschetti G, Rinaudo-Gaujous M, Moreau A, Del Tedesco E, Bonneau J, Presles E, Mounsef F, Clavel L, Genin C, Flourie B, Phelip JM, Nancey S, Roblin X. 2015. Association of Anti-glycan Antibodies and Inflammatory Bowel Disease Course. *J Crohns Colitis* 9: 445-51
32. Arnott ID, Landers CJ, Nimmo EJ, Drummond HE, Smith BK, Targan SR, Satsangi J. 2004. Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype. *Am J Gastroenterol* 99: 2376-84
33. Darlington GJ, Wilson DR, Lachman LB. 1986. Monocyte-conditioned medium, interleukin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. *J Cell Biol* 103: 787-93
34. Solem CA, Loftus EV, Jr., Tremaine WJ, Harmsen WS, Zinsmeister AR, Sandborn WJ. 2005. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm Bowel Dis* 11: 707-12
35. Vermeire S, Van Assche G, Rutgeerts P. 2004. C-reactive protein as a marker for inflammatory bowel disease. *Inflamm Bowel Dis* 10: 661-5

36. Lichtenstein GR, McGovern DPB. 2016. Using markers in IBD to predict disease and treatment outcomes: rationale and a review of current status. In *Am J Gastroenterol*, pp. 17-26
37. Henriksen M, Jahnsen J, Lygren I, Stray N, Sauar J, Vatn MH, Moum B, Group IS. 2008. C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study. *Gut* 57: 1518-23
38. Lewis JD. 2011. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. *Gastroenterology* 140: 1817-26 e2
39. Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, Ricchiuti A, Marchi S, Bottai M. 2005. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 54: 364-8
40. Gisbert JP, Bermejo F, Perez-Calle JL, Taxonera C, Vera I, McNicholl AG, Algaba A, Lopez P, Lopez-Palacios N, Calvo M, Gonzalez-Lama Y, Carneros JA, Velasco M, Mate J. 2009. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. *Inflamm Bowel Dis* 15: 1190-8
41. Mosli MH, Zou G, Garg SK, Feagan SG, MacDonald JK, Chande N, Sandborn WJ, Feagan BG. 2015. C-Reactive Protein, Fecal Calprotectin, and Stool Lactoferrin for Detection of Endoscopic Activity in Symptomatic Inflammatory Bowel Disease Patients: A Systematic Review and Meta-Analysis. *Am J Gastroenterol* 110: 802-19; quiz 20
42. Sipponen T, Savilahti E, Karkkainen P, Kolho KL, Nuutinen H, Turunen U, Farkkila M. 2008. Fecal calprotectin, lactoferrin, and endoscopic disease activity in monitoring anti-TNF-alpha therapy for Crohn's disease. *Inflamm Bowel Dis* 14: 1392-8
43. Ho GT, Lee HM, Brydon G, Ting T, Hare N, Drummond H, Shand AG, Bartolo DC, Wilson RG, Dunlop MG, Arnott ID, Satsangi J. 2009. Fecal calprotectin predicts the clinical course of acute severe ulcerative colitis. *Am J Gastroenterol* 104: 673-8
44. Gomollon F, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, Peyrin-Biroulet L, Cullen GJ, Daperno M, Kucharzik T, Rieder F, Almer S, Armuzzi A, Harbord M, Langhorst J, Sans M, Chowers Y, Fiorino G, Juillerat P, Mantzaris GJ, Rizzello F, Vavricka S, Gionchetti P, Ecco. 2017. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J Crohns Colitis* 11: 3-25
45. Langhorst J, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. 2008. Noninvasive markers in the assessment of intestinal inflammation in inflammatory

bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am J Gastroenterol* 103: 162-9

46. Engstrom J, Lonnkvist M, Befrits R, Ljung T, Diaz-Tartera H, Holst M, Hellstrom PM. 2019. Comparison of fecal calprotectin and serum C-reactive protein in early prediction of outcome to infliximab induction therapy. *Scand J Gastroenterol* 54: 1081-8
47. Schirbel A, Reichert A, Roll S, Baumgart DC, Buning C, Wittig B, Wiedenmann B, Dignass A, Sturm A. 2010. Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J Gastroenterol* 16: 3168-77
48. Lonnfors S, Vermeire S, Greco M, Hommes D, Bell C, Avedano L. 2014. IBD and health-related quality of life -- discovering the true impact. *J Crohns Colitis* 8: 1281-6
49. Zallot C, Peyrin-Biroulet L. 2013. Deep remission in inflammatory bowel disease: looking beyond symptoms. *Curr Gastroenterol Rep* 15: 315
50. Bossuyt P, Vermeire S. 2016. Treat to Target in Inflammatory Bowel Disease. *Curr Treat Options Gastroenterol* 14: 61-72
51. Froslie KF, Jahnsen J, Moum BA, Vatn MH, Group I. 2007. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 133: 412-22
52. Schnitzler F, Fidder H, Ferrante M, Noman M, Arijs I, Van Assche G, Hoffman I, Van Steen K, Vermeire S, Rutgeerts P. 2009. Mucosal healing predicts long-term outcome of maintenance therapy with infliximab in Crohn's disease. *Inflamm Bowel Dis* 15: 1295-301
53. Baert F, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P, Hommes D, Rutgeerts P, Vermeire S, D'Haens G, Belgian Inflammatory Bowel Disease Research G, North-Holland Gut C. 2010. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. *Gastroenterology* 138: 463-8; quiz e10-1
54. Harbord M, Eliakim R, Bettenworth D, Karmiris K, Katsanos K, Kopylov U, Kucharzik T, Molnar T, Raine T, Sebastian S, de Sousa HT, Dignass A, Carbonnel F, European Cs, Colitis O. 2017. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 2: Current Management. *J Crohns Colitis* 11: 769-84
55. Moja L, Danese S, Fiorino G, Del Giovane C, Bonovas S. 2015. Systematic review with network meta-analysis: comparative efficacy and safety of budesonide and mesalazine (mesalamine) for Crohn's disease. *Aliment Pharmacol Ther* 41: 1055-65

56. Punchard NA, Greenfield SM, Thompson RP. 1992. Mechanism of action of 5-arninosalicylic acid. *Mediators Inflamm* 1: 151-65
57. Nielsen OH, Munck LK. 2007. Drug insight: aminosalicylates for the treatment of IBD. *Nat Clin Pract Gastroenterol Hepatol* 4: 160-70
58. Kaiser GC, Yan F, Polk DB. 1999. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes. *Gastroenterology* 116: 602-9
59. Rubin DT, Bradette M, Gabalec L, Dobru D, Marquez J, Inglis S, Magee E, Solomon D, D'Haens G, Ulcerative Colitis Remission Study G. 2016. Ulcerative Colitis Remission Status After Induction With Mesalazine Predicts Maintenance Outcomes: the MOMENTUM Trial. *J Crohns Colitis* 10: 925-33
60. Derkx B, Taminiau J, Radema S, Stronkhorst A, Wortel C, Tytgat G, van Deventer S. 1993. Tumour-necrosis-factor antibody treatment in Crohn's disease. *Lancet* 342: 173-4
61. Rutgeerts P, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 353: 2462-76
62. Levin AD, Wildenberg ME, van den Brink GR. 2016. Mechanism of Action of Anti-TNF Therapy in Inflammatory Bowel Disease. *J Crohns Colitis* 10: 989-97
63. Reinisch W, Sandborn WJ, Hommes DW, D'Haens G, Hanauer S, Schreiber S, Panaccione R, Fedorak RN, Tighe MB, Huang B, Kampman W, Lazar A, Thakkar R. 2011. Adalimumab for induction of clinical remission in moderately to severely active ulcerative colitis: results of a randomised controlled trial. *Gut* 60: 780-7
64. Sandborn WJ, van Assche G, Reinisch W, Colombel JF, D'Haens G, Wolf DC, Kron M, Tighe MB, Lazar A, Thakkar RB. 2012. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 142: 257-65 e1-3
65. Sandborn WJ, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, Adedokun OJ, Guzzo C, Colombel JF, Reinisch W, Gibson PR, Collins J, Jarnerot G, Rutgeerts P, Group PU-MS. 2014. Subcutaneous golimumab maintains clinical response in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 146: 96-109 e1
66. Feagan BG, Rutgeerts P, Sands BE, Hanauer S, Colombel JF, Sandborn WJ, Van Assche G, Axler J, Kim HJ, Danese S, Fox I, Milch C, Sankoh S, Wyant T, Xu J, Parikh A, Group GS. 2013. Vedolizumab as induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 369: 699-710

67. Roda G, Jharap B, Neeraj N, Colombel JF. 2016. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 7: e135
68. Ben-Horin S, Kopylov U, Chowers Y. 2014. Optimizing anti-TNF treatments in inflammatory bowel disease. *Autoimmun Rev* 13: 24-30
69. Danese S, Vuitton L, Peyrin-Biroulet L. 2015. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 12: 537-45
70. Coskun M, Vermeire S, Nielsen OH. 2017. Novel Targeted Therapies for Inflammatory Bowel Disease. *Trends Pharmacol Sci* 38: 127-42
71. Verstockt B, Ferrante M, Vermeire S, Van Assche G. 2018. New treatment options for inflammatory bowel diseases. *J Gastroenterol* 53: 585-90
72. Komaki Y, Komaki F, Sakuraba A, Cohen R. 2016. Approach to Optimize Anti-TNF-alpha Therapy in Patients With IBD. *Curr Treat Options Gastroenterol* 14: 83-90
73. Papamichael K, Cheifetz AS, Melmed GY, Irving PM, Vande Casteele N, Kozuch PL, Raffals LE, Baidoo L, Bressler B, Devlin SM, Jones J, Kaplan GG, Sparrow MP, Velayos FS, Ullman T, Siegel CA. 2019. Appropriate Therapeutic Drug Monitoring of Biologic Agents for Patients With Inflammatory Bowel Diseases. *Clin Gastroenterol Hepatol* 17: 1655-68 e3
74. Hendy P, Hart A, Irving P. 2016. Anti-TNF drug and antidrug antibody level monitoring in IBD: a practical guide. *Frontline Gastroenterol* 7: 122-8
75. Papamichael K, Cheifetz AS. 2019. Is It Prime Time for Proactive Therapeutic Drug Monitoring of Anti-Tumor Necrosis Factor Therapy in Inflammatory Bowel Disease? *Gastroenterology* 157: 922-4
76. Torres J, Burisch J, Riddle M, Dubinsky M, Colombel JF. 2016. Preclinical disease and preventive strategies in IBD: perspectives, challenges and opportunities. *Gut* 65: 1061-9
77. Xavier RJ, Podolsky DK. 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448: 427-34
78. Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Farkkila M, Kontula K. 2006. Family and twin studies in inflammatory bowel disease. *World J Gastroenterol* 12: 3668-72
79. Gordon H, Trier Moller F, Andersen V, Harbord M. 2015. Heritability in inflammatory bowel disease: from the first twin study to genome-wide association studies. *Inflamm Bowel Dis* 21: 1428-34
80. Podolsky DK. 2002. Inflammatory bowel disease. *N Engl J Med* 347: 417-29
81. Piovani D, Danese S, Peyrin-Biroulet L, Nikolopoulos GK, Lytras T, Bonovas S. 2019. Environmental Risk Factors for Inflammatory Bowel Diseases: An Umbrella Review of Meta-analyses. *Gastroenterology* 157: 647-59 e4

82. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, Ripke S, Lee JC, Jostins L, Shah T, Abedian S, Cheon JH, Cho J, Dayani NE, Franke L, Fuyuno Y, Hart A, Juyal RC, Juyal G, Kim WH, Morris AP, Poustchi H, Newman WG, Midha V, Orchard TR, Vahedi H, Sood A, Sung JY, Malekzadeh R, Westra HJ, Yamazaki K, Yang SK, International Multiple Sclerosis Genetics C, International IBDGC, Barrett JC, Alizadeh BZ, Parkes M, Bk T, Daly MJ, Kubo M, Anderson CA, Weersma RK. 2015. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* 47: 979-86

83. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, et al. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 491: 119-24

84. Lees CW, Barrett JC, Parkes M, Satsangi J. 2011. New IBD genetics: common pathways with other diseases. *Gut* 60: 1739-53

85. Lee JC, Biasci D, Roberts R, Gearry RB, Mansfield JC, Ahmad T, Prescott NJ, Satsangi J, Wilson DC, Jostins L, Anderson CA, Consortium UIG, Traherne JA, Lyons PA, Parkes M, Smith KG. 2017. Genome-wide association study identifies distinct genetic contributions to prognosis and susceptibility in Crohn's disease. *Nat Genet* 49: 262-8

86. Lee HS, Yang SK, Hong M, Jung S, Kim BM, Moon JW, Park SH, Ye BD, Oh SH, Kim KM, Yoon YS, Yu CS, Baek J, Lee CH, Han B, Liu J, Haritunians T, McGovern DPB, Song K. 2018. An intergenic variant rs9268877 between HLA-DRA and HLA-DRB contributes to the clinical course and long-term outcome of ulcerative colitis. *J Crohns Colitis*

87. Kolho KL, Paakkanen R, Lepisto A, Wennerstom A, Meri S, Lokki ML. 2016. Novel Associations Between Major Histocompatibility Complex and Pediatric-onset Inflammatory Bowel Disease. *J Pediatr Gastroenterol Nutr* 62: 567-72

88. Billiet T, Vande Casteele N, Van Stappen T, Princen F, Singh S, Gils A, Ferrante M, Van Assche G, Cleynen I, Vermeire S. 2015. Immunogenicity to infliximab is associated with HLA-DRB1. *Gut* 64: 1344-5

89. Mirkov MU, Verstockt B, Cleynen I. 2017. Genetics of inflammatory bowel disease: beyond NOD2. *Lancet Gastroenterol Hepatol* 2: 224-34

90. Momozawa Y, Dmitrieva J, Theatre E, Deffontaine V, Rahmouni S, Charloteaux B, Crins F, Docampo E, Elansary M, Gori AS, Lecut C, Mariman R, Mni M, Oury C, Altukhov I, Alexeev D, Aulchenko Y, Amininejad L, Bouma G, Hoentjen F, Lowenberg M, Oldenburg B, Pierik MJ, Vander Meulen-de Jong AE, Janneke van der Woude C, Visschedijk MC, International IBDGC, Lathrop M, Hugot JP, Weersma RK, De Vos M, Franchimont D, Vermeire S, Kubo M, Louis E, Georges M. 2018.

IBD risk loci are enriched in multigenic regulatory modules encompassing putative causative genes. *Nat Commun* 9: 2427

91. Schwerk J, Savan R. 2015. Translating the Untranslated Region. *J Immunol* 195: 2963-71
92. Zwiers A, Kraal L, van de Pouw Kraan TC, Wurdinger T, Bouma G, Kraal G. 2012. Cutting edge: a variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production. *J Immunol* 188: 1573-7
93. Peterson LW, Artis D. 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* 14: 141-53
94. Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper LV. 2011. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science* 334: 255-8
95. Jager S, Stange EF, Wehkamp J. 2013. Inflammatory bowel disease: an impaired barrier disease. *Langenbecks Arch Surg* 398: 1-12
96. Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Buller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 131: 117-29
97. Garcia-Hernandez V, Quiros M, Nusrat A. 2017. Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. *Ann N Y Acad Sci* 1397: 66-79
98. Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. 2007. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 56: 61-72
99. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118: 229-41
100. Rugtveit J, Nilsen EM, Bakka A, Carlsen H, Brandtzaeg P, Scott H. 1997. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* 112: 1493-505
101. Bain CC, Mowat AM. 2014. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev* 260: 102-17
102. Mowat AM, Bain CC. 2011. Mucosal macrophages in intestinal homeostasis and inflammation. *J Innate Immun* 3: 550-64

103. Bouma G, Strober W. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 3: 521-33

104. Kmiec Z, Cyman M, Slebioda TJ. 2017. Cells of the innate and adaptive immunity and their interactions in inflammatory bowel disease. *Adv Med Sci* 62: 1-16

105. Lee GR, Kim ST, Spilianakis CG, Fields PE, Flavell RA. 2006. T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* 24: 369-79

106. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 154: 5071-9

107. Neurath MF, Fuss I, Kelsall BL, Stuber E, Strober W. 1995. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 182: 1281-90

108. Mosmann TR, Coffman RL. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7: 145-73

109. Zenewicz LA, Antov A, Flavell RA. 2009. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med* 15: 199-207

110. Vainer B, Nielsen OH, Hendel J, Horn T, Kirman I. 2000. Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease. *Cytokine* 12: 1531-6

111. Kadivar K, Ruchelli ED, Markowitz JE, Defelice ML, Strogatz ML, Kanzaria MM, Reddy KP, Baldassano RN, von Allmen D, Brown KA. 2004. Intestinal interleukin-13 in pediatric inflammatory bowel disease patients. *Inflamm Bowel Dis* 10: 593-8

112. Bernardo D, Vallejo-Diez S, Mann ER, Al-Hassi HO, Martinez-Abad B, Montalvillo E, Tee CT, Murugananthan AU, Nunez H, Peake ST, Hart AL, Fernandez-Salazar L, Garrote JA, Arranz E, Knight SC. 2012. IL-6 promotes immune responses in human ulcerative colitis and induces a skin-homing phenotype in the dendritic cells and Tcells they stimulate. *Eur J Immunol* 42: 1337-53

113. Galvez J. 2014. Role of Th17 Cells in the Pathogenesis of Human IBD. *ISRN Inflamm* 2014: 928461

114. Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116: 1310-6

115. Hue S, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS, Powrie F, Maloy KJ. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 203: 2473-83

116. Weaver CT, Elson CO, Fouser LA, Kolls JK. 2013. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annu Rev Pathol* 8: 477-512

117. Nielsen OH, Kirman I, Rudiger N, Hendel J, Vainer B. 2003. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. *Scand J Gastroenterol* 38: 180-5

118. Sugihara T, Kobori A, Imaeda H, Tsujikawa T, Amagase K, Takeuchi K, Fujiyama Y, Andoh A. 2010. The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease. *Clin Exp Immunol* 160: 386-93

119. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C, Pin JJ, Garrone P, Garcia E, Saeland S, Blanchard D, Gaillard C, Das Mahapatra B, Rouvier E, Golstein P, Banchereau J, Lebecque S. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 183: 2593-603

120. Schwarzenberger P, La Russa V, Miller A, Ye P, Huang W, Zieske A, Nelson S, Bagby GJ, Stoltz D, Mynatt RL, Spriggs M, Kolls JK. 1998. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J Immunol* 161: 6383-9

121. Bettelli E, Oukka M, Kuchroo VK. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8: 345-50

122. Siakavellas SI, Bamias G. 2012. Role of the IL-23/IL-17 axis in Crohn's disease. *Discov Med* 14: 253-62

123. Iwakura Y, Ishigame H, Saijo S, Nakae S. 2011. Functional specialization of interleukin-17 family members. *Immunity* 34: 149-62

124. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811-21

125. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. 2008. Regulation of inflammatory responses by IL-17F. *J Exp Med* 205: 1063-75

126. Tang C, Kakuta S, Shimizu K, Kadoki M, Kamiya T, Shimazu T, Kubo S, Saijo S, Ishigame H, Nakae S, Iwakura Y. 2018. Suppression of IL-17F, but not of IL-17A, provides protection against colitis by inducing Treg cells through modification of the intestinal microbiota. *Nat Immunol* 19: 755-65

127. Ogawa A, Andoh A, Araki Y, Bamba T, Fujiyama Y. 2004. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin Immunol* 110: 55-62

128. O'Connor W, Jr., Kamanaka M, Booth CJ, Town T, Nakae S, Iwakura Y, Kolls JK, Flavell RA. 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol* 10: 603-9

129. Song X, Dai D, He X, Zhu S, Yao Y, Gao H, Wang J, Qu F, Qiu J, Wang H, Li X, Shen N, Qian Y. 2015. Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. *Immunity* 43: 488-501

130. Lee JS, Tato CM, Joyce-Shaikh B, Gulen MF, Cayatte C, Chen Y, Blumenschein WM, Judo M, Ayanoglu G, McClanahan TK, Li X, Cua DJ. 2015. Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability. *Immunity* 43: 727-38

131. Maxwell JR, Zhang Y, Brown WA, Smith CL, Byrne FR, Fiorino M, Stevens E, Bigler J, Davis JA, Rottman JB, Budelsky AL, Symons A, Towne JE. 2015. Differential Roles for Interleukin-23 and Interleukin-17 in Intestinal Immunoregulation. *Immunity* 43: 739-50

132. Ohnmacht C, Marques R, Presley L, Sawa S, Lochner M, Eberl G. 2011. Intestinal microbiota, evolution of the immune system and the bad reputation of pro-inflammatory immunity. *Cell Microbiol* 13: 653-9

133. Wolk K, Sabat R. 2006. Interleukin-22: a novel T- and NK-cell derived cytokine that regulates the biology of tissue cells. *Cytokine Growth Factor Rev* 17: 367-80

134. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 118: 534-44

135. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 29: 947-57

136. Zenewicz LA, Flavell RA. 2011. Recent advances in IL-22 biology. *Int Immunol* 23: 159-63

137. Geremia A, Arancibia-Carcamo CV. 2017. Innate Lymphoid Cells in Intestinal Inflammation. *Front Immunol* 8: 1296

138. McVay LD, Li B, Biancaniello R, Creighton MA, Bachwich D, Lichtenstein G, Rombeau JL, Carding SR. 1997. Changes in human mucosal gamma delta T cell repertoire and function associated with the disease process in inflammatory bowel disease. *Mol Med* 3: 183-203

139. Kuhl AA, Loddenkemper C, Westermann J, Hoffmann JC. 2002. Role of gamma delta T cells in inflammatory bowel disease. *Pathobiology* 70: 150-5

140. Ferreira LM. 2013. Gammadelta T cells: innately adaptive immune cells? *Int Rev Immunol* 32: 223-48

141. Li M, Wang B, Sun X, Tang Y, Wei X, Ge B, Tang Y, Deng Y, He C, Yuan J, Li X. 2017. Upregulation of Intestinal Barrier Function in Mice with DSS-Induced Colitis by a Defined Bacterial Consortium Is Associated with Expansion of IL-17A Producing Gamma Delta T Cells. *Front Immunol* 8: 824
142. Kober OI, Ahl D, Pin C, Holm L, Carding SR, Juge N. 2014. gammadelta T-cell-deficient mice show alterations in mucin expression, glycosylation, and goblet cells but maintain an intact mucus layer. *Am J Physiol Gastrointest Liver Physiol* 306: G582-93
143. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. 2015. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* 74: 5-17
144. Tanoue T, Atarashi K, Honda K. 2016. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol* 16: 295-309
145. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 66: 5224-31
146. Saruta M, Yu QT, Fleshner PR, Mantel PY, Schmidt-Weber CB, Banham AH, Papadakis KA. 2007. Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. *Clin Immunol* 125: 281-90
147. Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A, Zeitz M, Duchmann R. 2005. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* 128: 1868-78
148. Yu QT, Saruta M, Avanesyan A, Fleshner PR, Banham AH, Papadakis KA. 2007. Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis. *Inflamm Bowel Dis* 13: 191-9
149. Brandtzaeg P, Johansen FE. 2005. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev* 206: 32-63
150. Wang X, Hao GL, Wang BY, Gao CC, Wang YX, Li LS, Xu JD. 2019. Function and dysfunction of plasma cells in intestine. *Cell Biosci* 9: 26
151. Herlands RA, Christensen SR, Sweet RA, Hershberg U, Shlomchik MJ. 2008. T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29: 249-60
152. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603-7
153. Macpherson AJ, Yilmaz B, Limenitakis JP, Ganal-Vonarburg SC. 2018. IgA Function in Relation to the Intestinal Microbiota. *Annu Rev Immunol* 36: 359-81

154. Gutzeit C, Magri G, Cerutti A. 2014. Intestinal IgA production and its role in host-microbe interaction. *Immunol Rev* 260: 76-85

155. Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, Degnan PH, Hu J, Peter I, Zhang W, Ruggiero E, Cho JH, Goodman AL, Flavell RA. 2014. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158: 1000-10

156. Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. 2008. The immune geography of IgA induction and function. *Mucosal Immunol* 1: 11-22

157. Pabst O. 2012. New concepts in the generation and functions of IgA. *Nat Rev Immunol* 12: 821-32

158. Brandtzaeg P, Carlsen HS, Halstensen TS. 2006. The B-cell system in inflammatory bowel disease. *Adv Exp Med Biol* 579: 149-67

159. Cupi ML, Sarra M, Marafini I, Monteleone I, Franze E, Ortenzi A, Colantoni A, Sica G, Sileri P, Rosado MM, Carsetti R, MacDonald TT, Pallone F, Monteleone G. 2014. Plasma cells in the mucosa of patients with inflammatory bowel disease produce granzyme B and possess cytotoxic activities. *J Immunol* 192: 6083-91

160. Vidarsson G, Dekkers G, Rispens T. 2014. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 5: 520

161. van der Waaij LA, Kroese FG, Visser A, Nelis GF, Westerveld BD, Jansen PL, Hunter JO. 2004. Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 16: 669-74

162. Harmsen HJ, Pouwels SD, Funke A, Bos NA, Dijkstra G. 2012. Crohn's disease patients have more IgG-binding fecal bacteria than controls. *Clin Vaccine Immunol* 19: 515-21

163. O'Mahony S, Barton JR, Crichton S, Ferguson A. 1990. Appraisal of gut lavage in the study of intestinal humoral immunity. *Gut* 31: 1341-4

164. Armstrong H, Alipour M, Valcheva R, Bording-Jorgensen M, Jovel J, Zaidi D, Shah P, Lou Y, Ebeling C, Mason AL, Lafleur D, Jerasi J, Wong GK, Madsen K, Carroll MW, Huynh HQ, Dieleman LA, Wine E. 2019. Host immunoglobulin G selectively identifies pathobionts in pediatric inflammatory bowel diseases. *Microbiome* 7: 1

165. Lin R, Chen H, Shu W, Sun M, Fang L, Shi Y, Pang Z, Wu W, Liu Z. 2018. Clinical significance of soluble immunoglobulins A and G and their coated bacteria in feces of patients with inflammatory bowel disease. *J Transl Med* 16: 359

166. Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. 1996. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* 38: 365-75

167. Baklien K, Brandtzaeg P. 1975. Comparative mapping of the local distribution of immunoglobulin-containing cells in ulcerative colitis and Crohn's disease of the colon. *Clin Exp Immunol* 22: 197-209

168. Castro-Dopico T, Dennison TW, Ferdinand JR, Mathews RJ, Fleming A, Clift D, Stewart BJ, Jing C, Strongili K, Labzin LI, Monk EJM, Saeb-Parsy K, Bryant CE, Clare S, Parkes M, Clatworthy MR. 2019. Anti-commensal IgG Drives Intestinal Inflammation and Type 17 Immunity in Ulcerative Colitis. *Immunity* 50: 1099-114 e10

169. Gouni-Berthold I, Baumeister B, Berthold HK, Schmidt C. 1999. Immunoglobulins and IgG subclasses in patients with inflammatory bowel disease. *Hepatogastroenterology* 46: 1720-3

170. Brandtzaeg P, Baklien K, Fausa O, Hoel PS. 1974. Immunohistochemical characterization of local immunoglobulin formation in ulcerative colitis. *Gastroenterology* 66: 1123-36

171. Keren DF, Appelman HD, Dobbins WO, 3rd, Wells JJ, Whisenant B, Foley J, Dieterle R, Geisinger K. 1984. Correlation of histopathologic evidence of disease activity with the presence of immunoglobulin-containing cells in the colons of patients with inflammatory bowel disease. *Hum Pathol* 15: 757-63

172. Goulet DR, Atkins WM. 2020. Considerations for the Design of Antibody-Based Therapeutics. *J Pharm Sci* 109: 74-103

173. van Schouwenburg PA, Krieckaert CL, Nurmohamed M, Hart M, Rispens T, Aarden L, Wouters D, Wolbink GJ. 2012. IgG4 production against adalimumab during long term treatment of RA patients. *J Clin Immunol* 32: 1000-6

174. Nimmerjahn F, Ravetch JV. 2008. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 8: 34-47

175. Furrie E, Macfarlane S, Cummings JH, Macfarlane GT. 2004. Systemic antibodies towards mucosal bacteria in ulcerative colitis and Crohn's disease differentially activate the innate immune response. *Gut* 53: 91-8

176. Uo M, Hisamatsu T, Miyoshi J, Kaito D, Yoneno K, Kitazume MT, Mori M, Sugita A, Koganei K, Matsuoka K, Kanai T, Hibi T. 2013. Mucosal CXCR4+ IgG plasma cells contribute to the pathogenesis of human ulcerative colitis through FcgammaR-mediated CD14 macrophage activation. *Gut* 62: 1734-44

177. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak

S, Dore J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Meta HITC, Bork P, Ehrlich SD, Wang J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464: 59-65

178. Sekirov I, Russell SL, Antunes LC, Finlay BB. 2010. Gut microbiota in health and disease. *Physiol Rev* 90: 859-904
179. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, Ley RE. 2014. Human genetics shape the gut microbiome. *Cell* 159: 789-99
180. Munger E, Montiel-Castro AJ, Langhans W, Pacheco-Lopez G. 2018. Reciprocal Interactions Between Gut Microbiota and Host Social Behavior. *Front Integr Neurosci* 12: 21
181. An R, Wilms E, Masclee AAM, Smidt H, Zoetendal EG, Jonkers D. 2018. Age-dependent changes in GI physiology and microbiota: time to reconsider? *Gut* 67: 2213-22
182. Belkaid Y, Hand TW. 2014. Role of the microbiota in immunity and inflammation. *Cell* 157: 121-41
183. Neish AS. 2009. Microbes in gastrointestinal health and disease. *Gastroenterology* 136: 65-80
184. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, Mele MC. 2019. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* 7
185. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. 2005. Diversity of the human intestinal microbial flora. *Science* 308: 1635-8
186. Morelli L. 2008. Postnatal development of intestinal microflora as influenced by infant nutrition. *J Nutr* 138: 1791S-5S
187. Li J, Butcher J, Mack D, Stintzi A. 2015. Functional impacts of the intestinal microbiome in the pathogenesis of inflammatory bowel disease. *Inflamm Bowel Dis* 21: 139-53
188. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 104: 13780-5
189. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner

F, Pedersen O, de Vos WM, Brunak S, Dore J, Meta HITC, Antolin M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Merieux A, Melo Minardi R, M'Rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. 2011. Enterotypes of the human gut microbiome. *Nature* 473: 174-80

190. Halfvarson J, Brislawn CJ, Lamendella R, Vazquez-Baeza Y, Walters WA, Bramer LM, D'Amato M, Bonfiglio F, McDonald D, Gonzalez A, McClure EE, Dunklebarger MF, Knight R, Jansson JK. 2017. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol* 2: 17004
191. Dickson I. 2017. Gut microbiota: Diagnosing IBD with the gut microbiome. *Nat Rev Gastroenterol Hepatol* 14: 195
192. Hornef M. 2015. Pathogens, Commensal Symbionts, and Pathobionts: Discovery and Functional Effects on the Host. *ILAR J* 56: 159-62
193. Tamboli CP, Neut C, Desreumaux P, Colombel JF. 2004. Dysbiosis in inflammatory bowel disease. *Gut* 53: 1-4
194. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. 2011. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 60: 631-7
195. Sokol H, Seksik P, Rigottier-Gois L, Lay C, Lepage P, Podglajen I, Marteau P, Dore J. 2006. Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis* 12: 106-11
196. Vigsnaes LK, van den Abbeele P, Sulek K, Frandsen HL, Steenholdt C, Brynskov J, Vermeiren J, van de Wiele T, Licht TR. 2013. Microbiotas from UC patients display altered metabolism and reduced ability of LAB to colonize mucus. *Sci Rep* 3: 1110
197. Nagao-Kitamoto H, Kamada N. 2017. Host-microbial Cross-talk in Inflammatory Bowel Disease. *Immune Netw* 17: 1-12
198. Hou JK, Abraham B, El-Serag H. 2011. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Am J Gastroenterol* 106: 563-73
199. Castro F, de Souza HSP. 2019. Dietary Composition and Effects in Inflammatory Bowel Disease. *Nutrients* 11
200. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ,

Huttenhower C. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 13: R79

201. Shaw SY, Blanchard JF, Bernstein CN. 2010. Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease. *Am J Gastroenterol* 105: 2687-92
202. Ungaro R, Bernstein CN, Gearry R, Hviid A, Kolho KL, Kronman MP, Shaw S, Van Kruiningen H, Colombel JF, Atreja A. 2014. Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. *Am J Gastroenterol* 109: 1728-38
203. Stecher B. 2015. The Roles of Inflammation, Nutrient Availability and the Commensal Microbiota in Enteric Pathogen Infection. *Microbiol Spectr* 3
204. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2: 119-29
205. Xu J, Chen N, Wu Z, Song Y, Zhang Y, Wu N, Zhang F, Ren X, Liu Y. 2018. 5-Aminosalicylic Acid Alters the Gut Bacterial Microbiota in Patients With Ulcerative Colitis. *Front Microbiol* 9: 1274
206. Kamada N, Seo SU, Chen GY, Nunez G. 2013. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* 13: 321-35
207. Darfeuille-Michaud A, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P, Gambiez L, Joly B, Cortot A, Colombel JF. 1998. Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 115: 1405-13
208. Sorbara MT, Pamer EG. 2019. Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunol* 12: 1-9
209. Medellin-Pena MJ, Wang H, Johnson R, Anand S, Griffiths MW. 2007. Probiotics affect virulence-related gene expression in Escherichia coli O157:H7. *Appl Environ Microbiol* 73: 4259-67
210. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331: 337-41
211. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500: 232-6

212. Round JL, Mazmanian SK. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* 107: 12204-9

213. O'Mahony C, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, Sherlock G, MacSharry J, Kiely B, Shanahan F, O'Mahony L. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog* 4: e1000112

214. Hayashi A, Sato T, Kamada N, Mikami Y, Matsuoka K, Hisamatsu T, Hibi T, Roers A, Yagita H, Ohteki T, Yoshimura A, Kanai T. 2013. A single strain of Clostridium butyricum induces intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. *Cell Host Microbe* 13: 711-22

215. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139: 485-98

216. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, Langella P. 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 105: 16731-6

217. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S. 2014. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* 63: 1275-83

218. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A* 111: 2247-52

219. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, Lee JR, Offermanns S, Ganapathy V. 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* 40: 128-39

220. Palmer R. 2011. Fecal matters. *Nat Med* 17: 150-2

221. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB. 2008. Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. *J Infect Dis* 197: 435-8

222. Hamilton MJ, Weingarden AR, Unno T, Khoruts A, Sadowsky MJ. 2013. High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut Microbes* 4: 125-35

223. Li SS, Zhu A, Benes V, Costea PI, Hercog R, Hildebrand F, Huerta-Cepas J, Nieuwdorp M, Salojarvi J, Voigt AY, Zeller G, Sunagawa S, de Vos WM, Bork P. 2016. Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science* 352: 586-9

224. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller JJ. 2013. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* 368: 407-15

225. Rossen NG, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JH, Duflou A, Lowenberg M, van den Brink GR, Mathus-Vliegen EM, de Vos WM, Zoetendal EG, D'Haens GR, Ponsioen CY. 2015. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. *Gastroenterology* 149: 110-8 e4

226. Colman RJ, Rubin DT. 2014. Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *J Crohns Colitis* 8: 1569-81

227. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, Armstrong D, Marshall JK, Kassam Z, Reinisch W, Lee CH. 2015. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. *Gastroenterology* 149: 102-9 e6

228. Varki A. 2017. Biological roles of glycans. *Glycobiology* 27: 3-49

229. Varki A, Kornfeld S. 2015. Historical Background and Overview. In *Essentials of Glycobiology*, ed. rd, A Varki, RD Cummings, JD Esko, P Stanley, GW Hart, M Aebi, AG Darvill, T Kinoshita, NH Packer, JH Prestegard, RL Schnaar, PH Seeberger, pp. 1-18. Cold Spring Harbor (NY)

230. Marth JD, Grewal PK. 2008. Mammalian glycosylation in immunity. *Nat Rev Immunol* 8: 874-87

231. Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell* 126: 855-67

232. Dias AM, Pereira MS, Padrao NA, Alves I, Marcos-Pinto R, Lago P, Pinho SS. 2018. Glycans as critical regulators of gut immunity in homeostasis and disease. *Cell Immunol* 333: 9-18

233. Spiro RG. 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12: 43R-56R

234. Moremen KW, Tiemeyer M, Nairn AV. 2012. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol* 13: 448-62
235. Braakman I, Hebert DN. 2013. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol* 5: a013201
236. Caramelo JJ, Parodi AJ. 2015. A sweet code for glycoprotein folding. *FEBS Lett* 589: 3379-87
237. Helenius A, Aebi M. 2001. Intracellular functions of N-linked glycans. *Science* 291: 2364-9
238. Dennis JW, Nabi IR, Demetriou M. 2009. Metabolism, cell surface organization, and disease. *Cell* 139: 1229-41
239. Lau KS, Partridge EA, Grigorian A, Silvescu CI, Reinhold VN, Demetriou M, Dennis JW. 2007. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* 129: 123-34
240. Johnson JL, Jones MB, Ryan SO, Cobb BA. 2013. The regulatory power of glycans and their binding partners in immunity. *Trends Immunol* 34: 290-8
241. Rabinovich GA, van Kooyk Y, Cobb BA. 2012. Glycobiology of immune responses. *Ann N Y Acad Sci* 1253: 1-15
242. Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrao NA, Pinto V, Fernandes A, Dias AM, Pinho SS. 2018. Glycans as Key Checkpoints of T Cell Activity and Function. *Front Immunol* 9: 2754
243. Liu FT, Rabinovich GA. 2005. Galectins as modulators of tumour progression. *Nat Rev Cancer* 5: 29-41
244. Schetters STT, Kruijssen LJW, Crommentuijn MHW, Kalay H, Ochando J, den Haan JMM, Garcia-Vallejo JJ, van Kooyk Y. 2018. Mouse DC-SIGN/CD209a as Target for Antigen Delivery and Adaptive Immunity. *Front Immunol* 9: 990
245. Gringhuis SI, Kaptein TM, Wevers BA, Mesman AW, Geijtenbeek TB. 2014. Fucose-specific DC-SIGN signalling directs T helper cell type-2 responses via IKKepsilon- and CYLD-dependent Bcl3 activation. *Nat Commun* 5: 3898
246. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB. 2009. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nat Immunol* 10: 1081-8
247. Eriksson M, Johannssen T, von Smolinski D, Gruber AD, Seeberger PH, Lepenies B. 2013. The C-Type Lectin Receptor SIGNR3 Binds to Fungi Present in Commensal Microbiota and Influences Immune Regulation in Experimental Colitis. *Front Immunol* 4: 196

248. Anderton SM. 2004. Post-translational modifications of self antigens: implications for autoimmunity. *Curr Opin Immunol* 16: 753-8

249. Purcell AW, van Driel IR, Gleeson PA. 2008. Impact of glycans on T-cell tolerance to glycosylated self-antigens. *Immunol Cell Biol* 86: 574-9

250. Chui D, Sellakumar G, Green R, Sutton-Smith M, McQuistan T, Marek K, Morris H, Dell A, Marth J. 2001. Genetic remodeling of protein glycosylation in vivo induces autoimmune disease. *Proc Natl Acad Sci U S A* 98: 1142-7

251. Green RS, Stone EL, Tenno M, Lehtonen E, Farquhar MG, Marth JD. 2007. Mammalian N-glycan branching protects against innate immune self-recognition and inflammation in autoimmune disease pathogenesis. *Immunity* 27: 308-20

252. Suzuki K, Yamada T, Yamazaki K, Hirota M, Ishihara N, Sakamoto M, Takahashi D, Iijima H, Hase K. 2018. Intestinal Epithelial Cell-specific Deletion of alpha-Mannosidase II Ameliorates Experimental Colitis. *Cell Struct Funct* 43: 25-39

253. Cobb BA, Wang Q, Tzianabos AO, Kasper DL. 2004. Polysaccharide processing and presentation by the MHCII pathway. *Cell* 117: 677-87

254. Johnson JL, Jones MB, Cobb BA. 2015. Polysaccharide A from the capsule of Bacteroides fragilis induces clonal CD4+ T cell expansion. *J Biol Chem* 290: 5007-14

255. Ryan SO, Bonomo JA, Zhao F, Cobb BA. 2011. MHCII glycosylation modulates Bacteroides fragilis carbohydrate antigen presentation. *J Exp Med* 208: 1041-53

256. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. 2001. Glycosylation and the immune system. *Science* 291: 2370-6

257. Wolfert MA, Boons GJ. 2013. Adaptive immune activation: glycosylation does matter. *Nat Chem Biol* 9: 776-84

258. Papa Gobbi R, De Francesco N, Bondar C, Muglia C, Chirdo F, Rumbo M, Rocca A, Toscano MA, Sambuelli A, Rabinovich GA, Docena GH. 2016. A galectin-specific signature in the gut delineates Crohn's disease and ulcerative colitis from other human inflammatory intestinal disorders. *Biofactors* 42: 93-105

259. Jensen-Jarolim E, Gscheidlinger R, Oberhuber G, Neuchrist C, Lucas T, Bises G, Radauer C, Willheim M, Scheiner O, Liu FT, Boltz-Nitulescu G. 2002. The constitutive expression of galectin-3 is downregulated in the intestinal epithelia of Crohn's disease patients, and tumour necrosis factor alpha decreases the level of galectin-3-specific mRNA in HCT-8 cells. *Eur J Gastroenterol Hepatol* 14: 145-52

260. Muller S, Schaffer T, Flogerzi B, Fleetwood A, Weimann R, Schoepfer AM, Seibold F. 2006. Galectin-3 modulates T cell activity and is reduced in the inflamed intestinal epithelium in IBD. *Inflamm Bowel Dis* 12: 588-97

261. Lippert E, Gunckel M, Brenmoehl J, Bataille F, Falk W, Scholmerich J, Obermeier F, Rogler G. 2008. Regulation of galectin-3 function in mucosal fibroblasts: potential role in mucosal inflammation. *Clin Exp Immunol* 152: 285-97

262. Lippert E, Stieber-Gunckel M, Dunger N, Falk W, Obermeier F, Kunst C. 2015. Galectin-3 Modulates Experimental Colitis. *Digestion* 92: 45-53

263. Tsai HF, Wu CS, Chen YL, Liao HJ, Chyuan IT, Hsu PN. 2016. Galectin-3 suppresses mucosal inflammation and reduces disease severity in experimental colitis. *J Mol Med (Berl)* 94: 545-56

264. Dennis JW, Lau KS, Demetriou M, Nabi IR. 2009. Adaptive regulation at the cell surface by N-glycosylation. *Traffic* 10: 1569-78

265. Demetriou M, Granovsky M, Quaggin S, Dennis JW. 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409: 733-9

266. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, Maia L, Delerue-Matos C, Taniguchi N, Lima M, Pedroto I, Marcos-Pinto R, Lago P, Reis CA, Vilanova M, Pinho SS. 2018. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 115: E4651-E60

267. Comelli EM, Sutton-Smith M, Yan Q, Amado M, Panico M, Gilmartin T, Whisenant T, Lanigan CM, Head SR, Goldberg D, Morris HR, Dell A, Paulson JC. 2006. Activation of murine CD4+ and CD8+ T lymphocytes leads to dramatic remodeling of N-linked glycans. *J Immunol* 177: 2431-40

268. Chen HL, Li CF, Grigorian A, Tian W, Demetriou M. 2009. T cell receptor signaling co-regulates multiple Golgi genes to enhance N-glycan branching. *J Biol Chem* 284: 32454-61

269. Cabral J, Hanley SA, Gerlach JQ, O'Leary N, Cunningham S, Ritter T, Ceredig R, Joshi L, Griffin MD. 2017. Distinctive Surface Glycosylation Patterns Associated With Mouse and Human CD4(+) Regulatory T Cells and Their Suppressive Function. *Front Immunol* 8: 987

270. Dias AM, Dourado J, Lago P, Cabral J, Marcos-Pinto R, Salgueiro P, Almeida CR, Carvalho S, Fonseca S, Lima M, Vilanova M, Dinis-Ribeiro M, Reis CA, Pinho SS. 2014. Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 23: 2416-27

271. Salvatore S, Heuschkel R, Tomlin S, Davies SE, Edwards S, Walker-Smith JA, French I, Murch SH. 2000. A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment Pharmacol Ther* 14: 1567-79

272. Shinzaki S, Ishii M, Fujii H, Iijima H, Wakamatsu K, Kawai S, Shiraishi E, Hiyama S, Inoue T, Hayashi Y, Kuwahara R, Takamatsu S, Kamada Y, Morii E, Tsujii M, Takehara T, Miyoshi E. 2016. N-Acetylglucosaminyltransferase V exacerbates murine colitis with macrophage dysfunction and enhances colitic tumorigenesis. *J Gastroenterol* 51: 357-69

273. Klasic M, Markulin D, Vojta A, Samarzija I, Birus I, Dobrinic P, Ventham NT, Trbojevic-Akmacic I, Simurina M, Stambuk J, Razdorov G, Kennedy NA, Satsangi J, Dias AM, Pinho S, Annese V, Latiano A, D'Inca R, consortium IBD, Lauc G, Zoldos V. 2018. Promoter methylation of the MGAT3 and BACH2 genes correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease. *Clin Epigenetics* 10: 75

274. Fujii H, Shinzaki S, Iijima H, Wakamatsu K, Iwamoto C, Sobajima T, Kuwahara R, Hiyama S, Hayashi Y, Takamatsu S, Uozumi N, Kamada Y, Tsujii M, Taniguchi N, Takehara T, Miyoshi E. 2016. Core Fucosylation on T Cells, Required for Activation of T-Cell Receptor Signaling and Induction of Colitis in Mice, Is Increased in Patients With Inflammatory Bowel Disease. *Gastroenterology* 150: 1620-32

275. Jones MB. 2018. IgG and leukocytes: Targets of immunomodulatory alpha2,6 sialic acids. *Cell Immunol* 333: 58-64

276. Nose M, Wigzell H. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc Natl Acad Sci U S A* 80: 6632-6

277. Sola RJ, Griebenow K. 2010. Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy. *BioDrugs* 24: 9-21

278. Plomp R, Bondt A, de Haan N, Rombouts Y, Wuhrer M. 2016. Recent Advances in Clinical Glycoproteomics of Immunoglobulins (Igs). *Mol Cell Proteomics* 15: 2217-28

279. Kiyoshi M, Tsumoto K, Ishii-Watabe A, Caaveiro JMM. 2017. Glycosylation of IgG-Fc: a molecular perspective. *Int Immunol* 29: 311-7

280. Bakovic MP, Selman MH, Hoffmann M, Rudan I, Campbell H, Deelder AM, Lauc G, Wuhrer M. 2013. High-throughput IgG Fc N-glycosylation profiling by mass spectrometry of glycopeptides. *J Proteome Res* 12: 821-31

281. Novokmet M, Lukic E, Vuckovic F, Ethuric Z, Keser T, Rajsl K, Remondini D, Castellani G, Gasparovic H, Gornik O, Lauc G. 2014. Changes in IgG and total plasma protein glycomes in acute systemic inflammation. *Sci Rep* 4: 4347

282. Simurina M, de Haan N, Vuckovic F, Kennedy NA, Stambuk J, Falck D, Trbojevic-Akmacic I, Clerc F, Razdorov G, Khon A, Latiano A, D'Inca R, Danese S, Targan S, Landers C, Dubinsky M, Inflammatory Bowel Disease Biomarkers C, McGovern DPB, Annese V, Wuhrer M, Lauc G. 2018. Glycosylation of Immunoglobulin G

Associates With Clinical Features of Inflammatory Bowel Diseases. *Gastroenterology* 154: 1320-33 e10

283. Trbojevic Akmacic I, Ventham NT, Theodoratou E, Vuckovic F, Kennedy NA, Kristic J, Nimmo ER, Kalla R, Drummond H, Stambuk J, Dunlop MG, Novokmet M, Aulchenko Y, Gornik O, Campbell H, Pucic Bakovic M, Satsangi J, Lauc G, Consortium I-B. 2015. Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome. *Inflamm Bowel Dis* 21: 1237-47
284. Plomp R, Ruhaak LR, Uh HW, Reiding KR, Selman M, Houwing-Duistermaat JJ, Slagboom PE, Beekman M, Wuhrer M. 2017. Subclass-specific IgG glycosylation is associated with markers of inflammation and metabolic health. *Sci Rep* 7: 12325
285. Matsumoto A, Shikata K, Takeuchi F, Kojima N, Mizuochi T. 2000. Autoantibody activity of IgG rheumatoid factor increases with decreasing levels of galactosylation and sialylation. *J Biochem* 128: 621-8
286. Vuckovic F, Kristic J, Gudelj I, Teruel M, Keser T, Pezer M, Pucic-Bakovic M, Stambuk J, Trbojevic-Akmacic I, Barrios C, Pavic T, Menni C, Wang Y, Zhou Y, Cui L, Song H, Zeng Q, Guo X, Pons-Estel BA, McKeigue P, Leslie Patrick A, Gornik O, Spector TD, Harjacek M, Alarcon-Riquelme M, Molokhia M, Wang W, Lauc G. 2015. Association of systemic lupus erythematosus with decreased immunosuppressive potential of the IgG glycome. *Arthritis Rheumatol* 67: 2978-89
287. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313: 670-3
288. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. 2007. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. *Mol Immunol* 44: 1524-34
289. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. 2008. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320: 373-6
290. Anthony RM, Wermeling F, Ravetch JV. 2012. Novel roles for the IgG Fc glycan. *Ann N Y Acad Sci* 1253: 170-80
291. Karsten CM, Pandey MK, Figge J, Kilchenstein R, Taylor PR, Rosas M, McDonald JU, Orr SJ, Berger M, Petzold D, Blanchard V, Winkler A, Hess C, Reid DM, Majoul IV, Strait RT, Harris NL, Kohl G, Wex E, Ludwig R, Zillikens D, Nimmerjahn F, Finkelman FD, Brown GD, Ehlers M, Kohl J. 2012. Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcgammaRIIB and dectin-1. *Nat Med* 18: 1401-6

292. Houde D, Peng Y, Berkowitz SA, Engen JR. 2010. Post-translational modifications differentially affect IgG1 conformation and receptor binding. *Mol Cell Proteomics* 9: 1716-28

293. Dashivets T, Thomann M, Rueger P, Knaupp A, Buchner J, Schlothauer T. 2015. Multi-Angle Effector Function Analysis of Human Monoclonal IgG Glycovariants. *PLoS One* 10: e0143520

294. Subedi GP, Barb AW. 2016. The immunoglobulin G1 N-glycan composition affects binding to each low affinity Fc gamma receptor. *MAbs* 8: 1512-24

295. Nakajima S, Iijima H, Shinzaki S, Egawa S, Inoue T, Mukai A, Hayashi Y, Kondo J, Akasaka T, Nishida T, Kanto T, Morii E, Mizushima T, Miyoshi E, Tsujii M, Hayashi N. 2011. Functional analysis of agalactosyl IgG in inflammatory bowel disease patients. *Inflamm Bowel Dis* 17: 927-36

296. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat Med* 1: 237-43

297. Nimmerjahn F, Anthony RM, Ravetch JV. 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. *Proc Natl Acad Sci U S A* 104: 8433-7

298. Dong X, Storkus WJ, Salter RD. 1999. Binding and uptake of agalactosyl IgG by mannose receptor on macrophages and dendritic cells. *J Immunol* 163: 5427-34

299. Knezevic A, Polasek O, Gornik O, Rudan I, Campbell H, Hayward C, Wright A, Kolcic I, O'Donoghue N, Bones J, Rudd PM, Lauc G. 2009. Variability, heritability and environmental determinants of human plasma N-glycome. *J Proteome Res* 8: 694-701

300. Masuda K, Kubota T, Kaneko E, Iida S, Wakitani M, Kobayashi-Natsume Y, Kubota A, Shitara K, Nakamura K. 2007. Enhanced binding affinity for FcgammaRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. *Mol Immunol* 44: 3122-31

301. Iida S, Misaka H, Inoue M, Shibata M, Nakano R, Yamane-Ohnuki N, Wakitani M, Yano K, Shitara K, Satoh M. 2006. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcgammaRIIIa. *Clin Cancer Res* 12: 2879-87

302. Niwa R, Hatanaka S, Shoji-Hosaka E, Sakurada M, Kobayashi Y, Uehara A, Yokoi H, Nakamura K, Shitara K. 2004. Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 Is independent of FcgammaRIIIa functional polymorphism. *Clin Cancer Res* 10: 6248-55

303. Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M. 2001. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. *Biotechnol Bioeng* 74: 288-94
304. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Hennig M, Ruf A, Rufer AC, Stihle M, Umana P, Benz J. 2011. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A* 108: 12669-74
305. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277: 26733-40
306. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, Hanai N, Shitara K. 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* 278: 3466-73
307. Medzhitov R, Janeway C, Jr. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol* 8: 452-6
308. Tan FY, Tang CM, Exley RM. 2015. Sugar coating: bacterial protein glycosylation and host-microbe interactions. *Trends Biochem Sci* 40: 342-50
309. Halim A, Anonsen JH. 2017. Microbial glycoproteomics. *Curr Opin Struct Biol* 44: 143-50
310. Martens EC, Roth R, Heuser JE, Gordon JI. 2009. Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *J Biol Chem* 284: 18445-57
311. Arike L, Holmen-Larsson J, Hansson GC. 2017. Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases. *Glycobiology* 27: 318-28
312. Pickard JM, Chervonsky AV. 2015. Intestinal fucose as a mediator of host-microbe symbiosis. *J Immunol* 194: 5588-93
313. Coyne MJ, Reinap B, Lee MM, Comstock LE. 2005. Human symbionts use a host-like pathway for surface fucosylation. *Science* 307: 1778-81
314. Comstock LE, Kasper DL. 2006. Bacterial glycans: key mediators of diverse host immune responses. *Cell* 126: 847-50

315. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, Bogatyrev SR, Ismagilov RF, Pamer EG, Turnbaugh PJ, Chervonsky AV. 2014. Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness. *Nature* 514: 638-41

316. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, Sperandio V. 2012. Fucose sensing regulates bacterial intestinal colonization. *Nature* 492: 113-7

317. Pham TA, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, Keane JA, Page AJ, Kumasaka N, Kane L, Mottram L, Harcourt K, Hale C, Arends MJ, Gaffney DJ, Sanger Mouse Genetics P, Dougan G, Lawley TD. 2014. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe* 16: 504-16

318. Van Tyne D, Gilmore MS. 2014. A delicate balance: maintaining mutualism to prevent disease. *Cell Host Microbe* 16: 425-7

319. Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, Lebrilla CB, Weimer BC, Mills DA, German JB, Sonnenburg JL. 2011. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe* 10: 507-14

320. Martens EC, Chiang HC, Gordon JI. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe* 4: 447-57

321. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, Naidu N, Choudhury B, Weimer BC, Monack DM, Sonnenburg JL. 2013. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* 502: 96-9

322. Huang YL, Chassard C, Hausmann M, von Itzstein M, Hennet T. 2015. Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. *Nat Commun* 6: 8141

323. Vimr ER. 2013. Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. *ISRN Microbiol* 2013: 816713

324. Perdicchio M, Ilarregui JM, Verstege MI, Cornelissen LA, Schetters ST, Engels S, Ambrosini M, Kalay H, Veninga H, den Haan JM, van Berkel LA, Samsom JN, Crocker PR, Sparwasser T, Berod L, Garcia-Vallejo JJ, van Kooyk Y, Unger WW. 2016. Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. *Proc Natl Acad Sci U S A* 113: 3329-34

325. Zhou JY, Oswald DM, Oliva KD, Kreisman LSC, Cobb BA. 2018. The Glycoscience of Immunity. *Trends Immunol* 39: 523-35
326. Poole J, Day CJ, von Itzstein M, Paton JC, Jennings MP. 2018. Glycointeractions in bacterial pathogenesis. *Nat Rev Microbiol* 16: 440-52
327. Oommen AM, Somaiya N, Vijayan J, Kumar S, Venkatachalam S, Joshi L. 2016. GlycoGAIT: A web database to browse glycogenes and lectins under gastric inflammatory diseases. *J Theor Biol* 406: 93-8
328. Lyons JJ, Milner JD, Rosenzweig SD. 2015. Glycans Instructing Immunity: The Emerging Role of Altered Glycosylation in Clinical Immunology. *Front Pediatr* 3: 54
329. Reily C, Stewart TJ, Renfrow MB, Novak J. 2019. Glycosylation in health and disease. *Nat Rev Nephrol* 15: 346-66
330. Zhou RW, Mkhikian H, Grigorian A, Hong A, Chen D, Arakelyan A, Demetriou M. 2014. N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* 15: 1038-45
331. Brown SJ, Miller AM, Cowan PJ, Slavin J, Connell WR, Moore GT, Bell S, Elliott PR, Desmond PV, d'Apice AJ. 2004. Altered immune system glycosylation causes colitis in alpha1,2-fucosyltransferase transgenic mice. *Inflamm Bowel Dis* 10: 546-56
332. McGovern DP, Jones MR, Taylor KD, Marciante K, Yan X, Dubinsky M, Ippoliti A, Vasiliauskas E, Berel D, Derkowski C, Dutridge D, Fleshner P, Shih DQ, Melmed G, Mengesha E, King L, Pressman S, Haritunians T, Guo X, Targan SR, Rotter JI, International IBDGC. 2010. Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. *Hum Mol Genet* 19: 3468-76
333. Tong M, McHardy I, Ruegger P, Goudarzi M, Kashyap PC, Haritunians T, Li X, Graeber TG, Schwager E, Huttenhower C, Fornace AJ, Jr., Sonnenburg JL, McGovern DP, Borneman J, Braun J. 2014. Reprograming of gut microbiome energy metabolism by the FUT2 Crohn's disease risk polymorphism. *ISME J* 8: 2193-206
334. Hu D, Zhang D, Zheng S, Guo M, Lin X, Jiang Y. 2016. Association of Ulcerative Colitis with FUT2 and FUT3 Polymorphisms in Patients from Southeast China. *PLoS One* 11: e0146557
335. Shen X, Klaric L, Sharapov S, Mangino M, Ning Z, Wu D, Trbojevic-Akmacic I, Pucic-Bakovic M, Rudan I, Polasek O, Hayward C, Spector TD, Wilson JF, Lauc G, Aulchenko YS. 2017. Multivariate discovery and replication of five novel loci associated with Immunoglobulin G N-glycosylation. *Nat Commun* 8: 447
336. Lauc G, Huffman JE, Pucic M, Zgaga L, Adamczyk B, Muzinic A, Novokmet M, Polasek O, Gornik O, Kristic J, Keser T, Vitart V, Scheijen B, Uh HW, Molokhia M, Patrick AL, McKeigue P, Kolcic I, Lukic IK, Swann O, van Leeuwen FN, Ruhaak LR,

Houwing-Duistermaat JJ, Slagboom PE, Beekman M, de Craen AJ, Deelder AM, Zeng Q, Wang W, Hastie ND, Gyllensten U, Wilson JF, Wuhrer M, Wright AF, Rudd PM, Hayward C, Aulchenko Y, Campbell H, Rudan I. 2013. Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet* 9: e1003225

337. Huffman JE, Knezevic A, Vitart V, Kattla J, Adamczyk B, Novokmet M, Igl W, Pucic M, Zgaga L, Johannson A, Redzic I, Gornik O, Zemunik T, Polasek O, Kolcic I, Pehlic M, Koeleman CA, Campbell S, Wild SH, Hastie ND, Campbell H, Gyllensten U, Wuhrer M, Wilson JF, Hayward C, Rudan I, Rudd PM, Wright AF, Lauc G. 2011. Polymorphisms in B3GAT1, SLC9A9 and MGAT5 are associated with variation within the human plasma N-glycome of 3533 European adults. *Hum Mol Genet* 20: 5000-11
338. Clerc F, Novokmet M, Dotz V, Reiding KR, de Haan N, Kammeijer GSM, Dalebout H, Bladergroen MR, Vukovic F, Rapp E, Consortium I-B, Targan SR, Barron G, Manetti N, Latiano A, McGovern DPB, Annese V, Lauc G, Wuhrer M. 2018. Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. *Gastroenterology* 155: 829-43
339. Murugan D, Albert MH, Langemeier J, Bohne J, Puchalka J, Jarvinen PM, Hauck F, Klenk AK, Prell C, Schatz S, Diestelhorst J, Sciskala B, Kohistani N, Belohradsky BH, Muller S, Kirchner T, Walter MR, Bufler P, Muise AM, Snapper SB, Koletzko S, Klein C, Kotlarz D. 2014. Very early onset inflammatory bowel disease associated with aberrant trafficking of IL-10R1 and cure by T cell replete haploidentical bone marrow transplantation. *J Clin Immunol* 34: 331-9
340. Sivanesan D, Beauchamp C, Quinou C, Lee J, Lesage S, Chemtob S, Rioux JD, Michnick SW. 2016. IL23R (Interleukin 23 Receptor) Variants Protective against Inflammatory Bowel Diseases (IBD) Display Loss of Function due to Impaired Protein Stability and Intracellular Trafficking. *J Biol Chem* 291: 8673-85
341. Vanhooren V, Vandenbroucke RE, Dewaele S, Van Hamme E, Haigh JJ, Hochepied T, Libert C. 2013. Mice overexpressing beta-1,4-Galactosyltransferase I are resistant to TNF-induced inflammation and DSS-induced colitis. *PLoS One* 8: e79883
342. Masoodi I, Kochhar R, Dutta U, Vaishnavi C, Prasad KK, Vaiphei K, Kaur S, Singh K. 2009. Fecal lactoferrin, myeloperoxidase and serum C-reactive are effective biomarkers in the assessment of disease activity and severity in patients with idiopathic ulcerative colitis. *J Gastroenterol Hepatol* 24: 1768-74

343. Siqueiros-Cendon T, Arevalo-Gallegos S, Iglesias-Figueroa BF, Garcia-Montoya IA, Salazar-Martinez J, Rascon-Cruz Q. 2014. Immunomodulatory effects of lactoferrin. *Acta Pharmacol Sin* 35: 557-66

344. Gornik O, Lauc G. 2008. Glycosylation of serum proteins in inflammatory diseases. *Dis Markers* 25: 267-78

345. Shinzaki S, Kuroki E, Iijima H, Tatsunaka N, Ishii M, Fujii H, Kamada Y, Kobayashi T, Shibukawa N, Inoue T, Tsujii M, Takeishi S, Mizushima T, Ogata A, Naka T, Plevy SE, Takehara T, Miyoshi E. 2013. Lectin-based immunoassay for aberrant IgG glycosylation as the biomarker for Crohn's disease. *Inflamm Bowel Dis* 19: 321-31

346. Miyahara K, Nouso K, Saito S, Hiraoka S, Harada K, Takahashi S, Morimoto Y, Kobayashi S, Ikeda F, Miyake Y, Shiraha H, Takaki A, Okada H, Amano M, Hirose K, Nishimura S, Yamamoto K. 2013. Serum glycan markers for evaluation of disease activity and prediction of clinical course in patients with ulcerative colitis. *PLoS One* 8: e74861

347. Dierckx T, Verstockt B, Vermeire S, van Weyenbergh J. 2019. GlycA, a Nuclear Magnetic Resonance Spectroscopy Measure for Protein Glycosylation, is a Viable Biomarker for Disease Activity in IBD. *J Crohns Colitis* 13: 389-94

Chapter II

Journal of Crohn's and Colitis, 2018, 1–11
doi:10.1093/ecco-jcc/jjy139
Advance Access publication September 20, 2018
Original Article

Original Article

A [Glyco]biomarker that Predicts Failure to Standard Therapy in Ulcerative Colitis Patients

Márcia S. Pereira,[a,b,c] Luís Maia,[d] Luís F. Azevedo,[e] Sara Campos,[a] Sandra Carvalho,[a,b] Ana M. Dias,[a,b,c] André Albergaria,[a,b,f] Jorge Lima,[a,b,f] Ricardo Marcos-Pinto,[c,d] Paula Lago,[d] Salomé S. Pinho[a,b,f]

[a]Institute of Molecular Pathology and Immunology of the University of Porto [IPATIMUP], Porto, Portugal [b]Institute for Research and Innovation in Health [i3S], University of Porto, Porto, Portugal [c]Institute of Biomedical Sciences of Abel Salazar [ICBAS], University of Porto, Porto, Portugal [d]Department of Gastroenterology, Porto Centre Hospital, Porto, Portugal [e]Department of Community Medicine, Information and Health Decision Sciences [MEDCIDS], and Center for Health Technology and Services Research [CINTESIS], University of Porto, Porto, Portugal [f]Department of Pathology, Medical Faculty, University of Porto, Portugal

Corresponding author: Salomé S. Pinho, DVM, PhD, Rua Alfredo Allen, 208 4200-135 Porto, Portugal. Tel.: +351220408800; email: salomep@ipatimup.pt

Abstract

Background and Aims: There is a clinical need to identify biomarkers able to select patients who are most likely to develop aggressive/complicated disease, for early selection for appropriate therapy. Changes in the glycosylation profile of intestinal lymphocytic infiltrate were previously demonstrated to regulate T cell activity, being associated with disease severity in ulcerative colitis [UC] patients. We interrogated whether this heterogeneous expression of branched N-glycans in intestinal inflammatory infiltrate predicts therapy response early in disease course.

Methods: The expression levels of the branched N-glycans in colonic biopsies collected around time of diagnosis from a well-characterised cohort of 131 UC patients were correlated with response to standard therapy. Receiver operating characteristic analysis and specificity/sensitivity were determined.

Results: Branched N-glycans levels around time of diagnosis predict non-response to conventional therapy with 75% specificity. Moreover, high levels of branched N-glycans predict 78% of UC patients who will display a favourable disease course [exclusively under 5-aminosalicylate therapy for more than 5 years of disease]. The best predictive performance was observed in severe UC patients with Mayo endoscopic subscore 3 and in those that were naïve to therapy. Multivariable analysis revealed that low levels of branched N-glycans and high levels of C-reactive protein [CRP] around time of diagnosis act as independent predictors of non-response to standard therapy. A powerful effect of the combined use of the branched N-glycans and CRP was observed.

Conclusions: Our results reveal a potential [glyco]biomarker that predicts, early in the disease course, patients who will fail to respond to standard therapy, benefiting thereby from other therapeutic strategies such as biologics.

Keywords: Ulcerative colitis; biomarker; glycosylation

1. Introduction

Inflammatory bowel diseases [IBD], comprising Crohn's disease [CD] and ulcerative colitis [UC], are multifactorial disorders characterised by a chronic and relapsing inflammation of the gastrointestinal tract.[1,2] IBD is considered an emergent global disease and a major health problem due to the increasing incidence and prevalence worldwide.[3] The prevalence of IBD is higher in more industrialised regions, affecting approximately 2.5 million people in Europe and 1 million people in the USA, with a peak of disease onset in young populations [aged 15–30 years].[4] Although the mortality rate does not significantly differ from that of unaffected population, the negative impact in IBD patients' quality of life is tremendous,[4,5] with patients facing a reduced ability to work and increased social stigma which leads to restrictions in career choices. In addition to the lifetime of care, there are high medical and societal costs associated with frequent hospitalisations and ongoing medications.[5,6] Taking all this together, IBD remains a major clinical challenge worldwide.

Despite recent advances in IBD therapeutic resources, a high proportion of patients remain refractory to conventional treatment, and overall half of the patients with UC do not achieve sustained remission.[7] There are currently potent therapeutic regimens available for the treatment of IBD, such as the anti-tumour necrosis factor [anti-TNF] therapy, but these are not an option for all patients early in the disease course.[7] Besides cost, the risk of adverse and toxic effects are of concern. Therefore, it is a priority to identify predictive biomarkers that help to select the patients who are most likely to develop an aggressive/complicated or a mild disease, and carefully stratify them for appropriate therapy. This personalised approach is fundamental, since there is evidence showing that some patients with IBD will have a favourable disease course when more potent therapy with biologics, such as anti-TNF and others, is administered early in the disease course.[7] This tailored approach will certainly improve the long-term course and outcomes of the disease and the success of the therapeutic options.

Glycosylation is a complex post-translational mechanism characterised by the addition of carbohydrate structures [glycans] to proteins and lipids in the endoplasmic reticulum/Golgi secretory pathway, by specific enzymes.[8,9] We and others have been contributing to the understanding of the key role that different glycans play in cell biology, particularly in cancer[8,10–13] and in the regulation of immune response.[14–16] Evidence suggests that immune signalling and activation are tightly regulated by glycosylation.[17,18] The dysregulation of the regulatory mechanism between immune cell functions and its glycosylation modifications have been described to increase the susceptibility to immune-mediated diseases, including IBD.[16,19] In IBD, we have recently demonstrated that UC patients display a deficiency in branched N-glycans [catalyzed by N-acetylglucosaminyltransferase V – GnT-V enzyme] on intestinal T cells.[14] Patients with severe disease exhibit low levels of branched N-glycans on mucosal T lymphocytes compared with healthy controls. This deficiency was observed in the T cell receptor [TCR] of the intestinal lymphocytes and was associated with a hyperimmune response and UC pathogenesis.[14,16]

Taking into consideration that UC patients display, at the level of intestinal mucosa, a heterogeneous expression of branched glycans that are correlated with disease severity,[14] we herein investigated whether the expression of branched N-glycans on the intestinal inflammatory infiltrate [glycobiomarker] can predict an unfavourable disease course in UC and thus identify, early in disease course, those patients who are likely to fail standard therapy and will benefit from other therapies such as biologics.

2. Methods

2.1. UC patients' cohort

A retrospective collection of 131 formalin-fixed paraffin-embedded [FFPE] colonic biopsies [collected from the most inflamed area] of 131 patients diagnosed with UC [median age 36 years; range 14–69] were obtained from scheduled colonoscopies at the Gastroenterology Department of Porto Centre Hospital—Hospital Santo António [CHP/HSA], Porto, Portugal, between 1981 and 2015 [Table 1]. The duration of storage of the biopsy samples since collection appears not to influence the immunohistochemistry analysis. In order to assess the predictive value of the branched N-glycans around time of diagnosis, we have analysed the colonic biopsies that were collected closer to diagnosis [Table 1]: 86 patients with biopsy at the time of diagnosis and up to 5 months after diagnosis, median (interquartile range [IQR] of 0 [0–0.1]); and 45 patients with biopsy adjacent to diagnosis more than 5 months after diagnosis, 13 [9–24]. Data from clinicopathological, therapeutic, and biochemical parameters from each UC patient were collected at the time of biopsy collection, except for disease extension and surgery which were collected in November 2016 [Table 1]. The data include: disease extension; Mayo endoscopic [MayoE] subscores; need of surgery [defined as colectomy/proctocolectomy due to a medically refractory disease or due to the development of toxic megacolon]; clinical remission [no rectal bleeding, normal stool frequency, and no abdominal pain]; biochemical remission (C-reactive protein [CRP] levels collected the closest to the time of biopsy collection with a median of 1.6 months [IQR 0.2–4.3] lower than 5 mg/L); and type of therapy (without therapy [naïve or with suspended therapy due to clinical reasons, no therapy] vs under specific therapy). Patients were classified either as responders or as non-responders to standard therapy (5-aminosalicylate [5-ASA], corticosteroids, and immunosuppressants) on November 2016, after at least 1–2 years of follow-up (median 12 years [IQR 7–19]) and following clinical and endoscopic guidelines.[20] Additionally, patients with more than 5 years after diagnosis were classified as having a favourable disease course if they responded to 5-ASA therapy [patients in monotherapy with 5-ASA with clinical/endoscopic response after 5 years since diagnosis], vs non-favourable disease course if they failed to respond to standard therapy after 5 years since diagnosis, with need of biologics. In a retrospective study, all UC patients available in the CHP/HSA cohort who met the eligible criteria were included in the study. A power calculation *a posteriori* indicated that our sample of 131 subjects, with 23 non-responders and 108 responders, had a 49% power to detect differences in the proportion of biomarker positives as large as the one found in our study—25.0% vs 47.8%, respectively]. The present study was approved by the ethics committee of the CHP/HSA.

2.2. Immunohistochemistry

The levels of expression of branched N-glycans in intestinal inflammatory infiltrate in lamina propria [glycobiomarker expression] were determined by performing histochemical analysis in 131 FFPE UC colonic biopsies using biotinylated *Phaseolus vulgaris* leucoagglutinin [L-PHA] lectin that specifically recognises the branched N-glycan structures, as previously described.[14] The negative control was performed by using phosphate-buffered saline [PBS] instead of the lectin. Colon carcinoma samples were used as positive controls for L-PHA staining.

The levels of expression of branched N-glycans on the intestinal infiltrate in lamina propria were evaluated by three independent observers and scored using a standard semi-quantitative method

Table 1. Characterisation of the UC cohort in terms of clinical and pathological parameters. Data on Mayo endoscopic score, clinical remission [no rectal bleeding, normal stool frequency, and no abdominal pain], and type of therapy were collected at the time of biopsy collection. Information on biochemical remission (C-reactive protein [CRP] levels lower than 5 mg/L) was collected the closest to the time of biopsy collection. Information on disease extension, therapy duration, responders vs non-responders [to the standard therapy], and disease course [UC patients with more than 5 years of diagnosis; favourable—always under monotherapy with 5-ASA with clinical/endoscopic response; non-favourable—do not respond to standard therapy] were collected in 2016.

		N [%]	Median [IQR]
Years old at diagnostis [*N* = 131]	≤36	28 [22.4]	36 [27–47]
	>36	97 [77.6]	
Years of follow-up [*N* = 131]		-	12 [7–19]
Months since diagnosis [*N* = 131]	≤5	86 [65.7]	0.0 [0.0–0.1]
	>5	45 [34.4]	13.0 [8.5–23.9]
Gender [*N* = 131]	M	66 [50.4]	-
	F	65 [49.6]	-
Smoking status [N = 128][a]	No	91 [71.1]	-
	Former	25 [19.5]	-
	Yes	12 [9.4]	-
Disease extension [*N* = 131]	Proctitis	22 [16.8]	-
	Left-sided	66 [50.4]	-
	Pancolitis	43 [32.8]	-
Mayo endoscopic score [*N* = 124][a]	Mayo 0	13 [10.5]	-
	Mayo 1	35 [28.2]	-
	Mayo 2	41 [33.1]	-
	Mayo 3	35 [28.2]	-
Surgery [*N* = 131]	No	127 [96.9]	-
	Yes	4 [3.1]	-
Clinical remission [*N* = 126][a]	No	81 [64.3]	-
	Yes	45 [35.7]	-
PCR [mg/L; *N* = 95][a]		-	4.4 [1.1–15.2]
PCR [months since biopsy; *N* = 95][a]		-	1.6 [0.2–4.3]
Biochemical remission [*N* = 95][a]	No	44 [46.3]	-
	Yes	51 [53.7]	-
Therapy [*N* = 131]	None/naive	63 [48.1]	-
	5-ASA	52 [39.7]	-
	Corticosteroids	12 [9.2]	-
	Immunosuppressants	3 [2.3]	-
	Anti-TNF	1 [0.8]	-
Therapy duration [months; *N* = 66][a]			7.2 [3.6–16.2]
Responders [*N* = 131]	Responder	108 [82.4]	-
	Non-responder	23 [17.6]	-
Therapy [*N* = 129][a]	Always with 5-ASA	71 [55.0]	-
	Anti-TNF	58 [45.0]	-
Disease course [*N* = 75]	Good	54 [72.0]	-
	Bad	21 [28.0]	-

UC, ulcerative colitis; 5-ASA, 5-aminosalicylate; IQR, interquartile range; M, male; F, female; PCR, polymerase chain reaction; TNF, tumour necrosis factor.
[a]Data not available due to follow-up loss.

as follows: 0–5%, 5–25%, 25–50%, 50%–75%, and more than 75% of positive L-PHA expression. Then and for statistical analysis, the data were regrouped in the following categories: less than 5%; 5–25%; and more than 25% of expression. A receiver operating characteristic [ROC] analysis was done and the cut-off of 25% was determined. Colonic biopsies presenting ≤25% of L-PHA lectin reactivity were classified as LOW expression and those with >25% were classified as HIGH expression.

2.3. Isolation of lamina propria lymphocytes and CD3⁺T cells from fresh colonic biopsies of UC patients

Lamina propria lymphocytes [LPLs] were isolated as described before,[14,16] using Percoll [Sigma] density gradients of 1.05–1.09 g/ml. T cells were isolated from a subset of fresh colon biopsies as previously described.[14,16] Briefly, colonic biopsies were mechanically dissociated to prepare single-cell suspensions using the Hanks' Balanced Salt solution modified medium and penicillin/streptomycin and gentamicin. $CD3^+$ T cells were magnetically sorted by using the EasySep™ Human T Cell Enrichment Kit [STEMCELL] following the manufacturer's instructions. $CD3^+$ T cells were cultured for 72 h with anti-CD3 mAb [clone OKT3] and soluble anti-CD28 mAb [clone CD28.2] [eBioscience].

2.4. Flow cytometry

The detection of branched N-glycans on *ex vivo* T cells was performed as described previously,[16] using fluorescein isothiocyanate [FITC]-conjugated L-PHA [Vector Lab]. Median fluorescence intensity [MFI] of L-PHA was determined by flow cytometry. Data acquisition was performed on a FACSCanto™ II system [BD Biosciences, San Jose, CA] using the FACSDiva™ software [BD] and compensated and analysed in FlowJo version 10.4. [Tree Star, Inc., Ashland,

OR]. Doublets were excluded from the analysis based on FSC-A vs FSC-H parameters, and dead cells were excluded based on propidium iodide incorporation.

2.5. Real-time polymerase chain reaction

The real-time polymerase chain reaction [PCR] was performed as previously described[14] using TaqMan Gene Expression Assays [Applied Biosystems]: MGAT5 [hs.00159136_m1] and GAPDH [hs.02758991; used as reference gene]. PCR reactions were performed on the ABI Prism 7000 Sequence Detection System.

2.6. Statistical analysis

The prediction capacity of L-PHA levels to discriminate patients who respond to standard therapy from those that do not respond was determined by plotting the receiver operating characteristic [ROC] curves and calculating the area under the curve [AUC]. The cut-off that revealed the best balance between sensitivity and specificity was selected for the subsequent statistical analysis. Sensitivity, specificity, and positive and negative predictive values were calculated. Univariate binary logistic regression analysis was performed to test L-PHA levels and clinical variables [age at diagnosis, gender, MayoE score, clinical remission, biochemical remission, and therapy at the time of the biopsy] in predicting failure to standard therapy. Moreover, multivariable logistic regression analysis [using a stepwise variable selection procedure, implemented with the stepAIC function of the MASS package of the R software, version 3.3.3] was used to determine the variable[s] that could independently predict failure in response to standard therapy. In logistic regressions, model goodness-of-fit was assessed by the Hosmer-Lemeshow statistic and test. The final multivariable models were also checked for influence of outlier data values on model fit, using leverage statistics [standardised Pearson residuals, Studentised residuals, and deviance], and possible multicollinearity, by evaluating the coefficients' correlation matrix. The results did not show any major concern regarding outliers or multicollinearity. All but one case had residuals or deviance in the range -2 to +2, indicating no major outliers or highly influential subjects. Moreover, all correlations between model coefficients were below 0.35, indicating no evidence of multicollinearity. Results are presented as odds ratios [ORs]for each category as compared with a predefined reference category, and their respective 95% confidence intervals [CIs]. Odds ratios above one and below one are indicative, respectively, of higher and lower odds of non-response to standard therapy as compared with a reference category. The predicted probabilities of non-response to standard therapy for the combination of L-PHA levels and CRP were calculated by performing a multivariable logistic regression model including these two variables, and a ROC curve analysis to assess the global predictive ability of the model and the sensitivity and specificity for different cut-off values. Subgroup analysis was performed for patients with severe UC [MayoE 3] around time of diagnosis. Influence of outlier data values on model fit was assessed using leverage statistics, and collinearity was assessed by evaluation of the coefficients correlation matrix.

Pearson's chinsquare [χ^2] test was performed to evaluate the associations between L-PHA levels and clinical features. A multivariable logistic regression analysis was also performed using L-PHA as a dependent variable, in which odd ratios above one and below one are indicative, respectively, of higher and lower odds of patients presenting low levels of L-PHA as compared with a reference category.

All the statistical analysis was performed using the statistical software SPSS version 25 [IBM Corp., IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY; released 2017.] and R version 3.3.3 [R Foundation for Statistical Computing, Vienna; released 2017]. The threshold used for statistical significance was $P < 0.05$. Adjustments for multiple testing were not applied in this case, as this is an exploratory study.

3. Results

3.1. Heterogenous expression of branched N-glycans in lamina propria inflammatory infiltrate among UC patients

Our previous evidence demonstrated that UC patients exhibit a significant decreased expression of branched N-glycans on intestinal lymphocytic infiltrate in lamina propria, which correlated with disease severity when compared with healthy controls.[14] Importantly, patients with a severe disease course showed the lowest levels of branched N-glycans expression on mucosal T cells.[14] Here we assessed whether different levels of branched N-glycans on lamina propria inflammatory infiltrate [Figure 1] from colonic biopsies of UC patients around time of diagnosis [Table 1] may have a prognostic value, predicting early in disease course the failure of standard therapy. The studied cohort comprises 131 UC patients with a median age at diagnosis of 36 years, who are equally distributed regarding gender [50% females and 50% males, Table 1], and in whom the majority of were former or non-smokers [116/128–90.6%]. The clinical-pathological features of the patients are summaried in Table 1. The therapeutic outcome of the 131 UC patients was determined on November 2016, after at least 1–2 years of follow-up; 82% [108/131] of the patients were always under standard therapy [5-ASA, corticosteroids, and/or immunosuppressants], among whom 71/129 [55%] maintained 5-ASA as monotherapy during disease course [Table 1]; 18% [23/131] failed to respond to standard therapy and thus stepped-up to biologics [anti-TNF].

Our analysis showed a heterogeneous expression of the branched N-glycans [Figure 1] in the colonic mucosa of UC biopsies, in which 10% [13/131] of the patients exhibited less than 5% of L-PHA reactivity, 19% [25/131] displayed 525% of positive expression, and 71% [93/131] showed more than 25% of reactivity of L-PHA [Figure 1B]. The levels of expression of branching N-glycans are similar when comparing colonic biopsies at diagnosis [up to 5 months after diagnosis] or adjacent to diagnosis [from 6 to 56 months] Supplementary Table 1, available as Supplementary data at *ECCO-JCC* online].

3.2. Expressions of the branched N-glycans around time of diagnosis reveal predictive capacity to select UC patients who will fail to respond to standard therapy

In order to determine the predictive capacity of the branched N-glycans expression around time of diagnosis in distinguishing UC patients who respond to standard therapy from those that will fail to respond to standard therapy, ROC analysis was performed for the different L-PHA levels [<5%, 5–25%, and >25% reactivity] [Figure 2A]. The cut-off [or threshold] value that presented the best balance between specificity and sensitivity [arrows in the Figure 2A] was at 25% of L-PHA reactivity. The overall accuracy in discriminating patients who fail to respond to standard therapy from those who respond was determined by calculating the sensitivity, specificity, and positive and negative predicted values [+PV and -PV, respectively], using the defined cut-off [Table 2]. Additionally, and considering the

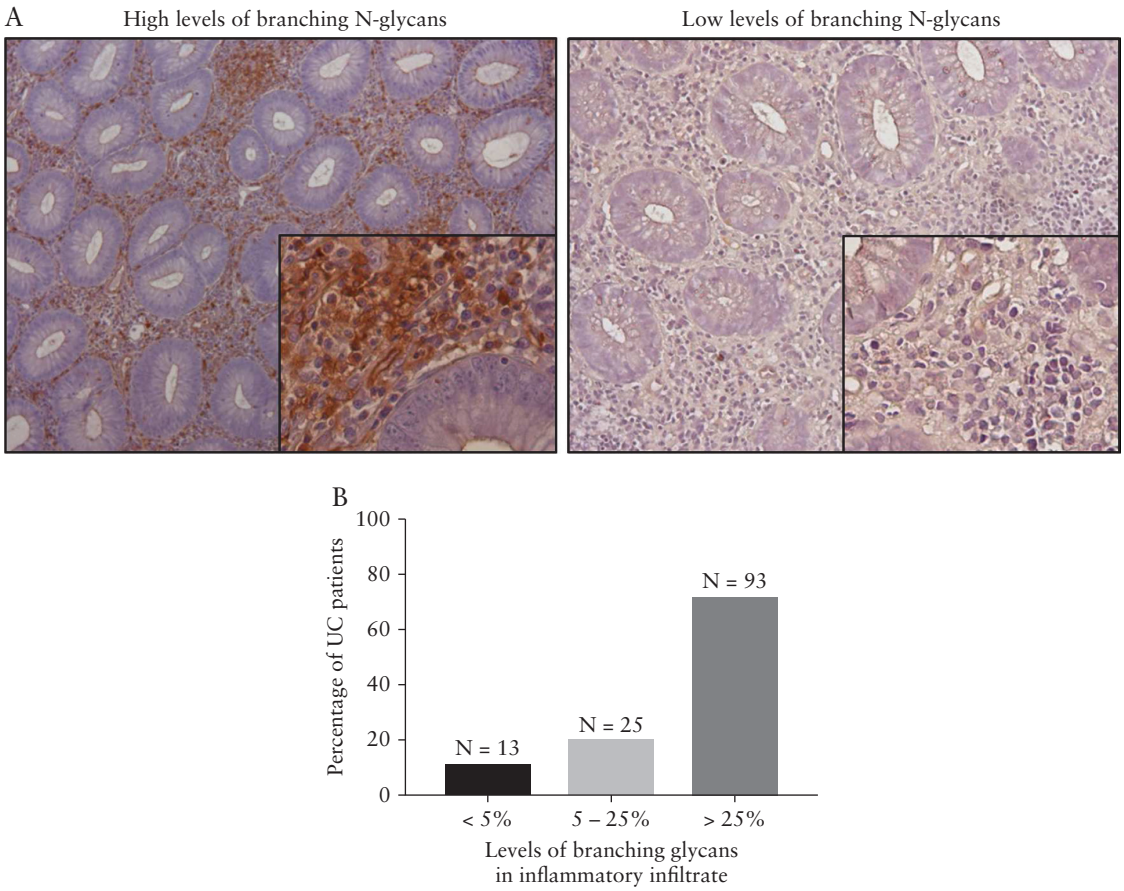


Figure 1. [A] Representative image of the intestinal inflammatory infiltrate positive to branched N-glycans expression [L-PHA staining] measured in lamina propria [biomarker] of ulcerative colitis [UC] biopsy patients. The levels of branched N-glycans were stratified into high [more than 25% of reactivity] and low [less than 25% reactivity]. The pictures were taken at amplifications of 10x and 40x. [B] Distribution of the levels of branched N-glycans expression.

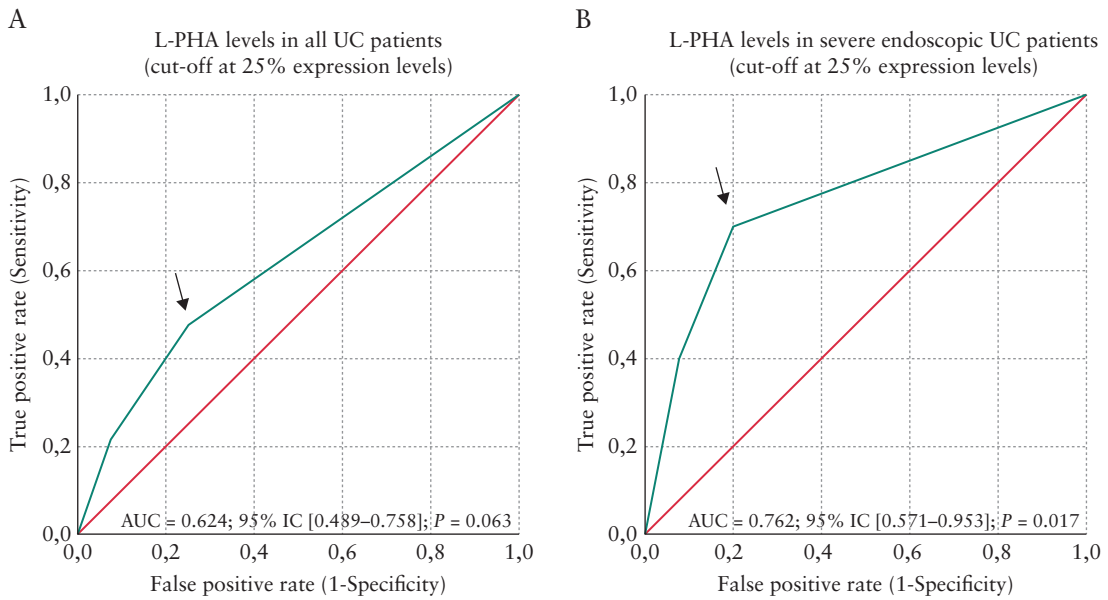


Figure 2. Receiver operating characteristic [ROC] curves plotted for the branched N-glycans [L-PHA in lamina propria] levels in all ulcerative colitis [UC] patients [A], and in those with endoscopic severe disease—MayoE 3— [B] do not respond to standard therapy [A and B]. The arrows indicate the cut-off [25% expression levels of branched N-glycans] that best discriminates patients who respond to standard therapy from those who need biologic therapies. The performance is increased in MayoE 3 UC patients.

importance of the MayoE subscores and the clinical and biochemical criteria for remission in the process of therapy decisions, the overall accuracy was also determined for these parameters [the best cut-off was determined for MayoE subscore; Supplementary Figure 1, available as Supplementary data at *ECCO-JCC* online and Table 2].

Interestingly, our results showed that levels of branched N-glycans around time of diagnosis are able to distinguish patients with different therapeutic outcomes, with a sensitivity of 48% [11/23] and a specificity of 75% [81/108; Table 2]. The same holds true when we analysed the predictive capacity of the branched N-glycans in the biopsies that were collected within 5 months after diagnosis (median of months after diagnosis of 0.0 [IQR 0.0–0.1]; Table 3), in which the sensitivity was 50% [7/14] and the specificity 76% [55/72]. Levels of the branched N-glycans and MayoE subscore showed a similar global capacity in patient stratification [Figure 2A; Supplementary Figure 1; Table 2], presenting the highest specificities. Clinical and biochemical remissions showed the highest sensitivities (78% [18/23] and 76% [16/21], respectively; Table 2). When severe UC patients with MayoE 3 were particularly analysed around time of diagnosis, the branched N-glycan expression was capable of predicting the failure of standard therapy with higher accuracy [AUC = 0.762, p = 0.017, Figure 2B] than in the analysis including patients with mild to moderate disease around time of diagnosis [AUC = 0.624, p = 0.063, Figure 2A]. The results showed that whenever the branched N-glycans are analysed in patients with severe disease around time of diagnosis, the sensitivity substantially increases from 48% [11/24] to 70% [7/10; Table 2], with a concomitant increase (double, from 29% [11/38] to 58% [7/12], Table 2) of the +PV (patients with low levels of branching N-glycans, who failed standard therapy, thus needing step-up therapies such as anti-TNF). The same holds true when the branched N-glycans were analysed in biopsies collected within 5 months after diagnosis [Table 3], displaying a sensitivity of 75% [3/4] and a specificity of 83% [19/23]. On the other hand, the levels of CRP which appeared to be a good predictor when considering all MayoE subscores showed a decrease in specificity (from 62% [46/74] to 41% [7/17]) when analysed only in patients with MayoE subscore 3 [Table 2].

Additionally, we showed that the levels of branched N-glycans are statistically associated with the response to standard therapy predominantly in naïve/no therapy UC patients [p = 0.018, Figure 3], in whom 82% (46/56 vs 18%[10/56]) of the responders display high levels of branched N-glycans and 57% (4/7 vs 43%[3/7]) of the non-responders display low levels.

Importantly, we further demonstrated that specifically $CD3^+$ mucosal T cells from non-responders display lower levels of branched N-glycans (mean MFI of 5.1 ± standard error of the mean [SEM] of 1.40; Figure 4A] compared with responders [27.3 ± 9.97]; Figure 4A). This result validates our immunohistochemistry observations, being in accordance with our previous evidences on the target-specific effects of branched N-glycans in T cell-mediated immune response in UC.[14,16] Moreover, we showed that patients exhibiting low levels of branched N-glycans in the intestinal inflammatory infiltrate display lower expression levels of *MGAT5* transcription [mean of 0.009 ± SEM of 0.003], compared with patients with high levels of branched N-glycans [0.024 ± 0.005] around time of diagnosis [Figure 4B], which is also in accordance with our previous observations.[14,16]

Taken together, these results showed that branched N-glycans are able to distinguish responders vs non-responders to standard therapy at and adjacent to diagnosis, especially in the setting of patients with severe endoscopic disease and naïve to therapy.

3.3. Branched N-glycans in the intestinal lamina propria as an independent predictor of therapy response in UC patients

We next evaluated the association between the response to standard therapy and different clinicopathological features of UC patients, by performing univariate and multivariable analysis [Table 4]. From a total of 93 UC patients with high levels of branched N-glycans,

Table 2. The predictive capacity of L-PHA [branched N-glycans] and other clinical pathological parameters to distinguish patients who fail standard therapy from patients who respond. The sensitivity, specificity, and positive [+PV] and negative predictive values [-PV] were calculated for all ulcerative colitis [UC] patients and for only UC patients with severe endoscopic disease around time of diagnosis.

All UC patients	Sensitivity	Specificity	+PV	-PV
L-PHA levels	48%	75%	29%	87%
Mayo score	43%	75%	29%	85%
Clinical remission	78%	39%	22%	89%
Biochemical remission	76%	62%	36%	90%
UC patients with severe endoscopic disease				
L-PHA levels	70%	80%	58%	87%
Clinical remission	100%	4%	29%	100%
Biochemical remission	90%	41%	47%	88%

Table 3. The predictive capacity of L-PHA [branched N-glycans] to distinguish patients who fail standard therapy from patients who respond, analysed only in patients with biopsy collected the closest to diagnosis [up to 5 months since diagnosis]. The sensitivity, specificity and positive [+PV] and negative predictive values [-PV] were calculated for all ulcerative colitis [UC] patients.

All UC patients	*N*	Responders *N* [%]	Non-responders *N* [%]	*p*-Value	Sensitivity	Specificity	+PV	-PV
High L-PHA	62	55[88.7]	7[11.3]	0.044	50%	76%	29%	89%
Low L-PHA	24	17[70.8]	7[29.2]					
UC patients with severe endoscopic disease								
High L-PHA	20	19[95.0]	1[5.0]	0.042	75%	83%	43%	95%
Low L-PHA	7	4[57.1]	3[42.9]					

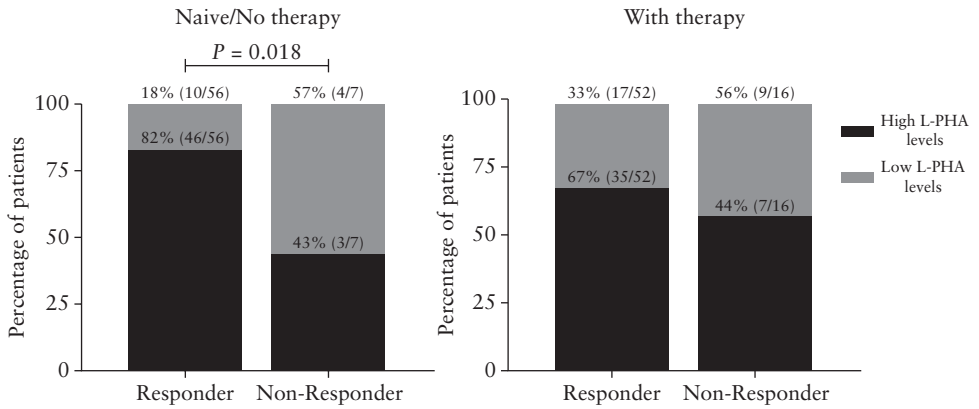


Figure 3. The expression levels of branched N-glycans in responder and non-responder ulcerative colitis [UC] patients [to standard therapy], who were naïve to therapy, vs those who were under therapy around the time of diagnosis.

Table 4. Predictive capacity of the branched N-glycans and other clinicopathological parameters in relationship with non-response to standard therapy in all ulcerative colitis [UC] patients and in only those displaying a severe endoscopic UC disease around time of diagnosis. The predictive capacity was assessed by univariate and multivariate analysis.

		All UC patients					UC patients with severe endoscopic disease				
		N	Responders *N* [%]	Non-responders *N* [%]	OR [95% CI]	*p*-Value	*N*	Responders *N* [%]	Non-responders *N* [%]	OR [95% CI]	*p*-Value
L-PHA levels	>25%[a]	93	81 [87.1]	12 [12.9]	1		23	20 [87.0]	3 [13.0]	1	
	0–25%	38	27 [71.1]	11 [28.9]	2.75 [1.09–6.95]	0.032	12	5 [41.7]	7 [58.3]	9.33 [1.76–49.6]	0.009
Age at diagnosis [years]	>36[a]	62	53 [85.5]	9 [14.5]	1		12	8 [66.7]	4 [33.3]	1	
	0–36	69	55 [79.7]	14 [20.3]	1.50 [0.60–3.76]	0.492	23	17 [73.9]	6 [26.1]	0.70 [0.16–3.22]	0.652
Gender	F[a]	65	58 [89.2]	7 [10.8]	1		11	8 [72.7]	3 [27.3]	1	
	M	66	50 [75.8]	16 [24.2]	2.65 [1.01–6.96]	0.048	24	17 [70.8]	7 [29.2]	1.10 [0.22–5.40]	0.908
Mayo score	0–2[a]	89	76 [85.4]	13 [14.6]	1		-	-	-	-	
	3	35	25 [71.4]	10 [28.6]	2.34 [0.91–5.99]	0.077	-	-	-	-	-
Clinical remission	Yes[a]	46	40 [88.9]	5 [11.1]	1		1	1 [100]	0 [0.0]	1	
	No	81	63 [77.8]	18 [22.2]	2.29 [0.79–6.64]	0.129	34	24 [70.6]	10 [29.4]	6x10^8 [0.00]	1.000
Biochemical remission	Yes[a]	51	46 [90.2]	5 [9.8]	1		8	7 [87.5]	1 [12.5]	1	
	No	44	28 [63.6]	16 [36.4]	5.26 [1.74–15.93]	0.003	19	10 [52.6]	9 [47.4]	6.30 [0.64–61.63]	0.114
Therapy	None/naive[a]	63	56 [88.9]	7 [11.1]	1		21	18 [85.7]	3 [14.3]	1	
	5-ASA	52	43 [82.7]	9 [17.3]	1.67 [0.58–4.86]		8	4 [50.0]	4 [50.0]	6.00 [0.95–38.08]	
	Other therapies	16	9 [56.3]	7 [43.8]	6.22 [1.76–21.98]	0.016	6	3 [50.0]	3 [50.0]	6.00 [0.80–44.95]	0.093
Multivariate analysis—stepwise					**OR [95% CI]**	***p*-Value**				**OR [95% CI]**	***p*-Value**
L-PHA levels					-	-				9.17 [1.24–68.0]	0.030
Biochemical remission					4.76 [1.46–13.60]	0.009				11.53 [0.87–152.95]	0.064

OR, odds ratio; CI, confidence interval; F, female; M, male; 5-ASA, 5-aminosalicylate.
[a]Reference variable.

87.1% were responders to the standard therapy and 12.9% were non-responders. Additionally, from 38 UC patients with low levels of branched N-glycans, 71.1% were responders to the standard therapy and 28.9% were non-responders. Univariate analysis showed that low levels of branched N-glycans around time of diagnosis increased 2.8 times the odds (95% CI = [1.09–6.95], $P = 0.032$) of UC patients failing standard therapy, and thus needing to step-up to biologics, comparing with patients with high levels of branched N-glycans [Table 4]. In addition, UC male patients (OR = 2.65 [1.01–6.96], p = 0.048; Table 4) and those with high levels of CRP around time of diagnosis (OR = 5.26 [1.74–6.64], $p = 0.003$; Table 4) are more prone to fail the response to standard therapy, compared with females and low levels of CRP, respectively. Moreover, UC patientswho were under other therapies, rather than 5-ASA, at the time of biopsy collection (median of months of therapy after diagnosis of 7.2, IQR [3.6–16.2]) are more prone to step-up to biologics (OR = 6.22 [1.76–21.98], $p = 0.016$; Table 4). Interestingly, in UC patients with severe endoscopic disease [MayoE 3], the branched N-glycans appear as the only parameter that influences the response to standard therapy, in which patients displaying low levels of branched N-glycans (58.3% [7/12] exhibit 9.33 higher odds of non-response to standard therapy compared with patients showing high levels of branched N-glycans 13.0% [3/23]; 95% CI = [1.76–49.6], $p = 0.009$; Table 4).

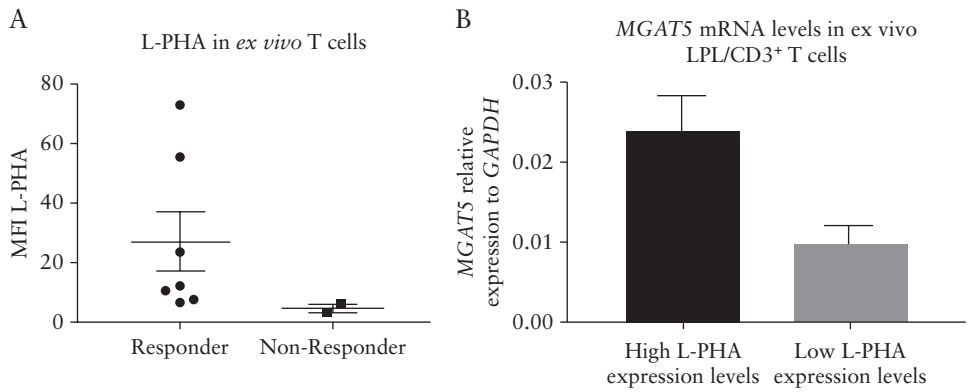


Figure 4. [A] Levels of branching N-glycans on CD3+T cells/lamina propria lymphocytes [LPL]. Median fluoresce intensity [MFI] for L-PHA inT cells isolated from fresh colonic biopsies of UC patients [*n* = 7 responders vs *n* = 2 non-responders]. [B] *MGAT5* mRNA expression levels on CD3+T cells/LPL in relationship with high [*n* = 8 UC patients] vs low [*n* = 4 UC patients] levels of branched N-glycans around time of diagnosis.

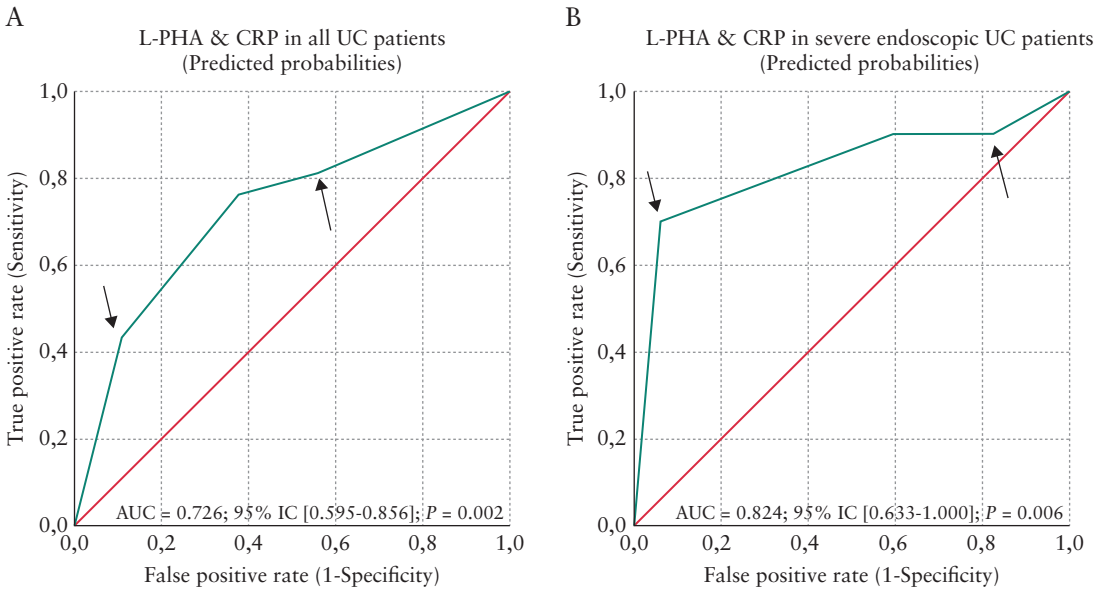


Figure 5. Receiver operating characteristic [ROC] curve plotted for the predicted probabilities of different combinations of L-PHA [branched N-glycans in lamina propria] and C-reactive protein [CRP] levels in all ulcerative colitis [UC] patients [A], and those with severe endoscopic disease [B] who do not respond to standard therapy. The arrows indicate the cut-offs that best discriminate patients who respond to standard therapy from those who need biologic therapies. The cut-offs correspond to low L-PHA + high CRP and high L-PHA + low CRP. The overall accuracy of the combination of the two molecular features is similar in the two groups.

Importantly, the multivariable analysis revealed that CRP levels (in which low levels indicate biochemical remission; OR = 4.76 [1.46–13.60], p = 0.009) and the branched N-glycans (low levels; OR = 9.17 [1.24–68.0], p = 0.030) are the only variables that appeared to predict the failure of standard therapy independently of the other clinical parameters [Table 4], in all UC patients and in UC patients with severe endoscopic disease, respectively.

In fact, univariate and multivariable analysis addressing which clinicopathological feature is associated with the branched N-glycans showed that response to therapy was the only variable independently associated with branched N-glycans levels when analysed in severe UC patients around time of diagnosis (OR = 5.60 [1.02–30.90], p = 0.048; Supplementary Table 2, available as Supplementary data at *ECCO-JCC* online). The results showed that 80% [20/25] of UC patients with a severe endoscopic disease around time of diagnosis, with high levels of the branched N-glycans, will respond to standard therapy vs 70% [7/10] of the patients with low levels of branched glycans who will need to step-up to biologics [p = 0.015; Supplementary Table 2]. Also, in this subgroup of patients [MayoE 3 patients], we verified that therapy vs no therapy is associated with lower levels of branched N-glycans [p = 0.014; Supplementary Table 2]. Multivariate analysis of all UC patients reveal as independent variables the response to therapy (non-response; 3.73 [1.14–12.22], p = 0.029] and the disease extension [proctitis/left-sided; 0.32 [0.10–1.05], p = 0.059; Supplementary Table 2).

3.4. Combined use of branched N-glycans and CRP as early predictors of therapy response in UC patients

Taking into account that the branched N-glycans and CRP levels around time of diagnosis were the only independent predictors of failure to response to standard therapy, we have computed the predicted probabilities of the two molecular markers when used in combination [Figure 5 and Supplementary Table 3, available as Supplementary data at *ECCO-JCC* online]. Remarkably, patients

with low levels of branched N-glycans and high levels of CRP around time of diagnosis display 46.6% and 76.5% probability of failure of standard therapy [Supplementary Table 3] in all and in severe [MayoE 3] UC patients, respectively.

Accordingly, the ROC curve showed a powerful effect when using the computed predicted probabilities for both molecular markers together [Figure 5]. The overall accuracy of the extreme combinations of the branched N-glycans and CRP levels [high and low vs low and high, respectively] to stratify patients according to therapy response was determined [Table 5]. The combination of the two molecular parameters appears to fill the gap in the predictive power of each marker alone. The combination revealed high sensitivity (69% [9/13] and 88% [7/8], Table 5]; and high specificity [80% [33/41] and 75% [3/4], Table 5). Low levels of the branched N-glycans together with high CRP are able to predict 53% [9/17; +PV] of UC patients who will exhibit failure of the standard therapy [Table 5], whereas high branched N-glycans with low CRP predicts 89% [33/37; -PV] of patients who will respond to standard therapy. Interestingly, the +PV is improved (88% [7/8]) when analysed in severe patients [MayoE 3] around time of diagnosis compared to the +PV obtained for all MayoE subscores [53%; Table 5]. Taken together, the combination of the branched N-glycan and CRP levels revealed powerful prediction for stratifying patients around time of diagnosis.

3.5. Branched N-glycans around time of diagnosis are able to stratify patients as having a favourable vs non-favourable disease course

In order to test the association of the branched N-glycans as a predictor of disease course around time of diagnosis, we have refined our analysis by including only UC patients with at least 5 years of disease duration since diagnosis. The results showed that high branched N-glycan levels predicted 81% [42/52, -PV; Table 6] of the patients who will exhibit a favourable disease course, in which 5-ASA was effective in controlling the disease. The sensitivity of the glycobiomarker to stratify patients with a non-favourable disease course [patients who step-up therapy to anti-TNF] increases from 52% [11/21] to 70% [7/10] when evaluated in severe UC patients [MayoE 3 around time of diagnosis], among whom, 88% of the patients with low levels of branched N-glycans will step-up to anti-TNF therapy [Table 6].

4. Discussion

In recent years, various therapeutic advances have been contributing to a paradigm shift in the clinical management of patients with IBD. This includes the TNF blockers, anti-adhesion molecules [such as α4β7 integrin blocker], cytokines inhibitors, and JAK inhibitors, among others.[21,22] In this regard, there is an urgent need to identify reliable biomarkers able to predict therapy response and assist therapy decision-making processes, particularly in the drug switching or swapping approaches, which will be fundamental to enable individualised therapy. In this study, we propose a new glycobiomarker with predictive capacity, early in disease course, for the selection of UC patients who will fail to respond to standard therapy, being those potentially eligible for benefiting from other therapeutic strategies such as biologics.

We have previously identified a new molecular mechanism in UC pathogenesis, in which a deficiency of branched N-glycans in intestinal T cells was observed and correlated with disease severity[14] and hyperimmune T cell response.[16] The heterogeneous expression of branched N-glycans, observed in intestinal lymphocytic infiltrate among UC patients with different severity scores,[14] raised the question of whether different levels of branched N-glycans on mucosal T cells could predict disease severity and therapy response early in disease course. Our results reveal that the levels of expression of branched N-glycans in intestinal inflammatory infiltrate [glycobiomarker] around time of diagnosis is able to predict non-response to conventional therapy with a specificity of 75%. These results were further corroborated when assessing the impact of the branched N-glycans in predicting a favourable vs non-favourable

Table 5. The sensitivity, specificity, and positive [+PV] and negative [-PV] predictive values of the two extreme combinations of L-PHA [branched N-glycans] and C-reactive protein [CRP] levels, in determining ulcerative colitis [UC] patients who will benefit from biologic therapies. The values were also calculated for only severe endoscopic UC patients around time of diagnosis.

All UC patients	*N*	Responders *N* [%]	Non-responders *N* [%]	*p*-Value	Sensitivity	Specificity	+PV	-PV
High L-PHA + low CRP	37	33 [89.2]	4 [10.8]	**0.002**	69%	80%	53%	89%
Low L-PHA + high CRP	17	8 [47.1]	9 [52.9]					
UC patients with severe endoscopic disease								
High L-PHA + low CRP	4	3 [75.0]	1 [25.0]	0.067	88%	75%	88%	75%
Low L-PHA + high CRP	8	1 [12.5]	7 [87.5]					

Table 6. The predicted value of branched N-glycans in relationship with ulcerative colitis [UC] disease course [favourable disease course—always under 5-ASA; non-favourable disease course—need biologics] in patients with more than 5 years of disease progression.

All UC patients	*N*	Only 5-ASA as therapy *N* [%]	Disease progression to the need for anti-TNF *N* [%]	*p*-Value	Sensitivity	Specificity	+PV	-PV
High L-PHA	52	42 [80.8]	10 [19.2]	0.011	52%	78%	48%	81%
Low L-PHA	23	12 [52.2]	11 [47.8]					
UC patients with severe endoscopic disease								
High L-PHA	14	11 [78.6]	3 [21.4]	0.006	70%	92%	88%	79%
Low L-PHA	8	1 [12.5]	7 [87.5]					

5-ASA, 5-aminosalicylic acid; TNF, tumour necrosis factor; +PV, positive predictive value; -PV, negative predictive value.

disease course, confirming a similar overall accuracy of the branched N-glycans as predictor of disease course, with a higher sensitivity [70%] and specificity [92%] when analysed in the subgroup of patients displaying a severe endoscopic disease at diagnosis [MayoE 3]. These observations suggest that low levels of branched N-glycans in severe UC patients around time of diagnosis can potentially be used in the prediction of patients who will fail the standard therapy, and thus may benefit from early biologics therapy.

The predictive ability of the branched N-glycans in discriminating responders vs non-responders to standard therapy around time of diagnosis appears to exhibit the best performance when analysed in treatment-naïve patients or those with suspended therapy, as well as in patients with severe endoscopic disease at diagnosis. In fact, we showed that the type of therapy around time of diagnosis might influence the levels of branched N-glycans, which is in line with previous reports.[23] Nevertheless, our results demonstrate that the determination of branched N-glycans around time of diagnosis is able to predict patients who will not respond to standard therapy independently of any other clinical parameter.

These observations are in accordance with our previous mechanistic evidences showing that low levels of branched N-glycans in intestinal T cells from UC patients are associated with T cell hyperactivity and with disease severity,[16] and that *MGAT5* gene is downregulated in UC patients. In accordance, we also showed that patients with low levels of branched N-glycans around time of diagnosis displayed low levels of *MGAT5* mRNA expression specifically in lamina propria lymphocytes.[14,16] Deficiency in *MGAT5* gene was associated with a decreased threshold of T cell activation due to an increase of TCR clustering.[24,25] Preliminary evidences from our group have further identified an association between *MGAT5* polymorphisms and UC severity.[26] Taken together, UC patients exhibiting low levels of branched N-glycans around time of diagnosis, due to a deficiency in *MGAT5* glycogene in mucosal T lymphocytes, exhibit a dysfunctional capacity in controlling the threshold of T cell activation, imposing thereby a preferential effector T cell-mediated immune response at the level of intestinal mucosa.[16] This mechanistic impact may, consequently, underlie the inability to respond to standard therapy.

CRP is a non-invasive biomarker commonly used in clinical practice as predictor of inflammation.[27] The sensitivity of CRP is high; however, the specificity is far from being the ideal, since it detects general inflammation associated with causes other than IBD inflammation, thus not reflecting specifically the endoscopic inflammation.[27] Moreover, only 50% of UC patients with active disease present high levels of CRP.[28] Our study showed that levels of CRP around time of diagnosis can predict the failure of standard therapy with high sensitivity, but the specificity was far from acceptable as a reliable predictor. Importantly, our results showed that the combination of CRP [high sensitivity] with the branched N-glycans [high specificity] revealed a significant increase in overall accuracy in the prediction of patients who would fail standard therapy. Patients with low branched N-glycans and high CRP around time of diagnosis are the ones that may benefit from an early aggressive therapy and a swapping approach. These results contribute to fulfilling an urgent need for the identification of patients who will benefit from biologic agents early in disease course, as this therapeutic decision will certainly avoid patient suffering, high costs due to hospitalisations, and unnecessary surgeries, further improving patients' quality of life. The combined use of these two molecular biomarkers [branched N-glycans and CRP] constitutes a powerful tool in the therapy decision-making process, which will improve prognostic accuracy in the clinical management of UC patients.

The prognostic implications of this glycobiomarker in the therapy-decision making process and in disease course need to be replicated in larger and multicentric cohorts, aiming to validate both retrospectively and, importantly, prospectively the prognostic power of this glycobiomarker in UC therapy decisions. Moreover, the predictive ability of the branched N-glycans in terms of response to biologics is also worth exploring. Importantly from the clinical practice standpoint, the overall accuracy of the branched N-glycans to distinguish patients' prognosis could be potentiated with a quantitative detection method.

Taken together, we herein propose a novel biomarker based in glycans—a glycobiomarker—that is able to stratify patients, early in the disease course, according to their therapeutic outcomes, showing a promising predictive ability as a valuable prognostic tool to be included in the therapeutic algorithm of IBD.

Funding

This work was supported by Portuguese funds through FCT-Fundação para a Ciência e Tecnologia: the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF) [NORTE-01-0145-FEDER-000029 to S.S.P.]; FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020 [POCI-01/0145-FEDER-016601 to S.S.P.]; Portuguese funds through FCT funded grants [PTDC/DTP-PIC/0560/2014] to S.S.P; PhD fellowships SFRH/BD/110148/2015 and PD/BD/105982/2014 to M.S.P and A.M.D, respectively; Grant from Abbvie international pharmaceutical company to S.S.P. Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP) integrates the i3S research unit, which is partially supported by the FCT.

Conflict of Interest

None declared.

Acknowledgement

We thank Alexandra Correia [i3S] for the help in the FACS analysis.

Author Contributions

MSP: manuscript writing, immunohistochemical analysis, staining evaluation, and data analysis; LM: clinical data collection; LFA: statistical analysis support and revision of the article; SC and SC: immunohistochemical analysis and staining evaluation; AMD: immunohistochemical analysis, T cell isolation from biopsies, and FACs analysis; AA and JL: help with intellectual content; RM-P and PL: patient recruitment and clinical selection of samples; SSP: study design, critical revision of the manuscript, and staining evaluation.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

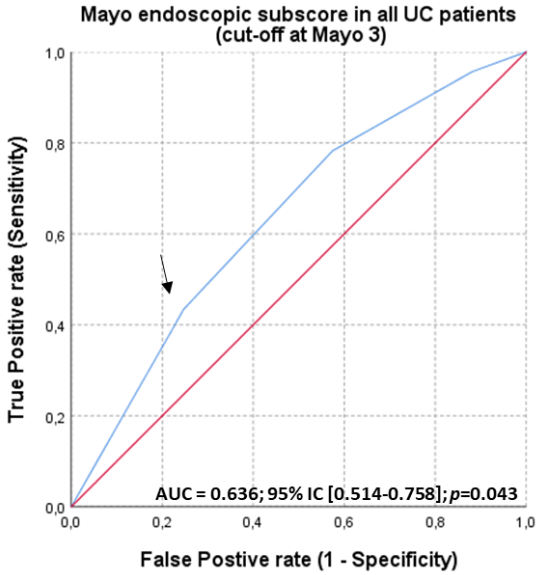
References

1. Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet* 2012;**380**:1606–19.
2. Baumgart DC, Sandborn WJ. Crohn's disease. *Lancet* 2012;**380**:1590–605.
3. Molodecky NA, Soon IS, Rabi DM, *et al*. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012;**142**:46–54.e42; quiz e30.
4. Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol* 2015;**12**:720–7.

5. Lönnfors S, Vermeire S, Greco M, Hommes D, Bell C, Avedano L. IBD and health-related quality of life—discovering the true impact. *J Crohns Colitis* 2014;**8**:1281–6.
6. Schirbel A, Reichert A, Roll S, *et al.* Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J Gastroenterol* 2010;**16**:3168–77.
7. D'Haens GR. Top-down therapy for IBD: rationale and requisite evidence. *Nat Rev Gastroenterol Hepatol* 2010;**7**:86–92.
8. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* 2015;**15**:540–55.
9. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell* 2006;**126**:855–67.
10. Carvalho S, Catarino TA, Dias AM, *et al.* Preventing E-cadherin aberrant N-glycosylation at Asn-554 improves its critical function in gastric cancer. *Oncogene* 2016;**35**:1619–31.
11. Carvalho S, Oliveira T, Bartels MF, *et al.* O-mannosylation and N-glycosylation: two coordinated mechanisms regulating the tumour suppressor functions of E-cadherin in cancer. *Oncotarget* 2016;**7**:65231–46.
12. Pinho SS, Carvalho S, Marcos-Pinto R, *et al.* Gastric cancer: adding glycosylation to the equation. *Trends Mol Med* 2013;**19**:664–76.
13. Pinho SS, Figueiredo J, Cabral J, *et al.* E-cadherin and adherens-junctions stability in gastric carcinoma: functional implications of glycosyltransferases involving N-glycan branching biosynthesis, N-acetylglucosaminyltransferases III and V. *Biochim Biophys Acta* 2013;**1830**:2690–700.
14. Dias AM, Dourado J, Lago P, *et al.* Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 2014;**23**:2416–27.
15. Dias AM, Almeida CR, Reis CA, Pinho SS. Studying T cells N-glycosylation by imaging flow cytometry. *Methods Mol Biol* 2016;**1389**: 167–76.
16. Dias AM, Correia A, Pereira MS, *et al.* Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 2018;**115**:E4651–60.
17. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the immune system. *Science* 2001;**291**:2370–6.
18. Marth JD, Grewal PK. Mammalian glycosylation in immunity. *Nat Rev Immunol* 2008;**8**:874–87.
19. Mkhikian H, Grigorian A, Li CF, *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nat Commun* 2011;**2**:334.
20. Harbord M, Eliakim R, Bettenworth D, *et al*. Third European evidence-based consensus on diagnosis and management of ulcerative colitis. Part 2: Current management. *J Crohns Colitis* 2017;**11**:1512.
21. Coskun M, Vermeire S, Nielsen OH. Novel targeted therapies for inflammatory bowel disease. *Trends Pharmacol Sci* 2017;**38**:127–42.
22. Danese S, Vuitton L, Peyrin-Biroulet L. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 2015;**12**:537–45.
23. Khare V, Lang M, Dammann K, Campregher C, Lyakhovich A, Gasche C. Modulation of N-glycosylation by mesalamine facilitates membranous E-cadherin expression in colon epithelial cells. *Biochem Pharmacol* 2014;**87**:312–20.
24. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 2001;**409**:733–9.
25. Morgan R, Gao G, Pawling J, Dennis JW, Demetriou M, Li B. N-acetylglucosaminyltransferase V [Mgat5]-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. *J Immunol* 2004;**173**:7200–8.
26. Pereira MS, Duraes C, Catarino TA, *et al*. P850: Genetic variants of MGAT5 gene are associated with ulcerative colitis severity and response to therapy. *J Crohns Colitis* 2018;**12**(Suppl 1):S546–S547.
27. Vermeire S, Van Assche G, Rutgeerts P. C-reactive protein as a marker for inflammatory bowel disease. *Inflamm Bowel Dis* 2004;**10**:661–5.
28. Tall AR. C-reactive protein reassessed. *N Engl J Med* 2004;**350**:1450–2.

Supplementary data

Supplementary *Figure 1*



Supplementary *Figure 1*_ Receiver-operator characteristic (ROC) curves plotted for Mayo endoscopic (MayoE) subscore in the prediction of the UC patients who do not respond to standard therapy. The arrow indicate the cut-offs that best discriminate patients who respond to standard therapy from those that need biologic therapies.

Supplementary Table 1

Supplementary Table 1_ Comparison of the expression levels of the branching N-glycans of colonic biopsies from UC patients at diagnosis (median months after diagnosis IQR 0.0 [0.0-0.1]) and adjacent to diagnosis (median IQR 13 [8.5-23.9]).

-	N	<5% L-PHA expression levels N(%)	5-25% L-PHA expression levels N(%)	>25% L-PHA expression levels N(%)	*p* value
At diagnosis	86	9(10.5)	15(17.4)	62(72.1)	**0.790**
Adjacent to diagnosis	45	4(8.9)	10(22.2)	31(68.9)	

Supplementary Table 2

Supplementary Table 2_ Association between branched N-glycans and other clinicopathological parameters.

		All UC patients				UC patients with severe endoscopic disease			
		N	**LPHA High N(%)**	**L-PHA Low N(%)**	***p* value**	**N**	**LPHA High N(%)**	**L-PHA Low N(%)**	***p* value**
Age at diagnosis	>36	62	43(69.4)	19(30.6)		12	7(58.3)	5(41.7)	
	0-36	69	50(72.5)	19(27.5)	0.695	23	16(69.6)	7(30.4)	0.506
Gender	F	65	48(73.8)	17(26.2)		11	9(81.8)	2(18.2)	
	M	66	45(68.2)	21(31.8)	0.564	24	14(58.3)	10(41.7)	0.259
Disease extension	Proctitis/ left-sided*	88	59(67.0)	29(33.0)		22	14(63.6)	8(36.4)	
	Pancolitis	43	34(79.1)	9(20.9)	0.218	13	9(69.2)	4(30.8)	1.000
MayoE score	0-2	89	65(73.0)	24(27.0)		-	-	-	-
	3	35	23(65.7)	12(34.3)	0.419	-	-	-	-
Clinical remission	Yes	45	33(73.3)	12(26.7)		1	0(0.0)	1(100)	
	No	81	56(69.1)	25(30.9)	0.686	34	23(67.6)	11(32.4)	0.343
Biochemical remission	Yes	51	37(72.5)	14(27.5)		8	4(40.0)	4(40.0)	
	No	44	27(61.4)	17(38.6)	0.278	19	11(57.9)	8(42.1)	0.706
Surgery	No	127	90(70.9)	37(29.1)		33	21(63.6)	12(36.4)	
	Yes	4	3(75.0)	1(25.0)	1.000	2	2(100)	0(0.0)	0.536
Therapy	No therapy/ Naive	63	49(77.8)	14(22.2)		21	17(81.0)	4(19.0)	
	5-ASA	52	36(69.2)	16(30.8)		8	5(62.5)	3(37.5)	
	Other therapy	16	8(50.0)	8(50.0)	0.086	6	1(16.7)	5(83.3)	**0.014**
Responder	Yes*	108	81(75.0)	27(25.0)		25	20(80.0)	5(20.0)	
	No	23	12(52.2)	11(47.8)	**0.029**	10	3(30.0)	7(70.0)	**0.015**
Multivariate analysis - Stepwise			**OR [95% IC]**		***p* value**		**OR [95% IC]**		***p* value**
Disease Extension			0.32 [0.10-1.05]		0.059		-		-
Responder			3.73 [1.14-12.22]		**0.029**		5.60 [1.02-30.90]		**0.048**

Supplementary Table 3

Supplementary Table 3_ Computed predictive probabilities of non-response to standard therapy using both branched N-glycans and C-reactive protein (CRP) levels. Highlighted in blue are the extreme predicted probabilities.

			L-PHA expression levels	
		N	**High %(N)**	**Low %(N)**
All UC patients				
CRP levels	**Low**	51	7.9(37)	14.9(14)
	High	41	30.0(25)	46.6(16)
UC patients with severe endoscopic disease				
CRP levels	**Low**	8	7.9(4)	22.0(4)
	High	19	26.2(11)	76.5(8)

Chapter III

Genetic Variants of the MGAT5 Gene Are Functionally Implicated in the Modulation of T Cells Glycosylation and Plasma IgG Glycome Composition in Ulcerative Colitis

Márcia S. Pereira, MSc[1,2], Cecília Durães, PhD[1], Telmo A. Catarino, MSc[1], José L. Costa, PhD[1,3], Isabelle Cleynen, PhD[4], Mislav Novokmet, PhD[5], Jasminka Kristić, PhD[5], Jerko Štambuk, PhD[5], Nádia Conceição-Neto, PhD[6], José C. Machado, PhD[1,3], Ricardo Marcos-Pinto, MD, PhD[2,7], Fernando Magro, MD, PhD[8], Séverine Vermeire, MD, PhD[9], Gordan Lauc, PhD[5], Paula Lago, MD[7] and Salomé S. Pinho, DVM, PhD[1,3]

OBJECTIVES: **The impact of genetic variants (single nucleotide polymorphisms [SNPs]) in the clinical heterogeneity of ulcerative colitis (UC) remains unclear. We showed that patients with UC exhibit a deficiency in *MGAT5* glycogene transcription in intestinal T cells associated with a hyperimmune response. Herein, we evaluated whether *MGAT5* SNPs might functionally impact on T cells glycosylation and plasma IgG glycome in patients with UC, as well as in UC clinical outcomes.**

METHODS: **Three selected *MGAT5* SNPs (rs3814022, rs4953911, and rs1257220), previously associated with severity of autoimmune disease or with plasma glycome composition in healthy individuals, were functionally evaluated in patients with UC through analysis of *MGAT5* mRNA levels in colonic (n = 14) and circulating (n = 24) T cells and through profiling the plasma IgG Fc glycosylation (n = 152). *MGAT5* SNPs were genotyped in 931 patients with UC from 2 European cohorts and further associated with patients' prognosis. Targeted next-generation sequencing for *MGAT5* coding and regulatory regions was also performed.**

RESULTS: ***MGAT5* SNPs were shown to be functionally associated with low transcription levels of *MGAT5* in colonic and circulating T cells from patients with UC and with agalactosylation of IgGs, often associated with a proinflammatory phenotype. The SNPs rs3814022 and rs4953911 were further associated with the need of biologics. Next-generation sequencing data further revealed a combination of *MGAT5* SNPs that stratify patients with UC according to their severity.**

DISCUSSION: **Our results revealed that *MGAT5* SNPs have a phenotypic impact on T cells glycosylation and in plasma IgG glycome composition associated with UC pathogenesis. *MGAT5* SNPs display a tendency in the association with a worse disease course in patients with UC.**

SUPPLEMENTARY MATERIAL accompanies this paper at: http://links.lww.com/CTG/A267, http://links.lww.com/CTG/A268, http://links.lww.com/CTG/A269, http://links.lww.com/CTG/A270, http://links.lww.com/CTG/A271, http://links.lww.com/CTG/A272, http://links.lww.com/CTG/A273, http://links.lww.com/CTG/A274, http://links.lww.com/CTG/A275, http://links.lww.com/CTG/A276, http://links.lww.com/CTG/A277, http://links.lww.com/CTG/A278

Clinical and Translational Gastroenterology 2020;11:e00166. https://doi.org/10.14309/ctg.0000000000000166

INTRODUCTION

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a multifactorial disorder in which genetically susceptible individuals develop an exacerbated immune response in the gut (1). The heterogeneity of IBD in terms of disease course, severity and therapeutic outcomes, highlights the urgent need in the clinics to identify reliable biomarkers that could help in patients' stratification, enabling

[1]Instituto de Investigação e Inovação em Saúde (I3S)/Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal; [2]Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal; [3]Faculty of Medicine, University of Porto, Porto, Portugal; [4]Department of Human Genetics, KU Leuven, Leuven, Belgium; [5]Genos Glycoscience Research Laboratory, Zagreb, Croatia; [6]Department of Microbiology and Immunology, KU Leuven-University of Leuven, Laboratory of Viral Metagenomics, Rega Institute, Leuven, Belgium; [7]Department of Gastroenterology, Porto Centre Hospital, Porto, Portugal; [8]Department of Gastroenterology, Centro Hospitalar de São João, Porto, Portugal; [9]Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven, Leuven, Belgium. **Correspondence:** Salomé S. Pinho, PhD. E-mail: salomep@ipatimup.pt.

Received November 12, 2019; accepted March 12, 2020; published online April 22, 2020

a personalized medicine through optimized preventive and therapeutic strategies. Genome-wide association studies have revolutionized our understanding of complex diseases. In fact, susceptibility to IBD is unequivocally a complex genetic trait with around 240 distinct genetic risk loci identified so far (2), most of them is associated with both CD and UC, whereas 54 are CD specific and 31 are UC specific. These loci are enriched in genes related with the immune system and the predisposition for host–microbiome interactions. The disease course and response to therapy are, however, less clearly defined by genetic factors (3,4), which remain as a major challenge for IBD research and clinical practice. Associations with some clinical features such as age at onset and disease location (*NOD2*, *MST1* 3p21, and major histocompatibility complex [MHC]) (5), need for surgery (*NOD2*) (6), and nonresponse to therapy (major histocompatibility complex and *MDR1*) (7–9) were shown. Curiously, most of the IBD risk single nucleotide polymorphisms (SNPs) are located in the noncoding regions, which make up 98% of the DNA (10–13). This highlights the importance of these noncoding regions in IBD immunopathogenesis, which is supported by the fact that those SNPs may be in linkage disequilibrium with relevant coding variants, something especially true in the HLA region on Chromosome 6 as shown for HLA variants (10,14). In fact, IBD-associated risk SNPs were shown to be located in regulatory regions of immune and epithelial cells (15) and to be enriched in activated DNA regulatory elements such as enhancers and promoters, in active disease (16).

Glycosylation is considered a pleotropic event in which the addition of glycans to proteins and lipids is essential for the regulation of both innate and adaptive immune responses (17–20). In humoral response, *N*-glycosylation of the conserved fraction (Fc) of immunoglobulin G (IgG) is known to regulate the effector functions of the Igs by modulating the binding affinity to FcγRs, instructing either a proinflammatory or an antiinflammatory response (21,22). In fact, alterations on IgG *N*-glycosylation were shown to predict the onset of immune-mediated diseases such as rheumatoid arthritis (23) and were recently associated with IBD clinical features (24–27). The role of protein glycosylation in the immunopathogenesis of chronic inflammatory gastrointestinal diseases, such as IBD, has been well-documented (28). In particular, the β-1,6 GlcNAc branched *N*-glycans (catalyzed by the *N*-acetylglucosaminyltransferase [GnT]-V enzyme that is encoded by *MGAT5*) were demonstrated to act as a critical determinant for T-cell function and activity (17) in major diseases such as multiple sclerosis (MS) (29–31) and type-I diabetes (32). In the context of gut immunity, we demonstrated that patients with UC display a deficiency on these branched *N*-glycans at the surface of intestinal T cells owing to a significant downregulation of the *MGAT5* glycogene (33), which encodes the GnT-V enzyme. This dysfunction in *MGAT5*/GnT-V–mediated branched *N*-glycosylation was associated with the hyperactivation of the T-cell–mediated immune response because of its effects in the regulation of the threshold of T-cell activation (34–36). We further showed that the levels of expression of branched *N*-glycans have prognostic applications by predicting the failure to standard therapy in UC (37).

Despite the clinical and pathogenic role of *MGAT5*-mediated glycosylation in UC, the underlying cause for the deficiency of *MGAT5* glycogene expression associated with the pathogenesis of UC remains unknown. Interestingly, genetic variants (polymorphisms) of *MGAT5* glycogene were associated with other immune-mediated disorders such as MS (38,39), which is a model for IBD understanding. Intronic variants of *MGAT5* were significantly associated with the clinical outcome of MS (38) and with variations in the human plasma *N*-glycome composition of healthy European adults (40).

In this study, we explored whether genetic variants of *MGAT5* gene are functionally correlated with the glycosylation alterations on T cells and with changes in plasma glycome composition associated with UC pathogenesis and clinical outcomes of patients, an issue that was never explored before.

METHODS

SNP genotyping

We have selected relevant *MGAT5* genetic variants based on previous associations with the severity of other immune-mediated diseases (such as MS) and with plasma glycome variations. Through literature search terms "MGAT5" AND ("genetic" or "polymorphisms" or "association study" or "autoimmune disease"), we have selected 3 intronic *MGAT5* SNPs: 2 SNPs (rs3814022 and rs4953911) previously associated with severity to MS (38,41) and 1 SNP (rs1257220) that was previously associated with alterations in human plasma *N*-glycome in a healthy European population (40). Genotyping of these 3 SNPs was performed using TaqMan SNP genotyping assays (c__7480346_10 for rs1257220; c__2163491_10 for rs3814022; and c__3258342_10 for rs4953911) and the ABI 7500 Fast real-time PCR system (Applied Biosystems, CA).

Quantification of mRNA from CD3+ T cells of the blood and biopsy

CD3+ T cells were isolated from 24 blood samples from patients with UC (18 with active disease—with Mayo endoscopic subscore > 0). A density gradient centrifugation (with Lymphoprep, STEMCELL) was used to obtain a peripheral blood mononuclear cells suspension, and CD3+ cells were magnetically sorted using the EasySep Human T Cell Enrichment Kit (STEMCELL) following the manufacturer's instructions. CD3+ T cells were also isolated from 14 fresh colon biopsies (12 with active disease), through mechanical dissociation (36) and posterior magnetically sorted with EasySep Human T Cell Enrichment Kit (STEMCELL).

RNA was isolated using RNAqueous-Mirco kit (Ambion), and *MGAT5* and *GAPDH* (used as reference genes) expression levels were detected using the Hs00159136_m1 and Hs02758991_g1 probes, respectively, by TaqMan Real-time PCR as previously described (33). The *MGAT5* expression levels were evaluated in patients who display different genetic variants of the SNPs rs1257220, rs3814022, and rs4953911 and were normalized to *GAPDH* housekeeping expression by delta-CT method, and the results are shown as relative expression.

IgG glycosylation evaluation

IgG was isolated from a human plasma using affinity chromatography coupled to mass spectrometry as described previously (42). Briefly, 100 μL of plasma was loaded onto Protein G monolithic plate (BIA Separations, Ljubljana, Slovenia) and washed 3 times with 1× PBS. IgG was eluted using 0.1% formic acid and immediately neutralized with 1 M ammonium bicarbonate. Isolated IgG was digested with trypsin, and glycopeptides were purified using solid phase extraction as described previously (43) with slight changes. Approximately 20 μg of IgG

was treated with 200 ng of trypsin (Promega, Fitchburg, WI) and incubated overnight at 37°C. Tryptic digests were loaded onto Chromabond C-18 stationary phase (Marcherey-Nagel, Düren, Germany) and washed 3 times with 0.1% TFA. Glycopeptides were eluted using 20% ACN and dried completely using a vacuum centrifuge. IgG glycopeptides were analyzed using M-class ACUITY UPLC system (Waters, Milford, MA) coupled to Compact mass spectrometer (Bruker Daltonics, Bremen, Germany). Dried glycopeptides were reconstituted in 80 μL of ultrapure water, and 5 μL were loaded onto Acclaim PepMap100 C8 (5 mm × 300 μm i.d.) trap column (Thermo Fisher Scientific, Waltham, MA) and washed for 1 minute with 0.1% TFA (solvent A) at a flow rate of 40 μL/min. Subclass-specific glycopeptides were separated on a Halo C18 nano-LC column (150 mm × 75 μm i.d., 2.7 μm HALO fused core particles; Advanced Materials Technology, Wilmington, DE) using a 3.5-minute gradient at a flow rate of 1 μL/min from 18% to 28% solvent B (80% ACN). Column temperature was maintained at 30°C. LC was coupled to MS through CaptiveSpray (Bruker) ion source equipped with NanoBooster (Bruker) using acetonitrile as a dopant in nebulizing gas (nitrogen). Mass spectra were recorded from m/z 800 to 2000 with 2 averages at a frequency of 0.5 Hz. Quadrupole ion energy and collision energy of the MS were set at 5 eV. Argon was used as collision gas. M-class ACUITY UPLC system was operated under MassLynx software version 4.1 (Waters), whereas Compact was controlled by HyStar software, version 4.2 (Bruker). Obtained raw data were converted into mzXML file format using ProteoWizard version 3 MSConvert tool. Data were extracted with LacyTools version 1.0.1. Alignment Parameters: alignment time window—25; mass window—0.1; signal-to-noise ratio—9; and alignment minimal peak—5. Calibration Parameters: mass window—0.5; signal-to-noise cutoff—9; and minimal peak number—3. Extraction Parameters: sum spectrum resolution—33; mass window—0.2; time window—25; minimum charge—2; and maximum charge—3. Data extraction was performed separately for doubly and triply charged species without subtraction. Absolute intensities of doubly and triply charged species were manually summed, and relative abundance of each of 20 glycoforms within each IgG subclass was calculated. Determination of relative abundance of agalactosylation, monogalactosylation, digalactosylation, sialylation, bisecting GlcNAc, and fucosylation were performed as stated in Table S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A272.

UC cohort

This study comprises a Portuguese discovery cohort with 432 patients with UC from Centro Hospitalar e Universitário do Porto/Hospital Santo António (CHUP/HSA) and Hospital São João and a Belgian validation cohort with 499 patients with UC from University Hospitals Leuven. In the discovery cohort, patients were diagnosed with UC between 1968 and 2014, with a median (interquartile range [IQR]) age at diagnosis of 35 (26–46) years and 55.8% female patients. Patients from the validation cohort were diagnosed between 1957 and 2012 with a median (IQR) age at diagnosis of 33 (24–43) years and 43.8% female patients. Family history of disease includes patients with at least one first-degree relative with IBD. All clinical information (need for colectomy and biologics) from both cohorts was collected through the hospital-based electronic medical record system and is provided in Table S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A273. Patients were classified as having *worse prognosis* when there was the need to step up to biologics within 2 years after diagnosis (a time in disease course that was consider to be an early introduction of more aggressive therapy owing to a more severe disease (44,45)), whereas those who did not need biologics or only stepped up to biologics after this period were considered as having a *better prognosis*. For next-generation sequencing (NGS), patients who only received 5-ASA or corticosteroid therapy (without experiencing neither resistance, who did not respond within 1 month of oral or 1 week of intravenous corticosteroid therapy, nor dependency, who initially responded but were unable to maintain control of symptoms without corticosteroids and needed low doses to remain symptom free (46,47)) were classified as having *low disease severity*, whereas a *severe/complicated disease* was defined when there was corticosteroid dependency, and/or corticosteroids resistance, and/or nonresponders to immunosuppressors (classified as patients keeping the activity and severity of disease despite optimal dose of thiopurine/methotrexate), and/or with need for biologics and/or need for colectomy. All participants gave informed consent about all clinical procedures and research protocols were approved by the ethics committee of the hospital.

Next-generation sequencing targeting the *MGAT5* gene

Patients with UC were sequenced by NGS for all coding (including few intronic nucleotides nearby the exons) and regulatory regions of the *MGAT5* gene, including a described promoter (48) and the 3′UTR. Primers used to amplify the mentioned regions are presented in Table S3, Supplementary Digital Content 3, http://links.lww.com/CTG/A274. NGS was performed in 31 patients with UC with a high genetic influence including 12 with family history (first-degree relatives with IBD) and 19 with a disease onset ≤18 years old (with a median [IQR] age at diagnosis of 15 [14–18] years), 8 patients with UC with a low genetic influence (without family history and with a late onset disease; median age of 22 [19–32] years), and 6 healthy individuals.

Sample DNA was quantified using the Qubit 3.0 fluorometer S/N 2321600945—Qubit dsDNA HS Assay kit P/N Q32851. Libraries were constructed according to Ion Xpress Plus Fragment Library Kit protocol and pooled: each sample was ligated to a unique barcode for sample identification and tracking. The pooled libraries were processed on Ion Chef System and sequenced on an Ion 314 chip using the Ion PGM System. FASTQ and/or BAM files were generated using the Torrent Suit plugin FileExporter v5. Data from the PGM run were processed using the Ion Torrent platform-specific pipeline software Torrent Suite v5 (Life Technologies) to generate sequence reads, trim adapter sequences, filter and remove poor signal reads, and split the reads according to the barcode. Reads assembly was performed by default settings with SeqMan NGen v14 (DNAStar, Madison, WI) using the FastQ files containing sequence reads, and the template references were adjusted for the covered amplicons. SeqMan Pro v14 (DNAStar) was used as postassembly analysis tool for the analysis of overall amplicon coverage, individual base depth of coverage, and variant identification.

Statistical analysis

Differences between *MGAT5* mRNA levels of the different genotypes were assessed with Dunn's multiple comparison test or Mann–Whitney test using GraphPad (7.0). For each genotype, the outliers were identified and excluded. In addition, a linear

logistic regression analysis for *MGAT5* expression levels was performed in SPSS (26), using dominant (at least 1 copy of the risk allele), recessive (2 copies of the risk allele), or additive (presenting 0, 1, or 2 copies of the risk allele) models (with the major allele from each SNP as reference) as variables. The model was also adjusted to disease activity based on Mayo endoscopic (E) subscore at the time of sample collection (MayoE = 0—inactive and MayoE ≥ 1—active). A linear logistic regression was also performed for IgG glycosylation, with dominant or recessive models as variables together with age at the time of sample collection and gender.

Association between the SNPs and the clinical parameters was independently assessed using the SNPassoc package in R (3.5.1) and considering additive, dominant, and recessive models. For each model, an unconditional logistic regression was performed adjusting for gender and age at diagnosis. Haplotype frequencies were inferred using the haplo.stats package in R. Multiple testing correction was performed using the false discovery rate (FDR) method (49). Any SNP association resists to multiple correction; thus, all the *P* values presented are uncorrected. Association of haplotypes with clinical features was assessed for those with a minimum haplotype frequency above 0.01 and using the most frequent haplotype as reference. NGS data were clustered in SPSS (25) using hierarchical clustering analysis to group patient with UC according to the identified genetic variants (0, 1, or 2 rare alleles), using the between-groups linkage method and the squared Euclidean distance as distance measure.

RESULTS

MGAT5 genetic variants are associated with lower *MGAT5* transcription in circulating T cells and impact on plasma IgG glycome composition in patients with UC

Impact of* MGAT5 *genotypes on mRNA transcription levels of colonic and circulating T cells from patients with UC. The deficiency on *MGAT5* transcription was previously associated with T-cell hyperactivation and UC disease severity (33,36). Moreover, levels of *MGAT5*-mediated branched glycosylation on the intestinal inflammatory infiltrate predict the nonresponse to standard therapy (37). We analyzed whether specific genetic variants of *MGAT5* gene, known to be associated with severity of autoimmune disease and with changes in glycome of healthy adults, are functionally correlated with changes in *MGAT5* mRNA transcription on lamina propria and circulating T cells from patients with UC. A logistic linear regression analysis was performed and adjusted to MayoE subscore, and the results showed that rs3814022 and rs4953911 are significantly associated with lower mRNA levels of the *MGAT5* glycogene both *in situ* (in colonic CD3+ T cells) and circulating CD3+ T cells from patients with UC (Table 1). The same result was observed in circulating T cells without adjusting to disease activity (Figure 1). No association was observed for SNP rs1257220. These results show the influence of *MGAT5* SNPs in *MGAT5* expression of T cells in patients with UC.

MGAT5 ***genetic variants influence plasma IgG Fc N-glycosylation profile of patients with UC.*** We further analyzed the functional implications of the different *MGAT5* genetic variants in IgG glycome composition of 152 patients with UC (see Table S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A272, and Figure S1, Supplementary Digital Content 4, http://links.lww.com/CTG/A267). Interestingly, in linear regression analysis, we found that rs3814022 and rs4953911 are significantly correlated with lower levels of monogalactosylation of the Fc domain of IgG2 and IgG3 (rs3814022 dominant model [CG + GG vs CC], Beta = −0.164, R^2 = 0.044, *P* = 0.044; rs4953911 dominant model [AT + TT vs AA], Beta = −0.184, R^2 = 0.051, *P* = 0.024; Table 2). The SNP rs4953911 was also found to be associated with agalactosylation of IgG1 (dominant model [AT + TT vs AA], Beta = 0.154, R^2 = 0.194, *P* = 0.040; Table 1), which is in accordance with the fact that agalactosylation of IgG is often associated with a proinflammatory effector functions of IgGs (50). In addition, rs4953911 is associated with higher levels of fucosylation on IgG2 and IgG3 (recessive model [TT vs AT + AA], Beta = 0.184; R^2 = 0.015, *P* = 0.027; Table 2). Similar to what we observed in relationship with *MGAT5* mRNA levels, the SNP rs1257220 did not show any association with IgG glycosylation profile in patients with UC.

Taken together, these observations demonstrate for the first time a direct correlation between genetic variants in a specific glycogene and its functional/phenotypic impact, through modulation of the glycosylation profile of key molecules involved in UC pathogenesis, such as T cells (28,33,36) and plasma IgGs (24).

MGAT5 SNPs are associated with UC clinical and therapeutic outcomes

To gain further insights on the clinical and prognostic relevance of the 3 SNPs in UC clinical outcome, we have genotyped the 3 SNPs in a Portuguese discovery cohort and validated in a Belgium validation cohort (see Table S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A273).

In the discovery cohort, SNP rs3814022, which phenotypically associates with alterations in the glycosylation of T cells and plasma IgG glycome in patients with UC, seemed to be associated with the need to step up to biologics (recessive model [GG vs CC + CG]: OR = 2.27, *P* = 0.032, and log-additive model: OR = 1.53, *P* = 0.021; Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275). This observation was, at least indirectly validated in the Belgium cohort, as the same variant (rs3814022 dominant model [CG + GG vs CC], OR = 2.21, *P* = 0.027; Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275), together with rs4953911 (dominant model [AT + TT vs AA]: OR = 2.51, *P* = 0.012; log-additive: OR = 1.84, *P* = 0.022; Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275), were associated with the need to step up therapy to biologics within the first 2 years after diagnosis (defined as worse prognosis). The combination of both cohorts suggest the association between rs3814022 and the need of biologics (dominant model: G carriers, OR = 1.36, *P* = 0.046; log-additive: OR = 1.31, *P* = 0.022; Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275) and rs4953911 with need to step up to biologics within 2 years of disease (recessive model: TT, OR = 1.75, *P* = 0.047). Once time—disease duration since diagnosis until the clinical and therapeutic characterization—was added in as a variable, the associations between rs3814022 and rs4953911 and the need of biologics remains and follow the same tendency (see Table S5, Supplementary Digital Content 6, http://links.lww.com/CTG/A276).

Furthermore, rs1257220 SNP (not correlated with changes in glycosylation) seemed to be associated with family history of IBD in the discovery cohort (dominant model [GA + AA vs GG]: OR = 4.2, *P* = 0.022), further validated in the Belgium cohort

Table 1. Associations of the polymorphisms rs1257220, rs3814022, and rs4953911 with *MGAT5* mRNA expression levels of circulating (N = 24) and lamina propria CD3+ T cells (N = 14) from patients with UC

		Adjusted *R* square	β	B	95% IC	*P* value
Circulating CD3+ T cells						
rs2257220	Dominant model G/G[a] vs A/G-A/A	−0.029	−0.236	−0.131	−0.375 to 0.113	0.277
	Recessive model G/G-A/G[a] vs A/A	−0.065	−0.153	−0.305	−1.212 to 0.601	0.491
	Additive model G[a]/A (0,1,2)	−0.024	−0.246	−0.211	−0.587 to 0.165	0.256
rs3814022	Dominant model C/C[a] vs C/G-G/G	0.147	−0.475	−0.278	−0.517 to −0.038	***0.025***
	Recessive model C/C-C/G[a] vs G/G	−0.084	−0.072	−0.107	−0.775 to 0.561	0.742
	Additive model C[a]/G (0,1,2)	0.048	−0.358	−0.288	−0.631 to 0.055	0.096
rs4953911	Dominant model A/A[a] vs T/A-T/T	0.158	−0.477	−0.289	−0.531 to −0.048	***0.021***
	Recessive model A/A-T/A[a] vs T/T	−0.09	−0.017	−0.025	−0.718 to 0.669	0.941
	Additive model A[a]/T (0,1,2)	0.033	−0.337	−0.28	−0.635 to 0.076	0.117
Colonic CD3+ T cells						
rs2257220	Dominant model G/G[a] vs A/G-A/A	0.216	−0.24	−0.003	−0.011 to 0.004	0.354
	Recessive model G/G-A/G[a] vs A/A	0.17	−0.133	−0.005	−0.026 to 0.016	0.614
	Additive model G[a]/A (0,1,2)	0.205	−0.216	−0.004	−0.013 to 0.006	0.4
rs3814022	Dominant model C/C[a] vs C/G-G/G	0.648	−0.608	−0.009	−0.015 to −0.003	***0.006***
	Recessive model C/C-C/G[a] vs G/G	0.233	−0.075	−0.004	−0.04 to 0.033	*0.831*
	Additive model C[a]/G (0,1,2)	0.601	−0.653	−0.016	−0.027 to −0.004	**0.012**
rs4953911	Dominant model A/A[a] vs T/A-T/T	0.648	−0.608	−0.009	−0.015 to −0.003	***0.006***
	Recessive model A/A-T/A[a] vs T/T	0.233	−0.075	−0.004	−0.04 to 0.033	*0.831*
	Additive model A[a]/T (0,1,2)	0.601	−0.653	−0.016	−0.027 to −0.004	**0.012**

Logistic linear regression was performed for dominant, recessive, and additive models, and it was adjusted to disease activity (based on Mayo subscore; Mayo 0: inactive and Mayo 1–3: active).
Bold-italic is to highlight the *P* values that are statistically significant.
CD, Crohn's disease; CI, confidence interval; UC, ulcerative colitis.
[a]Reference genotype.

(recessive model [AA vs GG + GA]: OR = 3.59, $P = 0.021$) (Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275) and in the combination of both cohorts (dominant model [GA + AA vs GG]: OR = 2.13, $P = 0.025$; recessive model [AA vs GG + GA]: OR = 2.13, $P = 0.025$; log-additive model: OR = 1.91, $P = 0.027$). In the combined cohort, rs1257220 was further associated with the need for colectomy (dominant model [GA + AA vs GG]: OR = 0.71, $P = 0.037$; Table 3 and see Table S4, Supplementary Digital Content 5, http://links.lww.com/CTG/A275).

The differences between the discovery and validation cohorts might reflect the fact that Leuven IBD center should be a tertiary referral center for IBD. The proportion of patients with extensive disease (pancolitis) who needed biologics is higher compared with Porto center hospital (see Table S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A273), which together with heterogeneity among some clinical/therapeutic features between both cohorts might contribute to explain the different associations between the 2 cohorts.

Haplotypes were further inferred for each cohort and associated with the same clinical parameters, using as a reference the most frequent haplotype—GCA (G for rs1257220; C for rs3814022; and A for rs4963911; Table 4). Two haplotypes were associated with clinical features: ACA haplotype (containing the

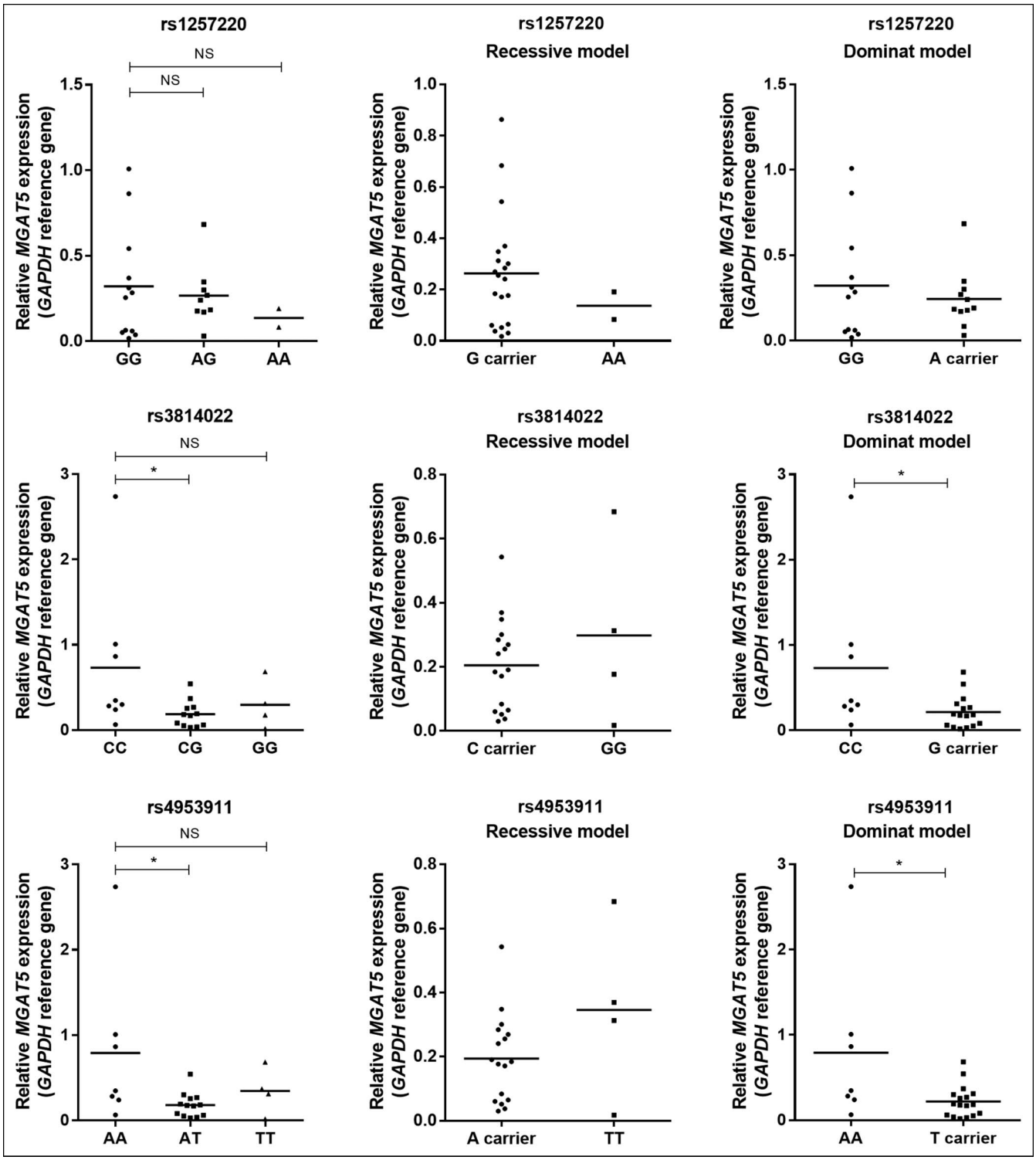


Figure 1. *MGAT5* mRNA expression levels on circulating CD3+ T cells from 24 blood samples of patients with UC determined for the different genotypes of rs1257220, rs3814022, and rs4953911. The mRNA of *MGAT5* expression levels were normalized to *GAPDH* expression by delta-CT method, and the data are the relative expression. G and T risk alleles of rs3814022 and rs4953911 showed lower levels of *MGAT5* mRNA than C and A alleles, respectively. Outliers in each genotype were identified and excluded. Dunn's multiple comparison test was used to determine the differences of *MGAT5* mRNA levels between genotypes and Mann–Whitney test to assess differences in dominant and recessive models. *P* values < 0.05 were considered as significant. NS, not significant; UC, ulcerative colitis.

A risk allele rs1257220) with family history (validation cohort: OR = 2.39, $P = 0.021$; combined study: OR = 2.26, $P = 0.011$; Table 4) and need of biologics within 2 years (discovery cohort: OR = 2.81, $P = 0.023$); and GGT haplotype with the need for biologics (discovery cohort: OR = 1.74 [1.03–2.94], $P = 0.037$; combined study: OR = 1.47, $P = 0.023$, Table 4). Taking into

Table 2. Associations of the *MGAT5* SNPs rs1257220, rs3814022, and rs4953911 with IgG Fc glycosylation profile (only the statistically significant associations are shown)

	IgG subtype	IgG glycosylation	Adjusted *R* square	β	95% CI	*P* value
rs3814022						
C/C[a] vs C/G-G/G	IgG2 and IgG3	Monogalactosylation	0.044	−0.164	−1.413 to −0.018	0.044
rs4953911						
A/A[a] vs T/A-T/T	IgG1	Agalactosylation	0.194	0.154	0.082 to 3.328	0.040
A/A[a] vs T/A-T/T	IgG2 and IgG3	Monogalactosylation	0.051	−0.184	−1.522 to 0.110	0.024
A/A-T/A[a] vs T/T	IgG2 and IgG3	Fucosylation	0.015	0.184	0.111 to 1.839	0.027

Linear regression was adjusted to age and gender.
CI, confidence interval; IgG, Immunoglobulin G; SNP, single nucleotide polymorphism.
[a]Reference genotype.

consideration that patients carrying the GGT haplotype have a higher chance to be refractory to the standard therapy than the ones with the GCA haplotype, we can infer that the combination of these 2 risk SNPs (rs3814022 (G) and rs4953911 (T)) is more valuable and accurate in the prediction of the therapeutic outcome than rs1257220 alone.

Altogether, these results suggest the clinical relevance of *MGAT5* genetic variants in UC clinical outcome and patients' stratification. Nevertheless, none of the SNPs survived after multiple testing correction (see Table S6, Supplementary Digital Content 7, http://links.lww.com/CTG/A277). These results, even if suggestive of *MGAT5* involvement in development of a severe/complicated UC, require a bigger sample size to confirm the role of this locus in UC disease course.

MGAT5 genetic variants from the 3′UTR are able to cluster patients with UC according to their disease severity

To further investigate other genetic variants on *MGAT5* that could be determining clinical outcomes of patients with UC, we screened coding (including few intronic nucleotides nearby the exons) and regulatory regions (including a described promoter (48) and the 3′UTR) of the gene. We selected patients with UC with a high genetic influence—patients with family history (first-degree relatives with IBD) and with age of IBD onset ≤ 18 years old, as well as UC patients with low genetic influence including late-onset patients without family history and healthy individuals.

Through targeted sequencing of all coding (including few intronic nucleotides nearby the exons) and regulatory regions (including a described promoter (48) and the 3′UTR) of the *MGAT5* gene, we identified a total of 46 genetic alterations. These included 1 deletion and 45 single nucleotide variants: 7 in the promoter, 28 in the 3′UTR, 9 in intronic regions, and 2 in exon regions (see Figure S2-S3, Supplementary Digital Content 8 and 9, http://links.lww.com/CTG/A268, http://links.lww.com/CTG/A269 and see Table S7, Supplementary Digital Content 10, http://links.lww.com/CTG/A278). The 2 SNPs identified in exons 9 (rs34876684) and 16 (rs2230908) are synonymous variants.

Clustering of the individuals according to genetic variants showed no discriminate pattern between healthy individuals and patients with UC (see Figure S3, Supplementary Digital Content 9, http://links.lww.com/CTG/A269). The distribution of the SNPs seems to be independent of the genetic burden of the patient (see Figure S3, Supplementary Digital Content 9, http://links.lww.com/CTG/A269). Focusing only on patients with UC, one of the main clusters (highlighted with a blue box; see Figure S4, Supplementary Digital Content 11, http://links.lww.com/CTG/A270) is completely (6 of 6) composed by patients with UC with low/mild severe disease (always on 5-ASA or previous corticosteroid therapy). The same is observed when including only genetic variants with a minor allele frequency ≥10% (Figure 2), which are predominantly constituted by SNPs from the 3′UTR region and the above-explored intronic SNPs. Moreover, another cluster (highlighted with an orange box; Figure 2 and see Figure S4, Supplementary Digital Content 11, http://links.lww.com/CTG/A270) was observed to be composed mainly by patients with UC with high severity/complicated disease. The specificity of the cluster increases when considering only the most frequent SNPs, from 68% (13/19; see Figure S4, Supplementary Digital Content 11, http://links.lww.com/CTG/A270) to 80% (12/15; Figure 2). Interestingly, the clustered group with patients with low severity is mainly composed by major alleles (rs3748900, rs34497810, rs62170036, rs7841, rs3762484, rs3214771, and rs1257198), including the 3 *MGAT5* SNPs studied. The minor allele (in homozygosity) of 6 SNPs located in the 3′UTR (rs2439568, rs626540, rs651970, rs681148, rs669740, and rs636975; Figure 2) is also mainly present in patients developing a mild disease (Table 5). In addition, the logistic regression model shows that the minor allele from rs3214771 (intronic region before exon 8) and both studied risk alleles (rs3814022 (G) and rs4953911 (T)) are statistically associated with high severity/complicated UC (Table 5). Overall, these data support the identification of novel genetic variants, mainly in the regulatory 3′UTR region of *MGAT5*, which stratify patients with UC in accordance with disease severity.

DISCUSSION

The susceptibility to IBD is unequivocally a complex genetic trait; however, the disease course, outcomes and response to therapy, is less clearly influenced by genes (51,52). In this study, we have identified a novel genetic risk locus that includes intronic SNPs in the glycogene *MGAT5* that are functionally correlated with glycosylation alterations on T cells and plasma IgGs, both previously shown to be implicated in the regulation of the immune response in UC (33,36) and with clinical features in IBD (24), respectively. These *MGAT5* genetic variants, together with other SNPs from

Table 3. Associations between rs1257220, rs3814022, and rs4953911, which are significant for at least one of the clinical and therapeutic outcomes in patients with UC

	Discovery cohort						Validation cohort						Combined cohort											
	Family history			Need for biologics			Family history			Prognosis			Family history			Need for colectomy			Need for biologics			Prognosis		
	No[a] vs Yes			No[a] vs Yes			No[a] vs Yes			Good[a] vs Bad			No[a] vs Yes			No[a] vs Yes			No[a] vs Yes			Good[a] vs Bad		
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
rs1257220																								
Dominant																								
G/G	**1.00**		**0.022**	1.00		0.921	1.00		0.284	1.00		0.846	**1.00**		**0.025**	**1.00**		**0.037**	1.00		0.915	1.00		0.675
A/G-A/A	**4.02**	**1.09–14.86**		0.97	0.58–1.64		1.57	0.69–3.60		0.93	0.46–1.87		**2.13**	**1.09–4.19**		**0.71**	**0.51–0.98**		1.02	0.75–1.38		1.12	0.65–1.93	
Recessive																								
G/G-A/G	1.00		0.534	1.00		0.466	**1.00**		**0.021**	1.00		0.368	**1.00**		**0.019**	1.00		0.447	1.00		0.102	1.00		0.996
A/A	1.69	0.35–8.12		1.41	0.57–3.47		**3.59**	**1.33–9.69**		0.54	0.12–2.33		**2.92**	**1.29–6.63**		0.80	0.43–1.45		1.54	0.93–2.55		1.00	0.38–2.59	
Log-additive																								
G[a]/A (0,1,2)	2.16	1.00–4.67	0.053	1.05	0.70–1.58	0.813	1.76	1.00–3.10	0.056	0.87	0.51–1.48	0.599	**1.92**	**1.22–3.01**	**0.006**	0.78	0.60–1.01	0.052	1.10	0.87–1.39	0.419	1.07	0.71–1.61	0.751
rs3814022																								
Dominant																								
C/C	1.00		0.156	1.00		0.082	1.00		0.605	**1.00**		**0.027**	1.00		0.182	1.00		0.980	**1.00**		**0.046**	1.00		0.063
C/G-G/G	2.27	0.70–7.42		1.59	0.94–2.70		1.25	0.54–2.91		**2.21**	**1.08–4.52**		1.57	0.80–3.07		1.00	0.72–1.38		**1.36**	**1.01–1.85**		1.68	0.97–2.90	
Recessive																								
C/C-C/G	1.00		0.663	**1.00**		**0.032**	1.00		0.061	1.00		0.663	1.00		0.430	1.00		0.554	1.00		0.090	1.00		0.674
G/G	1.43	0.31–6.65		**2.27**	**1.11–4.67**		0.00	0.00		1.33	0.38–4.63		0.58	0.14–2.48		0.84	0.46–1.53		1.56	0.94–2.59		0.80	0.28–2.29	
Log-additive																								
C[a]/G (0,1,2)	1.62	0.76–3.48	0.221	**1.56**	**1.07–2.27**	**0.021**	0.95	0.48–1.88	0.880	1.67	0.99–2.80	0.057	1.19	0.73–1.95	0.486	0.97	0.75–1.24	0.784	**1.31**	**1.04–1.65**	**0.022**	1.29	0.87–1.93	0.215
rs4953911																								
Dominant																								
A/A	1.00		0.352	1.00		0.219	1.00		0.834	**1.00**		**0.012**	1.00		0.475	1.00		0.670	1.00		0.141	**1.00**		**0.047**
T/A-T/T	1.72	0.53–5.63		1.40	0.81–2.41		1.09	0.47–2.55		**2.51**	**1.20–5.27**		1.28	0.65–2.50		1.07	0.77–1.49		1.26	0.93–1.71		**1.75**	**1.00–3.08**	
Recessive																								
A/A-T/A	1.00		0.818	1.00		0.059	**1.00**		**0.048**	1.00		0.392	1.00		0.289	1.00		0.697	1.00		0.106	1.00		0.793
T/T	1.20	0.26–5.58		1.99	1.00–3.97		**0.00**	**0.00**		1.66	0.55–5.04		0.49	0.12–2.09		0.90	0.51–1.56		1.49	0.93–2.41		0.88	0.34–2.28	
Log-additive																								
A[a]/T (0,1,2)	1.36	0.63–2.98	0.438	1.42	0.97–2.09	0.065	0.85	0.43–1.70	0.650	**1.84**	**1.10–3.07**	**0.022**	1.02	0.62–1.69	0.927	1.02	0.80–1.30	0.884	1.24	0.99–1.57	0.063	1.32	0.89–1.97	0.173

Highlighted in bold are the statistically significant associations.
CI, confidence interval; OR, odds ratio; UC, ulcerative colitis.
[a]Reference category.

Table 4. Associations between the haplotypes and the clinical parameters, with the most frequent haplotype, the GCA (G for rs1257220; C for rs3814022; and A for rs4963911), used as reference for the associations

	Family history				Need for colectomy				Need biologics				Prognosis			
	No[a] vs Yes				No[a] vs Yes				No[a] vs Yes				Good[a] vs Bad			
	Freq	OR	95% CI	*P* value	Freq	OR	95% CI	*P* value	Freq	OR	95% CI	*P* value	Freq	OR	95% CI	*P* value
Discovery cohort																
GCA[a]	0.511	1.00			0.510	1.00			0.513	1.00			0.5113	1.00		
ACA	0.126	1.89	0.49–7.34	0.357	0.128	0.38	0.09–1.61	0.188	0.126	1.04	0.55–1.97	0.910	**0.128**	**2.81**	**1.15–6.87**	**0.023**
AGT	0.152	2.35	0.92–6.05	0.076	0.154	1.27	0.58–2.77	0.547	0.152	1.26	0.72–2.19	0.416	0.152	1.36	0.82–2.24	0.235
GCT	0.043	0.00	—	—	0.044	1.52	0.44–5.27	0.511	0.045	0.50	0.13–1.88	0.308	0.045	0.70	0.26–1.89	0.484
GGT	0.162	1.20	0.29–4.97	0.800	0.157	1.06	0.47–2.40	0.881	**0.158**	**1.74**	**1.03–2.94**	**0.037**	0.158	1.92	0.69–5.32	0.211
Validation cohort																
GCA[a]	0.575	1.00			0.577	1.00			0.577	1.00			0.577	1.00		
ACA	**0.136**	**2.39**	**1.14–5.00**	**0.021**	0.135	0.86	0.57–1.28	0.449	0.134	1.25	0.85–1.85	0.258	0.136	0.85	0.36–2.02	0.711
AGT	0.122	1.07	0.26–4.39	0.926	0.123	0.83	0.52–1.32	0.427	0.124	1.36	0.87–2.12	0.177	0.125	1.43	0.67–3.07	0.356
GCT	0.023	0.00	—	—	0.023	2.33	0.92–5.90	0.075	0.022	1.51	0.57–3.97	0.404	0.023	2.58	0.66–10.14	0.175
GGT	0.135	1.44	0.47–4.38	0.524	0.133	1.27	0.82–1.96	0.286	0.133	1.48	0.95–2.31	0.084	**0.130**	**2.11**	**1.05–4.24**	**0.037**
Combined cohort																
GCA[a]	0.546	1.00			0.546	1.00			0.546	1.00			0.546	1.00		
ACA	**0.131**	**2.26**	**1.21–4.23**	**0.011**	0.131	0.76	0.52–1.12	0.166	0.131	1.17	0.84–1.62	0.357	0.131	1.39	0.78–2.47	0.259
AGT	0.136	1.71	0.85–3.44	0.136	0.138	0.80	0.55–1.17	0.255	0.137	1.20	0.85–1.67	0.296	0.137	1.11	0.58–2.12	0.755
GCT	0.032	0.00	—	—	0.032	1.23	0.65–2.34	0.528	0.033	0.78	0.39–1.57	0.487	0.033	1.33	0.45–4.00	0.606
GGT	0.147	1.31	0.56–3.04	0.534	0.144	1.05	0.73–1.49	0.808	**0.145**	**1.47**	**1.06–2.04**	**0.023**	**0.144**	**1.79**	**1.01–3.17**	**0.045**

The statistically significant associations are highlighted in bold.
CI, confidence interval; OR, odds ratio.
[a]Reference category.

regulatory regions of the gene, also display the potential to stratify patients with UC according to their likelihood of developing a severe/complicated disease. However, further confirmation in a bigger sample size and in a more recent cohort, avoiding possible historical bias of biologic management, is needed.

Glycosylation is fundamental for the fine-tuning of the immune system in general (18,53) and specifically in IBD (17,19,33,36,54). Genetic variants of specific glycogenes such as *MGAT1* and *MGAT5* were already correlated with susceptibility and severity of other immune-mediated diseases such as MS (31,38,39). Previous evidences from us have pinpointed the *MGAT5*/GnT-V–mediated branched *N*-glycosylation in the immunopathogenesis of UC. A deficiency in *MGAT5* gene was observed in mucosal T cells from patients with UC and associated with hyperactivation of immune response and disease severity (33,36). However, the genetic cause underlying this altered transcription of *MGAT5* glycogene associated with UC pathogenesis remained to be explored.

The observed functional impact of the SNPs on reduced expression levels of *MGAT5* transcription on *in situ* (colonic T cells) and circulating T cells from patients with UC constitutes a genetic explanation for the deficiency on branched *N*-glycans on T cells associated with a hyperimmune T-cell response (34,36). This is also in accordance with our previous observations in which low levels of *MGAT5*-mediated branched *N*-glycans on the inflammatory infiltrate of colonic biopsies from patients with UC predict the failure to standard therapy and thus the need of biologics (37).

The studied *MGAT5* SNPs are localized in a putative regulatory region because of the presence of histone and DNAse markers (55,56) (see Figure S5, Supplementary Digital Content 12, http://links.lww.com/CTG/A271), which suggest a putative function as an enhancer, although this needs to be further explored. Interestingly, a recent study identified regulatory modules that drive IBD associations for some risk loci and listed 3 regulatory models that operate specifically in *MGAT5* and in T cells and monocytes (57), highlighting the importance of *MGAT5* in IBD pathogenesis.

The functional relevance of *MGAT5* genetic variants in UC is also demonstrated here through its impact on IgG glycome composition. In fact, the increased production of proinflammatory IgGs in IBD is a reflection of the increased number of plasma cells in the intestinal lamina propria (58–60), which can be activated in a T-cell–dependent manner. The glycans profile of

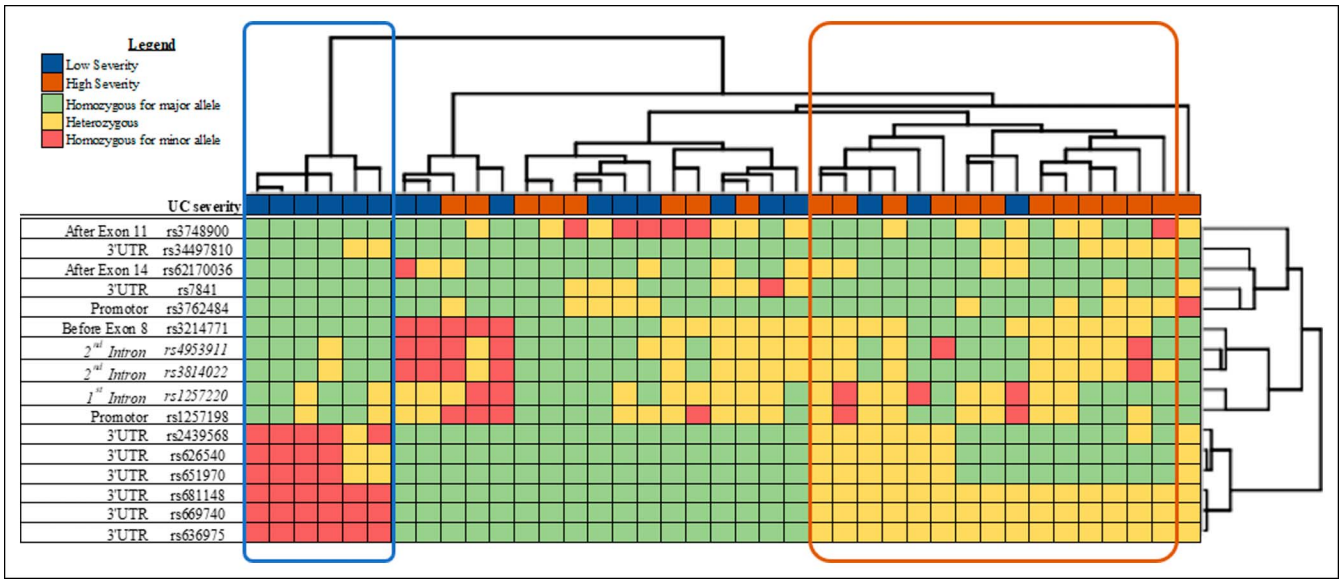


Figure 2. Genetic variants from regulatory and intron regions of *MGAT5* (with a minor allele frequency higher than 10%) and the association with severity in patients with UC. Clustering of patients with UC (hierarchical cluster on the upper side) according to SNPs from the promoter and intron regions (hierarchical cluster on the right side). Each SNP is colored according to the genotype: green, homozygous for the frequent allele; yellow, heterozygous; and red, homozygous for the rare allele. The 3 studied SNPs (rs1257220, rs3814022, and rs4953911) are included in the clustering and highlighted in italic. UC disease of each patient is classified according to the severity as mild (always with 5-ASA or with the need of previous corticotherapy, in blue) and severe (need hospitalization, corticodependent, corticoresistant, no responder to immunossupressors, and need biologics or need surgery, in orange), shown in the line above the cluster of patients. Two distinct clinical clusters were created, one comprising 100% of patients with a low severity (highlighted with blue) and the other with 81% of patients with a high severity (highlighted with orange). SNP, single nucleotide polymorphism; UC, ulcerative colitis.

the effector region of IgG (Fc domain) dictates its anti- or proinflammatory phenotype (22) through regulating the binding affinity to Fcγ receptors. Our results demonstrated for the first time that genetic variants of *MGAT5* (particularly rs3814022 and rs4953911) are correlated with alterations in plasma IgG glycome composition. A general increase of agalactosylation of IgG Fc was observed, which match with a proinflammatory glycoprofile of IgGs. In fact, the loss of terminal galactose (agalactosylation) on IgG was shown to enhance antibody-dependent phagocytosis (ADCC) (61) and has been seen in the circulation in patients with systemic lupus erythematosus (57, 58) and in patients suffering from rheumatoid arthritis years preceding disease onset (23). Moreover, this IgG agalactosylation profile was found to be different when comparing patients with IBD and healthy controls being associated with clinical severity of the disease (24–27,54).

Taken together, our results revealed a novel genetic cause that explains the changes in protein glycosylation on key players in IBD immunopathogenesis, such as T cells and plasma IgGs. *MGAT5* appears to be a common genetic factor that simultaneously regulates the function and activity of both humoral and adaptive components involved in IBD development through glycosylation modifications. We can here speculate that the role of *MGAT5* genetic variants on the regulation of IgG glycosylation profile might occur through a T-cell–dependent mechanism that needs to be further clarified. Under inflammatory conditions, T-cell hyperactivation (triggered by a deficiency on *MGAT5*-mediated branched glycosylation) may instruct B cells to produce proinflammatory agalactosylated IgGs. In addition, we cannot exclude the possible effect of therapy in glycosylation modifications associated with an active disease, a topic that needs to be clarified.

These functional *MGAT5* genetic variants exhibit a tendency in the association with UC clinical outcomes such as the likelihood of developing a severe/complicated disease, mainly because of the association with the need of biologics. In fact, the associations found between SNPs and the therapeutic outcomes are in the same trend in both discovery and validation cohorts. Moreover, the functional effects of the *MGAT5* SNPs, associated with a hyperreactive T-cell phenotype (related with low levels of *MGAT5* mRNA) and with a proinflammatory IgG glycome, biologically reinforce the relevance of these SNPs in UC clinical outcomes. Those associations need to be further validated in a larger and prospective cohort. In addition, *MGAT5* genetic variants mainly in regulatory regions appear to be able to cluster and to discriminate patients according to their disease severity. These results, despite supportive of the influence of *MGAT5* in UC disease course, outcomes and response to therapy, need further validation in a bigger sample size and in other cohorts.

In summary, our study identified a putative genetic cause for changes in protein glycosylation associated with the immunopathogenesis of IBD (28). *MGAT5* genetic variants were revealed to be correlated with pathologic changes in the glycosylation profile of T cells and plasma IgGs, potentially associated with the development of a severe/complicated UC disease.

CONFLICTS OF INTEREST

Guarantor of the article: Salomé S. Pinho, PhD.
Specific author contributions: M.S.P.: SNP genotyping, NGS experiment and molecular analysis, data analysis and interpretation, and manuscript writing; C.D.: statistical analysis and data interpretation; T.A.C.: SNP genotyping; J.L.C.: conducted NGS experience and collected the data; I.C.: provided clinical data and

Table 5. Association between 3′UTR SNPs in *MGAT5* and the severity of ulcerative colitis

	Severity/Complicated disease				
	Low, N (%)	High, N (%)	OR	95% CI	*P* value
rs3214771					
Dominant					
A/A[a]	10 (55.6)	8 (36.4)	1.00		**0.037**
A/T-T/T	8 (44.4)	14 (63.6)	5.26	1.01–27.32	
Recessive					
A/A-A/T[a]	15 (83.3)	20 (90.9)	1.00		0.450
T/T	3 (16.7)	2 (9.1)	0.43	0.05–3.90	
Log-additive					
A[a]/T (0,1,2)	18 (45.0)	22 (55.5)	1.95	0.61–6.27	0.243
rs681148					
Dominant					
T/T[a]	9 (50.0)	8 (36.4)	1.00		0.281
C/T-C/C	9 (50.0)	14 (63.6)	2.28	0.50–10.44	
Recessive					
T/T-C/T[a]	12 (66.7)	21 (95.5)	1.00		**0.026**
C/C	6 (33.3)	1 (4.5)	0.08	0.01–1.07	
Log-additive					
T[a]/C (0,1,2)	18 (45.0)	22 (55.0)	0.82	0.30–2.22	0.699
rs651970					
Dominant					
G/G[a]	10 (58.8)	16 (69.6)	1.00		0.657
A/G-A/A	7 (41.2)	7 (30.4)	0.72	0.17–3.05	
Recessive					
G/G-A/G[a]	13 (76.5)	23 (100)	1.00		**0.019**
A/A	4 (23.5)	0 (0.0)	0.0	0.0–0.0	
Log-additive					
G[a]/A (0,1,2)	17 (42.5)	23 (57.5)	0.50	0.17–1.52	0.211
rs626540					
Dominant					
G/G[a]	10 (58.8)	16 (69.6)	1.00		0.657
A/G-A/A	7 (41.2)	7 (30.4)	0.72	0.17–3.05	
Recessive					
G/G-A/G[a]	13 (76.5)	23 (100)	1.00		**0.019**
A/A	4 (23.5)	0 (0.0)	0.0	0.0–0.0	
Log-additive					
G[a]/A (0,1,2)	17 (42.5)	23 (57.5)	0.50	0.17–1.52	0.211
rs2439568					
Dominant					
A/A[a]	10 (55.6)	15 (68.2)	1.00		0.639
G/A-G/G	8 (44.4)	7 (31.8)	0.69	0.15–3.19	
Recessive					
A/A-G/A[a]	13 (72.2)	22 (100)	1.00		**0.022**
G/G	5 (27.8)	0 (0.0)	0.0	0.0–0.0	
Log-additive					
A[a]/G (0,1,2)	18 (45.0)	22 (55.0)	0.50	0.16–1.53	0.207
rs636975					
Dominant					
G/G[a]	9 (50.0)	8 (36.4)	1.00		0.281
A/G-A/A	9 (50.0)	14 (63.6)	2.28	0.50–10.44	
Recessive					
G/G-A/G[a]	12 (66.7)	21 (95.5)	1.00		**0.026**
A/A	6 (33.3)	1 (4.5)	0.08	0.01–1.07	
Log-additive					
G[a]/A (0,1,2)	18 (45.0)	22 (55.0)	0.82	0.30–2.22	0.699
rs669740					
Dominant					
T/T[a]	9 (50.0)	8 (36.4)	1.00		0.281
T/G-G/G	9 (50.0)	14 (63.6)	2.28	0.50–10.44	
Recessive					
T/T-T/G[a]	12 (66.7)	21 (95.5)	1.00		**0.026**
G/G	6 (33.3)	1 (4.5)	0.08	0.01–1.07	
Log-additive					
T[a]/G (0,1,2)	18 (45.0)	22 (55.0)	0.82	0.30–2.22	0.699
rs3814022					
Dominant					
C/C[a]	11 (61.1)	8 (36.4)	1.00		**0.016**
C/G-G/G	7 (38.9)	14 (63.6)	6.98	1.25–39.0	
Recessive					
C/C-C/G[a]	15 (83.3)	20 (90.9)	1.00		0.450
G/G	3 (16.7)	2 (9.1)	0.43	0.05–3.90	
Log-additive					
C[a]/G (0,1,2)	18 (45.0)	22 (55.0)	2.20	0.67–7.14	0.170
rs4953911					
Dominant					
A/A[a]	10 (55.6)	8 (38.1)	1.00		**0.027**
T/A-T/T	8 (44.4)	13 (61.9)	6.12	1.09–34.3	
Recessive					
A/A-T/A[a]	15 (83.3)	18 (85.7)	1.00		0.741
T/T	3 (16.7)	3 (14.3)	0.71	0.09–5.41	
Log-additive					
A[a]/T (0,1,2)	18 (46.2)	21 (53.8)	2.12	0.68–6.64	0.175

Bold-italic is to highlight the *P* values that are statistically significant.
CI, confidence interval; High severity/complicated disease, need for hospitalization, corticodependency, corticoresistance, nonresponders to immunossupressors, and/or with need of biologics and/or surgery; Low severity disease, always with 5-ASA or with previous corticotherapy without dependence or resistance; OR, odds ratio; SNP, single nucleotide polymorphism.
[a]Reference genotype.

revised critically the study; M.N., J.K., and J.S.: IgG glycome analysis; N.C.-N.: NGS analysis; J.C.M.: study discussion; R.M.-P., F.M., S.V., and P.L.: patient recruitment, clinical selection of samples, and clinical data collection; G.L.: intellectual content related with IgG glycome; S.S.P.: designed and conducted the study and critically revised the manuscript.

Financial support: Financial support from Portugal: Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP) integrates the i3S research unit, which is partially supported by the Portuguese Foundation for Science and Technology (FCT). This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). This work was also funded by FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the projects (POCI-01/0145-FEDER-016601; PTDC/DTP-PIC/0560/2014; POCI-01-0145-FEDER-028772). S.S.P. also acknowledges the European Crohn's and Colitis Organization (ECCO) for ECCO Grant; the Broad Medical Research Program at the Crohn's and Colitis Foundation of America, and the Portuguese Group of Study in IBD (GEDII) for funding. S.S.P. also acknowledges the US Department of Defense, US Army Medical Research Acquisition Activity, FY18 Peer Reviewed Medical Research Program Investigator-Initiated Research Award (award number W81XWH1920053). M.S.P. (SFRH/BD/110148/2015) acknowledges FCT for funding.

Potential competing interests: None to report.

Study Highlights

WHAT IS KNOWN

✓ *MGAT5* glycogene and its encoded branched *N*-glycan structure regulate the immunopathogenesis of IBD, in which low levels of MGAT5-mediated branching *N*-glycans on intestinal T cells result in a hyperimmune response and increased disease severity.

WHAT IS NEW HERE

✓ *MGAT5* genetic variants have a functional impact in the modulation of T cells glycosylation and plasma IgG glycome composition in patients with ulcerative colitis and show association with disease prognosis.

TRANSLATIONAL IMPACT

✓ Identification of a genetic risk locus in patients with ulcerative colitis, which is associated with immunopathogenesis of the disease and with clinical prognosis.

REFERENCES

1. Kaplan GG. The global burden of IBD: From 2015 to 2025. Nat Rev Gastroenterol Hepatol 2015;12(12):720–7.
2. Mirkov MU, Verstockt B, Cleynen I. Genetics of inflammatory bowel disease: Beyond NOD2. Lancet Gastroenterol Hepatol 2017;2(3):224–34.
3. Kopylov U, Boucher G, Waterman M, et al. Genetic predictors of benign course of ulcerative colitis-A North American inflammatory bowel disease genetics consortium study. Inflamm Bowel Dis 2016;22(10):2311–6.
4. Ananthakrishnan AN, Huang H, Nguyen DD, et al. Differential effect of genetic burden on disease phenotypes in crohn's disease and ulcerative colitis: Analysis of a North American cohort. Am J Gastroenterol 2014;109(3):395–400.
5. Cleynen I, Boucher G, Jostins L, et al. Inherited determinants of crohn's disease and ulcerative colitis phenotypes: A genetic association study. Lancet 2016;387(10014):156–67.
6. Yarur AJ, Strobel SG, Deshpande AR, et al. Predictors of aggressive inflammatory bowel disease. Gastroenterol Hepatol (NY) 2011;7(10):652–9.
7. Kolho KL, Paakkanen R, Lepisto A, et al. Novel associations between major histocompatibility complex and pediatric-onset inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2016;62(4):567–72.
8. Haritunians T, Taylor KD, Targan SR, et al. Genetic predictors of medically refractory ulcerative colitis. Inflamm Bowel Dis 2010;16(11):1830–40.
9. Potocnik U, Ferkolj I, Glavac D, et al. Polymorphisms in multidrug resistance 1 (MDR1) gene are associated with refractory Crohn disease and ulcerative colitis. Genes Immun 2004;5(7):530–9.
10. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491(7422):119–24.
11. Franke A, McGovern DP, Barrett JC, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42(12):1118–25.
12. Anderson CA, Boucher G, Lees CW, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat Genet 2011;43(3):246–52.
13. Elgar G, Vavouri T. Tuning in to the signals: Noncoding sequence conservation in vertebrate genomes. Trends Genet 2008;24(7):344–52.
14. Goyette P, Boucher G, Mallon D, et al. High-density mapping of the MHC identifies a shared role for HLA-DRB1*01:03 in inflammatory bowel diseases and heterozygous advantage in ulcerative colitis. Nat Genet 2015;47(2):172–9.
15. Mokry M, Middendorp S, Wiegerinck CL, et al. Many inflammatory bowel disease risk loci include regions that regulate gene expression in immune cells and the intestinal epithelium. Gastroenterology 2014;146(4):1040–7.
16. Boyd M, Thodberg M, Vitezic M, et al. Characterization of the enhancer and promoter landscape of inflammatory bowel disease from human colon biopsies. Nat Commun 2018;9(1):1661.
17. Pereira MS, Alves I, Vicente M, et al. Glycans as key checkpoints of T cell activity and function. Front Immunol 2018;9:2754.
18. Marth JD, Grewal PK. Mammalian glycosylation in immunity. Nat Rev Immunol 2008;8(11):874–87.
19. Dias AM, Pereira MS, Padrao NA, et al. Glycans as critical regulators of gut immunity in homeostasis and disease. Cell Immunol 2018;333:9–18.
20. Pinho SS, Reis CA. Glycosylation in cancer: Mechanisms and clinical implications. Nat Rev Cancer 2015;15(9):540–55.
21. Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr Opin Immunol 2008;20(4):471–8.
22. Biermann MH, Griffante G, Podolska MJ, et al. Sweet but dangerous—the role of immunoglobulin G glycosylation in autoimmunity and inflammation. Lupus 2016;25(8):934–42.
23. Ercan A, Cui J, Chatterton DE, et al. Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. Arthritis Rheum 2010;62(8):2239–48.
24. Simurina M, de Haan N, Vuckovic F, et al. Glycosylation of immunoglobulin G associates with clinical features of inflammatory bowel diseases. Gastroenterology 2018;154(5):1320–33 e1310.
25. Shinzaki S, Kuroki E, Iijima H, et al. Lectin-based immunoassay for aberrant IgG glycosylation as the biomarker for Crohn's disease. Inflamm Bowel Dis 2013;19(2):321–31.
26. Miyoshi E, Shinzaki S, Fujii H, et al. Role of aberrant IgG glycosylation in the pathogenesis of inflammatory bowel disease. Proteomics Clin Appl 2016;10(4):384–90.
27. Trbojevic Akmacic I, Ventham NT, Theodoratou E, et al. Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome. Inflamm Bowel Dis 2015;21(6):1237–47.

28. Verhelst X, Dias AM, Colombel JF, et al. Protein glycosylation as a diagnostic and prognostic marker of chronic inflammatory gastrointestinal and liver diseases. Gastroenterology 2019;158(6):95–110.
29. Grigorian A, Demetriou M. Mgat5 deficiency in T cells and experimental autoimmune encephalomyelitis. ISRN Neurol 2011;2011: 374314.
30. Lee SU, Grigorian A, Pawling J, et al. N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. J Biol Chem 2007;282(46):33725–34.
31. Mkhikian H, Grigorian A, Li CF, et al. Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. Nat Commun 2011;2:334.
32. Grigorian A, Lee SU, Tian W, et al. Control of T cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. J Biol Chem 2007;282(27):20027–35.
33. Dias AM, Dourado J, Lago P, et al. Dysregulation of T cell receptor N-glycosylation: A molecular mechanism involved in ulcerative colitis. Hum Mol Genet 2014;23(9):2416–27.
34. Demetriou M, Granovsky M, Quaggin S, et al. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. Nature 2001; 409(6821):733–9.
35. Morgan R, Gao G, Pawling J, et al. N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. J Immunol 2004;173(12):7200–8.
36. Dias AM, Correia A, Pereira MS, et al. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. Proc Natl Acad Sci USA 2018;115(20):E4651–E4660.
37. Pereira MS, Maia L, Azevedo LF, et al. A [Glyco]biomarker that predicts failure to standard therapy in ulcerative colitis patients. J Crohns Colitis 2019;13(1):39–49.
38. Brynedal B, Wojcik J, Esposito F, et al. MGAT5 alters the severity of multiple sclerosis. J Neuroimmunol 2010;220(1-2):120–4.
39. Li CF, Zhou RW, Mkhikian H, et al. Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively with MGAT1 and interleukin-2 and 7 receptor variants. J Neuroimmunol 2013;256(1-2):71–6.
40. Huffman JE, Knezevic A, Vitart V, et al. Polymorphisms in B3GAT1, SLC9A9 and MGAT5 are associated with variation within the human plasma N-glycome of 3533 European adults. Hum Mol Genet 2011; 20(24):5000–11.
41. Esposito F, Wojcik J, Rodegher M, et al. MGAT5 and disease severity in progressive multiple sclerosis. J Neuroimmunol 2011;230(1–2): 143–7.
42. Pucic M, Knezevic A, Vidic J, et al. High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. Mol Cell Proteomics 2011; 10(10):M111.010090.
43. Keser T, Vuckovic F, Barrios C, et al. Effects of statins on the immunoglobulin G glycome. Biochim Biophys Acta Gen Subj 2017; 1861(5 Pt A):1152–8.
44. Pillai N, Lupatsch JE, Dusheiko M, et al. Evaluating the cost-effectiveness of early compared to late or no biologic treatment to manage Crohn's disease using real world data. J Crohns Colitis 2019.
45. Ben-Horin S, Zhao Y, Guo J, et al. Efficacy of biological drugs in short-duration versus long-duration inflammatory bowel disease: A protocol for a systematic review and an individual-patient level meta-analysis of randomised controlled trials. BMJ Open 2019;9(1): e024222.
46. Faubion WA Jr, Loftus EV Jr, Harmsen WS, et al. The natural history of corticosteroid therapy for inflammatory bowel disease: A population-based study. Gastroenterology 2001;121(2):255–60.
47. Bianchi Porro G, Cassinotti A, Ferrara E, et al. Review article: The management of steroid dependency in ulcerative colitis. Aliment Pharmacol Ther 2007;26(6):779–94.
48. Ko JH, Miyoshi E, Noda K, et al. Regulation of the GnT-V promoter by transcription factor Ets-1 in various cancer cell lines. J Biol Chem 1999; 274(33):22941–8.
49. Benjamini Y, Hochberg Y. Controlling the false discovery rate—a practical and powerful approach to multiple testing. J R Stat Soc B 1995; 57(1):289–300.
50. Dekkers G, Rispens T, Vidarsson G. Novel concepts of altered immunoglobulin G galactosylation in autoimmune diseases. Front Immunol 2018;9:553.
51. Magro F, Dias CC, Portela F, et al. Development and validation of risk matrices concerning ulcerative colitis outcomes-Bayesian network analysis. J Crohns Colitis 2019;13(4):401–9.
52. Ananthakrishnan AN, Xavier RJ. How does genotype influence disease phenotype in inflammatory bowel disease? Inflamm Bowel Dis 2013; 19(9):2021–30.
53. Rudd PM, Elliott T, Cresswell P, et al. Glycosylation and the immune system. Science 2001;291(5512):2370–6.
54. Clerc F, Novokmet M, Dotz V, et al. Plasma N-glycan signatures are associated with features of inflammatory bowel diseases. Gastroenterology 2018;155(3):829–43.
55. Maurano MT, Humbert R, Rynes E, et al. Systematic localization of common disease-associated variation in regulatory DNA. Science 2012; 337(6099):1190–5.
56. Schaub MA, Boyle AP, Kundaje A, et al. Linking disease associations with regulatory information in the human genome. Genome Res 2012;22(9): 1748–59.
57. Momozawa Y, Dmitrieva J, Theatre E, et al. IBD risk loci are enriched in multigenic regulatory modules encompassing putative causative genes. Nat Commun 2018;9(1):2427.
58. Uzzan M, Colombel JF, Cerutti A, et al. B cell-activating factor (BAFF)-Targeted B cell therapies in inflammatory bowel diseases. Dig Dis Sci 2016;61(12):3407–24.
59. Zhou G, Song Y, Yang W, et al. ASCA, ANCA, ALCA and many more: Are they useful in the diagnosis of inflammatory bowel disease? Dig Dis 2016;34(1-2):90–7.
60. Magro F, Langner C, Driessen A, et al. European consensus on the histopathology of inflammatory bowel disease. J Crohns Colitis 2013; 7(10):827–51.
61. Nakajima S, Iijima H, Shinzaki S, et al. Functional analysis of agalactosyl IgG in inflammatory bowel disease patients. Inflamm Bowel Dis 2011; 17(4):927–36.

Supplementary figure legends

Figure S1

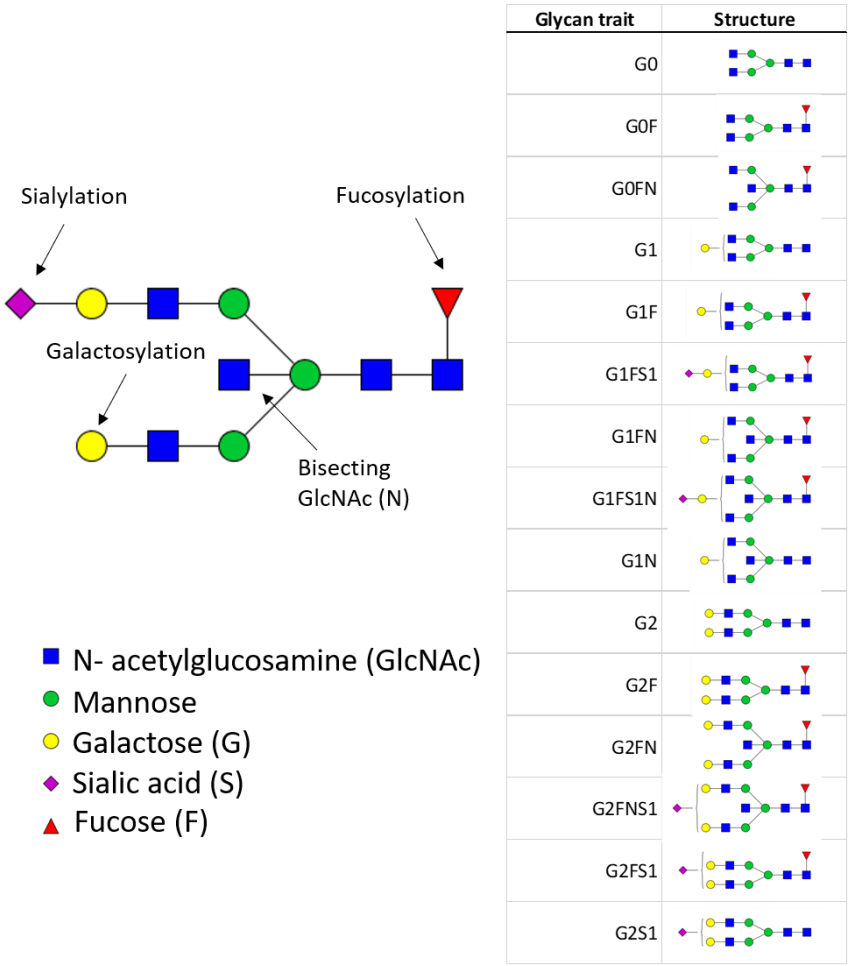


Figure S1_ General N-glycan structure and the different glycan traits.

Figure S2

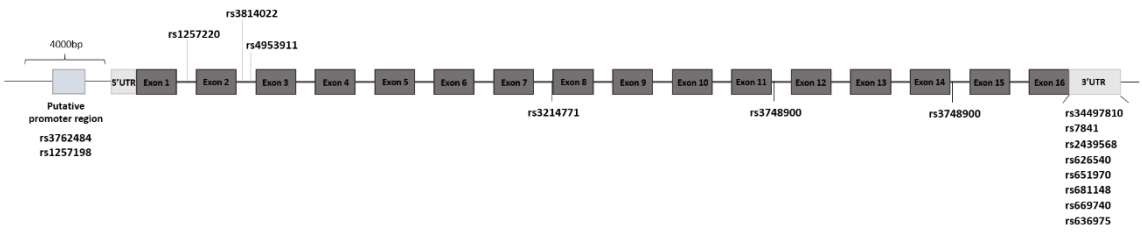


Figure S2_Representative image of the targeted regions in NGS (putative promoter region, 5'UTR, Exons and 3'UTR). Genetic variants from regulatory and intron regions of *MGAT5* with a minor allele frequency higher than 10% are highlighted.

Figure S3

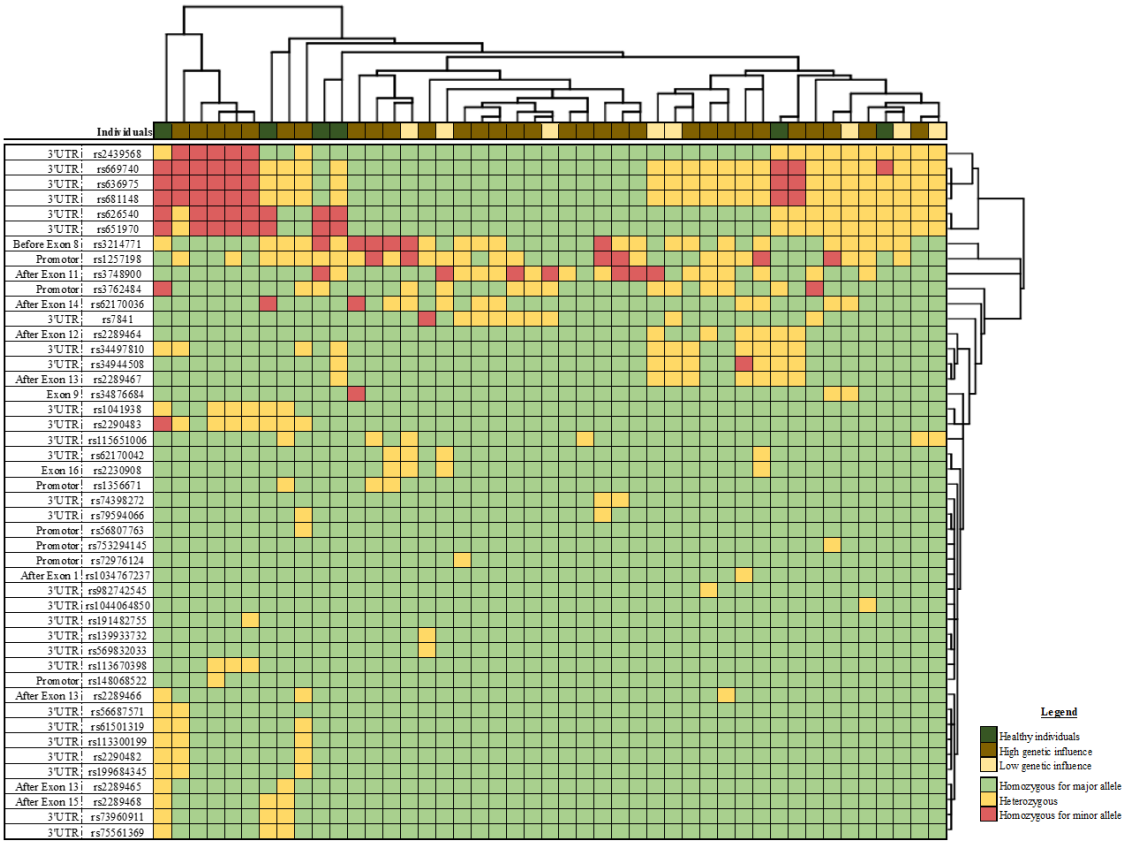


Figure S3_Genetic variants of *MGAT5* distributed within UC patients and healthy individuals. Clustering of individuals (hierarchical cluster on the upper side) according with SNPs from promoter, intron and exon regions (hierarchical cluster on the right side). Healthy individuals are colored in green, UC patients with high genetic influence (with 1st degree IBD relatives or with early disease onset ≤18 years old) are colored in brown and UC patients with low genetic influence (without family history and with a late onset disease >18 years old) are colored in beige. Each SNP is colored according to the genotype: green – homozygous for the frequent allele; yellow – heterozygous; red – homozygous for the rare allele.

Figure S4

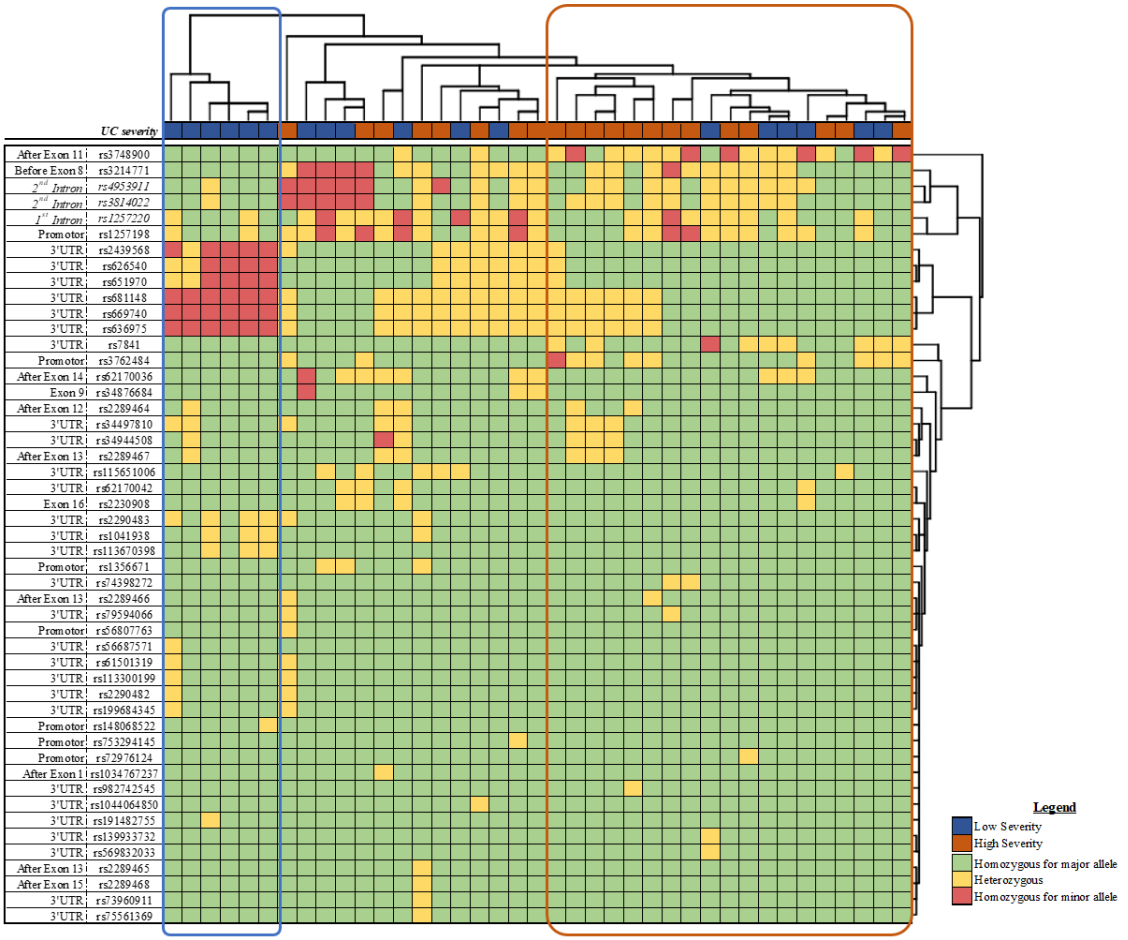


Figure S4_ Genetic variants from the regulatory regions of *MGAT5* and the association with UC patients' severity. Clustering of UC patients (hierarchical cluster on the upper side) according with SNPs from promoter, intron and exon regions (hierarchical cluster on the right side). Each SNP is colored according to the genotype: green – homozygous for the frequent allele; yellow – heterozygous; red – homozygous for the rare allele. The three studied SNPs (rs1257220, rs3814022 and rs4953911) are included in the clustering and are in italic. UC disease of each patient is classified according to the severity, in LOW (always with 5-ASA or with the need of previous corticotherapy, in blue) and HIGH (need hospitalization, corticodependent, corticoresistant, no responder to immunossupressors, need biologics or need surgery, in orange) shown in the line above the cluster of patients.

Figure S5

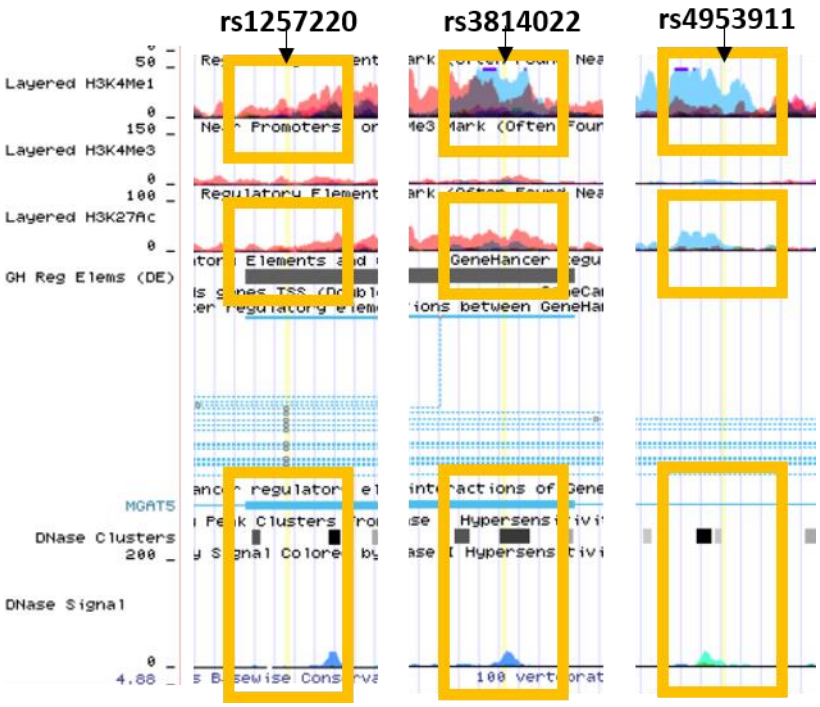


Figure S5_The regulatory potential of genetic regions that includes the SNPs of the study. Histone (H3K4Me1 and H3K27Ac) and DNAse markers show higher potential of rs3814022 and rs4953911 being included in a regulatory element.

Supplementary tables

Table S1_ Formula for the calculation of the glycan profile of each IgG subtype.

	Agalactosylation	Monogalactosylation	Digalactosylation	Sialylation	Bisecting GlcNAc	Fucosylation
IgG1	G0 + G0F + G0FN	G1 + G1F + G1N + G1FN	G2 + G2F + G2FN	G1FS + G1FNS + G2S + G2FS + G2FNS	G0FN + G1N + G1FN + G1FNS + G2FN + G2FNS	G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS
IgG23	G0F + G0FN	G1 + G1F + G1FN	G2F + G2FN	G1FS + G1FNS + G2S + G2FS + G2FNS	G0FN + G1FN + G1FNS + G2N + G2FN + G2FNS	G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS
IgG4	G0F + G0FN	G1F + G1FN	G2 + G2F + G2N	G1FS + G2S + G2FS	G0FN + G1FN + G2N	G0F + G0FN + G1F + G1FN + G1FS + G2F + G2FS

G – Galactose; **F** – Fucose; **N** – Bisecting; **S** – Sialic acid

Table S2_ Characterization of the UC discovery cohort (from Portugal) and validation cohort (from Belgium), alone and in combination, in terms of clinical and pathological parameters. Minor allele frequencies for each SNP in the different cohorts.

		Discovery Cohort (N= 432)		***Validation Cohort (N= 499)***		***Combined Cohort (N=931)***	
		n (%)	**Median [IQR]**	**n (%)**	**Median [IQR]**	**n (%)**	**Median [IQR]**
Years old at diagnostic		427	35 [26-46]	469	33 [24-43]	896	34 [25-45]
Years of follow up		381	12 [6-17]	469	21 [16-28]	850	17 [11-25]
Gender	F	241 (55.8)	-	217 (43.5)	-	458 (49.2)	-
	M	191 (44.2)	-	282 (56.5)	-	473 (50.8)	-
	Missing	0 (0.0)		0 (0.0)		0 (0.0)	
Family History*	No	389 (90.0)	-	469 (94.0)	-	858 (92.2)	-
	Yes	14 (3.2)	-	30 (6.0)	-	44 (4.7)	-
	Missing	29 (6.7)		0 (0.0)		29 (3.1)	
Disease extent	Proctitis	155 (35.9)	-	44 (8.8)	-	199 (21.4)	-
	Left-sided	129 (29.9)	-	157 (31.5)	-	286 (30.7)	-
	Pancolitis	144 (33.3)	-	262 (52.5)	-	406 (43.6)	-
	Missing	4 (0.9)		36 (7.2)		40 (4.3)	
Surgery	No	403 (93.3)	-	333 (66.7)	-	736 (79.1)	-
	Yes	25 (5.8)	-	161 (32.3)	-	186 (20.0)	-
	Missing	4 (0.9)		5 (1.0)		9 (1.0)	
Need for biologics	No	354 (81.9)	-	334 (66.9)	-	688 (73.9)	-
	Yes	73 (16.9)	-	165 (33.1)	-	238 (25.6)	-
	Missing	5 (1.2)		0 (0.0)		5 (0.5)	
Prognosis**	Good	407 (94.2)	-	449 (90.0)	-	856 (91.9)	-
	Bad	22 (5.1)	-	35 (7.0)	-	57 (6.1)	-
	Missing	3 (0.7)		15 (3.0)		18 (1.9)	
SNPs (alleles)	**Minor allele**	**MAF**		**MAF**		**MAF**	

rs1257220 (G/A)	A	0.282	0.278	0.280
rs3814022 (C/G)	G	0.314	0.259	0.285
rs4953911 (A/T)	T	0.359	0.279	0.316

IQR - interquartile range; ***Family history** was considered the presence of at least one first degree relatives with IBD; ****Prognosis** was defined as the need of biologics within the first 2 years after diagnosis, in which patients that needed biologics in up to 2 years after diagnosis were classified as displaying a bad prognosis; **MAF –** Minor frequency allele.

Table S3_Primers used to amplify the promoter, coding and 3'UTR regions of *MGAT5* gene.

Chr: bp-bp	Region	Primers
2: 134250287-134253254	Putative promoter	Fw_CAACAAGGTCTTCTGAGATCCACTG Rv_TCTTCTCTGCCCTTGCCTTG
		Fw_CAAATTTGCATGTCTCTGAACTGG Rv_GCACTTTATTCAGCCCTTCTAACTGC
		Fw_GAGCGGAGTGGAGGAAGGAA Rv_TGCTATCATAGGAGATAACAGAACAGTGG
		Fw_CTCTCCTCCCCACCCCTGGTA Rv_GGCACATGAGTGCTCTCTATTATGCTG
		Fw_GGCAAACAGGCTGGGTTTGA Rv_GCTTAATGGGTCATGAAAAGCAATAAAAA
		Fw_TGCTGATTCAGTGGGTGTGG Rv_GAGACTAACCCAGGCTGTACTTCAGA
2: 134254206-134254753	Exon 1	Fw_TTTATTTTGCTGTATTGTGCCATGA Rv_GCAGTGGCAAAGGACATTCA
2: 134270168-134270629	Exon 2	Fw_TTATTGCCAGACTTGCATTCCA Rv_ACCACTGCTCTCCTTTATTCTCTTCTT
2: 134317439-134317685	Exon 3	Fw_GTTTCTCTCCCTACCTCTTGGCTTAC Rv_GGAAAAGGAAGAGAAGGGAAAAAGA
2: 134318553-134318818	Exon 4	Fw_CATTCACTCCATCTTCACCATTCTATC Rv_GCTTTTCAAATTTCAGACTCAGCTACA
2: 134336128-134336339	Exon 5	Fw_TGTGTGCCAGGAGCTGTTCT Rv_GCATCTGACCTAAATGCACAAGTC
2: 134338076-134338495	Exon 6	Fw_CAGCCTTGGCTTCAGTTTGC Rv_AGTCTTGTTTCCAAGCAAAATCAAA
2: 134341492-134341961	Exon 7	Fw_AAACATGACTTTGGGATTGGTCA Rv_CCTTCAGCGACAATGAAAACATATTA

2: 134344840-134345134	Exon 8	Fw_GGCATTTGCCCATTAAAGTTGC Rv_CCCACAACCATGCAACCTTTG
2: 134349757-135247773	Exon 9	Fw_CTTTGGGGGCAAGTATGTAGTGTT Rv_CAAGGAAAAGGTTCAACAGCACA
2: 134362186-134362512	Exon 10	Fw_TGGGTAAGATGGCCTGTGTTAAA Rv_ATTAAGCCCTGGGCACTTGG
2: 134402843-134403255	Exon 11	Fw_GCAGTTTGATTGCCAAATTCCTG Rv_GCCCCAAAATAGCCACAAGAGTT
2: 134412697-134413173	Exon 12	Fw_TGGTGTAAGCTGAATTCTCTCTGC Rv_CTTGCAAGGTGATGTGATCAGG
2: 134422730-134423037	Exon 13	Fw_AGCATAGCACTCCATCTAGCACAG Rv_TCCAGAGTTAAGCTGATGTGGTAAAG
2: 134428197-134428576	Exon 14	Fw_CACACCTAGTAAGGATCAGCTTGAGA Rv_TTATCCAGCTTCTAGAGTCTCCCAAA
2: 134441675-134442028	Exon 15	Fw_TCCATTGCTAGGGTGGTTGG Rv_ATCCCCACAGCCCAGTAGCTT
2: 134448561-134454814	Exon 16 + 3'UTR	Fw_GCCTCAAGATGGGGGCTCA Rv_TCCTCCTGTTTTGTTTTAAAGGACTCTTG
		Fw_AGCTGCTCCAGGGCAAAAGA Rv_CAAGGTATGTGTCTGGGCATGG
		Fw_AGGCCCCTTTCCTCCATGTC Rv_TCAGGGGCTTTTCAGAAACTTACAA
		Fw_CCACCCCACTGAATCATTGCT Rv_TTTGGACTTCTGCCCTTCTTCCTA
		Fw_CTGCCCTGAGCATCATCACA Rv_AGTGTTCTTCTGGGCTGTCTGG
		Fw_GCCTCCCTACCTTCCCCTTCT Rv_GCGGTGTTTACCAGCATTCAAA
		Fw_CCCCATTGTGATCCTAAGCTCTTAAA Rv_TGATTCTGCCTCAGCCACACCTC
		Fw_CAACGTGTCAACCAGCCTGTG Rv_ATCACAGGAACTCACCCTCAGCTT
		Fw_TGCTCATTTAGGAAGCTGGGAGTT Rv_AAATGTTCCTTGAGGAAAACCCTTG
		Fw_GGCTGCAAGAATTTATGAACTCCA Rv_ACAAAAAGCAAACCGATATTTAGGTGA
		Fw_GCTGCTCATGACTGAATGTTTTCC Rv_CCCTCTCAGCTTTGGCAGATG
		Fw_TGTCAGGAACCTCAGAGCAGCTT Rv_TAGTAAAACCTTCCCCTCTCCACTCC

Chr – chromosome; **bp** – base pairs; **Fw** – forward; **Rv** – reverse

Table S4_Associations between rs1257220, rs3814022 and rs4953911 and the different clinical and therapeutic outcomes of UC patients. In grey the statistically significant associations are highlighted.

	Family history					**Need for colectomy**					**Need for Biologics**					**Prognosis**				
	No	**Yes**				**No**	**Yes**				**No**	**Yes**				**Good**	**Bad**			
	N (%)	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**
Discovery Cohort																				
rs1257220																				
Dominant																				
G/G	**205 (53.1)**	**3 (21.4)**	**1.00**		**0.022**	202 (50.6)	15 (60.0)	1.00		0.390	181 (51.4)	38 (52.8)	1.00		0.921	210 (52.4)	9 (40.9)	1.00		0.258
A/G-A/A	**181 (46.9)**	**11 (78.6)**	**4.02**	**1.09-14.86**		197 (49.4)	10 (40.0)	0.70	0.31-1.59		171 (48.6)	34 (47.2)	0.97	0.58-1.64		191 (47.6)	13 (59.1)	1.66	0.68-4.03	
Recessive																				
G/G-A/G	355 (92.0)	12 (85.7)	1.00		0.534	366 (91.7)	23 (92.0)	1.00		0.984	325 (92.3)	65 (90.3)	1.00		0.466	370 (92.3)	19 (86.4)	1.00		0.302
A/A	31 (8.0)	2 (14.3)	1.69	0.35-8.12		33 (8.3)	2 (8.0)	1.02	0.23-4.52		27 (7.7)	7 (9.7)	1.41	0.57-3.47		31 (7.7)	3 (13.6)	2.08	0.57-7.64	
log-Additive																				
G*/A (0,1,2)	386 (96.5)	14 (3.5)	2.16	1.00-4.67	0.053	399 (94.1)	25 (5.9)	0.80	0.41-1.56	0.499	352 (83.0)	72 (17.0)	1.05	0.70-1.58	0.813	401 (94.8)	22 (5.2)	1.52	0.82-3.00	0.182
rs3814022																				
Dominant																				
C/C	186 (48.2)	4 (28.6)	1.00		0.156	194 (48.6)	10 (40.0)	1.00		0.364	175 (49.9)	30 (41.1)	1.00		0.082	194 (48.4)	10 (45.5)	1.00		0.618
C/G-G/G	200 (51.8)	10 (71.4)	2.27	0.70-7.42		205 (51.4)	15 (60.0)	1.46	0.64-3.34		176 (50.1)	43 (58.9)	1.59	0.94-2.70		207 (51.6)	12 (54.5)	1.25	0.52-3.03	
Recessive																				

C/C-C/G	345 (89.4)	12 (85.7)	1.00		0.663	357 (89.5)	21 (84.0)	1.00		0.408	**318 (90.6)**	**60 (82.2)**	**1.00**		**0.032**	356 (88.8)	21 (95.5)	1.00		0.305
G/G	41 (10.6)	2 (14.3)	1.43	0.31-6.65		42 (10.5)	4 (16.0)	1.64	0.54-5.02		**33 (9.4)**	**13 (17.8)**	**2.27**	**1.11-4.67**		45 (11.2)	1 (4.5)	0.39	0.05-3.02	
log-Additive																				
C*/G (0,1,2)	386 (96.5)	14 (3.5)	1.62	0.76-3.48	0.221	399 (94.1)	25 (5.9)	1.37	0.77-2.44	0.290	**351 (82.8)**	**73 (17.2)**	**1.56**	**1.07-2.27**	**0.021**	401 (94.8)	22 (5.2)	0.98	0.51-1.89	0.955
rs4953911																				
Dominant																				
A/A	157 (41.1)	4 (28.6)	1.00		0.352	166 (41.9)	8 (33.3)	1.00		0.484	147 (42.2)	27 (37.5)	1.00		0.219	165 (41.6)	9 (40.9)	1.00		0.659
T/A-T/T	225 (58.9)	10 (71.4)	1.72	0.53-5.63		230 (58.1)	16 (66.7)	1.50	0.63-3.62		201 (57.8)	45 (62.5)	1.40	0.81-2.41		232 (58.4)	13 (59.1)	1.22	0.50-3.01	
Recessive																				
A/A-T/A	334 (87.4)	12 (85.7)	1.00		0.818	346 (87.4)	19 (79.2)	1.00		0.270	307 (88.2)	58 (80.6)	1.00		0.059	343 (86.4)	21 (95.5)	1.00		0.203
T/T	48 (12.6)	2 (14.3)	1.20	0.26-5.58		50 (12.6)	5 (20.8)	1.84	0.66-5.18		41 (11.8)	14 (19.4)	1.99	1.00-3.97		54 (13.6)	1 (4.5)	0.33	0.04-2.49	
log-Additive																				
A*/T (0,1,2)	382 (96.5)	14 (3.5)	1.36	0.63-2.98	0.438	396 (94.3)	24 (5.7)	1.45	0.81-2.60	0.220	348 (82.9)	72 (17.1)	1.42	0.97-2.09	0.065	397 (94.7)	22 (5.3)	0.93	0.48-1.78	0.815
Validation Cohort																				
rs1257220																				
Dominant																				
G/G	250 (56.7)	11 (45.8)	1.00		0.284	163 (54.0)	97 (60.2)	1.00		0.175	176 (57.3)	85 (53.8)	1.00		0.531	234 (55.7)	20 (57.1)	1.00		0.846
A/G-A/A	191 (43.3)	13 (54.2)	1.57	0.69-3.60		139 (46.0)	64 (39.8)	0.76	0.52-1.13		131 (42.7)	73 (46.2)	1.13	0.77-1.67		186 (44.3)	15 (42.9)	0.93	0.46-1.87	

Recessive																			
G/G-A/G	**402 (91.2)**	**18 (75.0)**	**1.00**		**0.021**	269 (89.1)	149 (92.5)	1.00		0.237	281 (91.5)	139 (88.0)	1.00		0.191	377 (89.8)	33 (94.3)	1.00		0.368
A/A	**39 (8.8)**	**6 (25.0)**	**3.59**	**1.33-9.69**		33 (10.9)	12 (7.5)	0.67	0.33-1.33		26 (8.5)	19 (12.0)	1.53	0.81-2.88		43 (10.2)	2 (5.7)	0.54	0.12-2.33	
log-Additive																			
G*/A (0,1,2)	441 (94.8)	24 (5.2)	1.76	1.00-3.10	0.056	302 (65.2)	161 (34.8)	0.79	0.59-1.07	0.124	307 (66.0)	158 (34.0)	1.17	0.88-1.56	0.292	420 (92.3)	35 (7.7)	0.87	0.51-1.48	0.599
rs3814022																			
Dominant																			
C/C	242 (55.0)	11 (47.8)	1.00		0.605	167 (55.5)	86 (53.8)	1.00		0.731	176 (57.7)	77 (48.7)	1.00		0.078	**236 (56.5)**	**13 (37.1)**	**1.00**		**0.027**
C/G-G/G	198 (45.0)	12 (52.2)	1.25	0.54-2.91		134 (44.5)	74 (46.2)	1.07	0.73-1.58		129 (42.3)	81 (51.3)	1.42	0.96-2.10		**182 (43.5)**	**22 (62.9)**	**2.21**	**1.08-4.52**	
Recessive																			
C/C-C/G	408 (92.7)	23 (100)	1.00		0.061	279 (92.7)	150 (93.8)	1.00		0.700	286 (93.8)	145 (91.8)	1.00		0.385	390 (93.3)	32 (91.4)	1.00		0.663
G/G	32 (7.3)	0 (0)	0.00	0.00		22 (7.3)	10 (6.2)	0.86	0.40-1.87		19 (6.2)	13 (8.2)	1.39	0.66-2.92		28 (6.7)	3 (8.6)	1.33	0.38-4.63	
log-Additive																			
C*/G (0,1,2)	440 (95.0)	23 (5.0)	0.95	0.48-1.88	0.880	301 (65.3)	160 (34.7)	1.02	0.75-1.39	0.906	305 (65.9)	158 (34.1)	1.32	0.97-1.80	0.078	418 (92.3)	35 (7.7)	1.67	0.99-2.80	0.057
rs4953911																			
Dominant																			
A/A	227 (51.6)	11 (47.8)	1.00		0.834	162 (53.6)	76 (47.5)	1.00		0.210	166 (54.4)	72 (45.6)	1.00		0.082	**223 (53.3)**	**11 (31.4)**	**1.00**		**0.012**
T/A-T/T	213 (48.4)	12 (52.2)	1.09	0.47-2.55		140 (46.4)	84 (52.5)	1.28	0.87-1.88		139 (45.6)	86 (54.4)	1.41	0.96-2.08		**195 (46.7)**	**24 (68.6)**	**2.51**	**1.20-5.27**	

Recessive																			
A/A-T/A	**404 (91.8)**	**23 (100)**	**1.00**		**0.048**	278 (92.1)	148 (92.5)	1.00		0.924	284 (93.1)	143 (90.5)	1.00		0.256	387 (92.6)	31 (88.6)	1.00		0.392
T/T	**36 (8.2)**	**0 (0)**	**0.00**	**0.00**		24 (7.9)	12 (7.5)	0.97	0.47-1.99		21 (6.9)	15 (9.5)	1.51	0.75-3.04		31 (7.4)	4 (11.4)	1.66	0.55-5.04	
log-Additive																			
A*/T (0,1,2)	440 (95.0)	23 (5.0)	0.85	0.43-1.70	0.650	302 (65.4)	160 (34.6)	1.16	0.86-1.57	0.3443	305 (65.9)	158 (34.1)	1.33	0.98-1.81	0.064	**418 (92.3)**	**35 (7.7)**	**1.84**	**1.10-3.07**	**0.022**
Combined Cohort																				
rs1257220																				
Dominant																				
G/G	**455 (55.0)**	**14 (36.8)**	**1.00**		**0.025**	**365 (52.1)**	**112 (60.2)**	**1.00**		**0.037**	357 (54.2)	123 (53.5)	1.00		0.915	444 (54.1)	29 (50.9)	1.00		0.675
A/G-A/A	**372 (45.0)**	**24 (63.2)**	**2.13**	**1.09-4.19**		**336 (47.9)**	**74 (39.8)**	**0.71**	**0.51-0.98**		302 (45.8)	107 (46.5)	1.02	0.75-1.38		377 (45.9)	28 (49.1)	1.12	0.65-1.93	
Recessive																				
G/G-A/G	**757 (91.5)**	**30 (78.9)**	**1.00**		**0.019**	635 (90.6)	172 (92.5)	1.00		0.447	606 (92.0)	204 (88.7)	1.00		0.102	747 (91.0)	52 (91.2)	1.00		0.996
A/A	**70 (8.5)**	**8 (21.1)**	**2.92**	**1.29-6.63**		66 (9.4)	14 (7.5)	0.80	0.43-1.45		53 (8.0)	26 (11.3)	1.54	0.93-2.55		74 (9.0)	5 (8.8)	1.00	0.38-2.59	
log-Additive																				
G*/A (0,1,2)	**827 (95.6)**	**38 (4.4)**	**1.92**	**1.22-3.01**	**0.006**	701 (79.0)	186 (21.0)	0.78	0.60-1.01	0.052	659 (74.1)	230 (25.9)	1.10	0.87-1.39	0.419	821 (93.5)	57 (6.5)	1.07	0.71-1.61	0.751
rs3814022																				
Dominant																				
C/C	428 (51.8)	15 (40.5)	1.00		0.182	361 (51.6)	96 (51.9)	1.00		0.980	**351 (53.6)**	**107 (46.3)**	**1.00**		**0.046**	430 (52.5)	23 (40.4)	1.00		0.063

C/G-G/G	398 (48.2)	22 (59.5)	1.57	0.80-3.07		339 (48.4)	89 (48.1)	1.00	0.72-1.38		**305 (46.5)**	**124 (53.7)**	**1.36**	**1.01-1.85**		389 (47.5)	34 (59.6)	1.68	0.97-2.90	
Recessive																				
C/C-C/G	753 (91.2)	35 (94.6)	1.00			636 (90.9)	171 (92.4)	1.00			604 (92.1)	205 (88.7)	1.00			746 (91.1)	53 (93.0)	1.00		
G/G	73 (8.8)	2 (5.4)	0.58	0.14-2.48	0.430	64 (9.1)	14 (7.6)	0.84	0.46-1.53	0.554	52 (7.9)	26 (11.3)	1.56	0.94-2.59	0.090	73 (8.9)	4 (7.0)	0.80	0.28-2.29	0.674
log-Additive																				
C*/G (0,1,2)	826 (95.7)	37 (4.3)	1.19	0.73-1.95	0.486	700 (79.1)	185 (20.9)	0.97	0.75-1.24	0.784	**656 (74.0)**	**231 (26.0)**	**1.31**	**1.04-1.65**	**0.022**	819 (93.5)	57 (6.5)	1.29	0.87-1.93	0.215
rs4953911																				
Dominant																				
A/A	384 (46.7)	15 (40.5)	1.00			328 (47.0)	84 (45.7)	1.00			313 (47.9)	99 (43.0)	1.00			**388 (47.6)**	**20 (35.1)**	**1.00**		
T/A-T/T	438 (53.3)	22 (59.5)	1.28	0.65-2.50	0.475	370 (53.0)	100 (54.3)	1.07	0.77-1.49	0.670	340 (52.1)	131 (57.0)	1.26	0.93-1.71	0.141	**427 (52.4)**	**37 (64.9)**	**1.75**	**1.00-3.08**	**0.047**
Recessive																				
A/A-T/A	738 (89.8)	35 (94.6)	1.00			624 (89.4)	167 (90.8)	1.00			591 (90.5)	201 (87.4)	1.00			730 (89.6)	52 (91.2)	1.00		
T/T	84 (10.2)	2 (5.4)	0.49	0.12-2.09	0.289	74 (10.6)	17 (9.2)	0.90	0.51-1.56	0.697	62 (9.5)	29 (12.6)	1.49	0.93-2.41	0.106	85 (10.4)	5 (8.8)	0.88	0.34-2.28	0.793
log-Additive																				
A*/T (0,1,2)	822 (95.7)	37 (4.3)	1.02	0.62-1.69	0.927	698 (79.1)	184 (20.9)	1.02	0.80-1.30	0.884	653 (74.0)	230 (26.0)	1.24	0.99-1.57	0.063	815 (93.5)	57 (6.5)	1.32	0.89-1.97	0.173

***Reference category; **OR** - odds ratio; **CI** - confidence interval*

Table S5_Associations between rs1257220, rs3814022 and rs4953911 and the different clinical and therapeutic outcomes of UC patients, adjusted to age, gender and time of follow-up. In grey the statistically significant associations are highlighted. - Not statistically significant differences.

	Family history					**Need for colectomy**					**Need for Biologics**					**Prognosis**				
	No	**Yes**				**No**	**Yes**				**No**	**Yes**				**Good**	**Bad**			
	N (%)	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**	**N (%)**	**N (%)**	**OR**	**[95% IC]**	***p* value**
Discovery Cohort																				
rs1257220																				
Dominant																				
G/G	**189 (53.5)**	**3 (23.1)**	**1.00**		**0.031**	-	-	-		-	-	-	-		-	-	-	-		-
A/G-A/A	**164 (46.5)**	**10 (76.9)**	**3.84**	**1.02-14.53**		-	-	-	-		-	-	-	-		-	-	-	-	
Recessive																				
G/G-A/G	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-		-
A/A	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
log-Additive																				
G*/A (0,1,2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rs3814022																				
Dominant																				
C/C	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-		-
C/G-G/G	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
Recessive																				
C/C-C/G	-	-	-		-	-	-	-		-	**282 (91.0)**	**55 (80.9)**	**1.00**		**0.018**	-	-	-		-
G/G	-	-	-	-		-	-	-	-		**28 (9.0)**	**13 (19.1)**	**2.57**	**1.21-5.42**		-	-	-	-	

log-Additive																				
C*/G (0,1,2)	-	-	-	-	-	-	-	-	-	-	**310 (82.0)**	**68 (18.0)**	**1.62**	**1.09-2.40**	**0.017**	-	-	-	-	-
rs4953911																				
Dominant																				
A/A	-	-	-			-	-	-	-		-	-	-			-	-	-		
T/A-T/T	-	-	-	-		-	-	-	-		-	-	-	-	-	-	-	-	-	-
Recessive																				
A/A-T/A	-	-	-			-	-	-			**273 (88.9)**	**53 (79.1)**	**1.00**			-	-	-		
T/T	-	-	-	-	-	-	-	-	-	-	**34 (11.1)**	**14 (20.9)**	**2.38**	**1.16-4.89**	**0.023**	-	-	-	-	-
log-Additive																				
A*/T (0,1,2)	-	-	-	-	-	-	-	-	-	-	**307 (82.1)**	**67 (17.9)**	**1.54**	**1.03-2.29**	**0.034**	-	-	-	-	-
Validation Cohort																				
rs1257220																				
Dominant																				
G/G	-	-	-			-	-	-			-	-	-			-	-	-		
A/G-A/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Recessive																				
G/G-A/G	**402 (91.2)**	**18 (75.0)**	**1.00**			-	-	-			-	-	-			-	-	-		
A/A	**39 (8.8)**	**6 (25.0)**	**3.64**	**1.34-9.91**	**0.021**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
log-Additive																				

G*/A (0,1,2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rs3814022																				
Dominant																				
C/C	-	-	-			-	-	-			-	-	-			236 (56.5)	13 (37.1)	1.00		
C/G-G/G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	182 (43.5)	22 (62.9)	2.13	0.92-4.93	0.071
Recessive																				
C/C-C/G	408 (92.7)	23 (100)	1.00			-	-	-			-	-	-			-	-	-		
G/G	32 (7.3)	0 (0)	0.00	0.00	0.063	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
log-Additive																				
C*/G (0,1,2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rs4953911																				
Dominant																				
A/A	-	-	-			-	-	-			-	-	-			223 (53.3)	11 (31.4)	1.00		
T/A-T/T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	195 (46.7)	24 (68.6)	2.21	0.94-5.22	0.063
Recessive																				
A/A-T/A	-	-	-			-	-	-			-	-	-			-	-	-		
T/T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
log-Additive																				
A*/T (0,1,2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	418 (92.3)	35 (7.7)	1.68	0.91-3.10	0.100

Combined Cohort																				
rs1257220																				
Dominant																				
G/G	**439 (55.3)**	**14 (37.8)**	**1.00**			**346 (52.3)**	**112 (60.2)**	**1.00**		**0.019**	-	-	-			-	-	-		
A/G-A/A	**355 (44.7)**	**23 (62.2)**	**2.08**	**1.05-4.12**	**0.032**	**315 (47.7)**	**70 (38.7)**	**0.66**	**0.46-0.94**		-	-	-	-	-	-	-	-	-	-
Recessive																				
G/G-A/G	**726 (91.4)**	**29 (78.4)**	**1.00**			-	-	-			-	-	-			-	-	-		
A/A	**68 (8.6)**	**8 (21.6)**	**3.07**	**1.34-7.04**	**0.015**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
log-Additive																				
G*/A (0,1,2)	**794 (95.5)**	**37 (4.5)**	**1.92**	**1.21-3.04**	**0.006**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rs3814022																				
Dominant																				
C/C	-	-	-			-	-	-			332 (54.0)	105 (46.5)	1.00			-	-	-		
C/G-G/G	-	-	-	-	-	-	-	-	-	-	283 (46.0)	121 (53.5)	1.36	0.98-1.83	0.067	-	-	-	-	-
Recessive																				
C/C-C/G	-	-	-			-	-	-			-	-	-			-	-	-		
G/G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
log-Additive																				
C*/G (0,1,2)	-	-	-	-	-	-	-	-	-	-	**615 (73.1)**	**226 (26.9)**	**1.30**	**1.03-1.65**	**0.029**	-	-	-	-	-
rs4953911																				

Dominant																				
A/A	-	-	-			-	-	-			-	-	-			372 (48.2)	19 (33.9)	1.00		0.077
T/A-T/T	-	-	-	-		-	-	-	-		-	-	-	-		399 (51.8)	37 (66.1)	1.70	0.93-3.08	
Recessive																				
A/A-T/A	-	-	-			-	-	-			-	-	-			-	-	-		-
T/T	-	-	-	-		-	-	-	-		-	-	-	-		-	-	-	-	
log-Additive																				
A*/T (0,1,2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table S6_ Bonferroni multiple testing correction for 13 variables (Family history, need colectomy, need biologics, prognosis and dominant, recessive and log-additive models for rs1257220, rs3814022 and rs4953911). Table presents the p values before and after Bonferroni correction.

	Before Bonferroni correction				*After Bonferroni correction*			
	Family history	**Need for colectomy**	**Need for Biologics**	**Prognosis**	**Family history**	**Need for colectomy**	**Need for Biologics**	**Prognosis**
Discovery Cohort					***Discovery Cohort***			
rs1257220					**rs1257220**			
Dominant	**0.022**	0.390	0.921	0.258	Dominant	**0.792**	1	1	1
Recessive	0.534	0.984	0.466	0.302	Recessive	1	1	1	1
log-Additive	0.053	0.499	0.813	0.182	log-Additive	1	1	1	1
rs3814022					**rs3814022**			
Dominant	0.156	0.364	0.082	0.618	Dominant	1	1	1	1
Recessive	0.663	0.408	**0.032**	0.305	Recessive	1	1	**1**	1
log-Additive	0.221	0.290	**0.021**	0.955	log-Additive	1	1	**0.756**	1
rs4953911					**rs4953911**			
Dominant	0.352	0.484	0.219	0.659	Dominant	1	1	1	1
Recessive	0.818	0.270	0.059	0.203	Recessive	1	1	1	1
log-Additive	0.438	0.220	0.065	0.815	log-Additive	1	1	1	1
Validation Cohort					***Validation Cohort***			
rs1257220					**rs1257220**			
Dominant	0.284	0.175	0.531	0.846	Dominant	1	1	1	1
Recessive	**0.021**	0.237	0.191	0.368	Recessive	**0.756**	1	1	1
log-Additive	0.056	0.124	0.292	0.599	log-Additive	1	1	1	1
rs3814022					**rs3814022**			
Dominant	0.605	0.731	0.078	**0.027**	Dominant	1	1	1	**0.972**
Recessive	0.061	0.700	0.385	0.663	Recessive	1	1	1	1
log-Additive	0.880	0.906	0.078	0.057	log-Additive	1	1	1	1
rs4953911					**rs4953911**			
Dominant	0.834	0.210	0.082	**0.012**	Dominant	1	1	1	**0.432**
Recessive	**0.048**	0.924	0.256	0.392	Recessive	**1**	1	1	1
log-Additive	0.650	0.3443	0.064	**0.022**	log-Additive	1	1	1	**0.792**
Combined Cohort					***Combined Cohort***			
rs1257220					**rs1257220**			
Dominant	**0.025**	**0.037**	0.915	0.675	Dominant	**0.900**	**1**	1	1
Recessive	**0.019**	0.447	0.102	0.996	Recessive	**0.684**	1	1	1
log-Additive	**0.006**	0.052	0.419	0.751	log-Additive	**0.216**	1	1	1
rs3814022					**rs3814022**			

Dominant	0.182	0.980	**0.046**	0.063	Dominant	1	1	**1**	1
Recessive	0.430	0.554	0.090	0.674	Recessive	1	1	1	1
log-Additive	0.486	0.784	**0.022**	0.215	log-Additive	1	1	**0.792**	1
rs4953911					**rs4953911**				
Dominant	0.475	0.670	0.141	**0.047**	Dominant	1	1	1	**1**
Recessive	0.289	0.697	0.106	0.793	Recessive	1	1	1	1
log-Additive	0.927	0.884	0.063	0.173	log-Additive	1	1	1	1

Table S7_ Identified SNPs in the coding and non-coding regions of *MGAT5* in UC patients mainly harboring a genetic background (either early onset disease or family history).

Chr: bp	rs nomenclature	Alleles	EUR MAF	CEU MAF	Gene region
2: 134250470	rs1257198	G/A	0.281 (G)	0.227 (G)	Intron
2: 134250861	rs3762484	T/C	0.063 (C)	0.061 (C)	Intron
2: 134251669	rs1356671	C/T	0.065 (T)	0.035 (T)	Intron
2: 134251775	rs56807763	**A**/**C**/G	0.018 (C)	0.035 (C)	Intron
2: 134252156	rs148068522	G/A	0.008 (A)	0.005 (A)	Intron
2: 134252340	rs753294145	G/T	-	-	Intron
2: 134252373	rs72976124	**G**/**A**/C	-	-	Intron
2: 134254718	rs1034767237	T/C	-	-	Intron
2: 134257776	rs1257220	A/G	0.260 (A)	0.260 (A)	Intron
2: 134290348	rs3814022	C/G	0.242 (G)	0.242 (G)	Intron
2: 134311223	rs4953911	**T**/**A**/C	0.493 (A)	0.493 (A)	Intron
2: 134344883	rs3214771	A/-	0.317 (-)	0.258 (-)	Intron
2: 134349829	rs34876684	A/G	0.012 (G)	0.005 (G)	Exon 9
2: 134403184	rs3748900	G/A	0.339 (A)	0.354 (A)	Intron
2: 134413128	rs2289464	A/G	0.086 (G)	0.071 (G)	Intron
2: 134422950	rs2289465	T/C	0.028 (C)	0.020 (C)	Intron
2: 134422955	rs2289466	A/G	0.041 (G)	0.020 (G)	Intron
2: 134422979	rs2289467	C/T	0.048 (T)	0.040 (T)	Intron
2: 134428486	rs62170036	**T**/A/**G**	0.097 (G)	0.066 (G)	Intron
2: 134441993	rs2289468	C/T	0.019 (T)	0.010 (T)	Intron
2: 134448769	rs2230908	C/A	0.054 (A)	0.035 (A)	Exon 16
2: 134448868	rs1454081815	C/T	-	-	3'UTR
2: 134449047	rs115651006	G/A	0.052 (A)	0.056 (A)	3'UTR
2: 134449068	rs62170042	G/T	0.055 (T)	0.035 (T)	3'UTR
2: 134449363	rs681148	C/T	0.443 (C)	0.470 (C)	3'UTR
2: 134450368	rs61501319	C/T	0.023 (T)	0.010 (T)	3'UTR
2: 134450393	rs34944508	C/T	0.051 (T)	0.051 (T)	3'UTR
2: 134450403	rs982742545	G/A	-	-	3'UTR
2: 134450506	rs651970	A/G	0.313 (A)	0.374 (A)	3'UTR

2: 134450642	rs626540	A/G	0.313 (A)	0.374 (A)	3'UTR
2: 134450809	rs113300199	T/A	0.024 (A)	0.010 (A)	3'UTR
2: 134450813	rs199684345	T/A	0.020 (A)	0.010 (A)	3'UTR
2: 134450828	rs1044064850	G/A	-	-	3'UTR
2: 134450904	rs75561369	G/A	0.026 (A)	0.020 (A)	3'UTR
2: 134451250	rs56687571	G/C	0.027 (C)	0.010 (C)	3'UTR
2: 134451322	rs569832033	T/C	0.001 (C)	0.005 (C)	3'UTR
2: 134451571	rs2439568	G/A	0.341 (G)	0.384 (G)	3'UTR
2: 134451583	rs636975	A/G	0.440 (A)	0.470 (A)	3'UTR
2: 134452020	rs669740	C/T	0.442 (C)	0.470 (C)	3'UTR
2: 134452046	rs113670398	C/T	0.059 (T)	0.035 (T)	3'UTR
2: 134452524	rs79594066	T/C	0.050 (C)	0.025 (C)	3'UTR
2: 134452535	rs191482755	A/G	0.006 (G)	0.005 (G)	3'UTR
2: 134452564	rs2290482	**G**/**A**/C	0.023 (A)	0.010 (A)	3'UTR
2: 134452819	rs2290483	A/G	0.107 (G)	0.066 (G)	3'UTR
2: 134453311	rs139933732	C/T	0.012 (T)	0.005 (T)	3'UTR
2: 134453452	rs74398272	T/C	0.016 (C)	0.010 (C)	3'UTR
2: 134453649	rs73960911	A/G	0.019 (G)	0.025 (G)	3'UTR
2: 134453973	rs1041938	A/T	0.085 (T)	0.056 (T)	3'UTR
2: 134453978	rs34497810	**G**/A/**C**	0.082 (C)	0.051 (C)	3'UTR
2: 134454290	rs7841	C/T	0.091 (T)	0.121 (T)	3'UTR

Chr – Chromosome; **MAF** – Minor frequent allele; **EUR** – Europe; **CEU** - Utah Residents (CEPH) with Northern and Western European Ancestry

Chapter IV

Glycans in the crosstalk between microbiome and immune response

Márcia S. Pereira[1,2], Inês Alves[1,3], Nuno Padrão[1], Vanda Pinto[1], Mariana Silva[1,2], Manuel Vicente[1,2], Aonghus Lavelle[4], Harry Sokol[4], Salomé S. Pinho[1,3]

[1]Instituto de Investigação e Inovação em Saúde (I3S) / Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal
[2]Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal
[3]Faculty of Medicine, University of Porto, Porto, Portugal
[4]UMRS 938, Centre de Recherche Saint-Antoine (CRSA), Inserm, Paris, France

Abstract

The role of protein glycosylation in Inflammatory Bowel Disease (IBD) has been gaining high relevance in the last few years. Human gut microbiota use glycans as a major source of nutrients and energy. However, whether and how an altered host glycoprofile exerts bifunctional roles in the perturbation of microbiome composition associated with dysbiosis remains to be elucidated.

We used glycoengineered mice models with absence of branched *N*-glycans (Mgat5$^{-/-}$ mice) that exhibit a higher susceptibility to DSS-induced colitis associated with an hyperactivation of T-cell immune response in the gut lamina propria. Microbiome analysis was performed by 16S rDNA gene sequencing both in WT and Mgat5$^{-/-}$ mice.

We demonstrated that the deficiency on branched *N*-glycans instructs a dysbiotic phenotype in Mgat5$^{-/-}$ mice characterized by a significant decrease in beneficial members of the Firmicutes phylum compared to WT mice. After 5 weeks of cohousing with WT mice, the Mgat5$^{-/-}$ mice exhibited a much less severe disease compared to single housed Mgat5$^{-/-}$ mice. Analysis of microbiome diversity and composition revealed an increased proportion of bacteria from the Firmicutes phylum after cohousing KO mice with WT. Inferred metagenomics analysis further revealed an increase in UDP-GlcNAc biosynthetic pathway that was apparently shared and gained upon co-housing the Mgat5$^{-/-}$ with WT. Cohoused Mgat5$^{-/-}$ mice also seem to exhibit an increased production of IL17 and IL22 by Th17 and γδT cells before DSS which pinpoints for a protective effect.

Altogether, these results demonstrate the essential roles of branched *N*-glycans in maintaining a homeostatic crosstalk between the microbiome and host immunity, which pave the way for innovative preventive intervention strategies using glycans in IBD.

Introduction

Gut homeostasis is driven by the symbiotic relation between microbiota and the host. The composition of microbial community is crucial for the instruction of a homeostatic innate and adaptative immune response, and the loss of this balance is associated with some immune-mediated disorders, such as inflammatory bowel disease (IBD) (1). Environment factors, such as diet, stress and antibiotics, can alter the microbiota abundance and diversity (termed dysbiosis), which might lead genetic susceptible individuals to develop an exacerbated immune response in the gut (2).

The competition between "beneficial" commensals and "harmful" commensals (pathobionts) or pathogens is crucial to maintain homeostasis. Host glycans from both mucus (mucins) and epithelial cell layer are fundamental in this competition, as an energy source (3, 4) or as adhesion molecules (5, 6). Bacteria are covered with glycans distinct to the host and use their complex biosynthetic pathways as a community to incorporate host glycans in order to mimic themselves as self, thus avoiding immune recognition (3, 4, 7, 8). Therefore, the number and composition of bacteria might depend on the glycans profile of the host.

Microbial community is genetically different, presenting a huge functional diversity which confer advantage to a specific community in a certain host condition. Commensal bacterial might trigger immune response indirectly through the release of metabolites such as short-chain fatty acids (SCFA), which induce immune tolerance by increasing the infiltration and differentiation of colonic regulatory T cells (Treg) and the production of anti-microbial peptides (9). Moreover, commensal bacteria are important for the differentiation of T cell repertoire, not only on Treg but also in the induction of CD4+ T helper cells that produce IL-17 and IL-22 by Th17 cells, which has been important in a homeostatic condition (1). Innate γδ T cells have been also shown to sense microbiota and to be important in regulating intestinal epithelial homeostasis, by inducing IL-17-producers (γδT17) cells (10), which are reduced in DSS-induced colitis (11). In a dysbiosis scenario, alterations on gut permeability associated with modulation of tight junctions (mediated by claudins) (12, 13) are observed and the booming of pathobionts that lead to a dysregulation of the immune system. Dysbiosis might also be triggered by previous immune dysregulation associated with gut inflammation, which might lead to the outgrowth of less common bacteria due to the uncontrolled clearance of the most abundant, the protective commensal bacteria, leading to the perpetuation of the inflammation (14).

Glycosylation plays a critical role in T cell biology and function, including T cell development, activation, differentiation and signaling (15). We previously showed that lamina propria T cells from ulcerative colitis patients display a disruption on branched *N*-glycans (catalyzed by GnT-V), which was associated with an hyperimmune response (16). In fact, *Mgat5* (gene

that encode GnT-V) knockout in mice (Mgat5$^{-/-}$) result in T cell hyperactivity due to the increased TCR clustering and in higher susceptibility of the mice to develop a severe DSS-induced colitis (17) and other immune-mediated diseases (18-20). Considering the importance of glycans in the crosstalk between host and bacteria, as well as in T cell function, in this study we aim to explore the effects of host branched *N*-glycans on microbiota composition and in the susceptibility to develop colitis.

Methods

Animal experiment

C57BL/6J mice (WT) and Mgat5-deficient C57BL/6J mice (Mgat5$^{-/-}$) were housed under specific pathoge*N*-free (SPF) conditions in i3S animal facility and fed with standard chow diet (2014S, Teklad). After weaning, WT and Mgat5$^{-/-}$ were single housed (SH-WT and SH-KO) or cohoused (CH-WT and CH-KO) in SPF conditions for 5 weeks (Figure S1). After 5 weeks, colitis was induced by supplementing 2% (wt/vol) dextran sulfate sodium (DSS; MP Biomedicals) in drinking water for 7 days and let mice to recover during 5 days with water. In the case of development of severe clinical symptoms, some Mgat5$^{-/-}$ mice only took 6 days of DSS. Body weight, stool consistency and blood in the stools or in rectum were examined daily. Disease activity index (DAI) was calculated to classify disease severity based on the criteria presented on Table S1. All experiments were performed in females due to the differential microbiome composition when compared with males. Animal experiments were performed according to European guidelines.

Microbiome analysis

Stools were collected before DSS treatment and snap frozen in liquid nitrogen. Microbiome composition was determined by 16S rDNA gene sequencing and analyzed as previously described (21). Metagenome imputation was performed using picrust2 software.

Claudins mRNA expression

RNA was isolated from the colon using RNAqueous - Micro kit (Ambion), according with manufacturer's instructions. DNAse was used to digest possible contamination of RNA with genomic DNA. A quantitative PCR was performed for Claudin (Cldn) 1, 2, 3, 4, 5, 7 and 8 expression, using the primers stated on Table S2 and the SybrGreen (BioRad) reagent. Gapdh was used as a reference gene.

Isolation of cells from colonic lamina propria

Colon was digested with 1 mg/mL of collagenase IV (Sigma) in RPMI-1640 GlutaMAX medium (Gibco) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin (pen/strep), 1mM $CaCl_2$ and 1mM $MgCl_2$, during 45 min with agitation at 37ºC. Cell suspension were filtered in 70 µm cell strainers (Corning) and mononuclear cells were enriched by Lymphoprep gradient density centrifugation at 800 g with no break for 30 min. Cell suspension was used to evaluate also epithelial cells.

Flow cytometry analysis

Cells isolated from colon and mesenteric lymph nodes (MLNs) were cultured during 4 hours in RPMI-1640 + 10% fetal bovine serum (FBS) + 1% Pen/strep supplemented with Phorbol 12-myristate 13-acetate (PMA; 20ng/ml), Ionomycin (200ng/ml) and Brefeldin (10ng/ml), for the stimulation of cytokine production.

Cell suspension was analyzed in FACSCanto™ II system (BD Biosciences, San Jose, CA) after staining with the following primary antibodies: CD45-FITC (30-F11), CD45-Pe-Cy7 (30-F11), CD3-eFluor 506 (17A2), CD4-eFluor 450 (RM4-5), CD8a-PE-Cy7 (53-6.7), CD25-PE-Cy5 (PC6.5), Foxp3-APC (FJK-16S), TCR$\gamma\delta$-APC (eBioGL3), IL-17-FITC (TC11-8H4), IL-22- PerCP-eFluor 710 (1H8PWSR). The lectin L-PHA (biotinylated) and further staining with streptavidin – PE, was used to detect β-1,6 GlcNAc branched *N*-glycans. Fixable Viability Dye (FVD) -eFluor 780 was used to stain dead cells and 2% rat serum was used for blocking. Figure S2 shows the gates used to detect the frequencies of CD3, CD4, CD8, Treg (CD25+Foxp3+) and $\gamma\delta$T cells.

Epithelial cells were considered those negative to CD45 (Pe-Cy7, 30-F11) and were stained with different lectins to evaluate the different glycans profile: L-PHA-FITC for β-1,6 GlcNAc branched *N*-glycans, biotinylated ULEx Europaeus Agglutinin I (UEA I) for α-1,2 linked fucose residues and Sambucus Nigra (SNA) – APC for α-2,6 linked sialic acids. Biotinylated lectins were further staining with streptavidin – PE.

Isolation of cells from mesenteric lymph nodes

MLNs were collected and maintained on ice in FACs buffer (PBS + 2% FBS) until cells isolation procedure. MLNs were gently teased between glass slides and cells were collected in FACs buffer and pass through a 70-um filter to obtain a single cell suspension. Cells ($1x10^6$ cells/well) were incubated in 200 µl RPMI-1640 + 10% FBS + 1% Pen/strep supplemented with 50 ng/ml PMA and 1µM ionomycin, at 37°C for 48 hours in a 96-well plate. Medium was collected and stored at -80°C.

Colonic tissue culture

The distal part of the colon was cultured in 500 µl RPMI-1640 supplemented with 1% Pen/strep and 2% Gentamycin for 24h in a 24-well plate. Medium was collected and immediately frozen and stored at -80°C.

Cytokine quantification

Cytokines released from MLNs and colon explants were quantified by ELISA, according with respective manufacturer' instructions: IL10 (R&D Systems), IL17a, TNFα, IFNγ and

IL1β (all from Invitrogen). Medium from colon explant was concentrated for a final volume of 200 µl using centrifugal filters (Amicon, Ultracel – 3k) before quantification. Quantified cytokines from explant supernatant were normalized to the weight of the explant.

Statistical analysis

Two-way ANOVA was performed to evaluate the profile of the disease severity over time. Kruskal-Wallis test from one-way ANOVA was used to evaluate the differences between single housed Mgat5$^{-/-}$ and single housed Mgat5$^{+/+}$ or co-housed with Mgat5$^{+/+}$. Statistical significance is considered with the p value < 0.05.

Results

Mgat5$^{-/-}$ mice display an alteration in microbiome composition compatible with dysbiosis

The *Mgat5*$^{-/-}$ in mice has been showed by our group to be associated with an early-onset DSS-induced colitis and increased disease severity (17). As expected, *Mgat5*$^{-/-}$ mice displayed higher weight loss and higher disease activity index (DAI) when compared with WT mice (Figure 1A). Considering the importance of glycans in microbiota composition and the influence of microbiome in colitis development, we compared the microbial composition of both WT and *Mgat5*$^{-/-}$ mice. Significant differences regarding microbial composition were observed, mainly in the abundance of bacteria belonging to *Firmicutes* phylum, such as *Clostridia* class and *Lachnospiraceae* family (Figure 1B), which were increased in WT mice comparing with *Mgat5*$^{-/-}$. In turn, *Mgat5*$^{-/-}$ displayed an increased abundance in bacteria from Tenericutes, Actinobacteria and Bacteroidetes, additionally to other Firmicutes bacteria (Figure 1B). This suggest a role of microbiome in the susceptibility of *Mgat5*$^{-/-}$ mice to colitis.

The higher susceptibility of *Mgat5*$^{-/-}$ mice to develop a severe colitis is controlled by co-housing with WT mice, apparently due to the sharing of the microbiota

To evaluate the possible implications of microbiome in the increased susceptibility of *Mgat5*$^{-/-}$ to DSS-induced colitis, we performed a co-housing experiment, in which microbiota was shared during approximately 5 weeks before DSS induction between WT and KO mice. Briefly, WT mice were cohousing after weaning with KO (termed as CH-WT) and Mgat5$^{-/-}$ mice with WT (termed as CH-KO) mice. Moreover, both WT and KO mice were also single housed (SH-WT and SH-KO) during the same period (used as controls; Figure S1). Results show that CH-KO mice developed a less severe disease (DAI score) comparing with SH-KO, displaying an intermediate disease behavior (together with CH-WT) when compared with SH-WT and SH-KO (Figure 2A and Figure S2A). The recovery phase was also faster in CH-KO (as well as in SH-WT and CH-WT) comparing with SH-KO, starting to recover on day 8 (*vs* day 11 in SH-KO, Figure 2A).

Analysis of the microbiome revealed a higher abundance and diversity in CH-KO comparing with SH-KO (Figure 2B). The overall composition of SH-KO and CH-KO are totally distinct. The CH-KO are more similar with CH-WT and SH-WT (Figure 2C). Interestingly, the comparison between SH-KO and CH-KO is partially recapitulated between SH-KO and SH-WT (Figure 1B), in which there is an increased abundance of *Firmicutes* species in CH-KO (Figure 2D). Specifically, while SH-KO microbiota is predominantly composed by *Proteobacteria* phylum, the microbiota of CH-KO is mainly increased by *Firmicutes* as

Ruminococcaceae, *Clostridium* and *Acetatifactor* genera (Figure 2D). The concordance on microbial community within SH-WT and CH-KO compared with SH-KO was observed and demonstrated in the Figure S2B, in which several genera from *Ruminococcaceae* and *Lachnospiraceae* family are increased. These results support the restore of microbial community upon cohousing, pinpointing relevant bacteria that might be responsible for the reduction of the susceptibility of *Mgat5*$^{-/-}$ mice to colitis.

Metagenome analysis were further performed and the results show that CH-KO mice display an enrichment with bacteria with higher capacity to synthesize UDP-GlcNAc (the substrate used for GnT-V; UDP-*N*-acetyl-D-glucosamine biosynthesis I pathway; Figure 2E) and potentially with higher UDP-GlcNAc production explained by higher peptidoglycan biosynthesis (peptidoglycan biosynthesis III and peptidoglycan biosynthesis I pathways; Figure 2E). An enrichment of bacteria in CH-KO with indirect and direct metabolic pathways involved in the production of acetate (glycolysis III (from glucose) and pyruvate fermentation to acetate and lactate II), was also observed (Figure 2E). These observations indicate the gain of a differential composition and functional microbiota by Mgat5$^{-/-}$ mice after co-housing with WT, which might explain the distinct clinical outcome after DSS induction.

WT microbiota seems to improve epithelial barrier of Mgat5$^{-/-}$ mice

In order to understand whether the rescue of microbial composition of the Mgat5$^{-/-}$ observed after co-housing with WT might be influencing epithelial barrier, we determined the mRNA expression levels of claudins in the colon. We verified an up-regulation of *CldN*-2 and *CldN*-8 in *Mgat5*$^{-/-}$ mice after co-housing with WT (Figure S3), suggesting a beneficial effect of microbiota the epithelial barrier.

Impact of microbiota in modulation of T cells glycosylation and adaptive immune response

In order to investigate the immunological impact of the different microbiota composition observed in *Mgat5*$^{-/-}$ before and after co-housing with WT mice, we collected immune cells from both mesenteric lymph nodes (MLNs) and colonic lamina propria. Although these are still preliminary results that will be further explored, the results suggest that sharing of microbiome seems to decrease the frequency of CD3+ T cells in both MLNs (from 79.3% in SH-KO to 65.2% in CH-KO) and colon (from 47.5% in SH-KO to 20.6% in CH-KO) from *Mgat5*$^{-/-}$ (Figure 3), almost reaching the levels from SH-WT (50% in MLNs and 27.6% in colon; Figure S4). Despite the decreased CD4+ and increased CD8+ T cells frequency in MLNs from SH-KO mice comparing with SH-WT, no differences on CD4+ and CD8+ T cells were observed upon co-housing (Figure 3 and Figure S4). Unexpectedly, *Mgat5*$^{-/-}$ mice who received microbiota from WT displayed an enhancement of the branched *N*-glycans

expression (recognized by L-PHA lectin) at the surface of all T cells subtypes (CD3, CD4, CD8 and Treg; Figure 3), mainly evident in MLNs, which can be associated with less reactivity of T cells, as previously shown by our group (17). However, the branched *N*-glycan levels seem to do not reach those observed in SH-WT (Figure S4). This enhancement of branched *N*-glycans expression seem to be specific of T cells since non-immune cells (enriched of epithelial cells) do not exhibit this enhanced expression (Figure S5).

Afterwards, we investigated whether microbiota from WT may instruct a protective immunological profile in *Mgat5*$^{-/-}$, by assessing IL17- and IL22-producing CD4 (Th17) and γδTCR T cells in MLNs. No differences on γδT cells or CD4+ T cells frequencies were observed, but an increase IL17- and IL22-producing T cells seem to occur (Figure S6). Altogether, our preliminary data suggest a protective effect of microbiota by inducing branching *N*-glycans on T cells and IL17 and IL22 immune response.

DISCUSSION

The presence of gut microbiota is a condition to develop intestinal inflammation, once germ-free mice fail to develop colitis (22, 23) and alterations on gut microbiota composition can precede colitis development (24). Host inflammation, either genetically promoted (as IL22- (25) and IL10- (26, 27) deficient mice) or chemically induced (DSS) (27), alters microbiota composition, favoring the outgrowth of less abundant species, more resistant to inflammation that turns-out to be pathobionts (27). In our study we found that the lack of *Mgat5* gene associated with the absence of branched *N*-glycans, *per se*, decreases the microbial biodiversity and abundance, culminating in an increased susceptibility to develop severe colitis. These *Mgat5*$^{-/-}$ mice exhibit a deficiency in some bacteria from *Firmicutes* phylum, mainly from *Clostridia* class, known to be composed by Treg inducing bacteria (28). This disturbance impacting homeostasis might be explaining the elevated susceptibility to DSS-colitis induction. The increased T cell reactivity of Mgat5$^{-/-}$ mice (19) that is also associated with susceptibility to auto-immune disorders (19, 20) might be a cause for the disturbance or a consequence of the microbiota composition.

The elevated susceptibility of MAGT5 $^{-/-}$ to colitis was hampered upon sharing microbiota with WT mice, whose microbiota was enriched with bacteria from *Firmicutes* phylum. This enrichment was accompanied with a decreased abundance of bacteria from *Proteobacteria* phylum, suggesting a protective competitive effect. In fact, the enrichment of microbiome with *Firmicutes* might be protecting mice against pathobionts outgrowth and thus impairing DSS-induced colitis, as it was previously demonstrated with the enrichment with bacteria from *Firmicutes* phylum (29) in *Citrobacter rodentium*-induced colitis. The increased ratio of Proteobacteria/Firmicutes is observed in IBD patients (30), in which some *Proteobacteria*, such as *Gammaproteobacteria*, increase in inflammatory conditions (31). The presence of bacteria from *Firmicutes* phylum were shown to restrict intestinal inflammation (32), and to suppress *C. difficile* colonization (33), which is in line the protective effect observed in MGAT5$^{-/-}$ upon co-housing with WT mice.

This homeostatic competition observed in *Mgat5*$^{-/-}$ mice seems to slow down T cell immune response by the potential stimulation of hexosamine biosynthetic pathway, upon incorporation of UDP-GlcNAc (produced by microbiota) in CD3+ cells and the consequent formation of branched *N*-glycans observed in *Mgat5*$^{-/-}$ mice after co-housing. Indeed, even in the absence of the enzyme Gnt-V (encoded by Mgat5 and responsible for the branching *N*-glycans), *in vivo* supplementation of the substrate UDP-GlcNAc leads to an increase of T cell *N*-glycan branching by the compensatory function of other glycosyltransferases as Gnt-Vb (17). This glycosylation modulation ameliorates clinical symptoms and reduces disease severity in DSS-induced colitis in Mgat5$^{-/-}$ mice, by promoting branched *N*-glycan

expression in intestinal inflammatory infiltrate and decreasing T cell activity through dow*N*-regulation of TCR signaling pathway and CD3 proliferation, as well as controlling Th1 and Th17 immune response (17). Thus, this T cell immune control by GlcNAc incorporation and branched *N*-glycan biosynthesis might be a way on how gut microbiota, or specifically *Firmicutes*, maintains mucosal T cell homeostasis.

Commensal bacteria induces mucosal homeostasis through the production of IgA and the instruction of T cell immune tolerance, through inducing Treg and IL17- and IL22-producing T cells (1). IL22 acts as an important regulator of the interplay between host and microbial community, by promoting the production of antimicrobial peptides (34, 35), and maintaining epithelial integrity (36) and mucus by increasing epithelial fucosylation through Fut2 expression (37). In fact, the loss of IL22 in mice leads to an aberrant immune response against commensals (25). In accordance, we observed a decreased frequency of IL17- and IL22-producing cells (CD4 and γδT cells) in Mgat5$^{-/-}$ mice comparing with WT. This might result in the imbalance of microbiota observed in Mgat5$^{-/-}$ mice. Both cytokines were increased in Mgat5$^{-/-}$ mice cohoused with WT, suggesting a rescue of the immune tolerance induced by the increased proportion of *Firmicutes*. Actually, bacteria consortium transplantation was shown to protect DSS-induced colitis in mice, promoting IL17A-producing γδ T cell expansion and improvement of intestinal barrier function (38). Also, IL-22 was shown to increase epithelial permeability through increasing expression of the Cld*N*-2 (39), which has been also stimulated through IL-17 (40) and microbial-derived butyrate (41). The increased permeability induced by Cld*N*-2 was shown to protect mice against DSS-induced colitis, promoting colonocyte proliferation and integrity and inducing immune tolerance (42). We observed that Mgat5$^{-/-}$ mice display a deficiency on Cld*N*-2 expression, which is increased after sharing microbiota with WT mice. We can speculate that Cld*N*-2 overexpression might be induced by the increased levels of IL-22 and IL-17, which might suggest another level of immune protection induced by microbiota.

Taken together, branching *N*-glycans are herein pinpointed as being a critical determinant for a balanced crosstalk between host immunity and microbiome, maintaining mucosal T cell homeostasis through a healthy microbiome community.

References

1. Belkaid Y, Hand TW. 2014. Role of the microbiota in immunity and inflammation. *Cell* 157: 121-41
2. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. 2017. Dysbiosis and the immune system. *Nat Rev Immunol* 17: 219-32
3. Hooper LV, Xu J, Falk PG, Midtvedt T, Gordon JI. 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proc Natl Acad Sci U S A* 96: 9833-8
4. Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM. 2004. Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev* 68: 132-53
5. Schroeder BO. 2019. Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterol Rep (Oxf)* 7: 3-12
6. Formosa-Dague C, Castelain M, Martin-Yken H, Dunker K, Dague E, Sletmoen M. 2018. The Role of Glycans in Bacterial Adhesion to Mucosal Surfaces: How Can Single-Molecule Techniques Advance Our Understanding? *Microorganisms* 6
7. Comstock LE, Kasper DL. 2006. Bacterial glycans: key mediators of diverse host immune responses. *Cell* 126: 847-50
8. Coyne MJ, Reinap B, Lee MM, Comstock LE. 2005. Human symbionts use a host-like pathway for surface fucosylation. *Science* 307: 1778-81
9. Parada Venegas D, De la Fuente MK, Landskron G, Gonzalez MJ, Quera R, Dijkstra G, Harmsen HJM, Faber KN, Hermoso MA. 2019. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol* 10: 277
10. Yang Y, Xu C, Wu D, Wang Z, Wu P, Li L, Huang J, Qiu F. 2018. gammadelta T Cells: Crosstalk Between Microbiota, Chronic Inflammation, and Colorectal Cancer. *Front Immunol* 9: 1483
11. Sun X, Cai Y, Fleming C, Tong Z, Wang Z, Ding C, Qu M, Zhang HG, Suo J, Yan J. 2017. Innate gammadeltaT17 cells play a protective role in DSS-induced colitis via recruitment of Gr-1(+)CD11b(+) myeloid suppressor cells. *Oncoimmunology* 6: e1313369
12. Yu LC. 2018. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. *J Biomed Sci* 25: 79
13. Zeissig S, Burgel N, Gunzel D, Richter J, Mankertz J, Wahnschaffe U, Kroesen AJ, Zeitz M, Fromm M, Schulzke JD. 2007. Changes in expression and distribution of

claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut* 56: 61-72

14. Zeng MY, Inohara N, Nunez G. 2017. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol* 10: 18-26
15. Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrao NA, Pinto V, Fernandes A, Dias AM, Pinho SS. 2018. Glycans as Key Checkpoints of T Cell Activity and Function. *Front Immunol* 9: 2754
16. Dias AM, Dourado J, Lago P, Cabral J, Marcos-Pinto R, Salgueiro P, Almeida CR, Carvalho S, Fonseca S, Lima M, Vilanova M, Dinis-Ribeiro M, Reis CA, Pinho SS. 2014. Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 23: 2416-27
17. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, Maia L, Delerue-Matos C, Taniguchi N, Lima M, Pedroto I, Marcos-Pinto R, Lago P, Reis CA, Vilanova M, Pinho SS. 2018. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 115: E4651-E60
18. Grigorian A, Demetriou M. 2011. Mgat5 deficiency in T cells and experimental autoimmune encephalomyelitis. *ISRN Neurol* 2011: 374314
19. Demetriou M, Granovsky M, Quaggin S, Dennis JW. 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409: 733-9
20. Lee SU, Grigorian A, Pawling J, Chen IJ, Gao G, Mozaffar T, McKerlie C, Demetriou M. 2007. N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *J Biol Chem* 282: 33725-34
21. Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, Bridonneau C, Jegou S, Hoffmann TW, Natividad JM, Brot L, Taleb S, Couturier-Maillard A, Nion-Larmurier I, Merabtene F, Seksik P, Bourrier A, Cosnes J, Ryffel B, Beaugerie L, Launay JM, Langella P, Xavier RJ, Sokol H. 2016. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat Med* 22: 598-605
22. Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM, Sartor RB. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 66: 5224-31
23. Feng T, Wang L, Schoeb TR, Elson CO, Cong Y. 2010. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J Exp Med* 207: 1321-32

24. Nagao-Kitamoto H, Shreiner AB, Gillilland MG, 3rd, Kitamoto S, Ishii C, Hirayama A, Kuffa P, El-Zaatari M, Grasberger H, Seekatz AM, Higgins PD, Young VB, Fukuda S, Kao JY, Kamada N. 2016. Functional Characterization of Inflammatory Bowel Disease-Associated Gut Dysbiosis in Gnotobiotic Mice. *Cell Mol Gastroenterol Hepatol* 2: 468-81
25. Zenewicz LA, Yin X, Wang G, Elinav E, Hao L, Zhao L, Flavell RA. 2013. IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic. *J Immunol* 190: 5306-12
26. Maharshak N, Packey CD, Ellermann M, Manick S, Siddle JP, Huh EY, Plevy S, Sartor RB, Carroll IM. 2013. Altered enteric microbiota ecology in interleukin 10-deficient mice during development and progression of intestinal inflammation. *Gut Microbes* 4: 316-24
27. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2: 119-29
28. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500: 232-6
29. Vong L, Pinnell LJ, Maattanen P, Yeung CW, Lurz E, Sherman PM. 2015. Selective enrichment of commensal gut bacteria protects against Citrobacter rodentium-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 309: G181-92
30. Sokol H, Seksik P. 2010. The intestinal microbiota in inflammatory bowel diseases: time to connect with the host. *Curr Opin Gastroenterol* 26: 327-31
31. Rizzatti G, Lopetuso LR, Gibiino G, Binda C, Gasbarrini A. 2017. Proteobacteria: A Common Factor in Human Diseases. *Biomed Res Int* 2017: 9351507
32. Chen L, Wilson JE, Koenigsknecht MJ, Chou WC, Montgomery SA, Truax AD, Brickey WJ, Packey CD, Maharshak N, Matsushima GK, Plevy SE, Young VB, Sartor RB, Ting JP. 2017. NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. *Nat Immunol* 18: 541-51
33. Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB. 2012. Suppression of Clostridium difficile in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the family Lachnospiraceae. *Infect Immun* 80: 3786-94
34. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. 2004. IL-22 increases the innate immunity of tissues. *Immunity* 21: 241-54

35. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 14: 282-9
36. Perusina Lanfranca M, Lin Y, Fang J, Zou W, Frankel T. 2016. Biological and pathological activities of interleukin-22. *J Mol Med (Berl)* 94: 523-34
37. Pham TA, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, Keane JA, Page AJ, Kumasaka N, Kane L, Mottram L, Harcourt K, Hale C, Arends MJ, Gaffney DJ, Sanger Mouse Genetics P, Dougan G, Lawley TD. 2014. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe* 16: 504-16
38. Li M, Wang B, Sun X, Tang Y, Wei X, Ge B, Tang Y, Deng Y, He C, Yuan J, Li X. 2017. Upregulation of Intestinal Barrier Function in Mice with DSS-Induced Colitis by a Defined Bacterial Consortium Is Associated with Expansion of IL-17A Producing Gamma Delta T Cells. *Front Immunol* 8: 824
39. Wang Y, Mumm JB, Herbst R, Kolbeck R, Wang Y. 2017. IL-22 Increases Permeability of Intestinal Epithelial Tight Junctions by Enhancing Claudin-2 Expression. *J Immunol* 199: 3316-25
40. Kinugasa T, Sakaguchi T, Gu X, Reinecker HC. 2000. Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology* 118: 1001-11
41. Zheng L, Kelly CJ, Battista KD, Schaefer R, Lanis JM, Alexeev EE, Wang RX, Onyiah JC, Kominsky DJ, Colgan SP. 2017. Microbial-Derived Butyrate Promotes Epithelial Barrier Function through IL-10 Receptor-Dependent Repression of Claudin-2. *J Immunol* 199: 2976-84
42. Ahmad R, Chaturvedi R, Olivares-Villagomez D, Habib T, Asim M, Shivesh P, Polk DB, Wilson KT, Washington MK, Van Kaer L, Dhawan P, Singh AB. 2014. Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. *Mucosal Immunol* 7: 1340-53

Figures

Figure 1

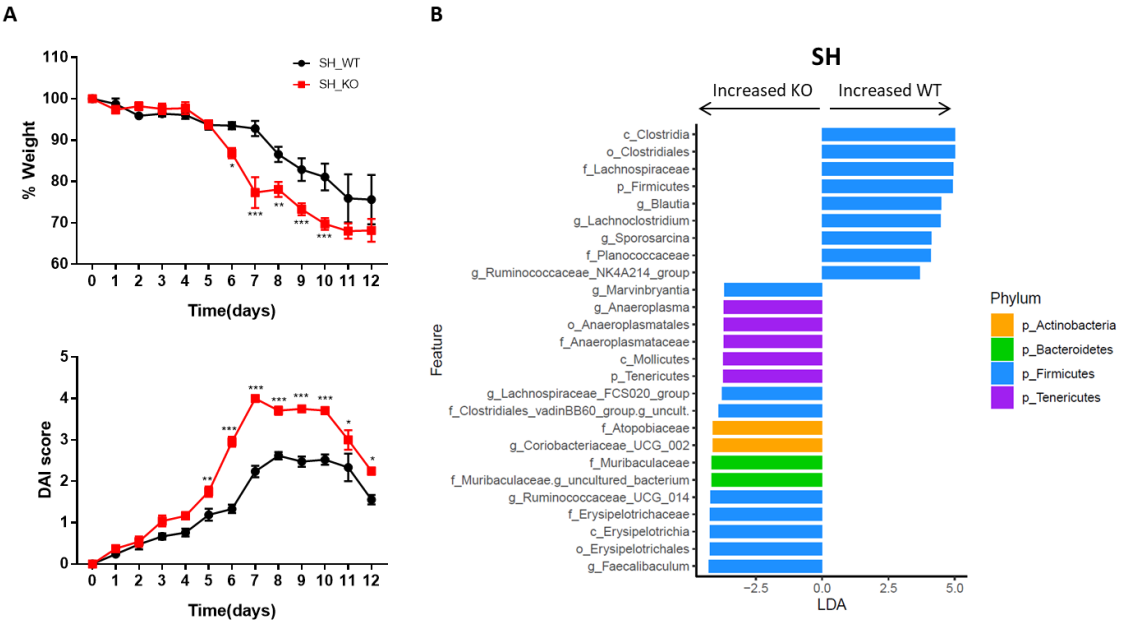


Figure 1_ Mgat5$^{-/-}$ mice is predisposed to develop severe colitis and display an altered microbiota comparing with wildtype. **A)** Females age-matched single-housed (SH) for 5 weeks received 2% DSS *ad libitum* for 7 days, with the exception of 6 SH-KO (received for 6 days) that develop severe clinical symptoms - SH-WT (n=7), SH-KO (n=8); **B)** Differential bacterial composition comparing SH-WT and SH-KO.

Figure 2

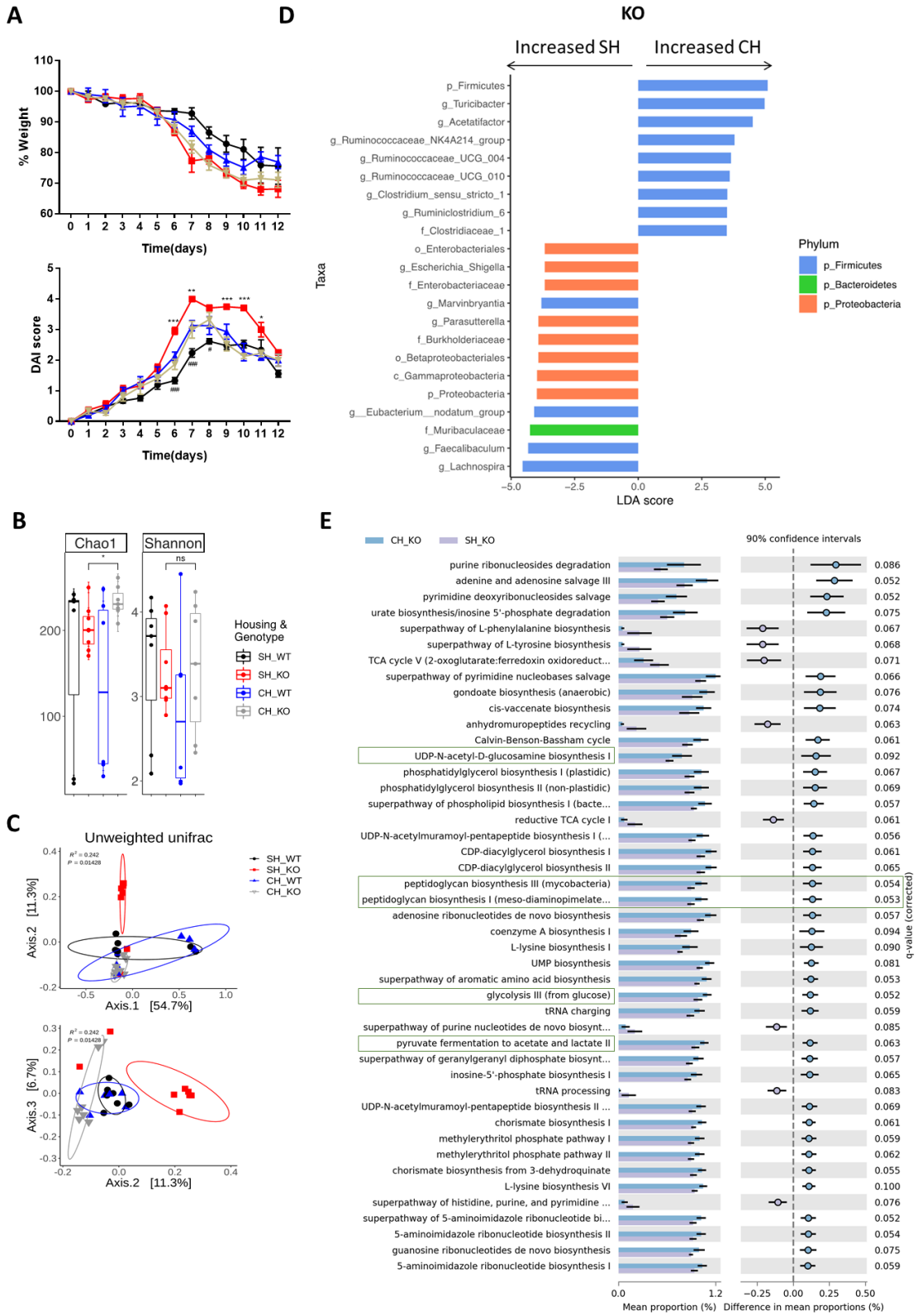


Figure 2_Dysbiosis of Mgat5$^{-/-}$ is rescued in co-housed mice, favoring the enrichment of *Firmicutes* and bacteria with capacity to synthesize UDP-*N*-acetyl-D-glucosamine. **A)** Females age-matched single- and co-housed (SH and CH) for 5 weeks received 2% DSS ad libitum for 7 days, with the exception of 6 SH-KO (received for 6 days) that develop

severe clinical symptoms - SH-WT (n=7), SH-KO (n=8), CH-WT (n=5) and CH-KO (n=5). **B)** Richness and evenness of fecal microbiota from females age-matched after single- and co-housing (SH and CH) for 5 weeks - SH-WT (n=7), SH-KO (n=8), CH-WT (n=7) and CH-KO (n=7); **C)** Discrimination of microbial composition - PCoA plot generated on unweighted UniFrac distance matrices; **D)** Differential bacterial composition comparing SH-KO and CH-KO; **E)** Metagenome analysis comparing both SH-KO and CH-KO. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 3

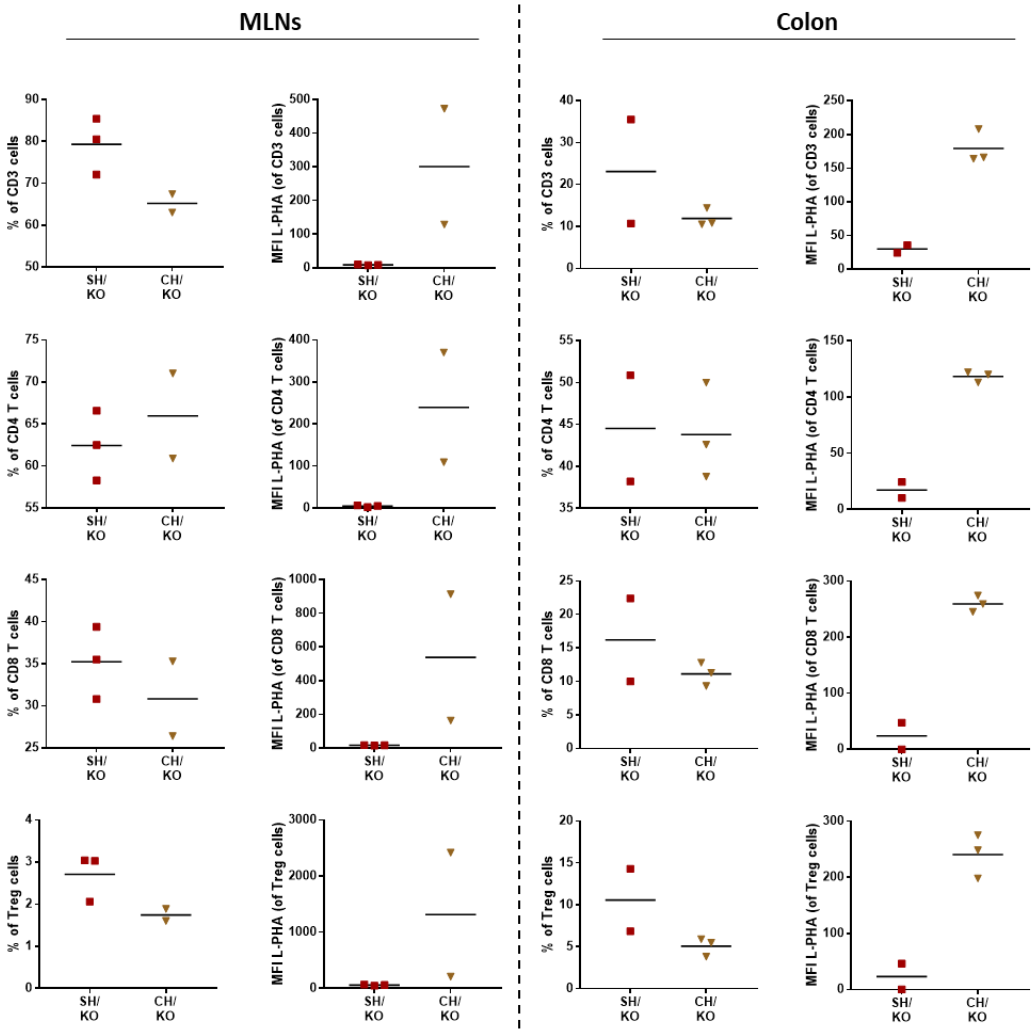


Figure 3_ Microbiota from wildtype mice increases *N*-glycan branching of T cells. T cells from mesenteric lymph nodes (MLNs) and colonic lamina propria were isolated from Mgat5$^{-/-}$ mice single- and co-housing (SH and CH) for 5 weeks. CD3+, CD4+, CD8+ and FOXP3+CD25+ T cells were analyzed in flow cytometry. L-PHA lectin were used to detect branched *N*-glycans and the median fluorescence intensity was determined.

Supplementary figures

Figure S1

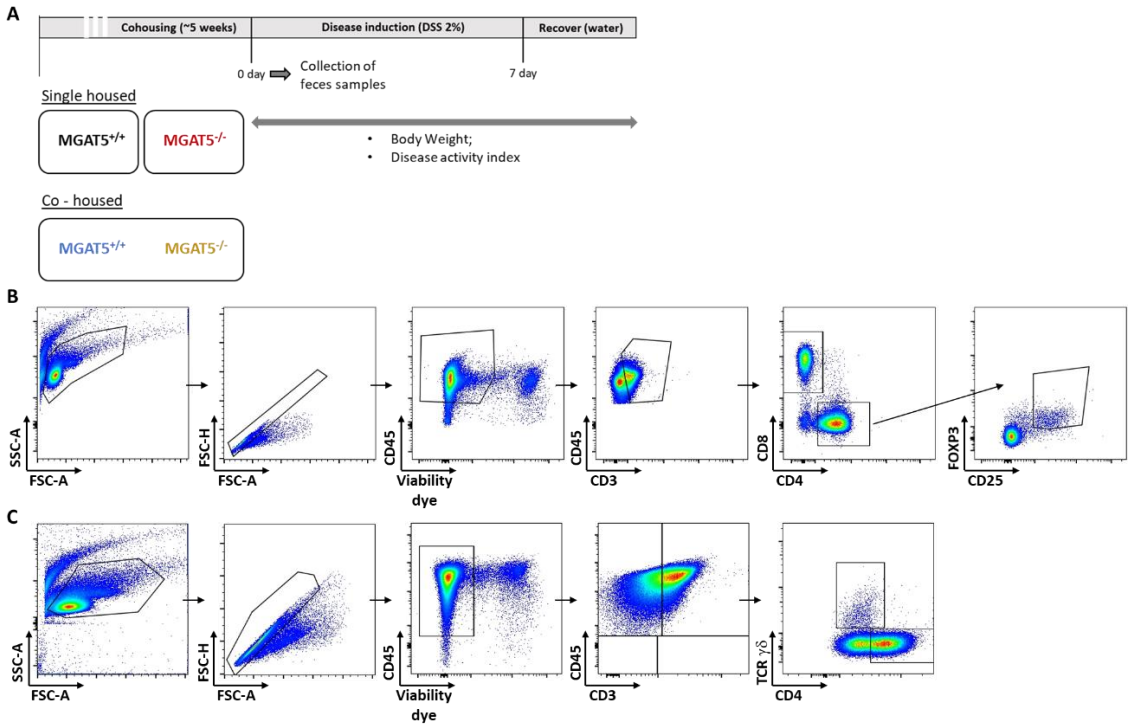


Figure S1_Animal experiment design **(A)** and gate strategy to determine the frequencies of **A)** CD3, CD4, CD8 T cells and Treg (CD25+Foxp3+) and **B)** CD4+ and γδ T cells producing IL17 and IL22. Median fluorescence intensity (MFI) of IL17 and IL22 and lectins (L-PHA, SNA and ULEx) were used for quantification.

Figure S2

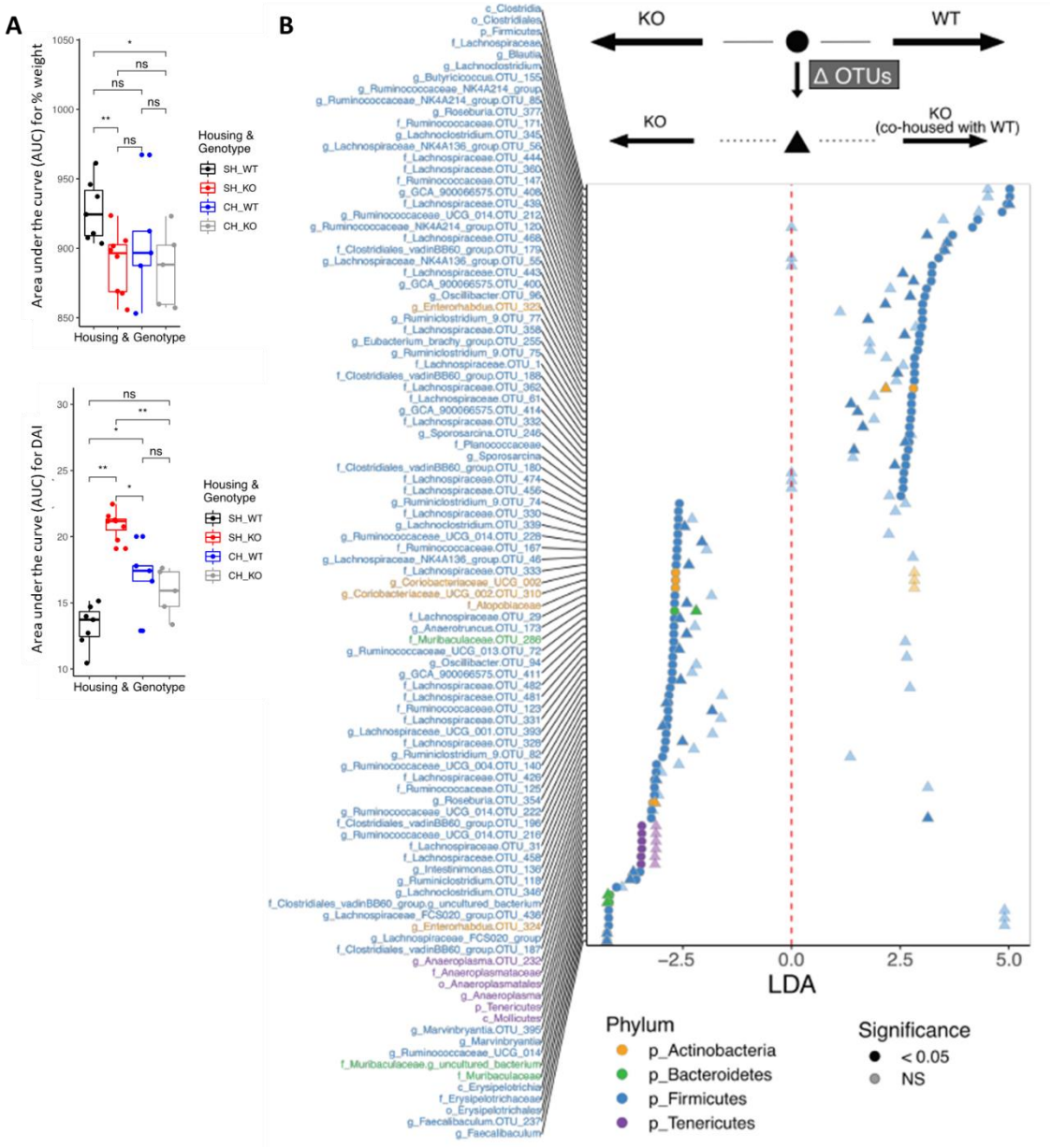


Figure S2_The predisposition of Mgat5$^{-/-}$ mice to develop severe colitis is decreased when co-housed with wildtype mice, promoting a similar microbiota composition to wildtype mice. **A)** Area under the curve (AUC) for females age-matched single- and co-housed (SH and CH) for 5 weeks received 2% DSS ad libitum for 7 days, with the exception of 6 SH-KO (received for 6 days) that develop severe clinical symptoms - SH-WT (n=7), SH-KO (n=8), CH-WT (n=5) and CH-KO (n=5); **B)** Differential bacterial composition based on microbial OTUs within two groups - SH-KO *vs* SH-WT (pointed with circles) and SH-KO *vs* CH-KO (pointed with triangles).

Figure S3

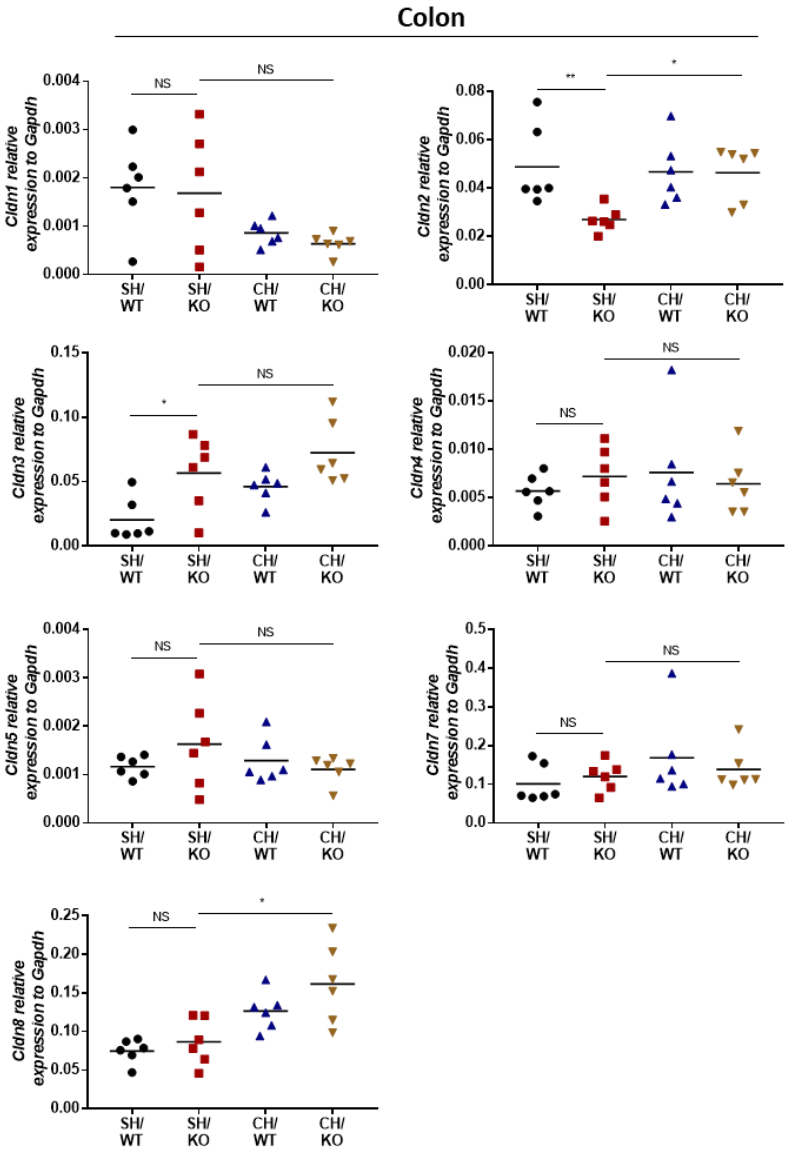


Figure S3_mRNA expression levels of claudins 1, 2, 3, 4, 5, 7 and 8 from mice before DSS. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure S4

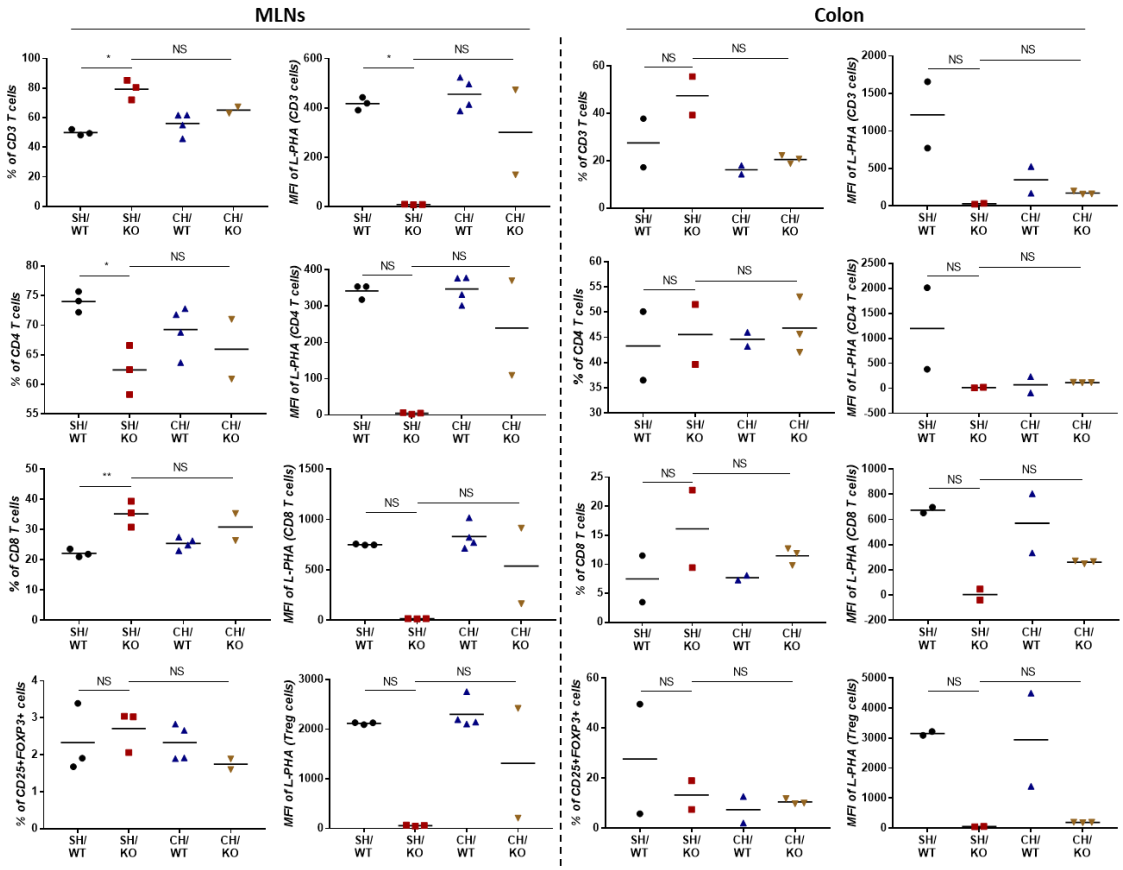


Figure S4_Microbiota from wildtype mice seems to increase *N*-glycan branching of T cells. T cells from mesenteric lymph nodes (MLNs) and colonic lamina propria were isolated from mice single- and co-housing (SH and CH) for 5 weeks. CD3+, CD4+, CD8+ and FOXP3+CD25+ T cells were analyzed in flow cytometry. L-PHA lectin were used to detect branched *N*-glycans and the median fluorescence intensity was determined. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure S5

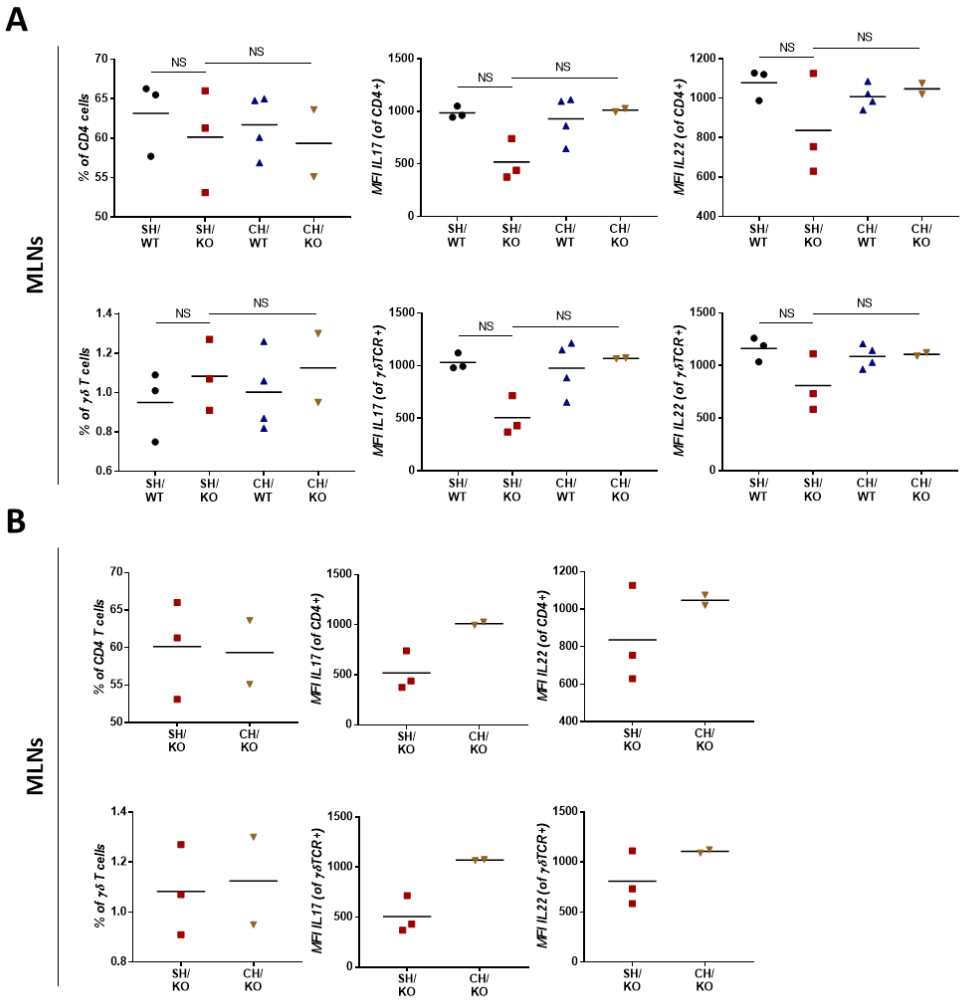


Figure S5_Microbiota from wildtype mice seem to alter adaptive immune response in Mgat5$^{-/-}$ mice. **A)** and **B)** CD4+ and γδ T cells producing IL17 and IL22 from mesenteric lymph nodes (MLNs) of mice single- and co-housing (SH and CH) for 5 weeks were detected by flow cytometry. IL17 and IL22 were measured by median fluorescence intensity (MFI).

Figure S6

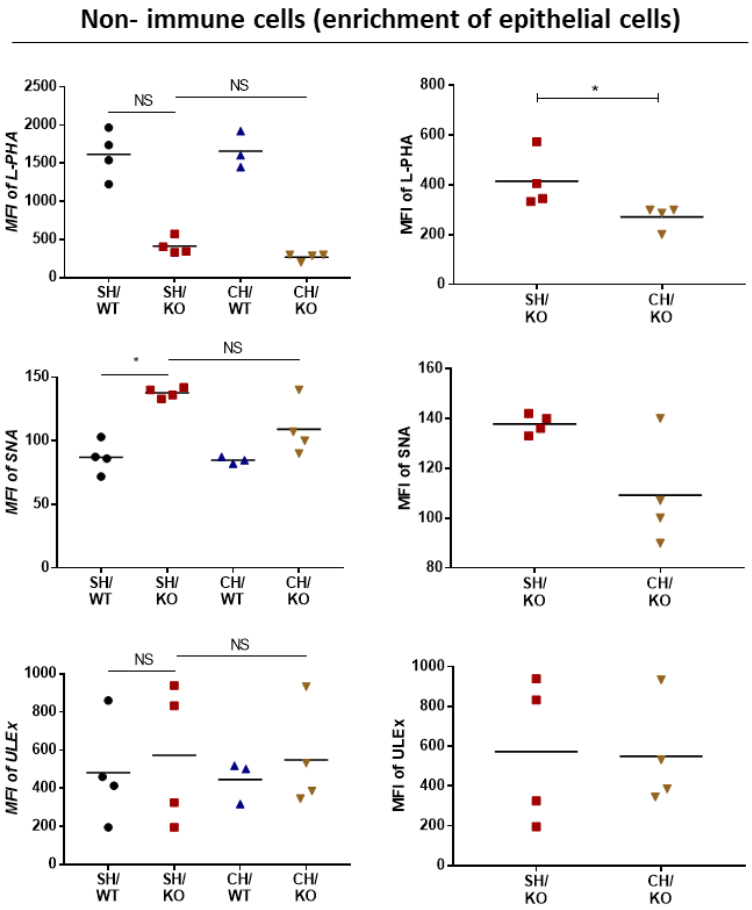


Figure S4_Mgat5$^{-/-}$ mice display low levels of sialic acids in and enrichment of epithelial cells. CD45- cells from colon were considered as epithelial cells and branched *N*-glycans (L-PHA), sialic acids (SNA) and fucose (ULEx) were detected by flow cytometry, in mice single- and co-housing (SH and CH) for 5 weeks were detected by flow cytometry. L-PHA, SNA and ULEx were measured by median fluorescence intensity (MFI). *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary tables

Table S1_Disease activity index (DAI) score used to classify disease severity in colitis-induced mice. DAI score is the mean of three parameters: weight loss, consistency of the feces and presence of blood. The presence of the blood was evaluated using a swab in the anus of the animal and in the cage.

DAI score	Weight Loss	Consistency of feces	Blood
0	Normal	WF	Any blood in the swab
1	1-5%	WF/P	Vestigious in the swab + Blood or no blood in the cage
2	6-10%	P	Blood in the swab + Blood or no blood in the cage
3	11-18%	P/L	Blood in the swab + high blood quatity in the cage
4	>18%	L	Blood in anus at naked eye + blood in the cage

WF – well formed; P – pasty; L - Liquid

Table S2_Primers used in quantitative real-time PCR to determine mRNA expression in mice.

Gene	Primers	bp	Tm (°C)	CG (%)	bp product
Claudin 1	Fw_GGCTTCTCTGGGATGGATCG	20	60	60	235
	Rv_CCCCAGCAGGATGCCAATTA	20	60.1	55	
Claudin 2	Fw_TTTTGGGGCTGTTAGGCACA	20	60.1	50	290
	Rv_AGAATCCTGGCAGAACACGG	20	60	55	
Claudin 3	Fw_GTACAAGACGAGACGGCCAA	20	60	55	462
	Rv_CGTAGTCCTTGCGGTCGTAG	20	60.3	60	
Claudin 4	Fw_ACGTCATCCGCGACTTCTAC	20	59.9	55	151
	Rv_TTGTCGTTGCTACGAGGTGG	20	60.3	55	
Claudin 5	Fw_GTGTCTGGTAGGATGGGTGG	20	59.5	60	179
	Rv_GCGCCAGCACAGATTCATAC	20	59.7	55	
Claudin 7	Fw_CCATGTACAAGGGGCTCTGG	20	60.1	60	224
	Rv_AGCTATTCGGGCCTTCTTCG	20	59.9	55	
Claudin 8	Fw_TTGCTGACAGCCGGAATCAT	20	60	50	295
	Rv_TCGGAGATCTCTTTTCGGCG	20	59.9	55	
Gapdh	Fw_GAAGGTCGGTGTGAACGGAT	20	60	55	230
	Rv_CTCGCTCCTGGAAGATGGTG	20	60.2	60	

bp – base pairs; Tm – Melting temperature.

Chapter V

Part I

Glycosylation of Immunoglobulin G is able to distinguish Crohn´s Disease patients that lost response to anti-TNF biologics.

[1,2]Márcia S Pereira, [3]Francesca Petralia, [4]Jerko Štambuk, [4]Elham Memarian, [4]Genadij Razdorov, [4]Mislav Novokmet, [4]Frano Vučković, [4]Irena Trbojevic-Akmacic, [5]Luís Maia, [1]Vanda Pinto, [1,6]Inês Alves, [5,2]Ricardo Marcos-Pinto, [5]Paula Lago, [4]Gordan Lauc, [7]Jean-Frederic Colombel, [1,6*]Salomé S. Pinho.

[1]Instituto de Investigação e Inovação em Saúde (I3S) / Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal
[2]Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal
[3]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai New York, New York, USA
[4]Genos Glycoscience Research Laboratory, Zagreb, Croatia
[5]Department of Gastroenterology, Porto Centre Hospital, Porto, Portugal
[6]Faculty of Medicine, University of Porto, Porto, Portugal
[7]Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

Abstract

In the last decades, we are assisting the development of a significant variety of novel therapies targeting several pathways and molecules that have been revolutionizing the treatment of patients with Inflammatory Bowel Disease (IBD). Anti-tumor necrosis factor (anti-TNF) agents remain the first line treatment in the majority of IBD patients. Nevertheless, almost half of the patients lose response over time (secondary loss of response (SLR)) or become intolerant to anti-TNFs, requiring alternative agents. Human immunoglobulin G (IgG) isolated from plasma is comprised of multiple glycoforms and different glycans variations have been detected on circulating IgGs. Whether this IgG glycome heterogeneity among the individuals translates to a better stratification of patients accordingly with their response to biologics therapy remains completely unknown. In this study, we measured IgG Fc subclass specific glycopeptides in Crohn´s disease (CD) patients under infliximab and adalimumab and the results suggest a unique glycoprofile of plasma IgGs that is able to distinguish responders from patients who lose response to anti-TNF therapy. Low levels of sialylated IgG was associated with secondary non responders (SNR), independently of anti-TNF drug type. Agalactosylated IgG was associated with loss of response to infliximab, while afucosylated IgG were correlated with loss of response to adalimumab. In summary, IgG glycome was revealed as a promising non-invasive biomarker able to assist a proactive therapy drug monitoring improving the therapy decision making process and thereby a personalized medicine in IBD.

Introduction

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and Ulcerative Colitis (UC), is a chronic immune-mediated disorder of the gastrointestinal tract that places a heavy burden on worldwide population. The heterogeneity in terms of disease onset, response to therapies and progression to complications gave rise to an explosion of new targeted therapies. Starting roughly 20 years ago with the clinical effectiveness and approval of infliximab, other anti-tumor necrosis factor (anti-TNF) blockers (adalimumab e.g.) (1), as well as alternative therapies targeting other molecules as integrins (vedolizumab e.g.) and IL12/IL23 (ustekinumab) (2-4) has been included in clinical practice and revolutionized the treatment of IBD.

Despite the significant efficacy of anti-TNF therapy, up to 30% of IBD patients do not respond to this therapeutic class in the induction phase (primary non-responders - PNR) and up to 50% display a transient efficacy of the drug, losing response over time (secondary loss of response - SLR) (5, 6). Moreover, patients who initiated anti-TNF can discontinue treatment because of serious adverse events (SAE), namely with infliximab. In fact, the evidence to guide decisions for dose intensification and swap to a different drug class is currently in discussion and was recently revised (7). The current strategy for PNR is to swap therapy to other drug class (such as anti-integrin therapy e.g.), while secondary non-responders (SNR) are subjected to adjustment in drug doses and interval times as well as therapy enforcement with immunosuppressants or swap therapy to other drug class if this strategy fails (5, 8, 9).

There is a pressing need in the field to identify reliable and minimally invasive biomarkers to select those patients that will fail to respond to anti-TNF therapies and elect them, early in disease course, to first-line treatment with another drug class of biologics rather than anti-TNF. This tailored approach will improve clinical remission, avoiding the risk of relapse and side effects, being a cost-effective approach to improve the long time course of the disease and patients´ quality of life.

Glycosylation is a highly-regulated post-translational mechanism characterized by the addition of carbohydrate structures (glycans) to proteins and lipids of essentially all cells (10). The diversity of the glycome (biological repertoire of glycans in an organism) is enormous reflecting their multiple biological functions in key cellular and molecular mechanisms of homeostasis and disease, including cancer and IBD (10-13).

In fact, almost all molecules involved in immune response are glycosylated, including immunoglobulins, in which the function and activity highly depends on their glycosylation profile. Human Immunoglobulin G (IgG) isolated from plasma is comprised of multiple glycoforms owing to the addition of a diverse type of glycan structures in the IgG Fc

(conserved domain) and Fab (variable domain) regions. Every IgG Fc domain contains a single, highly conserved, glycosylation site at Asn297 that carries complex N-glycans. Over 30 different glycans variations have been detected on circulating IgG in healthy individuals, which reflects a tremendous heterogeneity in IgG Fc glycome (14). The glycosylation pattern of IgG is dynamic and can vary depending on age, sex, as well as autoimmune and infectious disorders (15). In fact, the type of glycan attached to IgG´s Fc is critical for the proper effector functions of all IgGs, regulating the binding to FcγRs on immune cells and instructing either a pro-inflammatory (through binding to activating FcγRs) or an anti-inflammatory (through binding to inhibitory FcγRs) response (14). A seminal observation in the 1980´s found that IgG glycosylation differs in patients with Rheumatoid Arthritis (RA), in which loss of terminal galactose (agalactosylation) on IgG was seen in patients suffering from RA and appearing in circulation preceding disease onset (16, 17). Terminal sialylation on Fc glycans also modulates FcγR binding. The presence of α2,6 sialic acid on the Fc glycan significantly reduces FcγR affinity being associated with anti-inflammatory activity (18).

More recently, the glycosylation profile of IgG was found to be different comparing UC and CD patients. IBD patients showed a distinct pattern of plasma IgG glycosylation, comparing with healthy individuals, characterized by a decreased galactosylation, decreased sialylation and increased bisecting *N*-acetylglucosamine (GlcNAc) (19, 20) which was associated with clinical severity of the disease (19, 21).

Taking into consideration the growing body of evidence showing the prominent role of glycans in the immunopathogenesis of IBD (11, 13, 22, 23), in this study we evaluated whether changes in plasma IgG glycome are correlated with response to anti-TNF biologics therapy, as a proxy of a uncontrolled inflammatory disease. We tested whether the glycosylation profile of the different IgG subclasses in the plasma of CD patients is correlated with loss of response to anti-TNF (infliximab and adalimumab), envisioning the identification of a minimally invasive plasma biomarker to be used in the process of therapy decision-making benefiting a proactive therapy drug monitoring.

Methods

Crohn´s Disease patients' cohort

This was a retrospective observational study, which included a cohort of IBD patients that comprises 305 blood samples from 64 CD patients (Table 1 and Table S1) under anti-TNF therapy (infliximab or adalimumab). Patients were followed in the IBD unit of Porto Centre University Hospital (CHUP), where physician's global assessment (PGA) of disease activity and biochemical parameters as C-reactive protein (CRP) and calprotectin were retrieved. All information, including clinical and biochemical parameters, are from the time of sample collection (Table 1 and Table S1). Samples are from patients in maintenance phase of therapy that were collected with a median [interquartile range] of 61,2 [30,7-119] and 28,7 [16,3-54,9] months after infliximab and adalimumab initiation, respectively. Disease activity was defined according to PGA as active or inactive and was recorded in our electronic clinical information system. Response to biologics was defined using PGA of disease activity, biomarkers (CRP and calprotectin) and in some cases imaging and/or endoscopy. Those patients that relapsed during maintenance therapy and thus needed to increase drug dosages; shorten intervals, associate immunosuppressants to therapy or swap class of drug (such as vedolizumab or ustekinumab), were classified as SLR. SLR was defined according to PGA as worsening of symptoms after an initial response to infliximab or adalimumab and raised CRP, calprotectin and/or image activity and/or endoscopic activity. Patients who initiated anti-TNF and develop serious adverse events (SAE), were defined as those patients developing any acute or delayed infusion reaction necessitating anti-TNF discontinuation.

Sample preparation and IgG glycosylation analysis

Immunoglobulin G (IgG) was isolated from a human plasma using affinity chromatography on protein G as described previously (24). Briefly, human plasma (100 uL) was diluted seven times with 1X PBS and loaded onto Protein G (BIA Separations, Ljubljana, Slovenia). Bound IgG was washed with 1X PBS and eluted using a 0.1 M formic acid. Eluted protein was immediately neutralized with 1 M ammonium bicarbonate. IgG was trypsinized to obtain glycopeptides which were purified using solid phase extraction as described previously (25). In short, 200 ng of trypsin (Promega, Fitchburg, WI, USA) was added to 20 µg of IgG and incubated overnight at 37°C. Glycopeptides were purified on Chromabond C-18 stationary phase (Marcherey-Nagel, Düren, Germany). Samples loaded to stationary phase were washed three times with 0.1 % TFA and finally eluted using 20 % ACN. Samples were immediately dried completely using a vacuum centrifuge. Separation and measurements were performed using liquid chromatography coupled to Compact mass spectrometer

(Bruker Daltonics, Bremen, Germany). IgG Fc glycopeptides were separated on nanoACQUITY system (Waters, Milford, MA, USA) which was coupled to Compact using Apollo ion source (Bruker) equipped CE sprayer (Agilent, Santa Clara, CA, USA) or on ACQUITY M-class (Waters, Milford, MA, USA). M-class instrument was coupled to Compact using CaptiveSpray ion source equipped with NanoBooster (Bruker). Acetonitrile was introduced into nitrogen flow to increase ionisation of glycopeptides. Samples were reconstituted in 20 µL of ultrapure water and 4 µL were loaded onto Acclaim PepMap100 C8 (5 mm×300 µm i.d.) trap column (Thermo Fisher Scientific, Waltham, MA). On the trap column, glycopeptides were washed 1 min with 0.1% TFA (solvent A) at a flow rate of 40 µL/min. Separation of IgG subclasses was based on differences in their tryptic peptide backbone and was performed on a Halo C18 nano-LC column (150 mm×75 µm i.d., 2.7 µm HALO fused core particles; Advanced Materials Technology, Wilmington, DE, USA) in a 3.5 min gradient from 18% to 28% solvent B (80% ACN). Flow rate was 1 µL/min and column temperature was 30°C. Mass spectra were recorded with 2 averages at a frequency of 0.5 Hz in a mass range from m/z 800 to m/z 2000. Collision energy and ion energy were set at 5 eV. Argon was used as collision gas. M-class ACQUITY UPLC system was operated under MassLynx software version 4.1 (Waters) while Compact and nanoACQUITY were controlled by HyStar software, version 4.2 (Bruker). With use of MSConvert tool (ProteoWizard version 3), all raw data was converted into mzXML file format prior data extraction with LacyTools version 1.0.1. Relative intensities of extracted glycopeptide were calculated for each IgG subclass and correction for batch effects was performed. Quality control resulted in 19 IgG1 glycoforms, 20 IgG2&3 glycoforms and 18 IgG4 glycoforms. Derived glycan traits describing levels of agalactosylation, monogalactosylation, digalactosylation, sialylation, bisecting GlcNAc and core fucosylation were calculated for each IgG subclass (Table S2).

Statistical Analysis

Glycosylation profiles were log2 transformed to eliminate the skewness in the data. Consensus clustering was performed in order to identify group of samples with similar glycosylation profiles. Consensus clustering was performed based on K-means algorithm via the bioconductor package *ConsensusClusterPlus* (26). Each glycan abundance was first normalized to z-score. Then, glycans were modeled as function of different clusters via a linear model. P-values were adjusted for multiple comparison via Benjamini-Hochberg correction. Kaplan Meier curve was performed to determine the influence of glycans on the loss of response considering time (days) after therapy initiation. Analyses were performed separately in terms of anti-TNF therapy (adalimumab versus infliximab).

Results

The IgG-Fc glycome is able to stratify CD patients accordingly with clinical and therapeutic features

Considering the different patients' response to infliximab and adalimumab, we split the CD cohort into patients under infliximab or patients under adalimumab. The distribution of IgG glycosylation in our cohort (Table 1 and Table S1), both in patients under infliximab or adalimumab, suggests 3 main clusters (Figure 1A and Figure 2A) containing specific IgG glycosylation fingerprint. Interestingly, one cluster (the cluster 3; Figure 1A) from samples in patients under infliximab is predominantly composed by secondary non-responders to infliximab (Figure 1B). Samples from this cluster are all from males, who are older than the other two clusters (Figure 1B) and display higher levels of calprotectin and CRP (Figure 1C). Glycan distribution of this cluster contains lower proportion of galactosyled Fc in all subclasses (Monogalactosylated in IgG1 and IgG2&3; Digalactosylated in all IgG subclasses; Figure 1A) compared with the other clusters, together with lower sialylation in all IgG subclasses (predominantly in IgG1; Figure 1A). It is also evident a higher proportion of agalactosylated and fucosylated in all Fc IgG subclasses (with exception of fucosylation in IgG2&3; Figure 1A).

The constructed clusters for adalimumab therapy are similarly distributed regarding therapy response (Figure 2B). However, the cluster 1 that is mainly composed by the same Fc glycosylation profile of IgG1 and IgG2&3 observed in cluster 3 from patients under infliximab (low proportion of galactosylation and sialylation of Fc IgG1 and IgG2&3; Figure 2A), also contains older patients and higher values of CRP (Figure 2C) than the other clusters. This cluster is also composed high proportion of bisecting GlcNAc of Fc of all IgG subclasses (Figure 2A).

These results suggest a promisor association of low galactosylation and sialylation of Fc IgG1 and IgG2&3 with age and CRP levels, as well as with loss of response to adalimumab.

Differences in IgG-Fc glycosylation are associated with loss of response to anti-TNF therapy with a higher performance than C-Reactive Protein and calprotectin

In order to evaluate the overall capacity of IgG-Fc glycosylation in distinguishing samples of responders *versus* SNR, further comparing this potential biomarker with other current biomarkers such as CRP and calprotectin, we plotted a ROC curve (Figure 3). The analyses were split into patients under infliximab and under adalimumab. Interestingly, the overall performance of IgG Fc glycosylation in relationship with loss of response to anti-TNF therapy is better for infliximab than for adalimumab (AUC~0.8 vs AUC~0.6; Figure 3). This

was observed for each subclass-specific IgG glycopeptides considering the different glycan traits: agalactosylation, bisecting, fucosylation and sialylation (Figure 3B).

In the case of adalimumab therapy, IgG glycosylation displays a much higher efficacy in the association with loss of response to the drug (AUC-0.91; Figure 3A) than CRP and calprotectin together (AUC – 0.57; Figure 3A). This higher performance of IgG glycans is more evident in agalactosylation (AUC – 0.74; Figure 3B), bisecting (AUC – 0.77; Figure 3B) and fucosylation (AUC – 0.77; Figure 3B) profiles of IgG4 (Figure 3B) and the differences are even higher when considering all glycans traits from all IgG subclasses (AUC – 0.84; Figure 3A).

Overall, IgG glycosylation is able to distinguish samples from SNR to anti-TNF therapy displaying higher performance that CRP and calprotectin (in the case of adalimumab).

The correlation of IgG glycosylation with anti-TNF therapy response is maintained along time and disease course

Kaplan Meier curves were plotted to evaluate the profile of IgG Fc glycosylation over time (days after therapy initiation) according with the loss of response to anti-TNF therapy. The results showed that the association of specific IgG Fc glycan patterns with response to anti-TNF therapy appears to be maintained along time (Figure 3 and Figure S1-3). The results also showed that, in patients under infliximab, low levels (red lines in figure 3) of galactosylation in all IgG subclasses are associated with patients that lost response earlier than the ones with high levels (blue lines) of galactosylation. As example, at 3000 days after therapy initiation the probability to identify and stratify a SNR is higher if plasma IgG is agalactosylated (in all IgG subclasses; Figure 3). The same holds true for low levels of sialylation (in all IgG subclasses), low bisecting (IgG2&3 and IgG4) and high fucosylation (IgG1 and IgG4).

On the contrary, for adalimumab, we observed (Figure 3) that plasma with high proportion of galactosylated Fc glycans (in all IgG subclasses), high bisecting (IgG2&3 and IgG4) and low fucosylation (IgG4; except in IgG1 that is significantly increased) are mostly associated with a SNR over time. For both drugs, and at a given time, the probability to identify SNR is higher when IgGs display low levels of sialylation (IgG2&3 and IgG4). Altogether, we found that the Fc glycans´ profile of plasma IgG can be printing the clinical response to anti-TNF drugs, having the ability to distinguish responders and non-responders in a constant way, over time and along disease course and differently in the case of infliximab or adalimumab.

Discussion

Immunoglobulin G (IgG) antibodies are the predominant antibody class in circulation, being a key effector of the humoral immune system by triggering leukocyte activation and inflammation (27). The diverse type of glycans structures in the Fc region of IgG is responsible for modulating cellular response through interaction with FcγRs in immune cells. Decreased galactosylation of IgG is associated with a pro-inflammatory IgG function and was observed in IBD patients in comparison with healthy individuals (19-21, 28, 29). Nevertheless, whether this IgG glycome heterogeneity among individuals translates to a better stratification of patients accordingly with their response to biologics therapy in IBD remains completely unknown.

In this study we demonstrated that differences in plasma Fc IgG glycosylation profiles of CD patients are able to distinguish responders from patients that lost response to anti-TNF therapy, with higher accuracy than CRP and calprotectin together (predominantly for adalimumab), current markers used in IBD therapeutic management (30, 31). Different profiles of glycans on IgG Fc were correlated with SLR to infliximab or adalimumab in a drug-specific manner and in a stable manner along time and disease course.

We showed that samples from patients that lose response to anti-TNF therapy exhibited decreased levels of sialylation in all IgGs (with the exception of IgG1 for adalimumab) which is in accordance with a pro-inflammatory function of low sialylation on IgGs. In fact, the presence of sialylation promotes the anti-inflammatory activity of IgG by reducing antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) and increasing the activation threshold of innate effector cells to immune complexes by stimulating the upregulation of the inhibitory Fcγ receptor IIB (FcγRIIB) (18, 32). Furthermore, SNR to infliximab also presented lower levels of galactosylation comparing with the responders. Low galactosylated IgG was already observed in CD patients with active disease displaying problems in maintaining long-term remission and was also correlated with CRP (33, 34).

Our results further showed that low levels of galactose and sialic acid on plasma Fc IgG is associated with loss of response to infliximab in a cooperative way. This is in accordance with the pro-inflammatory effects of agalactosylated IgG through increased antibody-dependent phagocytosis (35) and increased pathogenicity of antibodies (36-39).

Our results also suggest that the capacity of IgG glycome to identify SNR appear to be higher in infliximab than in adalimumab. Nevertheless, SLR to adalimumab were significantly correlated with afucosylation of Fc IgG that is known to potentiate the enhancement of ADCC (40, 41). The presence of bisecting N-glycans, that also confer a pro-inflammatory effect on IgG by enhancing ADCC (42, 43), was also observed in

adalimumab scenario, as well as in patients under infliximab. These results suggest that the association of the profile of Fc IgG glycosylation with response to biologics therapy is specific and dependent on the type of anti-TNF drug.

Taken together, our results reveal a potential novel plasma biomarker able to distinguish responders *versus* SNR to anti-TNF. The validation of the performance of this glycomic plasma biomarker in a prospective study and in a larger cohort that includes samples collected at defined and synchronized time-points of therapy induction and maintenance phase is worth and needed, envisioning the integration of this biomarker in therapy decision and proactive TDM in IBD.

If validated, we are in front of a new and minimally invasive plasma biomarker with the capacity to identify patients that will fail to respond to anti-TNF therapy and thus benefiting, early in disease course, from other alternative therapies. This will improve the personalized medicine and a tailored therapeutic management in IBD.

References

1. Billiet T, Rutgeerts P, Ferrante M, Van Assche G, Vermeire S. 2014. Targeting TNF-alpha for the treatment of inflammatory bowel disease. *Expert Opin Biol Ther* 14: 75-101
2. Danese S, Vuitton L, Peyrin-Biroulet L. 2015. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 12: 537-45
3. Coskun M, Vermeire S, Nielsen OH. 2017. Novel Targeted Therapies for Inflammatory Bowel Disease. *Trends Pharmacol Sci* 38: 127-42
4. Verstockt B, Ferrante M, Vermeire S, Van Assche G. 2018. New treatment options for inflammatory bowel diseases. *J Gastroenterol* 53: 585-90
5. Roda G, Jharap B, Neeraj N, Colombel JF. 2016. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 7: e135
6. Ben-Horin S, Kopylov U, Chowers Y. 2014. Optimizing anti-TNF treatments in inflammatory bowel disease. *Autoimmun Rev* 13: 24-30
7. Vande Casteele N, Herfarth H, Katz J, Falck-Ytter Y, Singh S. 2017. American Gastroenterological Association Institute Technical Review on the Role of Therapeutic Drug Monitoring in the Management of Inflammatory Bowel Diseases. *Gastroenterology* 153: 835-57 e6
8. Komaki Y, Komaki F, Sakuraba A, Cohen R. 2016. Approach to Optimize Anti-TNF-alpha Therapy in Patients With IBD. *Curr Treat Options Gastroenterol* 14: 83-90
9. Papamichael K, Cheifetz AS, Melmed GY, Irving PM, Vande Casteele N, Kozuch PL, Raffals LE, Baidoo L, Bressler B, Devlin SM, Jones J, Kaplan GG, Sparrow MP, Velayos FS, Ullman T, Siegel CA. 2019. Appropriate Therapeutic Drug Monitoring of Biologic Agents for Patients With Inflammatory Bowel Diseases. *Clin Gastroenterol Hepatol* 17: 1655-68 e3
10. Pinho SS, Reis CA. 2015. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* 15: 540-55
11. Dias AM, Dourado J, Lago P, Cabral J, Marcos-Pinto R, Salgueiro P, Almeida CR, Carvalho S, Fonseca S, Lima M, Vilanova M, Dinis-Ribeiro M, Reis CA, Pinho SS. 2014. Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 23: 2416-27
12. Theodoratou E, Campbell H, Ventham NT, Kolarich D, Pucic-Bakovic M, Zoldos V, Fernandes D, Pemberton IK, Rudan I, Kennedy NA, Wuhrer M, Nimmo E, Annese V, McGovern DP, Satsangi J, Lauc G. 2014. The role of glycosylation in IBD. *Nat Rev Gastroenterol Hepatol* 11: 588-600

13. Verhelst X, Dias AM, Colombel JF, Vermeire S, Van Vlierberghe H, Callewaert N, Pinho SS. 2019. Protein Glycosylation as a Diagnostic and Prognostic Marker of Chronic Inflammatory Gastrointestinal and Liver Diseases. *Gastroenterology*
14. Anthony RM, Wermeling F, Ravetch JV. 2012. Novel roles for the IgG Fc glycan. *Ann N Y Acad Sci* 1253: 170-80
15. Lauc G, Pezer M, Rudan I, Campbell H. 2016. Mechanisms of disease: The human N-glycome. *Biochim Biophys Acta* 1860: 1574-82
16. Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D, Rademacher TW, Mizuochi T, Taniguchi T, Matsuta K, et al. 1985. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 316: 452-7
17. Parekh RB, Roitt IM, Isenberg DA, Dwek RA, Ansell BM, Rademacher TW. 1988. Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity. *Lancet* 1: 966-9
18. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313: 670-3
19. Simurina M, de Haan N, Vuckovic F, Kennedy NA, Stambuk J, Falck D, Trbojevic-Akmacic I, Clerc F, Razdorov G, Khon A, Latiano A, D'Inca R, Danese S, Targan S, Landers C, Dubinsky M, Inflammatory Bowel Disease Biomarkers C, McGovern DPB, Annese V, Wuhrer M, Lauc G. 2018. Glycosylation of Immunoglobulin G Associates With Clinical Features of Inflammatory Bowel Diseases. *Gastroenterology* 154: 1320-33 e10
20. Trbojevic Akmacic I, Ventham NT, Theodoratou E, Vuckovic F, Kennedy NA, Kristic J, Nimmo ER, Kalla R, Drummond H, Stambuk J, Dunlop MG, Novokmet M, Aulchenko Y, Gornik O, Campbell H, Pucic Bakovic M, Satsangi J, Lauc G, Consortium I-B. 2015. Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome. *Inflamm Bowel Dis* 21: 1237-47
21. Clerc F, Novokmet M, Dotz V, Reiding KR, de Haan N, Kammeijer GSM, Dalebout H, Bladergroen MR, Vukovic F, Rapp E, Consortium I-B, Targan SR, Barron G, Manetti N, Latiano A, McGovern DPB, Annese V, Lauc G, Wuhrer M. 2018. Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. *Gastroenterology* 155: 829-43
22. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, Maia L, Delerue-Matos C, Taniguchi N, Lima M, Pedroto I, Marcos-Pinto R, Lago P, Reis CA, Vilanova M, Pinho SS. 2018. Metabolic

control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 115: E4651-E60

23. Pereira MS, Maia L, Azevedo LF, Campos S, Carvalho S, Dias AM, Albergaria A, Lima J, Marcos-Pinto R, Lago P, Pinho SS. 2019. A [Glyco]biomarker that Predicts Failure to Standard Therapy in Ulcerative Colitis Patients. *J Crohns Colitis* 13: 39-49
24. Pucic M, Knezevic A, Vidic J, Adamczyk B, Novokmet M, Polasek O, Gornik O, Supraha-Goreta S, Wormald MR, Redzic I, Campbell H, Wright A, Hastie ND, Wilson JF, Rudan I, Wuhrer M, Rudd PM, Josic D, Lauc G. 2011. High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. *Mol Cell Proteomics* 10: M111 010090
25. Keser T, Vuckovic F, Barrios C, Zierer J, Wahl A, Akinkuolie AO, Stambuk J, Nakic N, Pavic T, Perisa J, Mora S, Gieger C, Menni C, Spector TD, Gornik O, Lauc G. 2017. Effects of statins on the immunoglobulin G glycome. *Biochim Biophys Acta Gen Subj* 1861: 1152-8
26. Wilkerson MD, Hayes DN. 2010. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. *Bioinformatics* 26: 1572-3
27. Vidarsson G, Dekkers G, Rispens T. 2014. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 5: 520
28. Miyoshi E, Shinzaki S, Fujii H, Iijima H, Kamada Y, Takehara T. 2016. Role of aberrant IgG glycosylation in the pathogenesis of inflammatory bowel disease. *Proteomics Clin Appl* 10: 384-90
29. Shinzaki S, Kuroki E, Iijima H, Tatsunaka N, Ishii M, Fujii H, Kamada Y, Kobayashi T, Shibukawa N, Inoue T, Tsujii M, Takeishi S, Mizushima T, Ogata A, Naka T, Plevy SE, Takehara T, Miyoshi E. 2013. Lectin-based immunoassay for aberrant IgG glycosylation as the biomarker for Crohn's disease. *Inflamm Bowel Dis* 19: 321-31
30. Lopetuso LR, Gerardi V, Papa V, Scaldaferri F, Rapaccini GL, Gasbarrini A, Papa A. 2017. Can We Predict the Efficacy of Anti-TNF-alpha Agents? *Int J Mol Sci* 18
31. Magro F. 2019. Anti-TNF treatment failure: drug levels, immunogenicity, or both? *Lancet Gastroenterol Hepatol* 4: 326-7
32. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, Wang LX, Munz C, Nimmerjahn F, Dalakas MC, Lunemann JD. 2015. Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J Clin Invest* 125: 4160-70
33. Dube R, Rook GA, Steele J, Brealey R, Dwek R, Rademacher T, Lennard-Jones J. 1990. Agalactosyl IgG in inflammatory bowel disease: correlation with C-reactive protein. *Gut* 31: 431-4

34. Shinzaki S, Iijima H, Nakagawa T, Egawa S, Nakajima S, Ishii S, Irie T, Kakiuchi Y, Nishida T, Yasumaru M, Kanto T, Tsujii M, Tsuji S, Mizushima T, Yoshihara H, Kondo A, Miyoshi E, Hayashi N. 2008. IgG oligosaccharide alterations are a novel diagnostic marker for disease activity and the clinical course of inflammatory bowel disease. *Am J Gastroenterol* 103: 1173-81

35. Nakajima S, Iijima H, Shinzaki S, Egawa S, Inoue T, Mukai A, Hayashi Y, Kondo J, Akasaka T, Nishida T, Kanto T, Morii E, Mizushima T, Miyoshi E, Tsujii M, Hayashi N. 2011. Functional analysis of agalactosyl IgG in inflammatory bowel disease patients. *Inflamm Bowel Dis* 17: 927-36

36. Rademacher TW, Williams P, Dwek RA. 1994. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *Proc Natl Acad Sci U S A* 91: 6123-7

37. Karsten CM, Pandey MK, Figge J, Kilchenstein R, Taylor PR, Rosas M, McDonald JU, Orr SJ, Berger M, Petzold D, Blanchard V, Winkler A, Hess C, Reid DM, Majoul IV, Strait RT, Harris NL, Kohl G, Wex E, Ludwig R, Zillikens D, Nimmerjahn F, Finkelman FD, Brown GD, Ehlers M, Kohl J. 2012. Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcgammaRIIB and dectin-1. *Nat Med* 18: 1401-6

38. Ito K, Furukawa J, Yamada K, Tran NL, Shinohara Y, Izui S. 2014. Lack of galactosylation enhances the pathogenic activity of IgG1 but Not IgG2a anti-erythrocyte autoantibodies. *J Immunol* 192: 581-8

39. Dong X, Storkus WJ, Salter RD. 1999. Binding and uptake of agalactosyl IgG by mannose receptor on macrophages and dendritic cells. *J Immunol* 163: 5427-34

40. Gasdaska JR, Sherwood S, Regan JT, Dickey LF. 2012. An afucosylated anti-CD20 monoclonal antibody with greater antibody-dependent cellular cytotoxicity and B-cell depletion and lower complement-dependent cytotoxicity than rituximab. *Mol Immunol* 50: 134-41

41. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277: 26733-40

42. Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M. 2001. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. *Biotechnol Bioeng* 74: 288-94

43. Zou G, Ochiai H, Huang W, Yang Q, Li C, Wang LX. 2011. Chemoenzymatic synthesis and Fcgamma receptor binding of homogeneous glycoforms of antibody

Fc domain. Presence of a bisecting sugar moiety enhances the affinity of Fc to FcgammaIIIa receptor. *J Am Chem Soc* 133: 18975-91

Figures

Figure 1

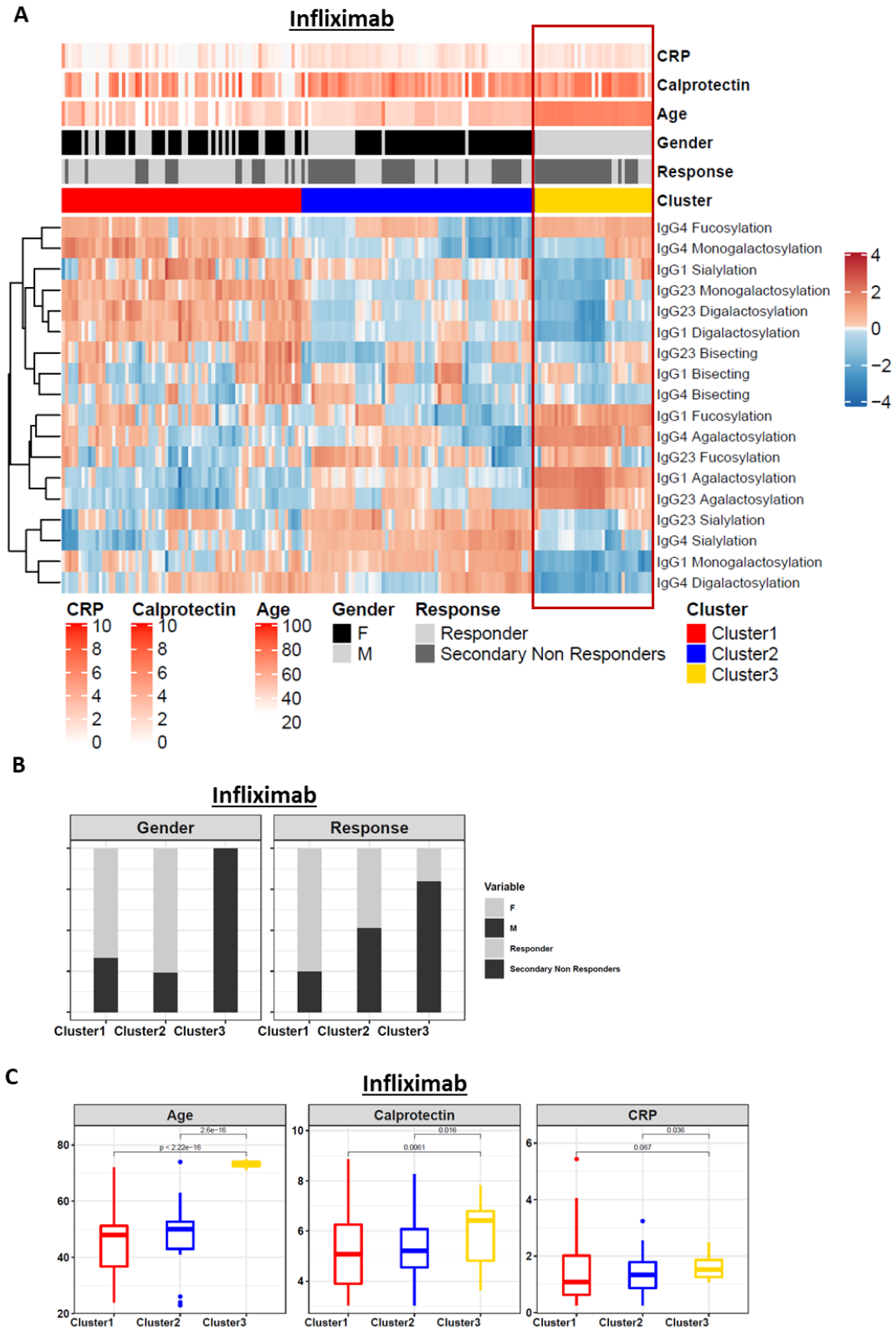


Figure 1_(A) Heatmap illustrating glycan profiles for different clusters in samples from patients under *Infliximab*. The seven tracks on the top annotate clusters identified by consensus clustering (Cluster), response to biologics (Response), maintenance (Maintenance), gender (Gender), age (Age), Calprotectin in log(x+1) scale (Calprotectin)and CRP in log(x+1) scale (CRP). (B) Proportion of Female/Male and proportion of Responder, Loss of Response and Non-Responder for different clusters. (C) Distribution of Age, Calprotectin in log(x+1) scale and CRP in log(x+1) scale for different clusters. P-values from wilcoxon test are reported.

Figure 2

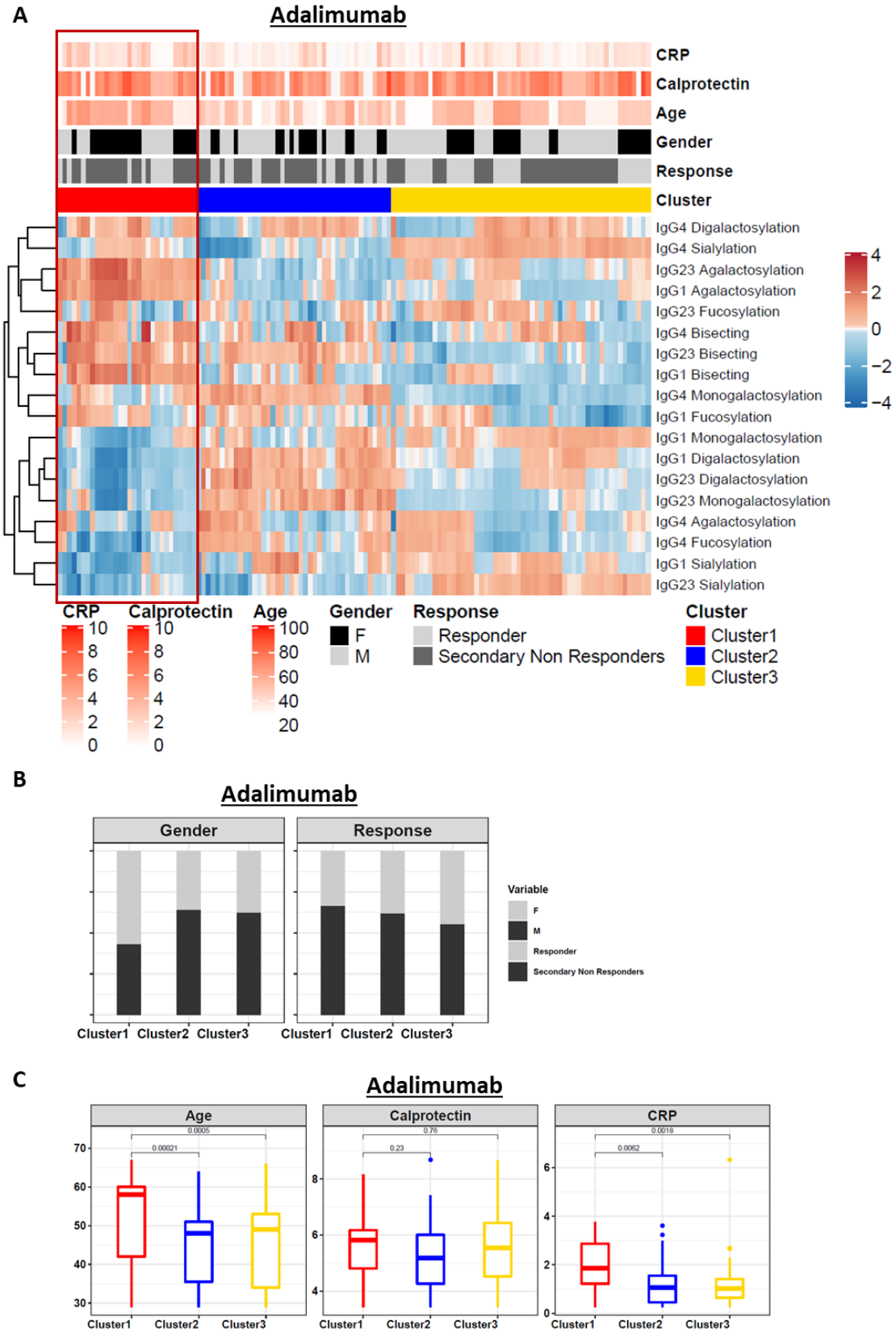


Figure 2_(A) Heatmap illustrating glycan profiles for different clusters in samples from patients under *Adalimumab*. The seven tracks on the top annotate clusters identified by

consensus clustering (Cluster), response to biologics (Response), maintenance (Maintenance), gender (Gender), age (Age), Calprotectin in log(x+1) scale (Calprotectin)and CRP in log(x+1) scale (CRP). (B) Proportion of Female/Male and proportion of Responder, Loss of Response and Non-Responder for different clusters. (C) Distribution of Age, Calprotectin in log(x+1) scale and CRP in log(x+1) scale for different clusters. P-values from wilcoxon test are reported.

Figure 3

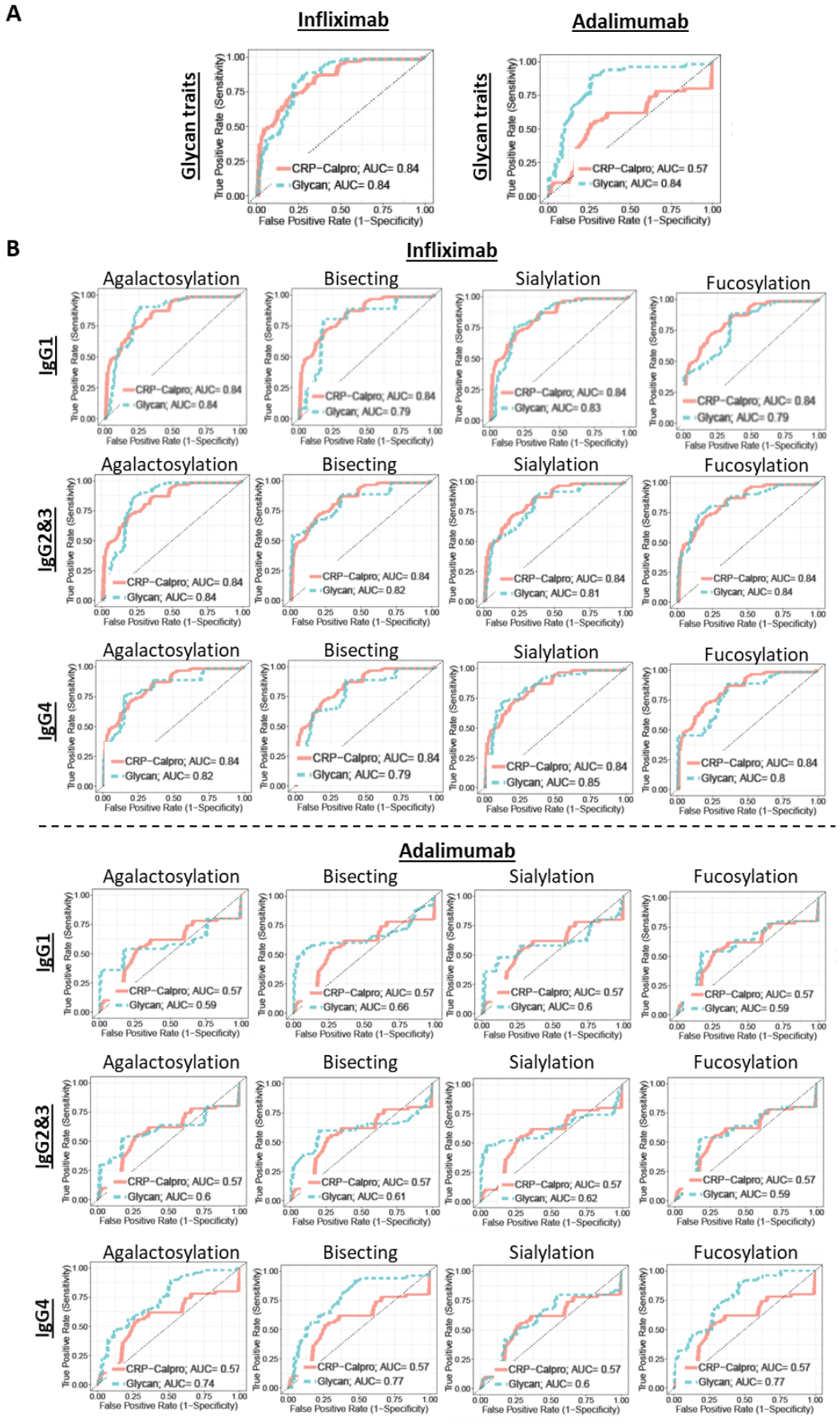

A
Infliximab
Adalimumab
Glycan traits
True Positive Rate (Sensitivity)
False Positive Rate (1−Specificity)
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.84
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.84
B
Infliximab
Agalactosylation
Bisecting
Sialylation
Fucosylation
IgG1
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.84
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.79
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.83
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.79
IgG2&3
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.84
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.82
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.81
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.84
IgG4
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.82
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.79
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.85
CRP−Calpro; AUC= 0.84
Glycan; AUC= 0.8
Adalimumab
IgG1
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.59
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.66
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.6
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.59
IgG2&3
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.6
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.61
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.62
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.59
IgG4
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.74
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.77
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.6
CRP−Calpro; AUC= 0.57
Glycan; AUC= 0.77

Figure 3_(A) Overall capacity of the combined Fc IgG glycans for loss of response to infliximab and adalimumab, comparing with C-reactive protein (CRP) and calprotectin. The association is showed for the combination of all glycan traits (all directly measured glycans for each IgG subtype have been taken into account). (B) Overall capacity of the different types of glycosylation (agalactosylation, bisecting, sialylation and fucosylation) in Fc IgG (per IgG subtype) to correlate with loss of response to infliximab and adalimumab. Area under the curve (AUC) of the plotted ROC curves are highlighted.

Figure 4

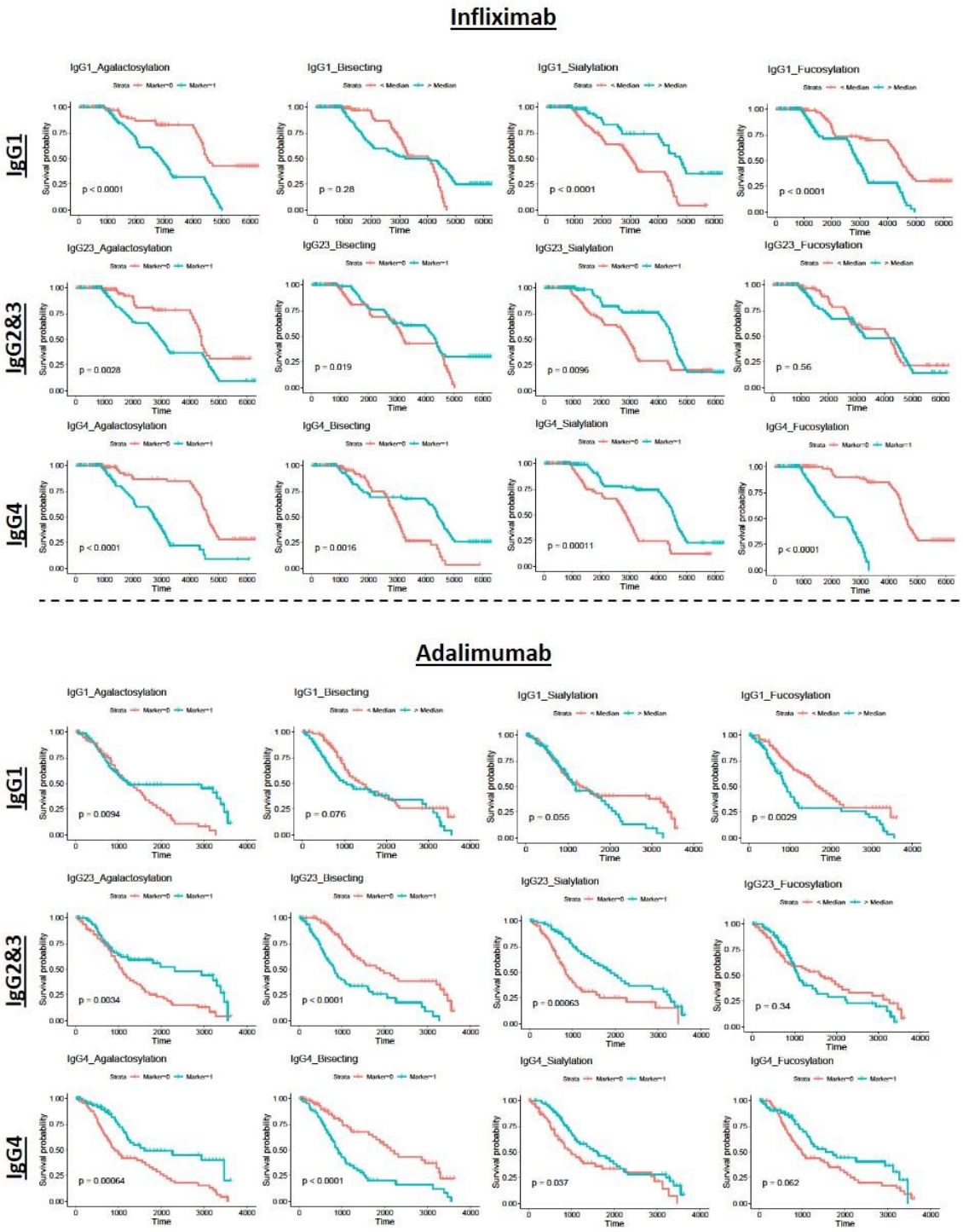


Figure 4_Kaplan Meier curves shows the profile of glycosylation of the IgGs during time (days) of therapy, in which the event is the loss of response to infliximab or adalimumab. Each glycan type has been stratified based on median value of each glycan profile proportion (lower than the median is scored as 0 – colored in red - and higher than the median is scored as 1 – colored in blue). In red are samples presenting lower levels of the median and in blue the ones with higher levels. For infliximab, 177 samples from 40 patients

were included (25 patients have only sample); For infliximab, 128 samples from 22 patients were included (7 patients have only sample). Time is at days after therapy initiation.

Supplementary Figures

Figure S1

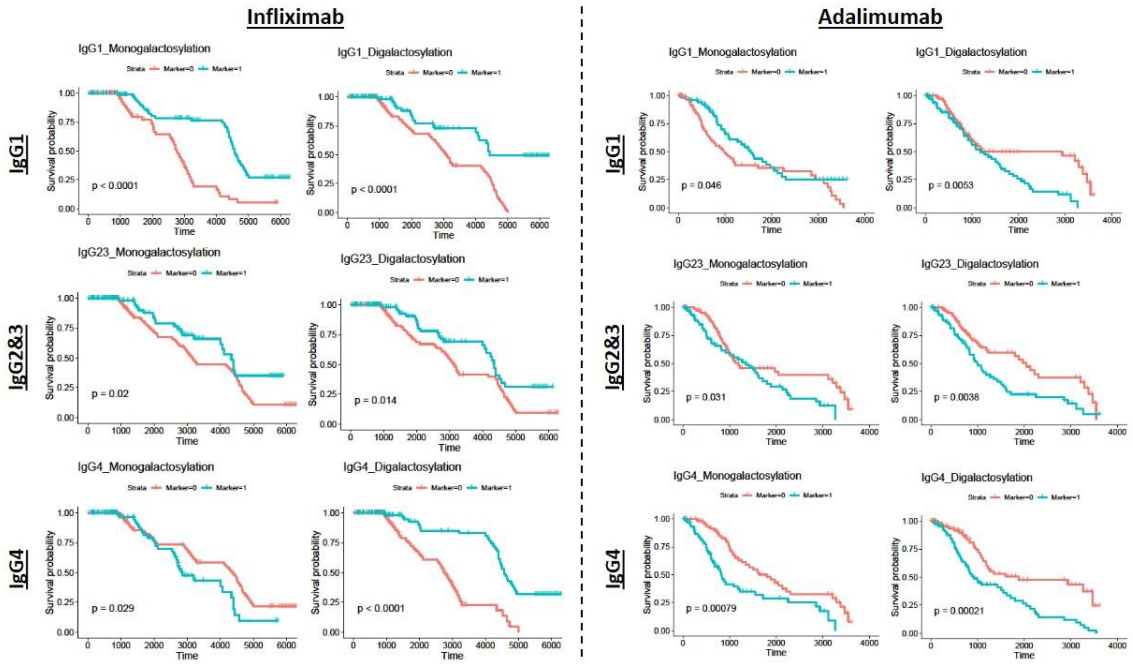


Figure S1_Kaplan Meier curves showing the profile of mono- and digalactosylation in Fc IgGs during time (days) of therapy, in which the event is the loss of response to infliximab or adalimumab. Each glycan type has been stratified based on median value of each glycan profile proportion (lower than the median is scored as 0 – colored in red - and higher than the median is scored as 1 – colored in blue). In red are samples presenting lower levels of the median and in blue the ones with higher levels. For infliximab, 177 samples from 40 patients were included (25 patients have only sample); For infliximab, 128 samples from 22 patients were included (7 patients have only sample). Time is at days after therapy initiation.

Figure S2

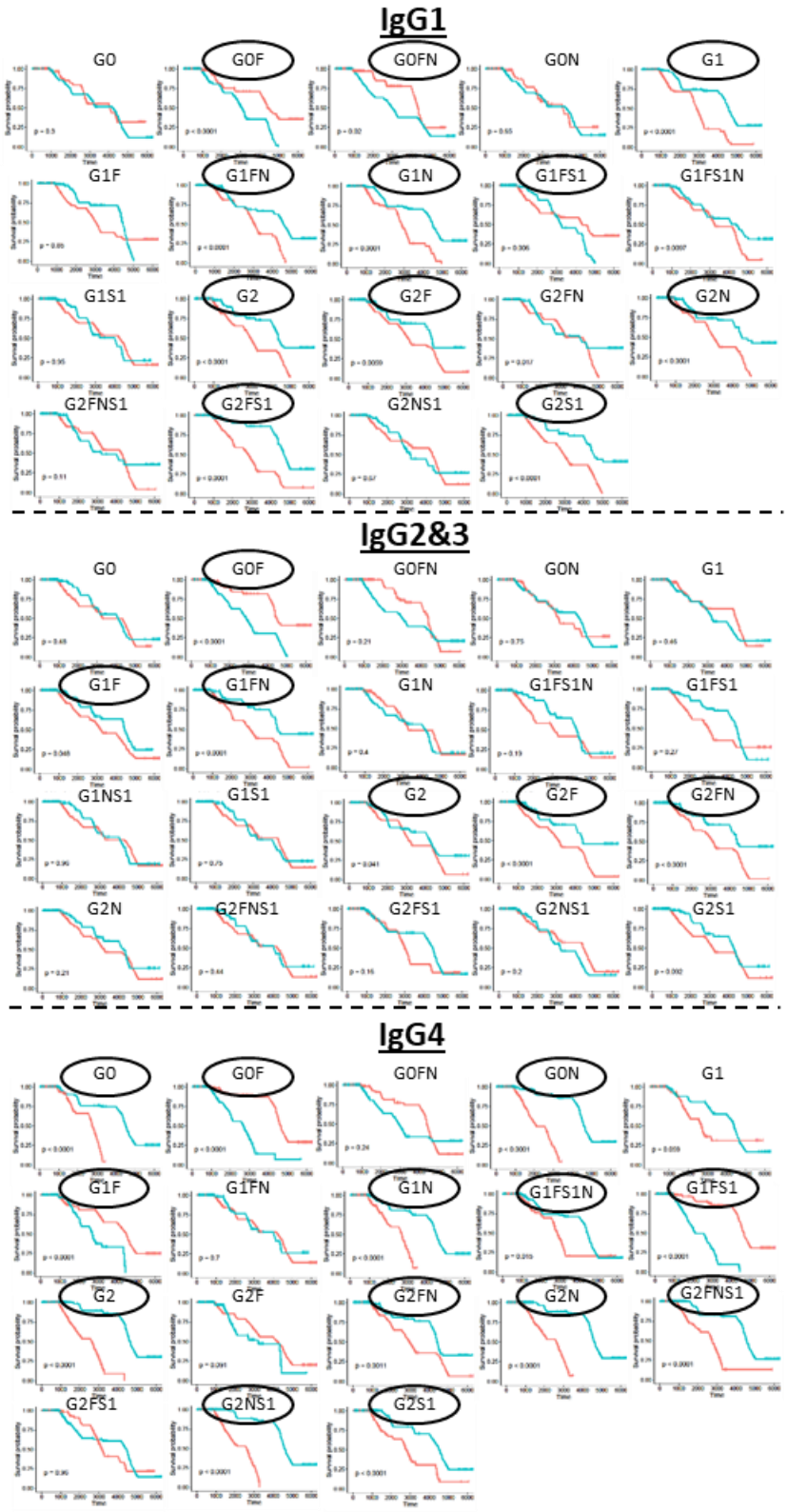


Figure S2_Kaplan Meier curves showing the glycan profile traits of Fc IgGs during time (days) of *infliximab*, in which the event is the loss of response to the therapy. Each glycan trait has been stratified based on median of the intensity value (lower than the median is

colored in red and higher than the median in blue). In red are samples presenting lower levels of the median and in blue the ones with higher levels. For infliximab, 177 samples from 40 patients were included (25 patients have only sample); For infliximab, 128 samples from 22 patients were included (7 patients have only sample). Time is at days after therapy initiation.

Figure S3

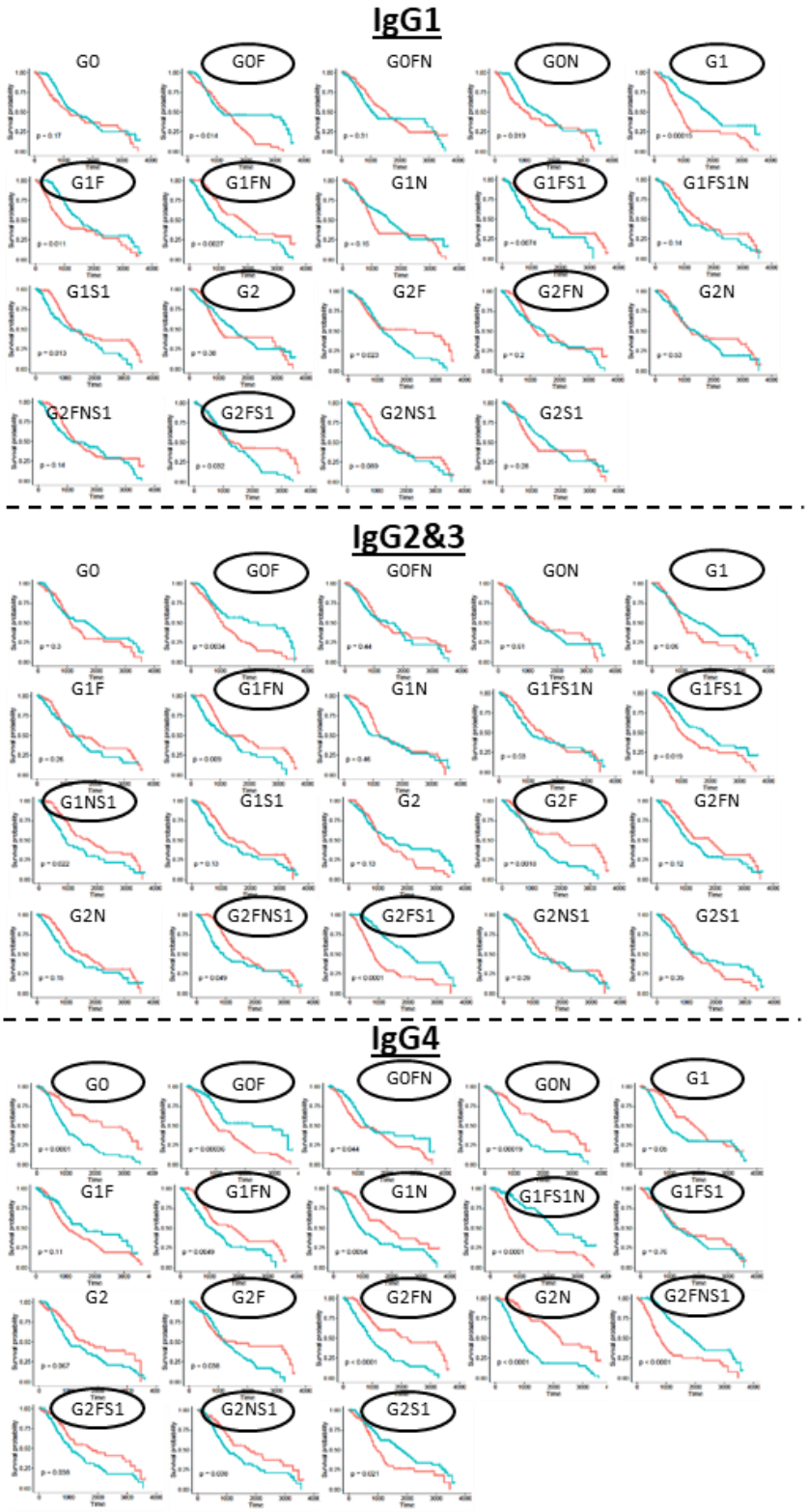


Figure S3_ Kaplan Meier curves showing the glycan profile traits of Fc IgGs during time (days) of *adalimumab*, in which the event is the loss of response to the therapy. Each glycan trait has been stratified based on median of the intensity value (lower than the median is

colored in red and higher than the median in blue). In red are samples presenting lower levels of the median and in blue the ones with higher levels. For infliximab, 177 samples from 40 patients were included (25 patients have only sample); For infliximab, 128 samples from 22 patients were included (7 patients have only sample). Time is at days after therapy initiation.

Tables

Table 1. Characterization of the Crohn´s disease (CD) cohort in terms of clinical and biochemical parameters. All samples are from CD patients under infliximab or adalimumab in maintenance phase. The cohort includes 177 samples from 40 patients under infliximab and 128 samples from 22 patients under adalimumab. The samples from CD patients were collected at different time points during maintenance therapy. With the exception of 2 patients, all secondary non-responders entry after losing response to the therapy. All information was retrieved at the time of sample collection. The time since diagnosis until sample collection is referred in years of follow-up, and the time since infliximab or adalimumab initiation is referred in months after therapy. Physician's global assessment (PGA) was used to determine disease activity using the cut-off of 4. Patients were classified as responders or secondary non-responders to the biologic therapy that are subjected at the time of sample collection.

		Infliximab				*Adalimumab*			
		Patients (N= 40)		***Samples (N= 177)***		***Patients (N= 22)***		***Samples (N= 128)***	
		n (%)	**Median [IQR]**	**n (%)**	**Median [IQR]**	**n (%)**	**Median [IQR]**	**n (%)**	**Median [IQR]**
Age (in years old)		40	43,5 [31-52]	177	50 [43-55]	22	47,5 [36,5-53,8]	128	49 [36-56]
Years of follow up		40	11 [4,3-18]	177	17,9 [6,9-19,5]	22	17,8 [11-24,1]	128	20,4 [14,8-24,5]
Months after therapy		40	29,2 [9,4-83,8]	177	61,2 [30,7-119]	22	18,9 [7,1-53,4]	140	28,7 [16,3-54,9]
Gender	F	20 (50)	-	101 (57,1)	-	13 (59,1)	-	53 (41,4)	-
	M	20 (50)	-	76 (42,9)	-	9 (40,9)	-	75 (58,6)	-
HBS index		17	2 [1-4]	151	2 [1-4]	17	2 [0-3,5]	135	2 [1-4]
Clinical symptoms	Remission	14 (35)	-	127 (71,8)	-	14 (63,6)	-	105 (82,0)	-
	Activity	3 (7,5)	-	24 (13,6)	-	3 (13,6)	-	18 (14,1)	-
	Missing data	23 (57,5)	-	26 (14,7)	-	5 (22,7)	-	5 (3,9)	-
Response to biologics	Responder	31 (77,5)	-	95 (53,7)	-	9 (40,9)	-	51 (39,8)	-
	Secondary Non Responder	9 (22,5)	-	82 (46,3)	-	13 (59,1)	-	77 (60,2)	-
CRP (mg/L)		16	1,64 [0,9-13,6]	146	2,97 [1,4-5,5]	16	2 [1-5,3]	131	2,2 [0,9-5,5]
Calprotectin (µg/g)		11	355 [51-760]	141	214 [72-605,5]	12	260 [107-710,1]	125	254,5 [96-554]

Supplementary Tables

Table S1_ Distribution of samples per patient and therapy.

	Infliximab		*Adalimumab*	
	Nº Samples	**Nº Patients**	**Nº Samples**	**Nº Patients**
	1	25	1	7
	2	4	2	1
	4	1	3	1
	5	1	6	1
	12	2	7	1
	13	1	8	3
	15	3	9	3
	16	1	10	3
	18	1	11	2
	19	1		
Total	177	40	128	22

Table S2_ Formula for the calculation of the glycan profile of each immunoglobulin G (IgG) subclass.

	Agalactosylation	**Monogalactosylation**	**Digalactosylation**	**Sialylation**	**Bisecting GlcNAc**	**Fucosylation**
IgG1	G0 + G0F + G0N + G0FN	G1 + G1F + G1N + G1FN	G2 + G2F + G2N + G2FN	G1S + G1FS + G1FNS + G2S + G2FS + G2FNS + G2NS	G0N + G0FN + G1N + G1FN + G1FNS + G2N + G2FN + G2FNS + G2NS	G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS
IgG2&3	G0 + G0F + G0N + G0FN	G1 + G1F + G1N + G1FN	G2 + G2F + G2N + G2FN	G1S + G1FS + G1FNS + G1NS + G2S + G2FS + G2FNS + G2NS	G0N + G0FN + G1N + G1FN + G1FNS + G1NS + G2N + G2FN + G2FNS + G2NS	G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS
IgG4	G0 + G0F + G0N + G0FN	G1 + G1F + G1N + G1FN	G2 + G2F + G2N + G2FN	G1FS + G1FNS + G2S + G2FS + G2FNS + G2NS	G0N + G0FN + G1N + G1FN + G1FNS + G2N + G2FN + G2FNS + G2NS	G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS

G – Galactose; **F** – Fucose; **N** – Bisecting; **S** – Sialic acid

Part II

Glycosylation of IgG as a blood biomarker to predict immunogenicity to anti-TNF in Crohn´s Disease patients.

[1,2]Márcia S Pereira, [3]Francesca Petralia, [4]Jerko Štambuk, [4]Elham Memarian, [4]Genadij Razdorov, [4]Mislav Novokmet, [4]Frano Vučković, [4]Irena Trbojevic-Akmacic, [5]Luís Maia, [1]Vanda Pinto, [1,6]Inês Alves, [5,2]Ricardo Marcos-Pinto, [5]Paula Lago, [4]Gordan Lauc, [7]Jean-Frederic Colombel, [1,6*]Salomé S. Pinho.

[1]Instituto de Investigação e Inovação em Saúde (I3S) / Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal
[2]Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal
[3]Department of Genetics and Genomic Sciences, Icahn Institute of Genomics and Multiscale Biology, Icahn School of Medicine at Mount Sinai New York, New York, USA
[4]Genos Glycoscience Research Laboratory, Zagreb, Croatia
[5]Department of Gastroenterology, Porto Centre Hospital, Porto, Portugal
[6]Faculty of Medicine, University of Porto, Porto, Portugal
[7]Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

Introduction

In the last decades, we are assisting the development of a significant variety of novel therapies targeting several pathways and molecules that have been revolutionizing the treatment of patients with Inflammatory Bowel Disease (IBD). Anti-tumor necrosis factor (anti-TNF) therapies remain the first line treatment in the majority of IBD patients. Nevertheless, almost half of the patients lose response over time (secondary loss of response (SLR)) or become intolerant to anti-TNFs, requiring alternative agents. The loss of response to anti-TNF can be accompanied by the formation of anti-drug antibodies due to immunogenicity issues related with the drug, being a major cause for treatment failure and non-response to therapy (1).

The mechanisms underlying formation of anti-drug antibodies is not fully understood. Recently, an association between HLA-DQA1*05 carriage and the development of antibodies against anti-TNF agents were reported (2).

Glycosylation is a highly-regulated post-translational mechanism characterized by the addition of carbohydrate structures (glycans) to proteins and lipids of essentially all cells. The diversity of the glycome (biological repertoire of glycans in an organism) is enormous reflecting their multiple biological functions in key cellular and molecular mechanisms of homeostasis and disease, including cancer and IBD (3-5).

Human immunoglobulin G (IgG) isolated from plasma is comprised of multiple glycoforms and different glycans variations have been detected on circulating IgGs in IBD patients (6). Whether this IgG glycome heterogeneity is associated with likelihood of developing immunogenicity to biologic therapies remains completely unknown.

The main aim of this study was to evaluate whether changes in plasma IgG glycosylation are correlated with the development of anti-drug antibodies to anti-TNF therapies and thus with the risk of developing immunogenicity.

Methods

We performed a glycoproteomics study to measure plasma IgG-Fc subclass-specific glycopeptides in Crohn´s disease (CD) patients under anti-TNF maintenance therapy and correlated our findings with the development of anti-drug antibodies and thus immunogenicity. This observational study includes 40 CD patients under therapy with infliximab (n=21) or adalimumab (n= 19) for a total of 251 blood samples that were analyzed at different time points during maintenance therapy. Samples were collected with a median [interquartile range] of 61 [31-119] and 29 [16-55] months after infliximab and adalimumab initiation, respectively. At each visit, blood samples were collected (at trough) just before the administration of the biologic. The serum infliximab or adalimumab drug levels and anti-drug antibody levels were analyzed by Elisa (LISA-Tracker, Theradiag which is able to detect antibodies in presence of the drug). Phenotypic characterization of the cohort is presented in Supplementary Table 1. Immunogenicity was defined as an anti-drug antibody (ADA) titer ≥ 9 µg/ml for infliximab and ≥ 4 µg/ml for adalimumab using a drug-tolerant enzyme-linked immunosorbent assay. Drug trough levels were measured and were considered adequate when being ≥ 3 µg/ml for infliximab and ≥ 5 µg/ml for adalimumab (7, 8). Using the algorithm for reactive therapeutic drug monitoring for patients with loss of response and based on measurements of anti-TNF levels and ADA, three groups of patients were classified accordingly: 1) patients with low levels of the drug and the presence of ADA that needed to be switched were classified as "**YES**"; 2) patients with low levels of the drug and negative for ADA (despite increase of anti-TNF dose and/or decrease of intervals between infusions), were classified as "**NO_a**"; 3) patients with adequate levels of the drug and negative for ADA were classified as "**NO_b**".

IgGs were isolated from human plasma using affinity chromatography on protein G and measurements were performed using liquid chromatography coupled to Compact mass spectrometer (Bruker Daltonics, Bremen, Germany) as described previously [6]. Quality control resulted in 19 IgG1 glycoforms, 20 IgG2&3 glycoforms and 18 IgG4 glycoforms. Derived glycan traits describing levels of agalactosylation, sialylation, bisecting GlcNAc and core fucosylation were calculated for each IgG subclass. The glycans profiles of Infliximab and Adalimumab were jointly analyzed. Specifically, each glycan abundance was first adjusted for covariates such as gender and age, as well as for drug therapy. In order to assess the association between immunogenicity issues and glycan profile, glycans profiles were modeled as a function of immunogenicity score via linear model. The p-values and Benjamini-Hochberg adjusted p-values are reported (Supplementary Table 2). Given that for Adalimumab more patients developed immunogenicity, we also report the association between glycans profile and immunogenicity specifically for Adalimumab alone. Similarly to the joint analysis, glycan profiles were first adjusted for covariates such as gender and age. Then, glycans profiles were modeled as a linear function of immunogenicity index.

Results

From n=40 CD patients under maintenance therapy with infliximab or adalimumab 37.5% of the patients had a loss of response to the drug. A total of 252 samples were analyzed and among them, 6/252 were classified as “YES”; 46/252 as “NO_a” and 200/252 as “NO_b” according to our definition above. IgG-Fc glycosylation profile was significantly associated with the development of anti-drugs antibodies (ADA) to infliximab and adalimumab (Figure 1; Supplementary Figure 1; Supplementary Table 1). Specifically, decreased levels of sialylation in IgG4 were associated with the development of immunogenicity to anti-TNF therapy. Moreover, increased levels of bisecting N-glycans together with low fucosylated glycans in IgG2&3 were associated with immunogenicity to anti-TNFs. The same was observed specifically for adalimumab (Supplementary Figure 1). An overall increased galactosylation of IgGs was also observed to be associated with the development of anti-drug antibodies to anti-TNF therapy (Figure 1).

Discussion

This is the first exploratory study revealing plasma IgG glycome as significantly associated with immunogenicity to anti-TNF therapy. We demonstrated an overall decrease on the complexity of *N*-glycans in plasma IgGs, verified by a decrease of terminal sialylation, decreased fucosylation and presence of bisecting N-glycans on IgG, significantly associated with the development of anti-drug antibodies to anti-TNF therapy.

We also showed that presence of galactose on Fc IgG is consistently associated with development of immunogenicity. The decrease on terminal sialylation and fucosylation associated with immunogenicity to anti-TNF therapy is in accordance with a pro-inflammatory function of low sialylation/fucosylation on IgGs (9, 10). Particularly, the absence of sialylation promotes the anti-inflammatory activity of IgG by promoting antibody-dependent cellular cytotoxicity (ADCC). Presence of sialylation in IgG stimulates the upregulation of the inhibitory Fcγ receptor IIB (FcγRIIB) (11). Moreover, the binding to activating FcγRIIIa was also shown to be enhanced with the presence of galactosylated IgGs inducing ADCC and complement mediated cytotoxicity (12-15). Taken together, this unique IgG glycome profile, mainly low proportion of sialylated and fucosylated glycoforms, as well as elevated GlcNAc bisecting and presence of galactose were found to be associated with the likelihood of developing immunogenicity.

In summary, changes in plasma IgG glycome may constitute a new non-invasive biomarker able to predict the development of immunogenicity to anti-TNF. Prospective studies on larger sample size are needed to evaluate whether the plasma IgG glycans profile of the patients is printed before the therapy initiation and if it is also associated with immunogenicity to other biologic drugs.

Figure 1

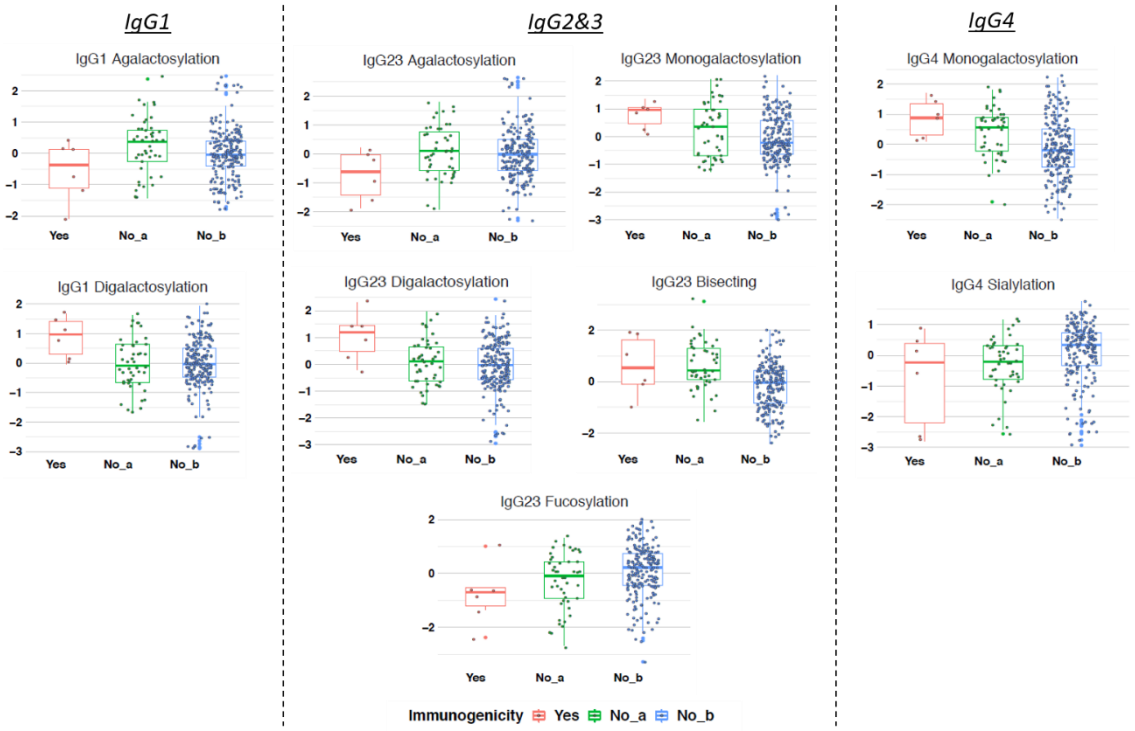


Figure 1_Glycosylation profile of IgG-Fc associated with the development of anti-drugs antibodies to infliximab and adalimumab in CD patients. Abundance adjusted by age, gender and drug therapy is shown. To detect association, glycans profile in Fc IgG subclass was modeled as function of immunogenicity after adjusting for covariates such as age and gender and type of drug via a linear model. In this analysis n=150 and n=102 samples for infliximab and adalimumab were analyzed jointly. Significance was considered with a p-value lower than 0.05 and FDR < 15%.

Supplementary Material

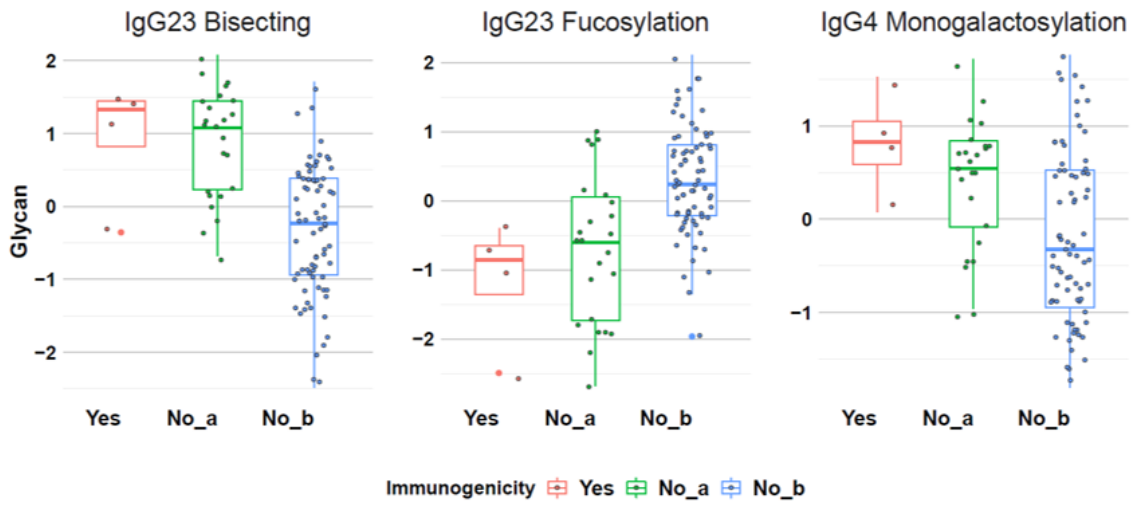
Methods

Sample preparation and IgG glycosylation analysis

Immunoglobulin G (IgG) was isolated from a human plasma using affinity chromatography on protein G as described previously (16). Briefly, human plasma (100 uL) was diluted seven times with 1X PBS and loaded onto Protein G (BIA Separations, Ljubljana, Slovenia). Bound IgG was washed with 1X PBS and eluted using a 0.1 M formic acid. Eluted protein was immediately neutralized with 1 M ammonium bicarbonate. IgG was trypsinized to obtain glycopeptides which were purified using solid phase extraction as described previously (17). In short, 200 ng of trypsin (Promega, Fitchburg, WI, USA) was added to 20 µg of IgG and incubated overnight at 37°C. Glycopeptides were purified on Chromabond C-18 stationary phase (Marcherey-Nagel, Düren, Germany). Samples loaded to stationary phase were washed three times with 0.1 % TFA and finally eluted using 20 % ACN. Samples were immediately dried completely using a vacuum centrifuge. Separation and measurements were performed using liquid chromatography coupled to Compact mass spectrometer (Bruker Daltonics, Bremen, Germany). IgG Fc glycopeptides were separated on nanoACQUITY system (Waters, Milford, MA, USA) which was coupled to Compact using Apollo ion source (Bruker) equipped CE sprayer (Agilent, Santa Clara, CA, USA) or on ACQUITY M-class (Waters, Milford, MA, USA). M-class instrument was coupled to Compact using CaptiveSpray ion source equipped with NanoBooster (Bruker). Acetonitrile was introduced into nitrogen flow to increase ionisation of glycopeptides. Samples were reconstituted in 20 µL of ultrapure water and 4 µL were loaded onto Acclaim PepMap100 C8 (5 mm×300 µm i.d.) trap column (Thermo Fisher Scientific, Waltham, MA). On the trap column, glycopeptides were washed 1 min with 0.1% TFA (solvent A) at a flow rate of 40 µL/min. Separation of IgG subclasses was based on differences in their tryptic peptide backbone and was performed on a Halo C18 nano-LC column (150 mm×75 µm i.d., 2.7 µm HALO fused core particles; Advanced Materials Technology, Wilmington, DE, USA) in a 3.5 min gradient from 18% to 28% solvent B (80% ACN). Flow rate was 1 µL/min and column temperature was 30°C. Mass spectra were recorded with 2 averages at a frequency of 0.5 Hz in a mass range from m/z 800 to m/z 2000. Collision energy and ion energy were set at 5 eV. Argon was used as collision gas. M-class ACQUITY UPLC system was operated under MassLynx software version 4.1 (Waters) while Compact and nanoACQUITY were controlled by HyStar software, version 4.2 (Bruker). With use of

MSConvert tool (ProteoWizard version 3), all raw data was converted into mzXML file format prior data extraction with LacyTools version 1.0.1. Relative intensities of extracted glycopeptide were calculated for each IgG subclass and correction for batch effects was performed. Quality control resulted in 19 IgG1 glycoforms, 20 IgG2&3 glycoforms and 18 IgG4 glycoforms. Derived glycan traits describing levels of *agalactosylation* (G0 + G0F + G0N + G0FN), *monogalactosylation* (G1 + G1F + G1N + G1FN), *digalactosylation* (G2 + G2F + G2N + G2FN), *sialylation* ([G1S + G1FS + G1FNS + G2S + G2FS + G2FNS + G2NS] for IgG1; [G1S + G1FS + G1FNS + G1NS + G2S + G2FS + G2FNS + G2NS] for IgG2&3; [G1FS + G1FNS + G2S + G2FS + G2FNS + G2NS] for IgG4), *bisecting N-acetylglucosamine* ([G0N + G0FN + G1N + G1FN + G1FNS + G2N + G2FN + G2FNS + G2NS] for IgG1 and IgG4 and [G0N + G0FN + G1N + G1FN + G1FNS + G1NS + G2N + G2FN + G2FNS + G2NS] for IgG2&3) and *core fucosylation* (G0F + G0FN + G1F + G1FN + G1FS + G1FNS + G2F + G2FN + G2FS + G2FNS) were calculated for each IgG subclass.

Supplementary Figure



Supplementary Figure 1_ Glycosylation profile of IgG-Fc associated with the development of anti-drugs antibodies to adalimumab in CD patients. Abundance adjusted by age and gender. To detect association, glycans profile in Fc IgG subclass was modeled as function of immunogenicity after adjusting for covariates such as age and gender via a linear model. In this analysis n=102 samples were analyzed. Significance was considered with a *p* value lower than 0.05 and FDR < 15%.

Supplementary Tables

Supplementary Table 1_ Characterization of the Crohn´s disease (CD) cohort in terms of clinical and biochemical parameters. All samples are from CD patients under infliximab or adalimumab in maintenance phase. The samples from CD patients were collected at different time points during maintenance therapy. All information was retrieved at the time of sample collection. The time since diagnosis until sample collection is referred in years of follow-up, and the time since infliximab or adalimumab initiation is referred in months after therapy. Physician's global assessment (PGA) was used to determine disease activity using the cut-off of 4. Patients were classified as responders or secondary non-responders to the biologic therapy that are subjected at the time of sample collection. Immunogenicity issues were classified as "Yes" – presence of anti-drug antibodies (ADA: > 9 µg/ml for infliximab and > 4 µg/ml for adalimumab) and low levels of the drug (<3 µg/ml for infliximab and 5 µg/ml for adalimumab); "No_a" - low levels of the drug and no presence of ADA; "No_b" – no presence of ADA and high levels of the drug.

		Patients under anti-TNF therapy		*Samples from 21 CD patients under infliximab*		*Samples from 19 CD patients under adalimumab*	
		N (%)	**Median [IQR]**	**N (%)**	**Median [IQR]**	**N (%)**	**Median [IQR]**
Age (in years old)		40	48,5 [37,3-53]	150	51 [43-57]	102	49,5 [36,8-56]
Years of follow up		40	17,3 [7,9-21,7]	150	18,3 [7,7-19,6]	102	20,8 [14,2-24,7]
Months after therapy		40	19,8 [7,0-65,1]	150	66,8 [35,4-136,7]	102	24,5 [14,2-51]
Gender	F M	22 (55,0) 18 (45,0)	- -	90 (60) 60 (40)	- -	42 (41,2) 60 (58,8)	- -
HBS index		31	2 [0-4]	143	2 [1-4]	100	3 [1-4]
Clinical symptoms	Remission Activity Missing data	26 (65,0) 5 (12,5) 9 (22,5)	- - -	119 (79,3) 24 (16,0) 7 (4,7)	- - -	84 (82,4) 16 (15,7) 2 (2)	- - -
Therapy	Infliximab Adalimumab	21 (52,5) 19 (47,5)	- -	- -	- -	- -	- -
Response to biologics	Responder Secondary Non Responder	25 (62,5) 15 (37,5)	- -	74 (49,3) 76 (50,7)	- -	41 (40,2) 61 (59,8)	- -
CRP (mg/L)		31	2,0 [0,9-5,9]	140	3,0 [1,5-5,5]	98	1,9 [0,6-5,5]
Calprotectin (µg/g)		23	355 [93-763]	135	214 [72-605,5]	96	276 [93,8-539,3]
Drug level (µg/ml)		40	7,2 [1,1-10,9]	150	5,1 [3,1-7,4]	102	9 [4,1-18,4]
Ab anti-drug (µg/ml)		40	0,5 [0,3-1,8]	142	0,6 [0,3-1,1]	100	0,5 [0,4-1,1]
Immunogenicity issues	Yes No_a No_b	4 (10,0) 12 (30,0) 24 (60,0)	- - -	2 (1,3) 22 (14,7) 126 (84)	- - -	4 (3,9) 24 (23,5) 74 (72,5)	- - -

Supplementary Table 2_ P-values comparing glycans of baseline (immunogenecity = Yes) to glycans profile in No_a and No_b group. For each glycan, -log10 of p-values times the sign of association with baseline is reported. Highlighted in bold are the associations with marginal p-values < 0.05 and FDR < 0.15.

		Infliximab + Adalimumab				**Adalimumab**			
		No_a		No_b		No_a		No_b	
		p value	FDR	p value	FDR	p value	FDR	p vaue	FDR
IgG1	**Agalactosylation**	***1,629426***	***0,903864***	0,962607	0,587602	0,693504	0,168841	0,214736	0,089354
	Monogalactosylation	-0,36006	-0,22973	-0,05944	-0,01725	0,131258	0,080105	0,407665	0,122429
	Digalactosylation	***-1,83017***	***-0,94287***	***-1,92286***	***-0,94287***	-0,54418	-0,16884	-0,48754	-0,16884
	Fucosylation	0,554532	0,354894	0,05003	0,016606	0,174651	0,080105	-0,08979	-0,04759
	Sialylation	-0,5641	-0,35489	-0,25902	-0,14987	-0,66859	-0,16884	-0,24759	-0,09931
	Bisecting	0,872165	0,554745	0,000849	0,000849	0,298333	0,099313	-0,56447	-0,16884
IgG2&3	**Agalactosylation**	***1,570671***	***0,903864***	***1,337937***	***0,835992***	0,761557	0,168841	0,554421	0,168841
	Monogalactosylation	-0,82454	-0,53019	***-1,72173***	***-0,90386***	-0,25868	-0,09931	-0,75922	-0,16884
	Digalactosylation	***-1,88658***	***-0,94287***	***-2,42725***	***-0,94287***	-0,58656	-0,16884	-0,81562	-0,16884
	Fucosylation	0,589815	0,354894	***1,458231***	***0,871965***	0,486261	0,168841	***2,826999***	***1,094605***
	Sialylation	0,091118	0,030822	0,958268	0,587602	-0,27347	-0,09931	0,349705	0,099313
	Bisecting	-0,01866	-0,00323	***-1,40258***	***-0,85252***	-0,05983	-0,03501	***-2,32856***	***-0,8972***
IgG4	**Agalactosylation**	0,361777	0,229727	0,49028	0,326088	-0,01421	-0,01421	0,208964	0,089354
	Monogalactosylation	-0,56594	-0,35489	***-1,86636***	***-0,94287***	-0,42579	-0,12476	***-1,5836***	***-0,48342***
	Digalactosylation	-0,52668	-0,35059	-0,89365	-0,55919	0,177023	0,080105	-0,26984	-0,09931
	Fucosylation	-0,07711	-0,02596	-0,01135	-0,00323	-0,50162	-0,16884	0,041768	0,025378
	Sialylation	0,54322	0,354894	***1,522314***	***0,903864***	0,240642	0,099313	1,071509	0,184213
	Bisecting	-0,13254	-0,0389	-0,90748	-0,55919	0,135248	0,080105	-0,9476	-0,16884

References

1. Danese S, Vuitton L, Peyrin-Biroulet L. 2015. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 12: 537-45
2. Sazonovs A, Kennedy NA, Moutsianas L, Heap GA, Rice DL, Reppell M, Bewshea CM, Chanchlani N, Walker GJ, Perry MH, McDonald TJ, Lees CW, Cummings JRF, Parkes M, Mansfield JC, Irving PM, Barrett JC, McGovern D, Goodhand JR, Anderson CA, Ahmad T, Consortium P. 2020. HLA-DQA1*05 Carriage Associated With Development of Anti-Drug Antibodies to Infliximab and Adalimumab in Patients With Crohn's Disease. *Gastroenterology* 158: 189-99
3. Pinho SS, Reis CA. 2015. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* 15: 540-55
4. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, Maia L, Delerue-Matos C, Taniguchi N, Lima M, Pedroto I, Marcos-Pinto R, Lago P, Reis CA, Vilanova M, Pinho SS. 2018. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 115: E4651-E60
5. Verhelst X, Dias AM, Colombel JF, Vermeire S, Van Vlierberghe H, Callewaert N, Pinho SS. 2020. Protein Glycosylation as a Diagnostic and Prognostic Marker of Chronic Inflammatory Gastrointestinal and Liver Diseases. *Gastroenterology* 158: 95-110
6. Simurina M, de Haan N, Vuckovic F, Kennedy NA, Stambuk J, Falck D, Trbojevic-Akmacic I, Clerc F, Razdorov G, Khon A, Latiano A, D'Inca R, Danese S, Targan S, Landers C, Dubinsky M, Inflammatory Bowel Disease Biomarkers C, McGovern DPB, Annese V, Wuhrer M, Lauc G. 2018. Glycosylation of Immunoglobulin G Associates With Clinical Features of Inflammatory Bowel Diseases. *Gastroenterology* 154: 1320-33 e10
7. Roda G, Jharap B, Neeraj N, Colombel JF. 2016. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 7: e135
8. Papamichael K, Cheifetz AS, Melmed GY, Irving PM, Vande Casteele N, Kozuch PL, Raffals LE, Baidoo L, Bressler B, Devlin SM, Jones J, Kaplan GG, Sparrow MP, Velayos FS, Ullman T, Siegel CA. 2019. Appropriate Therapeutic Drug Monitoring of Biologic Agents for Patients With Inflammatory Bowel Diseases. *Clin Gastroenterol Hepatol* 17: 1655-68 e3
9. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves

binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277: 26733-40

10. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313: 670-3
11. Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. 2011. Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature* 475: 110-3
12. Kiyoshi M, Caaveiro JMM, Tada M, Tamura H, Tanaka T, Terao Y, Morante K, Harazono A, Hashii N, Shibata H, Kuroda D, Nagatoishi S, Oe S, Ide T, Tsumoto K, Ishii-Watabe A. 2018. Assessing the Heterogeneity of the Fc-Glycan of a Therapeutic Antibody Using an engineered FcgammaReceptor IIIa-Immobilized Column. *Sci Rep* 8: 3955
13. Peschke B, Keller CW, Weber P, Quast I, Lunemann JD. 2017. Fc-Galactosylation of Human Immunoglobulin Gamma Isotypes Improves C1q Binding and Enhances Complement-Dependent Cytotoxicity. *Front Immunol* 8: 646
14. Thomann M, Reckermann K, Reusch D, Prasser J, Tejada ML. 2016. Fc-galactosylation modulates antibody-dependent cellular cytotoxicity of therapeutic antibodies. *Mol Immunol* 73: 69-75
15. Dekkers G, Treffers L, Plomp R, Bentlage AEH, de Boer M, Koeleman CAM, Lissenberg-Thunnissen SN, Visser R, Brouwer M, Mok JY, Matlung H, van den Berg TK, van Esch WJE, Kuijpers TW, Wouters D, Rispens T, Wuhrer M, Vidarsson G. 2017. Decoding the Human Immunoglobulin G-Glycan Repertoire Reveals a Spectrum of Fc-Receptor- and Complement-Mediated-Effector Activities. *Front Immunol* 8: 877
16. Pucic M, Knezevic A, Vidic J, Adamczyk B, Novokmet M, Polasek O, Gornik O, Supraha-Goreta S, Wormald MR, Redzic I, Campbell H, Wright A, Hastie ND, Wilson JF, Rudan I, Wuhrer M, Rudd PM, Josic D, Lauc G. 2011. High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. *Mol Cell Proteomics* 10: M111 010090
17. Keser T, Vuckovic F, Barrios C, Zierer J, Wahl A, Akinkuolie AO, Stambuk J, Nakic N, Pavic T, Perisa J, Mora S, Gieger C, Menni C, Spector TD, Gornik O, Lauc G. 2017. Effects of statins on the immunoglobulin G glycome. *Biochim Biophys Acta Gen Subj* 1861: 1152-8

Chapter VI

frontiers in Immunology

REVIEW
published: 27 November 2018
doi: 10.3389/fimmu.2018.02754

Glycans as Key Checkpoints of T Cell Activity and Function

Márcia S. Pereira[1,2,3†], *Inês Alves*[1,2,4†], *Manuel Vicente*[1,2,3], *Ana Campar*[1,2,3,5], *Mariana C. Silva*[1,2], *Nuno A. Padrão*[1,2,4], *Vanda Pinto*[1,2], *Ângela Fernandes*[1,2], *Ana M. Dias*[1,2] *and Salomé S. Pinho*[1,2,4*]

[1] *Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal,* [2] *Institute for Research and Innovation in Health (I3S), Porto, Portugal,* [3] *Institute of Biomedical Sciences of Abel Salazar, University of Porto, Porto, Portugal,* [4] *Medical Faculty, University of Porto, Porto, Portugal,* [5] *Centro Hospitalar do Porto, Porto, Portugal*

The immune system is highly controlled and fine-tuned by glycosylation, through the addition of a diversity of carbohydrates structures (glycans) to virtually all immune cell receptors. Despite a relative backlog in understanding the importance of glycans in the immune system, due to its inherent complexity, remarkable findings have been highlighting the essential contributions of glycosylation in the regulation of both innate and adaptive immune responses with important implications in the pathogenesis of major diseases such as autoimmunity and cancer. Glycans are implicated in fundamental cellular and molecular processes that regulate both stimulatory and inhibitory immune pathways. Besides being actively involved in pathogen recognition through interaction with glycan-binding proteins (such as C-type lectins), glycans have been also shown to regulate key pathophysiological steps within T cell biology such as T cell development and thymocyte selection; T cell activity and signaling as well as T cell differentiation and proliferation. These effects of glycans in T cells functions highlight their importance as determinants of either self-tolerance or T cell hyper-responsiveness which ultimately might be implicated in the creation of tolerogenic pathways in cancer or loss of immunological tolerance in autoimmunity. This review discusses how specific glycans (with a focus on *N*-linked glycans) act as regulators of T cell biology and their implications in disease.

Keywords: *N*-glycosylation, glycans, T cells, immune response, autoimmunity, self-tolerance

OPEN ACCESS

Edited by:
Jasmeen S. Merzaban, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Martin J. Richer, McGill University, Canada
Yvette Van Kooyk, VU University Medical Center, Netherlands

***Correspondence:**
Salomé S. Pinho
salomep@ipatimup.pt

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *31 July 2018*
Accepted: *08 November 2018*
Published: *27 November 2018*

Citation:
Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrão NA, Pinho V, Fernandes Â, Dias AM and Pinho SS (2018) Glycans as Key Checkpoints of T Cell Activity and Function. Front. Immunol. 9:2754. doi: 10.3389/fimmu.2018.02754

INTRODUCTION

The immune system is highly regulated by a series of stimulatory and inhibitory pathways that are crucial to maintain a healthy and balanced system. Disruption of the control of this immunological balance can result in abnormal stimulatory signals associated with the loss of immune tolerance in autoimmunity or in the creation of aberrant immunosuppressive networks that occur in cancer. Accumulating evidences have been demonstrating the importance of glycans and glycans binding proteins [including galectins (1, 2), C-type lectins (3), and sialic acid-binding immunoglobulin-type lectins (siglecs) (4, 5)] in the regulation of both innate and adaptive immune responses. In fact, all cells are covered with a dense coat of glycans that constitute a major molecular interface between cells and their environment. The diversity of glycans presentation at cell surface is enormous, encoding a myriad of important biological information that remains to be fully characterized. Glycosylation is the enzymatic process responsible for the attachment of glycans (carbohydrates) to

proteins or lipids (predominantly via nitrogen (*N*) and oxygen (*O*) linkages), a process that occurs in the Endoplasmic Reticulum/Golgi compartment of essentially all cells being mediated by the coordinated action of a portfolio of different glycosyltransferases and glycosidases enzymes (6). The proper development and function of the immune system relies both on the dynamic regulation of the expression of glycan-structures and glycan-binding proteins, and the interactions between them (7). This review discusses the role of glycans (with a focus on *N*-linked glycans) on T cells biology and function, including T cell development, activation, differentiation, and signaling. This dynamic interplay between glycans and T cells activity controlling both auto-reactivity and self-tolerance will be presented and discussed (**Figure 1**).

GLYCANS IN T CELL DEVELOPMENT AND THYMUS SELECTION

T cells are developed in the thymus where a microenvironment is set, which enables the selection of T cell receptors (TCRs) to generate a diverse repertoire of potential antigen recognition (8). Lymphoid progenitors from the bone marrow enter into the cortical tissue of the thymus, where they start to expand and develop (9, 10). Despite the fact that the role of glycosylation in T cell development and thymus selections still remains to be fully understood, some important findings highlight the relevance of glycans in this process (**Figure 2**).

Role of Glycans in Thymus Seeding and T Cell Lineage Commitment

The initial step of T cell development, the trafficking of thymus-seeding progenitors (TSPs) to the thymus, is an active process that relies on the expression of P-selectin in the thymic epithelium and its partner, P-selectin glycoprotein ligand-1 (PSGL-1), expressed by circulating TSPs-derived from the bone marrow (11). The expression and post-translational modifications of PSGL-1 are regulated in bone marrow progenitors. The deficiency of α1,3 fucosylation on PSGL-1, required for its binding to P-selectin, was shown to be associated with the impairment of TSPs homing into the thymus (12). Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$ double negative 1 (DN1) population, which give rise to multiple lymphoid lineages (8). The conserved Notch signaling pathway is responsible for the commitment of DN1 thymocytes to the T cell lineage (13). The glycosylation profile of Notch receptors (and ligands) was shown to regulate Notch-dependent intracellular signal transduction. The lunatic, manic, and radical Fringe are the glycosyltransferases that modify Notch receptors by transferring *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans of epidermal growth factor-like (EGF-like) repeats, present in the extracellular domain of Notch, and described to regulate its cell-surface signaling and function (14, 15). Loss of the three Fringe glycosyltransferases leads to a reduced binding of Notch to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T cell subsets in the thymus (16). The first indication that Fringe-mediated Notch glycosylation was involved in T cell development was shown when the lunatic Fringe gene, *Lfng*, was misexpressed under a *lck*-proximal promoter (17). This alteration of the Notch glycosylation profile (lack of GlcNAc in the EGF-like repeats) resulted in a large B cell population developed from lymphoid progenitors in the thymus. In fact, further work showed that *Lfng* is poorly expressed in $CD4^{+}CD8^{+}$ double positive (DP) thymocytes, but when ectopically expressed in that population (under *lck*-proximal promoter), led to an increased binding of Notch to its ligands on stromal cells, blocking DN development, and enabling B cell differentiation (18). These studies also revealed that changes in the glycosylation of Notch across T cell development also impacts on its signaling pathway. At DN stages, the reactions that drive development are dependent on Notch interactions with DLLs, which exist at functionally limiting concentrations. The high levels of *Lfng* expression in DNs facilitate Notch interactions with DLLs and the dramatic downregulation of *Lfng* in DPs coincides with Notch-independent reactions of T cell development. The final commitment to the T cell lineage occurs at the DN3 stage, where a recombination-activating genes (RAG)-mediated productive rearrangement of the *Tcrb* leads to the expression of the ß chain of the TCR (TCRß) and the formation of a pre-TCR signaling complex (13, 19).

Role of Glycans in Thymocyte ß Selection

Together with Notch and Interleukin (IL)-7, the pre-TCR signaling initiates ß-selection, by inducing the downregulation of the RAG complex expression (*Rag1* and *Rag2*) in quiescent DN3 (DN3a), becoming large cycling DN3 thymocytes (DN3b), which differentiate into DN4 cells. A deficient pre-TCR signaling in *lck*-null cells is rescued by *Lfng* overexpression, but not in a $Rag2^{-/-}$ background, indicating a pre-TCR dependency for development (20). Upon ß-selection, it was recently demonstrated that DN4 cells upregulate glucose and glutamine metabolites that enter into the hexosamine pathway, increasing the production of UDP-GlcNAc, which is needed to undergo clonal expansion (8, 21). The UDP-GlcNAc is also the substrate of the *O*-GlcNAc transferase (OGT) in the process of *O*-GlcNAcylation of intracellular proteins on serine and threonine residues (22). Recent evidences showed that *O*-GlcNAcylation regulates the process of T cell development (23). Using a conditional knockout mouse model of OGT in the DN stage, it was shown a reduced population of DPs, indicating either a deficiency on ß-selection or in clonal expansion of DN4s. The absence of OGT appeared not to impact self-renewal of DNs, or their differentiation into DPs, but to promote the failure of the clonal expansion of DN4, in response to Notch ligands. A feedback mechanism was proposed in which the metabolic changes (the shift to glycolysis) that support the DN-to-DP stage of thymocyte differentiation, controlled by Notch, induces c-Myc expression, which in turn controls the rate of T cell nutrient uptake as well as the expression of OGT and consequently the abundance of *O*-GlcNAc (15). The *O*-GlcNAcylation of c-Myc was also shown to increase its stability (24), further contributing to the feedback loop.

In the stage of post-β selected DN4 thymocytes, it was seen a 10-fold increase in expression of ST6 β-Galactoside

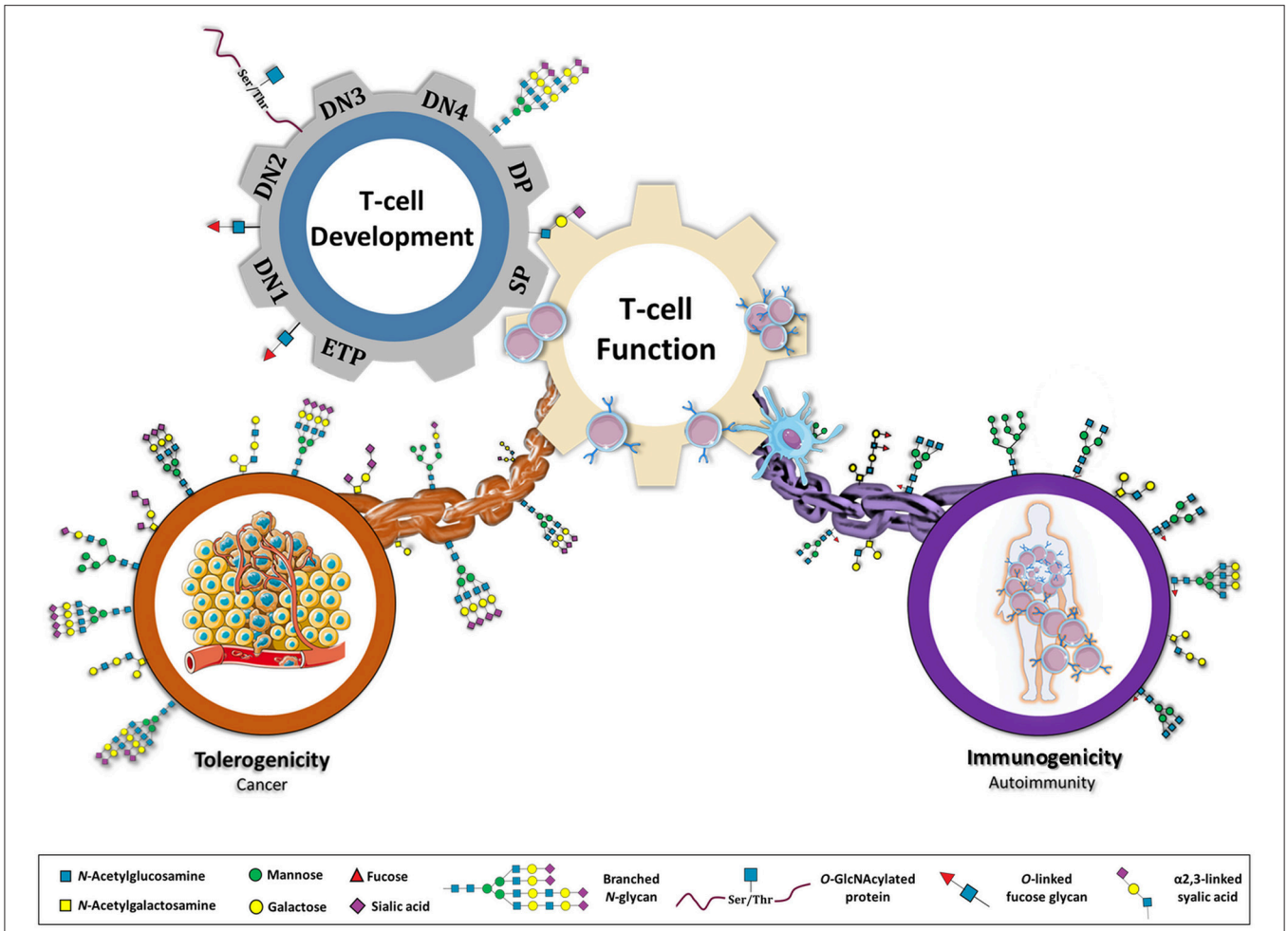


FIGURE 1 | Glycans as a major connective chain that controls T cell response in either a tolerogenic or immunostimulatory scenario. Glycosylation appears to be central in regulating several steps of a T cell's life. During T cell development, different population of T cells (ETP, early thymocyte progenitor; DN1, 2, 3, and 4, double negative; DP, double positive; SP, single positive) display specific glycosylation patterns. The normal glycosylation of SP population results in an educated T cell function. However, by genetic, environmental or metabolic constrains, T cell glycosylation can be compromised re-directing immune system toward an immunostimulatory or tolerogenic response. Glycans are proposed here as key players in immune-unbalanced diseases, such as autoimmunity and cancer.

α2,6-Sialyltransferase 1 (ST6Gal I) when comparing to the DN3 population, which resulted in an increase in α2,6-linked sialic acid (25). Accordingly, in *ST6Gal1* deficient mice, the DN populations were decreased, beginning at the DN1 subset. Microarray data showed a downregulation of CD96 (receptor molecule of nectin-1, that plays a putative role in cell migration) in the DN2 and DN3 populations in the *ST6Gal1* deficiency background, and a disruption of thymopoiesis in these mice was proposed. Moreover, ST3 β-Galactoside α2,3-Sialyltransferase 1 (ST3Gal I) expression is decreased in most DN and in all DP, only increasing in single-positive (SP) thymocytes (26). In *ST3Gal1*$^{-/-}$ mice, the TCR repertoire was significantly altered, indicating a role for sialylation in thymocyte selection (27).

Role of Glycans in Positive and Negative Selection in the Thymus

The ß-selected DN4 cells undergo rapid self-renewal, giving rise to a clonally expanded population, that differentiate into DP $CD4^+CD8^+$ thymocytes (8). In this developmental stage, mature TCRαß receptors are formed (28) and the expression of the co-receptors CD4 and CD8 confer a MHC class II and class I restriction of TCR activation, respectively. The newly formed mature TCRs are then screened by thymic epithelial cells (TECs) by the specificity and binding strength for the MHC ligands presented. The next developmental process is named positive selection, where the DP population is enriched for cells that express an immunocompetent TCR (8, 29). The selected DPs then commit to the SP $CD4^+$ or $CD8^+$ lineage and go through a process called negative selection, which eliminates autoreactive T cells (8, 29). The affinity of the correctly assembled TCRαβ for the MHC-antigen complexes determines cell survival and differentiation. Glycosylation modifications of the TCR may provide an alternative mechanism to control positive and negative selection by directly affecting the TCR-MHC-antigen binding, TCR interaction with its co-receptors and the threshold of activation (30), an issue that is far from being fully elucidated.

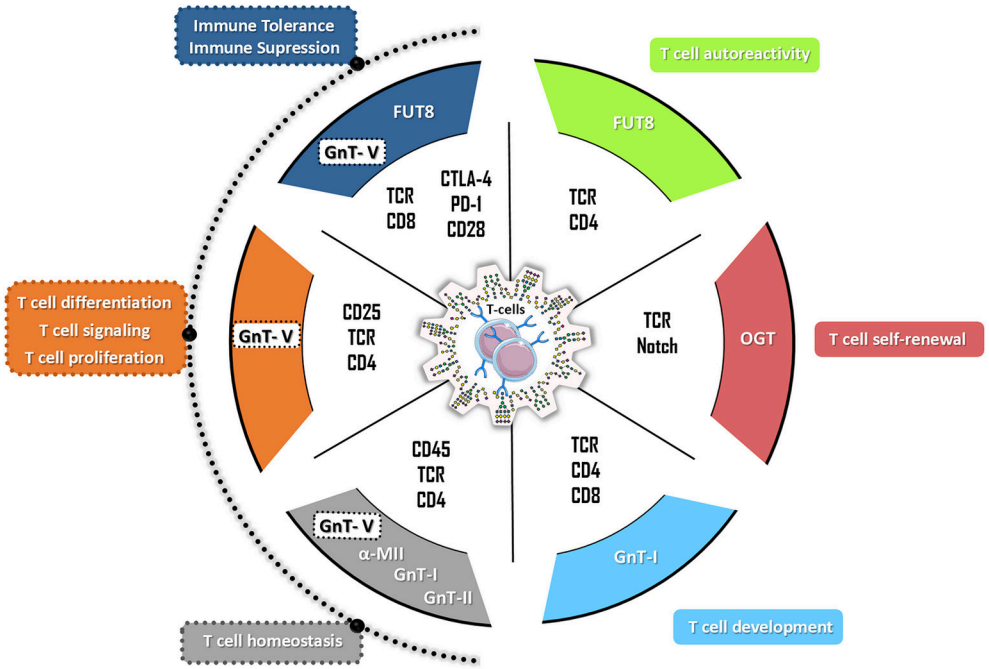


FIGURE 2 | The hallmarks of glycans in T cell biology. *N*-glycans have a broad effect on the multiple T cell functions with impact both in autoreactivity and in immune tolerance. Particularly, the complex branched *N*-glycans catalyzed by beta 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) (encoded by *MGAT5* gene) have been demonstrated to control different T cells functions by targeting different T cells receptors (such as TCR, CD25, and CD4) and therefore regulating T cell proliferation, T cell differentiation, T cell signaling as well as the production of inflammatory cytokines. Alterations on GnT-V activity but also in alpha-mannosidase II (α-MII) as well as in *N*-acetylglucosaminyltransferase I (GnT-I, *MGAT1* gene) and II (GnT-II, *MGAT2* gene) activity were shown to compromise T cell homeostasis being associated with the development of several autoimmune disorders in humans and mouse models (such as EAE, IBD, SLE, TID). The FUT8-mediated core fucosylation of TCR was associated with hyperactivation of $CD4^+$ T cells (T cells autoreactivity) whereas the modification of the co-inhibitory receptors (CTLA-4 and PD-1) by FUT8-mediated core fucose results in immune tolerance. The T cell development and T cell self-renewal are controlled by GnT-I-mediated glycosylation and by *O*-GlcNAcylation through OGT (*O*-GlcNAc transferase), respectively.

The subunits of the TCRαβ contain at least 7 potential sites for *N*-linked glycosylation and the TCR-CD3 complex is estimated to have 12 *N*-glycan addition sites that contribute to TCR folding and functions (31, 32). Indeed, selective removal of conserved *N*-glycosylation sites of the constant regions of the TCR, enhanced its functional avidity (the sensitivity of the T cell response to other cell which carries the respective MHC-peptide) (32). However, whether *N*-glycosylation in the variable regions of the TCR affect its selection remains to be addressed. Moreover, low levels of sialylation in DPs are associated with binding to Major Histocompatibility Complex (MHC) class I (common to all nucleated cells) and the increased expression of sialic-acid linkages on differentiated SP $CD8^+$ thymic T cells was shown to decrease the binding avidity of CD8 for MHC class I molecules, which acts as a regulation for a TCR affinity dependent negative selection (33).

Furthermore the deficiency of the *Mgat5* gene, that encodes for a Golgi branching enzyme *N*-acetylglucosaminyltransferase V (GnT-V) was shown to markedly increases TCR clustering and signaling at the immune synapse, resulting in a lower T cell activation threshold and increased incidence of autoimmune disease *in vivo* and in human (30). In a model of positive selection, it was demonstrated that branching *N*-glycosylation dynamically expands the affinity spectrum of positive selection by differentially controlling both the lower and upper limits of positively selected TCR-MHC-antigen interactions (34). The intracellular domains of CD4 and CD8 co-receptors bind Lck, enhancing TCR responses to low-affinity MHC-antigen complexes when coupled to the TCR (35). Both co-receptors have *N*-glycosylation sites and it was shown that the branching deficiency in $Mgat1^{f/f}$Lck-Cre^+ T cells resulted in decreased surface expression of CD4 and CD8 receptors (34). The lack of branched *N*-glycans in the same genetic background also decreased TCR threshold signaling (30). These evidences supported that branching *N*-glycans display an important role in the maturation of DN cells and/or TCR selection.

Changes in the expression of *O*-linked glycans also impact T cell development by modulating galectin binding. Galectin-1 was shown to induce apoptosis of immature thymocytes through binding to core 2 *O*-glycans expressed in CD43 and CD45 (36). In contrast, CD45 on mature thymocytes bears core 1 *O*-glycans as well as *N*-glycans capped with α2,6-linked sialic acid, which inhibits galectin-1 binding (36).

Overall, glycosylation appears to play a critical role in the different stages of thymocyte development and in the generation of an efficient immune system. Nevertheless, further research is

needed in order to understand how glycans control each stage of thymocytes development, differentiation and selection, which might reveal novels insights on the influence of the glycome in major diseases, such as autoimmunity and cancer.

GLYCANS IN THE REGULATION OF T CELL ACTIVITY AND FUNCTIONS

The proper function of T lymphocytes is highly dependent on their surface receptors, which in turn are highly mediated by glycosylation. Although *O*-glycan structures have been shown to play important roles on immune-associated molecules (37), the prominent role of *N*-linked glycans is emphasized in this section (**Figure 2**).

As previously mentioned, MHC I is expressed by almost all nucleated cells and interacts with TCRs on $CD8^+$ T cells; in turn, MHC II is expressed by professional antigen presenting cells (APCs) (dendritic cells - DC, macrophages, B cells and TECs) and is recognized by $CD4^+$ T cells (7, 38). More than 3 decades ago, it was demonstrated that blocking MHC1a *N*-glycosylation, through acceptor site mutation, results in significant increases in intracellular misfolded protein along with decreases in cell surface expression (39). MHC II is assembled by two glycoproteins, α and β chains. The α chain contains *N*-linked high-mannose and complex glycans whereas the β chain is only constituted by complex *N*-glycans (40). In contrast to the role of MHC I, MHC II glycosylation was shown to have a particular impact on the effective antigen binding, as well as in the presentation of microbial carbohydrate antigens, which consequently influences downstream T cell responses. This was demonstrated by the depletion of the *Mgat2* gene, which compromises *N*-glycan branching, decreasing carbohydrate antigen presentation by MHC class II and leading to loss of T cell stimulatory activity (41).

During TCR signal transduction, glycans play a key role in stabilizing individual molecules in the complexes at the immunological synapse and by protecting them from the action of proteases during T cell engagement (31). Additionally, glycans can also restrict nonspecific protein-protein interactions, like aggregation of TCRs on the membrane, helping to orient the interactions of the proteins in the central clusters (31). Demetriou et al. demonstrated that β1,6-GlcNAc branched *N*-glycans structures (catalyzed by GnT-V) regulate T cell activity, namely in $CD4^+$ T cells by increasing the threshold of T cell activation, suppressing T cell growth and signaling (30, 42). Moreover, core-fucosylation, which refers to fucose attached to the innermost *N*-acetylglucosamine of *N*-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8), was also shown to affect T cell activity in immune mediated disorders (42, 43).

The T cell activity is also dependent on glycosylation of co-receptors, such as the complex formation between TCR and CD45. Galectin-3 is a key mediator of this complex, by establishing a molecular lattice through binding to polylactosamine structures in branched *N*-glycans. Consequently, CD45 phosphatase activity induces downregulation of T cell signaling, preventing T cell activation (44). Furthermore, CD45 is alternatively spliced into five different isoforms on human leukocytes (CD45ABC, CD45AB, CD45BC, CD45B, and CD45RO) (45–47), all decorated with up to 11 *N*-glycans in the membrane proximal region. Importantly, all isoforms present different glycosylation profiles (48, 49), that change during T cell differentiation and activation (50, 51), as reviewed in (36). CD28 is another T cell surface glycoprotein acting as a secondary signaling molecule of T cell activation. Interestingly, nearly 50% of the molecular mass of CD28 is constituted by *N*-glycans (52). Previous studies reported that *N*-glycosylation of human CD28 can negatively regulate CD28-mediated T cell adhesion and co-stimulation, namely the interaction between CD28/CD80. Mutation of all potential *N*-linked glycosylation sites of CD28 as well as treatment of Jurkat cells with inhibitors of *N*-glycosylation pathway resulted in a defective CD28 glycosylation with enhancement of the binding to CD80 expressed on APCs (52). The branching *N*-glycosylation of CD25 receptor also modulates its cell surface retention controlling T differentiation with impact in immune tolerance. Recently, it was demonstrated that a decreased UDP-GlcNAc and complex branching *N*-glycosylation induces a decreased cell surface retention of CD25 and IL-2 signaling, promoting a T helper (T_H) 17 over induced regulatory T cell (iTreg) differentiation (53) (**Figure 2**).

Importantly, the co-inhibitory receptors are likewise modulated by *N*-glycosylation. One of the major negative regulators of T cell response is the cytotoxic T-lymphocyte protein 4 (CTLA-4), that comprises two *N*-glycosylation sites described to modulate its cell surface retention on T cells and thereby its affinity for CD80/CD28 on APCs (54–56). The impact of *N*-glycosylation in the modulation of the inhibitory functions of CTLA-4 and programmed cell death protein-1 (PD-1) is discussed in more detail in section "Glycans in tolerogenic/immunosuppressive responses". Nonetheless, other co-inhibitory receptors like Lymphocyte-activation gene 3 (Lag-3), mucin-domain-containing molecule-3 (Tim-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) may also undergo glycans-mediated regulation, as they exhibit *N*-glycan-binding sites, however the role of glycans on these molecules remains to be explored (57).

Taken together, *N*-glycosylation plays an instrumental role in the regulation of T cell activation and functions by targeting not only TCR but also its co- receptors (**Figure 2**).

GLYCANS AS MODULATORS OF HYPER-REACTIVE/AUTOIMMUNE RESPONSES

Autoimmunity is characterized by the loss of self-tolerance and development of an autoreactive immune response toward the individual's own organism. Glycan motifs play a crucial role in the determination of self/non-self antigens. Specific glycan structures, expressed by microbial pathogens, are commonly responsible for the primary activation of the innate immune system; however, the mechanisms involved in the self/non-self discrimination, mediated by glycans are far from being fully

elucidated. Abnormal levels of branched *N*-glycans have been associated with exacerbated immune responses in murine models (58). Particularly, the dysregulation of the *N*-glycosylation pathway has been associated with autoimmune-like phenotypes. The inability to synthetize β1,6-GlcNAc antennae, in *Mgat5*$^{-/-}$ mice has been associated with an increased susceptibility to immune-mediated disorders such as an increased delayed-type hypersensitivity responses, as well as increased susceptibility to develop experimental autoimmune encephalomyelitis (EAE) (30, 59) and severe forms of colitis (60). The lack of β1,6 branching *N*-glycans favors TCR clustering, leading to a decrease of the TCR threshold and consequently increased T cell activation (30) associated with the hyperimmune response observed in these mice (**Figure 2**). This hyperimmune phenotype is also due to an abnormal formation of lattices between TCR-branched glycosylation and galectins (61, 62). Accordingly, β3 GnT2-deficient mice show T cell hypersensitivity due to the reduction of polylactosamine on the *N*-glycans (ligands of galectins), similarly to what is observed in *Mgat5* deficient mice (30, 61). Furthermore, absence of α-mannosidase II (which catalyses the last hydrolysis of the α-mannose), was shown to result in signs of glomerulonephritis, deposits of glomerular IgM immunocomplexes and complement component 3 as well as high levels of anti-nuclear antibodies (63, 64), which is consistent with a Lupus-like syndrome (**Figure 2**). Taken together, these evidences support the role of *N*-glycosylation in the perspective of T cell biology.

The role of *N*-glycans in antigen presentation and recognition is still elusive, and in fact abnormal glycoantigen presentation might also impact T cell activity. Abnormal accumulation of high-mannose, paucimannose, and agalactosyl bi-antennary glycans, have been detected in kidney tissue from MRL-lpr mouse (a well-stablished murine model of SLE) (65). Moreover, evidences have been showing that *Mgat1*$^{f/f}$Syn1-Cre mice, with *Mgat1* deletion at the Synapsin I-*expressing cells* (abundant in neural tissues), presented neurological defects, with high levels of neuronal apoptosis and caspase 3 activation (66). These high levels of apoptosis are observed in several autoimmune diseases, which results in activation of immune system (67) (**Figure 2**). Although highly unexplored, rare autoimmune diseases are also associated with *N*-glycosylation dysfunctions. As example, idiopathic inflammatory myopathies (IIM) are a group of rare diseases of autoimmune nature, whose etiopathogenesis is far from being totally understood (68). Muscle cells surface is enriched with glycoproteins and several lines of evidence provide support for a fundamental role of glycosylation in muscle homeostasis and function (69, 70). Glucosamine (UDP-*N*-Acetyl)-2-Epimerase/*N*-Acetylmannosamine Kinase (GNE) genetic mutations (a gene that encodes *N*-acetylmannosamine (ManNAc) kinase enzyme, responsible for the biosynthesis of *N*-acetylneuraminic acid) results in hypo-sialylation of muscle glycoproteins; the prophylactic supplementation with sialic acid precursor (ManNAc) was shown to prevent the muscle phenotype in mice with gene mutations that cause hereditary inclusion-body myositis (hIBM), a muscle phenotype that resembles one type of IIM (71). Altogether, these findings highlight the importance of further studies addressing the role of *N*-glycosylation in the perspective of *neoautoantigens*, since autoantigens contain a significant amount of glycoantigens due to the increased number of *N*-glycosylation sites comparing with other proteins (72).

The Glycan binding proteins (GBPs) are expressed in the APCs being characterized by a carbohydrate recognition domain which specifically recognizes glycan structures present at the cell surface receptors. This glycan-GBPs engagement results in either an anti- or pro-inflammatory response (73). C-type lectins, siglecs, and galectins are examples of GBPs, that are instructors of immune responses (5, 73). As example, SIGN1R (expressed by APCs and the analogous of the human dendritic cell-specific ICAM-grabbing non-integrin - DC-SIGN) signaling was shown to result in the expansion of IL-10-secreting Treg cells, preventing the development of autoimmune diseases such as EAE and type 1 diabetes (T1D) (74). Galectin-1 also plays an important immune-regulatory role in EAE (75) as mice deficient in galectin-1 (*Lgals1*$^{-/-}$) have increased T_H1 and T_H17 responses being more susceptible to EAE when compared with wild type mice (76). More recently, Galectin-1 was shown to modulate the cytolytic activity of $CD8^+$ T cell. The interaction of Galectin-1 and Fas ligand seems to be responsible for the retention of this glycoprotein at the surface of cytotoxic T lymphocytes hampering the cytolytic ability of these cells (77). Overall, GBPs-glycoprotein interaction is essential to instruct a T cell—mediated immune response.

Notably, one of the first evidences addressing the relationship between the dysregulation of *N*-glycosylation and human autoimmunity was observed in multiple sclerosis (MS) patients. During active, relapse or in very early stages of remission, peripheral blood mononuclear cells from MS patients display a significant decrease of the enzymatic activity of Golgi β1,6 *N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), compared to healthy subjects (78). Moreover, *MGAT5* polymorphisms were associated with MS severity (79) together with *MGAT1*, *IL2R,* and *IL7R* Single Nucleotide Polymorphisms (80–82). Additionally, in Inflammatory Bowel Disease (IBD), it was also demonstrated that *lamina propria* T lymphocytes from ulcerative colitis (UC) patients exhibited a deficiency in β1,6-GlcNAc branching *N*-glycans due to decreased levels of *MGAT5* gene expression (83). Importantly, low levels of branched *N*-glycans in lamina propria early at diagnosis were shown to predict UC patients that will fail the response to standard therapy, thus displaying a bad disease course (84). The supplementation of intestinal T cells from UC patients and mouse models with colitis with GlcNAc promoted the enhancement of β1,6 branching *N*-glycans on T cells, suppressing TCR signaling and reducing the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Pre-clinical studies both in IBD and MS demonstrated the immunomodulatory properties of *N*-glycans in the control of T cell-mediated immune response (60, 85), paving the way for the development of human clinical trials, that are currently on going (53, 60). Less explored but of utmost importance is the study of *N*-glycosylation profile in rare autoimmune disorders, since its etiopathogenesis is still very elusive. Glycosylation changes in muscle-associated human disease have focused in muscular dystrophies (86) and congenital

disorders of glycosylation (87). Recent studies have shown that muscle cell surface glycosylation is finely regulated and subjected to alterations under inflammatory conditions (88), pointing to a possible interaction between muscle glycocalyx and the extracellular milieu, which is particularly enriched in immune cells and antibodies in IIM patients (89).

Overall, glycans are critical determinants in autoreactive responses both by directly regulating T cell activity and also through the creation of abnormal glycoantigens that may unleash an autoreactive immune response.

GLYCANS IN TOLEROGENIC/IMMUNOSUPPRESSIVE RESPONSES

Recent studies have been highlighted that alterations on the glycosylation pattern of T cells' receptors, as well as the alterations of the glycosylation profile of tumor cells (tumor glyco-code), are implicated in the modulation of the immune response leading to immunosuppressive pathways, known to occur in the tumor microenvironment associated with tumor immunoescape (90).

Role of Glycans in the Modulation of Inhibitory T Cell Receptors

PD-1, as already introduced, is a cell surface inhibitory T cell receptor responsible for immune-inhibitory responses associated with the so-called "T cell exhaustion" (91). The expression of this cell surface receptor, as well as Tim-3, was described to be positively regulated by the core fucosylation pathway, catalyzed by FUT8 enzyme (92). The inhibition of core fucosylation in PD-1 was demonstrated to lead to an anti-tumor immune response mediated by T cells activation, being a new attractive target for enhancing anti-tumor immunity in future clinical settings (**Figure 2**). This was a pioneer study that supported the importance of PD-1 post-translational modifications by glycosylation on T cell-mediated immunosuppression (92). Additionally, the glycosylation of programmed death ligand-1 (PD-L1), a PD-1 ligand, was described to have an important role in its cellular stabilization. The interaction of non-glycosylated PD-L1 with glycogen synthase kinase 3β (GSK3β), a key enzyme on glycogenesis, leads to the degradation of this molecule (93). In triple-negative breast cancer cells, it was further shown that the β1,3-*N*-acetylglucosaminyl transferase (B3GNT3), involved in the biosynthesis of poly-*N*-acetyllactosamine chains, is important for the interaction between PD-1 and its ligand PD-L1 (94). The use of an antibody targeting the glycosylated form of PD-L1 resulted in its degradation and internalization, with the blockage of PD-L1/PD-1 interaction and consequently the inducement of anti-tumor activity in triple-negative breast cancer *in vitro* and *in vivo* models (94). In accordance, Tregs from healthy humans and mice were shown to display an increased variability on its *N*-glycosylation pattern when compared with $CD4^+$ T cells. The levels of the complex branched *N*-glycans were shown to be correlated with the expression of proteins involved in Treg suppressive functions, including PD-1, PD-L1, and also other negative regulators of T cell response, namely CTLA-4 (95). In fact, the CTLA-4 protein, comprises multiple *N*- and *O*-glycosylation sites known to modulate its retention at T cell surface and consequently affecting its function (56). The TCR activation is associated with an increased β1,6-GlcNAc branched *N*-glycosylation of CTLA-4, which enhances CTLA-4 retention at the T cell surface and thereby suppresses T cell activation promoting immune tolerance (96) (**Figure 2**). Accordingly, the presence of Thr17Ala polymorphism in human *CTLA-4* was shown to result in the reduction of the *N*-glycosylation sites from one to two sites, which limited CTLA-4 retention at T cell surface (80). Supplementations with GlcNAc and Vitamin D promoted an enhancement of *N*-glycans branching expression, increasing the cell surface retention of CTLA-4, culminating in immunosuppression (80).

Glycans as Instructors of Immunosuppressive Responses

Tumor cells aberrantly express different types of glycans structures when compared with normal counterparts, such as an increased sialylation, an expression of truncated glycans and an overexpression of branched *N*-glycans (97). This alteration in the cellular glycosylation profile governs several steps of tumor development and progression, such as tumor cell dissociation, proliferation, invasion, metastasis, angiogenesis, with recent evidences pointing toward its effects in tumor immunoediting and immunosurveillance (98). GBPs expressed on immune cells are able to recognize altered glycan structures expressed at tumor cell surfaces instructing either immunostimulatory or immunoinhibitory responses.

The expression of sialylated glycans, such as Tn antigen and Lewis antigens, aberrantly expressed in tumor cells, were described to be recognized by DC-SIGN, expressed by macrophages and immature DCs, which lead to immunosuppression (99). The fucose residues present in Lewis structures (Lewis x and Lewis y), attached to carcinoembryonic antigen (CEA) (100), were described to trigger the upregulation of the anti-inflammatory cytokines IL-10 and IL-27 by APCs and the induction of T_H2, follicular (T_Hf), and Treg immune responses (101, 102). Besides, antigen-containing liposomes modified with DC-SIGN-binding Lewis b and x resulted in glycans recognition and internalization through DCs with consequent activation of $CD4^+$ and $CD8^+$ T cells (103). Furthermore, macrophage galactose binding lectin (MGL) was found to be able to recognize Tn antigen and *N*-acetylgalactosamine (GalNAc) residues, resulting in an increased recognition by Toll-like receptor 2, ultimately resulting in the secretion of cytokines (IL-10 and TNF-α). (104). Its interaction with terminal GalNAc residues on CD45 glycoprotein negatively regulates TCR signaling, with consequent decrease of T cell proliferation and increased T cell death (105). Moreover, by blocking the tumor-infiltrated macrophages (responsible for the high levels of IL-10), it was observed an effective $CD8^+$ T cells response, highlighting the importance of combining anti-tumor immune therapy with conventional chemotherapy (106). Furthermore, it was recently demonstrated in chronic infection that IL-10 induces the upregulation of the *Mgat5*

gene increasing branched *N*-glycans on $CD8^+$ T cells, which in turn decreases T cell activity and allows viral persistence (107). Despite the different context in which this hypothesis was studied, *Mgat5*-mediated branching glycosylation can constitute a potential mechanism by which IL-10 is suppressing $CD8^+$T cells in cancer.

In addition, sialylated glycans also play a role in immunosuppression, mediated by siglecs, a family of lectin receptors that predominantly exhibit immune-inhibitory functions. In *in vitro* and *in vivo* studies, the binding to sialylated antigens by siglec-E expressed on DCs promoted an increase of antigen-specific Treg response and a reduced numbers of antigen-specific Teff cell response, associated with tumor growth (108, 109). Indeed, the sialylated tumor antigens, such as Sialyl-Tn (sTn) and Sialyl-T (sT) expressed in mucins, namely MUC1, were associated with tumor immune tolerance. The recognition of MUC1-ST by siglec-9 on tumor-infiltrating macrophages was shown to initiate inhibitory immune pathways mediated by MEK-ERK signaling (110). Moreover, siglec-binding to sTn-expressing mucins, led to the maturation of DCs and DC-mediated induction of $FOXP3^+$ Treg cells and reduced INFγ-producing T cells (111, 112). A recent study also demonstrates that siglec-9 expressed by $CD8^+$ tumor infiltrating lymphocytes (TILs) in non-small cell lung cancer (NSCLC) patients was associated with reduced survival. Accordingly, siglec-9 polymorphisms were associated with the risk of developing lung and colorectal cancer. Additionally, the characterization of siglec-9^+ $CD8^+$ TILs revealed that these cells concomitantly express several inhibitory receptors, including PD-1, TIM-3, Lag3, and others. In addition, the same study further reveals that lack of sialic acid-containing glycans in tumor cells led to a delay of tumor growth and an increased infiltration of $CD3^+$ and $CD8^+$ T cells (113).

Another important GBP that have been pointed out as a crucial checkpoint in T cell viability and activity are galectins. Galectin-1, 3, and 9 were predominantly described in T cell immunosuppression. Galectin-1, was demonstrated to be expressed by tolerogenic DCs (75) and $CD4^+CD25^+$ T cells (114), triggering T cell apoptosis through binding to *N*-glycans and *O*-glycans on CD45, CD43, and CD7 or by sensitizing resting T cells to FAS-induced death (115, 116). The T_H1 and T_H17 activated cells are susceptible to galectin-1-induced cell death once these cells express the repertoire of glycans required for galectin-1 binding, while T_H2 cells are protected via α2,6-sialylation on cell surface glycoproteins, which was described to preclude galectin-1 recognition and binding (76). In addition, several tumors have the capacity to secrete galectin-1 in order to promote immunosuppression, through a mechanism that involves a bias toward a T_H2 cytokine profile and activation of tolerogenic circuits mediated by IL-27-producing DCs and IL-10-producing type 1 Treg cells (117). On other hand, galectin-3 has an ambiguous role in T cell viability: when it is localized at intracellular level, this protein presents a protective role through a cell death inhibition pathway that involves B-cell lymphoma 2 (Bcl-2) (118), whereas extracellular galectin-3 induces cell death in activated T cells, by binding to glycosylated receptors of T cells through a distinct way than galectin-1 (115). Moreover, galectin-3 has the capacity to bind to *N*-glycans on CTLA-4 prolonging the inhibitory signals (119), as well as to Lag-3 on the surface of $CD8^+$ T cells, suppressing its function (120). Finally, galectin-9 abrogates T_H1, T_H17, and $CD8^+$ T cells through glycosylation-dependent binding to Tim-3 (121–123), whereas may regulate pro-inflammatory cytokine production by binding with other receptors (124).

Altogether, these findings support the relevance of glycans on T cells-mediated immunosuppressive/tolerogenic pathways which have relevant implications in tumor progression. Targeting the abnormal glycosylation pattern of cancer cells constitutes a promising strategy to instruct an effective anti-tumor immune response, an issue that needs to be further explored.

GLYCANS AS METABOLIC REGULATORS OF T CELL FUNCTION

The impact of glycosylation on T cell development and functions is enormous, as revealed by the critical roles of glycans in the development and progression of major diseases such as auto-immunity and cancer, as described herein. In order to accompany the bioenergetic and biosynthetic demands required for T cell proliferation and activation, a shift in the T cell metabolism is required. While naïve T cells are in a metabolic quiescent state, mainly using oxidative phosphorylation to maximize ATP production, T cells under clonal expansion or under differentiation, reprogram their metabolic status to aerobic glycolysis and glutaminolysis in order to increase the availability of glycolytic precursors for the biosynthesis of nucleotides, amino acids and lipids (125–127). During T cell activation, the hexosamine biosynthetic pathway (HBP—a branch of the glucose metabolism) is upregulated in order to generate the nucleotide sugar-donor substrate UDP-GlcNAc, required for *N*-glycosylation, O-GlcNAcylation, and glycosaminoglycans production that are needed for a proper T cell function (128).

Mediators from the glycolytic pathway such as glucose (Glc), glutamine (Gln), acetyl CoA are known to interfere with the availability of the UDP-GlcNAc in the cell (129–131). Together Glc and Gln were shown to increase UDP-GlcNAc in nutrient-starved T cells. In the same setup, the supplementation of both Glc and glucosamine (GlcN–a metabolite of the HBP) further increased the UDP-GlcNAc cellular content, demonstrating the sensitivity of the HBP to nutrients that enter directly (GlcN) or through a precursor pathway (Glc in glycolysis) (130). Despite the general use of the UDP-GlcNAc as a substrate donor of HBP, there are some glycosyltransferases that are more susceptible to nutrient changing than others, such as the case of OGT (132). In fact, the supply with Glc and Gln are crucial for protein O-GlcNAcylation, that is important during T cell development, being associated with T cell malignant transformation (23). Among the *N*-acetylglucosaminyltransferases (GnTs) that participate in the HBP, the less sensitive to nutrient changing (and thus substrate availability) are GnT1, GnT2, and GnT3, due to lower *Michaelis Constant* (K_m) levels, meaning that these enzymes require low

levels of the substrate to synthetize the specific glycans. In contrast, GnT4 and GnT5 present higher K_m and therefore their activity is highly dependent on the availability of the UDP-GlcNAc substrate (119, 133). Therefore, these two enzymes are sensitive to alterations in glucose and HBP metabolism (as the GlcN or *N*-acetyl glucosamine–GlcNAc) (62), which ultimately will interfere in the *N*-glycan branching biosynthesis on T cells with impact in their activity, as detailed in section "Glycans in the regulation of T cell activity and functions" (60). In fact, supplementation with Glc, Gln, and GlcNAc increases branching *N*-glycans on Jurkat cells and resting T cells from mice (85, 119, 130). Moreover, $CD4^+$ T cells from $MGAT5^{+/+}$ or $MGAT5^{+/--}$ mice supplemented with oral GlcNAc also results in up to 40% increase of branching *N*-glycans, detected by L-PHA (130). This enhancement of branching *N*-glycosylation upon GlcNAc supplementation was shown to functionally impact on T cells activity by reducing T cell activation, decreasing T_H1 differentiation, and increasing retention of the growth inhibitory receptor CTLA-4 at T cell surface (85, 130).

Importantly, evidences suggest that the glycolysis and glutaminolysis compete with HBP pathway for the same metabolites. Recently, Araujo et al showed that, during T_H17 differentiation the existence of common mediators shared between HBP, glycolysis (fructose-6-phosphate) and glutaminolysis (Gln) results in a starvation of the HBP mediators, translated in a reduction of *N*-glycan branching due to the limitation on the UDP-GlcNAc availability (53). Fueling HBP with GlcNAc switched the cell fate from T_H17 to iTreg differentiation, through stimulation of IL2-Rα signaling (53). This interplay between metabolic pathways was further demonstrated by the increase on Glc, Gln, fatty-acids uptake, and lipid storage upon stimulation of the HBP with GlcNAc supplementation, suggesting a reprogramming of the cellular metabolism upon GlcNAc flux (53, 134).

The impact of glycans as metabolic regulators of T cells is also testified by its effects in *ex vivo* and *in vivo* models of autoimmune diseases. The metabolic supplementation with GlcNAc in *ex vivo* human colonic T cells from IBD patients resulted in an enhancement of the branching *N*-glycosylation pathway that was accompanied by a significant reduction of T cell proliferation, supression of T_H1/T_H17 immune response (through decreased production of IFN-γ and IL-17A pro-inflammatory cytokines) and decreased TCR signaling (60). Accordingly, the GlcNAc supplementation of mice models with auto-immune diseases such as EAE, TID, and IBD results in inhibition of T_H1, T_H17 immune response concomitantly with a significant improvement of the clinical symptoms (60, 85). Treatment with GlcNAc after disease onset also demonstrate inhibitory effects on the development of the EAE, by reducing the secretion of INF-γ, TNF-α, IL-17, and IL-22 (85). Interestingly, a dual role of GlcN (the precursor of GlcNAc) on the progression of autoimmune disorders was shown, by demonstrating its impact in preventing T_H1-mediated Type I diabetes (through the reduction of IFN-γ producing $CD4^+$ T cells), but also the GlcN effects in exacerbating T_H1/T_H17–mediated EAE symptoms (trough stimulation of T_H17 response) (135). In contrast, another study showed that GlcN suppresses acute EAE through the blockage of T_H1 and induction of T_H2 response (136). GlcN supplementation was further shown to mediate T cell activation by decreasing the *N*-glycosylation of CD25 (IL-2Rα) from $CD4^+$ T cell (135). This down-regulation of *N*-glycosylation might be explained by the competition between GlcN and Glc for the same glucose transporter which might impact in the reduction of the GlcNAc concentration.

Altogether, alterations on the glucose metabolism and partially changes in the metabolic flux of HBP have a direct impact on T cells *N*-glycosylation profile with major consequences in their function and activity. Ultimately, the modulation of the HBP constitutes an important metabolic target able to control both autoreactive and immunosuppressive responses known to occur, respectively, in autoimmunity and cancer.

CONCLUDING REMARKS

The contribution of the glycome as a major regulator of the immune system is clear. Glycans actively participate in the cellular and molecular mechanisms underlying the genesis of the loss of immunological tolerance associated with (auto)immunity, from one hand, participating also in the creation of tolerogenic pathways associated with cancer progression, from the other. The importance of glycans in immune response spans from its role in the modulation the T cell development; their importance as a source of glycoantigens presentation; as well as their role as fine tuners of T cell response. In this context, glycans can exert a dual role, acting either as immune inhibitory checkpoints or as immune stimulatory signals. Understanding in depth the influence of glycans in the immune regulatory circuits that mediate the pathophysiology of autoimmunity and cancer will generate a platform with extraordinary potential to illuminate the identification of novel biomarkers and targets for the development of efficient immunomodulatory strategies with applications in the clinical setting.

AUTHOR CONTRIBUTIONS

All the authors wrote the manuscript. AD and NP created the figures. SP performed the critical review of the manuscript.

FUNDING

The Institute of Molecular Pathology and Immunology of the University of Porto integrates the i3S research unit, which is partially supported by the Portuguese Foundation for Science and Technology (FCT). This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund. This work was also funded by Fundo Europeu de Desenvolvimento Regional (FEDER) funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalization (POCI), Portugal

2020, and by Portuguese funds through the FCT in the framework of the project (POCI-01/ 0145-FEDER-016601 and PTDC/DTP-PIC/0560/2014, as well as POCI-01-0145-FEDER-028772). SSP acknowledges the European Crohn's and Colitis Organization (ECCO) for ECCO Grant 2017, the Broad Medical Research Program at the Crohn's and Colitis Foundation of America, and the Portuguese Group of Study in IBD (GEDII) for funding. MSP [SFRH/BD/110148/2015], IA [SFRH/BD/128874/2017], MV [PD/BD/135452/2017], received funding from the FCT.

ACKNOWLEDGMENTS

AC acknowledges Group of Studies for Autoimmune diseases from Portuguese Society of Internal Medicine (NEDAI) for funding.

REFERENCES

1. Di Lella S, Sundblad V, Cerliani JP, Guardia CM, Estrin DA, Vasta GR, et al. When galectins recognize glycans: from biochemistry to physiology and back again. *Biochemistry* (2011) 50:7842–57. doi: 10.1021/bi201121m
2. Vasta GR, Feng C, González-Montalbán N, Mancini J, Yang L, Abernathy K, et al. Functions of galectins as 'self/non-self'-recognition and effector factors. *Pathog Dis.* (2017) 75:ftx046. doi: 10.1093/femspd/ftx046
3. Brown GD, Willment JA, Whitehead L. C-type lectins in immunity and homeostasis. *Nat Rev Immunol.* (2018) 18:374–89. doi: 10.1038/s41577-018-0004-8
4. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol.* (2014) 14:653–66. doi: 10.1038/nri3737
5. Bochner BS, Zimmermann N. Role of siglecs and related glycan-binding proteins in immune responses and immunoregulation. *J Allergy Clin Immunol.* (2015) 135:598–608. doi: 10.1016/j.jaci.2014.11.031
6. Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, et al., editors. *Essentials of Glycobiology*. 3rd ed. New York, NY: Cold Spring Harbor (2015).
7. Johnson JL, Jones MB, Ryan SO, Cobb BA. The regulatory power of glycans and their binding partners in immunity. *Trends Immunol.* (2013) 34:290–8. doi: 10.1016/j.it.2013.01.006
8. Koch U, Radtke F. Mechanisms of T cell development and transformation. *Annu Rev Cell Dev Biol.* (2011) 27:539–62. doi: 10.1146/annurev-cellbio-092910-154008
9. Kumar BV, Connors TJ, Farber DL. Human T cell development, localization, and function throughout life. *Immunity* (2018) 48:202–13. doi: 10.1016/j.immuni.2018.01.007
10. Krueger A, Zietara N, Łyszkiewicz M. T cell development by the numbers. *Trends Immunol.* (2017) 38:128–39. doi: 10.1016/j.it.2016.10.007
11. Rossi FMV, Corbel SY, Merzaban JS, Carlow DA, Gossens K, Duenas J, et al. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol.* (2005) 6:626–34. doi: 10.1038/ni1203
12. Sultana DA, Zhang SL, Todd SP, Bhandoola A. Expression of functional P-selectin glycoprotein ligand 1 on hematopoietic progenitors is developmentally regulated. *J Immunol.* (2012) 188:4385–93. doi: 10.4049/jimmunol.1101116
13. Shah DK, Zú-iga-Pflücker JC. An overview of the intrathymic intricacies of T cell development. *J Immunol.* (2014) 192:4017–23. doi: 10.4049/jimmunol.1302259
14. Rampal R, Li ASY, Moloney DJ, Georgiou SA, Luther KB, Nita-Lazar A, et al. Lunatic fringe, manic fringe, and radical fringe recognize similar specificity determinants in O-fucosylated epidermal growth factor-like repeats. *J Biol Chem.* (2005) 280:42454–63. doi: 10.1074/jbc.M509552200
15. Matsuura A, Ito M, Sakaidani Y, Kondo T, Murakami K, Furukawa K, et al. O-linked N-acetylglucosamine is present on the extracellular domain of notch receptors. *J Biol Chem.* (2008) 283:35486–95. doi: 10.1074/jbc.M806202200
16. Song Y, Kumar V, Wei HX, Qiu J, Stanley P. Lunatic, manic, and radical fringe each promote T and B cell development. *J Immunol.* (2016) 196:232–43. doi: 10.4049/jimmunol.1402421
17. Koch U, Lacombe TA, Holland D, Bowman JL, Cohen BL, Egan SE, et al. Subversion of the T/B lineage decision in the thymus by lunatic fringe-mediated inhibition of notch-1. *Immunity* (2001) 15:225–36. doi: 10.1016/S1074-7613(01)00189-3
18. Visan I, Yuan JS, Tan JB, Cretegny K, Guidos CJ. Regulation of intrathymic T-cell development by lunatic fringe? Notch1 interactions. *Immunol Rev.* (2006) 209:76–94. doi: 10.1111/j.0105-2896.2006.00360.x
19. Boudil A, Matei IR, Shih HY, Bogdanoski G, Yuan JS, Chang SG, et al. IL-7 coordinates proliferation, differentiation and Tcra recombination during thymocyte β-selection. *Nat Immunol.* (2015) 16:397–405. doi: 10.1038/ni.3122
20. Visan I, Yuan JS, Liu Y, Stanley P, Guidos CJ. Lunatic Fringe enhances competition for Delta-like Notch ligands but does not overcome defective pre-TCR signaling during thymocyte β-selection *in vivo*. *J Immunol.* (2010) 185:4609–17. doi: 10.4049/jimmunol.1002008
21. Hart GW, Housley MP, Slawson C. Cycling of O-linked β-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* (2007) 446:1017–22. doi: 10.1038/nature05815
22. Hart GW. Minireview series on the thirtieth anniversary of research on O-GlcNAcylation of nuclear and cytoplasmic proteins: nutrient regulation of cellular metabolism and physiology by O-GlcNAcylation. *J Biol Chem.* (2014) 289:34422–3. doi: 10.1074/jbc.R114.609776
23. Swamy M, Pathak S, Grzes KM, Damerow S, Sinclair L V, van Aalten DMF, et al. Glucose and glutamine fuel protein O-GlcNAcylation to control T cell self-renewal and malignancy. *Nat Immunol.* (2016) 17:712–20. doi: 10.1038/ni.3439
24. Chou TY, Hart GW, Dang CV. c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. *J Biol Chem.* (1995) 270:18961–5. doi: 10.1074/jbc.270.32.18961
25. Marino JH, Tan C, Davis B, Han ES, Hickey M, Naukam R, et al. Disruption of thymopoiesis in ST6Gal I-deficient mice. *Glycobiology* (2008) 18:719–26. doi: 10.1093/glycob/cwn051
26. Bi S, Baum LG. Sialic acids in T cell development and function. *Biochim Biophys Acta* (2009) 1790:1599–610. doi: 10.1016/j.bbagen.2009.07.027
27. Moody AM, Chui D, Reche PA, Priatel JJ, Marth JD, Reinherz EL. Developmentally regulated glycosylation of the CD8αβ coreceptor stalk modulates ligand binding. *Cell* (2001) 107:501–12. doi: 10.1016/S0092-8674(01)00577-3
28. Shih HY, Hao B, Krangel MS. Orchestrating T-cell receptor α gene assembly through changes in chromatin structure and organization. *Immunol Res.* (2011) 49:192–201. doi: 10.1007/s12026-010-8181-y
29. Takaba H, Takayanagi H. The mechanisms of T cell selection in the thymus. *Trends Immunol.* (2017) 38:805–16. doi: 10.1016/j.it.2017.07.010
30. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* (2001) 409:733–9. doi: 10.1038/35055582
31. Rudd PM, Wormald MR, Stanfield RL, Huang M, Mattsson N, Speir JA, et al. Roles for glycosylation of cell surface receptors involved in cellular immune recognition. *J Mol Biol.* (1999) 293:351–66. doi: 10.1006/jmbi.1999.3104
32. Kuball J, Hauptrock B, Malina V, Antunes E, Voss RH, Wolfl M, et al. Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *J Exp Med.* (2009) 206:463–75. doi: 10.1084/jem.20082487
33. Daniels MA, Devine L, Miller JD, Moser JM, Lukacher AE, Altman JD, et al. CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation. *Immunity* (2001) 15:1051–61. doi: 10.1016/S1074-7613(01)00252-7
34. Zhou RW, Mkhikian H, Grigorian A, Hong A, Chen D, Arakelyan A, et al. N-glycosylation bidirectionally extends the boundaries of thymocyte positive

selection by decoupling Lck from Ca2+ signaling. *Nat Immunol*. (2014) 15:1038–45. doi: 10.1038/ni.3007
35. Artyomov MN, Lis M, Devadas S, Davis MM, Chakraborty AK. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc Natl Acad Sci USA*. (2010) 107:16916–21. doi: 10.1073/pnas.1010568107
36. Clark MC, Baum LG. T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. *Ann N Y Acad Sci*. (2012) 1253:58–67. doi: 10.1111/j.1749-6632.2011.06304.x
37. Marth JD, Grewal PK. Mammalian glycosylation in immunity. *Nat Rev Immunol*. (2008) 8:874–87. doi: 10.1038/nri2417
38. Rossy J, Williamson DJ, Benzing C, Gaus K. The integration of signaling and the spatial organization of the T cell synapse. *Front Immunol*. (2012) 3:352. doi: 10.3389/fimmu.2012.00352
39. Barbosa JA, Santos-Aguado J, Mentzer SJ, Strominger JL, Burakoff SJ, Biro PA. Site-directed mutagenesis of class I HLA genes. Role of glycosylation in surface expression and functional recognition. *J Exp Med*. (1987) 166:1329–50. doi: 10.1084/jem.166.5.1329
40. Unanue ER, Turk V, Neefjes J. Variations in MHC Class II antigen processing and presentation in health and disease. *Annu Rev Immunol*. (2016) 34:265–97. doi: 10.1146/annurev-immunol-041015-055420
41. Ryan SO, Bonomo JA, Zhao F, Cobb BA. MHCII glycosylation modulates Bacteroides fragilis carbohydrate antigen presentation. *J Exp Med*. (2011) 208:1041–53. doi: 10.1084/jem.20100508
42. Dias AM, Pereira MS, Padrão NA, Alves I, Marcos-Pinto R, Lago P, et al. Glycans as critical regulators of gut immunity in homeostasis and disease. *Cell Immunol*. (2018). doi: 10.1016/j.cellimm.2018.07.007. [Epub ahead of print].
43. Fujii H, Shinzaki S, Iijima H, Wakamatsu K, Iwamoto C, Sobajima T, et al. Core Fucosylation on T cells, required for activation of T-cell receptor signaling and induction of colitis in mice, is increased in patients with inflammatory bowel disease. *Gastroenterology* (2016) 150:1620–32. doi: 10.1053/j.gastro.2016.03.002
44. Wolfert MA, Boons GJ. Adaptive immune activation: glycosylation does matter. *Nat Chem Biol*. (2013) 9:776–84. doi: 10.1038/nchembio.1403
45. Hermiston ML, Xu Z, Weiss A. CD45: a critical regulator of signaling thresholds in immune cells. *Annu Rev Immunol*. (2003) 21:107–37. doi: 10.1146/annurev.immunol.21.120601.140946
46. Ohta T, Kitamura K, Maizel AL, Takeda A. Alterations in CD45 glycosylation pattern accompanying different cell proliferation states. *Biochem Biophys Res Commun*. (1994) 200:1283–9. doi: 10.1006/bbrc.1994.1590
47. Rogers PR, Pilapil S, Hayakawa K, Romain PL, Parker DC. CD45 alternative exon expression in murine and human CD4+ T cell subsets. *J Immunol*. (1992) 148:4054–65.
48. Furukawa K, Funakoshi Y, Autero M, Horejsi V, Kobata A, Gahmberg CG. Structural study of the O-linked sugar chains of human leukocyte tyrosine phosphatase CD45. *Eur J Biochem*. (1998) 251:288–94. doi: 10.1046/j.1432-1327.1998.2510288.x
49. Zapata JM, Pulido R, Acevedo A, Sanchez-Madrid F, de Landazuri MO. Human CD45RC specificity. A novel marker for T cells at different maturation and activation stages. *J Immunol*. (1994) 152:3852–61.
50. Daniels MA, Hogquist KA, Jameson SC. Sweet "n" sour: the impact of differential glycosylation on T cell responses. *Nat Immunol*. (2002) 3:903–10. doi: 10.1038/ni1002-903
51. Garcia GG, Berger SB, Sadighi Akha AA, Miller RA. Age-associated changes in glycosylation of CD43 and CD45 on mouse CD4 T cells. *Eur J Immunol*. (2005) 35:622–31. doi: 10.1002/eji.200425538
52. Aruffo A, Seed B. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc Natl Acad Sci USA*. (1987) 84:8573–7. doi: 10.1073/pnas.84.23.8573
53. Araujo L, Khim P, Mkhikian H, Mortales CL, Demetriou M. Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *Elife* (2017) 6:1–16. doi: 10.7554/eLife.21330
54. Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* (1992) 71:1093–102. doi: 10.1016/S0092-8674(05)80059-5
55. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol*. (2001) 1:220–8. doi: 10.1038/35105024
56. Zhu L, Guo Q, Guo H, Liu T, Zheng Y, Gu P, et al. Versatile characterization of glycosylation modification in CTLA4-Ig fusion proteins by liquid chromatography-mass spectrometry. *MAbs* (2014) 6:1474–85. doi: 10.4161/mabs.36313
57. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) 415:536–41. doi: 10.1038/415536a
58. Maverakis E, Kim K, Shimoda M, Gershwin ME, Patel F, Wilken R, et al. Glycans in the immune system and the altered glycan theory of autoimmunity: a critical review. *J Autoimmun*. (2015) 57:1–13. doi: 10.1016/j.jaut.2014.12.002
59. Lee SU, Grigorian A, Pawling J, Chen IJ, Gao G, Mozaffar T, et al. N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *J Biol Chem*. (2007) 282:33725–34. doi: 10.1074/jbc.M704839200
60. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, et al. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci USA*. (2018) 115:E4651–60. doi: 10.1073/pnas.1720409115
61. Togayachi A, Kozono Y, Ishida H, Abe S, Suzuki N, Tsunoda Y, et al. Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation. *Proc Natl Acad Sci USA*. (2007) 104:15829–34. doi: 10.1073/pnas.0707426104
62. Mkhikian H, Mortales CL, Zhou RW, Khachikyan K, Wu G, Haslam SM, et al. Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *Elife* (2016) 5:e14814. doi: 10.7554/eLife.14814
63. Chui D, Sellakumar G, Green R, Sutton-Smith M, McQuistan T, Marek K, et al. Genetic remodeling of protein glycosylation in vivo induces autoimmune disease. *Proc Natl Acad Sci USA*. (2001):1142–7. doi: 10.1073/pnas.98.3.1142
64. Green RS, Stone EL, Tenno M, Lehtonen E, Farquhar MG, Marth JD. Mammalian N-glycan branching protects against innate immune self-recognition and inflammation in autoimmune disease pathogenesis. *Immunity* (2007) 27:308–20. doi: 10.1016/j.immuni.2007.06.008
65. Hashii N, Kawasaki N, Itoh S, Nakajima Y, Kawanishi T, Yamaguchi T. Alteration of N-glycosylation in the kidney in a mouse model of systemic lupus erythematosus: relative quantification of N-glycans using an isotope-tagging method. *Immunology* (2009) 126:336–45. doi: 10.1111/j.1365-2567.2008.02898.x
66. Ye Z, Marth JD. N-glycan branching requirement in neuronal and postnatal viability. *Glycobiology* (2004) 14:547–58. doi: 10.1093/glycob/cwh069
67. Darrah E, Andrade F. NETs: the missing link between cell death and systemic autoimmune diseases? *Front Immunol*. (2013) 3:428. doi: 10.3389/fimmu.2012.00428
68. Miller FW, Lamb JA, Schmidt J, Nagaraju K. Risk factors and disease mechanisms in myositis. *Nat Rev Rheumatol*. (2018) 14:255–68. doi: 10.1038/nrrheum.2018.48
69. McMorran BJ, McCarthy FE, Gibbs EM, Pang M, Marshall JL, Nairn A V, et al. Differentiation-related glycan epitopes identify discrete domains of the muscle glycocalyx. *Glycobiology* (2016) 26:1120–32. doi: 10.1093/glycob/cww061
70. Townsend D. Finding the sweet spot: assembly and glycosylation of the dystrophin-associated glycoprotein complex. *Anat Rec*. (2014) 297:1694–705. doi: 10.1002/ar.22974
71. Malicdan MCV, Noguchi S, Hayashi YK, Nonaka I, Nishino I. Prophylactic treatment with sialic acid metabolites precludes the development of the myopathic phenotype in the DMRV-hIBM mouse model. *Nat Med*. (2009) 15:690–5. doi: 10.1038/nm.1956
72. Szabó TG, Palotai R, Antal P, Tokatly I, Tóthfalusi L, Lund O, et al. Critical role of glycosylation in determining the length and structure of T cell epitopes. *Immunome Res*. (2009) 5:4. doi: 10.1186/1745-7580-5-4

73. van Kooyk Y, Rabinovich GA. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol*. (2008) 9:593–601. doi: 10.1038/ni.f.203
74. Diana J, Moura IC, Vaugier C, Gestin A, Tissandie E, Beaudoin L, et al. Secretory IgA induces tolerogenic dendritic cells through SIGNR1 dampening autoimmunity in mice. *J Immunol*. (2013) 191:2335–43. doi: 10.4049/jimmunol.1300864
75. Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME, et al. Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat Immunol*. (2009) 10:981–91. doi: 10.1038/ni.1772
76. Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, et al. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol*. (2007) 8:825–34. doi: 10.1038/ni1482
77. Clemente T, Vieira NJ, Cerliani JP, Adrain C, Luthi A, Dominguez MR, et al. Proteomic and functional analysis identifies galectin-1 as a novel regulatory component of the cytotoxic granule machinery. *Cell Death Dis*. (2017) 8:e3176. doi: 10.1038/cddis.2017.506
78. Orlacchio A, Sarchielli P, Gallai V, Datti A, Saccardi C, Palmerini CA. Activity levels of a beta1,6 N-acetylglucosaminyltransferase in lymphomonocytes from multiple sclerosis patients. *J Neurol Sci*. (1997) 151:177–83. doi: 10.1016/S0022-510X(97)00117-2
79. Brynedal B, Wojcik J, Esposito F, Debailleul V, Yaouanq J, Martinelli-Boneschi F, et al. MGAT5 alters the severity of multiple sclerosis. *J Neuroimmunol*. (2010) 220:120–4. doi: 10.1016/j.jneuroim.2010.01.003
80. Mkhikian H, Grigorian A, Li CF, Chen HL, Newton B, Zhou RW, et al. Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nat Commun*. (2011) 2:334. doi: 10.1038/ncomms1333
81. Grigorian A, Mkhikian H, Li CF, Newton BL, Zhou RW, Demetriou M. Pathogenesis of multiple sclerosis via environmental and genetic dysregulation of N-glycosylation. *Semin Immunopathol*. (2012) 34:415–24. doi: 10.1007/s00281-012-0307-y
82. Li CF, Zhou RW, Mkhikian H, Newton BL, Yu Z, Demetriou M. Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively with MGAT1 and interleukin-2 and 7 receptor variants. *J Neuroimmunol*. (2013) 256:71–6. doi: 10.1016/j.jneuroim.2012.12.008
83. Dias AM, Dourado J, Lago P, Cabral J, Marcos-Pinto R, Salgueiro P, et al. Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet*. (2014) 23:2416–27. doi: 10.1093/hmg/ddt632
84. Pereira MS, Maia L, Azevedo LF, Campos S, Carvalho S, Dias AM, et al. A [Glyco]biomarker that predicts failure to standard therapy in ulcerative colitis patients. *J Crohns Colitis* (2018). doi: 10.1093/ecco-jcc/jjy139. [Epub ahead of print].
85. Grigorian A, Araujo L, Naidu NN, Place DJ, Choudhury B, Demetriou M. N -Acetylglucosamine Inhibits T-helper 1 (Th1)/T-helper 17 (Th17) cell responses and treats experimental autoimmune encephalomyelitis. *J Biol Chem*. (2011) 286:40133–41. doi: 10.1074/jbc.M111.277814
86. McMorran BJ, Miceli MC, Baum LG. Lectin-binding characterizes the healthy human skeletal muscle glycophenotype and identifies disease-specific changes in dystrophic muscle. *Glycobiology* (2017) 27:1134–43. doi: 10.1093/glycob/cwx073
87. Balasubramanian M, Johnson DS, DDD Study. MAN1B-CDG: Novel variants with a distinct phenotype and review of literature. *Eur J Med Genet*. (2018). doi: 10.1016/j.ejmg.2018.06.011. [Epub ahead of print].
88. Wiendl H, Hohlfeld R, Kieseier BC. Immunobiology of muscle: advances in understanding an immunological microenvironment. *Trends Immunol*. (2005) 26:373–80. doi: 10.1016/j.it.2005.05.003
89. Afzali AM, Müntefering T, Wiendl H, Meuth SG, Ruck T. Skeletal muscle cells actively shape (auto)immune responses. *Autoimmun Rev*. (2018) 17:518–29. doi: 10.1016/j.autrev.2017.12.005
90. RodrÍguez E, Schetters STT, van Kooyk Y. The tumour glyco-code as a novel immune checkpoint for immunotherapy. *Nat Rev Immunol*. (2018) 18:204–11. doi: 10.1038/nri.2018.3
91. Blank C, Mackensen A. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. *Cancer Immunol Immunother*. (2007) 56:739–45. doi: 10.1007/s00262-006-0272-1
92. Okada M, Chikuma S, Kondo T, Hibino S, Machiyama H, Yokosuka T, et al. Blockage of core Fucosylation reduces cell-surface expression of PD-1 and promotes anti-tumor immune responses of T cells. *Cell Rep*. (2017) 20:1017–28. doi: 10.1016/j.celrep.2017.07.027
93. Li CW, Lim SO, Xia W, Lee HH, Chan LC, Kuo CW, et al. Glycosylation and stabilization of programmed death ligand-1 suppresses T-cell activity. *Nat Commun*. (2016) 7:12632. doi: 10.1038/ncomms12632
94. Li CW, Lim SO, Chung EM, Kim YS, Park AH, Yao J, et al. Eradication of triple-negative breast cancer cells by targeting Glycosylated PD-L1. *Cancer Cell*. (2018) 33:187–201.e10. doi: 10.1016/j.ccell.2018.01.009
95. Cabral J, Hanley SA, Gerlach JQ, O'Leary N, Cunningham S, Ritter T, et al. Distinctive surface Glycosylation patterns associated with mouse and human CD4+ regulatory T cells and their suppressive function. *Front Immunol*. (2017) 8:987. doi: 10.3389/fimmu.2017.00987
96. Dennis JW, Lau KS, Demetriou M, Nabi IR. Adaptive regulation at the cell surface by N-glycosylation. *Traffic* (2009) 10:1569–78. doi: 10.1111/j.1600-0854.2009.00981.x
97. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* (2015) 15:540–55. doi: 10.1038/nrc3982
98. Rodrigues JG, Balmaña M, Macedo JA, Poças J, Fernandes Â, De-Freitas-Junior JCM, et al. Glycosylation in cancer: selected roles in tumour progression, immune modulation and metastasis. *Cell Immunol*. (2018). doi: 10.1016/j.cellimm.2018.03.007. [Epub ahead of print].
99. van Liempt E, Bank CMC, Mehta P, Garci'a-Vallejo JJ, Kawar ZS, Geyer R, et al. Specificity of DC-SIGN for mannose- and fucose-containing glycans. *FEBS Lett*. (2006) 580:6123–31. doi: 10.1016/j.febslet.2006.10.009
100. van Gisbergen KPJM, Aarnoudse CA, Meijer GA, Geijtenbeek TBH, van Kooyk Y. Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin. *Cancer Res*. (2005) 65:5935–44. doi: 10.1158/0008-5472.CAN-04-4140
101. Gringhuis SI, Kaptein TM, Wevers BA, van der Vlist M, Klaver EJ, van Die I, et al. Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production. *Nat Commun*. (2014) 5:5074. doi: 10.1038/ncomms6074
102. García-Vallejo JJ, Ilarregui JM, Kalay H, Chamorro S, Koning N, Unger WW, et al. CNS myelin induces regulatory functions of DC-SIGN-expressing, antigen-presenting cells via cognate interaction with MOG. *J Exp Med*. (2014) 211:1465–83. doi: 10.1084/jem.20122192
103. Unger WWJ, van Beelen AJ, Bruijns SC, Joshi M, Fehres CM, van Bloois L, et al. Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells. *J Control Release* (2012) 160:88–95. doi: 10.1016/j.jconrel.2012.02.007
104. van Vliet SJ, Bay S, Vuist IM, Kalay H, García-Vallejo JJ, Leclerc C, et al. MGL signaling augments TLR2-mediated responses for enhanced IL-10 and TNF-α secretion. *J Leukoc Biol*. (2013) 94:315–23. doi: 10.1189/jlb.1012520
105. van Vliet SJ, Gringhuis SI, Geijtenbeek TBH, van Kooyk Y. Regulation of effector T cells by antigen-presenting cells via interaction of the C-type lectin MGL with CD45. *Nat Immunol*. (2006) 7:1200–8. doi: 10.1038/ni1390
106. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CMT, Pryer N, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell* (2014) 26:623–37. doi: 10.1016/j.ccell.2014.09.006
107. Smith LK, Boukhaled GM, Condotta SA, Mazouz S, Guthmiller JJ, Vijay R, et al. Interleukin-10 directly inhibits CD8(+) T cell function by enhancing N-Glycan branching to decrease antigen sensitivity. *Immunity* (2018) 48:299–312 e5. doi: 10.1016/j.immuni.2018.01.006
108. Perdicchio M, Ilarregui JM, Verstege MI, Cornelissen LAM, Schetters STT, Engels S, et al. Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and *de novo* induction of regulatory T cells. *Proc Natl Acad Sci USA*. (2016) 113:3329–34. doi: 10.1073/pnas.1507706113
109. Perdicchio M, Cornelissen LAM, Streng-Ouwehand I, Engels S, Verstege MI, Boon L, et al. Tumor sialylation impedes T cell mediated anti-tumor responses while promoting tumor associated-regulatory T cells. *Oncotarget* (2016) 7:8771–82. doi: 10.18632/oncotarget.6822

110. Beatson R, Tajadura-Ortega V, Achkova D, Picco G, Tsourouktsoglou TD, Klausing S, et al. The mucin MUC1 modulates the tumor immunological microenvironment through engagement of the lectin Siglec-9. *Nat Immunol*. (2016) 17:1273–81. doi: 10.1038/ni.3552
111. Carrascal MA, Severino PF, Guadalupe Cabral M, Silva M, Ferreira JA, Calais F, et al. Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. *Mol Oncol*. (2014) 8:753–65. doi: 10.1016/j.molonc.2014.02.008
112. Julien S, Videira PA, Delannoy P. Sialyl-tn in cancer: (how) did we miss the target? *Biomolecules* (2012):435–66. doi: 10.3390/biom2040435
113. Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, von Gunten S, et al. Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells. *J Clin Invest*. (2018) 128:4912–23. doi: 10.1172/JCI120612
114. Garín MI, Chu C-C, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* (2007) 109:2058–65. doi: 10.1182/blood-2006-04-016451
115. Stillman BN, Hsu DK, Pang M, Brewer CF, Johnson P, Liu F-T, et al. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol*. (2006) 176:778–89. doi: 10.4049/jimmunol.176.2.778
116. Matarrese P, Tinari A, Mormone E, Bianco GA, Toscano MA, Ascione B, et al. Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J Biol Chem*. (2005) 280:6969–85. doi: 10.1074/jbc.M409752200
117. Rabinovich GA, van Kooyk Y, Cobb BA. Glycobiology of immune responses. *Ann N Y Acad Sci*. (2012) 1253:1–15. doi: 10.1111/j.1749-6632.2012.06492.x
118. Yang RY, Hsu DK, Liu FT. Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci USA*. (1996) 93:6737–42. doi: 10.1073/pnas.93.13.6737
119. Lau KS, Partridge EA, Grigorian A, Silvescu CI, Reinhold VN, Demetriou M, et al. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* (2007) 129:123–34. doi: 10.1016/j.cell.2007.01.049
120. Kouo T, Huang L, Pucsek AB, Cao M, Solt S, Armstrong T, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of Plasmacytoid dendritic cells. *Cancer Immunol Res*. (2015) 3:412–23. doi: 10.1158/2326-6066.CIR-14-0150
121. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. (2005) 6:1245–52. doi: 10.1038/ni1271
122. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner. *Clin Immunol*. (2012) 143:51–8. doi: 10.1016/j.clim.2012.01.004
123. Kang CW, Dutta A, Chang LY, Mahalingam J, Lin YC, Chiang JM, et al. Apoptosis of tumor infiltrating effector TIM-3+CD8+ T cells in colon cancer. *Sci Rep*. (2015) 5:15659. doi: 10.1038/srep15659
124. Su EW, Bi S, Kane LP. Galectin-9 regulates T helper cell function independently of Tim-3. *Glycobiology* (2011) 21:1258–65. doi: 10.1093/glycob/cwq214
125. Almeida L, Lochner M, Berod L, Sparwasser T. Metabolic pathways in T cell activation and lineage differentiation. *Semin Immunol*. (2016) 28:514–24. doi: 10.1016/j.smim.2016.10.009
126. Wang T, Marquardt C, Foker J. Aerobic glycolysis during lymphocyte proliferation. *Nature* (1976) 261:702–5. doi: 10.1038/261702a0
127. Wang R, Green DR. Metabolic checkpoints in activated T cells. *Nat Immunol*. (2012) 13:907–15. doi: 10.1038/ni.2386
128. Wellen KE, Thompson CB. A two-way street: reciprocal regulation of metabolism and signalling. *Nat Rev Mol Cell Biol*. (2012) 13:270–6. doi: 10.1038/nrm3305
129. Abdel Rahman AM, Ryczko M, Pawling J, Dennis JW. Probing the hexosamine biosynthetic pathway in human tumor cells by multitargeted tandem mass spectrometry. *ACS Chem Biol*. (2013) 8:2053–62. doi: 10.1021/cb4004173
130. Grigorian A, Lee SU, Tian W, Chen IJ, Gao G, Mendelsohn R, et al. Control of T cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. *J Biol Chem*. (2007) 282:20027–35. doi: 10.1074/jbc.M701890200
131. Love DC, Hanover JA. The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE* (2005) 2005:re13. doi: 10.1126/stke.3122005re13
132. Kreppel LK, Hart GW. Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. *J Biol Chem*. (1999) 274:32015–22. doi: 10.1074/jbc.274.45.32015
133. Sasai K, Ikeda Y, Fujii T, Tsuda T, Taniguchi N. UDP-GlcNAc concentration is an important factor in the biosynthesis of beta1,6-branched oligosaccharides: regulation based on the kinetic properties of N-acetylglucosaminyltransferase V. *Glycobiology* (2002) 12:119–27. doi: 10.1093/glycob/12.2.119
134. Ryczko MC, Pawling J, Chen R, Abdel Rahman AM, Yau K, Copeland JK, et al. Metabolic reprogramming by hexosamine biosynthetic and golgi N-Glycan branching pathways. *Sci Rep*. (2016) 6:23043. doi: 10.1038/srep23043
135. Chien MW, Lin MH, Huang SH, Fu SH, Hsu CY, Yen BLJ, et al. Glucosamine modulates T cell differentiation through down-regulating N-linked Glycosylation of CD25. *J Biol Chem*. (2015) 290:29329–44. doi: 10.1074/jbc.M115.674671
136. Zhang GX, Yu S, Gran B, Rostami A. Glucosamine abrogates the acute phase of experimental autoimmune encephalomyelitis by induction of Th2 response. *J Immunol*. (2005) 175:7202–8. doi: 10.4049/jimmunol.175.11.7202

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter VII

General Discussion

The heterogeneity that characterize IBD is driven by the complex interaction between genetic, microbial and environmental factors, which trigger an abnormal immune response. The complex mechanisms underlying the pathogenesis of IBD explain the differences in disease severity as well as in the therapy outcome of IBD patients. As described in Chapter I and Chapter VI, glycosylation is a dynamic process that is exhibited in most proteins from immune system and is involved in the regulation of cellular immunity, host-pathogen interaction, maintenance of homeostasis as well as in the loss of immune tolerance (1-4). Previous work of our group has shown that UC patients display a dysregulation on *MGAT5* and branched N-glycans expression in lamina propria T cells, which is associated with an exacerbated immune response (5, 6). In this thesis we focused our attention on *MGAT5* - mediated branched N-glycans and explore its relevance in the three major points of IBD: genetics, microbiome and immune response related with IgG, always envisioning clinical applications. We found that dysregulation on branching *N*-glycans increases the probability to develop a severe outcome of IBD, being associated with the need for more aggressive therapies. We propose glycan- based biomarkers with the ability to detect this dysregulation early in disease course, as well as, a biomarker based on IgG glycome that is associated with immunogenicity issues against the drug.

The histological information obtained from colonic biopsies is routinely performed in the clinical practice helping IBD diagnosis and monitoring (7). Taking into consideration that a colonic biopsy is a snapshot of the inflammatory state of the patient, relevant *in situ* information can be further explored from biopsies. On this regard, the *in situ* glycosylation-signature is able to provide important molecular information that can be translated into clinical and prognostic value. The results presented in this thesis (Chapter II) supports the identification of a novel glycobiomarker - based on branched N-glycans on inflammatory infiltrate of lamina propria (8) - that might be useful for clinicians to take into consideration at the time of UC diagnosis, for a guidance on the therapy management.

Our results (presented on chapter II) suggest that UC patients displaying low levels of branching N-glycans early at diagnosis have higher probability to fail standard therapy, with the need for a more aggressive drug, such as anti-TNF. Moreover, we found that T cells isolated from lamina propria of non-responders to the conventional therapy display lower levels of branched N-glycans than T cells from responders. Furthermore, in chapter III, we found that UC patients might be genetically predisposed

to express low levels of *MGAT5* mRNA in both colonic and circulating T cells, in the case they possess specific SNPs from the intronic region of *MGAT5*. This genetic alteration might explain the deficiency on branched N-glycans on T cells associated with a hyperimmune T cell response (6, 9, 10), which is supported by similar evidences in MS that showed the influence of the same SNPs on the levels of branching N-glycans of serum T cells (11). Moreover, these functional genetic variants of *MGAT5* seem to be associated with the therapeutic outcome of the UC patients, specifically with the need to step-up to biologics during disease course, similarly to the *in situ* glycobiomarker. However, results should be validated in a larger and more recent cohort. It would be interesting to evaluate whether these SNPs might influence T cell activation. In fact, these genetic variants seem to predispose UC patients to a higher pro-inflammatory scenario, due to its association with a pro-inflammatory phenotype of IgG glycome. Once Fc from IgG only contains biantennary *N*-linked glycan, the influence of *MGAT5* SNPs, which catalyze the formation of tetra antennary complex *N*-glycans, can not be direct. We suggest that IgG glycosylation profile could be mediated by T cell hyperactivation. Indeed, under inflammatory conditions, T cell hyperactivation (which can be triggered by a deficiency on *MGAT5*-mediated branched glycosylation) may induce B cell activation and plasma cells to produce pro-inflammatory IgGs with agalactosylation (12). Accordingly, increased agalactosylation on IgG from UC patients had been shown to be associated with higher severity of the disease, specifically with higher disease extension and with need for colectomy (13), being also shown to be increased in UC patients treated with anti-TNF when comparing with those under azathioprine/6-mercaptopurine (13). Furthermore, naïve and memory B cells were reported to express *MGAT5*-mediated branched N-glycans at the surface, replete with poly-N-acetyllactosamine (poly-LacNAc), which serve as binding sites to many galectins (14, 15). Galectin-9 were found to be highly binding to both naïve and memory B cells. Interestingly, galectin-9 was found to regulate BCR signaling, antagonizing naïve B cell activation (14, 15). It would be worth to explore whether *MGAT5* SNPs might be interfering with B cell N-glycosylation and whether UC patients display this deficiency.

The genetic variants that we identified as being associated with lower levels of *MGAT5* mRNA expression in circulating T cells, were located in intronic regions of the glycogene. Data from our group support the existence of an enhancer activity of the regions containing the studied SNPs, particularly in the thymus and brain, using zebrafish as model. This activity is lost in the presence of the genotypes associated with lower mRNA levels, suggesting the SNPs as potential sites for transcription factors binding (data developed in a master thesis of the group). UC patients with predisposition to

exhibit a deficiency in *MGAT5* expression T cell might display an impairment in T cell development, once branching N-glycans were already demonstrated to be important in both positive and negative T cell selection in the thymus, thus impairing responsiveness and self-tolerance of T cells (16). Also, the association with the inability of UC patients to respond to the standard therapy indicate an incapacity to control T cell growth and the threshold of T cell activation, which imposes a preferential effector T cell-mediated immune response. In fact, branching N-glycans in T regulatory cells were demonstrated to be crucial for its suppressive activity against CD4+ and CD8+ T cells (17), supporting that the deficiency on MAGT5/branching N-glycan will also impair immune tolerance.

In addition to the intronic SNPs, we also identified genetic variants in regulatory regions of *MGAT5*, as 3'UTR, that were associated with patients who developed a severe UC. 3'UTR are targeted by microRNAs, leading to translation repression and/or degradation of the mRNA (18). SNPs within the site of microRNA recognition might impact on its repressive regulation, or create a new seed site for the molecule, thus inducing degradation. Despite the functional effects of these SNPs were not explored, our results suggest that genetic variants of *MGAT5* might be implicated in the predisposition of an individual to be unable to ensure the branched *N*-glycan demanding of a specific cell in a certain condition. Thus, genetic variants on *MGAT5* might impact on T cell glycosylation and consequently on its activity, being also associated with disease severity of the patients. These results suggest the importance of a genetic screening of *MGAT5* as a way to extrapolate possible *MGAT5* gene regulation issues, which might indicate higher predisposition to develop a severe disease.

In addition to these evidences, in chapter IV we further showed for the first time that deficiency in *MGAT5* increases susceptibility to develop a severe DSS-induced colitis in mice potentially due to the predisposition of a dysbiotic scenario. We observed low abundance of bacteria belonging to *Firmicutes* phyla, mainly *Laschnospiraceae* family and a *Ruminococcaceae* genera, which are shown to be under-represented in IBD patients (19-22). These bacteria are producers of short chain fatty acids that serve as carbon source for intestinal epithelial cells and induce Treg cells (23, 24). The natural dysbiotic condition of a mice lacking *MGAT5* might result from or promote the characteristic hyperactivated profile of the T cells (6, 9, 10). Whether dysbiosis contributes to the initiation of IBD or is a secondary change caused by intestinal inflammation and/or medication is still unclear. The threshold of activation of the T cells and its capacity to control activation are lower in Mgat5$^{-/-}$ mice than in wt (6, 9, 10). Thus, the basal micro-environment that microbiota inhabit is more stringent, only remaining the most resistant and the more adapted to an inflammatory- related scenario.

How is the microbiota of individuals predisposed to express lower levels of *MGAT5* and branched *N*-glycans? Is their microbiota more susceptible to external factors? Upon antibiotic exposure, are they able to restore microbiota composition properly? Normal colon is possibly in a continuous state of low-grade inflammation due to the microbial flora. It is tempting to suggest that host predisposition to display altered branching *N*-glycans might confer a risk to develop IBD, once they might display a more reactive immune response that may hamper the restore of microbiota upon challenged. It would be interesting to evaluate the microbiome composition of IBD patients carrying the risk alleles of *MGAT5* gene in order to understand whether these patients are more prone to possess a dysbiotic condition. Also, and considering the findings in DSS-induced colitis model, IBD patients with a deficiency in *MGAT5* expression might benefit from fecal microbiota transplantation (FMT), once we verified that Mgat5$^{-/-}$ co-housing with WT decrease the susceptibility to develop a severe disease and recover faster than singe housing Mgat5$^{-/-}$.

A promisor finding was the discovery of a community of bacteria that are able to induce branching N-glycans on T cells from the host, controlling the T cell immune response. The bacteria behind this mechanism belong to *Firmicutes* phylum, including bacteria that is known to be benefic for host homeostasis and IBD disease outcome, as those from *Laschnospiraceae* family (25, 26). This mechanism and the bacteria behind it need to be further explored; however, our results suggest that host might be taking advantage from the UDP-N-acetyl-D-glucosamine (GlcNAc), the substrate of GnT-V enzyme, released by bacteria once there is an enrichment of the bacteria with GlcNAc biosynthesis capacity. In fact, rectal administration of GlcNAc in DSS- induced Mgat5$^{-/-}$ mice were shown to decrease colitis severity and to induce branching N-glycans production in colonic T cells (6). The improvement of the IBD symptoms in children refractory to treatment were also observed when GlcNAc was giving orally or rectally (27). A probiotic containing higher abundance of bacteria with capacity to produce UDP-N-acetyl-D-glucosamine biosynthesis would improve self-tolerance in IBD patients who display deficiency in *MGAT5* or branching N-glycans expression. Taking advantage for the fact that branching N-glycosylation can be changed upon metabolic supplementation directly or indirectly by microbiota and that this supplementation ameliorates colitis symptoms and slows-down the T cell immune response, it would be worthy to consider as an option in patients displaying a deficit in *MGAT5* expression or branching N-glycans.

The explosion of a new era of targeted-specific drugs that aimed to overcome the ineffectiveness of the standard medication, give rise to a panoply of IBD clinical choices (28, 29). Anti-TNF therapy (mainly infliximab and adalimumab) remains as a first-line

biologic agent given the elevated efficacy to control of immune response. However, the effectiveness of anti-TNF therapy is not transversal to all IBD patients, given that some do not respond and others lose response over time (30).

In chapter V, in this case in Crohn's disease, we identified a distinct *N*-glycan profile in the constant fraction of the IgG suggestive of being associated with loss of response to Anti-TNF and the development of immunogenicity against the drug. The IgG effector function is dictated by the glycan profile that the Fc fraction of the IgG possess, which might switch from anti-inflammatory to pro-inflammatory function, usually associated with increased antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity (31-35). Less elongated N-glycans from IgG (low galactosylation, sialylation and high bisecting), associated with pro-inflammatory IgG function, has been observed in IBD patients in comparison with healthy individuals (13, 36-39). However, not all patients carry the same glycan profile, which might reflect the highly heterogeneity among IBD patients in terms of disease clinical and therapeutic outcomes.

We verify that the profile of IgG glycosylation is distinct in secondary non-responders to infliximab and adalimumab. A low proportion of sialylated IgGs along time of therapy, which is associated with IgGs that loss the anti-inflammatory function (40, 41), was observed in secondary non-responders to both anti-TNF therapies. The increased production of pro-inflammatory IgGs against microbial elements or autoantigens in IBD (42, 43) is a reflection of the increased number of plasma cells in the intestinal lamina propria (44), which can be activated in a T cell-dependent manner and might impair the control of the disease. The lack of anti-inflammatory activity of non-sialylated IgG Fc, might be leading to higher clearance of gut microorganisms for instance, which might impact on the outcome of the disease. In fact, alterations on microbiota have been shown to be associated with refractory CD patients to anti-TNF therapy (26). The proinflammatory scenario of the IgG potentiated by the alterations on IgG might per se overcome the effectiveness of anti-TNF therapies, explaining the loss of response to anti-TNF therapy.

The development of antibodies against anti-TNF drug may also occur in IBD patients and is associated with the reduction of efficacy and a loss of response to the drug (45). Biologic therapies carry an intrinsic risk of immunogenicity, which might be dependent on intrinsic factors of the drug, as well as, in the genetic background of the patient (45, 46). Genetic markers have been identified to be associated with IBD patients who developed immunogenicity against the anti-TNF drugs (47, 48). Our results suggest an association between IgG Fc glycosylation and immunogenicity issues. The IgG fc from patients with elevated levels of antibodies anti-drugs is mainly composed by

elevated galactosylation and IgG4 subtype display low sialylation, which is in accordance with the profile observed in secondary non responders to adalimumab. The possible mechanism behind this association should be explored and the results should be confirmed in a bigger cohort.

IgG glycosylation has been reported to be partially contributed by genetic predisposition (49-51). Indeed, we had also identified genetic variants on *MGAT5* that are associated with lower galactosylation in IgG in the context of UC. The same genetic variants seem to predispose UC patients to develop a severe disease, with the need of more aggressive therapy to control inflammation. In fact, in rheumatoid arthritis, the low levels of galactosylated IgG can be detected years before diagnosis (52). This reinforces our results and the importance of IgG glycome as a helpful biomarker in the identification of CD patients that will fail anti-TNF response (or infliximab or adalimumab), redirecting patients early in disease course for the therapy that will benefit the most. It would be interesting to evaluate whether the same observations that we verified in CD patients concerning response to adalimumab and infliximab are transversal to UC patients. In a positive scenario, would UC patients with the *MGAT5* SNPs associated with low galactosylated IgG benefit for adalimumab rather than infliximab?

This study should be validated in a larger cohort, only including one sample per patient, preferentially at the same time of therapy duration. Most of the secondary non-responders' patients entered in the study already with the failure in response. This does not allow to understand whether the profile of glycosylation is printed in patient since the beginning, once genetic predisposition might be one explanation behind this phenotype. As so, a prospective study including samples from induction phase and maintenance phase, as well as, samples before and after loss of response, would be important.

Taken together, our results support the proposal of a non-invasive marker, a specific glycosylation pattern of a subset of IgGs, with the potential to identify secondary non-responders or even causing the loss of effect of infliximab and adalimumab.

Concluding Remarks

How does this thesis help the clinical practice of IBD patients? All evidences reported in this doctoral thesis tapered to the same point: N-glycosylation is a critical process in IBD, which is frequently dysregulated in lamina propria lymphocyte and circulating T cells and IgGs. This dysregulation might trigger a hyperimmune activation and microbiome alterations, which might underlie the immunopathogenesis of IBD.

With this thesis we identified *in situ* and serum glycan-based biomarkers with high potential to be included in clinical practice of IBD patients, mainly in therapy decision-making process. We believe that the findings of this thesis will lead to the refinement of the clinical management of patients with IBD:

- At diagnosis, patients might be guided for a step-up or step-down therapy, depending on the levels of *MGAT5* mediated branching *N*-glycans in colonic lamina propria;
- Patients carrying specific genetic variants on *MGAT5* are more prone to harbor *MGAT5* transcription alterations in T cells associated with T cell hyperactivity and concomitantly a pro-inflammatory IgG glycome, suggesting their guidance to a more portent therapy as biologics;
- Branching *N*-glycans mediated by *MGAT5* are involved in the interplay between host and microbiome, being important for the maintenance of a homeostatic microbial community;
- IgG glycome seems to be able to stratify patients according to their response to anti-TNF and to be associated with immunogenicity issues, which might be useful in a proactive therapy drug monitoring.

The early detection, either by *MGAT5* genetic screening or by determination of the levels of branching *N*-glycans *in situ* or IgG glycome, at diagnosis, it would be a valuable tool for therapy decision. Summing up, *N*-glycans might be used as promising biomarkers to be used in the clinical practice of IBD.

References

1. Marth JD, Grewal PK. 2008. Mammalian glycosylation in immunity. *Nat Rev Immunol* 8: 874-87
2. Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell* 126: 855-67
3. Pereira MS, Alves I, Vicente M, Campar A, Silva MC, Padrao NA, Pinto V, Fernandes A, Dias AM, Pinho SS. 2018. Glycans as Key Checkpoints of T Cell Activity and Function. *Front Immunol* 9: 2754
4. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. 2001. Glycosylation and the immune system. *Science* 291: 2370-6
5. Dias AM, Dourado J, Lago P, Cabral J, Marcos-Pinto R, Salgueiro P, Almeida CR, Carvalho S, Fonseca S, Lima M, Vilanova M, Dinis-Ribeiro M, Reis CA, Pinho SS. 2014. Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 23: 2416-27
6. Dias AM, Correia A, Pereira MS, Almeida CR, Alves I, Pinto V, Catarino TA, Mendes N, Leander M, Oliva-Teles MT, Maia L, Delerue-Matos C, Taniguchi N, Lima M, Pedroto I, Marcos-Pinto R, Lago P, Reis CA, Vilanova M, Pinho SS. 2018. Metabolic control of T cell immune response through glycans in inflammatory bowel disease. *Proc Natl Acad Sci U S A* 115: E4651-E60
7. Canavese G, Villanacci V, Sapino A, Rocca R, Daperno M, Suriani R, Maletta F, Cassoni P, Members of the Piedmont IBDG. 2015. The diagnosis of inflammatory bowel disease is often unsupported in clinical practice. *Dig Liver Dis* 47: 20-3
8. Pereira MS, Maia L, Azevedo LF, Campos S, Carvalho S, Dias AM, Albergaria A, Lima J, Marcos-Pinto R, Lago P, Pinho SS. 2019. A [Glyco]biomarker that Predicts Failure to Standard Therapy in Ulcerative Colitis Patients. *J Crohns Colitis* 13: 39-49
9. Demetriou M, Granovsky M, Quaggin S, Dennis JW. 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409: 733-9
10. Morgan R, Gao G, Pawling J, Dennis JW, Demetriou M, Li B. 2004. N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. *J Immunol* 173: 7200-8
11. Li CF, Zhou RW, Mkhikian H, Newton BL, Yu Z, Demetriou M. 2013. Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively

with MGAT1 and interleukin-2 and 7 receptor variants. *J Neuroimmunol* 256: 71-6

12. Hess C, Winkler A, Lorenz AK, Holecska V, Blanchard V, Eiglmeier S, Schoen AL, Bitterling J, Stoehr AD, Petzold D, Schommartz T, Mertes MM, Schoen CT, Tiburzy B, Herrmann A, Kohl J, Manz RA, Madaio MP, Berger M, Wardemann H, Ehlers M. 2013. T cell-independent B cell activation induces immunosuppressive sialylated IgG antibodies. *J Clin Invest* 123: 3788-96
13. Simurina M, de Haan N, Vuckovic F, Kennedy NA, Stambuk J, Falck D, Trbojevic-Akmacic I, Clerc F, Razdorov G, Khon A, Latiano A, D'Inca R, Danese S, Targan S, Landers C, Dubinsky M, Inflammatory Bowel Disease Biomarkers C, McGovern DPB, Annese V, Wuhrer M, Lauc G. 2018. Glycosylation of Immunoglobulin G Associates With Clinical Features of Inflammatory Bowel Diseases. *Gastroenterology* 154: 1320-33 e10
14. Giovannone N, Liang J, Antonopoulos A, Geddes Sweeney J, King SL, Pochebit SM, Bhattacharyya N, Lee GS, Dell A, Widlund HR, Haslam SM, Dimitroff CJ. 2018. Galectin-9 suppresses B cell receptor signaling and is regulated by I-branching of N-glycans. *Nat Commun* 9: 3287
15. Giovannone N, Smith LK, Treanor B, Dimitroff CJ. 2018. Galectin-Glycan Interactions as Regulators of B Cell Immunity. *Front Immunol* 9: 2839
16. Zhou RW, Mkhikian H, Grigorian A, Hong A, Chen D, Arakelyan A, Demetriou M. 2014. N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* 15: 1038-45
17. Cabral J, Hanley SA, Gerlach JQ, O'Leary N, Cunningham S, Ritter T, Ceredig R, Joshi L, Griffin MD. 2017. Distinctive Surface Glycosylation Patterns Associated With Mouse and Human CD4(+) Regulatory T Cells and Their Suppressive Function. *Front Immunol* 8: 987
18. Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215-33
19. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. 2011. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 60: 631-7
20. Sokol H, Seksik P, Rigottier-Gois L, Lay C, Lepage P, Podglajen I, Marteau P, Dore J. 2006. Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis* 12: 106-11
21. Vigsnaes LK, van den Abbeele P, Sulek K, Frandsen HL, Steenholdt C, Brynskov J, Vermeiren J, van de Wiele T, Licht TR. 2013. Microbiotas from UC patients

display altered metabolism and reduced ability of LAB to colonize mucus. *Sci Rep* 3: 1110

22. Nagao-Kitamoto H, Kamada N. 2017. Host-microbial Cross-talk in Inflammatory Bowel Disease. *Immune Netw* 17: 1-12
23. Topping DL, Clifton PM. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev* 81: 1031-64
24. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504: 451-5
25. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, Giannoukos G, Ciulla D, Tabbaa D, Ingram J, Schauer DB, Ward DV, Korzenik JR, Xavier RJ, Bousvaros A, Alm EJ. 2012. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. *PLoS One* 7: e39242
26. Yilmaz B, Juillerat P, Oyas O, Ramon C, Bravo FD, Franc Y, Fournier N, Michetti P, Mueller C, Geuking M, Pittet VEH, Maillard MH, Rogler G, Swiss IBDCI, Wiest R, Stelling J, Macpherson AJ. 2019. Microbial network disturbances in relapsing refractory Crohn's disease. *Nat Med* 25: 323-36
27. Salvatore S, Heuschkel R, Tomlin S, Davies SE, Edwards S, Walker-Smith JA, French I, Murch SH. 2000. A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment Pharmacol Ther* 14: 1567-79
28. Danese S, Vuitton L, Peyrin-Biroulet L. 2015. Biologic agents for IBD: practical insights. *Nat Rev Gastroenterol Hepatol* 12: 537-45
29. Coskun M, Vermeire S, Nielsen OH. 2017. Novel Targeted Therapies for Inflammatory Bowel Disease. *Trends Pharmacol Sci* 38: 127-42
30. Roda G, Jharap B, Neeraj N, Colombel JF. 2016. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* 7: e135
31. Kaneko Y, Nimmerjahn F, Ravetch JV. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313: 670-3
32. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, Wang LX, Munz C, Nimmerjahn F, Dalakas MC, Lunemann JD. 2015. Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J Clin Invest* 125: 4160-70
33. Zou G, Ochiai H, Huang W, Yang Q, Li C, Wang LX. 2011. Chemoenzymatic synthesis and Fcgamma receptor binding of homogeneous glycoforms of

antibody Fc domain. Presence of a bisecting sugar moiety enhances the affinity of Fc to FcgammaIIIa receptor. *J Am Chem Soc* 133: 18975-91

34. Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M. 2001. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. *Biotechnol Bioeng* 74: 288-94
35. Nimmerjahn F, Anthony RM, Ravetch JV. 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. *Proc Natl Acad Sci U S A* 104: 8433-7
36. Clerc F, Novokmet M, Dotz V, Reiding KR, de Haan N, Kammeijer GSM, Dalebout H, Bladergroen MR, Vukovic F, Rapp E, Consortium I-B, Targan SR, Barron G, Manetti N, Latiano A, McGovern DPB, Annese V, Lauc G, Wuhrer M. 2018. Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. *Gastroenterology* 155: 829-43
37. Miyoshi E, Shinzaki S, Fujii H, Iijima H, Kamada Y, Takehara T. 2016. Role of aberrant IgG glycosylation in the pathogenesis of inflammatory bowel disease. *Proteomics Clin Appl* 10: 384-90
38. Shinzaki S, Kuroki E, Iijima H, Tatsunaka N, Ishii M, Fujii H, Kamada Y, Kobayashi T, Shibukawa N, Inoue T, Tsujii M, Takeishi S, Mizushima T, Ogata A, Naka T, Plevy SE, Takehara T, Miyoshi E. 2013. Lectin-based immunoassay for aberrant IgG glycosylation as the biomarker for Crohn's disease. *Inflamm Bowel Dis* 19: 321-31
39. Trbojevic Akmacic I, Ventham NT, Theodoratou E, Vuckovic F, Kennedy NA, Kristic J, Nimmo ER, Kalla R, Drummond H, Stambuk J, Dunlop MG, Novokmet M, Aulchenko Y, Gornik O, Campbell H, Pucic Bakovic M, Satsangi J, Lauc G, Consortium I-B. 2015. Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome. *Inflamm Bowel Dis* 21: 1237-47
40. Gudelj I, Lauc G, Pezer M. 2018. Immunoglobulin G glycosylation in aging and diseases. *Cell Immunol* 333: 65-79
41. Anthony RM, Kobayashi T, Wermeling F, Ravetch JV. 2011. Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature* 475: 110-3
42. Uzzan M, Colombel JF, Cerutti A, Treton X, Mehandru S. 2016. B Cell-Activating Factor (BAFF)-Targeted B Cell Therapies in Inflammatory Bowel Diseases. *Dig Dis Sci* 61: 3407-24

43. Zhou G, Song Y, Yang W, Guo Y, Fang L, Chen Y, Liu Z. 2016. ASCA, ANCA, ALCA and Many More: Are They Useful in the Diagnosis of Inflammatory Bowel Disease? *Dig Dis* 34: 90-7
44. Magro F, Langner C, Driessen A, Ensari A, Geboes K, Mantzaris GJ, Villanacci V, Becheanu G, Borralho Nunes P, Cathomas G, Fries W, Jouret-Mourin A, Mescoli C, de Petris G, Rubio CA, Shepherd NA, Vieth M, Eliakim R, European Society of P, European Cs, Colitis O. 2013. European consensus on the histopathology of inflammatory bowel disease. *J Crohns Colitis* 7: 827-51
45. Vermeire S, Gils A, Accossato P, Lula S, Marren A. 2018. Immunogenicity of biologics in inflammatory bowel disease. *Therap Adv Gastroenterol* 11: 1756283X17750355
46. Dreesen E, Gils A, Vermeire S. 2018. Pharmacokinetic Modeling and Simulation of Biologicals in Inflammatory Bowel Disease: The Dawning of a New Era for Personalized Treatment. *Curr Drug Targets* 19: 757-76
47. Sazonovs A, Kennedy NA, Moutsianas L, Heap GA, Rice DL, Reppell M, Bewshea CM, Chanchlani N, Walker GJ, Perry MH, McDonald TJ, Lees CW, Cummings JRF, Parkes M, Mansfield JC, Irving PM, Barrett JC, McGovern D, Goodhand JR, Anderson CA, Ahmad T, Consortium P. 2020. HLA-DQA1*05 Carriage Associated With Development of Anti-Drug Antibodies to Infliximab and Adalimumab in Patients With Crohn's Disease. *Gastroenterology* 158: 189-99
48. Lee HS, Cleynen I. 2019. Molecular Profiling of Inflammatory Bowel Disease: Is It Ready for Use in Clinical Decision-Making? *Cells* 8
49. Shen X, Klaric L, Sharapov S, Mangino M, Ning Z, Wu D, Trbojevic-Akmacic I, Pucic-Bakovic M, Rudan I, Polasek O, Hayward C, Spector TD, Wilson JF, Lauc G, Aulchenko YS. 2017. Multivariate discovery and replication of five novel loci associated with Immunoglobulin G N-glycosylation. *Nat Commun* 8: 447
50. Lauc G, Huffman JE, Pucic M, Zgaga L, Adamczyk B, Muzinic A, Novokmet M, Polasek O, Gornik O, Kristic J, Keser T, Vitart V, Scheijen B, Uh HW, Molokhia M, Patrick AL, McKeigue P, Kolcic I, Lukic IK, Swann O, van Leeuwen FN, Ruhaak LR, Houwing-Duistermaat JJ, Slagboom PE, Beekman M, de Craen AJ, Deelder AM, Zeng Q, Wang W, Hastie ND, Gyllensten U, Wilson JF, Wuhrer M, Wright AF, Rudd PM, Hayward C, Aulchenko Y, Campbell H, Rudan I. 2013. Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet* 9: e1003225
51. Menni C, Keser T, Mangino M, Bell JT, Erte I, Akmacic I, Vuckovic F, Pucic Bakovic M, Gornik O, McCarthy MI, Zoldos V, Spector TD, Lauc G, Valdes AM.

2013. Glycosylation of immunoglobulin g: role of genetic and epigenetic influences. *PLoS One* 8: e82558

52. Ercan A, Cui J, Chatterton DE, Deane KD, Hazen MM, Brintnell W, O'Donnell CI, Derber LA, Weinblatt ME, Shadick NA, Bell DA, Cairns E, Solomon DH, Holers VM, Rudd PM, Lee DM. 2010. Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. *Arthritis Rheum* 62: 2239-48

Appendix I

Cellular Immunology 333 (2018) 9–18

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Glycans as critical regulators of gut immunity in homeostasis and disease

Ana M. Dias[a,b,c], Márcia S. Pereira[a,b,c], Nuno A. Padrão[a,b,d], Inês Alves[a,b,d], Ricardo Marcos-Pinto[c,e,f], Paula Lago[e], Salomé S. Pinho[a,b,d,*]

[a] Institute for Research and Innovation in Health (I3S), Porto, Portugal
[b] Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal
[c] Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, Porto, Portugal
[d] Medical Faculty, University of Porto, Porto, Portugal
[e] Department of Gastroenterology, Centro Hospitalar do Porto, Porto, Portugal
[f] Medical Faculty, Centre for Research in Health Technologies and Information Systems (CINTESIS), Porto, Portugal

A B S T R A C T

The diversity of glycans expression within a cell or an organism is enormous and the amount of relevant biological information that each glycan structure encodes is far from being clarified. The importance of glycans in health and life sciences is highlighted by their multiple functional implications in different cellular and molecular biology processes with impact in homeostasis and diseases, such as cancer and inflammatory conditions. Glycans actively participate in the regulatory circuits that govern both innate and adaptive immune response. Changes in the glycans repertoire occur during the transition from normal to inflamed conditions and the aberrant expression of glycans dictates either pro-inflammatory or anti-inflammatory responses. This review summarizes how glycans integrate the regulatory networks of immune response with a focus on gut immunity.

1. Introduction

Inflammation is a complex biological process that occurs as a normal host response to tissue damage inflicted by infections or other stimuli. An inadequate resolution of inflammation and/or an unchecked inflammatory reaction can evoke chronic inflammation, predisposing the host to various diseases, including chronic immune-mediated disorders, such as intestinal inflammation and cancer.

A compelling body of evidence has demonstrated that glycans (carbohydrates or sugar chains) integrate into the canonical circuits that govern immune response as demonstrated by the fact that alterations in the cellular glycosylation repertoire have remarkable effects in the development and progression of different immune-mediated disorders [1,2].

The intestinal mucosa is considered a major physical and biological barrier that supports the bodý s immune system. The glycanś composition of the gut mucosa is considered a key factor that guides the establishment of the microbial community [3], shaping the course of immune response both in homeostasis and in disease.

This review summarizes the importance of glycosylation as a fundamental player in gut immunity, discussing how changes in the mucosal glycome, with a focus on *N*-linked glycans, impact in the regulatory circuits that triggers either a pro-inflammatory or anti-inflammatory response.

2. Glycosylation as a major biological barrier of the gut mucosa

Glycosylation is a well-orchestrated post-translational process that occurs in essentially all cells. It is characterized by the addition of carbohydrate structures (glycans or sugar chains) to proteins or lipids through the synchronized action of a diverse repertoire of glycosyltransferase and glycosidase enzymes. The genes encoding the portfolio of those enzymes contribute to more than 1–2% of the total human genome which results in a huge diversity of glycosylation modifications in which the same protein or lipid can exhibit different glycoforms and consequently different biological functions [4].

The glycan composition of the gut mucosa is determined by different sources, such as endogenous microorganisms (i.e. their capsules), glycans influx from breast milk and diet, and the host mucus (rich in heavily glycosylated proteins such as mucins) [3]. Secreted mucus is considered a carbon and energy source when dietary fibers are not sufficiently available [5,6]. Glycans can also derive from the process of mucin glycan foraging, as a strategy of mucin-degrading bacteria to utilize host glycans as a nutrient source [7–9].

In fact, one important component of the intestinal glycocalyx is the oligomeric mucus gel-forming glycoprotein Mucin 2 (MUC2) which is a major intestinal mucin produced by goblet cells. Curiously, alterations of MUC2 glycosylation are associated with inflammation [10,11]. The core 3–derived *O*-glycans also play an important role in the intestinal

* Corresponding author.
E-mail address: salomep@ipatimup.pt (S.S. Pinho).

https://doi.org/10.1016/j.cellimm.2018.07.007
Received 10 January 2018; Received in revised form 4 July 2018; Accepted 17 July 2018
Available online 18 July 2018

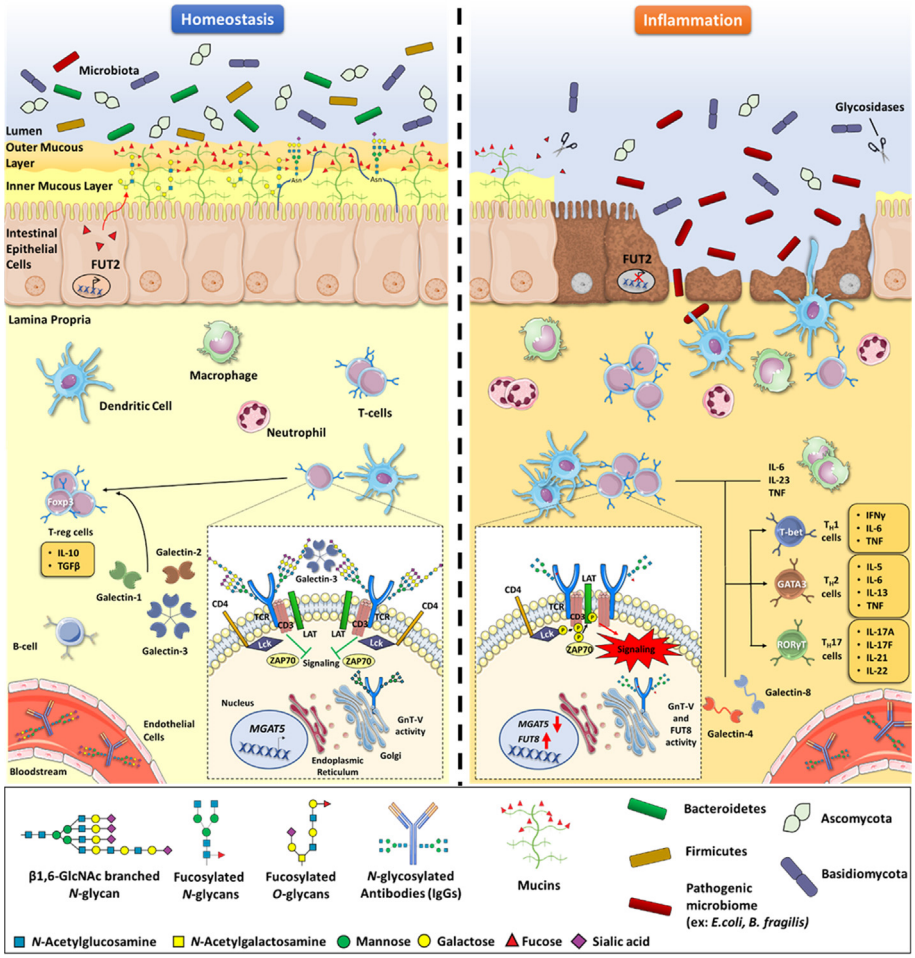


Fig. 1. Impact of glycosylation in gut homeostasis and inflammation. During the transition from normal to inflamed gut mucosa changes in the glycans repertoire occur. The aberrant expression of specific glycans that accompany intestinal inflammation impacts not only in the hosťs immune response but also in the microbiota content and functions. Glycans composition namely in mucins are crucial to maintain the protective function of the intestinal barrier against pathogenic microbiota. Particularly, the α-1,2 fucosylation catalyzed by FUT2 in epithelial cells is crucial in maintaining commensal microbiota protecting against pathogenic microorganisms. The loss of mucosal integrity results in pathogens invasion concomitantly with a cascade of inflammatory events mediated by dendritic cells, macrophages, neutrophils and later by T cells. These pathogenic microorganisms can took advantage from host's glycans, through glycosidases activity as strategies to "escape" recognition by hosťs immune system. This selective process gives rise to dysbiosis that in intestinal disorders, such as in IBD, is characterized by a decreased in microbiota diversity (less Firmicutes and more Proteobacteria) and a higher Fungi ratio of Basidiomycota/Ascomycota. At the level of lamina propria, glycosylation alterations are known to regulate T cell-mediated immune response. In homeostasis, the expression of β1,6 GlcNAc branched *N*-glycans, catalyzed by GnT-V is crucial to control T cell receptor (TCR) function and signaling. On this process, specific carbohydrate recognition proteins such as galectins (Galectin-3) recognizes the elongated chain (polylactosamine) of the branched *N*-glycans forming a "lattice" that precludes the TCR clustering and in this way control the threshold of T cell activation. Other galectins, such as Galectin-1 and Galectin-2, are also important in controlling T cell-mediated immune response towards a T regulatory response. Importantly, in homeostatic conditions, the profile of IgG glycosylation released in the serum has been described to exhibit predominantly galactosylated and terminal sialylated *N*-glycans in the Fc portion. In intestinal inflammation, specific alterations on the expression of glycans, particularly the upregulation of α1-6 fucosyltransferase (FUT8) with overexpression of core fucose and the downregulation of *MGAT5* gene with decreased expression of GnT-V-mediated branched *N*-glycans negatively impact in T cell response. This altered expression of *N*-glycans (branched and core-fucosylated) lead to T cell hyperactivation and signaling of intestinal T lymphocytes, being also associated with a T cell differentiation towards Th1 and Th17 immune responses. In addition, the expression of galectins 4 and 8 further stimulate T cell proliferation in colitis. Moreover, in intestinal inflammation (such as in IBD) the expression profile of serum IgG is different from homeostasis, being predominantly characterized by agalatosylation.

barrier, as C3GnT-deficient mice revealed a reduction of MUC2 glycoprotein expression with a consequent impairment of mucosal integrity and susceptibility to develop colitis and colorectal adenocarcinoma [12]. The protein misfolding of intestinal MUC2 was shown to induce ER stress which impacts gut homeostasis leading to inflammation [13–15]. The IL-10 expression was shown to prevent MUC2 protein misfolding and ER stress maintaining MUC2 production by goblet cells, and consequently preserving the intestinal mucus barrier [16]. Additionally, few studies exist on the importance of *C*-mannosylation of mucins, that occurs in the first tryptophan (Trp) of the consensus aminoacids Trp-X-X-Trp motif in proteins of the Cys-D domain of mucins [17]. This unique type of glycosylation appears to play an important role in MUC5AC and MUC5B normal development and secretion, representing another mechanism in the regulation of mucus layer formation [18–20].

The glycosylation of intestinal epithelial cells (IECs) has been shown to be important in the modulation of the host immune response. An elegant study demonstrated that expression of α-1,2 fucose on the apical side of IECs protects against pathogenic bacteria being essential in the maintenance of the commensal microbiota [21] (Fig. 1). This process of fucosylation was found to be mediated by group 3 innate lymphoid cells (ILC3s), which constitutes the most recently identified component of the innate immune system [22]. It was demonstrated that commensal and pathogenic bacteria as well as bacterial products (*lipopolysaccharide* (LPS)) stimulate ILC3s to produce IL-22 which in turn induces α-1,2 fucosylation of IECs. Accordingly, inactivating polymorphisms of fucosyltransferase (FUT) 2 (non-secretor status) were associated with inflammatory diseases [23,24].

Another component of the gut mucosa are the Paneth cells (PCs), secretory epithelial cells that act as guardians of small intestinal crypts, controlling the microbiome. They are also functionally and structurally involved in formation of the stem cell zone of small intestinal crypts and in the morphogenesis of the crypt-villus axis (CVA). The crypt compartment harbors abundant mannose rich glycoproteins whereas the mature villus zone contains mainly complex-type glycoproteins [25]. Recently, Rouhanifard and colleagues [26] applied an *in situ* glycan editing method [27] using crypt organoids in order to study the impact of glycosylation on stem cell proliferation and differentiation in a complex multicellular system. They demonstrated that abundant terminal *N*-acetyllactosamine (LacNAc) of PCs contribute to the regulation of stem cell behavior. The *in situ* glycan editing, that blocks LacNAc (which is highly expressed in PCs) promotes hyperproliferation phenotype of adjacent $Lgr5^{+}$ (leucinerich-repeat-containing G-protein-coupled receptor 5) stem cells [26]. These evidences support the

instrumental role of glycosylation in the maintenance of mucosal homeostasis through controlling growth and differentiation of stems cells, as a critical event for intestinal epithelial layer self-renewal.

Gut mucosa is also composed by other cell type, such as microfold cells (M cells). They are found in the gut-associated lymphoid tissue of the Peyer's patches in the small intestine, and in the mucosa-associated lymphoid tissue of other parts of the gastrointestinal tract. These cells are known to initiate mucosal immunity responses and are also involved in the transport of microbes and particles across the epithelial cell layer from the gut lumen to the lamina propria where interactions with immune cells can take place [28]. The role of glycans in M cells is still poorly characterized. Fucosylation mediated by FUT1 was shown to act as an exclusive and reliable marker of these type of cells [29]. Given the functional role of M cells in intestinal mucosa immunity, the study of its glycosignature is worth exploring.

Overall, glycosylation is a central regulatory mechanism of gut homeostasis being implicated in many different processes, spanning from epithelial self-renewal, maintenance of integrity of intestinal mucus barrier to control of gut immunity.

3. Glycosylation in the regulatory circuits of the gut immune response

Changes in cellular glycosylation occur during the transition from normal to inflamed conditions. This altered expression of glycans during inflammation has major implications in the regulation of both innate and adaptive immune responses [30–32].

3.1. Innate immunity

Glycans are key mediators of innate immune response due to their interaction with a particular family of receptors called C-type lectin receptors (CLRs) that bind to carbohydrates in a calcium-dependent manner. CLRs, such as dendritic cell specific intracellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) (that binds mainly to high-mannose but also to fucose moieties) [33,34], sialic acid-binding immunoglobulin-type lectins (siglecs) and macrophage mannose receptor (MMR), are expressed in various innate immune cells including DCs and macrophages. These CLRs are involved in glycan-mediated pathogen recognition and internalization of antigen for loading on Major Histocompatibility Complex (MHC) class I and II molecules inducing a T cell response [35–38]. The sialylation of antigens was shown to induce antigen-specific tolerogenic immune response through recognition by DCs with consequent induction of T regulatory (Treg) cells and suppression of IFN-γ production [39]. Conversely, the sialic acid composition of the gut mucosa (glycocalyx) was associated with dysbiotic conditions. The sialic acid catabolism was reported to mediate intestinal inflammation concomitantly with the expansion of *E. coli* during colitis. The overgrowth of *E. coli* that occurs in colitis was demonstrated to be dependent on the sialic acid release from the hosťs glycans after sialidase activity [40]. The dependence of *E. coli* on sialidases secreted by *Bacteroides* spp. (abundant in intestinal inflammation) contribute to the overgrowth of *E. coli* and thereby to dysbiosis in patients with colitis [41]. In line with this, it was proposed that sialidase inhibitors can constitute a glycan-mediated strategy to decrease *E. coli* outgrowth preventing dysbiosis and thereby controlling the severity of colitis [40].

Moreover, higher galactosylation of *N*-glycans on mucus proteins were associated with increased ratio of Firmicutes/Bacteroidetes in healthy conditions, conferring protection against TNF-induced systemic inflammation and DSS-induced colitis [42].

The anti-fungal innate immunity is also dependent on the polysaccharides components of fungal surfaces, such as α- and β-glucans, chitin, and both *N*- and *O*-linked glycoproteins [43].

The antimicrobial peptides (AMPs) are also essential components of the gut innate immunity. Glycosylation may offer a means to tailor an AMP to a precise molecular target. Interestingly, *N*-linked glycosylation has been shown to be effective in modifying the serum half-life and bioavailability of peptides and other compounds [44,45]. For example, Enfuvirtide is an AMP, acting as a novel antiretroviral drug in which glycosylation with sialic acid residues extends its half-life more than 10-fold, without affecting its sensitivity towards the target [46]. Several reports have been showing the importance of AMPs (some of them glycosylated) in the regulation of gut microbial community and in gut immunity, which highlights their importance in gut homeostasis [47–49].

Complement cascade is also a potent innate immune mechanism which plays an important role in maintaining the homeostasis of intestinal barrier [50]. *C*-mannosylation is highly present in the complement alternative pathway (namely in properdin and membrane attack protein complex), which is the first encoded response to extracellular stimuli [51].

3.2. Adaptive immunity

Protein glycosylation can also influence the adaptive immune response. The MHC I and II can display glycosylated peptides that are recognized by T cells, supporting the impact of glycans in the modulation of T helper (Th) cells (by MHC II) and cytotoxic T cells (by MHC I) responses [52]. Recently, it was demonstrated that cytotoxic immune response, mediated by $CD8^+$ T cells, during chronic viral infection is mediated by branching *N*-glycans [53]. The induction of IL-10 by chronic infections, such as those mediated by human immunodeficiency virus (HIV) and hepatitis C virus (HCV), was shown to restrict $CD8^+$ T cell activation and function through enhancement of branched *N*-glycans expression on T cell surface. In chronic infection, higher levels of IL-10 increases the threshold of T cell receptor (TCR) activation inducing, via STAT3, an increased expression of *MGAT5* gene leading to enhanced branched *N*-glycans on glycoproteins, including TCR. Thereby decreasing $CD8^+$ T cells antigen sensitivity through branched *N*-glycosylation, enables the pathogen to outpace the immune response and establish viral persistence [53]. This enhancement of branched *N*-glycans promotes the formation of lattices through galectin-3 binding, which precludes the capacity of TCR and CD8 co-receptor to interact. The disruption of galectin binding might restore T cell function inducing the control of chronic infection [53].

Indeed, this important functional relationship between branched *N*-glycans and *T*-cell mediated immune response was also documented in $CD4^+$ T cells in the context of autoimmune disorders [54–56]. In fact, a compelling body of evidence has been pointing toward the importance of complex branched *N*-glycans catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V) in controlling T cell activity and functions in different immune-mediated disorders as demonstrated in mouse models of Multiple sclerosis (MS), Type I diabetes [54,57,58] and Ulcerative Colitis (UC) [55,56]. Particularly, the GnT-V-mediated branched glycosylation of intestinal T cells was found to play a role in UC pathogenesis. Patients with UC exhibit, at the level of intestinal mucosa, a deficiency in the expression of branched *N*-glycans on the TCR that was associated with disease severity. This decreased expression of branched *N*-glycans was found to be due to a reduced transcription of *MGAT5* glycogene in intestinal T lymphocytes (Fig. 1) [55]. Nevertheless, whether this dysregulation of *MGAT5*/GnT-V-mediated glycosylation on T cells is a cause or consequence of intestinal inflammation in UC remains unclear. Evidences suggest that GnT-V-mediated branching *N*-glycans are implicated in the development of Th2 over Th1 responses [59], further showing an impact in the differentiation of induced Treg cells over Th17 differentiation [60].

Importantly, the metabolic supplementation of mouse models of experimental autoimmune encephalomyelitis (EAE), type I non-obese diabetic mice, colitis mouse model and T cells with *N*-acetylglucosamine (GlcNAc) resulted in the enhancement of β1,6 GlcNAc branched *N*-glycans on T cells catalyzed by GnT-V activity. This

enhanced branching glycosylation on T cells was shown to increase the threshold of T cell activation, suppressing T cell growth and inhibiting Th1 differentiation which resulted in a decreased disease severity [56,57,61]. These results demonstrate that GnT-V-mediated branching *N*-glycosylation plays a key role in the regulation of T cell activity and signaling in immune-mediated disorders.

Besides the TCR, the enhancement of branched *N*-glycosylation can also modify other receptors like CD4 and CD8 co-receptors as well as the growth inhibitory receptor CTLA-4 [54,58,59]. Moreover, CD45 and CD25 are also potential targets of branched *N*-glycosylation modifications with impact in the regulation of T cell-mediated immune response [60,62].

The branched *N*–glycans can be further modified and elongated with poly–*N*–acetyllactosamine (repeats of Galβ1,4GlcNAcβ1,3) that can be capped with sialic acid, fucose, galactose, *N*-acetylgalactosamine, and sulfate [63]. This poly–*N*–acetyllactosamine structure is the preferred ligand for galectins, a family of soluble conserved carbohydrate-binding proteins, forming galectin–glycan structures termed “lattices” [30]. This molecular complex contributes to restrict the interactions between receptors and co-receptors on immune cells regulating intracellular signaling pathways, apoptosis, proliferation and migration [64]. For instance, the production of these polylactosamine extensions in T cells glycoproteins is central to the control of the immune response [65]. Accordingly, β3GnT2-deficient mice show a T cell hypersensitivity due to the reduction of polylactosamine on the *N*-glycan similarly to the observations in *MGAT5*-deficient mice [54,65]. Moreover, in mice lacking specific *N*-acetylglucosaminyltransferases (GnTs) enzymatic activity (due to genetic knockout of *MGAT2* and *MGAT5*), the disruption of the branching *N*-glycosylation pathway produces fewer LacNAc branches glycans due to reduction of UDP-GlcNAc consumption at *medial* Golgi. This unused UDP-GlcNAc was shown to be driven forward to *trans* Golgi where β3GnTs enzymes appears to produce bioequivalent poly-LacNAc structures. Accordingly, the authors proposed that loss of LacNAc branches is balanced by increased production of linear LacNAc polymers, as a Golgi self-correcting ability to control cell surface LacNAc density and thereby the galectin-glycoprotein lattices, as a way to control homeostasis [66].

In fact, galectins can be found in activated T and B cells, being significantly upregulated in activated macrophages and Treg cells [67,68]. These carbohydrate-recognizing proteins have been implicated in a wide range of key biological processes including: regulation of host-pathogen interactions; innate and adaptive immune responses; acute and chronic inflammation, and immune tolerance [69].

Interestingly, galectins family can play an opposite role in immune response as they can act as negative or positive regulators of T cell function. Galectin-1 and -3 are known to suppress inflammation and T cell response [54,70,71]. Galectin-1 is described to negatively regulate Th1 and Th17 effector cells by inducing cell death [70]. Galectin-3 has been described to limit TCR clustering due to lattice formation in *MGAT5*-expressing cells controlling thereby the threshold of T cell activation [54,72]. Similarly, galectin-2 also exhibits a suppressive effect by inducing apoptosis of lamina propria T lymphocytes attenuating acute and chronic mouse colitis [73]. In contrast, galectin-8 and galectin-4 act in opposite way. When binding to T cells, galectin-8 promotes *T*-cell proliferation, possibly through unique interactions with CD45 [74]. Galectin-4 mediates $CD4^+$ T cells stimulation (through IL-6 production) leading to exacerbation of T cell-mediated chronic colitis [75]. These opposite responses mediated by different galectins are not yet fully clarified, deserving further investigation.

Interestingly, an association between environmental factors (sunlight/vitamin D_3 and metabolism) combined with multiple genetic variants (*IL17RA, IL2RA, MGAT1* and *CTLA-4*) was described to dysregulate Golgi *N*-glycosylation with impact in MS development and severity [76]. These observations support the impact of branched *N*-glycosylation in development and progression of autoimmune disorders at a genetic level. Accordingly, GWAS studies revealed that *MGAT5* polymorphisms cooperatively with *MGAT1* and interleukin-2 and -7 receptor variants were associated with susceptibility to MS [77,78]. Preliminary evidences also point towards an association between *MGAT5* genetic variants and UC severity [79] which support the prominent role of *MGAT5* (and other glycogenes) in determining genetic susceptibility to immune-mediated disorders.

In addition to genetics, the epigenetic alterations of key glycogenes in a tissue/cell-specific manner were also implicated in immune-mediated disorders, such as IBD [80].

Another important type of glycosylation with impact in T cell-mediated immune response is core fucosylation which refers to fucose attached to the innermost *N*-acetylglucosamine of *N*-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8). A dysregulation of FUT8 was described to be associated with IBD pathogenesis [81]. In mouse models of induced colitis, T cells were found to display an increased expression of core fucosylation when compared with healthy mice. Moreover, the $Fut8^{-/-}$ mice developed a less severe colitis than $Fut8^{+/+}$ mice, and T cells from $Fut8^{-/-}$ mice produced lower levels of Th1 and Th2 cytokines. Accordingly, colonic samples from IBD patients also revealed that inflamed mucosa exhibited higher levels of core fucosylation comparing to non-inflamed mucosa and healthy colon (Fig. 1) [81].

Several examples exist in literature testifying the role of genetic alterations of glycosylation (modelled in different mice models) in triggering the activation of immune-mediated responses, with a focus on intestinal inflammation (Table 1).

It is important to highlight that the extrapolation of data from mice to human have some caveats [86,87] being fundamental the clinical validation (in human clinical samples) for translational applicability of the findings.

Protein *O*-GlcNAcylation has been also implicated in T cell self-renewal, differentiation and proliferation [88]. It consists in the addition of *O*-GlcNAc moieties to serine or threonine residues of nuclear and cytoplasmic proteins by the *O*-GlcNAc transferase (OGT) through UDP-GlcNAc [89]. The mechanisms underlying T cells activation were shown to be accompanied by increased nutrients uptake (glucose and glutamine) that increases UDP-GlcNAc availability enhancing OGT activity and *O*-GlcNAcylation of multiple proteins such as c-Myc. The glycosylated form of c-Myc was shown to be an important factor that controls the nutrients uptake and UDP-GlcNAc production thereby

Table 1
Examples of genetic alterations of glycosylation associated with intestinal inflammation.

Type of alterations	Colitis association	Intestinal feature	References
Core $3^{-/-}$	Susceptible to DSS	Mucins were more susceptible to proteolysis; Loss of mucus barrier	[12,82]
Core $1^{-/-}$	Spontaneously develop colitis	Mucins were more susceptible to proteolysis; Loss of mucus barrier	[82]
$FX^{-/-}$ (fucosylation deficiency)	Develop colitis	Altered the composition of the fecal microbiota, epithelial barrier dysfunction and altered epithelial proliferation	[83]
MUC $2^{-/-}$	Spontaneously develop colitis	Associated with epithelial barrier dysfunction	[84]
FUT $8^{+/+}$	Develop severe colitis	Increased TCR signaling and production of inflammatory cytokines	[21]
$MGAT5^{-/-}$	Develop severe colitis	Increased TCR signaling and production of inflammatory cytokines	[56]
Humanized FUT 1	Spontaneously develop colitis	Influence on T cell development	[85]

modulating *O*-GlcNAcylation, and thus constituting a key factor in the regulation of T cells activity and their precursors. The loss of OGT enzyme was shown to block T cell progenitor renewal, controlling peripheral T cell clonal expansion and suppressing malignant transformation [88].

Complex *O*-glycans have been also demonstrated to be important in the regulation of T cells trafficking and homing [90]. The capacity for memory T cells to rapidly traffic into a site of infection for protective immunity is highly dependent on *de novo* synthesis of core 2*O*-glycans. Stimulation of core 2*O*-glycans on tumor-specific T cells was suggested to contribute to enhance T cell trafficking and ultimately improve cancer immunotherapy [90].

Importantly, the T cell response is also controlled by the interplay between glycans and the microbiota. As example, glycans in helminths act as a conserved molecular pattern that instructs DCs functions to drive Th2-polarized responses [91]. Interestingly, it was postulated that helminths might have a protective effect by modulating the host immune response. Using colitis-induced mouse models, it was demonstrated that the helminth, *Heligmosomoides polygyrus bakeri* prevents colitis by inhibiting antigen-specific gut T cell response through alterations of DCs function [92]. This mechanism of suppression of inflammation through helminthś glycans has been explored [93,94] and recently, it was suggested that IL-4Rα signaling is an important pathway required for an effective suppression of immune response [95]. These evidences have been raising the interest in helminthś antigen cell-based therapy [96], which were already tested in small clinic trials in UC [97]. The importance of helminths in immunity, and particularly the glycanś component as regulatory elements in inducing tolerogenicity, is an important topic [98–100] that deserves further investigation. In fact, it has been suggested that helminths develop "glycan gimmickry" (as an alternative concept of "molecular mimicry") to target DCs, thereby contributing to the modulation of inflammatory T cell responses towards an anti-inflammatory response [101]. Glycan gimmickry can be regarded as an active strategy of helminths to use their glycans to target host glycan-binding proteins to prolong their survival. Therefore, there is a need to identify novel glycan structures in parasitic helminths to fully understand this strategy of immune escape.

Taken together, the integration of glycans in the regulatory networks that govern both innate and adaptive immune response emphasizes their mechanistic importance both in homeostasis and in pathological conditions. The rapid advance of cutting edge (glyco) technological approaches that can be used to decode the human glycome [102–107] will be an asset to disclose how glycans impact in immunity and autoimmunity, paving the way for the development of optimized (glyco)biomarkers and novel glycan-based and targeted specific therapeutic approaches. Importantly, the research community can take advantage of two major online repositories for reference of all glycan structures and their proteins of interaction: CaZy (Carbohydrate-Active enZYmes database) (http://www.cazy.org) and Consortium for functional Glycomics (http://functionalglycomics.org).

4. GlycoMedicine: A window of opportunity for clinical applications in different immune-mediated diseases?

The complex mechanisms underlying the pathogenesis of different immune-mediated disorders, including IBD, MS, type-I diabetes, rheumatoid arthritis (RA) and Systemic Lupus Erythematosus (SLE) explain the substantial heterogeneity of these diseases with respect to disease onset, course, response to therapies and progression to complications [108–112]. Consequently, there is a pressing need to identify novel molecular markers of disease that can be therapeutically targeted. This tailored approach will certainly improve patientś risk stratification and prognosis and the success of the therapeutic results [113–115].

In fact, a compelling body of evidence has been supporting the promising role of glycans as essential elements integrated in the algorithm of the etiopathogenesis of different immune-mediated diseases

Table 2
Summary of glycosylation-based alterations associated with etiopathogenesis of intestinal immune-mediated diseases with potential clinical applications.

Disease	Species	Target	Function	Alteration	Clinical relevance	References
IBD	Human	MGAT3	Genetic	SNP	Associated with IgG glycosylation	[153]
IBD	Human	ST6GAL1	Genetic	SNP	Associated with IgG glycosylation	[153]
IBD	Human	FUT8	Genetic; mucosal	SNP Overexpression	Associated with IgG glycosylation; Increased TCR core fucosylation on T cells in inflamed mucosa.	[81,153]
IBD	Human and Mice	B4GALT1	Genetic	SNP Overexpression	Associated with IgG glycosylation; Drastic change in composition of gut microbiota conferring protection against TNF-induced inflammation and DDS-induced colitis.	[42,153]
IBD	Human and Mice	MAN2A1	Genetic	SNP; Deletion	Associated with the UC onset; Lack of MAN2A1 in intestinal epithelial cells is associated with less susceptibility to develop colitis in mice and less neutrophil infiltration	[154]
CD	Human	FUT2	Genetic	Loss of function	Increased susceptibility to CD by altering host-microbial interactions	[23,155,156]
UC	Human	FUT3	Genetic	Polymorphism	Increased susceptibility	[157]
UC	Human	Intestinal inflammatory infiltrate	Mucosal	Low levels of branched *N*-glycans	Discriminate UC patients according to their therapeutic outcome	[117]
UC	Human	MUC2	Mucosal	Shorter *O*-glycans -STn antigen	UC activity and severity	[11]
IBD	Human	Lactoferrin	Serologic	Fecal glycosylated protein	Act as selective anti-microbial, and is able to distinguish active IBD from inactive IBD	[158–160]
IBD	Human	IgG	Serologic	Decrease galactosylation; decrease sialylation	IgG glycosylation alterations and disease severity	[127,134,161]
Celiac disease	Human	IgG	Serologic	Galactose deficiency in IgG	Hypogalactosylation of serum IgG in patients with coeliac disease	[162]
Celiac disease	Human	IgA1	Serologic	Galactose deficiency in IgA1	Associated with Celiac disease	[163]
IBD	Mice	Fut8 inhibitors	Potential therapy	Aim to decrease core fucosylation	Decrease TCR signaling and production of inflammatory cytokines	[81]
IBD	Mice	Sialidases inhibitors	Potential therapy	Aim to decrease core sialylation	Decrease *E. coli* outgrowth, controlling colitis severity	[40]
UC	Human and Mice	UDP-GlcNAc	Therapy (on going clinical trial)	Increase branched *N*-Glycans	Decrease TCR signaling, decrease pro-inflammatory cytokines	[56]

Table 3
Summary of glycosylation-based alterations associated with etiopathogenesis of other autoimmune diseases with potential clinical applications.

Disease	Species	Target	Function	Alteration	Clinical relevance	References
RA	Human	IgG	Serologic	Decreased galactosylation; Decreased sialylation	Increased autoantibody activity; higher risk to a future diagnosis;	[128,129]
SLE	Human	IgG	Serologic	Decreased galactosylation, sialylation and core fucose and increased bisecting *N*-acetylglucosamine	Associated with SLE	[131]
SLE	Human	MAN2B1	Genetic	2548C deletion in exon 21	Association with SLE (Case report)	[164]
SLE	Mice	MAN2A1$^{-/-}$	Experimental model of disease	Ablation of complex *N*-glycans	Spontaneous development of autoimmune lupus-like syndrome	[165]
SLE	Mice	MGAT5$^{-/-}$	Experimental model of disease	Ablation of Tetraantennary complex *N*-glycans	Age-associated development of glomerulonephritis	[54]
SLE	Mice	Recombinant Gal1	Potential therapy	Prevent TCR activation by reorganize the synaptic contact	Amelioration of Lupus-induced mouse model disease;	[166]
SLE and Type I diabetes	Mice	Recombinant Gal9	Potential therapy	Expanded Regulatory T cells; decreased Th1 and Th17 effector cells and apoptosis of plasma cells	Amelioration of Lupus-induced disease; Amelioration of Type I diabetes	[167–170]
Phenylketonuria	Mice	Glycomacropeptide	Therapy	Prebiotic based on extensive glycosylation with sialic acid, galactose, and galactosamine	Reduces Desulfovibrio bacteria, increases cecum short-chain fatty acids; anti-inflammatory characteristics.	[171]
Chronic inflammatory and autoimmune diseases	Human	Intravenous immunoglobulin (IVIG)	Therapy	Terminal sialic acid of Fc revealed to play a key role in the immuno-modulatory effects of IgG	Used as infusion therapy to modulate the immune response	[119]

(Table 2, a focus on gastrointestinal disorders), (Table 3, other immune-mediated diseases), constituting promising prognostic biomarkers as well as providing a source for the development of new therapeutic strategies with clinical applications.

The determination of the glycosylation signature associated with the pathogenesis of immune-mediated diseases have been paving the way for improving personalized medicine. In fact, glycogenes constitute important genetic determinants with functional impact in the etiopathogenesis of many autoimmune diseases with applications in prognosis and risk stratification, as summarized in Tables 2 and 3. As example, the emerging role of glycoconjugates in IBD pathogenesis gave rise to the creation of an online database called Glycosylation and Gut Associated Immune Tolerance (GlycoGAIT-https://apps.connexios.com/glycogait/), in which the profile of the expression pattern of glycome can be analyzed under different inflammatory conditions [116].

Furthermore, the analysis of *in situ* glycosignature has been shown to provide a plethora of important biological information that can be translated in relevant molecular parameters with clinical and therapeutic applications [55]. For instance, the levels of expression of branched *N*-glycans in intestinal lamina propria appear to be associated with the therapeutic outcome of IBD patients. At the time of diagnosis, low levels of branched *N*-glycans on intestinal inflammatory infiltrate are able to predict patients that do not respond to standard therapy, thus needing to step-up to biologics [117]. The predictive capacity of this glycobiomarker was shown to be independent, and improved when used together with C-reactive protein (CRP). Importantly, the best performance (best predictive capacity) of this glycobiomarker was observed in patients displaying a severe endoscopic Mayo subscore (Mayo 3) at diagnosis [117]. These results are in accordance with previous evidences showing that, low levels of branching *N*-glycans on intestinal T lymphocytes are associated with severe disease phenotypes [55].

Alterations in the glycosylation of immunoglobulin gamma (IgG) have been also associated with the pathogenesis of autoimmune diseases. In fact, *N*-glycosylation alterations in the fragment crystallizable region (Fc) of the IgG has been demonstrated to modulate the inflammatory functions of the IgG, by altering the affinity to Fcγ receptors (FcγRs), present in the leukocytes [118–120], interfering with the activation of the complement pathway. These alterations can thus trigger the canonical effector function of IgG as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP), as well as, complement-dependent cytotoxicity (CDC) [118,121]. For instance, the core fucose plays an important role in regulating ADCC, once the loss of core fucose enhances affinity to the activating FcγR IIIA [122]. Agalactosylated IgG also displays an increased affinity to activating FcγR III [118,123,124], enhancing antibody-dependent phagocytosis [125]. Moreover, terminal sialylation on Fc glycan also modulates FcγR binding. The presence of α2,6-sialylation on the Fc glycan significantly reduces FcγR affinity being associated with anti-inflammatory activity [119,126].

In fact, the analysis of the IgG glycome revealed significant differences comparing healthy individuals and patients with autoimmune diseases (Fig. 1), such as IBD [127], RA [128,129], MS [130] and SLE [131,132] which highlight their importance in susceptibility to autoimmune disorders, as well as different disease severity scores [133–137].

The importance of glycosylation in the regulation of effector function of IgG is also highlighted by the success of glycoengineered therapeutic monoclonal antibodies (mAb) [138,139]. The quality of an antibody relies not only in the primary structure, charge and purity but also in the type, quantity and position of glycosylation. The Fc glycosylation influences the clearance rate, pharmacokinetic, pharmacodynamic and the binding to Fc receptors [140]. Moreover, antigen-binding fragment (Fab) glycosylation also influences the binding to antigens and glycan binding proteins [141].

Importantly, the intravenous immunoglobulin (IVIG) administration, used as an infusion therapy to modulate the immune response in a

variety of chronic inflammatory and autoimmune diseases [142–144], can also be glycoengineered to potentiate its anti-inflammatory functions. In fact, the anti-inflammatory activity of IVIG results from the small portion of IgGs that contains sialylated (α2,6-sialylated Fc) IgG glycovariants in which increased sialylation of Fc showed a 100-fold increase anti-inflammatory activity in a mouse model of arthritis [145,146]. Sialylation of IgG was demonstrated to result in Treg cell expansion as well as suppression of T cell pro-inflammatory response in models of EAE and experimental colitis [147].

Additionally, the therapeutic effects of specific glycans (as supplements) have been reaching the clinical settings, showing therapeutic efficacy in the control of MS and IBD, being tested in ongoing clinical trials on autoimmune disorders (MS and IBD) [56,60] (Table 2).

Conversely, the therapeutic potential of glycans might also be related with microbiome. For instance, fecal microbiota transplantation (FMT) (which aims to restore abnormal microbial composition of the gut by introducing fecal microbiota obtained from a healthy donor into a diseased individual) is a strategy that has been widely explored in the context of intestinal inflammation [148,149]. In this regard, the understanding of the glycophenotype of microbiome might represent an important target of research that could improve FMT therapeutic strategy [150–152]. Future studies are warranted to define the role of donor-recipient matching based on microbial glycoprofile.

5. Concluding remarks

Glycosylation is now considered an important “building block” of life. Glycans encode a huge diversity of different biological functions within a cell or an organism with remarkable implications both in homeostasis and in disease. Particularly, glycans are essential regulatory elements that finely tune the immune response.

The abundance and diversity of glycan structures that compose the glycocalix reveal its prominent role at the interface of host-pathogen interaction in which glycans can bi-directionally regulate microbiota content and function and the host immune response. This holistic implication of glycans in the mucosal homeostasis and immunity, place them as fundamental molecules with major implications in the pathogenesis of immune-mediated diseases.

The detailed identification and characterization of the impact of aberrant expression of specific glycans will bring to light new mechanisms underlying autoreactive and autoinflammatory responses and consequently new biomarkers of disease as well as novel targeted-specific therapeutic strategies.

Acknowledgments

Financial support from Portugal: Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP) integrates the i3S research unit, which is partially supported by the Portuguese Foundation for Science and Technology (FCT). This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund (ERDF). This work was also funded by FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT—Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação in the framework of the project (POCI-01/0145-FEDER-016601; PTDC/DTP-PIC/0560/2014). SSP acknowledges the European Crohńs and Colitis Organization (ECCO) for the ECCO Grant 2017 that contributed to the funding of this research project. SSP also acknowledges the “Broad Medical Research program at Crohńs and Colitis Foundation of America-CCFA” for funding. SSP acknowledges the Portuguese Group of Study on IBD (GEDII) for funding. AMD [PD/BD/105982/2014], MSP [SFRH/BD/110148/2015] and IA [SFRH/BD/128874/2017] acknowledge FCT for funding.

References

[1] P.M. Rudd, T. Elliott, P. Cresswell, I.A. Wilson, R.A. Dwek, Glycosylation and the immune system, Science 291 (2001) 2370–2376.
[2] J.D. Marth, P.K. Grewal, Mammalian glycosylation in immunity, Nat. Rev. Immunol. 8 (2008) 874–887.
[3] N.M. Koropatkin, E.A. Cameron, E.C. Martens, How glycan metabolism shapes the human gut microbiota, Nat. Rev. Microbiol. 10 (2012) 323–335.
[4] P. Gagneux, M. Aebi, A. Varki, Evolution of glycan diversity, in: A. Varki, R.D. Cummings, J.D. Esko, P. Stanley, G.W. Hart, M. Aebi, A.G. Darvill, T. Kinoshita, N.H. Packer, J.H. Prestegard, R.L. Schnaar, P.H. Seeberger (Eds.), Essentials of glycobiology, 2015, pp. 253–264 Cold Spring Harbor (NY).
[5] A.L. Kau, P.P. Ahern, N.W. Griffin, A.L. Goodman, J.I. Gordon, Human nutrition, the gut microbiome and the immune system, Nature 474 (2011) 327–336.
[6] B.D. Muegge, J. Kuczynski, D. Knights, J.C. Clemente, A. Gonzalez, L. Fontana, B. Henrissat, R. Knight, J.I. Gordon, Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans, Science 332 (2011) 970–974.
[7] D.A. Ravcheev, I. Thiele, Comparative genomic analysis of the human gut microbiome reveals a broad distribution of metabolic pathways for the degradation of host-synthetized mucin glycans and utilization of mucin-derived monosaccharides, Front. Genet. 8 (2017) 111.
[8] J.L. Sonnenburg, J. Xu, D.D. Leip, C.H. Chen, B.P. Westover, J. Weatherford, J.D. Buhler, J.I. Gordon, Glycan foraging in vivo by an intestine-adapted bacterial symbiont, Science 307 (2005) 1955–1959.
[9] L.E. Tailford, E.H. Crost, D. Kavanaugh, N. Juge, Mucin glycan foraging in the human gut microbiome, Front. Genet. 6 (2015) 81.
[10] J.M. Larsson, H. Karlsson, H. Sjovall, G.C. Hansson, A complex, but uniform O-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn, Glycobiology 19 (2009) 756–766.
[11] J.M. Larsson, H. Karlsson, J.G. Crespo, M.E. Johansson, L. Eklund, H. Sjovall, G.C. Hansson, Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation, Inflam. Bowel Dis. 17 (2011) 2299–2307.
[12] G. An, B. Wei, B. Xia, J.M. McDaniel, T. Ju, R.D. Cummings, J. Braun, L. Xia, Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans, J. Experim. Med. 204 (2007) 1417–1429.
[13] X. Ma, Z. Dai, K. Sun, Y. Zhang, J. Chen, Y. Yang, P. Tso, G. Wu, Z. Wu, Intestinal epithelial cell endoplasmic reticulum stress and inflammatory bowel disease pathogenesis: an update review, Front. Immunol. 8 (2017) 1271.
[14] A. Kaser, R.S. Blumberg, Endoplasmic reticulum stress and intestinal inflammation, Mucosal Immunol. 3 (2010) 11–16.
[15] A. Kaser, E. Martinez-Naves, R.S. Blumberg, Endoplasmic reticulum stress: implications for inflammatory bowel disease pathogenesis, Curr. Opin. Gastroenterol. 26 (2010) 318–326.
[16] S.Z. Hasnain, S. Tauro, I. Das, H. Tong, A.C. Chen, P.L. Jeffery, V. McDonald, T.H. Florin, M.A. McGuckin, IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells, Gastroenterology 144 (2013) 357–368 e359.
[17] K. Julenius, NetCGlyc 1.0: prediction of mammalian C-mannosylation sites, Glycobiology 17 (2007) 868–876.
[18] J. Perez-Vilar, S.H. Randell, R.C. Boucher, C-Mannosylation of MUC5AC and MUC5B Cys subdomains, Glycobiology 14 (2004) 325–337.
[19] J.L. Desseyn, Mucin CYS domains are ancient and highly conserved modules that evolved in concert, Mol. Phylogen. Evol. 52 (2009) 284–292.
[20] D. Boltin, T.T. Perets, A. Vilkin, Y. Niv, Mucin function in inflammatory bowel disease: an update, J. Clin. Gastroenterol. 47 (2013) 106–111.
[21] Y. Goto, T. Obata, J. Kunisawa, S. Sato, A. Ivanov II, N. Lamichhane, M. Takeyama, M. Kamioka, T. Sakamoto, H. Matsuki, A. Setoyama, S. Imaoka, S. Uematsu, S.E. Akira, P. Domino, B. Kulig, J.C. Becher, C. Renauld, Y. Sasakawa, Y. Umesaki, H. Kiyono Benno, Innate lymphoid cells regulate intestinal epithelial cell glycosylation, Science 345 (2014) 1254009.
[22] D. Artis, H. Spits, The biology of innate lymphoid cells, Nature 517 (2015) 293–301.
[23] D.P. McGovern, M.R. Jones, K.D. Taylor, K. Marciante, X. Yan, M. Dubinsky, A. Ippoliti, E. Vasiliauskas, D. Berel, C. Derkowski, D. Dutridge, P. Fleshner, D.Q. Shih, G. Melmed, E. Mengesha, L. King, S. Pressman, T. Haritunians, X. Guo, S.R. Targan, J.I. Rotter, I.B.D.G.C. International, Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease, Hum. Mol. Genet. 19 (2010) 3468–3476.
[24] A. Franke, D.P. McGovern, J.C. Barrett, K. Wang, G.L. Radford-Smith, T. Ahmad, C.W. Lees, T. Balschun, J. Lee, R. Roberts, C.A. Anderson, J.C. Bis, S. Bumpstead, D. Ellinghaus, E.M. Festen, M. Georges, T. Green, T. Haritunians, L. Jostins, A. Latiano, C.G. Mathew, G.W. Montgomery, N.J. Prescott, S. Raychaudhuri, J.I. Rotter, P. Schumm, Y. Sharma, L.A. Simms, K.D. Taylor, D. Whiteman, C. Wijmenga, R.N. Baldassano, M. Barclay, T.M. Bayless, S. Brand, C. Buning, A. Cohen, J.F. Colombel, M. Cottone, L. Stronati, T. Denson, M. De Vos, R. D'Inca, M. Dubinsky, C. Edwards, T. Florin, D. Franchimont, R. Gearry, J. Glas, A. Van Gossum, S.L. Guthery, J. Halfvarson, H.W. Verspaget, J.P. Hugot, A. Karban, D. Laukens, I. Lawrance, M. Lemann, A. Levine, C. Libioulle, E. Louis, C. Mowat, W. Newman, J. Panes, A. Phillips, D.D. Proctor, M. Regueiro, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, F. Seibold, A.H. Steinhart, P.C. Stokkers, L. Torkvist, G. Kullak-Ublick, D. Wilson, T. Walters, S.R. Targan, S.R. Brant,

J.D. Rioux, M. D'Amato, R.K. Weersma, S. Kugathasan, A.M. Griffiths, J.C. Mansfield, S. Vermeire, R.H. Duerr, M.S. Silverberg, J. Satsangi, S. Schreiber, J.H. Cho, V. Annese, H. Hakonarson, M.J. Daly, M. Parkes, Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci, Nat. Genet. 42 (2010) 1118–1125.
[25] E. Kottgen, B. Volk, F. Kluge, W. Gerok, Gluten, a lectin with oligomannosyl specificity and the causative agent of gluten-sensitive enteropathy, Biochem. Biophys. Res. Commun. 109 (1982) 168–173.
[26] S.H. Rouhanifard, A. Lopez Aguilar, L. Meng, K.W. Moremen, P. Wu, Engineered glycocalyx regulates stem cell proliferation in murine crypt organoids, Cell Chem. Biol. 25 (2018) 439–446 e435.
[27] H. Jiang, A. Lopez-Aguilar, L. Meng, Z. Gao, Y. Liu, X. Tian, G. Yu, B. Ovryn, K.W. Moremen, P. Wu, Modulating cell-surface receptor signaling and ion channel functions by in situ glycan editing, Angew. Chem. 57 (2018) 967–971.
[28] D. Rios, M.B. Wood, J. Li, B. Chassaing, A.T. Gewirtz, I.R. Williams, Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria, Mucosal Immunol. 9 (2016) 907–916.
[29] K. Terahara, T. Nochi, M. Yoshida, Y. Takahashi, Y. Goto, H. Hatai, S. Kurokawa, M.H. Jang, M.N. Kweon, S.E. Domino, T. Hiroi, Y. Yuki, Y. Tsunetsugu-Yokota, K. Kobayashi, H. Kiyono, Distinct fucosylation of M cells and epithelial cells by Fut1 and Fut2, respectively, in response to intestinal environmental stress, Biochem. Biophys. Res. Commun. 404 (2011) 822–828.
[30] Y. van Kooyk, G.A. Rabinovich, Protein-glycan interactions in the control of innate and adaptive immune responses, Nat. Immunol. 9 (2008) 593–601.
[31] J.L. Johnson, M.B. Jones, S.O. Ryan, B.A. Cobb, The regulatory power of glycans and their binding partners in immunity, Trends Immunol. 34 (2013) 290–298.
[32] M.A. Wolfert, G.J. Boons, Adaptive immune activation: glycosylation does matter, Nat. Chem. Biol. 9 (2013) 776–784.
[33] H. Feinberg, D.A. Mitchell, K. Drickamer, W.I. Weis, Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR, Science 294 (2001) 2163–2166.
[34] U. Svajger, M. Anderluh, M. Jeras, N. Obermajer, C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity, Cell. Signal. 22 (2010) 1397–1405.
[35] S.K. Singh, J. Stephani, M. Schaefer, H. Kalay, J.J. Garcia-Vallejo, J. den Haan, E. Saeland, T. Sparwasser, Y. van Kooyk, Targeting glycan modified OVA to murine DC-SIGN transgenic dendritic cells enhances MHC class I and II presentation, Mol. Immunol. 47 (2009) 164–174.
[36] Y. van Kooyk, T.B. Geijtenbeek, DC-SIGN: escape mechanism for pathogens, Nat. Rev. Immunol. 3 (2003) 697–709.
[37] K.P. van Gisbergen, M. Sanchez-Hernandez, T.B. Geijtenbeek, Y. van Kooyk, Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN, J. Experim. Med. 201 (2005) 1281–1292.
[38] G.A. Rabinovich, D.O. Croci, Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer, Immunity 36 (2012) 322–335.
[39] M. Perdicchio, J.M. Ilarregui, M.I. Verstege, L.A. Cornelissen, S.T. Schetters, S. Engels, M. Ambrosini, H. Kalay, H. Veninga, J.M. den Haan, L.A. van Berkel, J.N. Samsom, P.R. Crocker, T. Sparwasser, L. Berod, J.J. Garcia-Vallejo, Y. van Kooyk, W.W. Unger, Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells, PNAS 113 (2016) 3329–3334.
[40] Y.L. Huang, C. Chassard, M. Hausmann, M. von Itzstein, T. Hennet, Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice, Nat. Commun. 6 (2015) 8141.
[41] U. Gophna, K. Sommerfeld, S. Gophna, W.F. Doolittle, S.J. Veldhuyzen van Zanten, Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis, J. Clin. Microbiol. 44 (2006) 4136–4141.
[42] V. Vanhooren, R.E. Vandenbroucke, S. Dewaele, E. Van Hamme, J.J. Haigh, T. Hochepied, C. Libert, Mice overexpressing beta-1,4-Galactosyltransferase I are resistant to TNF-induced inflammation and DSS-induced colitis, PloS One 8 (2013) e79883.
[43] E. Barreto-Bergter, R.T. Figueiredo, Fungal glycans and the innate immune recognition, Front. Cell. Infect. Microbiol. 4 (2014) 145.
[44] B. Byrne, G.G. Donohoe, R. O'Kennedy, Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells, Drug Discov. Today 12 (2007) 319–326.
[45] V. Pozsgay, C. Chu, L. Pannell, J. Wolfe, J.B. Robbins, R. Schneerson, Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from Shigella dysenteriae type 1, Proceedings of the National Academy of Sciences of the United States of America, 1999, pp. 5194–5197.
[46] S. Cheng, X. Chang, Y. Wang, G.F. Gao, Y. Shao, L. Ma, X. Li, Glycosylated enfuvirtide: a long-lasting glycopeptide with potent anti-HIV activity, J. Med. Chem. 58 (2015) 1372–1379.
[47] N.G. Bednarska, B.W. Wren, S.J. Willcocks, The importance of the glycosylation of antimicrobial peptides: natural and synthetic approaches, Drug Discov. Today 22 (2017) 919–926.
[48] J.L. Fox, Antimicrobial peptides stage a comeback, Nat. Biotechnol. 31 (2013) 379–382.
[49] N.H. Salzman, K. Hung, D. Haribhai, H. Chu, J. Karlsson-Sjoberg, E. Amir, P. Teggatz, M. Barman, M. Hayward, D. Eastwood, M. Stoel, Y. Zhou, E. Sodergren, G.M. Weinstock, C.L. Bevins, C.B. Williams, N.A. Bos, Enteric defensins are essential regulators of intestinal microbial ecology, Nat. Immunol. 11 (2010) 76–83.
[50] U. Jain, A.R. Otley, J. Van Limbergen, A.W. Stadnyk, The complement system in inflammatory bowel disease, Inflam. Bowel Dis. 20 (2014) 1628–1637.
[51] S. Hartmann, J. Hofsteenge, Properdin, the positive regulator of complement, is highly C-mannosylated, J. Biol. Chem. 275 (2000) 28569–28574.
[52] J. Neefjes, M.L. Jongsma, P. Paul, O. Bakke, Towards a systems understanding of MHC class I and MHC class II antigen presentation, Nat. Rev. Immunol. 11 (2011) 823–836.
[53] K. Smith, G.M. Boukhaled, S.A. Condotta, S. Mazouz, J.J. Guthmiller, R. Vijay, N.S. Butler, J. Bruneau, N.H. Shoukry, C.M. Krawczyk, M.J. Richer, Interleukin-10 directly inhibits CD8(+) T Cell function by enhancing N-glycan branching to decrease antigen sensitivity, Immunity 48 (2018) 299–312 e295.
[54] M. Demetriou, M. Granovsky, S. Quaggin, J.W. Dennis, Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation, Nature 409 (2001) 733–739.
[55] A.M. Dias, J. Dourado, P. Lago, J. Cabral, R. Marcos-Pinto, P. Salgueiro, C.R. Almeida, S. Carvalho, S. Fonseca, M. Lima, M. Vilanova, M. Dinis-Ribeiro, C.A. Reis, S.S. Pinho, Dysregulation of T cell receptor N-glycosylation: a molecular mechanism involved in ulcerative colitis, Hum. Mol. Genet. 23 (2014) 2416–2427.
[56] A.M. Dias, A. Correia, M.S. Pereira, C.R. Almeida, I. Alves, V. Pinto, T.A. Catarino, N. Mendes, M. Leander, M.T. Oliva-Teles, L. Maia, C. Delerue-Matos, N. Taniguchi, M. Lima, I. Pedroto, R. Marcos-Pinto, P. Lago, C.A. Reis, M. Vilanova, S.S. Pinho, Metabolic control of T cell immune response through glycans in inflammatory bowel disease, PNAS 115 (2018) E4651–E4660.
[57] A. Grigorian, S.U. Lee, W. Tian, I.J. Chen, G. Gao, R. Mendelsohn, J.W. Dennis, M. Demetriou, Control of T Cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis, J. Biol. Chem. 282 (2007) 20027–20035.
[58] K.S. Lau, E.A. Partridge, A. Grigorian, C.I. Silvescu, V.N. Reinhold, M. Demetriou, J.W. Dennis, Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation, Cell 129 (2007) 123–134.
[59] R. Morgan, G. Gao, J. Pawling, J.W. Dennis, M. Demetriou, B. Li, N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells, J. Immunol. 173 (2004) 7200–7208.
[60] L. Araujo, P. Khim, H. Mkhikian, C.L. Mortales, M. Demetriou, Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation, eLife 6 (2017).
[61] A. Grigorian, L. Araujo, N.N. Naidu, D.J. Place, B. Choudhury, M. Demetriou, N-acetylglucosamine inhibits T-helper 1 (Th1)/T-helper 17 (Th17) cell responses and treats experimental autoimmune encephalomyelitis, J. Biol. Chem. 286 (2011) 40133–40141.
[62] I.J. Chen, H.L. Chen, M. Demetriou, Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling, J. Biol. Chem. 282 (2007) 35361–35372.
[63] P. Stanley, N. Taniguchi, M. Aebi, N-Glycans, in: A. Varki, R.D. Cummings, J.D. Esko, P. Stanley, G.W. Hart, M. Aebi, A.G. Darvill, T. Kinoshita, N.H. Packer, J.H. Prestegard, R.L. Schnaar, P.H. Seeberger (Eds.), Essentials of glycobiology, 2015, pp. 99–111 Cold Spring Harbor (NY).
[64] F.T. Liu, G.A. Rabinovich, Galectins: regulators of acute and chronic inflammation, Ann. N. Y. Acad. Sci. 1183 (2010) 158–182.
[65] A. Togayachi, Y. Kozono, H. Ishida, S. Abe, N. Suzuki, Y. Tsunoda, K. Hagiwara, A. Kuno, T. Ohkura, N. Sato, T. Sato, J. Hirabayashi, Y. Ikehara, K. Tachibana, H. Narimatsu, Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation, PNAS 104 (2007) 15829–15834.
[66] H. Mkhikian, C.L. Mortales, R.W. Zhou, K. Khachikyan, G. Wu, S.M. Haslam, P. Kavarian, A. Dell, M. Demetriou, Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis, eLife 5 (2016).
[67] G.A. Rabinovich, M.A. Toscano, S.S. Jackson, G.R. Vasta, Functions of cell surface galectin-glycoprotein lattices, Curr. Opin. Struct. Biol. 17 (2007) 513–520.
[68] F.T. Liu, G.A. Rabinovich, Galectins as modulators of tumour progression, Nat. Rev. Cancer 5 (2005) 29–41.
[69] V. Sundblad, L.G. Morosi, J.R. Geffner, G.A. Rabinovich, Galectin-1: a jack-of-all-trades in the resolution of acute and chronic inflammation, J. Immunol. 199 (2017) 3721–3730.
[70] M.A. Toscano, G.A. Bianco, J.M. Ilarregui, D.O. Croci, J. Correale, J.D. Hernandez, N.W. Zwirner, F. Poirier, E.M. Riley, L.G. Baum, G.A. Rabinovich, Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death, Nat. Immunol. 8 (2007) 825–834.
[71] C.D. Chung, V.P. Patel, M. Moran, L.A. Lewis, M.C. Miceli, Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction, J. Immunol. 165 (2000) 3722–3729.
[72] H.Y. Chen, A. Fermin, S. Vardhana, I.C. Weng, K.F. Lo, E.Y. Chang, E. Maverakis, R.Y. Yang, D.K. Hsu, M.L. Dustin, F.T. Liu, Galectin-3 negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse, PNAS 106 (2009) 14496–14501.
[73] D. Paclik, U. Berndt, C. Guzy, A. Dankof, S. Danese, P. Holzloehner, S. Rosewicz, B. Wiedenmann, B.M. Wittig, A.U. Dignass, A. Sturm, Galectin-2 induces apoptosis of lamina propria T lymphocytes and ameliorates acute and chronic experimental colitis in mice, J. Mol. Med. 86 (2008) 1395–1406.
[74] M.V. Tribulatti, V. Cattaneo, U. Hellman, J. Mucci, O. Campetella, Galectin-8 provides costimulatory and proliferative signals to T lymphocytes, J. Leukocyte Biol. 86 (2009) 371–380.
[75] A. Hokama, E. Mizoguchi, K. Sugimoto, Y. Shimomura, Y. Tanaka, M. Yoshida, S.T. Rietdijk, Y.P. de Jong, S.B. Snapper, C. Terhorst, R.S. Blumberg, A. Mizoguchi, Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation, Immunity 20 (2004) 681–693.
[76] H. Mkhikian, A. Grigorian, C.F. Li, H.L. Chen, B. Newton, R.W. Zhou, C. Beeton, S. Torossian, G.G. Tatarian, S.U. Lee, K. Lau, E. Walker, K.A. Siminovitch, K.G. Chandy, Z. Yu, J.W. Dennis, M. Demetriou, Genetics and the environment

converge to dysregulate N-glycosylation in multiple sclerosis, Nat. Commun. 2 (2011) 334.
[77] B. Brynedal, J. Wojcik, F. Esposito, V. Debailleul, J. Yaouanq, F. Martinelli-Boneschi, G. Edan, G. Comi, J. Hillert, H. Abderrahim, MGAT5 alters the severity of multiple sclerosis, J. Neuroimmunol. 220 (2010) 120–124.
[78] C.F. Li, R.W. Zhou, H. Mkhikian, B.L. Newton, Z. Yu, M. Demetriou, Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively with MGAT1 and interleukin-2 and 7 receptor variants, J. Neuroimmunol. 256 (2013) 71–76.
[79] M.S. Pereira, C. Duraes, T.A. Catarino, I. Cleynen, R. Marcos-Pinto, J.C. Machado, F. Magro, S. Vermeire, P. Lago, S.S. Pinho, Genetic variants of MGAT5 gene are associated with ulcerative colitis severity and response to therapy, J. Crohns Colitis 12 (2018) S546–S547.
[80] M. Klasić, D. Markulin, A. Vojta, I. Samaržija, I. Biruš, P. Dobrinić, N.T. Ventham, I. Trbojević-Akmačić, M. Šimurina, J. Štambuk, G. Razdorov, N.A. Kennedy, J. Satsangi, A.M. Dias, S. Pinho, V. Annese, A. Latiano, R. D'Inca, G. Lauc, V. Zoldoš, Promoter methylation of the MGAT3 and BACH2 genes correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease, Clin. Epigenet. 10 (2018) 75.
[81] H. Fujii, S. Shinzaki, H. Iijima, K. Wakamatsu, C. Iwamoto, T. Sobajima, R. Kuwahara, S. Hiyama, Y. Hayashi, S. Takamatsu, N. Uozumi, Y. Kamada, M. Tsujii, N. Taniguchi, T. Takehara, E. Miyoshi, Core fucosylation on T cells, required for activation of T-cell receptor signaling and induction of colitis in mice, is increased in patients with inflammatory bowel disease, Gastroenterology 150 (2016) 1620–1632.
[82] K. Bergstrom, J. Fu, M.E. Johansson, X. Liu, N. Gao, Q. Wu, J. Song, J.M. McDaniel, S. McGee, W. Chen, J. Braun, G.C. Hansson, L. Xia, Core 1- and 3-derived O-glycans collectively maintain the colonic mucus barrier and protect against spontaneous colitis in mice, Mucosal Immunol. 10 (2017) 91–103.
[83] Y. Wang, D. Huang, K.Y. Chen, M. Cui, W. Wang, X. Huang, A. Awadellah, Q. Li, A. Friedman, W.W. Xin, L. Di Martino, F. Cominelli, A. Miron, R. Chan, J.G. Fox, Y. Xu, X. Shen, M.F. Kalady, S. Markowitz, I. Maillard, J.B. Lowe, W. Xin, L. Zhou, Fucosylation deficiency in mice leads to colitis and adenocarcinoma, Gastroenterology 152 (2017) 193–205 e110.
[84] M. Van der Sluis, B.A. De Koning, A.C. De Bruijn, A. Velcich, J.P. Meijerink, J.B. Van Goudoever, H.A. Buller, J. Dekker, I. Van Seuningen, I.B. Renes, A.W. Einerhand, Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection, Gastroenterology 131 (2006) 117–129.
[85] S.J. Brown, A.M. Miller, P.J. Cowan, J. Slavin, W.R. Connell, G.T. Moore, S. Bell, P.R. Elliott, P.V. Desmond, A.J. d'Apice, Altered immune system glycosylation causes colitis in alpha1,2-fucosyltransferase transgenic mice, Inflam. Bowel Dis. 10 (2004) 546–556.
[86] M.S. Macauley, N. Kawasaki, W. Peng, S.H. Wang, Y. He, B.M. Arlian, R. McBride, R. Kannagi, K.H. Khoo, J.C. Paulson, Unmasking of CD22 Co-receptor on germinal center B-cells occurs by alternative mechanisms in mouse and man, J. Biol. Chem. 290 (2015) 30066–30077.
[87] I.W. Mak, N. Evaniew, M. Ghert, Lost in translation: animal models and clinical trials in cancer treatment, Am. J. Transl. Res. 6 (2014) 114–118.
[88] M. Swamy, S. Pathak, K.M. Grzes, S. Damerow, L.V. Sinclair, D.M. van Aalten, D.A. Cantrell, Glucose and glutamine fuel protein O-GlcNAcylation to control T cell self-renewal and malignancy, Nat. Immunol. 17 (2016) 712–720.
[89] G.W. Hart, M.P. Housley, C. Slawson, Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins, Nature 446 (2007) 1017–1022.
[90] S.J. Hobbs, J.C. Nolz, Regulation of T cell trafficking by enzymatic synthesis of O-glycans, Front. Immunol. 8 (2017) 600.
[91] H.H. Smits, B. Everts, F.C. Hartgers, M. Yazdanbakhsh, Chronic helminth infections protect against allergic diseases by active regulatory processes, Curr. Allergy Asthma Rep. 10 (2010) 3–12.
[92] A.M. Blum, L. Hang, T. Setiawan, J.P. Urban Jr., K.M. Stoyanoff, J. Leung, J.V. Weinstock, Heligmosomoides polygyrus bakeri induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses, J. Immunol. 189 (2012) 2512–2520.
[93] L.M. Kuijk, I. van Die, Worms to the rescue: can worm glycans protect from autoimmune diseases? IUBMB Life 62 (2010) 303–312.
[94] R.M. Maizels, M. Yazdanbakhsh, Immune regulation by helminth parasites: cellular and molecular mechanisms, Nat. Rev. Immunol. 3 (2003) 733–744.
[95] C.E. Matisz, B. Faz-Lopez, E. Thomson, A. Al Rajabi, F. Lopes, L.I. Terrazas, A. Wang, K.A. Sharkey, D.M. McKay, Suppression of colitis by adoptive transfer of helminth antigen-treated dendritic cells requires interleukin-4 receptor-alpha signaling, Sci. Rep. 7 (2017) 40631.
[96] R.M. Maizels, Parasitic helminth infections and the control of human allergic and autoimmune disorders, Clin. Microbiol. Infect. 22 (2016) 481–486.
[97] R.W. Summers, D.E. Elliott, J.F. Urban Jr., R.A. Thompson, J.V. Weinstock, Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial, Gastroenterology 128 (2005) 825–832.
[98] L. Eligio-Garcia, C.V. Maria del Pilar, F.L. Andres, C.E. Apolinar, C.C. Adrian, J.C. Enedina, Giardia intestinalis: expression of ubiquitin, glucosamine-6-phosphate and cyst wall protein genes during the encystment process, Experim. Parasitol. 127 (2011) 382–386.
[99] M.E. Giorgi, R.M. de Lederkremer, Trans-sialidase and mucins of Trypanosoma cruzi: an important interplay for the parasite, Carbohydr. Res. 346 (2011) 1389–1393.
[100] S.Z. Hasnain, M.A. McGuckin, R.K. Grencis, D.J. Thornton, Serine protease(s) secreted by the nematode Trichuris muris degrade the mucus barrier, PLoS Neglect. Trop. Dis. 6 (2012) e1856.
[101] I. van Die, R.D. Cummings, Glycan gimmickry by parasitic helminths: a strategy for modulating the host immune response? Glycobiology 20 (2010) 2–12.
[102] C.D. Rillahan, J.C. Paulson, Glycan microarrays for decoding the glycome, Ann. Rev. Biochem. 80 (2011) 797–823.
[103] D.F. Smith, R.D. Cummings, Application of microarrays for deciphering the structure and function of the human glycome, Mol. Cell. Proteom: MCP 12 (2013) 902–912.
[104] S. Holst, B. Heijs, N. de Haan, R.J. van Zeijl, I.H. Briaire-de Bruijn, G.W. van Pelt, A.S. Mehta, P.M. Angel, W.E. Mesker, R.A. Tollenaar, R.R. Drake, J.V. Bovee, L.A. McDonnell, M. Wuhrer, Linkage-specific in situ sialic acid derivatization for N-glycan mass spectrometry imaging of formalin-fixed paraffin-embedded tissues, Anal. Chem. 88 (2016) 5904–5913.
[105] H. Hinneburg, F. Schirmeister, P. Korac, D. Kolarich, N- and O-glycomics from minor amounts of formalin-fixed, paraffin-embedded tissue samples, Meth. Mol. Biol. 1503 (2017) 131–145.
[106] P.H. Jensen, N.G. Karlsson, D. Kolarich, N.H. Packer, Structural analysis of N- and O-glycans released from glycoproteins, Nat. Protoc. 7 (2012) 1299–1310.
[107] S. Purohit, T. Li, W. Guan, X. Song, J. Song, Y. Tian, L. Li, A. Sharma, B. Dun, D. Mysona, S. Ghamande, B. Rungruang, R.D. Cummings, P.G. Wang, J.X. She, Multiplex glycan bead array for high throughput and high content analyses of glycan binding proteins, Nat. Commun. 9 (2018) 258.
[108] J. Cosnes, C. Gower-Rousseau, P. Seksik, A. Cortot, Epidemiology and natural history of inflammatory bowel diseases, Gastroenterology 140 (2011) 1785–1794.
[109] H. Lassmann, J. van Horssen, D. Mahad, Progressive multiple sclerosis: pathology and pathogenesis, Nat. Rev. Neurol. 8 (2012) 647–656.
[110] J.A. Bluestone, K. Herold, G. Eisenbarth, Genetics, pathogenesis and clinical interventions in type 1 diabetes, Nature 464 (2010) 1293–1300.
[111] J.S. Smolen, D. Aletaha, I.B. McInnes, Rheumatoid arthritis, Lancet 388 (2016) 2023–2038.
[112] V.R. Moulton, A. Suarez-Fueyo, E. Meidan, H. Li, M. Mizui, G.C. Tsokos, Pathogenesis of human systemic lupus erythematosus: a cellular perspective, Trends Mol. Med. 23 (2017) 615–635.
[113] G.R. D'Haens, Top-down therapy for IBD: rationale and requisite evidence, Nat. Rev. Gastroenterol. Hepatol. 7 (2010) 86–92.
[114] S. Siebert, D. Porter, C. Paterson, R. Hampson, D. Gaya, A. Latosinska, H. Mischak, J. Schanstra, W. Mullen, I. McInnes, Urinary proteomics can define distinct diagnostic inflammatory arthritis subgroups, Sci. Rep. 7 (2017) 40473.
[115] C.C. Mok, Towards new avenues in the management of lupus glomerulonephritis, Nat. Rev. Rheumatol. 12 (2016) 221–234.
[116] A.M. Oommen, N. Somaiya, J. Vijayan, S. Kumar, S. Venkatachalam, L. Joshi, GlycoGAIT: a web database to browse glycogenes and lectins under gastric inflammatory diseases, J. Theoret. Biol. 406 (2016) 93–98.
[117] M. Pereira, S. Carvalho, L. Azevedo, A. Albergaria, J. Lima, C. Reis, I. Pedroto, L. Maia, R. Marcos-Pinto, P. Lago, S.S. Pinho, P299 Identification of a prognostic biomarker able to predict ulcerative colitis patients that will not respond to standard therapy, J. Crohn's Colitis 11 (2017) S229-S229.
[118] I. Quast, B. Peschke, J.D. Lunemann, Regulation of antibody effector functions through IgG Fc N-glycosylation, Cell. Mol. Life Sci. CMLS 74 (2017) 837–847.
[119] Y. Kaneko, F. Nimmerjahn, J.V. Ravetch, Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation, Science 313 (2006) 670–673.
[120] R.L. Shields, J. Lai, R. Keck, L.Y. O'Connell, K. Hong, Y.G. Meng, S.H. Weikert, L.G. Presta, Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity, J. Biol. Chem. 277 (2002) 26733–26740.
[121] E.C. Franklin, Structure and function of immunoglobulins, Supplementum, Acta Endocrinol 194 (1975) 77–95.
[122] C. Ferrara, S. Grau, C. Jager, P. Sondermann, P. Brunker, I. Waldhauer, M. Hennig, A. Ruf, A.C. Rufer, M. Stihle, P. Umana, J. Benz, Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose, PNAS 108 (2011) 12669–12674.
[123] R. Malhotra, M.R. Wormald, P.M. Rudd, P.B. Fischer, R.A. Dwek, R.B. Sim, Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein, Nat. Med. 1 (1995) 237–243.
[124] F. Nimmerjahn, R.M. Anthony, J.V. Ravetch, Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity, PNAS 104 (2007) 8433–8437.
[125] S. Nakajima, H. Iijima, S. Shinzaki, S. Egawa, T. Inoue, A. Mukai, Y. Hayashi, J. Kondo, T. Akasaka, T. Nishida, T. Kanto, E. Morii, T. Mizushima, E. Miyoshi, M. Tsujii, N. Hayashi, Functional analysis of agalactosyl IgG in inflammatory bowel disease patients, Inflam. Bowel Dis. 17 (2011) 927–936.
[126] J.D. Pagan, M. Kitaoka, R.M. Anthony, Engineered sialylation of pathogenic antibodies in vivo attenuates autoimmune disease, Cell 172 (2018) 564–577 e513.
[127] I. Trbojevic Akmacic, N.T. Ventham, E. Theodoratou, F. Vuckovic, N.A. Kennedy, J. Kristic, E.R. Nimmo, R. Kalla, H. Drummond, J. Stambuk, M.G. Dunlop, M. Novokmet, Y. Aulchenko, O. Gornik, H. Campbell, M. Pucic Bakovic, J. Satsangi, G. Lauc, I.-B. Consortium, Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome, Inflam. Bowel Dis. 21 (2015) 1237–1247.
[128] A. Matsumoto, K. Shikata, F. Takeuchi, N. Kojima, T. Mizuochi, Autoantibody activity of IgG rheumatoid factor increases with decreasing levels of galactosylation and sialylation, J. Biochem. 128 (2000) 621–628.
[129] I. Gudelj, P.P. Salo, I. Trbojevic-Akmacic, M. Albers, D. Primorac, M. Perola, G. Lauc, Low galactosylation of IgG associates with higher risk for future diagnosis of rheumatoid arthritis during 10years of follow-up, Biochim. Biophys. Acta 2018 (1864) 2034–2039.
[130] M. Wuhrer, M.H. Selman, L.A. McDonnell, T. Kumpfel, T. Derfuss, M. Khademi, T. Olsson, R. Hohlfeld, E. Meinl, M. Krumbholz, Pro-inflammatory pattern of IgG1 Fc glycosylation in multiple sclerosis cerebrospinal fluid, J. Neuroinflam. 12

(2015) 235.
[131] F. Vuckovic, J. Kristic, I. Gudelj, M. Teruel, T. Keser, M. Pezer, M. Pucic-Bakovic, J. Stambuk, I. Trbojevic-Akmacic, C. Barrios, T. Pavic, C. Menni, Y. Wang, Y. Zhou, L. Cui, H. Song, Q. Zeng, X. Guo, B.A. Pons-Estel, P. McKeigue, A. Leslie Patrick, O. Gornik, T.D. Spector, M. Harjacek, M. Alarcon-Riquelme, M. Molokhia, W. Wang, G. Lauc, Association of systemic lupus erythematosus with decreased immunosuppressive potential of the IgG glycome, Arthrit. Rheumatol. 67 (2015) 2978–2989.
[132] I. Gornik, G. Maravic, J. Dumic, M. Flogel, G. Lauc, Fucosylation of IgG heavy chains is increased in rheumatoid arthritis, Clin. Biochem. 32 (1999) 605–608.
[133] K. Miyahara, K. Nouso, S. Saito, S. Hiraoka, K. Harada, S. Takahashi, Y. Morimoto, S. Kobayashi, F. Ikeda, Y. Miyake, H. Shiraha, A. Takaki, H. Okada, M. Amano, K. Hirose, S. Nishimura, K. Yamamoto, Serum glycan markers for evaluation of disease activity and prediction of clinical course in patients with ulcerative colitis, PloS One 8 (2013) e74861.
[134] M. Simurina, N. de Haan, F. Vuckovic, N.A. Kennedy, J. Stambuk, D. Falck, I. Trbojevic-Akmacic, F. Clerc, G. Razdorov, A. Khon, A. Latiano, R. D'Inca, S. Danese, S. Targan, C. Landers, M. Dubinsky, C. Inflammatory Bowel Disease Biomarkers, D.P.B. McGovern, V. Annese, M. Wuhrer, G. Lauc, glycosylation of immunoglobulin G associates with clinical features of inflammatory bowel diseases, Gastroenterology 154 (2018) 1320–1333 e1310.
[135] G.A. Rook, J. Steele, R. Brealey, A. Whyte, D. Isenberg, N. Sumar, J.L. Nelson, K.B. Bodman, A. Young, I.M. Roitt, et al., Changes in IgG glycoform levels are associated with remission of arthritis during pregnancy, J. Autoimmun. 4 (1991) 779–794.
[136] R.B. Parekh, I.M. Roitt, D.A. Isenberg, R.A. Dwek, B.M. Ansell, T.W. Rademacher, Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity, Lancet 1 (1988) 966–969.
[137] A. Ercan, J. Cui, D.E. Chatterton, K.D. Deane, M.M. Hazen, W. Brintnell, C.I. O'Donnell, L.A. Derber, M.E. Weinblatt, N.A. Shadick, D.A. Bell, E. Cairns, D.H. Solomon, V.M. Holers, P.M. Rudd, D.M. Lee, Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis, Arthrit. Rheum. 62 (2010) 2239–2248.
[138] F. Gomollon, Biosimilars: are they bioequivalent? Digest. Dis. 32 (Suppl 1) (2014) 82–87.
[139] S. Sha, C. Agarabi, K. Brorson, D.Y. Lee, S. Yoon, N-glycosylation design and control of therapeutic monoclonal antibodies, Trends Biotechnol. 34 (2016) 835–846.
[140] R.J. Sola, K. Griebenow, Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy, BioDrugs Clin. Immunother. Biopharmaceut. Gene Ther. 24 (2010) 9–21.
[141] R. Plomp, A. Bondt, N. de Haan, Y. Rombouts, M. Wuhrer, Recent advances in clinical glycoproteomics of immunoglobulins (Igs), Mol. Cell. Proteom. MCP 15 (2016) 2217–2228.
[142] I. Schwab, F. Nimmerjahn, Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Nat. Rev. Immunol. 13 (2013) 176–189.
[143] F. Nimmerjahn, J.V. Ravetch, Anti-inflammatory actions of intravenous immunoglobulin, Ann. Rev. Immunol. 26 (2008) 513–533.
[144] P. Imbach, S. Barandun, V. d'Apuzzo, C. Baumgartner, A. Hirt, A. Morell, E. Rossi, M. Schoni, M. Vest, H.P. Wagner, High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood, Lancet 1 (1981) 1228–1231.
[145] R.M. Anthony, F. Wermeling, M.C. Karlsson, J.V. Ravetch, Identification of a receptor required for the anti-inflammatory activity of IVIG, PNAS 105 (2008) 19571–19578.
[146] R.M. Anthony, T. Kobayashi, F. Wermeling, J.V. Ravetch, Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway, Nature 475 (2011) 110–113.
[147] B.M. Fiebiger, J. Maamary, A. Pincetic, J.V. Ravetch, Protection in antibody- and T cell-mediated autoimmune diseases by antiinflammatory IgG Fcs requires type II FcRs, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) E2385–E2394.
[148] A. Khoruts, M.J. Sadowsky, Understanding the mechanisms of faecal microbiota transplantation, Nat. Rev. Gastroenterol. Hepatol. 13 (2016) 508–516.
[149] S. Paramsothy, R. Paramsothy, D.T. Rubin, M.A. Kamm, N.O. Kaakoush, H.M. Mitchell, N. Castano-Rodriguez, Faecal microbiota transplantation for inflammatory bowel disease: a systematic review and meta-analysis, J. Crohn's Colitis 11 (2017) 1180–1199.
[150] P.K. Kump, H.P. Grochenig, S. Lackner, S. Trajanoski, G. Reicht, K.M. Hoffmann, A. Deutschmann, H.H. Wenzl, W. Petritsch, G.J. Krejs, G. Gorkiewicz, C. Hogenauer, Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis, Inflam. Bowel Dis. 19 (2013) 2155–2165.
[151] C.J. Damman, S.I. Miller, C.M. Surawicz, T.L. Zisman, The microbiome and inflammatory bowel disease: is there a therapeutic role for fecal microbiota transplantation? Am. J. Gastroenterol. 107 (2012) 1452–1459.
[152] S. Vermeire, M. Joossens, K. Verbeke, J. Wang, K. Machiels, J. Sabino, M. Ferrante, G. Van Assche, P. Rutgeerts, J. Raes, Donor species richness determines faecal microbiota transplantation success in inflammatory bowel disease, J. Crohn's Colitis 10 (2016) 387–394.
[153] G. Lauc, J.E. Huffman, M. Pucic, L. Zgaga, B. Adamczyk, A. Muzinic, M. Novokmet, O. Polasek, O. Gornik, J. Kristic, T. Keser, V. Vitart, B. Scheijen, H.W. Uh, M. Molokhia, A.L. Patrick, P. McKeigue, I. Kolcic, I.K. Lukic, O. Swann, F.N. van Leeuwen, L.R. Ruhaak, J.J. Houwing-Duistermaat, P.E. Slagboom, M. Beekman, A.J. de Craen, A.M. Deelder, Q. Zeng, W. Wang, N.D. Hastie, U. Gyllensten, J.F. Wilson, M. Wuhrer, A.F. Wright, P.M. Rudd, C. Hayward, Y. Aulchenko, H. Campbell, I. Rudan, Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers, PLoS Genet. 9 (2013) e1003225.
[154] K. Suzuki, T. Yamada, K. Yamazaki, M. Hirota, N. Ishihara, M. Sakamoto, D. Takahashi, H. Iijima, K. Hase, Intestinal epithelial cell-specific deletion of alpha-mannosidase II ameliorates experimental colitis, Cell Struct. Funct. 43 (2018) 25–39.
[155] M. Tong, I. McHardy, P. Ruegger, M. Goudarzi, P.C. Kashyap, T. Haritunians, X. Li, T.G. Graeber, E. Schwager, C. Huttenhower, A.J. Fornace Jr., J.L. Sonnenburg, D.P. McGovern, J. Borneman, J. Braun, Reprograming of gut microbiome energy metabolism by the FUT2 Crohn's disease risk polymorphism, ISME J. 8 (2014) 2193–2206.
[156] P. Rausch, A. Rehman, S. Kunzel, R. Hasler, S.J. Ott, S. Schreiber, P. Rosenstiel, A. Franke, J.F. Baines, Colonic mucosa-associated microbiota is influenced by an interaction of Crohn disease and FUT2 (Secretor) genotype, PNAS 108 (2011) 19030–19035.
[157] D. Hu, D. Zhang, S. Zheng, M. Guo, X. Lin, Y. Jiang, Association of ulcerative colitis with FUT2 and FUT3 polymorphisms in patients from Southeast China, PloS One 11 (2016) e0146557.
[158] P.L. Lakatos, M. Papp, F. Rieder, Serologic antiglycan antibodies in inflammatory bowel disease, Am. J. Gastroenterol. 106 (2011) 406–412.
[159] I. Masoodi, R. Kochhar, U. Dutta, C. Vaishnavi, K.K. Prasad, K. Vaiphei, S. Kaur, K. Singh, Fecal lactoferrin, myeloperoxidase and serum C-reactive are effective biomarkers in the assessment of disease activity and severity in patients with idiopathic ulcerative colitis, J. Gastroenterol. Hepatol. 24 (2009) 1768–1774.
[160] T. Siqueiros-Cendon, S. Arevalo-Gallegos, B.F. Iglesias-Figueroa, I.A. Garcia-Montoya, J. Salazar-Martinez, Q. Rascon-Cruz, Immunomodulatory effects of lactoferrin, Acta Pharmacol. Sin. 35 (2014) 557–566.
[161] S. Shinzaki, E. Kuroki, H. Iijima, N. Tatsunaka, M. Ishii, H. Fujii, Y. Kamada, T. Kobayashi, N. Shibukawa, T. Inoue, M. Tsujii, S. Takeishi, T. Mizushima, A. Ogata, T. Naka, S.E. Plevy, T. Takehara, E. Miyoshi, Lectin-based immunoassay for aberrant IgG glycosylation as the biomarker for Crohn's disease, Inflam. Bowel Dis. 19 (2013) 321–331.
[162] J.A. Cremata, L. Sorell, R. Montesino, R. Garcia, M. Mata, G. Cabrera, J.A. Galvan, G. Garcia, R. Valdes, J.A. Garrote, Hypogalactosylation of serum IgG in patients with coeliac disease, Clin. Experim. Immunol. 133 (2003) 422–429.
[163] K. Lindfors, H. Suzuki, J. Novak, P. Collin, P. Saavalainen, L.L. Koskinen, M. Maki, K. Kaukinen, Galactosylation of serum IgA1 O-glycans in celiac disease, J. Clin. Immunol. 31 (2011) 74–79.
[164] M. Urushihara, S. Kagami, K. Yasutomo, M. Ito, S. Kondo, A. Kitamura, D. Malm, H. Klenow, O. Nilssen, Y. Kuroda, Sisters with alpha-mannosidosis and systemic lupus erythematosus, Eur. J. Pediat. 163 (2004) 192–195.
[165] D. Chui, G. Sellakumar, R. Green, M. Sutton-Smith, T. McQuistan, K. Marek, H. Morris, A. Dell, J. Marth, Genetic remodeling of protein glycosylation in vivo induces autoimmune disease, PNAS 98 (2001) 1142–1147.
[166] S.D. Liu, S. Lee, A. La Cava, C.C. Motran, B.H. Hahn, M.C. Miceli, Galectin-1-induced down-regulation of T lymphocyte activation protects (NZB x NZW) F1 mice from lupus-like disease, Lupus 20 (2011) 473–484.
[167] S.K. Panda, V. Facchinetti, E. Voynova, S. Hanabuchi, J.L. Karnell, R.N. Hanna, R. Kolbeck, M.A. Sanjuan, R. Ettinger, Y.J. Liu, Galectin-9 inhibits TLR7-mediated autoimmunity in murine lupus models, J. Clin. Investig. 128 (2018) 1873–1887.
[168] M. Moritoki, T. Kadowaki, T. Niki, D. Nakano, G. Soma, H. Mori, H. Kobara, T. Masaki, M. Kohno, M. Hirashima, Galectin-9 ameliorates clinical severity of MRL/lpr lupus-prone mice by inducing plasma cell apoptosis independently of Tim-3, PloS One 8 (2013) e60807.
[169] S. Oomizu, T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, M. Hirashima, Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner, Clin. Immunol. 143 (2012) 51–58.
[170] M. Kanzaki, J. Wada, K. Sugiyama, A. Nakatsuka, S. Teshigawara, K. Murakami, K. Inoue, T. Terami, A. Katayama, J. Eguchi, H. Akiba, H. Yagita, H. Makino, Galectin-9 and T cell immunoglobulin mucin-3 pathway is a therapeutic target for type 1 diabetes, Endocrinology 153 (2012) 612–620.
[171] E.A. Sawin, T.J. De Wolfe, B. Aktas, B.M. Stroup, S.G. Murali, J.L. Steele, D.M. Ney, Glycomacropeptide is a prebiotic that reduces Desulfovibrio bacteria, increases cecal short-chain fatty acids, and is anti-inflammatory in mice, American journal of physiology, Gastrointest. Liver Physiol. 309 (2015) G590–G601.

Appendix II

Metabolic control of T cell immune response through glycans in inflammatory bowel disease

Ana M. Dias[a,b,c], Alexandra Correia[a,d], Márcia S. Pereira[a,b,c], Catarina R. Almeida[a,e,f], Inês Alves[a,b], Vanda Pinto[a,b], Telmo A. Catarino[a,b], Nuno Mendes[a,b], Magdalena Leander[g,h], M. Teresa Oliva-Teles[i], Luís Maia[j], Cristina Delerue-Matos[i], Naoyuki Taniguchi[k,1], Margarida Lima[g,h], Isabel Pedroto[j], Ricardo Marcos-Pinto[c,j,l], Paula Lago[j], Celso A. Reis[a,b,c,m], Manuel Vilanova[c,d], and Salomé S. Pinho[a,b,m,2]

[a]Institute for Research and Innovation in Health (i3S), University of Porto, 4200-135 Porto, Portugal; [b]Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP), 4200-135 Porto, Portugal; [c]Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal; [d]Institute for Molecular and Cell Biology (IBMC), Immunobiology Group, University of Porto, 4200-135 Porto, Portugal; [e]Institute of Biomedical Engineering (INEB), 4200-135 Porto, Portugal; [f]Institute for Biomedicine, Department of Medical Sciences, University of Aveiro, 3810-193 Aveiro, Portugal; [g]Hematology Department, Centro Hospitalar do Porto, 4099-001 Porto, Portugal; [h]Multidisciplinary Unit for Biomedical Research, ICBAS, University of Porto, 4050-313 Porto, Portugal; [i]REQUIMTE/LAQV, Instituto Superior de Engenharia, Instituto Politécnico do Porto, 4200-072 Porto, Portugal; [j]Department of Gastroenterology, Centro Hospitalar do Porto, Porto, Portugal; [k]Disease Glycomics Team, RIKEN, Tokyo 351-0198, Japan; [l]Medical Faculty, Centre for Research in Health Technologies and Information Systems, 4200-450 Porto, Portugal; and [m]Medical Faculty, University of Porto, 4200-319 Porto, Portugal

Edited by Kiyoshi Takeda, Graduate School of Medicine, Osaka University, Suita, Japan, and accepted by Editorial Board Member Tadatsugu Taniguchi April 6, 2018 (received for review November 25, 2017)

Mucosal T lymphocytes from patients with ulcerative colitis (UC) were previously shown to display a deficiency in branched N-glycosylation associated with disease severity. However, whether this glycosylation pathway shapes the course of the T cell response constituting a targeted-specific mechanism in UC remains largely unknown. In this study, we demonstrated that metabolic supplementation of ex vivo mucosal T cells from patients with active UC with *N*-acetylglucosamine (GlcNAc) resulted in enhancement of branched N-glycosylation in the T cell receptor (TCR), leading to suppression of T cell growth, inhibition of the T helper 1 (Th1)/Th17 immune response, and controlled T cell activity. We further demonstrated that mouse models displaying a deficiency in the branched N-glycosylation pathway (*MGAT5*$^{-/-}$, *MGAT5*$^{+/-}$) exhibited increased susceptibility to severe forms of colitis and early-onset disease. Importantly, the treatment of these mice with GlcNAc reduced disease severity and suppressed disease progression due to a controlled T cell-mediated immune response at the intestinal mucosa. In conclusion, our human ex vivo and preclinical results demonstrate the targeted-specific immunomodulatory properties of this simple glycan, proposing a therapeutic approach for patients with UC.

T lymphocytes | T cell receptor | adaptive immune response | branched N-glycosylation | intestinal inflammation

Inflammatory bowel diseases (IBDs), encompassing Crohn's disease and ulcerative colitis (UC), are chronic, relapsing, and life-long inflammatory disorders of the gastrointestinal tract affecting mainly young populations. The incidence of IBD is increasing worldwide, and the disease remains incurable, placing a heavy burden on populations by reducing patients' quality of life and increasing disability (1). The current therapeutic strategies for IBD are limited by reduced effectiveness, high costs, and/or side effects. This scenario highlights the urgent need in the clinic of identifying novel molecular markers capable of being selectively targeted with new and optimized therapies. Future progress in IBD monitoring and therapy mostly depends on the identification of key mechanism(s) mediating intestinal inflammation that could be therapeutically targeted.

The immune system is tightly regulated by glycosylation, through the addition of carbohydrate structures (glycans) to key molecules (proteins) involved in innate and adaptive immune responses (2). The *N*-acetylglucosaminyltransferase V (GnT-V) is a glycosyltransferase encoded by the human *MGAT5* gene that catalyzes the synthesis of β1,6-*N*-acetylglucosamine (GlcNAc) branched N-glycans, which are known to play pivotal roles in many glycoproteins in cancer (3–6) and also in T cell activity and function (7, 8). In homeostasis and self-tolerance, T cell activation [via T cell receptor (TCR) signaling] induces up-regulation of the *MGAT5* gene, which, in turn, leads to GnT-V–mediated glycosylation of the TCR (9). Consequently, it can promote growth arrest of T cells early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse (and restricting TCR signaling), and, later, by increasing surface retention of growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (9).

In fact, mice deficient in the *MGAT5* gene display an increased susceptibility to autoimmune diseases (7, 10, 11). These mice lacking GnT-V function (no synthesis of β1,6-GlcNAc branched N-glycan structures) display an increased TCR clustering and increased T helper 1 (Th1) differentiation that result in a hyperimmune response in mouse models of multiple sclerosis (7, 10). Interestingly, in mouse models of experimental autoimmune encephalomyelitis (EAE) and type I diabetes, it was shown that supplementation with GlcNAc induces increased N-glycan branching, through increasing the hexosamine pathway, that was associated with inhibition of T cell growth and differentiation (12, 13), leading to delayed disease progression. Moreover, N-glycan branching was also found to regulate T cell development (14). Recently, it was demonstrated that

Significance

Our findings demonstrate that metabolic supplementation of mucosal T cells, isolated from patients with active ulcerative colitis (UC), with *N*-acetylglucosamine (GlcNAc) leads to the enhancement of branched N-glycosylation on the T cell receptor, which was associated with the control of T cell activation and function. These results were validated in "glycoengineered" mouse models with severe colitis. Overall, our results open new avenues for a targeted-specific therapy in inflammatory bowel disease (IBD). The therapeutic use of GlcNAc (either alone or in combination with other antiinflammatory therapies) represents a simple immunomodulatory strategy in IBD, with absence of side effects, low costs, and the possibility of being used as a simple rescue therapy to avoid unnecessary toxic effects and step-up therapies in IBD.

Author contributions: A.M.D. and S.S.P. designed research; A.M.D., A.C., M.S.P., C.R.A., I.A., V.P., T.A.C., N.M., M. Leander, and L.M. performed research; A.M.D., C.R.A., M.T.O.-T., C.D.-M., N.T., M. Lima, and I.P. contributed new reagents/analytic tools; L.M., I.P., R.M.-P., and P.L. provided and characterized the clinical samples; A.M.D., A.C., C.R.A., M. Leander, M.T.O.-T., and M. Lima analyzed data; and A.M.D. and S.S.P. wrote the manuscript with contributions from A.C., C.A.R., and M.V.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. K.T. is a guest editor invited by the Editorial Board.

[1]Present address: Osaka International Cancer Institute, Chuoku, Osaka 541-8567, Japan.

[2]To whom correspondence should be addressed. Email: salomep@ipatimup.pt.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1720409115/-/DCSupplemental.

branching N-glycans potentiate the differentiation of induced regulatory (iTreg) T cells over Th17 differentiation (15). Importantly, and in the setting of human immune-mediated disorders, we have recently found that patients with UC exhibit a deficiency in branched glycosylation (catalyzed by GnT-V) in mucosal T cells that was associated with disease severity. Patients with UC who have severe disease showed the most pronounced defect on branched N-glycans in intestinal T cells, together with a significant reduction of *MGAT5* gene transcription in these cells (16).

In the present study, and building upon our previous findings in patients with UC (16), we have evaluated the impact of glycosylation, particularly the branched N-glycosylation pathway, in the regulation of the T cell-mediated immune response in patients with UC. We further explored whether this mechanism could be therapeutically targeted in vivo through a simple glycan-based strategy. Our results showed that metabolic supplementation of mucosal T cells, isolated from patients with active UC, with GlcNAc led to the enhancement of branched N-glycosylation on the TCR, controlling T cell activation and function. Preclinical data further demonstrated that GlcNAc treatment of *MGAT5* null or heterozygous mice developing severe forms of induced colitis significantly controlled disease severity and progression due to suppression of the intestinal T cell-mediated immune response, with good clinical effects when GlcNAc was topically administered by enemas. Altogether, this study highlights the potential of glycans as novel immunomodulatory agents in IBD, warranting validation in human clinical trials.

Results

Ex Vivo GlcNAc Supplementation Increased Branched N-Glycosylation of T Cells from Patients with Active UC. We have previously demonstrated that patients with UC display reduced branched N-glycosylation on mucosal T cells (16). To assess the ability of glycans as repairers of the above-mentioned mechanistic defect, we herein promoted, ex vivo, the hexosamine biosynthetic pathway (*SI Appendix*, Fig. S1*A*) in purified intestinal T cells by metabolic supplementation with GlcNAc. Previous studies showed that supplementation with GlcNAc increases the availability of the substrate (UDP-GlcNAc) to Golgi enzymes such as GnT-V, enhancing β1,6-GlcNAc branching N-glycans, particularly in T cells (13). To test this hypothesis, T cells ($CD3^+$) were isolated ex vivo from both the intestinal lamina propria of fresh colonic biopsies and peripheral blood of patients with UC who have active disease and were supplemented with increasing doses of GlcNAc. Different GlcNAc concentrations (40 mM, 80 mM, and 100 mM) were tested, and 40 mM did not reveal major alterations compared with nontreated T cells (*SI Appendix*, Fig. S1*B*). The expression of β1,6-GlcNAc branched N-glycans on colonic T cells was evaluated by flow cytometry using *Phaseolus vulgaris* leukoagglutinating (L-PHA) lectin. We observed a dose-dependent increase of branched N-glycans on intestinal T cells upon GlcNAc supplementation across different patients (Fig. 1*A*). This increased modification with branched N-glycans was also observed in T cells isolated from peripheral blood mononuclear cells of patients with active UC displaying FSC^{high} and SSC^{high} light-scattering parameters, characteristic of activated T lymphocytes (Fig. 1*B*). The increased expression of β1,6-GlcNAc branched N-glycans was detected both on $CD4^+$ and $CD8^+$ T cells (*SI Appendix*, Fig. S1 *C* and *D*). No effects of GlcNAc treatment in the proportion of $CD4^+$ and $CD8^+$ T cell subsets in the cultures were observed (*SI Appendix*, Fig. S1 *E* and *F*), supporting that GlcNAc supplementation leads to a specific modification with branched glycans on T cells in a dose-dependent manner. Importantly, the enhancement of branched N-glycans was only observed in T cells from patients with active UC (Fig. 1*C* and *SI Appendix*, Fig. S1*G*). T cells from healthy controls and from patients with inactive disease did not show alterations in the levels of branched glycans upon treatment with increasing concentrations of GlcNAc (Fig. 1*C* and *SI Appendix*, Fig. S2), possibly due to the higher baseline branching comparing with patients with active UC. These results were further confirmed by other technical approaches. Increased expression of β1,6-GlcNAc branched N-glycans on a band the same size as the TCR β-chain (TCRβ) after GlcNAc supplementation was also detected by L-PHA blotting (Fig. 1*C*) and by TCR immunoprecipitation using lysates of lamina propria T lymphocytes (LPLs) purified from patients with UC (Fig. 1*D*). Interestingly, this increased branching of N-glycans after GlcNAc supplementation was found to occur in intestinal T cells from patients with UC with different Mayo subscores (with Mayo endoscopic subscores 1, 2, and 3) with a trend association with disease severity, as depicted in *SI Appendix*, Fig. S1*G*. The internalization of externally given GlcNAc was already demonstrated in cell lines (17). The specific effects of GlcNAc in enhancing branched glycosylation on T cells from patients with active UC was further demonstrated by the reversed effects on L-PHA mean fluorescence intensity when T cells were treated with the N-glycan branching inhibitors kifunensine (KF) and swainsonine (SW) (*SI Appendix*, Fig. S3) in T cells from biopsies and blood of patients with active UC. Moreover, the specific effects of GlcNAc in the enhancement of branched glycosylation in T cells was further validated by supplementation of T cells from patients with active UC with other glycan types such as D-mannose, which revealed no impact in branched N-glycan expression (*SI Appendix*, Fig. S3). To further validate these observations, we also performed imaging flow cytometry showing that $TCR\alpha/\beta^+$ cells display an increase of fluorescence intensity due to staining with L-PHA on the cell membrane. This increase was observed in T cells displaying blast-like morphology (Fig. 1 *E* and *E1*). Taken together, these results demonstrate that treating ex vivo T cells from patients with active UC with GlcNAc promotes the hexosamine biosynthetic pathway enhancing β1,6-GlcNAc branched N-glycans on the TCR, and thus restoring the deficiency on branched N-glycans previously shown in mucosal T cells from patients with UC (16). Next, we have determined the specificity of this enhancement of β1,6-GlcNAc N-glycan branching by analyzing the correspondent GnT-V enzymatic activity. Interestingly, and in line with our previous observations on *MGAT5* gene transcription (16), T cells from patients with active UC displayed reduced GnT-V enzymatic activity compared with healthy controls (*SI Appendix*, Fig. S4). Our results showed that this reduced GnT-V enzymatic activity of T cells could be significantly recovered after metabolic supplementation with GlcNAc (Fig. 2*A*), which further supports the effects of GlcNAc in the enhancement of N-glycan branching mediated by GnT-V. In the N-glycosylation branching pathway, the β1,6-GlcNAc branched N-glycan catalyzed by GnT-V can be further extended with polylactosamine structures (ligands for galectins), which, in turn, can be terminally sialylated (*SI Appendix*, Fig. S1*A*). Our results showed that GlcNAc supplementation of ex vivo activated T cells led to increased expression of β1,6-GlcNAc branched N-glycans (as detected by L-PHA lectin) (Figs. 1*E* and 2 *B* and *B1*) with a trend of increased extension with polylactosamine structures, as indicated by staining with the *Lycopersicon esculentum* agglutinin (LEL) (Fig. 2 *B* and *B1*). Additionally, we determined whether there was a terminal addition of α2,6-linked sialic acid, recognized by binding of *Sambucus nigra* agglutinin (SNA), and/or α2,3-sialic acid, recognized by *Maackia amurensis* agglutinin (MAL-II). The results demonstrated a trend of increase in α2,6-linked sialic acid, and no consistent alterations in α2,3-sialic acid linkages were detected (Fig. 2 *B* and *B1*). Overall, our results support that ex vivo supplementation of T cells from patients with UC with GlcNAc has the potential to enhance the branched N-glycosylation on T cells, remodeling the T cell glycoprofile, which is ultimately expected to have an impact on the regulation of the immune response.

Shaping the T Cell-Mediated Immune Response in UC Through Increased Branching N-Glycans. After demonstrating the ability of GlcNAc supplementation to repair the deficiency of branched N-glycans on ex vivo T cells, we next evaluated its impact on the modulation of T cell responsiveness. The metabolic supplementation with GlcNAc of ex vivo activated T cells from naive patients (without therapy) resulted in significant suppression of their proliferative response to anti-CD3/CD28 mAb stimulation (Fig. 3 *A* and *A1*). In addition, and importantly, the increased N-glycan branching on T cells resulted in a significant reduction in the production of the proinflammatory cytokines TNF-α, IFN-γ, and IL-17A, which are associated with UC pathogenesis (18),

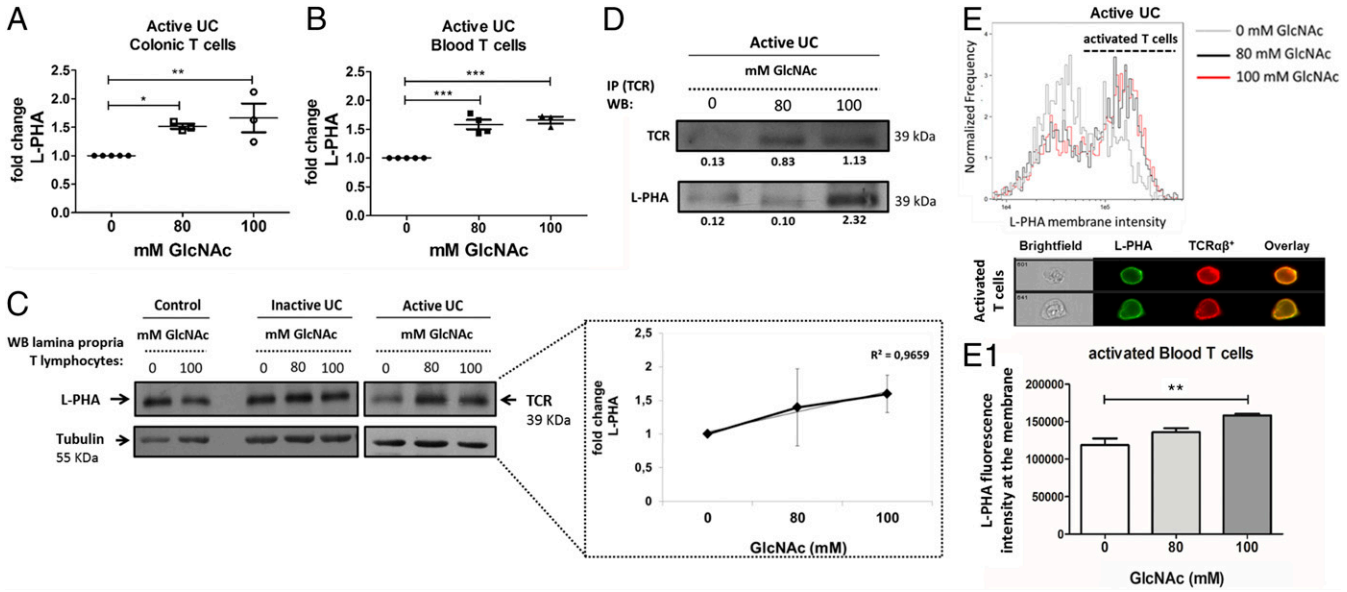


Fig. 1. Ex vivo GlcNAc supplementation of T cells from patients with UC resulted in increased branching N-glycans. (*A* and *B*) CD3$^+$ T cells from patients with active UC were cultured with different concentrations (millimolar) of GlcNAc, and the fold change of mean fluorescence intensity due to L-PHA staining was determined by flow cytometry. The scatter plots illustrate the mean ± SEM of five biological replicates. One-way ANOVA using the Newman–Keuls multiple comparison posttest: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. (*C*) Protein lysates of purified CD3$^+$ T cells under GlcNAc treatment were subjected to L-PHA lectin blotting to evaluate the expression levels of β1,6-GlcNAc branched N-glycans on a protein band corresponding to the migration profile of the TCRβ. WB, Western blot. (*Inset*) Linear regression using mean values per treatment condition. (*D*) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition with L-PHA. The density of bands is indicated below each band. (*E*) Imaging flow cytometry analysis (on an ImageStreamX) of L-PHA membrane distribution on TCR$^+$ cells after GlcNAc supplementation in T cells isolated from blood of patients with active UC. Representative images of activated T cells display blast-like morphology showing colocalization (overlaid images) of TCRαβ and L-PHA staining on the cell membrane. (*E1*) Bars depict the mean ± SEM of L-PHA staining intensity on gated TCRαβ$^+$ L-PHA^{high} cells from three independent experiments. One-way ANOVA using Dunnett's multiple comparison posttest: **$P \leq 0.01$. In all experiments, results are normalized to the corresponding untreated condition (0 mM).

in a GlcNAc dose-dependent manner (Fig. 3*B*), supporting its effect on the control of Th1/Th17-type immune responses. The effects of GlcNAc on the suppression of proinflammatory cytokine production were found to be independent of cell death and/or decreased T cell proliferation. After normalization of cytokine concentrations to the respective T cell proliferation index (index of division), we still observed a reduction in the production of proinflammatory cytokines (*SI Appendix*, Fig. S5*A*). Moreover, the percentage of IFN-γ– and TNF-α–expressing cells among total CD4$^+$ T cells, evaluated by intracellular flow cytometry, was decreased upon GlcNAc treatment (Fig. 3*B1*). The impact of GlcNAc supplementation on other cytokines, such as TGF-β and IL-10, is not significant (*SI Appendix*, Fig. S5 *B* and *C*). The specific effects of GlcNAc in suppressing proinflammatory cytokine production is further confirmed by inhibitors (KF and SW), which blocked the regulatory impact of branched N-glycans in T cell function (*SI Appendix*, Fig. S6). Next, we have evaluated the expression of the transcription factors (T-bet, RORγt, Foxp3, and Gata3) in T cells (CD3$^+$) under ex vivo GlcNAc treatment. The results showed that GlcNAc supplementation was associated with a reduction in the expression of T-bet and RORγt, which corroborates the negative impact on the Th1/Th17-type response (Fig. 3 *C* and *C1*). The expression of Foxp3, a transcription factor associated with regulatory T cells as well as with human activated T cells (18), was also found to be reduced upon GlcNAc treatment. To gain insight into the molecular basis of this modulation of the T cell response through metabolic supplementation with GlcNAc, we analyzed its impact on the TCR signaling pathway. We observed that GlcNAc supplementation led to an inhibition of the phosphorylation of ZAP70 and LAT, thereby hindering T cell activity by controlling the TCR signaling pathway (Fig. 3*D*). Additionally, we evaluated whether GlcNAc supplementation had an impact on T cell apoptosis. Our results showed that T cells from patients with active UC treated with GlcNAc displayed an increased susceptibility to apoptosis compared with nontreated T lymphocytes as soon as 3 h after stimulation (*SI Appendix*, Fig. S7*A*). This effect was not seen in T cells from controls and patients with inactive UC, which demonstrated no differences, or even a trend to decrease apoptosis, respectively, at the same time points upon GlcNAc supplementation. This argues against a putative effect of hyperosmolarity in the observed increase of apoptosis/cell death in GlcNAc-supplemented T cells from patients with active UC and in controls (16) (*SI Appendix*, Fig. S7*A*). Treatment with the branched N-glycan inhibitors (KF and SW) did not reverse the effects of GlcNAc in apoptosis (*SI Appendix*, Fig. S7*B*). At 72 h, a significant decrease in branched glycosylation levels was observed with KF and SW (*SI Appendix*, Fig. S3); however, no differences in the apoptosis were detected (*SI Appendix*, Fig. S7*B*). The percentage of cell death was unchanged or even higher with SW and KF than with treatment with GlcNAc only (*SI Appendix*, Fig. S7*B1*). Taken together, these data collectively demonstrate that ex vivo GlcNAc supplementation of T cells from patients with active UC enhances the branched N-glycans and resulted in a significant suppression of T cell proliferation and TCR signaling, as well as controlled Th1/Th17-type immune responses.

Treatment with GlcNAc Reduces Disease Severity and Ameliorates Clinical Signs of Disease in Mice with Colitis. To determine whether dysregulation of branched N-glycans on TCR occurs in different experimental mouse models of colitis, we have evaluated two different chemically induced colitis mouse models, the dextran sodium sulfate (DSS)-induced and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced models (19), in C57BL/6 wild-type mice. In both models, colitis was successfully induced, and we have observed a similar impact on the dysregulation of branched N-glycans in the TCR (*SI Appendix*, Fig. S8 *A* and *B*), which is in accordance with our previous observations on human patients with UC (16). The DSS-induced colitis model was selected as the ideal one to proceed with, since the rectal induction of disease in the TNBS model would interfere with the enema administration of GlcNAc. After disease

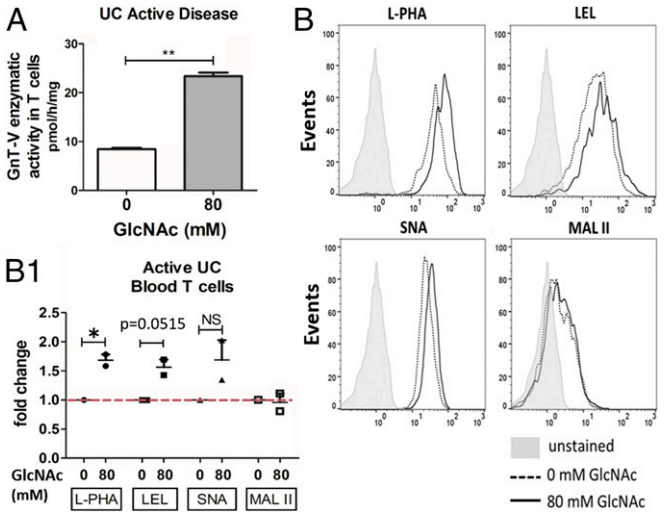


Fig. 2. Remodeling of the glycosylation phenotype upon metabolic supplementation with GlcNAc. (*A*) Impact of GlcNAc supplementation on GnT-V activity was determined using a pool of lysates from treated vs. nontreated peripheral blood T cells, in three biological replicates of patients with active UC, from two independent technical experiments. Student's *t* test: $^{**}P \leq 0.01$. (*B*) Flow cytometry evaluation of glycophenotype of T cells upon GlcNAc supplementation. (*B1*) Scatter plot: fold change of MFI due to staining with each lectin on T cells, in two biological replicates with different stages of disease severity (Mayo endoscopic scores 1 and 2), from two independent experiments. Results are normalized to the untreated condition, which was taken as 1. Student's *t* test: $^{*}P \leq 0.05$. NS, not significant.

onset using 2% DSS, GlcNAc was administered through two different routes: orally, by supplementing the drinking water with 0.25 mg/mL GlcNAc, and/or rectally, using 0.5 mg/mL GlcNAc enemas. With this approach, we assessed the therapeutic effects of GlcNAc on disease activity and on the control of intestinal inflammation. The experiment design is summarized in Fig. 4*A*. Our results showed that mice with colitis and treated with GlcNAc exhibited lower body weight loss (Fig. 4*B*) and significant improvements in disease activity index (DAI) (Fig. 4*C*) in comparison to mice with colitis not treated with GlcNAc (DSS control). Importantly, whenever animals received GlcNAc topically by enema administration (either alone or in combination with oral GlcNAc), the body weight changes were lower in comparison to animals receiving only oral GlcNAc. These results suggest that GlcNAc enemas may have promising topical effects on the control of disease severity (Fig. 4 *B* and *C*, *Insets*). Accordingly, macroscopic observation of the colon showed that mice with colitis displayed visible colonic edema (swelling of the bowel wall) that recovered upon GlcNAc treatment (*SI Appendix*, Fig. S8*C*). In addition, mice with colitis displayed extensive lymphocytic infiltrates (Fig. 4*D*, arrowhead) presenting with $CD3^{+}$ cells (Fig. 5*A*) in the intestinal lamina propria, together with notable alterations of the glandular morphology. Furthermore, these animals also showed a reduced mucus layer (produced by glycoproteins at the glycocalyx), a natural barrier that confers protection from microbiota, preventing disease aggressiveness and progression (20). When animals with colitis were treated with GlcNAc, there was a decrease in the lymphocytic infiltrate and an improvement of the glandular architecture (Fig. 4*D*), which is compatible with disease remission (21). To further investigate the relationship between this overall improvement of disease severity through GlcNAc treatment and the levels of β1,6-GlcNAc branched N-glycans on LPLs, we performed L-PHA and CD3 histochemistry in the formalin-fixed, paraffin-embedded (FFPE) colonic specimens from the different groups of animals (Fig. 5*A*). The results showed that induction of colitis was accompanied by a notable intestinal lymphocytic infiltrate in the lamina propria, including CD3-expressing cells that expressed low levels of branched N-glycans. When mice were treated with GlcNAc, an increased expression of β1,6-branched glycans in the inflammatory infiltrate was observed (Fig. 5*A*, arrowheads). Importantly, the administration of GlcNAc also resulted in increased mucus lining, with augmented expression of branched glycans in glycoproteins in the superficial mucus layer, which is compatible with disease remission. Afterward, we investigated whether the TCR was particularly targeted by the observed enhancement of branched N-glycans in vivo. The results showed that GlcNAc treatment resulted in increased branching glycosylation on intestinal T cells, particularly in the TCR (Fig. 5 *B* and *B1*). To further address the mechanistic basis of the clinical efficacy of GlcNAc, we have evaluated the impact on T cell activity and signaling. Interestingly, LPLs from GlcNAc-treated mice displayed a decrease in the phosphorylation levels of ZAP70 and LAT that was more pronounced in mice treated with GlcNAc enemas (Fig. 5*C*). This result supports control of T cell activity through GlcNAc treatment, with effective results in topical/enema administration. Next, the effects of GlcNAc administration on the expression levels of T-bet, the transcription factor associated with proinflammatory Th1 cell polarization, were assessed in situ in the intestinal lamina propria. We have observed abundant cells expressing T-bet in lymphocytic infiltrates in mice with colitis that were markedly reduced in GlcNAc-treated mice (Fig. 5*D*, *Insets*). Furthermore, mice treated with GlcNAc revealed a significant reduction of IFN-γ production and a trend in the suppression of IL-17A secretion, further supporting that the enhancement of branched N-glycans by GlcNAc treatment controls Th1/Th17-type immune responses in vivo (Fig. 5*E*). Taken together, our in vivo results demonstrate a therapeutic effect of GlcNAc in a colitis-induced mouse model, revealing the immunomodulatory properties of this agent in the control of intestinal inflammation and, consequently, in the control of disease severity and progression.

Deficiency in Branched Glycosylation in *MGAT5* Null or Heterozygous Mice Is Associated with Early-Onset Disease and Increased Severity of Colitis that Is Suppressed by GlcNAc Treatment. To gain insights into the targeted-specific therapeutic effects of GlcNAc, we used *MGAT5* null or heterozygous mice that display a deficiency in branched N-glycosylation, mimicking the mechanistic defect described in humans (16). The impact on DSS-induced colitis onset and severity was assessed in *MGAT5* heterozygous ($^{+/-}$, partial deficiency on branched N-glycosylation) and *MGAT5* knockout ($^{-/-}$, absence of branched N-glycans) mice. Those genotypes represent intermediate N-glycans and the loss of one branch (β1,6-branching) of N-glycans, which may mimic mild/moderate versus severe deficiency on branched glycosylation. Our results showed that after DSS induction, *MGAT5* null or heterozygous mice exhibited increased susceptibility to early-onset disease and to severe forms of colitis. At day 2 after DSS induction, more that 50% of $MGAT5^{-/-}$ mice developed both mild and moderate forms of colitis compared with WT mice, which only developed clinical signs of colitis at day 6 postinduction (Fig. 6*A*). Moreover, at the end of DSS induction period (day 7), and based on the DAI, we observed increased susceptibility to severe forms of colitis in *MGAT5* null or heterozygous mice (Fig. 6*A*). As depicted in the pie chart in Fig. 6*A*, on day 7, more than 50% of $MGAT5^{-/-}$ mice exhibited severe forms of disease (scores ≥3) and 29% of $MGAT5^{+/-}$ mice developed severe colitis. In contrast, WT mice presented only mild/moderate forms of the disease. These results reinforce that *MGAT5*-mediated branched glycosylation has a strong impact on UC disease onset and progression.

Afterward, we tested the effect of GlcNAc treatment on the control of disease severity in mice with the different *MGAT5* genotypes. As shown in Fig. 6 *B* and *B1*, $MGAT5^{+/-}$ mice with colitis and treated with GlcNAc presented lower body weight loss and lower DAI compared with nontreated controls. With regard to body weight changes and DAI scores, the same tendency was observed in $MGAT5^{-/-}$ mice treated with GlcNAc (Fig. 6 *C* and *C1*). Due to their higher susceptibility to colitis, nontreated $MGAT5^{-/-}$ animals were euthanized on day 10 since they reached the established humane end points. $MGAT5^{-/-}$ mice, besides developing a much more aggressive disease phenotype,

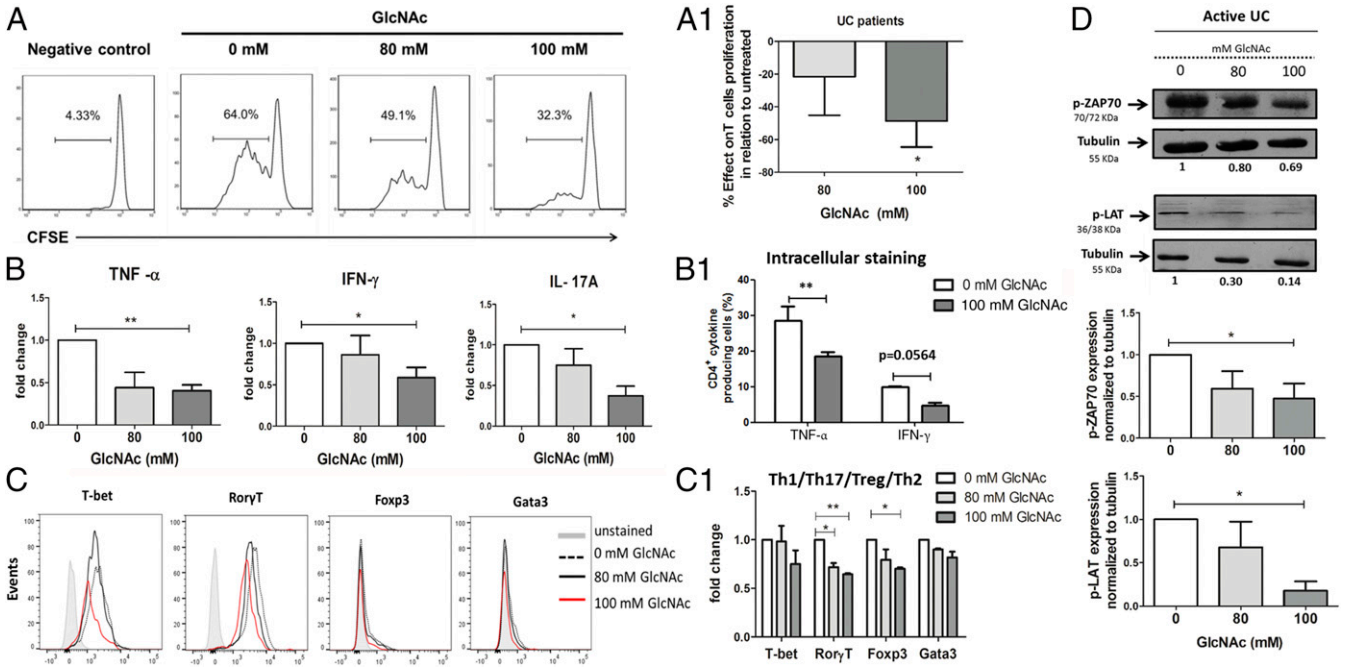


Fig. 3. Control of T cell-mediated immune response through enhancing branching N-glycosylation. (*A*) Purified CD3$^+$ T cells from fresh biopsies of naive patients with active UC were labeled with CFSE and cultured with GlcNAc treatment. The gated cells in the histograms correspond to the percentage of live cells. (*A1*) Bar plot: the mean percentage of effect ± SEM due to GlcNAc supplementation on T cell proliferation in comparison to untreated cells. Results include four biological replicates. Student's *t* test: $*P \leq 0.05$. (*B*) Cytokine profile assessed by flow cytometry in the supernatants from ex vivo T cell cultures under GlcNAc supplementation. Bar plots: mean fold change ± SEM of cytokine concentrations (picograms per milliliter) in six biological replicates. Student's *t* test: $*P \leq 0.05$; $**P \leq 0.01$. (*B1*) Evaluation of the percentage of IFN-γ– and TNF-α–producing CD4$^+$ T cells treated vs. nontreated with GlcNAc. Bar plots: mean ± SEM percentage of CD4$^+$cytokine-producing cells in three biological replicates from two independent experiments. Two-way ANOVA with Bonferroni postcorrection: $**P \leq 0.01$. (*C*) Expression of the transcription factors (TFs) in CD4$^+$CD45$^+$ T cells isolated from patients with UC and analyzed by flow cytometry. Histogram overlays correspond to the expression of the indicated TFs observed upon GlcNAc supplementation (gray-shadowed histograms depict the respective unstained control). (*C1*) Bar plots: mean fold change in TF mean fluorescence intensity ± SEM in two biological replicates, from two independent experiments. Two-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $**P \leq 0.01$. (*D*) Western blot analysis of TCR signaling, p-ZAP70, and p-LAT assessed in T cell lysates from cultures supplemented with GlcNAc. Bar plots: mean ± SEM fold change of p-ZAP70 and p-LAT densities normalized to tubulin in five biological replicates, from three independent experiments. Student's *t* test: $*P \leq 0.05$. In all experiments, results are normalized to the corresponding untreated condition (0 mM), which was taken as 1.

were also less responsive to GlcNAc, which makes GlcNAc therapy in these mice more challenging. Nevertheless, the results in *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice (Fig. 6 *B–D*) showed that even in these mice, treatment with GlcNAc improved clinical scores compared with the scores of DSS mice. This beneficial effect was pronounced whenever GlcNAc was administered topically by enema (single or in combination with oral administration), as evidenced by lower body weight loss and lower colitis scores compared with DSS mice. Interestingly, when GlcNAc treatment was initiated earlier in disease course of *MGAT5*$^{-/-}$ mice, at day 5, when animals started to develop severe colitis, we observed a decrease of the colitis scores compared with nontreated diseased animals (DSS), which supports the benefits of treating these susceptible animals earlier with GlcNAc, as demonstrated in Fig. 6*D*.

GlcNAc Treatment of MGAT5 Null or Heterozygous Mice Attenuates Disease Progression by Controlling Th1/Th17-Type Immune Responses. *MGAT5* null and heterozygous mice showed disorganization of the glandular architecture and an increase of inflammatory infiltrates in the colonic mucosa after DSS-induced colitis that was improved overall upon GlcNAc treatment (Fig. 7*A*). Notably, the evaluation of β1,6-GlcNAc branched N-glycan levels on CD3$^+$ LPLs confirmed that the induction of colitis was accompanied by decreased expression of branched N-glycans in LPLs from *MGAT5*$^{+/-}$ mice and by the absence of branched N-glycans in *MGAT5*$^{-/-}$ mice with induced colitis (DSS) (Fig. 7*A*). Interestingly, when mice of both *MGAT5* genotypes were treated with GlcNAc, a recovery of β1,6-branched glycan expression was observed in the intestinal inflammatory infiltrate (Fig. 7*A*, arrowhead) and in the superficial mucus layer, which is compatible with mucosal healing. The positive detection of L-PHA staining in *MGAT5* null mice upon GlcNAc supplementation was unexpected, as these mice lack the GnT-V enzyme. This positive detection was confirmed at the protein level by L-PHA blot (Fig. 7*B* and *SI Appendix*, Fig. S9*A*). In fact, the reactivity of L-PHA in *MGAT5*$^{-/-}$ mice is the lowest, compared with heterozygous and WT mice, but it is still positive. These observations may be in line with redundant effects of other GnTs at the Golgi (22–24) that, within an activated hexosamine pathway, may compensate for the absence of *MGAT5* by producing the β1,6-GlcNAc branched glycans, although with a much lower yield of synthesis, as we have observed. In an attempt to explore the potential compensatory synthesis of β1,6-GlcNAc branched N-glycans in *MGAT5* null mice, interestingly, we have observed that the *MGAT5b* gene [a homologous gene of MGAT5a that codifies the GnT-IX or GnT-Vb enzyme (25)] is apparently up-regulated in colonic T cells from *MGAT5* null mice treated with GlcNAc compared with control mice (nontreated mice) (*SI Appendix*, Fig. S9*B*). *MGAT5* null mice with DSS-induced colitis do not express *MGAT5b*. This preliminary evidence suggests GnT-IX/Vb as a potential candidate that might compensate for the synthesis of β1,6-GlcNAc branched glycans in *MGAT5* null mice. This issue needs further investigation. We then assessed the enhancement of branched N-glycans specifically on T cells after GlcNAc treatment. *MGAT5* null or heterozygous mice treated with GlcNAc showed an enhanced expression of branched N-glycans in the TCR compared with nontreated diseased animals (DSS) (Fig. 7 *C* and *D*). This effect was highlighted when animals were treated topically with GlcNAc enemas (Fig. 7 *C* and *D*). To explore the mechanistic effects of GlcNAc treatment in the T cell-mediated immune response, the

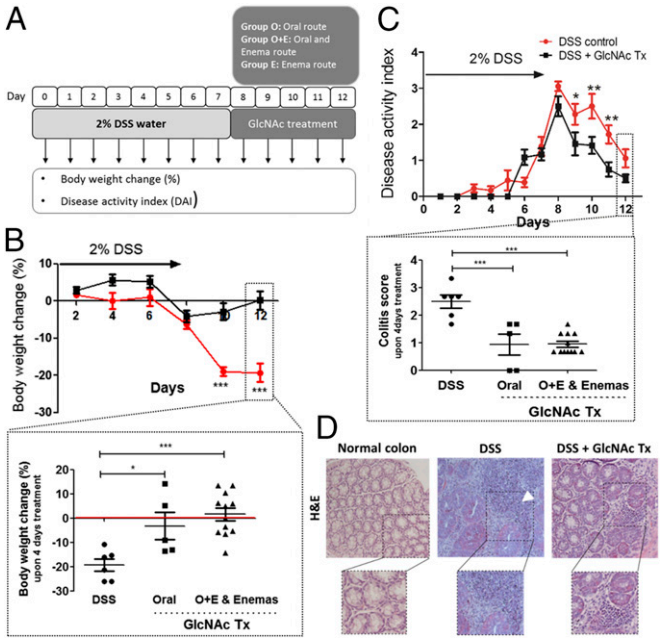


Fig. 4. Colitis-induced mouse model treated with GlcNAc displays significant control of disease severity and recovery from clinical signs. (*A*) Schematic representation of the in vivo study performed on C57BL/6 mice. (*B* and *C*) Body weight changes and DAI. (*B*) Effects of GlcNAc on body weight change (%) comparing DSS control and GlcNAc-treated animals. Graphs depict the mean ± SEM. Two-way ANOVA with Bonferroni postcorrection: $***P \leq 0.001$. (*Inset*) Discrimination of the effects of GlcNAc on body weight change (%) using different routes of administration. Scatter plots include the mean ± SEM. One-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $***P \leq 0.001$. (*C*) DAI comparing mice with colitis that were untreated with those that were treated with GlcNAc. Plots depict the mean ± SEM. Two-way ANOVA with Bonferroni postcorrection: $*P \leq 0.05$; $**P \leq 0.01$. (*Inset*) Discrimination of the effect of GlcNAc on colitis scores using different routes of administration. Plots depict the mean ± SEM. One-way ANOVA with Bonferroni post correction: $***P \leq 0.001$. (*D*) Representative histological images (H&E) of colonic samples from mice [normal colon, DSS (DSS-induced colitis), and GlcNAc treatment (Tx) (DSS + GlcNAc Enema Tx)]. (Magnification: 40×.)

impact on TCR signaling was evaluated in *MGAT5* null and heterozygous mice. An overall decrease of ZAP70 phosphorylation, indicative of reduced TCR signaling, was detected in colonic T cells from GlcNAc-treated mice that was evident when GlcNAc was administered topically (Fig. 7*E*). This topical effect was particularly observed in $MGAT5^{-/-}$ mice, where oral treatment did not affect TCR signaling. The more marked effect achieved through GlcNAc enema administration suggests that this molecule may be more efficiently taken up by cells in this way, likely by increasing its local concentration, thus facilitating its entry into the hexosamine pathway and usage by glycosyltransferases other than GnT-V (*SI Appendix*, Fig. S9*A*) that may redundantly catalyze the branched N-glycans. These redundant effects need further investigation. Additionally, the evaluation of the Th1 proinflammatory response revealed that GlcNAc treatment in both genotypes was associated with reduced proportions of cells expressing T-bet in lymphocytic infiltrates compared with control mice with colitis (Fig. 7*F*). Notably, similar to our observations of GlcNAc treatment in T cells from patients with UC, colonic explants from *MGAT5* null or heterozygous mice treated with GlcNAc indicate a trend for IFN-γ suppression, but with a more pronounced effect in reducing IL-17A. These results further support the impact of GlcNAc treatment and, consequently, the enhancement of branched N-glycans in controlling Th1/Th17-type immune responses also in the IBD in vivo model (Fig. 7*G*). Regarding the impact of GlcNAc on regulatory T cells, no apparent difference in the numbers of FoxP3-expressing cells was observed at the intestinal lamina propria comparing non-treated versus GlcNAc-treated mice of $MGAT5^{wt}$ and $MGAT5^{-/-}$ genotypes (*SI Appendix*, Fig. S9*C*). Nevertheless, further studies are needed to better characterize the regulatory effects of GlcNAc treatment in the different components of the immune response, such as in macrophages (*SI Appendix*, Fig. S9 *D* and *E*), as proposed by previous reports (26), and other T cell populations. Taken together, these data support the targeted-specific effects of GlcNAc that were able to repair the deficiency in branched glycosylation on T cells associated with *MGAT5* deficiency, thus controlling progression of colitis.

Discussion

IBD is characterized by a substantial heterogeneity concerning disease onset, course, response to therapy, and progression to complications (e.g., hospitalization, need for surgery, cancer) (1). Moreover, and despite recent advances in IBD therapeutic resources, a high proportion of patients remain refractory to conventional treatment, and approximately half of the patients with UC do not achieve

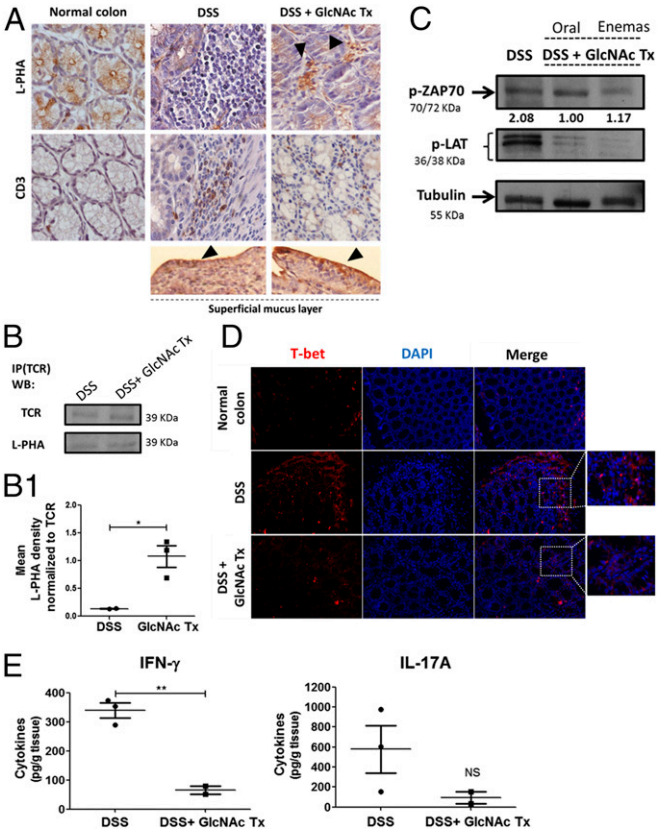


Fig. 5. Colitis-induced mouse model treated with GlcNAc showed increased branched N-glycosylation associated with suppression of T cell function. (*A*) L-PHA histochemistry and CD3 immunohistochemistry. L-PHA lectin reactivity showed an increased expression of β1,6-branched structures in the intestinal inflammatory infiltrate (positive to CD3) as well as an increase in mucus lining in mice treated with GlcNAc enemas (arrowheads). (Magnification: 63×.) (*B*) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition in mouse colon, DSS (DSS-induced colitis) vs. DSS + GlcNAc treatment (Tx). WB, Western blot. (*B1*) Scatter plot: ratio of densities of L-PHA reactivity normalized to that of TCR depicted as the mean ± SEM comparing DSS ($n = 2$) mice with DSS + GlcNAc Tx ($n = 3$) mice. Student's *t* test: $*P \leq 0.05$. (*C*) TCR signaling by Western blot analysis of the phosphorylation levels of ZAP70 and LAT in LPLs. Values of pZAP70 densities normalized to tubulin are indicated. (*D*) Immunofluorescence of T-bet in colonic sections of DSS vs. DSS + GlcNAc Tx. (*Insets*) T-bet–expressing cells at intestinal inflammatory infiltrate are highlighted. (Magnification: 20×.) (*E*) Concentration of IFN-γ and IL-17A in the supernatants of 24-h colonic explant cultures from DSS and DSS + GlcNAc Tx $MGAT5^{wt}$ ($n = 5$) mice by ELISA. Plots depict the mean ± SEM of two to three animals per group. Student's *t* test: $**P \leq 0.01$. NS, not significant.

sustained remission (27). In addition, issues related to side effects and failure in therapy response highlight the need for more effective and targeted-specific drugs (28). We have recently demonstrated that patients with UC exhibit a deficiency in branched glycosylation on intestinal T cells due to a transcriptional reduction of the *MGAT5* gene that accompanied disease severity (16).

Herein, we uncovered a prominent role for the branched glycosylation pathway in IBD pathogenesis, by shaping the course of the T cell response. This pathway is thus an attractive target for novel therapies. Indeed, we have shown here that checkpoints of T cell immune response in UC could be modulated by metabolic supplementation with the simple sugar GlcNAc. We showed that GlcNAc therapy concomitantly increased branched N-glycosylation on T cells and down-regulated T cell proinflammatory responses both ex vivo and in vivo. In line with these observations, it was previously reported that lack of β1,6-GlcNAc branched N-glycans, by targeted deletion of the locus encoding GnT-V, results in enhanced TCR signaling and increased susceptibility to multiple sclerosis (7, 10). Moreover, and in accordance with our results, the increase of N-glycan branching through GlcNAc salvage into the hexosamine pathway was associated with a decreased threshold in T cell activation and more stable CTLA-4 surface expression, which resulted in the inhibition of adoptively transferred EAE (7, 13).

The evidence presented here suggests that GlcNAc supplementation of T cells isolated from patients with active UC resulted in remodeling of the glycophenotype of T lymphocytes through a marked increase of β1,6-GlcNAc branched N-glycans and an increase of polylactosamine structures, the ligand for galectins (29), that can then be terminally sialylated, predominantly with α2,6-sialic acid residues. This glycan reprogramming on T cells was shown to translate into key immunomodulatory effects in UC. Importantly, the enhancement of branched N-glycans on T cells induced by GlcNAc supplementation led to the suppression of T cell proliferation; inhibition of T cell signaling; reduced production of the proinflammatory cytokines TNF-α, INF-γ, and IL1-7A; and controlled Th1- and Th17-type responses. Both Th responses have been associated with IBD pathogenesis (30). Furthermore, these results are in accordance with a very recent report using mouse T cell cultures, which showed the ability of GlcNAc to promote iTreg over Th17 differentiation (15).

The glycosylation of T cells can also have an impact on the susceptibility to cell death (31). Accordingly, treatment of T cells with GlcNAc induced an increased susceptibility to apoptosis, which is at the core of different regulatory processes controlling T cell activation and expansion, thus avoiding exacerbated inflammation (32). This effect of GlcNAc on apoptosis was dose-dependent and limited to T cells from patients with active UC (*SI Appendix*, Fig. S7*B*). In agreement, previous reports have shown that extension with polylactosamine structures, which are ligands for galectins (such as galectin 1), was associated with proapoptotic effects of $CD4^+$ T cells (31, 33). Nevertheless, and given that treatment with KF and SW did not reverse the apoptotic effects induced by GlcNAc in the cells of patients with active UC, it cannot be excluded that factors other than branching glycans can also contribute to GlcNAc-mediated regulation of in vitro T cell apoptosis. Importantly, besides the TCR, the enhancement of branched N-glycosylation can also modify other receptors like the coreceptors CD4 and CD8, as well as the growth inhibitory receptor CTLA-4 (7, 10, 22). Moreover, CD45 and CD25 are also potential targets of branched glycosylation modification that can further contribute to the regulation of the T cell-mediated immune response through branching N-glycans (15, 34). Immunomodulation through GlcNAc-mediated enhancement of branched glycosylation, as described here, is a promising therapeutic approach to restore T cell homeostasis in IBD (*SI Appendix*, Fig. S10). Indeed, metabolic regulation of T cell function has been highlighted by recent research (35) and may be manipulated to reduce T cell-mediated inflammation (15, 36).

The preclinical data reported here provide the proof of concept supporting such a therapeutic approach in IBD. Deficiency of the *MGAT5* gene was associated with higher susceptibility to severe

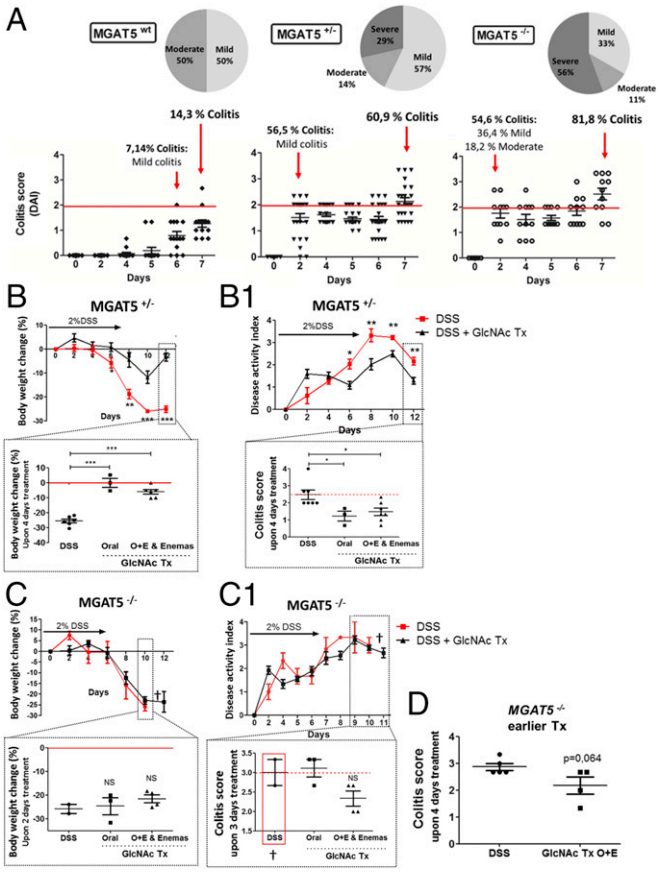


Fig. 6. *MGAT5* null or heterozygous mice develop early-onset colitis and an increase in disease severity that is suppressed by GlcNAc treatment. (*A*) Evaluation of colitis onset and disease severity in *MGAT5* null or heterozygous mice: C57BL/6 WT (n = 14), $MGAT5^{+/-}$ (n = 23), and $MGAT5^{-/-}$ (n = 11) mice. Active disease was defined when animals showed a DAI of ≥2, and three stages of severity were defined: mild (≥2 and <2.5), moderate (≥2.5 and <3), and severe (≥3). Average results of body weight change (*B* and *C*) and DAI (*B1* and *C1*) of $MGAT5^{+/-}$ (n = 23) and $MGAT5^{-/-}$ (n = 9) mice, respectively, randomly distributed in controls and GlcNAc treatment groups are shown. DSS-induced colitis (DSS) vs. DSS treated with GlcNAc treatment (DSS + GlcNAc Tx). Animals showing severe signs of disease were euthanized (†). (*B* and *B1*, *Insets*) Discrimination of the efficiency of GlcNAc treatment (colitis scores) with different routes of administration upon 4 d of treatment. Graphs correspond to the mean ± SEM of 17 animals (three to seven animals per route of administration). Student's t test (*B* and *B1*) and one-way ANOVA with Bonferroni postcorrection (*B* and *B1*, *Insets*): $*P \leq 0.05$*; $**P \leq 0.01$; $***P \leq 0.001$. Body weight changes of $MGAT5^{-/-}$ mice treated through different routes vs. nontreated upon 2 d of treatment (*C*) and DAI scores of $MGAT5^{-/-}$ mice treated (n = 7) vs. nontreated (n = 2) (*C1*) are shown. (C and *C1*, *Insets*) Discrimination of the efficiency of GlcNAc treatment (colitis scores) with different routes of administration upon 3 d of treatment. (*D*) Evaluation of the impact of early oral route (O) + enema route (E) GlcNAc treatment (starting on the second day of disease onset: 5–6 d after DSS induction) on the colitis scores (DAI of animals per group) of $MGAT5^{-/-}$ mice, comparing DSS (n = 5) with GlcNAc treated mice (n = 4).

forms of colitis and early-onset disease. These data highlight the prominent role of branched N-glycosylation in the pathogenesis of IBD, and are in accordance with previous studies on multiple sclerosis (11). GlcNAc supplementation improved clinical scores and was associated with a better disease course in mice developing the most severe disease phenotype ($MGAT5^{-/-}$). These immunosuppressive effects catalyzed by GlcNAc were observed by both oral and enema administration routes, with promising effects when mice received GlcNAc topically via enemas. This topical effect of GlcNAc is in line with the ability of GlcNAc to be more efficiently taken up by the intestinal mucosa, thereby entering directly into the

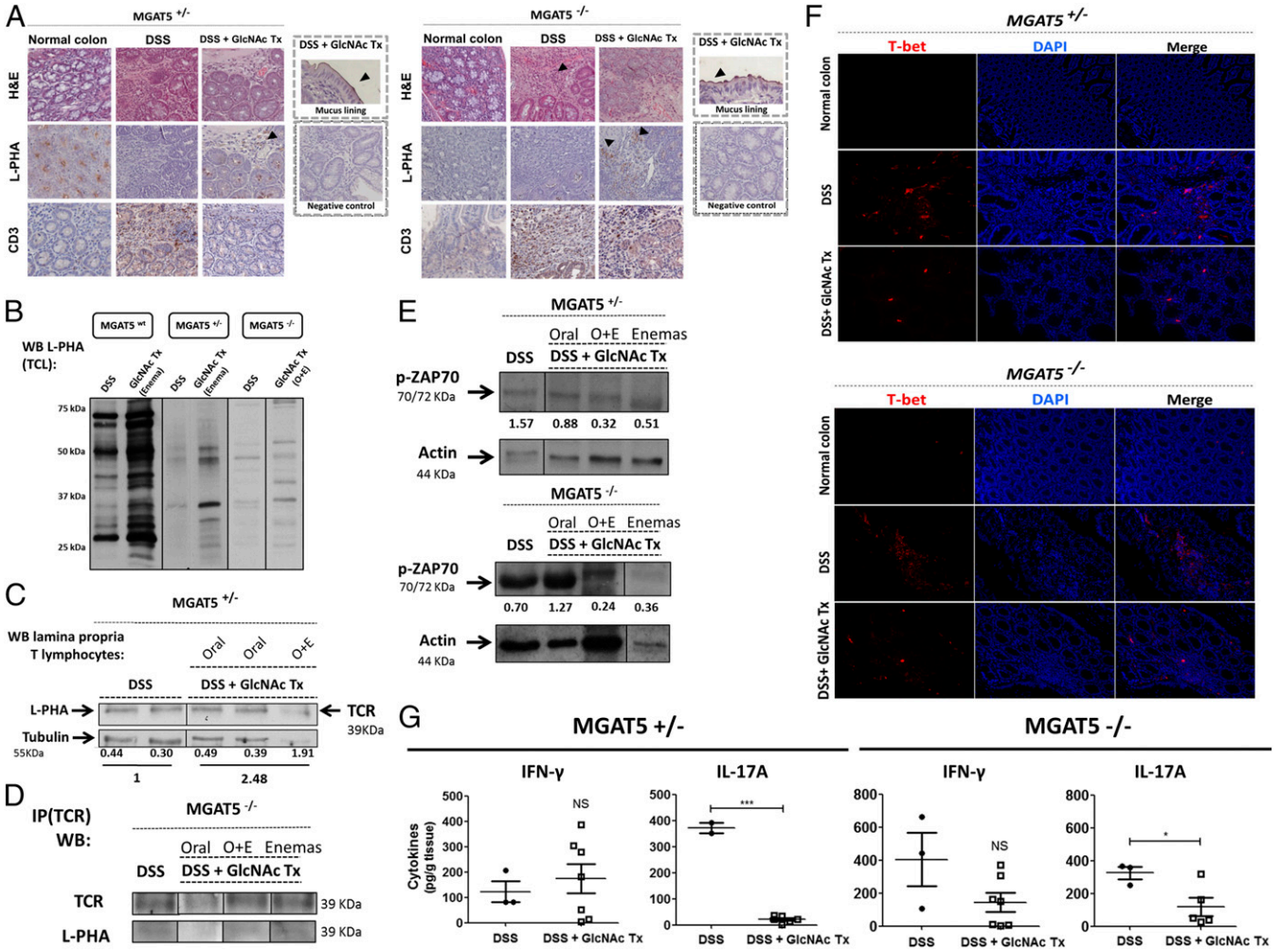


Fig. 7. GlcNAc treatment of *MGAT5* null or heterozygous mice attenuates disease progression by controlling Th1/Th17-type immune responses. (*A*) Representative histological images (H&E) of colonic sections from *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ [normal colon, DSS-induced colitis (DSS), and GlcNAc treatment (DSS + GlcNAc Tx)] (Magnification: 20×.) DSS mice displayed visible signs of lymphocytic infiltrate in the intestinal lamina propria (arrowheads). L-PHA histochemistry and CD3 immunohistochemistry of mouse colon from the different groups. (Magnification: 20×.) (*B*) Evaluation of branching N-glycans on colonic total cell lysates from *MGAT5*^{wt}, *MGAT5*$^{+/-}$, and *MGAT5*$^{-/-}$ mice comparing DSS control with GlcNAc Tx enema by Western blot (WB). TCL, total cell lysate. (*C*) Protein lysates from LPLs isolated from the colon of *MGAT5*$^{+/-}$ mice with DSS (colitis) or treated (DSS + GlcNAc Tx) mice were subjected to L-PHA lectin blotting to evaluate the expression of β1,6-GlcNAc branched N-glycans on the TCR (39 kDa). L-PHA density normalized to tubulin is indicated for each case, and fold change differences of DSS vs. DSS + GlcNAc Tx are highlighted. (*D*) Immunoprecipitation (IP) of the TCR in total cell lysates from *MGAT5*$^{-/-}$ mouse colon followed by β1,6-GlcNAc branched N-glycan recognition. DSS vs. DSS + GlcNAc Tx with different routes of administration. (*E*) Analysis of the phosphorylation levels of ZAP70 in LPL lysates from colon of *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice. Values of pZAP70 normalized to actin in *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice are indicated. (*F*) Immunofluorescence of T-bet in *MGAT5*$^{+/-}$ and *MGAT5*$^{-/-}$ mice comparing normal colon, DSS, and DSS + GlcNAc Tx mice. (Magnification: 20×.) (*G*) Concentration of IFN-γ and IL-17A in the supernatants of 24-h colonic explant cultures from DSS and DSS + GlcNAc Tx *MGAT5* heterozygous ($n = 10$) and null ($n = 10$) mice by ELISA. Plots depict the mean ± SEM of two to seven animals per group. Student's *t* test: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. NS, not significant.

hexosamine pathway to increase branched glycosylation. Intriguingly, in *MGAT5*$^{-/-}$, with the absence of *MGAT5*/GnT-V, expression of the β1,6-GlcNAc branched N-glycans detected by L-PHA lectin histochemistry and blotting was positive and slightly increased with GlcNAc treatment. This unexpected result may be in accordance with the fact that several glycosyltransferase-deficient mice exhibit no or only mild phenotypes due to redundancy or compensation of glycan functions. In fact, several family members of glycosyltransferases are known to be functionally redundant (37, 38). This potential redundant effect observed in *MGAT5*$^{-/-}$ mice treated with GlcNAc might be due to the fact that other Golgi glycosyltransferases within an activated hexosamine pathway triggered by UDP-GlcNAc may compensate for the lack of this specific glycan structure, thereby guaranteeing immune homeostasis. One of the candidate enzymes is the homologous GnT-Vb that may compensate for the synthesis of the branched glycans; however, further studies are needed to clarify this issue. Similar examples of the redundancy of glycosyltransferases were described, such as for FUT8-deficient mice (39). Nevertheless, this redundant effect observed upon GlcNAc supplementation needs to be further explored.

The clinical effects of GlcNAc were further evidenced by the decreased frequency of T-bet–expressing cells in colonic mucosa of treated mice, together with reduced release of the proinflammatory cytokine IL-17A of respective colonic explants.

GlcNAc is a naturally occurring amino sugar for which no adverse effects were reported in humans (40). It is available as a dietary supplement, and oral GlcNAc administration showed no toxicity in rats (41). Interestingly, 17 y ago, oral GlcNAc was described to promote intestinal lining through mucus production in children with severe treatment-resistant IBD (21). In that study, eight of the 12 children studied went into clinical remission, which could have resulted from the immunomodulatory effects of

GlcNAc proposed here. Taken together, the combined evidence from both ex vivo and preclinical data provides proof of concept for the therapeutic use of GlcNAc (either alone or in combination with other antiinflammatory therapies) as a simple immunomodulatory strategy in IBD. Assessment of this strategy in clinical studies is currently ongoing. Some of the most relevant properties associated with GlcNAc treatment are the absence of side effects, low cost, and possibility of being used as a simple rescue therapy to avoid unnecessary toxic effects and step-up therapies in IBD.

Materials and Methods

Patient Selection and Colonic Biopsy Collection. Fresh colonic biopsies were obtained from 75 patients (three patients in remission) diagnosed with UC and normal controls ($n = 3$) who underwent a scheduled colonoscopy (2014–2017) at the Gastroenterology Department of Centro Hospitalar do Porto–Hospital de Santo António (CHP-HSA), Porto, Portugal. All participants gave informed consent, and procedures were approved by the Ethics Committee of CHP/HSA [233/12(179-DEFI/177-CES)].

Isolation of $CD3^+$ T Cells from Fresh Colonic Biopsies and Blood of Patients with Active UC: Ex Vivo Culture of T Cells. After mechanical dissociation of colonic biopsies and blood density gradient centrifugation using Lymphoprep, $CD3^+$ T cells (from biopsies and blood) were magnetically sorted using an EasySep Human T Cell Enrichment Kit (STEMCELL Technologies) following the manufacturer's instructions. $CD3^+$ T cells were cultured for 72 h with anti-CD3 mAb (clone OKT3) and soluble anti-CD28 mAb (clone CD28.2) (eBioscience). T cell cultures were supplemented with GlcNAc (Sigma and Wellesley Therapeutics, Inc.).

Imaging Flow Cytometry. Imaging flow cytometry analysis was performed as previously described (42).

Flow Cytometry. $CD3^+$ T cells were stained with CD4 and CD8 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated L-PHA (Vector Laboratories), as well as with cell surface markers (CD4, CD45), intracellular antigens (T-bet, RORγt, Foxp3, and Gata3), and cytokine intracellular staining (TNF-α, IFN-γ). Various antibodies used for staining are described in *SI Appendix*, Table S1.

Proliferation Assay. $CD3^+$ T cells were purified from colonic biopsies and labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) using a CellTrace CFSE Cell Proliferation Kit (Invitrogen), as described by Oliveira et al. (43).

Cytokine Production. Supernatants from colonic T cell cultures were analyzed by flow cytometry using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) following the manufacturer's instructions. Human TGF-β1 quantification was performed using ELISA kits (R&D Systems) according to the manufacturer's instructions. The supernatants from mouse colonic explant cultures were concentrated using Amicon Ultra-2 mL Centrifugal Filters (Merck Millipore), according to manufacturer's instructions. The levels of IFN-γ and IL-17A (anti-mouse, Ready-SET-Go! kits; eBioscience) and TNF-α and IL-6 (anti-mouse; Biolegend) were quantified by ELISA, according to the manufacturers' instructions.

Western Blot and TCR Signaling. TCR signaling and L-PHA lectin blot analysis (44), using T cell protein lysates (extracted with radioimmunoprecipitation buffer), were performed as described by Dias et al. (16). Incubation of phospho–Zap-70 [Tyr319/Syk (Tyr352)] rabbit mAb and anti–phospho-LAT (Tyr191) rabbit mAb (Cell Signaling Technologies) was performed. Goat anti-rabbit IgG-HRP mAb was used as a secondary antibody, and rabbit IgG antiactin (Santa Cruz Biotechnology) or mouse IgG antitubulin (Sigma) was used as a loading control.

Immunoprecipitation. TCR immunoprecipitation, using total cell lysates obtained from mouse colons or from ex vivo human T cell cultures, was performed as previously described (16).

Glycophenotype. T cells were incubated with biotinylated L-PHA, biotinylated LEL, biotinylated SNA, or biotinylated MAL-II (Vector Labs). Lectins were revealed with FITC-conjugated streptavidin.

Apoptosis Assays. Apoptotic cells were identified by flow cytometry, using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), following the manufacturer's instructions.

Enzymatic Reaction and HPLC Analysis. The GnT-V enzymatic activity analyses in T cells from patients with UC and controls were performed as previously described by Takamatsu et al. (45).

DSS- and TNBS-Induced Colitis and in Vivo GlcNAc Treatment. Colitis was induced with DSS in C57BL/6, MGAT5 wild-type, heterozygous, and knockout mice (kindly provided by Michael Pierce, University of Georgia, Athens, GA) (19). The TNBS model was also performed using C57BL/6 mice (19). After disease onset, DSS mice were treated with GlcNAc (Sigma and Wellesley Therapeutics, Inc.) (12). LPLs were isolated from mouse colon as previously described (16).

Tissue Immunohistochemistry and Immunofluorescence. FFPE colonic tissue slides were used for H&E staining and for immunohistochemistry with L-PHA and anti-CD3 mAb as well as Foxp3 and F4/80, as described, respectively, by Dias et al. (16) and Teixeira et al. (46). For T-bet immunofluorescence, mouse IgG_1 T-bet–specific mAb (clone 4B10; Santa Cruz Biotechnology), goat anti-mouse Alexa 594 secondary antibody (Invitrogen), and DAPI staining were used.

Real-Time PCR. Total RNA from isolated LPLs was extracted and the quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems), as previously described (16). qRT-PCR was carried out in triplicates for the target gene MGAT5b (Taqman probe: Mm01252571_m1) and the endogenous control 18S (Hs99999901_s1) (Applied Biosystems) (16).

Statistics. Statistical significance was assessed by one-way or two-way ANOVA using a Bonferroni's, Dunnett's, or Newman–Keuls multiple comparison post-test and, where appropriate, by an unpaired Student's t test (two-tailed) using GraphPad Prism 5. P values of <0.05 were considered statistically significant.

More details can be found in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Hiroaki Korekane and Fumi (RIKEN) for support in preparation of the fluorescent oligosaccharide acceptor substrate. We thank Dr. Michael Pierce for kindly providing the MGAT5 knockout mice. We also thank Paula Paíga (REQUIMTE/LAQV) for technical support with the HPLC system. The Institute of Molecular Pathology and Immunology of the University of Porto integrates the i3S research unit, which is partially supported by the Portuguese Foundation for Science and Technology (FCT). This article is a result of the project NORTE-01-0145-FEDER-000029, supported by the Norte Portugal Regional Programme (NORTE 2020) under the PORTUGAL 2020 Partnership Agreement through the European Regional Development Fund. This work was also funded by Fundo Europeu de Desenvolvimento Regional (FEDER) funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through the FCT in the framework of the project (POCI-01/0145-FEDER-016601 and PTDC/DTP-PIC/0560/2014). S.S.P. acknowledges the European Crohn's and Colitis Organization (ECCO) for ECCO Grant 2017, the Broad Medical Research Program at the Crohn's and Colitis Foundation of America, and the Portuguese Group of Study in IBD (GEDII) for funding. A.M.D. [PD/BD/105982/2014], A.C. [SFRH/BPD/91623/2012], and M.S.P. [SFRH/BD/110148/2015] received funding from the FCT. M. Lima thanks the CHP for the research support.

1. Cosnes J, Gower-Rousseau C, Seksik P, Cortot A (2011) Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 140:1785–1794.
2. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA (2001) Glycosylation and the immune system. *Science* 291:2370–2376.
3. Pinho SS, et al. (2009) The role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin. *Hum Mol Genet* 18:2599–2608.
4. Pinho SS, et al. (2013) E-cadherin and adherens-junctions stability in gastric carcinoma: Functional implications of glycosyltransferases involving N-glycan branching biosynthesis, N-acetylglucosaminyltransferases III and V. *Biochim Biophys Acta* 1830:2690–2700.
5. Pinho SS, Reis CA (2015) Glycosylation in cancer: Mechanisms and clinical implications. *Nat Rev Cancer* 15:540–555.
6. Carvalho S, Reis CA, Pinho SS (2016) Cadherins glycans in cancer: Sweet players in a bitter process. *Trends Cancer* 2:519–531.
7. Demetriou M, Granovsky M, Quaggin S, Dennis JW (2001) Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409:733–739.
8. Marth JD, Grewal PK (2008) Mammalian glycosylation in immunity. *Nat Rev Immunol* 8:874–887.
9. Chen HL, Li CF, Grigorian A, Tian W, Demetriou M (2009) T cell receptor signaling co-regulates multiple Golgi genes to enhance N-glycan branching. *J Biol Chem* 284:32454–32461.
10. Morgan R, et al. (2004) N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. *J Immunol* 173:7200–7208.

11. Lee SU, et al. (2007) N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *J Biol Chem* 282:33725–33734.
12. Grigorian A, et al. (2011) N-acetylglucosamine inhibits T-helper 1 (Th1)/T-helper 17 (Th17) cell responses and treats experimental autoimmune encephalomyelitis. *J Biol Chem* 286:40133–40141.
13. Grigorian A, et al. (2007) Control of T cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. *J Biol Chem* 282:20027–20035.
14. Zhou RW, et al. (2014) N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca^{2+} signaling. *Nat Immunol* 15: 1038–1045.
15. Araujo L, Khim P, Mkhikian H, Mortales CL, Demetriou M (2017) Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *eLife* 6:e21330.
16. Dias AM, et al. (2014) Dysregulation of T cell receptor N-glycosylation: A molecular mechanism involved in ulcerative colitis. *Hum Mol Genet* 23:2416–2427.
17. Nakajima K, et al. (2013) Mass isotopomer analysis of metabolically labeled nucleotide sugars and N- and O-glycans for tracing nucleotide sugar metabolisms. *Mol Cell Proteomics* 12:2468–2480.
18. Neurath MF (2014) Cytokines in inflammatory bowel disease. *Nat Rev Immunol* 14: 329–342.
19. Wirtz S, Neufert C, Weigmann B, Neurath MF (2007) Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2:541–546.
20. Johansson ME (2014) Mucus layers in inflammatory bowel disease. *Inflamm Bowel Dis* 20:2124–2131.
21. Salvatore S, et al. (2000) A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. *Aliment Pharmacol Ther* 14:1567–1579.
22. Lau KS, et al. (2007) Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* 129:123–134.
23. Ryczko MC, et al. (2016) Metabolic reprogramming by hexosamine biosynthetic and Golgi N-glycan branching pathways. *Sci Rep* 6:23043.
24. Dennis JW, Brewer CF (2013) Density-dependent lectin-glycan interactions as a paradigm for conditional regulation by posttranslational modifications. *Mol Cell Proteomics* 12:913–920.
25. Stanley P, Taniguchi N, Aebi M (2015) N-Glycans. *Essentials of Glycobiology*, eds Varki A, et al. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), 3rd Ed, pp 99–111.
26. Shinzaki S, et al. (2016) N-acetylglucosaminyltransferase V exacerbates murine colitis with macrophage dysfunction and enhances colitic tumorigenesis. *J Gastroenterol* 51: 357–369.
27. D'Haens GR (2010) Top-down therapy for IBD: Rationale and requisite evidence. *Nat Rev Gastroenterol Hepatol* 7:86–92.
28. Plevy SE, Targan SR (2011) Future therapeutic approaches for inflammatory bowel diseases. *Gastroenterology* 140:1838–1846.
29. Rabinovich GA, Toscano MA (2009) Turning 'sweet' on immunity: Galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* 9:338–352.
30. Fujino S, et al. (2003) Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52:65–70.
31. Toscano MA, et al. (2007) Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* 8:825–834.
32. Maniati E, Potter P, Rogers NJ, Morley BJ (2008) Control of apoptosis in autoimmunity. *J Pathol* 214:190–198.
33. Santucci L, et al. (2003) Galectin-1 suppresses experimental colitis in mice. *Gastroenterology* 124:1381–1394.
34. Chen IJ, Chen HL, Demetriou M (2007) Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. *J Biol Chem* 282:35361–35372.
35. Buck MD, O'Sullivan D, Pearce EL (2015) T cell metabolism drives immunity. *J Exp Med* 212:1345–1360.
36. Gerriets VA, et al. (2015) Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J Clin Invest* 125:194–207.
37. Orr SL, et al. (2013) A phenotype survey of 36 mutant mouse strains with gene-targeted defects in glycosyltransferases or glycan-binding proteins. *Glycobiology* 23:363–380.
38. Mkhikian H, et al. (2016) Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *eLife* 5:e14814.
39. Kurimoto A, et al. (2014) The absence of core fucose up-regulates GnT-III and Wnt target genes: A possible mechanism for an adaptive response in terms of glycan function. *J Biol Chem* 289:11704–11714.
40. Levin RM, Krieger NN, Winzler RJ (1961) Glucmsumine and acetylglucosamine tolerance in man. *J Lab Clin Med* 58:927–932.
41. Lee KY, et al. (2004) Subchronic toxicity study of dietary N-acetylglucosamine in F344 rats. *Food Chem Toxicol* 42:687–695.
42. Dias AM, Almeida CR, Reis CA, Pinho SS (2016) Studying T cells N-glycosylation by imaging flow cytometry. *Methods Mol Biol* 1389:167–176.
43. Oliveira L, et al. (2015) Deficits in endogenous adenosine formation by ecto-5′-nucleotidase/CD73 impair neuromuscular transmission and immune competence in experimental autoimmune myasthenia gravis. *Mediators Inflamm* 2015:460610.
44. Li WP, Roth J (1997) Expression of beta 1,6 branched asparagine-linked oligosaccharides in non-mitotic and non-migratory cells of normal human and rat tissues. *Int J Cancer* 71:483–490.
45. Takamatsu S, et al. (2013) N-acetylglucosaminyltransferase (GnT) assays using fluorescent oligosaccharide acceptor substrates: GnT-III, IV, V, and IX (GnT-Vb). *Methods Mol Biol* 1022:283–298.
46. Teixeira L, et al. (2015) Immune response in the adipose tissue of lean mice infected with the protozoan parasite Neospora caninum. *Immunology* 145:242–257.

Appendix III

PLOS ONE

RESEARCH ARTICLE

Association between Polymorphisms in Antioxidant Genes and Inflammatory Bowel Disease

Cristiana Costa Pereira[1,2,3☯], Cecília Durães[4,5☯], Rosa Coelho[6], Daniela Grácio[7], Marco Silva[6], Armando Peixoto[6], Paula Lago[8], Márcia Pereira[4,5,9], Telmo Catarino[4,5], Salomé Pinho[4,5], João Paulo Teixeira[1,3], Guilherme Macedo[6], Vito Annese[10], Fernando Magro[2,6,7]*

1 Department of Environmental Health, National Institute of Health Dr. Ricardo Jorge, Oporto, Portugal, **2** MedInUP – Centre for Drug Discovery and Innovative Medicines, University of Porto, Oporto, Portugal, **3** EPIUnit – Institute of Public Health, University of Porto, Oporto, Portugal, **4** Ipatimup – Institute of Molecular Pathology and Immunology of the University of Porto, Oporto, Portugal, **5** i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Oporto, Portugal, **6** Department of Gastroenterology, Faculty of Medicine, Centro Hospitalar São João, Oporto, Portugal, **7** Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto, Oporto, Portugal, **8** Department of Gastroenterology, HSA – Centro Hospitalar do Porto, Oporto, Portugal, **9** Institute for the Biomedical Sciences Abel Salazar, University of Porto, Oporto, Portugal, **10** Emergency Department, Gastroenterology Unit, AOU Careggi, Florence, Italy

☯ These authors contributed equally to this work.
* fm@med.up.pt

OPEN ACCESS

Citation: Costa Pereira C, Durães C, Coelho R, Grácio D, Silva M, Peixoto A, et al. (2017) Association between Polymorphisms in Antioxidant Genes and Inflammatory Bowel Disease. PLoS ONE 12(1): e0169102. doi:10.1371/journal.pone.0169102

Editor: John Green, University Hospital Llandough, UNITED KINGDOM

Received: October 24, 2016

Accepted: December 12, 2016

Published: January 4, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by a grant from the Portuguese Study Group for Inflammatory Bowel Disease (GEDII) [http://www.gedii.pt/_bolsas_e_premios_a_decorrer]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Inflammation is the driving force in inflammatory bowel disease (IBD) and its link to oxidative stress and carcinogenesis has long been accepted. The antioxidant system of the intestinal mucosa in IBD is compromised resulting in increased oxidative injury. This defective antioxidant system may be the result of genetic variants in antioxidant genes, which can represent susceptibility factors for IBD, namely Crohn's disease (CD) and ulcerative colitis (UC). Single nucleotide polymorphisms (SNPs) in the antioxidant genes *SOD2* (rs4880) and *GPX1* (rs1050450) were genotyped in a Portuguese population comprising 436 Crohn's disease and 367 ulcerative colitis patients, and 434 healthy controls. We found that the AA genotype in *GPX1* is associated with ulcerative colitis (OR = 1.93, adjusted *P-value* = 0.037). Moreover, we found nominal significant associations between *SOD2* and Crohn's disease susceptibility and disease subphenotypes but these did not withstand the correction for multiple testing. These findings indicate a possible link between disease phenotypes and antioxidant genes. These results suggest a potential role for antioxidant genes in IBD pathogenesis and should be considered in future association studies.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) characterized by inflammation of the intestinal mucosa triggered by the action of

Competing Interests: Prof. Magro has received fees for speaking engagements from SchéringPlough/MSD, Abbvie, Lab Vitoria, and Dr. Falk Pharma Portugal, and fees for consultancies and honoraria from MSD. The other authors state no conflicts of interest concerning this paper. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

environmental factors in genetically predisposed individuals [1]. The accepted notion is that the two major classifications of inflammatory bowel disease (IBD), known as Crohn's disease (CD) and ulcerative colitis (UC), are indeed distinct entities and have different causes and discrete mechanisms of tissue inflammation and damage. UC results in inflammation and ulcerations in the mucosal lining of the colon and rectum [2–6]. Crohn's disease differs in that it may result in inflammation deeper within the intestinal wall (transmural) and can occur in any parts of the digestive system (the mouth, esophagus, stomach, duodenum, small intestine, colon and rectum). Further, CD may also involve other organs outside the GI system through fistulization [7, 8].

Reactive oxygen species (ROS) have been suggested as key molecules in mediating the tissue injuries promoted by the inflammatory processes occurring in IBD [9, 10], and oxidative stress has been recognized as a potential etiological factor for IBD [11]. The detrimental effects of oxidative stress may be promoted and/or exacerbated by impairments of cellular antioxidant systems. The activation of inflammatory cells and consequent oxidative stress are mechanisms that have already been associated with carcinogenic processes, with 25% of all cancer cases worldwide attributed to chronic inflammation [12]. Recent reports refer 1.9- and 2.4-fold increased risk for CRC among CD and UC patients, respectively [13].

The antioxidant defence systems protect cells against ROS by regulating their intracellular concentrations through the activity of a number of enzymes, including superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPX1). These two enzymes integrate a common detoxification pathway, in which SOD2 (a tetrameric manganese-containing enzyme expressed in mitochondria) first catalyses the dismutation of superoxide anion to hydrogen peroxide and oxygen. The GPX1 enzyme (a cytosolic and ubiquous selenoenzyme) catalyses the subsequent conversion of hydrogen peroxide to water and oxygen [14]. Their combined action promotes the detoxification of mitochondrial ROS and a balance is expected to exist between these two enzymes, as a deranged activity would result in the accumulation of toxic levels of hydrogen peroxide in the cells [15]. Genetic polymorphisms can modify the activity of these critical enzymes and thus promote imbalances in the cellular oxidative burden. Diseases such as diabetic nephropathy, cardiomyopathy, Behcet's disease, and various cancers have already been associated with polymorphisms in antioxidant genes [16]. Genetic polymorphisms in *SOD2* have also been referred [17] as a potential mechanism interfering in the pharmaco-response of cells exposed to methotrexate (MTX), an immunomodulator commonly prescribed to IBD patients, and an interdependent mechanism of action between *SOD2* and *GPX1* has been suggested, indicating that these genes could also have an important role for therapeutic management.

Genome wide association studies (GWAS) have identified more than 200 genetic risk loci for IBD [18, 19] but only few were associated with clinical phenotype [20, 21]. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations [19]. Moreover, several genetic polymorphisms have already been linked to IBD etiopathogenesis and to response to therapy [22–24]. Based on the described interactions between inflammation, ROS and oxidative damage, this study aimed to assess the association between IBD (CD and UC) and polymorphisms in the antioxidant genes *SOD2* (rs4880, c.47T>C, Val16Ala) and *GPX1* (rs1050450, c.596C>T, Pro198Leu). The association of these polymorphisms with IBD has not been studied before.

Materials and Methods

Population

The study comprised 803 IBD patients (436 CD and 367 UC) and 434 controls (Table 1). The control group included 434 unmatched samples obtained from unrelated healthy blood donors

Table 1. Clinical characteristics of patients with Crohn's disease and ulcerative colitis.

Patients characteristics	Crohn's disease		ulcerative colitis	
	n	%	n	%
Total	436	-	367	-
Family history of IBD (yes/no*)	37/348	9.6/90.4	16/231	6.5/93.5
Sex (male/female)	225/211	51.6/48.4	162/205	44.1/55.9
Smoking habits	-	-	-	-
Never	189	46.2	222	63.8
Former	108	26.4	91	26.2
Current	112	27.4	35	10.1
Age at diagnosis	-	-	-	-
A1 (<17 years)	56	12.8	14	3.8
A2 (17–40 years)	306	70.2	215	58.6
A3 (>40)	74	17.0	138	37.6
Location	436	-	na	-
L1* (ileal)	195	44.8	na	-
L2 (colonic)	60	13.8	na	-
L3 (ilealcolonic)	180	41.4	na	-
L4 ($L1_4$+$L2_4$+$L3_4$)** (involvement of the upper digestive tract)	47	(10.8)	na	-
Location	na	-	364	-
E1* (proctitis + rectosigmoid)	na	-	134	36.8
E2 (distal)	na	-	101	27.8
E3 (pancolitis)	na	-	129	35.4
Behaviour	431	-	na	-
B1* (nonstricturing, nonpenetrating)	187	43.4	na	-
B2 (stricturing)	90	20.9	na	-
B3 (penetrating)	154	35.7	na	-
P ($B1_P$+$B2_P$+$B3_P$)** (perianal disease)	41	(9.5)	na	-
Rectal involvement (yes/no*)	99/337	22.7/77.3	228/8	96.6/3.4
Colonic involvement (yes/no*)	229/207	52.5/47.5	198/38	83.9/16.1
Abdominal surgery (yes/no*)	226/208	52.1/47.9	23/342	6.3/93.7
Extraintestinal manifestations (yes/no*)	53/383	12.2/87.8	7/230	3.0/97.0
Previous ongoing corticosteroids (yes/no*)	398/36	91.7/8.3	225/141	61.5/38.5
Steroid dependency (yes/no*)	189/243	43.8/56.3	81/206	28.2/71.8
Steroid resistance (yes/no*)	11/422	2.5/97.5	26/261	9.1/90.9
Need for immunosuppressant (yes/no*)	359/50	87.8/12.2	130/236	35.5/64.5
Response to immunosuppressant (yes*/no)	229/138	62.4/37.6	72/51	58.5/41.5
Response to biologics (yes*/no)	198/42	82.5/17.5	50/19	72.5/27.5

* Represents the reference in the case-case association studies;

** For L4 and P phenotypes the reference is absence of phenotype;

na: not applicable

doi:10.1371/journal.pone.0169102.t001

(mean age 47.2±18.7 years; female:male ratio: 1.4:1). This group consisted of permanent residents in the catchment area of Hospital of S. João (Porto, Portugal), selected during the assembling of the EpiPorto cohort [25]. Enrollment of participants was performed under approval of Centro Hospitalar S. João ethic committee (Comissão de Ética para a Saúde do Centro Hospitalar S. João) and included written informed consent for data and DNA usage, obtained by

trained personnel (nurses and doctors) upon collection. The participants included did not present any apparent infectious and/or chronic disorders.

The IBD group included 436 CD and 237 UC patients enrolled at Centro Hospitalar São João, Porto, Portugal; these patients attended their routine IBD specialist medical appointment and all were prospectively followed-up in a national database from Portuguese IBD group (GEDII, https://gediibasedados.med.up.pt/). An additional 130 UC patients were enrolled at Centro Hospitalar do Porto, Hospital de Santo António (CHP-HSA), Porto, Portugal, prospectively followed at the same hospital. The diagnosis of IBD was made according to the ECCO (European Crohn's and Colitis Organisation) guidelines for CD [26] and UC [27] diagnosis, and the Lennard-Jones criteria [28]. Information on patients' characteristics was obtained, namely smoking habits, age at diagnosis, years of follow-up, location and behaviour of disease, extra-intestinal manifestations, rectal and colonic involvement, previous abdominal surgery, previous corticotherapy, steroid dependency and resistance, need for immunosuppressant, response to immunosuppressant and response to biologic therapy (Table 1). Location, behaviour and age at diagnosis, were classified according to the Montreal Classification [29]. Patients were defined as steroid-dependent when incapable to reduce steroids below the equivalent of prednisolone of 10mg per day within three months of starting steroids without recurrent active disease or disease relapse within three months of stopping steroids. The steroid resistance was considered as the presence of active disease despite of a prednisolone dose of up to 0.75mg/kg per day over a period of four weeks [30]. Patients who had treatment with azathioprine, or methotrexate were considered in the group of 'need for immunosuppressant'. The 'response to immunosuppressant' was defined as positive when long-term sustained improvement of the symptoms was achieved, lasting at least one year without any further modifications in the therapeutic regime; the response was considered negative whenever no symptom improvement was verified after three months of full dose with azathioprine or methotrexate or by decision of the physician to add steroids or biologic therapy (anti-TNFα) or to refer for surgery. The 'response to biologic therapy' was defined as positive when long-term sustained improvement of the symptoms lasting at least one year without any further modifications in the therapeutic regimen was observed; failure of the therapeutic regimen was defined by an absence of improvement of the symptoms of disease and by decision of the physician to add steroids, add an immunosuppressant, switch to an alternative biologic therapy medication or to refer for surgery. S1 Table shows the clinical characteristics defining the phenotypes tested for association (reference categories used in the association analyses are marked with an asterisk). The Ethics Committee of both institutions—Comissão de Ética para a Saúde do Centro Hospitalar São João, and Comissão de Ética para a Saúde do Centro Hospitalar do Porto—approved the protocol and all patients or their legal guardians gave their written informed consent, complying with the principles laid down in the Declaration of Helsinki.

SNP selection and genotyping

We identified two potential functional polymorphisms involved in antioxidant pathways–*SOD2* A/G (rs4880) and *GPX1* G/A (rs1050450)–whose main effects have already been described for other conditions. These SNPs have a reported minor allele frequency of ≥ 0.1 for the European Caucasian population (S2 Table). Patients' and controls' genomic DNA was isolated from blood using the QIAcube system and the QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Venlo, The Netherlands) following the supplier's instructions. The SNPs were genotyped on the complementary DNA strand using TaqMan Pre-Designed SNP Genotyping Assay (Life Technologies, Carlsbad, CA, USA) (S3 Table). DNA amplification and allelic discrimination were performed according to product specifications with the ABI 7500 Fast real-

time PCR system (Applied Biosystems, Carlsbad, USA). Cases and controls were randomized during genotyping and 5% of the samples were genotyped in duplicate to assess the genotyping error rate. Concordance of genotypes was 100%.

Statistical analysis

Genotyping results were evaluated with the SNPassoc 1.6–0 package in the statistical software suite R. Compliance of alleles at individual loci with Hardy-Weinberg equilibrium was measured at the level of the control group using a χ^2 test (P-value$<$0.05). Power calculations conducted before the study indicated there was more than 80% power to detect significant associations of OR between 1.3 and 2.0 for both SNPs and both disease groups. Calculations were performed using CaTS software with the following settings: *SOD2* (rs4880): MAF = 0.47, OR = 1.25–2.00, CD and UC prevalence Portugal = 0.07% [31], CD case/control = 340/330, UC case/control = 370/330; *GPX1* (rs1050450): MAF = 0.34, OR = 1.25–2.00, CD and UC prevalence Portugal = 0.07%[31], case/control = 340/330, UC case/control = 370/330. A post-hoc power analysis showed that the power to detect significant associations is 100% for both disease groups since the ORs observed fall within the 1.5–2.0 range (or the equivalent 0.5–1.0 range).

Comparison of genotype frequencies between groups defined by status (patients vs. controls) and clinical characteristics were assessed by unconditional logistic regression (level of significance set to P-value$<$0.05) using the SNPassoc library in R and SPSS 23 (IBM SPSS statistics). The models included adjustment by sex and age. The codominant and recessive models of inheritance were considered. Odds ratios (OR) with respective confidence intervals (95% CI) were calculated for the allele (one copy or genotype) with minor frequency. The association of SNPs with CD and UC clinical characteristics was subsequently assessed using case-case analyses (S3 Table). The IBD phenotypes and reference categories are defined in Table 1. The Bonferroni correction was used to adjust for multiple testing in the analysis of overall association of SNPs with CD and UC (Table 2) and in the case-case analysis (Table 3). The correction was applied separately to each disease group.

Results

Genotypic frequencies and overall association with CD and UC

We genotyped two SNPs in antioxidant enzyme genes (*SOD2* and *GPX1*). In the control group, the frequencies of all SNPs did not deviate significantly from those expected under Hardy-Weinberg equilibrium ($P>0.05$). Table 2 summarizes the genotype frequencies for the SNPs rs4880 in *SOD2* and rs1050450 in *GPX1*, and overall associations with CD and UC (OR, 95% CI and respective *P*-values).

Based on the two models analysed—codominant and recessive—SNP rs1050450 in *GPX1* achieved nominal significant association with UC with *P*-values of 0.006, in the codominant, and 0.012, for the recessive model. In the codominant model the homozygous AA showed an OR (CI) of 1.93 (1.20–3.12) and in the recessive model an OR (CI) of 1.78 (1.13–2.80) is observed. After correction for multiple testing (Bonferroni correction) the homozygous AA genotype of *GPX1* (rs1050450) withstood the association with UC with an adjusted P-value of 0.037. SNP rs4880 in *SOD2* achieved a nominal significant association with CD in both models analysed, codominant and recessive, with *P*-values of 0.013 and 0.033, respectively. The homozygous GG showed an OR (CI) of 0.57 (0.37–0.89) in the codominant model and an OR (CI) of 0.66 (0.45–0.97) in the recessive model. These associations were lost when corrected for multiple testing. The SNPs were also considered for analysis with CD and UC phenotypes (genotype frequencies are reported in S4 and S5 Tables, respectively).

Table 2. Genotypic frequencies and overall association of genetic variants in *SOD2* and *GPX1* with Crohn's disease and ulcerative colitis.

Locus	Model	Controls n = 434	Crohn's disease n = 436	OR (95% CI)	P-value	P-value adjusted	Ulcerative colitis n = 367	OR (95% CI)	P-value	P-value adjusted
SOD2 rs4880		n = 426	n = 435				n = 367			
	AA*	119 (27.9)	142 (32.6)	1.00			102 (27.8)	1.00		
	GA	198 (46.5)	214 (49.2)	0.79 (0.55–1.13)	0.193	1.00	184 (50.1)	1.07 (0.75–1.51)	0.700	1.00
	GG	109 (25.6)	79 (18.2)	0.57 (0.37–0.89)	0.013	0.077	81 (22.1)	0.89 (0.59–1.34)	0.547	1.00
	A carrier* *vs.* GG	317 (74.4)/109 (25.6)	356 (81.8)/79 (18.2)	0.66 (0.45–0.97)	0.033	0.197	286 (77.9)/81 (22.1)	0.85 (0.60–1.20)	0.366	1.00
GPX1 rs1050450		n = 428	n = 430				n = 367			
	GG*	199 (46.5)	191 (44.4)	1.00			146 (39.8)	1.00		
	GA	187 (43.7)	187 (43.5)	0.96 (0.69–1.34)	0.802	1.00	164 (44.7)	1.18 (0.86–1.61)	0.277	1.00
	AA	42 (9.8)	52 (12.1)	1.39 (0.80–2.40)	0.242	1.00	57 (15.5)	**1.93 (1.20–3.12)**	**0.006**	0.037
	G carrier* *vs.* AA	386 (90.2)/42 (9.8)	378 (87.9)/52 (12.1)	1.39 (0.83–1.38)	0.210	1.00	310 (84.5)/57 (15.5)	**1.78 (1.13–2.80)**	**0.012**	0.070

* Reference;

ORs and 95% CIs were calculated considering the codominant and recessive models, adjusted for gender and age; bold font indicates nominally significant results; **p-value cutoff = 0.0083** (after Bonferroni correction applied separately to each disease group); SNPs were genotyped on the complementary DNA strand.

doi:10.1371/journal.pone.0169102.t002

Bonferroni correction for the genotype-phenotype case-case analyses in CD established a p-value of 0.00063 (Table 3), and none of the associations found could remain significant. Nevertheless, nominal p-values were found significant for some phenotypes, as follows:

Genotype association with CD phenotypes

Under the recessive model (Table 3), we found significant associations between SNP rs4880 in *SOD2* and 'rectal involvement' [OR (CI) = 1.83 (1.03–1.98), $P = 0.034$], and 'colonic involvement' [OR(CI) 1.93 (1.15–3.22), $P = 0.011$]. In the codominant model (Table 3), *SOD2* homozygous GG was associated with 'rectal involvement' [OR (CI) = 2.08 (1.09–3.96), $P = 0.026$]. *GPX1* homozygous AA associated with 'response to biologics' [OR (CI) = 3.10 (1.10–8.70), $P = 0.032$], and the heterozygous GA genotype was found significantly associated with 'location (L3 vs L2)' [OR (CI) = 2.70 (1.39–5.24), $P = 0.003$],

Genotype association with UC phenotypes

Within UC, under the recessive model we found significant associations between SNP rs4880 in SOD2 and location (E2 vs E1) [OR (CI) = 0.50 (0.26–0.96), $P = 0.032$] (data not shown in table).

Combined genotypes and risk profiles

Joint effects of the risk genotypes observed for CD and UC phenotypes have been assessed, however, no significant associations were found ($P>0.05$ for all the combinations tested; data not shown).

Table 3. Association of SNPs in the antioxidant system genes *SOD2* and *GPX1* with Crohn's disease clinical characteristics.

Locus		Ileal colonic (L3)**		Rectal involvement		Colonic involvement		Responds to biologics	
	SNP / Model	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
SOD2 rs4880	**AA**	1.00		1.00		1.00		1.00	
	GA	0.82 (0.41–1.65)	0.577	1.23 (0.72–2.10)	0.457	0.84 (0.55–1.30)	0.442	1.48 (0.65–3.35)	0.347
	GG	0.71 (0.31–1.63)	0.422	**2.08 (1.09–3.96)**	**0.026**	1.74 (0.98–3.10)	0.059	1.08 (0.37–3.10)	0.890
	A carrier* vs. GG	0.80 (0.40–1.63)	0.546	**1.83 (1.06–3.16)**	**0.034**	**1.93 (1.15–3.22)**	**0.011**	0.83 (0.34–2.03)	0.682
GPX1 rs1050450	**GG***	1.00		1.00		1.00		1.00	
	GA	**2.70 (1.39–5.24)**	**0.003**	1.01 (0.62–1.63)	0.965	0.86 (0.57–1.30)	0.132	1.57 (0.72–3.43)	0.26
	AA	1.81 (0.66–4.95)	0.250	0.61 (0.26–1.40)	0.093	0.62 (0.33–1.16)	0.475	**3.10 (1.10–8.70)**	**0.032**
	G carrier* vs. AA	1.17 (0.44–3.10)	0.750	0.60 (0.27–1.34)	0.196	0.66 (0.37–1.20)	0.175	2.42 (0.96–6.10)	0.071

* Reference; phenotype reference categories were defined as in Table 1;

** Reference category is "colonic" location;

ORs and 95% CIs were calculated considering the recessive and codominant models, adjusted for gender and age;

Bold font indicates nominally significant results;

P-value cutoff = 0.00063 (after Bonferroni correction applied to 80 tests performed in the case-case analysis in the CD group);

SNPs were genotyped on the complementary DNA strand.

doi:10.1371/journal.pone.0169102.t003

Discussion

The characterization of susceptibility genes in IBD is expected to bring benefit for the identification of primary pathogenic pathways, and possible environmental drivers, as well as new therapeutic targets. To clarify whether polymorphisms in antioxidant enzyme genes were associated with IBD (CD and UC) we conducted case-control and case-case studies for two SNPs in antioxidant genes (*SOD2* Val16Ala and *GPX1* Pro198Leu). To our best knowledge, this is the first study investigating the association between these genetic variants and IBD pathogenesis. Among the already published GWAS studies in IBD the only reference found related to these two genes is for GPX1 that has been suggested [32] to be in linkage disequilibrium with macrophage stimulating protein-1 (MST1); the authors propose that SNP rs1050450 (c.596C>T) in *GPX1* is the pathophysiologic link between *IBD12* locus and IBD, rather than the macrophage stimulating protein-1 (*MST1*), as previously described.

In our study the allele A in *GPX1* (rs1050450) significantly associated with UC in the recessive model with an ORs of 1.93, and an adjusted *P*-value of 0.037. Antioxidant enzymes maintain cellular redox homeostasis. Glutathione peroxidase (GPX) is a selenoenzyme that catalyses the breakdown of hydrogen peroxide (H_2O_2), and other organic peroxides, into water (H_2O) and oxygen (O_2). GPX1 is cytosolic and produced in all tissues. The SNP rs1050450 (or *GPX1* Pro198Leu) has been studied extensively in human disease and has already been linked to cancer risk [33], and oxidative stress related diseases [34]. The Leu198 variant results in a 10% reduced enzyme activity compared with the Pro198 variant [35], interfering with the overall capacity to respond to oxidative damage [34]. Individuals with reduced GPX1 activity exhibit an increased incidence of oxidative stress-related diseases such as breast,

colon, prostate, bladder and lung cancers, coronary artery disease, and also, low bone mineral density [36] and osteoarthropathy [37].

Studies using genetically altered mice with reduced GPX1 activity have shown a link to chronic and acute gastrointestinal inflammation [38]. In this study, we found that the variant with lower activity *GPX1* 198Leu associates with UC (OR 1.93, $P = 0.006$). In the CD group, a 3.1-fold increased odds for 'responding to biologics' was found for carriers of the homozygous variant Leu198Leu ($P = 0.03$). These findings along with the recent observation that SNP rs1050450 in *GPX1* is the pathophysiological link for IBD locus 12 [32], suggest that this gene is a good candidate as a biomarker for disease and treatment management purposes.

The SOD2 enzyme, also known as manganese superoxide dismutase (MnSOD), is one of the major antioxidant defence systems against mitochondrial superoxide radicals [39] and is one of the first in a chain of enzymes to mediate the ROS generated by the partial reduction of O_2 to hydrogen peroxide (H_2O_2). The MnSOD expression has been suggested as a potential biomarker in UC for predicting disease activity and severity [40]. While the valine-containing SOD2 (rs4880) is partially arrested in the inner mitochondrial membrane, the alanine-containing SOD2 (rs4880) is actively targeted to the mitochondrial matrix resulting in a 30–40% increase in SOD2 activity for the Ala16Ala variant, due to more efficient transport of the protein into the mitochondrial matrix [41] and to a 4-fold higher and stable mRNA expression [42]. Crawford et al. (2012) [16] reviewed 79 studies addressing a potential link between the *SOD2* Val16Ala genotype and various diseases or disorders, and almost half of those studies reported relationships with various types of cancers, e.g. gastric, lung, prostate, bladder and breast, to diabetes type I, nephropathy, chronic kidney disease, and to chemotherapy responses. The Ala16Ala genotype is generally associated with a protective effect, yet important differences are described in the literature, which indicates a complex role for the presence of risk allele C. Also, it has been suggested that *SOD2* 16Val variant is associated with an increased production of pro-inflammatory cytokines [43], and that its expression can be modulated through NF-kB binding to the promoter region of the gene [44].

In this study, although significances were lost after correction for multiple testing, nominal significant associations suggest a potential protective role regarding CD. Also, *SOD2* Ala16Ala variant in the CD group presented a 2.08-fold risk for rectal involvement ($P = 0.026$), and in the recessive model, it also presented 1.93 odds for colonic involvement, These findings might indicate that although this variant is uncommon among CD patients, when present it confers some susceptibility for rectal and colonic involvement. We suggest that overall, this gene might be implicated in disease location, and the fact that the pro-inflammatory prone variant (16Val) is more common among CD, the variant 16Ala is associated with colonic involvement in CD. Also, the referred putative effects of *SOD2* in inflammatory pathways, together with the fact that *SOD2* expression can be induced by dietary intake of antioxidants [45], indicate a potential therapeutic targeting for this gene as well as for dietary intervention benefits.

Conclusion

This aim of this study was to explore possible associations between IBD pathogenesis (CD and UC) and gene polymorphisms implicated in other oxidative stress conditions, taking into account several clinical characteristics. We found a significant association between the variant *GPX1* (rs1050450) and UC. This association needs to be tested in an independent cohort to validate these findings. Genetic association studies support the idea that disease location is genetically determined and many important loci that could explain disease

heterogeneity are still undetermined [20]. Also, the work of Hauser et al. [32] recently published suggesting a pathophysiological role for *GPX1* (rs1050450) is as well suggested with our findings. Overall, our results point out to a potential role of antioxidant genes in IBD pathogenesis.

Supporting Information

S1 File. Dataset.
(XLSX)

S1 Table. Phenotype categories and number of tests performed for Crohn's disease and ulcerative colitis case-case analysis (according to the clinical characteristics described in Table 1).
(PDF)

S2 Table. Characteristics of the SNPs genotyped.
(PDF)

S3 Table. TaqMan SNP genotyping assay details.
(PDF)

S4 Table. Summary of genotypic frequencies of SNPs in, *SOD2*, and *GPX1* [n (%)] in the Crohn's disease group. Cases subdivided according to location, rectal involvement, colonic involvement and response to biologics.
(PDF)

S5 Table. Summary of genotypic frequencies of SNPs in, *SOD2*, and *GPX1* [n (%)] in the ulcerative colitis group. Cases subdivided according to 'location.
(PDF)

Author Contributions

Conceptualization: CCP FM.

Data curation: CCP CD FM.

Formal analysis: CCP CD FM.

Funding acquisition: CCP.

Investigation: CCP CD RC DG MS AP PL MP TC SP FM.

Methodology: CCP CD FM.

Project administration: FM.

Resources: RC DG MS AP PL MP TC FM.

Supervision: FM.

Validation: CD VA.

Visualization: CCP CD FM.

Writing – original draft: CCP CD.

Writing – review & editing: CCP CD SP JPT GM VA FM.

References

1. Abraham C, Cho JH. Inflammatory Bowel Disease. New England Journal of Medicine. 2009; 361 (21):2066–78. doi: 10.1056/NEJMra0804647 PMID: 19923578
2. Podolsky DK. Inflammatory Bowel Disease. New England Journal of Medicine. 2002; 347(6):417–29. doi: 10.1056/NEJMra020831 PMID: 12167685
3. Cobrin GM, Abreu MT. Defects in mucosal immunity leading to Crohn's disease. Immunological reviews. 2005; 206:277–95. Epub 2005/07/29. doi: 10.1111/j.0105-2896.2005.00293.x PMID: 16048555
4. Targan SR, Karp LC. Defects in mucosal immunity leading to ulcerative colitis. Immunological reviews. 2005; 206:296–305. Epub 2005/07/29. doi: 10.1111/j.0105-2896.2005.00286.x PMID: 16048556
5. Hyams JS. Crohn's disease in children. Pediatric clinics of North America. 1996; 43(1):255–77. Epub 1996/02/01. PMID: 8596683
6. Hyams JS, Davis P, Grancher K, Lerer T, Justinich CJ, Markowitz J. Clinical outcome of ulcerative colitis in children. The Journal of pediatrics. 1996; 129(1):81–8. Epub 1996/07/01. PMID: 8757566
7. Nosti PA, Stahl TJ, Sokol AI. Surgical repair of rectovaginal fistulas in patients with Crohn's disease. European journal of obstetrics, gynecology, and reproductive biology. 2013; 171(1):166–70. Epub 2013/09/10. doi: 10.1016/j.ejogrb.2013.08.011 PMID: 24011379
8. Nielsen OH, Rogler G, Hahnloser D, Thomsen OO. Diagnosis and management of fistulizing Crohn's disease. Nat Clin Pract Gastroenterol Hepatol. 2009; 6(2):92–106. Epub 2009/01/21. doi: 10.1038/ncpgasthep1340 PMID: 19153563
9. Jurjus A, Eid A, Al Kattar S, Zeenny MN, Gerges-Geagea A, Haydar H, et al. Inflammatory bowel disease, colorectal cancer and type 2 diabetes mellitus: The links. BBA clinical. 2016; 5:16–24. Epub 2016/04/07. doi: 10.1016/j.bbacli.2015.11.002 PMID: 27051585
10. Alzoghaibi MA. Concepts of oxidative stress and antioxidant defense in Crohn's disease. World J Gastroenterol. 2013; 19(39):6540–7. doi: 10.3748/wjg.v19.i39.6540 PMID: 24151379
11. Pereira C, Gracio D, Teixeira JP, Magro F. Oxidative Stress and DNA Damage: Implications in Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015; 21(10):2403–17. Epub 2015/07/21. PMID: 26193347
12. Vendramini-Costa DB, Carvalho JE. Molecular link mechanisms between inflammation and cancer. Current pharmaceutical design. 2012; 18(26):3831–52. Epub 2012/05/29. PMID: 22632748
13. Annese V, Beaugerie L, Egan L, Biancone L, Bolling C, Brandts C, et al. European Evidence-based Consensus: Inflammatory Bowel Disease and Malignancies. J Crohns Colitis. 2015; 9(11):945–65. Epub 2015/08/22. doi: 10.1093/ecco-jcc/jjv141 PMID: 26294789
14. Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med. 2002; 33(3):337–49. Epub 2002/07/20. PMID: 12126755
15. Degoul F, Sutton A, Mansouri A, Cepanec C, Degott C, Fromenty B, et al. Homozygosity for alanine in the mitochondrial targeting sequence of superoxide dismutase and risk for severe alcoholic liver disease. Gastroenterology. 2001; 120(6):1468–74. Epub 2001/04/21. PMID: 11313317
16. Crawford A, Fassett RG, Geraghty DP, Kunde DA, Ball MJ, Robertson IK, et al. Relationships between single nucleotide polymorphisms of antioxidant enzymes and disease. Gene. 2012; 501(2):89–103. Epub 2012/04/25. doi: 10.1016/j.gene.2012.04.011 PMID: 22525041
17. Barbisan F, Motta JdR, Trott A, Azzolin V, Dornelles EB, Marcon M, et al. Methotrexate-Related Response on Human Peripheral Blood Mononuclear Cells May Be Modulated by the Ala16Val-SOD2 Gene Polymorphism. PLoS ONE. 2014; 9(10):e107299. doi: 10.1371/journal.pone.0107299 PMID: 25330300
18. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012; 491(7422):119–24. http://www.nature.com/nature/journal/v491/n7422/abs/nature11582.html#supplementary-information. doi: 10.1038/nature11582 PMID: 23128233
19. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet. 2015; 47(9):979–86. Epub 2015/07/21. doi: 10.1038/ng.3359 PMID: 26192919
20. Cleynen I, Boucher G, Jostins L, Schumm LP, Zeissig S, Ahmad T, et al. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. Lancet (London, England). 2016; 387(10014):156–67. Epub 2015/10/23.
21. Alonso A, Domenech E, Julia A, Panes J, Garcia-Sanchez V, Mateu PN, et al. Identification of risk loci for Crohn's disease phenotypes using a genome-wide association study. Gastroenterology. 2015; 148 (4):794–805. Epub 2015/01/06. doi: 10.1053/j.gastro.2014.12.030 PMID: 25557950

22. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006; 3(7):390–407. doi: 10.1038/ncpgasthep0528 PMID: 16819502

23. Ladislava B, Michal K, Katerina W, Milan B. Gene Polymorphisms and Inflammatory Bowel Diseases. In: (Ed.) DIS, editor. Inflammatory Bowel Disease. InTech2012.

24. Achkar JP, Fiocchi C. Gene-gene interactions in inflammatory bowel disease: biological and clinical implications. Am J Gastroenterol. 2009; 104(7):1734–6. Epub 2009/05/14. doi: 10.1038/ajg.2009.179 PMID: 19436278

25. Ramos E, Lopes C, Barros H. Investigating the Effect of Nonparticipation Using a Population-Based Case—Control Study on Myocardial Infarction. Ann Epidemiol. 2004; 14:437–41. doi: 10.1016/j.annepidem.2003.09.013 PMID: 15246333

26. Van Assche G, Dignass A, Panes J, Beaugerie L, Karagiannis J, Allez M, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. J Crohns Colitis. 2010; 4(1):7–27. Epub 2010/12/03. doi: 10.1016/j.crohns.2009.12.003 PMID: 21122488

27. Dignass A, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. J Crohns Colitis. 2012; 6(10):965–90. Epub 2012/10/09. doi: 10.1016/j.crohns.2012.09.003 PMID: 23040452

28. Lennard-Jones JE. Classification of inflammatory bowel disease. Scandinavian journal of gastroenterology Supplement. 1989; 170:2–6; discussion 16–9. Epub 1989/01/01. PMID: 2617184

29. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Canadian journal of gastroenterology = Journal canadien de gastroenterologie. 2005; 19 Suppl A:5a–36a. Epub 2005/09/10.

30. Dignass A, Van Assche G, Lindsay JO, Lemann M, Soderholm J, Colombel JF, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. J Crohns Colitis. 2010; 4(1):28–62. Epub 2010/12/03. doi: 10.1016/j.crohns.2009.12.002 PMID: 21122489

31. Azevedo LF, Magro F, Portela F, Lago P, Deus J, Cotter J, et al. Estimating the prevalence of inflammatory bowel disease in Portugal using a pharmaco-epidemiological approach. Pharmacoepidemiology and drug safety. 2010; 19(5):499–510. Epub 2010/03/23. doi: 10.1002/pds.1930 PMID: 20306455

32. Hauser F, Rossmann H, Laubert-Reh D, Wild PS, Zeller T, Muller C, et al. Inflammatory bowel disease (IBD) locus 12: is glutathione peroxidase-1 (GPX1) the relevant gene? Genes Immun. 2015; 16(8):571–5. Epub 2015/09/12. doi: 10.1038/gene.2015.35 PMID: 26355565

33. Hong Z, Tian C, Zhang X. GPX1 gene Pro200Leu polymorphism, erythrocyte GPX activity, and cancer risk. Mol Biol Rep. 2013; 40(2):1801–12. doi: 10.1007/s11033-012-2234-3 PMID: 23073788

34. Meplan C. Selenium and chronic diseases: a nutritional genomics perspective. Nutrients. 2015; 7 (5):3621–51. doi: 10.3390/nu7053621 PMID: 25988760

35. Ravn-Haren G, Olsen A, Tjonneland A, Dragsted LO, Nexo BA, Wallin H, et al. Associations between GPX1 Pro198Leu polymorphism, erythrocyte GPX activity, alcohol consumption and breast cancer risk in a prospective cohort study. Carcinogenesis. 2006; 27(4):820–5. Epub 2005/11/17. doi: 10.1093/carcin/bgi267 PMID: 16287877

36. Mlakar SJ, Osredkar J, Prezelj J, Marc J. The antioxidant enzyme GPX1 gene polymorphisms are associated with low BMD and increased bone turnover markers. Dis Markers. 2010; 29(2):71–80. doi: 10.3233/DMA-2010-0728 PMID: 21045266

37. Xiong YM, Mo XY, Zou XZ, Song RX, Sun WY, Lu W, et al. Association study between polymorphisms in selenoprotein genes and susceptibility to Kashin-Beck disease. Osteoarthritis Cartilage. 2010; 18 (6):817–24. doi: 10.1016/j.joca.2010.02.004 PMID: 20178852

38. Chu FF, Esworthy RS, Doroshow JH. Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. Free Radic Biol Med. 2004; 36(12):1481–95. Epub 2004/06/09. doi: 10.1016/j.freeradbiomed.2004.04.010 PMID: 15182851

39. Ascencio-Montiel I, Parra E., Salgado A., Gomez-Zamudio J., Kumate-Rodriguez J., Escobedo-de-la-Peña J., Cruz M. SOD2 gene Val16ALa polymorphism is associated with macroalbuminuria in Mexican Type 2 Diabetes patients: a comparative study and meta-analysis. BMC Medical Genetics. 2013; 14.

40. Ikumoto T, Hayashi S, Tomita S, Miwa S, Mitomi H, Fujimori T, et al. Manganese superoxide dismutase plays an important role in the inflammatory process and predicts disease severity and activity in patients with ulcerative colitis. Acta Pathologica, Microbiologica et immunologica Scandinavica. 2014; 122:512–7.

41. Sutton A, Khoury H, Prip-Buus C, Cepanec C, Pessayre D, Degoul F. The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. Pharmacogenetics. 2003; 13(3):145–57. Epub 2003/03/06. PMID: 12618592

42. Sutton A, Imbert A, Igoudjil A, Descatoire V, Cazanave S, Pessayre D, et al. The manganese superoxide dismutase Ala16Val dimorphism modulates both mitochondrial import and mRNA stability. Pharmacogenetics and genomics. 2005; 15(5):311–9. Epub 2005/05/03. PMID: 15864132

43. Montano MA, da Cruz IB, Duarte MM, Krewer Cda C, da Rocha MI, Manica-Cattani MF, et al. Inflammatory cytokines in vitro production are associated with Ala16Val superoxide dismutase gene polymorphism of peripheral blood mononuclear cells. Cytokine. 2012; 60(1):30–3. Epub 2012/06/13. doi: 10.1016/j.cyto.2012.05.022 PMID: 22688013

44. Li C, Zhou HM. The role of manganese superoxide dismutase in inflammation defense. Enzyme Res. 2011; 2011:387176. doi: 10.4061/2011/387176 PMID: 21977313

45. St Clair D. Manganese superoxide dismutase: genetic variation and regulation. J Nutr. 2004; 134 (11):3190s–1s. Epub 2004/10/30. PMID: 15514302