Glikozilacija imunoglobulina G u predviđanju terapijskoga odgovora i tijekom anti-TNF terapije u Crohnovoj bolesti

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DOKTORSKI RAD

Zagreb, 2024.

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Zagreb, 2024.

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IMMUNOGLOBULIN G GLYCOSYLATION IN THE PREDICTION OF THERAPEUTIC RESPONSE AND DURING ANTI-TNF THERAPY IN CROHN'S DISEASE

DOCTORAL DISSERTATION

Supervisors:
Prof Gordan Lauc, PhD
Irena Trbojević Akmačić, PhD

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SAŽETAK

Crohnova bolest (CB) jedan je od oblika upalne bolesti crijeva koja može zahvatiti stijenku čitavog gastrointestinalnog sustava, od usta do rektuma, no najčešće je ograničena na distalni dio tankog crijeva (ileum) i debelo crijevo. Za takvu upalu kažemo da je kroničnog, diskontinuiranog i transmuralnog karaktera. Smatra se da je CB, odnosno upalna bolest crijeva općenito, posljedica pretjeranog imunosnog odgovora u probavnom traktu zbog narušene crijevne barijere, promijenjenog sastava crijevne mikrobiote i utjecaja okolišnih čimbenika kod osoba koja imaju genetičku predispoziciju za razvoj bolesti. Glavni ciljevi u liječenju CB-a jesu kontrola upale, postizanje remisije i njezino održavanje. U tu svrhu koriste se i biološki lijekovi poput antagonista faktora nekroze tumora, odnosno anti-TNF lijekovi. Ipak, takva je terapija izrazito skupa, ne mora biti prikladna za svakog bolesnika, a često dio bolesnika ne odgovora zadovoljavajuće na terapiju ili kasnije tijekom režima gubi odgovor na istu. Isto tako, mogu se razviti i nuspojave ili protutijela na sam lijek. U središtu ovog doktorskog rada jest imunoglobulin G (IgG). Istraživanja u području glikobiologije su pokazala da složeni oligosaharidi, odnosno glikani vezani na IgG imaju izravan učinak na njegovu izvršnu funkciju te da osobe oboljele od CB-a imaju drugačiji sastav ukupnih *N*-glikana serumskog IgG-a u odnosu na zdrave osobe. Stoga ova doktorska disertacija ima za cilj ispitati mogućnost predviđanja terapijskog odgovora na anti-TNF terapiju temeljem analize sastava *N*-glikoma serumskog IgG-a prije započinjanja terapije te istražiti longitudinalnu promjenu u sastavu *N*-glikoma serumskog IgG-a tijekom te iste terapije. Pritom je za istraživanje korišteno ukupno 1513 uzoraka krvnog seruma bolesnika s CB-om regrutiranih u sklopu studije PANTS (engl. *Personalised Anti-TNF therapy in Crohn's disease*). U svrhu predviđanja terapijskog odgovora na anti-TNF terapiju korišteno je 1315 uzoraka krvnog seruma bolesnika s od CB-om, a dodatno prikupljenih 198 uzoraka krvnog seruma korišteno je kao druga vremenska točka zbog ispitivanja longitudinalne promjene u sastavu *N*-glikoma serumskog IgG-a kod istih bolesnika tijekom terapije. Iz uzoraka krvnog seruma pročišćen je IgG te je njegova *N*-glikozilacija analizirana metodom tekućinske kromatografije. Dobiveni rezultati pokazali su da tijekom primjene anti-TNF terapije dolazi do promjene u sastavu *N*-glikoma serumskog IgG-a bez obzira na terapijski odgovor. Preciznije, dolazi do smanjenja zastupljenosti agalaktoziliranih, a povećanja mono- i digalaktoziliranih te sijaliniziranih *N*-glikana IgG-a, što upućuje na smanjenje proupalnog potencijala serumskog IgG-a tijekom terapije. Nadalje, rezultati upućuju da sastav *N*-glikoma serumskog IgG-a prije započinjanja anti-TNF terapije ne sadrži informaciju o budućem terapijskom odgovoru te da se ne može koristiti u svrhu identificiranja bolesnika s CB-om koji neće primjereno odgovoriti na terapiju anti-TNF lijekovima.

KLJUČNE RIJEČI

Crohnova bolest, imunoglobulin G, *N*-glikozilacija, anti-TNF terapija, HILIC-UHPLC-FLD

SUMMARY

Introduction: Inflammatory bowel disease (IBD) has a substantial impact on a global population, significantly influencing the well-being of numerous individuals. Furthermore, its prevalence increases in less developed countries, leading to a great burden on both healthcare systems and economies worldwide. IBD is thought to be caused by an abnormal immune response, decreased gut microbiota diversity, and a interplay of genetic and environmental factors. Glycans have also been implicated in the development of IBD, specifically in the form of altered glycosylation of immunoglobulin G (IgG), a crucial component of humoral immunity. Glycans are complex oligosaccharides bound to proteins or lipids, and their molecular structure is a product of a complex cooperation of genetic and environmental factors. This fact enables us to study the role of glycans not only in homeostatic conditions but also in various inflammatory conditions and carcinomas, where they already proved their potential as diagnostic and prognostic biomarkers. Previous studies have demonstrated a shift of IgG *N*-glycome towards a pro-inflammatory pattern in patients diagnosed with Crohn's disease (CD) compared to healthy individuals. The major goal of IBD treatment is to achieve remission, which is commonly accomplished with tumour necrosis factor-alpha inhibitors (anti-TNF), such as infliximab and adalimumab. Nonetheless, this approach may not be suitable for every patient, given the frequent occurrence of primary non-response (PNR) to the treatment, loss of response (LOR), or adverse drug reactions (ADR) associated with this medications. As a result, this study explores the predictive potential of IgG *N*-glycome composition at baseline to detect PNR to anti-TNF therapy among patients with severe CD. Furthermore, this study will investigate the longitudinal change of IgG *N*-glycome composition in CD patients during 14-week therapy with anti-TNF drugs infliximab and adalimumab.

Materials and methods: CD patients were enrolled as participants in the Personalized Anti-TNF Therapy in Crohn's Disease (PANTS) study, conducted across various hospitals in the United Kingdom. This study was designed as a prospective uncontrolled observational cohort study to investigate the molecular mechanisms behind PNR, LOR and ADR to infliximab and adalimumab in anti-TNF naïve patients with severe active luminal CD. To enter the study, participants needed to meet subsequent requirements: age of 6 years or older, presence of active luminal CD affecting the colon and/or small intestine (classified as L1, L2 or L3 according to the Montreal classification), elevated levels of C-reactive protein (CRP) and/or faecal calprotectin, and no prior exposure to the anti-TNF medication.

A total of 1513 serum samples were collected from CD patients at two time points: before the initial dose of anti-TNF therapy (T0, baseline/week 0, N = 1315), and for a subset of patients, immediately before the next scheduled anti-TNF injection/infusion (T1, week 14, N = 198). PNR was assessed at week 14 by the patient's gastroenterologist.

Collected serum samples were subjected to IgG isolation using an affinity purification method and protein G as a ligand. Isolated IgG was further denatured and deglycosylated by overnight incubation with enzyme PNGase F. Released *N*-glycans were labelled with a mixture of fluorescent dye 2-aminobenzamide and a reducing agent 2-picoline borane. Labelled glycans were purified from the excess dye and other reagents through the process of solid-phase extraction. Fluorescently labelled IgG *N*-glycans were analyzed by ultra-high performance liquid chromatography based on hydrophilic interactions and fluorescence detection (HILIC-UHPLC-FLD). Obtained IgG *N*-glycoprofiles were separated into 24 glycan peaks (GP1-GP24) by an automatic integration algorithm. The amount of *N*-glycans in each glycan peak was expressed as a percentage of the total integrated area. Furthermore, six derived traits of IgG *N*-glycosylation were calculated based on the glycans with similar structural features. Linear mixed effect models were utilised to analyse longitudinaly collected data at the derived trait level, and a general linear model to assess the predictive potential of IgG *N*-glycome in therapy response at baseline. The false discovery rate was controlled with Benjamini-Hochberg, with an adjusted *P*-value < 0.05 considered significant.

Results: Obtained results suggest that anti-TNF therapy has a significant impact on the composition of IgG *N*-glycome during the 14-week treatment of 198 CD patients, regardless of the therapy response. To elaborate, 14 weeks after the first dose of anti-TNF therapy with both infliximab and adalimumab, there is a significant decrease in the level of agalactosylated IgG *N*-glycans and an increase in the levels of monogalactosylated, digalactosylated and sialylated glycans, all of which indicate that anti-TNF therapy diminishes the pro-inflammatory potential of IgG in CD patients 14 weeks after the start of treatment. Furthermore, the same trend is observed at the level of infliximab and adalimumab separately. However, infliximab causes more pronounced changes compared to adalimumab on the same derived IgG traits.

The predictive potential of IgG *N*-glycome in response to anti-TNF therapy at baseline was also evaluated in 1315 CD patients, whose serum samples were collected before the start of anti-TNF therapy. Results suggest that there are no associations between baseline IgG *N*-glycome composition and future therapy response to infliximab and adalimumab.

Conclusion: Anti-TNF therapy reverts the composition of IgG *N*-glycome in CD patients toward less pro-inflammatory patterns during the 14 weeks of treatment. Moreover, infliximab

causes more pronounced changes compared to adalimumab during the same period, which can insinuate its greater capacity to diminish inflammation. However, baseline IgG *N*-glycan measurements do not possess a predictive potential to detect PNR before the commencement of anti-TNF treatment in CD patients.

KEYWORDS

Crohn's disease, immunoglobulin G, *N*-glycosylation, anti-TNF therapy, HILIC-UHPLC-FLD

POPIS KRATICA

2-AB = 2-aminobenzamid

2-PB = 2-pikolin boran

ACN = acetonitril

ADCC = stanicama posredovana citotoksičnost ovisna o protutijelima (engl. *antibody-dependent cell-mediated cytotoxicity*)

ADCP = stanicama posredovana fagocitoza ovisna o protutijelima (engl. *antibody-dependent cell-mediated phagocytosis*)

AMP = antimikrobni peptidi

Anti-TNF = antagonist faktora nekroze tumora

ASCA = protutijela usmjerena protiv *Saccharomyces cerevisiae* (engl. *anti-Saccharomyces cerevisiae antibodies)*

Asn = asparagin

ITM = indeks tjelesne mase

CB = Crohnova bolest

CDC = citotoksičnost ovisna o komplementu (engl. *complement-dependent cytotoxicity*)

CRP = C-reaktivni protein

DMSO = dimetil sulfoksid

Dol-P = dolikol fosfat

ER = endoplazmatski retikul

Fab = fragment koji veže antigen (engl. *fragment antigen-binding*)

Fc = fragment koji kristalizira (engl. *fragment crystallizable*)

FcγR = Fc receptori za IgG

FLD = fluorescencijski detektor

GA = Golgijev aparat

Glc = glukoza

GlcNAc = *N*-acetilglukozamin

GP = glikanski vršak (engl. *glycan peak*)

GU = glukozna jedinica

HILIC = tekućinska kromatografija temeljena na hidrofilnim interakcijama (engl. *hydrophilic interaction liquid chromatography*)

IBD = upalna bolest crijeva (engl. *inflammatory bowel disease*)

IgA = imunoglobulin A

IgG = imunoglobulin G

IgM = imunoglobulin M

IL = interleukin

INF = interferon

IQR = interkvartilni raspon (engl. *interquartile range*)

LOR = gubitak odgovora (engl. *loss of response*)

Man = manoza

NF-κB = nuklearni faktor kapa B

NK-stanice = stanice prirodne ubojice (engl. *natural killer cells*)

OST = oligosahariltransferaza

PANTS = engl. *Personalised Anti-TNF therapy in Crohn's disease*

PBS = fosfatni pufer (engl. *phosphate buffer saline*)

PNR = izostanak primarnog odgovora (engl. *primary non-response*)

Pro = prolin

SDS = natrijev dodecil sulfat (engl. *sodium dodecyl sulphate*)

Ser = serin

Th = pomoćnički limfociti T (engl. *helper T cells*)

Thr = treonin

TNF = faktor nekroze tumora (engl. *tumor necrosis factor*)

TNFR1 = receptor faktora nekroze tumora 1 (engl. *tumor necrosis factor receptor 1*)

TNFR2 = receptor faktora nekroze tumora 2 (engl. *tumor necrosis factor receptor 2*)

T_{reg} = regulacijski limfociti T (engl. *regulatory T cells*)

UHPLC = tekućinska kromatografija ultra-visoke djelotvornosti (engl. *ultra-high performance liquid chromatography*)

UK = ulcerozni kolitis

V_{inj} = volumen injektiranja

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1. UVOD

1.1. LITERATURNI PREGLED

1.1.1. Patofiziologija Crohnove bolesti

Upalna bolest crijeva (IBD, engl. *inflammatory bowel disease*) jedna je od široko rasprostranjenih bolesti modernog doba koja zahvaća više od 2,5 milijuna Europljana i više od milijun Amerikanaca. Donedavno se smatrala bolešću koja zahvaća stanovništvo razvijenijih zemalja, no povećana pojavnost počela se uočavati i u manje razvijenim dijelovima svijeta te raste upravo s njihovom industrijalizacijom (1,2). Na temelju dostupnih dijagnostičkih postupaka, IBD se dijeli na ulcerozni kolitis (UK), indeterminirani (intermedijarni) kolitis te Crohnovu bolest (CB), na kojoj je ujedno i naglasak u ovoj doktorskoj disertaciji.

CB je kronična, kompleksna, idiopatska i multifaktorna bolest koja za posljedicu ima stvaranje upale u stijenki gastrointestinalnog trakta od usta do rektuma, no najčešće zahvaća ileocekalno područje. U odnosu na UK, koji je ograničen na površinu stijenke kolona, upala u CB-u je transmuralnog, segmentalnog i asimetričnog karaktera, što znači da zahvaća i dublje slojeve stijenke crijeva te se upaljeni dijelovi stijenke izmjenjuju s naizgled nepromijenjenim segmentima stijenke crijeva (3). CB ima više fenotipova, točnije upalni, strikturirajući i penetrirajući, a oboljele osobe mogu imati jedan ili više fenotipova koji se izmjenjuju tijekom bolesti, najčešće od upalnog prema strikturirajućem ili penetrirajućem (4). Simptomi bolesti uključuju abdominalnu bol, gubitak na težini, mučninu i povraćanje, povišenu tjelesnu temperaturu, a kod težih oblika dolazi i do razvoja opstrukcije crijeva, fistula i apscesa (4). U odnosu na UK, vancrijevne manifestacije izraženije su kod CB-a i prisutne kod gotovo polovice bolesnika, mogu zahvatiti više sustava u tijelu, od lokomotornog i hepatobilijarnog, pa sve do očiju i kože, a često se takve manifestacije pojavljuju i prije pojave gastrointestinalnih simptoma (5).

Glavni izvor upalne kaskade koja potencira uništavanje crijevne sluznice nije u potpunosti jasan, no rezultati većine znanstvenih radova slažu se u činjenici da patogeneza CB-a uključuje narušenu crijevnu barijeru, promijenjeni sastav crijevne mikrobiote, stanjivanje luminalnog sloja mucina, poremećen imunosni odgovor u sluznici crijeva, preosjetljivost te stimulaciju određenim antigenima i hranom, prvenstveno kod osoba s genskom predispozicijom za razvoj bolesti (6,7). Crijevna sluznica je fizička, biokemijska i imunosna barijera odgovorna za održavanje crijevne homeostaze selektivnim propuštanjem nutrijenata i vode te pružanje zaštite od crijevnih komenzala, patogena i antigena iz hrane u crijevnom sadržaju. Ona se sastoji od tri sloja. S luminalne strane crijeva prvo se nalazi sloj sluzi ili mukusa, građen od izrazito

glikoziliranih proteina (mucina) koji tvore strukturu nalik gelu, a koji sadrži komenzalnu crijevnu mikrobiotu, antibakterijske peptide (AMP, engl. *antimicrobial peptides*) te sekrecijski imunoglobulin A (IgA). Slijedi sloj epitelnih stanica od kojih su najzastupljeniji enterociti, a zatim specijalizirane vrčaste, Panethove i enteroendokrine stanice, koje zajedno tvore jednoslojnu barijeru koja odvaja lumen od lamine proprije. Slijedi sloj lamine proprije koji je sadrži stanice urođene i stečene imunosti poput limfocita T, limfocita B, makrofaga i dendritičnih stanica (8). Kompromitiranje bilo koje od navedenih razina može dovesti do upale u gastrointestinalnom sustavu (9,10).

Povećana propusnost crijevne barijere jedan je od prvih faktora koji se razmatrao kao mogući uzrok CB-a (11), a danas je i povezana s razvojem ove bolesti (12). Može, također biti prisutna ne samo kod bolesnika s aktivnim oblikom bolesti nego i kod rođaka iz prvog koljena što upućuje na činjenicu da je upravo povećana propusnost crijevne barijere jedan od bitnih faktora rizika za razvoj ove bolesti u obiteljskoj anamnezi (13,14).

Od okolišnih čimbenika koji povećavaju relativni rizik za razvoj IBD-a, najčešće se spominju pušenje, uporaba antibiotika (metronidazol, flurokinoloni) posebice u mlađoj životnoj dobi, stupanj urbanizacije područja življenja, zagađenost zraka, sastav prehrane te uporaba drugih lijekova poput nesteroidnih antireumatika (15). Novije studije pokazale su da je pušenje zaista povezano s povećanim rizikom od razvoja CB-a, ali u manjoj mjeri no što se prije smatralo (13% umjesto prijašnjih 50%). Nadalje, pokazano je da je za razvoj CB-a prediktivniji povišen postotak tjelesne masti, indeks tjelesne mase (ITM) te smanjen unos omega-3 masnih kiselina (16), a da tjelesna aktivnost može imati zaštitni učinak od razvoja bolesti (17). Navedeni čimbenici mogu utjecati i na crijevnu mikrobiotu, čiji se sastav određuje i mijenja tijekom cijelog života na temelju ne samo genetičkog zapisa, nego i izloženosti pojedinim okolišnim čimbenicima.

Upravo se crijevna mikrobiota zbog svoje uloge u održavanju crijevne homeostaze nalazi u središtu patogeneze IBD-a (15). Disbioza nastaje kada se sastav crijevne mikrobiote mijenja u smjeru povećane zastupljenosti potencijalno patogenih mikroorganizama te kada dolazi do narušavanja ravnoteže između same mikrobiote i domaćina (18). Pokazano je da crijevna mikrobiota karakteristična za IBD ima smanjenu bioraznolikost te povećanu zastupljenost bakterija iz koljena *Bacteroidetes* i *Proteobacteria* u odnosu na zdrave pojedince te smanjenu zastupljenost bakterija *Firmicutes*. Jedna od značajnih bakterija iz koljena *Firmicutes* je *Faecalibacterium prausnitzii,* metabolički vrlo aktivna komenzalna bakterija za koju je pokazano da svojim metabolitima potpomaže protuupalni učinak na način da inhibira aktivaciju nuklearnog faktora-κB (NF-κB) i sekreciju interleukina-8 (IL-8) u *in vitro* modelima

te uzrokuje smanjenu sekreciju proupalnog IL-12 i povećanu razinu protuupalnog IL-10 u *in vivo* modelima. Kod bolesnika oboljelih od CB-a snižena razina ove bakterije je faktor rizika za pojavu recidiva šest mjeseci nakon operacije, a suplementacija ove bakterije u vidu probiotika bi mogla biti obećavajući pristup u liječenju CB-a (19). Zbog mogućnosti adherencije na crijevne epitelne stanice, patogena *Escherichia coli* kod bolesnika oboljelih od CB-a u velikoj mjeri kolonizira sluznicu crijeva te utječe na patogenezu bolesti, dijelom zbog umnožavanja u crijevnim makrofazima te dijelom zbog stimuliranja izlučivanja faktora nekroze tumora alfa (TNFα) i IL-6 (20).

Do danas je cijelogenomskim studijama povezanosti (engl. g*enome-wide association study*) otkriveno više od 200 genskih lokusa povezanih s rizikom za nastanak IBD-a. Prvi gen povezan s povećanim rizikom od nastanka CB-a bio je *NOD2*, gen koji kodira za unutarstanični receptor koji prepoznaje komponentu stanične stijenke bakterija, a čije mutacije dovode do pretjerane aktivacije signalnog puta NF-κB u monocitima te ometaju prepoznavanje patogenih mikroorganizama i njihov klirens (21). Nadalje, i druge su genske varijante pojedinih gena intenzivnije proučavane kako bi se produbilo znanje o genskoj podlozi u patofiziologiji CB-a. Neke od njih su primjerice polimorfizmi gena *ATG16L1* koji rezultiraju povećanom produkcijom proupalnih citokina iz makrofaga, smanjenom autofagijom, abnormalnim Panethovima stanicama s poremećajem u izlučivanju AMP-a i posljedično smanjenog klirensa bakterija (22). Veliki trud se i dalje ulaže kako bi se otkrile funkcionalne (23) i rijetke genske varijante (24) uključene u genetičku arhitekturu CB-a. Produkt gena *CARD9* važan je u zaštiti domaćina protiv gljivičnih infekcija i upale crijeva, a više genskih varijanti povezano je s razvojem IBD-a zbog narušenog sastava i funkcije crijevne mikrobiote te poremećene produkcije AMP-a (25), iako postoje i varijante gena koje djeluju zaštitno (26). Nedavna studija upozorila je i na novu ulogu mezenhimskih stanica u razvoju CB-a koje pod utjecajem proupalnih signala sudjeluju u razvoju i održavanju upale u crijevima i nastanku fibroznih promjena, a koja dosad nije bilo uočena u ranijim genetičkim studijama (24).

U konačnici, navedeni čimbenici dovode do upalnog odgovora, odnosno abnormalnog lučenja citokina i pretjerane aktivacije imunosnog sustava u crijevnoj sluznici, a koji pridonose patogenezi IBD-a. Glavne stanice stečene imunosti, limfociti T, aktiviraju se i diferenciraju u efektorske, regulacijske (T_{reg}, engl. *regulatory T cells*) i pomoćničke limfocite T (Th, engl. *helper T cells*) s odgovarajućim receptorima, kao odgovor na stimulaciju specifičnim antigenima u limfnom tkivu (27). Smatra se da je CB prvenstveno posredovana Th1/Th17 imunosnim odgovorom koji potencira upalu crijeva. Limfociti Th1 mogu biti aktivirani IL-12, interferonom-γ (INF-γ) i IL-2 te u konačnici dovesti do regrutiranja makrofaga, NK-stanica

(engl. *natural killer*) i $CD8^+$ T limfocita. Povećana razina limfocita Th17 i pripadajućih citokina također potiče upalu u crijevima kod IBD-a, dok manjak limfocita T_{reg} pomaže održati upalu (27). Stanice urođene imunosti, prvenstveno neutrofili, monociti, makrofagi, dendritične stanice, NK-stanice, ali i stanice koje ne pripadaju imunosnom sustavu, poput stromalnih i epitelnih, luče citokine, kemokine i antimikrobne agense kako bi inicirali upalu i pritom dovode do fagocitoze, prezentacije antigena i aktivacije stečene imunosti (27). Kao odgovor na stimulaciju u IBD-u, stanice urođene imunosti pojačano luče proupalne citokine, a to su prvenstveno IL-1, IL-6, IL-18, IL-12, IL-23, IL-27, IL-35, TNFα, interferon-α (INF-α) i interferon-β (INF-β) (28). U odnosu na UK, kod CB-a dolazi do povećane produkcije IL-12 od strane antigen-prezentirajućih stanica (engl. *antigen presenting cells*) poput dendritičnih stanica i makrofaga u lamini propriji, što sugerira da takve aktivirane stanice preferiraju imunosni odgovor posredovan pomoćničkim limfocitima Th1 (28,29). Povećane razine IL-23 pospješuju i infiltraciju patogenih limfocita Th17 u upaljenoj sluznici crijeva kod bolesnika s CB-om, dok signalni putevi koji uključuju IL17 i IL23 mogu inducirati kaskadu proupalnih citokina poput TNF-a, INF-γ, IL-22 i drugih. (30). U CB-u se također povećava razina jedinstvenih crijevnih makrofaga $CD14^+$ koji stvaraju povećane količine IL-6, IL-23 i TNF-a i potenciraju upalu, a koji nisu prisutni kod zdravih pojedinaca niti bolesnika s UK-om (31).

Premda su prvenstveno limfociti T odgovorni za upalu u crijevnoj sluznici, stanice humoralne imunosti također igraju važnu ulogu u patogenezi CB-a. Diferencijacija plazma stanica potaknuta je $CD4^+$ T limfocitima i IL-2 koji se pretjerano stvara u crijevima bolesnika s CB-om. Nadalje IL-21 stimulira sazrijevanje B stanica koje eksprimiraju granzim-B, citokin snažnog citotoksičnog djelovanja na crijevnu sluznicu (32,33). Smatra se da limfociti B mogu utjecati i na T_{reg} limfocite produkcijom protuupalnog IL-10 pa je moguće da imaju i regulacijsku ulogu (34). Postoji naznaka da u CB-u dolazi do disregulacije funkcije limfocita B odnosno do njihove infiltracije oko granulomatoznog tkiva, pretjerane stimulacije i sazrijevanja protutijela IgG i IgA, dok se populacija IgM memorijskih limfocita B smanjuje (34). Bolesnici oboljeli od CB-a često pokazuju pojačan imunosni odgovor posredovan antimikrobnim IgG-om i IgA-om, poput protutijela koje je usmjereno protiv *Saccharomyces cerevisiae* (ASCA, engl. *anti–Saccharomyces cerevisiae antibody*), kvasca kojeg često nalazimo u hrani. Razine IgG^+ B limfocita i plazma stanica također su povišene u upaljenoj sluznici kod bolesnika s CB-om i UK-om, a IgG je usmjeren protiv crijevne mikrobiote (35).

Važno mjesto u upalnoj kaskadi ima i citokin TNFα koji je u manjoj ili većoj mjeri povezan s kliničkom slikom CB-a i UK-a. TNFα je pleiotropni citokin i produkt stanica urođene imunosti poput makrofaga, monocita i diferenciranih limfocita T. Kod CB-a, njegova pojava

uglavnom je posljedica primarnog Th1/Th17 imunosnog odgovora kao rezultat stimulacije stanica urođene imunosti, prvenstveno makrofaga, epitelnih stanica i mastocita. Sudjeluje u upalnom odgovoru aktivacijom NF-kB i protein-kinaza aktiviranih mitogenom (engl. *mitogen-activated protein kinase*) i održavanju upale crijeva, izlučivanju proupalnih citokina INF-γ, IL-6, IL-1β i reaktivnih kisikovih radikala, akumulaciji neutrofila, nastanku edema i granuloma te povećanoj propusnosti crijevnog epitela (36–38). Povećana ekspresija TNF-a uočena je u crijevnim makrofagima kod bolesnika s CB-om i UK-om, a serumske koncentracije TNF-a koreliraju s aktivnošću bolesti (38). Na njegovu važnost upućuju i antagonisti TNF-a odnosno anti-TNF lijekovi, koji ne samo da pridonose olakšavanju simptoma nego pružaju i endoskopsko cijeljenje sluznice crijeva (39), a o kojima će biti nešto više riječi kasnije. S obzirom na to da za TNF kažemo da je pleiotropni citokin, često se spominje i njegova moguća zaštitna funkcija. Postoje saznanja da sudjeluje u održavanju homeostaze u crijevima i integriteta crijevne barijere te da stimulira proizvodnju glukokortikoida koji imaju snažan protuupalni učinak u sluznici crijeva. O konačnom učinku ovog citokina odlučuje i sam tip stanica koje ga proizvode i njihova lokalizacija, kao i ciljane molekule TNF-a (37).

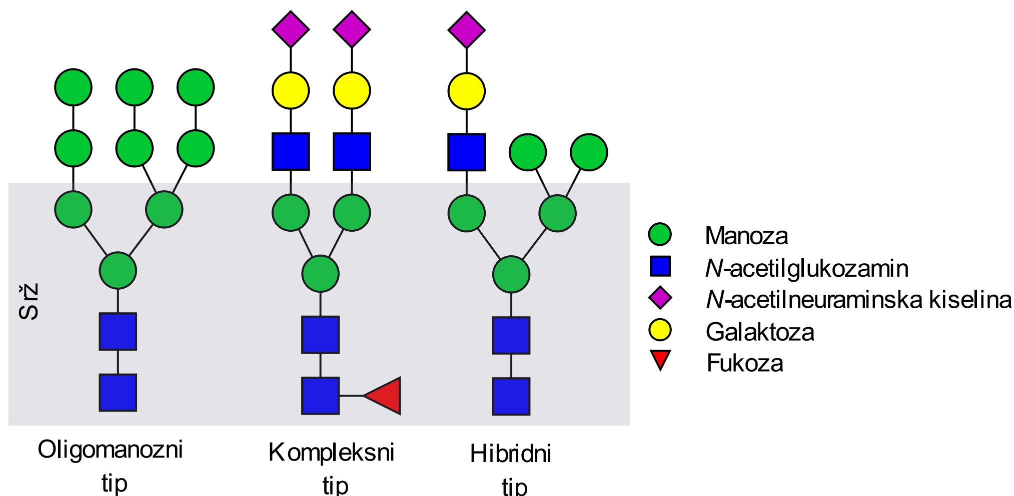
1.1.2. Glikozilacija

Glikobiologija je relativno mlada znanstvena grana koja se počela razvijati 6o.-tih godina prošlog stoljeća. Do danas je prepoznata kao grana koja se ubrzano razvija, a koja proučavajući ugljikohidratnu komponentu proteina ili lipida može ponuditi dodatnu informaciju o njihovoj ulozi u fiziološkim i patofiziološkim uvjetima. Uz nukleinske kiseline, proteine i lipide, ugljikohidrati predstavljaju osnovu svakom živom organizmu, a njihova uloga posebice je vidljiva kod viših eukariota. Složene ugljikohidrate sastavljene od različitih monosaharida raspoređenih u prostoru, u ovisnosti o konfiguraciji i poziciji glikozidne veze, nazivamo glikanima, dok glikokonjugatom nazivamo tvorbu glikana i aglikona, odnosno ne-ugljikohidratnog dijela poput proteina ili lipida. Površina svih eukariotskih stanica prekrivena je slojem glikana, a oni sudjeluju ne samo u staničnoj interakciji unutar organizma, nego i u interakciji s različitim simbiotima i patogenima (40). Za razliku od proteina čiji je aminokiselinski slijed rezultat isključivo genskog zapisa, sastav i raspored monosaharida, odnosno konačan izgled glikana kroje složeni biosintezni putevi pod utjecajem genskih zapisa i okolišnih čimbenika (40).

Glikozilacija predstavlja proces ko- i posttranslacijske modifikacije proteina i lipida u endoplazmatskom retikulu (ER) i Golgijevom aparatu (GA), čime se utječe na njihova svojstva

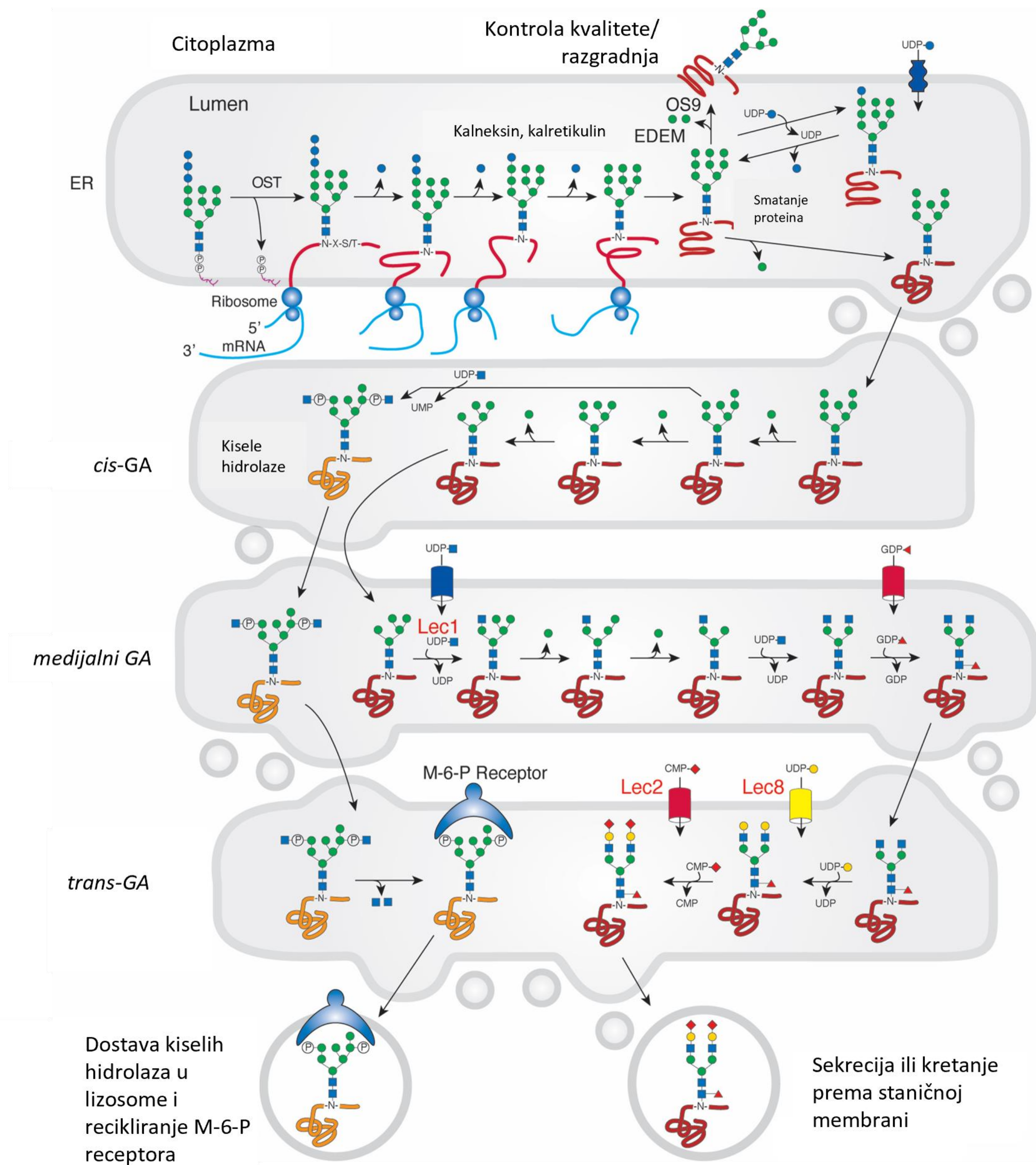
i funkciju. Razlikujemo više vrsta glikozilacije poput *N*- i *O*-glikozilacije proteina, *C*-manozilacije, dodatka glikozaminoglikana i glikozilfosfatidilinozitolnog sidra na polipeptidnu okosnicu ili nastanak glikosfingolipida u slučaju lipida (41). Velika varijabilnost glikanskih struktura vezanih za proteine posljedica je tkivno-specifične regulacije gena koji kodiraju za proteine uključene u proces glikozilacije, dostupnosti sastavnica nužnih za sam proces u određenom staničnom odjeljku poput aktiviranih donora šećera, akceptora (monosaharid ili amino kiselina unutar glikozilacijskog mjesta), odgovarajućih glikoziltransferaza i glikozidaza i njihove međusobne kompeticije te strukture samog glikopeptida i mikrookoliša unutar kojeg se odvija glikozilacija. Iz tog razloga, različite glikanske strukture nisu prisutne samo kod različitih glikoproteina, već isti glikoprotein i sam dolazi u obliku više različitih glikoformi, a glikozilacija se može značajno razlikovati i unutar samog glikoproteina ukoliko postoji više glikozilacijskih mjesta (42).

Danas najproučavaniji tip glikozilacije proteina je *N*-glikozilacija. *N*-glikozilacija označava prijenos glikana na dušikov atom asparagina (Asn) polipeptidne okosnice, koji se nalazi unutar slijeda Asn-X-serin(Ser)/treonin(Thr) (X je bilo koja aminokiselina osim prolina, Pro). No nije svaki Asn unutar navedenog evolucijski očuvanog aminokiselinskog slijeda okupiran *N*-glikanom, na što utječe i prostorna konformacija proteina (43). Svi *N*-glikani dijele istu sržnu strukturu koja se sastoji od dva *N*-acetilglukozamina (GlcNAc) i tri manoze (Man), a ovisno o vrsti vezanih monosaharida odnosno njihovom rasporedu razlikujemo oligomanozni, hibridni i kompleksni tip glikana (Slika 1).



Slika 1. Tipovi *N*-glikana (oligomanozni, kompleksni i hibridni). *N*-glikani su svojim nereducirajućim krajem vezani na Asn unutar slijeda Asn-X-Ser/Thr (X bilo koja aminokiselina osim Pro) na polipeptidnoj okosnici proteina. Svi *N*-glikani dijele istu sržnu strukturu $GlcNAc_2Man_3$. Izrađeno prema Stanley i sur., 2022. (44).

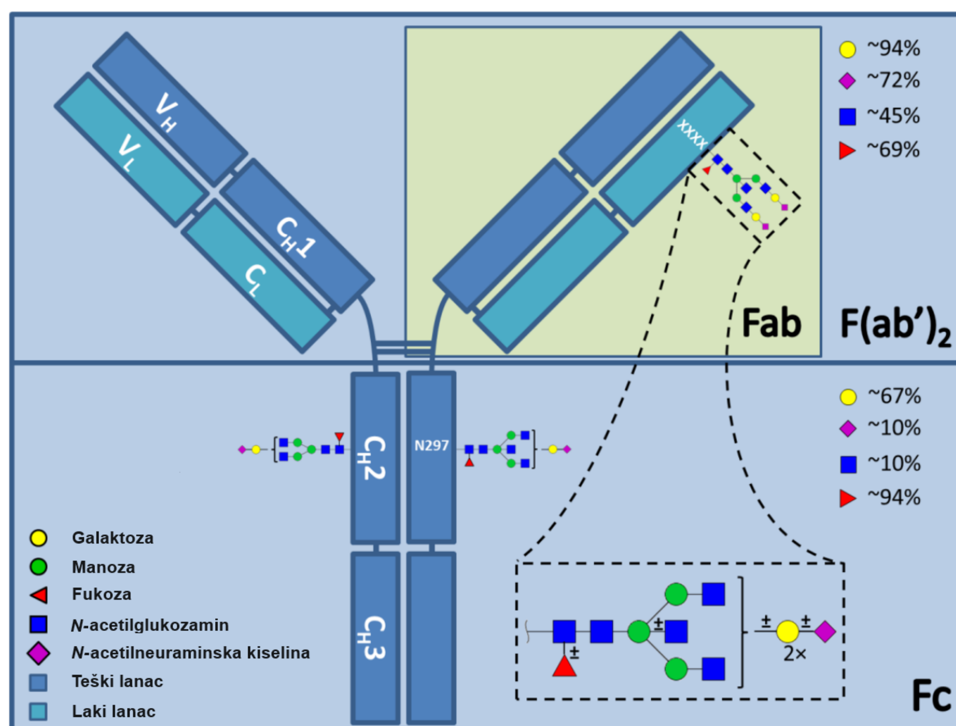
Biosinteza *N*-glikana (Slika 2) započinje sklapanjem lipidnog prekursora s citoplazmatske strane ER-a. Prebacivanjem *N*-acetilglukozamin-1-fosfata (GlcNAc-1-P) na lipidni nosač dolikol fosfat (Dol-P), nastaje dolikol pirofosfat *N*-acetilglukozamin (Dol-P-P-GlcNAc). Dodatkom monosaharidnih jedinica GlcNAc i Man nastaje prekursor Dol-P-P-$GlcNAc_2Man_5$ koji se potom prebacuje na luminalnu stranu ER-a uz pomoć enzima flipaze. Daljnjim dodavanjem jedinica glukoze (Glc) i Man tvori se zreli prekursor *N*-glikana Dol-P-P-$Glc_3Man_9GlcNAc_2$. Djelovanjem enzima oligosahariltransferaze (OST), zreli prekursor prebacuje se u cjelini (franc. *en bloc*) na polipeptid u nastajanju tijekom njegove translokacije u ER i to unutar aminokiselinskog slijeda Asn-X-Ser/Thr (X je bilo koja aminokiselina osim Pro). Djelovanjem enzima α-glukozidaza I i α-glukozidaza II, oligosaharidu se uklanjaju jedinice Glc, a α-manozidaza I uklanja jedinice Man. Ovaj korak važan je i za pravilno smatanje samog proteina te služi kao kontrola kvalitete proteina u nastajanju. Kada preostane jedna Glc na ugljikohidratnom prekursoru, on veže lektine kalneksin i kalretikulin koji promoviraju smatanje proteina. Nepravilno smotani proteini se ili reglukoziliraju i vraćaju u sustav kalneksina-kalretikulina ili se eliminiraju degradacijom u citoplazmi. Sporo uklanjanje jedinica Man na nepravilno smotanim glikoproteinima rezultira vezanjem lektina OS9 i usmjeravanjem glikoproteina u citoplazmu radi degradacije. Pravilno sintetizirani i smotani *N*-glikozilirani proteini dalje se procesiraju u *cis*-GA. Oligosaharid se skraćuje djelovanjem α1-2 manozidaza IA, IB i IC čime nastaju *N*-glikani oligomanoznog tipa, ali i ključni intermedijar u sintezi hibridnih i kompleksnih glikana, $Man_5GlcNAc_2$. Prelaskom u medijalni i *trans*-GA, djelovanjem odgovarajućih glikoziltransferaza koje dodaju šećere, grananjem te dekoracijom antena (poput dodatka sijalinskih kiselina, sulfatacije, *O*-acetilacije) nastaju *N*-glikani hibridnog i kompleksnog tipa, a nastali glikoproteini pakiraju se u transportne vezikule i putuju prema staničnoj membrani ili se izlučuju u izvanstanični prostor (41,42,44).



Slika 2. Biosinteza *N*-glikana. Biosinteza započinje u ER-u *en bloc* prebacivanjem ugljikohidratnog prekursora na polipeptidni lanac u nastajanju djelovanjem enzima oligosahariltranferaze (OST), unutar aminokiselinskog slijeda Asn-X-Ser/Thr. Jedinice Glc i Man se odstranjuju, a nakon zadovoljene kontrole kvalitete, *N*-glikozilirani glikoprotein putuje do GA radi daljnjeg procesiranja, tijekom kojeg nastaju oligomanozni, kompleksni i hibridni tipovi *N*-glikana. Naposlijetku se *N*-glikozilirani glikoproteini transportnim vezikulama distribuiraju na ciljna mjesta. Prikazan je i slučaj gdje receptori za manozu-6-fosfat (M-6-P) vežu kisele hidrolaze koje sadrže M-6-P marker i transportiraju ih iz GA u lizosome, a nakon čega se M-6-P receptor u kiselom okolišu lizosoma reciklira natrag u GA. Kratice: N – asparagin, S - serin, T - treonin, Gal – galaktoza, Glc – glukoza, Man – manoza, GlcNAc – *N*-acetilglukozamin, EDEM – engl. *ER-degradation-enhancing α-mannosidase I−like proteins*, CMP – citidin monofosfat, UDP – uridin difosfat. Preuzeto i prilagođeno iz Stanley i sur., 2019. (44) u skladu s uvjetima Creative Commons CC-BY-NC-ND licence.

1.1.3. N-glikozilacija serumskog IgG-a

U području glikobiologije, velika pozornost posvećuje se središnjoj molekuli humoralne imunosti, protutijelu IgG-u, glavnom oružju u obrani organizama od patogena. Molekula IgG-a je ujedno i najzastupljenije protutijelo u serumu čija prosječna koncentracija iznosi 6-15 g/L, manja je kod djece, a raste s povećanjem starosti osobe (45). Svaka molekula IgG-a sastoji se od dva teška i dva laka lanca međusobno povezanih disulfidnim mostovima. Zbog razlika u aminokiselinskom slijedu razlikujemo i četiri potklase, IgG1, koja je ujedno i najzastupljenija, IgG2, IgG3 i IgG4. Pritom u molekuli IgG-a razlikujemo dvije funkcionalne regije, Fc (engl. *fragment crystallisable*) i Fab (engl. *fragment antigen-binding*). Svaki teški lanac Fc regije IgG-a nosi diantenarni *N*-glikan vezan za visoko konzervirano glikozilacijsko mjesto, točnije aminokiselinski ostatak Asn297. Upravo taj glikan određuje funkciju molekule IgG-a tako što utječe na konformaciju Fc regije i modulira interakciju IgG-a i receptora na efektorskim stanicama (46,47). Nadalje, smatra se da je oko 20% IgG-a *N*-glikozilirano i u varijabilnom dijelu Fab regije, što ima utjecaj na interakciju protutijela sa specifičnim antigenima te s različitim lektinima (Slika 3) (48).



Slika 3. Pojednostavljeni prikaz molekule IgG-a. Diantenarni *N*-glikani vezani su za visoko konzervirano glikozilacijsko mjesto (Asn297) domene Fc i unutar varijabilne regije domene Fab (prisutno kod otprilike 20% poliklonalnog IgG-a). Prikazana je i zastupljenost monosaharida u strukturi *N*-glikana u pojedinoj domeni molekule IgG-a. Preuzeto i prilagođeno iz Bondt i sur., 2014. (49) u skladu s uvjetima Creative Commons CC-BY licence.

U konačnici, molekula IgG-a preko varijabilnog dijela Fab domene ima sposobnost prepoznavanja i neutralizacije enormnog broja ciljanih molekula odnosno antigena. Uništavanje transformiranih stanica ili bakterija posredovano je Fc regijom i to sljedećim mehanizmima: opsonizacijom, stanicama posredovanoj citotoksičnosti ovisnoj o protutijelima (ADCC, engl. *antibody-dependent cell-mediated cytotoxicity*), stanicama posredovanoj fagocitozi ovisnoj o protutijelima (ADCP, engl. *antibody-dependent cell-mediated phagocytosis*) te citotoksičnosti ovisnoj o komplementu (CDC, engl. *complement-dependent cytotoxicity*) (47). Upravo je efektorska funkcija IgG-a posredovana Fc regijom uvelike ovisna o strukturi *N*-glikana vezanog unutar same Fc regije. Većina glikana IgG-a prisutnog u serumu sadrži tzv. sržnu fukozu koja prevenira moguću pretjeranu aktivnost ADCC-a u homeostaznim uvjetima, na način da smanjuje afinitet IgG-a za aktivirajuće Fc receptore, FcγRIIIA i FcγRIIIB (50). Prisutnost *N*-glikana IgG-a bez galaktoze (agalaktozilirani IgG) često je opažena karakteristika starenja (50–52) i mnogih upalnih bolesti (50,53). Smatra se da agalaktozilirani IgG pridonosi upali putem povećane aktivacije alternativnog puta sustava komplementa. S druge strane, uloga glikana s terminalnom galaktozom je nešto kompleksnija. Postoje dokazi da IgG s terminalnom galaktozom ima povećani afinitet za inhibitorni FcγRIIB te da inhibira proupalnu funkciju sastavnice komplementa C5a, što dovodi do smanjenja upale (54). Tijekom biosinteze glikani s terminalnom galaktozom služe kao supstrat za sijaliltransferaze koje dodaju *N*-acetilneuraminsku (sijalinsku) kiselinu, što pridonosi protuupalnom učinku IgG-a, pa se može reći da su galaktozilirani glikani odgovorni i za protuupalni učinak. S druge strane, galaktozilacija glikana IgG-a može pospješiti heksamerizaciju IgG-a povećavajući afinitet prema C1q sastavnici komplementa i posljedično povećati aktivnost CDC-a, a time i potencirati upalu (55). Sijalinizacija glikana IgG-a odnosno prisutnost *N*-acetilneuraminske kiseline kod ljudi često se smatra prekidačem koji regulira efektorsku ulogu IgG-a i posljedičnu aktivaciju pro- odnosno protuupalnog odgovora. Glikani s terminalnim sijalinskim kiselinama dovode do smanjenog afiniteta takvog IgG-a za aktivirajuće FcγRIIIa što rezultira smanjenom aktivnosti ADCC-a (56), dok stimuliranjem ekspresije inhibitornih FcγRIIB, inhibiraju sustav komplementa (57). Račvajući GlcNAc na glikanima IgG-a smatra se proupalnom karakteristikom s obzirom na to da pridonosi povećanoj aktivnosti ADCC-a (58).

1.1.4. N-glikozilacija proteina u Crohnovoj bolest

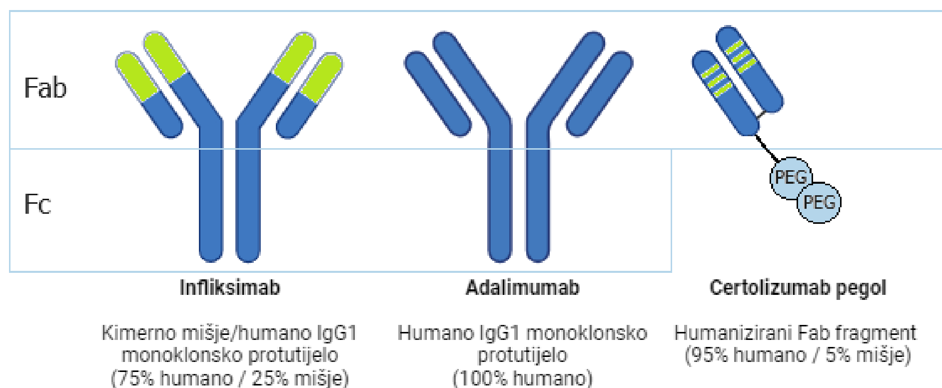
Cijelogenomske studije povezanosti pokazale su da postoji 16 gena koji reguliraju *N*-glikozilaciju IgG-a, a od tog broja njih pet (*BACH2, IL6ST, LAMB1, IKZF1 i MGAT3*) povezano je i s razvojem IBD-a (59). Nadalje, pokazano je da kod istih bolesnika postoji značajna korelacija između metilacije promotorske regije *MGAT3* gena i galaktozilacije, sijalinizacije i razine *N*-glikana IgG-a s račvajućim GlcNAc-om, što sugerira da je aktivnost produkta ovog gena, glikoziltransferaze GnT-III promijenjena te da epigenetičke modifikacije mogu imati značajan utjecaj na glikozilaciju IgG-a kod IBD-a (60).

Što se tiče promjena u *N*-glikozilaciji IgG-a u krvnoj plazmi/serumu bolesnika s CB-om, povišena razina *N*-glikana IgG-a bez galaktoze rano je prepoznata kao karakteristika CB-a (61), što je kasnije i potvrđeno drugim istraživanjima (62–64). Nadalje, utvrđeno je da bolesnici s CB-om u odnosu na zdrave pojedince imaju smanjenu razinu sijaliniziranih glikana serumskog IgG-a (63,64) i povećanu razinu glikana s račvajućim GlcNAc-om (64) te da je na temelju sastava *N*-glikana serumskog IgG-a moguće razlučiti bolesnike s CB-om od bolesnika s UK-om ili pak od zdravih pojedinaca (63). Sličan trend pokazan je i u novijoj studiji gdje bolesnici s kroničnim upalnim bolestima, među kojima su bili uključeni i bolesnici s CB-om, također pokazuju povećanu razinu *N*-glikana serumskog IgG-a bez terminalne galaktoze te smanjenu sijalinizaciju i galaktozilaciju u odnosu na kontrolnu skupinu (65). Promjene u glikozilaciji nisu ograničene samo na IgG, one su vidljive i u *N*-glikomu proteina plazme. Kod bolesnika s IBD-om, proteini plazme sadrže povećanu razinu složenih, razgranatih glikana i sijaliniziranih glikana u odnosu na zdrave pojedince, smanjenu razinu hibridnih i oligomanoznih glikana i smanjenu ukupnu fukozilaciju i galaktozilaciju. Nadalje, bolesnici s CB-om u odnosu na bolesnike s UK-om imaju povećanu razinu glikana s račvajućim GlcNAc-om, smanjenu galaktozilaciju te povećanu razinu glikana s α2-3 sijalinskom kiselinom (66). Istraživanja su također, upozorila na važnost mucina, visoko *O*- i *N*-glikoziliranih glikoproteina, koji tvore sloj sluzi u crijevima i podmazuju crijevni sadržaj, a kao izvor hrane izravno utječu na sastav i očuvanje zdrave crijevne mikrobiote (67). Glavni sekretorni mucin MUC2 u svojoj strukturi sadrži dva tipa *O*-glikana koji su od iznimne važnosti za očuvanje integriteta sloja sluzi te preveniraju razvoj kolitisa izazvanog mogućim kontaktom crijevne mikrobiote sa epitelnim stanicama crijeva (68). Kod bolesnika s UK-om vidljiva promjena u *O*-glikozilacijskom profilu MUC2 povezana je s upalom u crijevima, a ulaskom bolesnika u remisiju *O*-glikani poprimaju vrijednosti kao kod zdravih osoba (69).

1.1.5. Terapijski pristup i predviđanje odgovora na terapiju u Crohnovoj bolesti

Glavni cilj u liječenju CB-a je postizanje remisije i njezino održavanje. U tu se svrhu koriste lijekovi, suplementi, operacija ili njihova kombinacija radi kontrole upale, nadomjeska hranjivih tvari te suzbijanja ostalih simptoma bolesti (70). Lijekovi koji se koriste u svrhu liječenja CB-a mogu se podijeliti u pet skupina: kortikosteroidi (budezonid, prednizon), aminosalicilati (mesalazin), imunosupresivi (azatioprin/5-merkaptopurin, metotreksat), biološki lijekovi (monoklonska protutijela infliksimab, adalimumab, certolizumab, vedolizumab, ustekinumab) i antibiotici (metronidazol, ciprofloksacin) (71).

Terapija se određuje za pojedinca na temelju povijesti bolesti, lokalizacije i ozbiljnosti bolesti te ostalih komorbiditeta (72). Kortikosteroidi poput budezonida mogu se koristiti kod blažih oblika bolesti radi postizanja remisije te u kombinaciji s biološkim lijekovima ili imunomodulatorima radi održavanja remisije, no njihova dulja primjena nije opravdana zbog izraženih nuspojava poput razvoja infekcija ili osteopenije (72). Dugotrajna strategija kontrole CB-a uključuje lijekove koji djeluju na pretjerani imunosni odgovor, odnosno imunomodulatore te biološke lijekove. Imunomodulatori se prvenstveno koriste radi održavanja remisije i sprečavanja relapsa te se ne bi trebali koristiti u monoterapiji u svrhu induciranja remisije, već se u tom slučaju kombiniraju s kortikosteroidima (73). Kombinacija bioloških lijekova i imunomodulatora (primjerice infliksimab i azatioprin) pokazala se učinkovitijom u održavanju remisije u odnosu na monoterapiju istim lijekovima (72). Uporaba antibiotika poput metronidazola također se pokazala uspješnom zbog kontrole pretjeranog rasta bakterija u crijevima i lučenja antigena, no dugotrajna uporaba nije preporučljiva zbog izraženih nuspojava (74). Navedeni lijekovi predstavljaju tzv. konvencionalnu terapiju. S uvođenjem biološke terapije započeta je nova era u tretmanu CB-a (Slika 4), a odgovarajuća glikozilacija monoklonskih protutijela ključna je za njihovu učinkovitost i sigurnost primjene (75). Infliksimab je kimerno IgG1 monoklonsko protutijelo, antagonist citokina TNFα i prvi biološki lijek odobren za liječenje CB-a koji može učinkovito inducirati i održati remisiju, pospješiti zacjeljivanje sluznice crijeva te smanjiti potrebu za hospitalizacijom. U kombinaciji s imunomodulatorom učinkovitost mu se povećava (74). Zbog činjenice da se radi o lijeku čija je struktura dijelom ljudskog, a dijelom mišjeg porijekla, veća je vjerojatnost razvoja protutijela na takav lijek, a time i njegove smanjenje učinkovitosti te nastanka nuspojava. Iz toga razloga razvijeno je i potpuno humano IgG1 monoklonsko protutijelo adalimumab, za koje se smatra da je rizik od nastanka protutijela na lijek odnosno, imunogenost niža (74).



Slika 4. Pojednostavljeni prikaz strukture antagonista TNF-a u terapiji Crohnove bolesti. Fc regija odgovorna je za efektorsku funkciju monoklonskog protutijela, dok je Fab regija odgovorna za specifičnost i afinitet prema antigenu. Plavi dijelovi molekule prikazuju proteinski dio humanog podrijetla, zeleni dijelovi mišjeg podrijetla, a koji ujedno predstavlja i moguće imunogene regije. Izrađeno pomoću alata BioRender.com prema Chang i Lichtenstien, 2006. (76).

Mehanizam djelovanja ovih bioloških lijekova prvenstveno se sastoji od neutralizacije transmembranskog i slobodnog TNF-a koji je posredovan Fab domenom monoklonskog protutijela. Nadalje, ovi lijekovi uništavaju stanice koje proizvode TNF mehanizmima CDC i ADCC, a koji su pak posredovani Fc domenom. Stoga je djelotvornost ovih lijekova, ali i moguća imunogenost uzrokovana prisutnošću *N*-glikolilneuraminske kiseline (77) , ovisna i o njihovom *N*-glikozilacijskom profilu (78,79). Kao anti-TNF monoklonsko protutijelo dostupan je i certolizumab pegol koji dolazi u obliku dijela molekule IgG-a, odnosno humanizirane Fab domene uz dodatak polietilen glikola radi produljenja poluvremena života, a koristi se uglavnom kod bolesnika koji su izgubili odgovor na terapiju infliksimabom ili adalimumabom ili su pak razvili preosjetljivost na njih (70). Zbog nedostatka Fc regije smatra se manje učinkovitim u liječenju CB-a u odnosu na druge anti-TNF lijekove (80), iako je primjerice u reumatoidnom artritisu pokazano da bi upravo zbog nedostatka Fc regije takve molekule lijeka mogle biti učinkovitije kod bolesnika s visokom titrom reumatoidnog faktora (81). Stoga, navedeni lijekovi neutralizacijom aktivnosti slobodnog TNF-a sprječavaju njegovo vezivanje na TNFα receptor 1 (TNFR1) koji je eksprimiran na gotovo svim tipovima stanica i odgovoran za proupalni i citotoksični učinak te TNFR2 koji je eksprimiran prvenstveno na monocitima, T stanicama i mezenhimskim stanicama, a patogenu ulogu ispoljava u sinovijalnim fibroblastima važnima u razvoju reumatoidnog artritisa (82). Vezanjem transmembranskog TNF-a anti-TNF lijekovi utječu na unutarstanično signaliziranje i programiranu staničnu smrt, što može rezultirati smanjenom produkcijom citokina ili zaustavljanjem staničnog rasta (83). Zaključno,

anti-TNF lijekovi svoj terapijski učinak ispoljavaju smanjenjem ekspresije proupalnih citokina, kemokina, proteina akutne faze i adhezijskih molekula, povećanjem broja cirkulirajućih limfocita T te smanjenjem migracije upalnih stanica iz krvi u upaljeno tkivo (82).

Od bioloških lijekova za liječenje CB-a na tržištu dostupni su još i humanizirano IgG1 monoklonsko protutijelo vedolizumab kao antagonist α4β7 integrina i humano monoklonsko IgG1 protutijelo ustekinumab usmjereno protiv p40 podjedinice IL12 i IL23. Oni se koriste uglavnom u slučaju srednjih do teških oblika bolesti kada bolesnik ne odgovara na terapiju ili pokazuje intoleranciju na kortikosteroide, imunosupresive ili anti-TNF terapiju, iako njihovu sigurnost tijekom dugotrajne uporabe tek treba utvrditi (71).

Ipak, u periodu od 8. do 12. tjedna od početka anti-TNF terapije oko jedne trećine liječenih bolesnika s IBD-om ne odgovara zadovoljavajuće na primijenjenu terapiju (PNR, engl. *primary non-response*), a nakon godine dana 30-50% bolesnika koji su prvotno odgovarali na terapiju, gubi odgovor na istu (LOR, engl. *loss of response*) (84). Jedan od razloga je imunogenost kada se zbog stvaranja protutijela na lijek nakon izlaganja anti-TNF lijekovima tvore imunokompleksi koji rezultiraju povećanim klirensom lijeka (39). Uz debljinu, pušenje, nisku koncentraciju albumina i povišenih markera aktivnosti bolesti pri baznom mjerenju, pojava imunogenosti utječe i na nisku koncentraciju lijeka u 14. tjednu terapije koja se pak povezuje s PNR-om (85). Ova se pojava može smanjiti istovremenom upotrebom drugih lijekova poput azatioprina i metotreksata te povećanjem doze anti-TNF lijekova tijekom faze indukcije kod osoba s visokim rizikom za razvoj PNR-a (39,85). Iz navedenih razloga postoji stalna potreba za pronalaskom kliničkih biomarkera koji bi omogućili identifikaciju bolesnika koji ne bi primjereno odgovorili na anti-TNF terapiju, što bi omogućilo racionalniju upotrebu ovih skupih lijekova, brži odgovor na terapiju, smanjenje nuspojava i općenito bolju suradnju između bolesnika i liječnika i bolju kvalitetu života bolesnika. Dosad je identificirano više biljega čijom bi se uporabom mogli probrati bolesnike s povećanim rizikom od razvoja PNR-a poput dobi i spola osobe, duljine trajanja bolesti i lokalizacije u gastrointestinalnom sustavu, fenotipa bolesti, prisutnosti drugih komorbiditeta, razine C-reaktivnog proteina (CRP), genskih polimorfizama gena *NOD2, IBD5, HLA-DQA1*05* i *FCGR3A,* razine TNF-a u serumu i sluznici crijeva, ali do danas niti jedan nije ušao u kliničku praksu (84). Pereira i sur. pokazali su da je na temelju razine razgranatih glikana u bioptatu debelog crijeva moguće predvidjeti odgovor na konvencionalnu terapiju kod bolesnika s UK-om i koji ranije nisu primali terapiju te da je na taj način, u periodu oko dijagnoze bolesti, moguće stratificirati bolesnike koji bi primjereno odgovorili na konvencionalnu terapiju od onih koji bi više profitirali primjenom bioloških lijekova (86). Na temelju sastava *N*-glikoma proteina seruma bolesnika s IBD-om moguće je

predvidjeti potrebu za eskalacijom terapije, odnosno prelazak s lijekova prve linije na terapiju biološkim lijekovima ili na operaciju i to na temelju sedam deriviranih glikanskih svojstava, u prvom redu fukozilacije tetraantenarnih glikana i diantenarnih glikana s račvajućim GlcNAc-om te galaktozilacije i sijalinizacije diantenarnih glikana (87). Zanimljivo je da je kod bolesnika s uznapredovanim stadijem melanoma prije početka terapije inhibitorima kontrolnih točaka imunosnog sustava, *N*-glikom proteina seruma koji sadrži veći udio manje razgranatih glikana i manji stupanj antenarne fukozilacije povezan s povoljnim odgovorom na istu terapiju i produljenim preživljenjem (88).

1.2. OBRAZLOŽENJE TEME

Točna etiologija CB-a nije u potpunosti razjašnjena, no poznato je da se radi o multifaktornoj upalnoj bolesti crijeva koja je okarakterizirana diskontinuiranom i transmuralnom upalom stijenke gastrointestinalnog sustava, koja nastaje prvenstveno kao posljedica poremećenog imunosnog odgovora u probavnom sustavu osoba s genetičkom predispozicijom za razvoj bolesti, pod utjecajem određenih okolišnih čimbenika. Sve opsežnija istraživanja u području glikomike, odnosno glikozilacije proteina i lipida određene stanice, tkiva ili organizma, stoje uz bok istraživanjima iz područja genomike i proteomike (89) te produbljuju znanje o ulozi glikana u patofiziologiji IBD-a općenito (90,91). Dosadašnja istraživanja na primjeru IgG-a pokazala su da postoje značajne razlike u zastupljenosti određenih *N*-glikana kod bolesnika s CB-a u odnosu na bolesnike s UK-om i u odnosu na zdrave pojedince, koje mogu odražavati i aktivnost same bolesti. Danas se u liječenju CB-a koriste različite skupine lijekova, a jedna od njih su i biološki lijekovi poput anti-TNF lijekova. Premda se radi o učinkovitim lijekovima čije je uvođenje u praksu iz korijena promijenilo pristup u liječenju CB-a, izuzetno su skupi i pritom značajan broj bolesnika ne odgovara zadovoljavajuće na terapiju unutar 8 - 12 tjedana (PNR), gubi odgovor na istu nakon godine dana ili osjeća nuspojave. Stoga je rana identifikacija bolesnika koji neće odgovoriti na terapiju anti-TNF lijekovima imperativ u razvoju isplative personalizirane skrbi za bolesnike s ciljem povećanja učinkovitosti primijenjene terapije i smanjenja nuspojava.

Upravo su dosad otkrivene promjene u *N*-glikozilaciji serumskog IgG-a te mogući dijagnostički i prognostički potencijal *N*-glikana serumskog IgG-a u IBD-u i drugim bolestima bile poticaj za izradu ove doktorske disertacije koja ima postavljena dva cilja: 1) utvrditi je li moguće na temelju razlika u *N*-glikanskom profilu serumskog IgG-a bolesnika s CB-om

predvidjeti odgovor na terapiju anti-TNF lijekovima prije njezinog početka te 2) utvrditi mijenja li se zastupljenost *N*-glikanskih struktura serumskog IgG-a bolesnika s CB-om koji su tijekom 14 tjedana primali anti-TNF terapiju te ispitati je li odgovor na terapiju povezan s tipom promjene.

Na temelju zadanih ciljeva postavljene su dvije hipoteze: 1) na temelju razlika u sastavu *N*-glikoma serumskog IgG-a bolesnika s CB-om moguće je predvidjeti odgovor na terapiju anti-TNF lijekovima prije njezinog početka, te 2) anti-TNF lijekovi infliksimab i adalimumab mijenjaju sastav *N*-glikoma serumskog IgG-a bolesnika s aktivnim luminalnim CB-om.

Stoga, očekivani znanstveni doprinos ovog istraživanja uključuje unaprjeđenje saznanja o *N*-glikozilaciji serumskog IgG-a u CB-u prije djelovanja anti-TNF lijekova, moguće pružanje novog alata u predviđanju odgovora na anti-TNF terapiju prije njezinog započinjanja te definiciju odnosa između primjene anti-TNF terapije i longitudinalne promjene *N*-glikozilacije serumskog IgG-a u CB-u.

2. ISPITANICI, MATERIJALI I METODOLOGIJA ISTRAŽIVANJA

2.1. Ispitanici

Ispitanici ovog istraživanja ranije su regrutirani u sklopu studije PANTS provedene u Ujedinjenom Kraljevstvu. Radi se o četverogodišnjoj prospektivnoj nekontroliranoj opservacijskoj kohortnoj studiji čija je misija razvoj personalizirane terapije za bolesnike s CB-om te ispitivanje bioloških mehanizama PNR-a, LOR-a ili nastanka neželjenih reakcija na anti-TNF lijekove, točnije infliksimab i adalimumab.

Bolesnici su pozvani na sudjelovanje u studiji nakon što je odluka o započinjanju anti-TNF terapije infliksimabom ili adalimumabom donesena od strane njihovog gastroenterologa. S obzirom na to da se radi o opservacijskoj studiji, odluka o odabiru anti-TNF lijeka za pojedinog bolesnika bila je na timu kliničara. Kako bi bili uključeni u studiju, bolesnici su morali zadovoljiti nekoliko kriterija uključenja: biti stariji od 6 godina, imati aktivni luminalni CB koji uključuje tanko i/ili debelo crijevo (Montrealska klasifikacija L1, L2 ili L3), a da pritom primarna indikacija za anti-TNF terapiju nije fistulizirajući oblik bolesti, zatim imati povišenu razinu CRP-a ili fekalni kalprotektin kao dokaz aktivnosti upalne bolesti, biti bez povijesti uzimanja anti-TNF terapije te potpisati informirani pristanak (bolesnik ili roditelj/skrbnik). S druge strane, bolesnici koji nisu bili spremni sudjelovati u studiji ili nisu potpisali informirani pristanak, koji su imali kontraindikacije za liječenje anti-TNF lijekovima, čije su razine CRP-a i fekalni kalprotektin bile unutar referentnih vrijednosti tijekom probira ili za koje se smatralo da nisu prikladni za sudjelovanje u studiji, isključeni su iz studije.

S ciljem utvrđivanja mogućnosti predviđanja terapijskog odgovora na temelju profila *N*-glikana serumskog IgG-a prije započinjanja anti-TNF terapije, analizirali su se uzorci krvnog seruma prikupljeni od ukupno 1315 ispitanika regrutiranih u sklopu studije PANTS (T0, prva vremenska točka). Na temelju prikupljenih kliničkih podataka ispitani su broj muških i ženskih ispitanika, dobni raspon, vrijeme trajanja bolesti od dijagnoze do prve doze lijeka, broj ispitanika koji prima pojedinu anti-TNF terapiju (infliksimab ili adalimumab), udio ispitanika kod kojih je uočen izostanak odgovora na terapiju te dodatni klinički podaci poput indeksa tjelesne mase, lokacije bolesti i slično. Izostanak odgovora ispitanika na terapiju, odnosno PNR, utvrđen je u 14. tjednu i definiran je na sljedeći način: procjena liječnika sugerirala je izostanak odgovora na terapiju, Harvey-Bradshaw indeks nije bio manji za tri ili više bodova u odnosu na nulti tjedan, a razine CRP-a i fekalnog kalprotektina nisu se našle unutar referentnog raspona (ili se spustili za 50% u odnosu na nulti tjedan).

Kod dijela ispitanika, točnije njih 198, dodatno je izuzet krvni serum u 14. tjednu terapije (T1, druga vremenska točka), neposredno prije sljedeće zakazane doze. Zajedno s

pripadajućim uzorcima iz T0, ovi uzorci analizirani su u svrhu utvrđivanja promjene u zastupljenosti *N*-glikana serumskog IgG-a bolesnika s CB-om koji su tijekom 14 tjedana primali anti-TNF terapiju te je utvrđeno je li promjena ovisna o odgovoru na terapiju. Na temelju prikupljenih kliničkih podataka za ovu skupinu analizirani su udio muških i ženskih ispitanika, dobni raspon, udio ispitanika koji prima pojedinu anti-TNF terapiju, te udio ispitanika kod kojih je uočen izostanak odgovora na terapiju.

Studija je registrirana u registru ClinicalTrials.gov (broj NCT03088449) te posjeduje odgovarajuće etičko odobrenje (NHS REC 12/SW/0323), a istraživanje za potrebe izrade doktorskog rada odobreno je od strane Povjerenstva za etičnost eksperimentalnog rada Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu (broj odobrenja 643-02/19-01/02, dana 17. travnja 2019. godine).

2.2. Materijali

2.2.1. Kemikalije

2-aminobenzamid (2-AB) (Sigma-Aldrich), 2-amino-2-hidroksimetil-1,3-propandiol (TRIS) (Sigma-Aldrich), 2-pikolin boran (2-PB) (Sigma-Aldrich), acetonitril (ACN) (J. T. Baker), amonijev hidrogenkarbonat (NH_4HCO_3) (Sigma-Aldrich), dimetil-sulfoksid (DMSO) (Sigma-Aldrich), etanol (Carlo Erba), Igepal CA-630 (Sigma-Aldrich), kalijev dihidrogenfosfat (KH_2PO_4) (Sigma-Aldrich), kalijev klorid (KCl) (Gram-Mol), kloridna kiselina (HCl) (Kemika), mravlja kiselina (HCOOH) (Merck), natrijev dodecil sulfat (SDS) (Sigma-Aldrich), natrijev hidrogenfosfat (Na_2HPO_4) (Acros Organics), natrijev hidrogenkarbonat ($NaHCO_3$) (Merck), natrijev klorid (NaCl) (Gram-Mol), octena kiselina (CH_3COOH) (Merck), otopina amonijaka (Merck), ultra čista voda (18 MΩ cm pri 25 °C).

2.2.2. Enzimi

N-glikozidaza F (PNGaza F, Promega) – 10 U μL^{-1} (1 U - engl. *unit*, količina PNGaze F koja katalizira oslobađanje *N*-vezanih oligosaharida s 1 μmol denaturirane ribonukleaze B u jednoj minuti pri 37 °C).

2.2.3. Kromatografske kolone, pločice za afinitetnu izolaciju IgG-a

Kromatografska kolona ACQUITY UPLC Glycan BEH Amide dimenzija 2.1 mm × 100 mm, promjer čestica 1,7 μm (Waters); pločica monolita s 96 jažica CIM® r-Protein G LLD 0.2 mL, veličine pora 2 μm (Sartorius BIA Separations).

2.2.4. Ostali laboratorijski potrošni materijal

0,20 μm GHP AcroPrep filtar pločica (Pall Corporation), 0,45 μm GHP AcroPrep filtar pločica (Pall Corporation), pločica za sakupljanje uzoraka volumena 1 mL (Waters), pločica za sakupljanje uzoraka volumena 2 mL (Waters), PCR pločica (Thermo-Fisher Scientific), tubice različitih volumena (VWR International), polipropilenske vijale s čepovima (Chrom4).

2.2.5. Instrumenti

Analitička vaga (EX124/AD, Ohaus Explorer), tehnička vaga (Mettler Toledo), magnetska miješalica (RCT Basic, IKA), pipete i mikropipete (Rainin), Savant SpeedVac centrifuga za ukoncentriravanje (Thermo Fischer-Scientific), pH metar (Mettler Toledo), inkubator (DNI30, MRC), pećnica (DNO30, MRC), tresilica (GFL), vakumska pumpa (Pall Corporation), centrifuga (5804, Eppendorf), UV-VIS spektrofotometar NanoDrop 8000 (Thermo-Fisher Scientific), tekućinski kromatograf Acquity UPLC H-class s detektorom fluorescencije (Waters).

2.3. Metode

Korištene metode pripreme i analize *N*-glikana serumskog IgG-a ranije su opisane (92–94) te uključuju izolaciju IgG-a iz krvnog seruma, oslobađanje *N*-glikana IgG-a enzimskom hidrolizom pomoću enzima PNGaze F, njihovo fluorescentno obilježavanje i pročišćavanje, razdvajanje tekućinskom kromatografijom ultra-visoke djelotvornosti baziranoj na hidrofilnim interakcijama te detekciju obilježenih glikana fluorescentnim detektorom (HILIC-UHPLC-FLD, engl. *hydrophilic interaction ultra-high performance liquid chromathography with fluorescence detection*).

2.3.1. Izolacija IgG-a iz krvnog seruma

Izolacija serumskog IgG-a provedena je uz pomoć pločice monolita s vezanim proteinom G koji specifično veže Fc regiju IgG-a te ga na taj način izdvaja od ostatka složenog matriksa seruma. Sam monolit kao stacionarni sustav isprepletenih kanala i pora, pospješuje vezivanje Fc regije IgG-a i smanjuje nespecifične interakcije. Izolacija IgG-a provedena je na uređaju za filtriranje uz pomoć vakuuma (engl. *vacuum manifold*).

Sama pločica monolita najprije je prekondicionirana dodatkom 2 mL ultra čiste vode, zatim dodatkom 1 mL 0.1 M mravlje kiseline (pH 2,5), 2 mL 10×PBS (0,37 M NaCl; 27 mM Na_2HPO_4; 97 mM KH_2PO_4; 22 mM KCl; pH 6,8) i 4 mL 1×PBS (37 mM NaCl; 2,7 mM Na_2HPO_4; 9,7 mM KH_2PO_4; 2,2 mM KCl; titrirano s 1 M NaOH do pH 7,4), uz filtriranje vakuumom od 17 mmHg.

Za izolaciju IgG-a upotrijebljen je volumen od 100 µL krvnog seruma po uzorku. Uzroci su najprije razrijeđeni dodatkom 700 µL 1×PBS te filtrirani kroz AcroPrep GHP filtar pločice veličine pora 0,45 µL kako bi se odstranio višak lipida i ostalih nečistoća iz seruma, a koji bi kasnije mogli dovesti to začepljenja pločice monolita. Razrijeđeni i filtrirani uzorci potom su nanoseni na pločicu monolita s vezanim proteinom G, a monolit je ispran tri puta dodatkom 2 mL 1×PBS kako bi se uklonili preostali nevezani proteini seruma. Pročišćeni IgG eluiran je dodatkom 1 mL 0.1 M mravlje kiseline (pH 2,5) uz pomoć vakuuma (10 mmHg) te brzo neutraliziran dodatkom 170 µL 1 M amonijevog hidrogenkarbonata, kako bi se spriječio mogući gubitak labilnih sijalinskih kiselina. Pločica je potom ispirana dodatkom 1 mL 1 M mravlje kiseline, 2 mL 10×PBS i 4 mL 1×PBS uz pomoć vakuuma (17 mmHg), a nakon ispiranja i dodatka 1 mL pufera za skladištenje po jažici (20% etanol, 20 mM TRIS, 0.1 M NaCl; pH 7,4) pospremljena u hladnjak. U svakom eluatu izmjerena je koncentracija izoliranog IgG-a uz pomoć UV-VIS spektrofotometra (Nanodrop 8000) na način da se mjeri apsorbancija eluata pri 280 nm. Potom je od svakog eluata odvojeno 300 µL, osušeno u centrifugi za ukoncentriravanje i pospremljeno na -20°C do daljnje metode pripreme oslobođenih *N*-glikana.

2.3.2. Oslobađanje N-glikana serumskog IgG-a

Osušeni uzorci IgG-a resuspendirani su dodatkom 30 µL 1,33% *(w/v)* SDS te suinkubirani 10 minuta na 65°C kako bi se IgG denaturirao i tako pospješio pristup enzima glikozilacijskim mjestima u kasnijem koraku. Nakon inkubacije, uzorci su ohlađeni na tresilici tijekom 30 minuta, a potom je dodano 10 µL 4% (*v/v*) detergenta Igepal i ostavljeno na tresilici

narednih 15 minuta. Oslobađanje *N*-glikana IgG-a provedeno je se dodatkom 1,2 U enzima PNGaze F u 10 mL 5x PBS-a po uzorku uz inkubaciju na 37 °C tijekom 18 sati.

2.3.3. Obilježavanje oslobođenih N-glikana fluorescentnom bojom

Zbog nepostojanja kromofora ili fluorofora u molekuli glikana, oslobođene *N*-glikane IgG-a potrebno je fluorescentno obilježiti kako bi se mogli detektirati tijekom analize tekućinskom kromatografijom, a fluorescentna boja od izbora bila je 2-AB. Smjesa za obilježavanje svježe je pripremljena otapanjem boje 2-AB (konačna masena koncentracija 19,2 mg mL^{-1}) i reducensa 2-PB (konačna masena koncentracija 44,8 mg mL^{-1}) u smjesi 30% octene kiseline u DMSO (*v/v*). Svakom uzorku oslobođenih *N*-glikana IgG-a dodano je 25 µL pripremljene smjese te su nakon miješanja na tresilici 10 minuta glikani fluorescentno obilježeni inkubacijom s bojom i reducensom tijekom dva sata na 65°C .

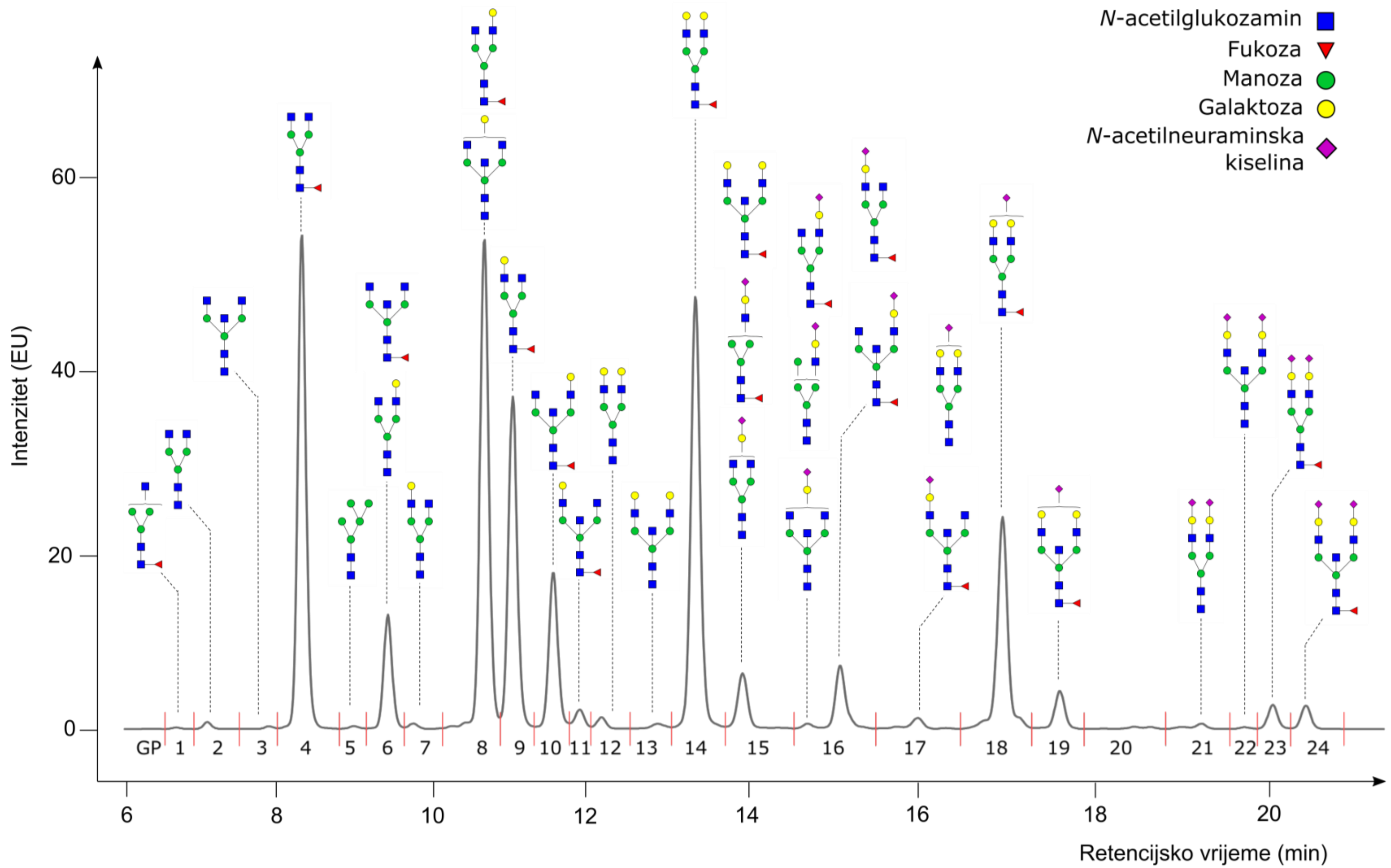
2.3.4. Pročišćavanje obilježenih N-glikana

Nakon obilježavanja fluorescentno bojom, glikane je potrebno očistiti od viška boje, reducensa, soli, proteinskih ostataka i ostalih reagensa. Pročišćavanje je provedeno na hidrofilnoj AcroPrep GHP filtar pločici veličine pora 0,20 µm koja u organskom mediju veže hidrofilne glikane, a otpušta ih dodatkom vode. Filtar pločica je najprije prekondicionirana dodatkom 200 µL svježe pripremljenog 70% etanola (*v/v*), 200 µL ultra čiste vode i 200 µL hladnog 96% ACN-a (*v/v*), a nakon svakog koraka otapala su propuštena uz pomoć vakuuma (2 mmHg). Nakon obilježavanja i hlađenja reakcijske smjese iz prethodnog koraka na sobnoj temperaturi tijekom 30 minuta, svakom uzorku obilježenih glikana dodano je 700 µL hladnog ACN-a nakon čega su uzorci premješteni na GHP filtar pločicu i inkubirani dvije minute. Suvišak reagensa uklonjen je ispiranjem hladnim 96% ACN-om pet puta, a zadnje ispiranje provedeno je centrifugiranjem pet minuta pri 165×*g*. Elucija pročišćenih i fluorescentno obilježenih *N*-glikana serumskog IgG-a obavljena je dodatkom 90 µL ultra čiste vode u dva navrata uz miješanje na tresilici tijekom 15 minuta i centrifugiranjem pet minuta pri 165×*g*. Ukupni volumen eluata u konačnici iznosio je 180 µL te je pohranjen na -20°C do analize tekućinskom kromatografijom.

2.3.5. HILIC-UHPLC-FLD analiza N-glikana serumskog IgG-a

Fluorescentno obilježeni i pročišćeni *N*-glikani serumskog IgG-a odvojeni su i analizirani metodom HILIC-UHPLC-FLD na instrumentu Acquity UPLC H-class koji se sastoji od modula za upravljanje otapalima (engl. *quartenary solvent manager*), modula za upravljanje uzorcima (engl. *sample manager*), detektora fluorescencije (FLD, engl. *fluorescence detector*) te kromatografske kolone.

Za upravljanje sustavom i analizu dobivenih kromatograma korišten je program *Empower 3* verzije 3471 (Waters). Biološki uzorci pripremljeni su za analizu instrumentom na način da je uzorak pomiješan s ACN-om u omjeru 20:80, a volumen injekcije (V_{inj}) na kolonu iznosio je 40 µL. Dekstran i standardni uzorak *N*-glikana IgG-a pripremljen je na isti način. Svaki set uzoraka je analiziran na način da je najprije injektirana ultra čista voda (V_{inj} = 10 µL) kako bi se ispitala stabilnost kromatografskog sustava, zatim dekstran (V_{inj} = 10 µL) radi vanjske kalibracije sustava, standardni uzorak *N*-glikana IgG (V_{inj} = 40 µL) radi ispitivanja i kontrole razdvajanja kromatografskih vršaka na kromatografskoj koloni, te do najviše 16 bioloških uzoraka nakon čega se redoslijed injektiranja ponovno ponavlja. Pokretnu fazu činio je 100 mM amonijev formijat (pH 4,4) kao otapalo A i ACN kao otapalo B. Primijenjen je linearni gradijent od 25% do 38% volumnog udjela otapala A pri protoku od 0,4 mL min^{-1} kroz 28 minuta. Zatim se udio otapala A podiže na 100% kako bi se tijekom dvije minute u potpunosti isprala kolona, da bi se potom sastav otapala vratio na početne uvjete odnosno 25% otapala A i 75% otapala B, radi uravnoteženja kolone prije injekcije sljedećeg uzorka. Svi uzorci držani su na temperaturi od 10°C prije injektiranja, a samo razdvajanje na koloni odvijalo se pri temperaturi od 60 °C. Fluorescentno obilježeni glikani detektirani su pri valnoj duljini pobude od 250 nm i valnoj duljini emisije od 428 nm. Sustav je kalibriran vanjskim standardom kojeg čine hidrolizirani i fluorescentno 2-AB obilježeni oligomeri glukoze različitog broja gradivnih elemenata (dekstran) prema kojem su vremena zadržavanja pojedinih glikana pretvorena u GU jedinice (engl. *glucose units*). Dobiveni kromatogrami integrirani su na isti način u 24 glikanska vrška (GP, engl. *glycan peak*), od GP1 do GP24 (Slika 5), a sastav pojedinog glikanskog vrška već je ranije utvrđen i prikazan u Tablici 1 (92).



Slika 5. Reprezentativni kromatogram *N*-glikana IgG-a razdvojen u 24 kromatografska vrška (od GP1 do GP24) metodom HILIC-UHPLC-FLD. Kratice: EU – jedinice emisije. Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).

Tablica 1. Sastav glikanskih vršaka u kromatogramu *N*-glikana serumskog IgG-a (GP1-GP24).

HILIC-UHPLC-FLD glikanski vršak	Notacija*	Opis strukture glikana
GP1	FA1	sržno fukoziliran s jednom granom (monoantenar)
GP2	A2	agalaktoziliran s dvije grane (diantenar)
GP3	A2B	diantenar s račvajućim GlcNAc-om
GP4	FA2	sržno fukoziliran diantenar
GP5	M5	glikan s pet manoznih ostatka
GP6	FA2B; A2[6]G1	sržno fukoziliran diantenar s račvajućim GlcNAc-om; monogalaktoziliran diantenar
GP7	A2[3]G1	monogalaktoziliran diantenar
GP8	A2BG1; FA2[6]G1	monogalaktoziliran diantenar s račvajućim GlcNAc-om; sržno fukoziliran, monogalaktoziliran diantenar
GP9	FA2[3]G1	sržno fukoziliran, monogalaktoziliran diantenar
GP10	FA2[6]BG1	sržno fukoziliran, monogalaktoziliran diantenar s račvajućim GlcNAc-om
GP11	FA2[3]BG1	sržno fukoziliran, monogalaktoziliran diantenar s račvajućim GlcNAc-om
GP12	A2G2	digalaktoziliran diantenar
GP13	A2BG2	digalaktoziliran diantenar s račvajućim GlcNAc-om
GP14	FA2G2	sržno fukoziliran, digalaktoziliran diantenar
GP15	A2G1S1; FA1G1S1; FA2BG2	monogalaktoziliran i monosijaliniziran diantenar; sržno fukoziliran, monogalaktoziliran i monosijaliniziran monoantenar; sržno fukoziliran, digalaktoziliran diantenar s račvajućim GlcNAc-om
GP16	A2BG1S1; FA2[6]G1S1; M1A1G1S1; FA2[6]BG1S1; FA2[3]G1S1	monogalaktoziliran i monosijaliniziran diantenar s račvajućim GlcNAc-om; sržno fukoziliran, monogalaktoziliran i monosijaliniziran diantenar; monomanoziliran, monogalaktoziliran i monosijaliniziran diantenar; sržno fukoziliran, monogalaktoziliran i monosijaliniziran diantenar s račvajućim GlcNAc-om; sržno fukoziliran, monogalaktoziliran i monosijaliniziran diantenar
GP17	FA2[3]BG1S1; A2G2S1	sržno fukoziliran, monogalaktoziliran i monosijaliniziran diantenar s račvajućim GlcNAc-om; digalaktoziliran i monosijaliniziran diantenar

GP18	A2BG2S1; FA2G2S1	digalaktoziliran i monosijaliniziran diantenar s račvajućim GlcNAc-om; sržno fukoziliran, digalaktoziliran i monosijaliniziran diantenar
GP19	FA2BG2S1	sržno fukoziliran, digalaktoziliran i monosijaliniziran diantenar s račvajućim GlcNAc-om
GP20	struktura nije određena	
GP21	A2G2S2	digalaktoziliran i disijaliniziran diantenar
GP22	A2BG2S2	digalaktoziliran i disijaliniziran diantenar s račvajućim GlcNAc-om
GP23	FA2G2S2	sržno fukoziliran, digalaktoziliran i disijaliniziran diantenar
GP24	FA2BG2S2	sržno fukoziliran, digalaktoziliran i disijaliniziran diantenar s račvajućim GlcNAc-om

*Kratice: F – sržna fukoza vezana α(1-6) vezom; Mx - broj manoza; Ax - broj antena/grana; B - račvajući *N*-acetilglukozamin (GlcNAc); Gx - broj galaktoza; Sx - broj sijalinskih kiselina; [3] i [6] - oznaka antene/grane.

2.3.6. Izračun izravnih i deriviranih svojstava N-glikozilacije serumskog IgG-a i statistička analiza podataka

Kako bi sirovi glikanski podaci postali usporedivi, provedena je normalizacija na ukupnu integriranu površinu kromatograma (engl. *total area normalization*), odnosno površina ispod svakog od 24 kromatografskog vrška podijeljena je s ukupnom integriranom površinom pripadajućeg kromatograma te je udio pojedine glikanske strukture izražen kao postotak (%) od ukupne integrirane površine kromatograma. Takve podatke nazivamo izravno mjerenim svojstvima *N*-glikozilacije IgG-a, a koja su korištena i za izračun šest deriviranih svojstava za *N*-glikane IgG-a koji dijele slične strukturne značajke (Tablica 2).

Tablica 2. Izračun deriviranih svojstava *N*-glikozilacije serumskog IgG-a.

Derivirano svojstvo	Opis	Formula za izračun (izraženo u %)
G0	Agalaktozilacija (udio glikana bez galaktoze u ukupnim glikanima IgG-a)	GP1 + GP2 + GP3 + GP4 + GP6
G1	Monogalaktozilacija (udio glikana s jednom galaktozom u ukupnim glikanima IgG-a)	GP7 + GP8 + GP9 + GP10 + GP11

G2	Digalaktozilacija (udio glikana s dvije galaktoze u ukupnim glikanima IgG-a)	GP12 + GP13 + GP14 + GP15
S	Sijalinizacija (udio sijaliniziranih glikana u ukupnim glikanima IgG-a)	GP16 + GP17 + GP18 + GP19 + GP21 + + GP22 + GP23 + GP24
B	Udio glikana s račvajućim GlcNAc-om u ukupnim glikanima IgG-a	GP3 + GP6 + GP10 + GP11 + GP13 + GP15 + + GP19 + GP22 + GP24
F	Fukozilacija (udio glikana sa sržnom fukozom u ukupnim glikanima IgG-a)	GP1 + GP4 + GP6 + GP8 + GP9 + GP10 + + GP11 + GP14 + GP15 + GP16 + GP18 + + GP19 + GP23 + GP24

Statistička analiza i vizualizacija podataka provedena je u programskom jeziku R (verzija 4.0.2). S obzirom na to da glikanski podaci prate asimetričnu raspodjelu nagnutu udesno, mjerenja su transformirana logaritmiranjem kako bi se približila normalnoj (Gaussovoj) raspodjeli. Radi umanjena utjecaja eksperimentalne varijacije na mjerenja, provedena je korekcija serijskih učinaka (engl. *batch correction*) metodom ComBat (R paket sva) te su podaci vraćeni nazad iz logaritamske transformacije. Prije statističkog modeliranja podaci su transformirani metodom inverzne transformacije rangova u varijable s normalnom raspodjelom što omogućava usporedbu procijenjenih učinaka različitih glikana u različitim skupinama, jer na ovaj način transformirane glikanske varijable imaju istu standardiziranu varijancu (R paket GenABEL, funkcija rntransform).

Longitudinalna analiza *N*-glikana serumskog IgG-a u periodu od 14 tjedana provedena je linearnim modelom s mješovitim učincima (engl. *linear mixed-effect model*). Pritom su izravno mjerena glikanska svojstva ili derivirana svojstva (Tablica 1, Tablica 2) uključena u model kao zavisna varijabla, vrijeme odnosno vremenske točke praćenja kao fiksni efekt, odnosno nezavisna varijabla, informacija o bolesniku kao nasumični odsječak, a dob i spol kao dodatni kovarijati. Utjecaj pojedine anti-TNF terapije na *N*-glikom serumskog IgG-a tijekom 14 tjedana također je analiziran linearnim modelom s mješovitim učincima. Analiza asocijacije *N*-glikoma serumskog IgG-a prije početka terapije i odgovora na terapiju utvrđenog u 14. tjednu provedena je koristeći generalni linearni model (engl. *general linear model*) gdje su dob, spol, ITM, duljina trajanja bolesti, lokacija bolesti i ponašanje uključeni kao dodatni kovarijati. Zbog višestrukog testiranja hipoteza i kontrole stope lažno pozitivnih otkrića, dobivene P-vrijednosti korigirane su koristeći Benjamini - Hochberg metodu (funkcija p.adjust(method = "BH"), a razlika čija je P-vrijednost manja od 0,05 smatrana je statistički značajnom.

3. REZULTATI

Koristeći visoko-protočnu HILIC-UHPLC-FLD metodu analize *N*-glikozilacije serumskog IgG-a, uspješno je analizirano i kvantificirano ukupno 1513 uzoraka IgG-a izoliranog iz krvnog seruma 1315 bolesnika s CB-om u sklopu kohorte PANTS. Pritom je kod 1315 bolesnika s CB-om ispitana mogućnost predviđanja odgovora na terapiju na temelju *N*-glikanskog profila serumskog IgG-a prije započete terapije infliksimabom ili adalimumabom, u vremenskoj točki T0. U svrhu ispitivanja longitudinalne promjene sastava *N*-glikoma serumskog IgG-a tijekom anti-TNF terapije, za dio bolesnika, preciznije njih 198, prikupljen je dodatan uzorak krvnog seruma u vremenskoj točki T1, a dobiveni *N*-glikanski profili serumskog IgG-a ovih bolesnika u vremenskim točkama T0 i T1 međusobno su uspoređeni.

3.1. Osobine ispitanika

Detaljan opis karakteristika 1315 bolesnika s CB-om za koje su bili dostupni klinički podaci prikazan je u Tablici 3.

Tablica 3. Deskriptivna statistika ispitivane populacije.

	Prva vremenska točka (bazna linija, T0)	**Druga vremenska točka (T1)**
Broj ispitanika (N)	1315	198
Broj žena (n)	673	86
Broj muškaraca (n)	642	112
Dob pri prvoj dozi; medijan [IQR], g	33 [23-47]	33 [24-46]
Trajanje bolesti pri prvoj dozi; medijan [IQR], g	2,5 [0,7-9,0]	2,0 [0,6-10,1]
ITM, medijan [IQR], kg/m^2	23,36 [20,23 – 27,66]	23,04 [20,09 – 26,69]
Lokalizacija bolesti* (n)		
Ileum (L1 ± L4)	371	54
Kolon (L2 ± L4)	313	46
Ileum i kolon (L3 ± L4)	605	95
Izolirana bolest u gornjem GIT-u (L4)	12	1
Fenotip bolesti* (n)		
Luminalni (B1)	799	118

Strikturirajući (B2)	388	58
Penetrirajući (B3)	118	20
Anti-TNF lijek (n)		
Infliksimab	820	100
Adalimumab	495	98
Odgovor na terapiju (n)		
Da	926	165
Ne	258	33

*Prema Montrealskoj klasifikaciji.

3.2. Longitudinalna promjena N-glikozilacije serumskog IgG-a kod bolesnika s Crohnovom bolesti tijekom primjene anti-TNF terapije

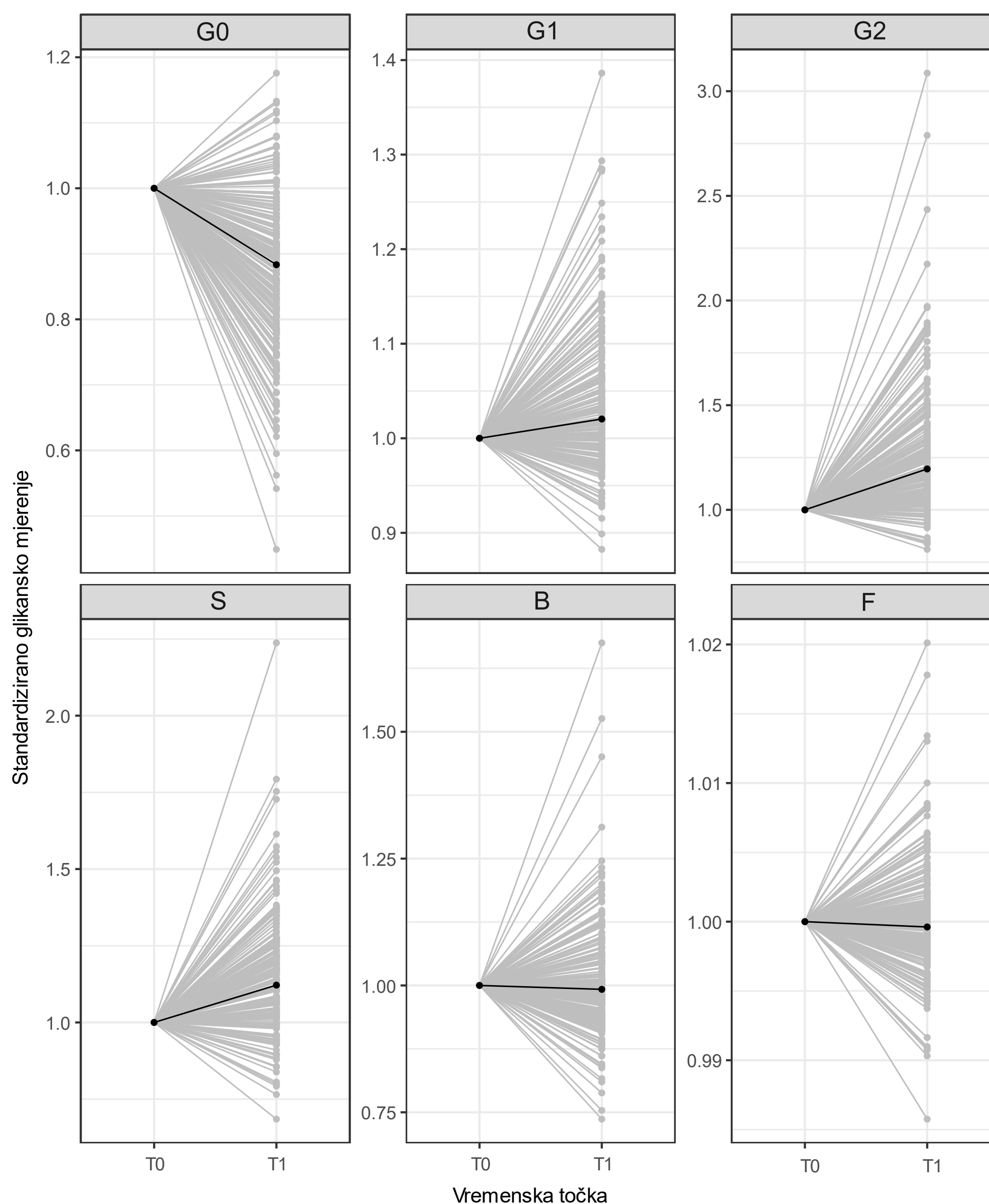
Ispitana je promjena *N*-glikoma serumskog IgG-a kod 198 bolesnika s CB-om na terapiji anti-TNF lijekovima infliksimabom i adalimumabom u dvije vremenske točke tijekom 14 tjedana. U tu svrhu korišten je linearni model s mješovitim učincima na razini deriviranih i izravno mjerenih svojstava *N*-glikozilacije serumskog IgG-a. Pritom je uočeno da kod četiri od šest ispitivanih deriviranih svojstava dolazi do statistički značajne promjene nakon 14 tjedana terapije. Preciznije, dolazi do statistički značajnog pada razine agalaktoziliranih glikana te rasta razine monogalaktoziranih, digalaktoziliranih i sijaliniziranih glikana (Tablica 4, Slika 6). Nadalje, uočeno je da ne postoji razlika u promjeni *N*-glikoma serumskog IgG-a kod skupine bolesnika koja je primjereno odgovorila na terapiju i kod skupine bolesnika koja nije odgovorila na terapiju (PNR) što je pokazano na razini izravno mjerenih svojstava *N*-glikozilacije serumskog IgG-a u Tablici 5.

Tablica 4. Utjecaj anti-TNF lijekova infliksimaba i adalimumaba na derivirana svojstva *N*-glikozilacije serumskog IgG-a bolesnika oboljelih od Crohnove bolesti u periodu od 14 tjedana. Za analizu je korišten linearni model s mješovitim učincima. P-vrijednost je korigirana na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05 (podebljano).

Derivirano svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
G0	**-0,55**	0,04	**$1,34 \times 10^{-29}$**	**$4,03 \times 10^{-29}$**
G1	**0,35**	0,05	**$1,74 \times 10^{-12}$**	**$2,61 \times 10^{-12}$**

G2	**0,60**	0,04	**$1,51 \times 10^{-32}$**	**$9,07 \times 10^{-32}$**
S	**0,46**	0,04	**$1,88 \times 10^{-21}$**	**$3,75 \times 10^{-21}$**
B	0,02	0,04	0,63	0,63
F	-0,02	0,04	0,58	0,63

Učinak – promjena u deriviranom svojstvu (izražena u jedinicama standardne devijacije) između dvije vremenske točke (T0 i T1); SE – standardna pogreška; G0 – agalaktozilacija, G1 – monogalaktozilacija, G2 – digalaktozilacija, S – sijalinizacija, F – sržna fukozilacija, B - učestalost račvajućeg *N*-acetilglukozamina (GlcNAc). Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).



Slika 6. Promjena deriviranih svojstava *N*-glikozilacije serumskog IgG-a kod bolesnika s CB-om tijekom 14 tjedana terapije anti-TNF lijekovima infliksimabom i adalimumabom. Medijan za svaku

vremensku točku prikazan je podebljano. Apscisa - vremenska točka (T0 - prva vremenska točka, nulti tjedan, bazno mjerenje; T1 - druga vremenska točka, 14. tjedan); Ordinata - relativna promjena glikanskih mjerenja normaliziranih na bazno mjerenje u T0; G0 – agalaktozilacija, G1 – monogalaktozilacija, G2 – digalaktozilacija, S – sijalinizacija, F – sržna fukozilacija, B - učestalost račvajućeg *N*-acetilglukozamina (GlcNAc). Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).

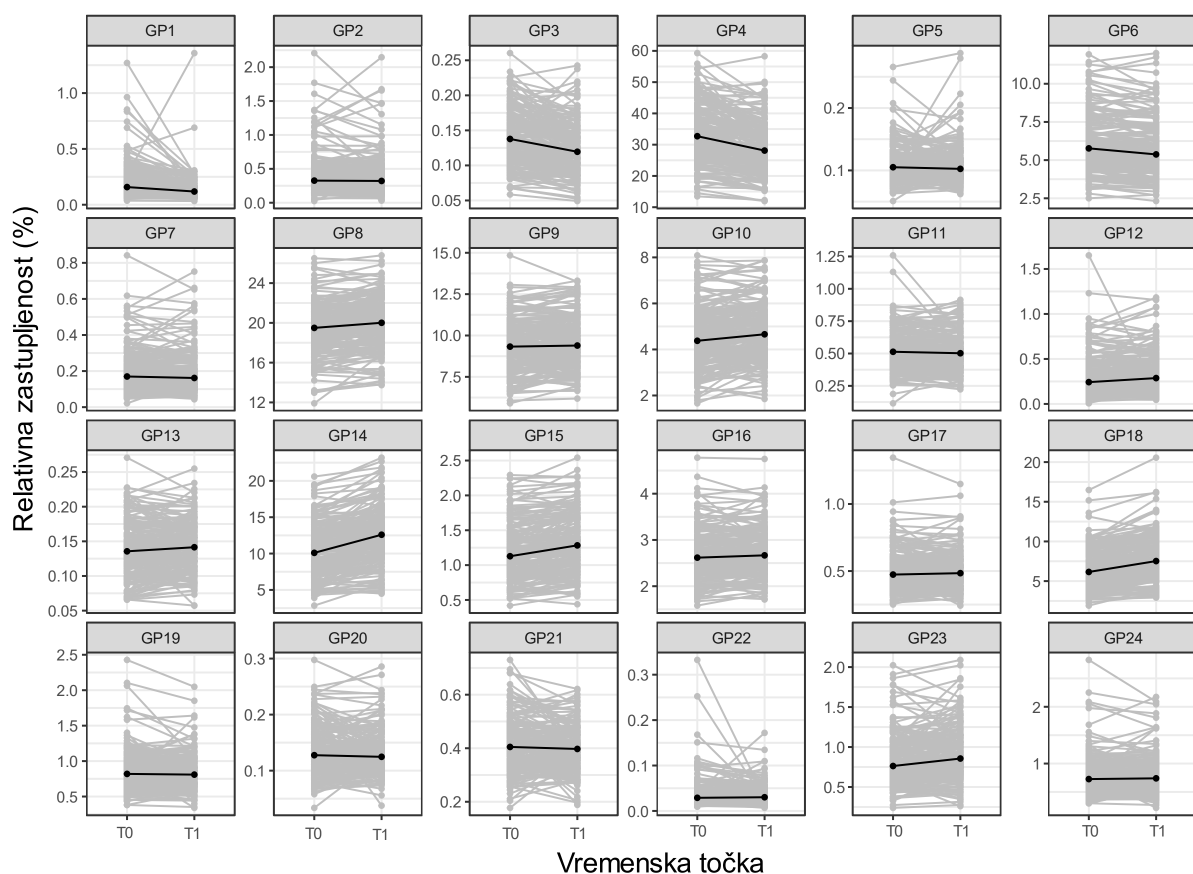
Tablica 5. Promjena u razini pojedinog izravno mjerenog svojstva *N*-glikozilacije serumskog IgG-a (GP1 - GP24) tijekom 14. tjedana terapije anti-TNF lijekovima između skupina bolesnika koja odgovora odnosno ne odgovara na terapiju. Za analizu je korišten linearni model s mješovitim učincima. P-vrijednost korigirana je na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05 (podebljano).

Izravno mjereno glikansko svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
GP1	-0,57	0,05	0,58	0,97
GP2	-0,10	0,05	0,60	0,97
GP3	-0,52	0,05	0,09	0,97
GP4	-0,58	0,05	0,21	0,97
GP5	-0,01	0,09	0,36	0,97
GP6	-0,22	0,03	0,38	0,97
GP7	0,05	0,04	0,53	0,97
GP8	0,31	0,04	0,97	0,97
GP9	0,14	0,04	0,61	0,97
GP10	0,23	0,04	0,76	0,97
GP11	-0,03	0,05	0,42	0,97
GP12	0,23	0,05	0,50	0,97
GP13	0,15	0,06	0,89	0,97
GP14	0,63	0,05	0,22	0,97
GP15	0,37	0,04	0,23	0,97
GP16	0,08	0,04	0,26	0,97
GP17	0,01	0,06	0,76	0,97
GP18	0,59	0,04	0,72	0,97
GP19	-0,03	0,07	0,82	0,97
GP20	0,03	0,07	0,43	0,97
GP21	-0,09	0,07	0,91	0,97
GP22	-0,01	0,07	0,94	0,97
GP23	0,29	0,06	0,78	0,97
GP24	0,01	0,06	0,57	0,97

Učinak – razlika između dva koeficijenta modela, gdje svaki koeficijent prikazuje promjenu izravno

mjerenih svojstava između dvije vremenske točke (T0 i T1) između skupina bolesnika koji primjereno odgovaraju i onih koji ne odgovaraju na primijenjenu terapiju; SE – standardna pogreška.

Longitudinalna promjena u relativnoj zastupljenosti glikana za svako od 24 izravno mjerena glikanska svojstva *N*-glikozilacije serumskog IgG-a (GP1 - GP24) između dvije vremenske točke prikazana je na Slici 7. Pritom je došlo do statistički značajne promjene u 14 izravno mjerenih glikanskih svojstava tijekom primjene anti-TNF terapije, koja je u skladu s promjenama opaženima kod deriviranih svojstava. Izravno mjerena glikanska svojstva od GP1 do GP4 i GP6 prvenstveno sadrže agalaktozilirane *N*-glikane te dolazi do smanjenja njihove zastupljenosti nakon 14 tjedana liječenja anti-TNF lijekovima. Svojstva počevši od GP8 do GP10 i od GP12 do GP14 prvenstveno sadrže *N*-glikane s terminalnom galaktozom te dolazi do njihovog porasta, kao i kod GP15, GP16 i GP18 koji predstavljaju uglavnom sijalinizirane glikane (Tablica 6).



Slika 7. Promjena u relativnoj zastupljenosti svakog od 24 izravno mjerenih glikanskih svojstava *N*-glikozilacije serumskog IgG-a (GP1 - GP24) u periodu od 14 tjedana terapije anti-TNF lijekovima infliksimabom i adalimumabom, kod bolesnika s CB-om. Medijan mjerenja za svaku vremensku točku prikazan je podebljano. Apscisa - vremenska točka (T0 - prva vremenska točka, nulti tjedan, bazno

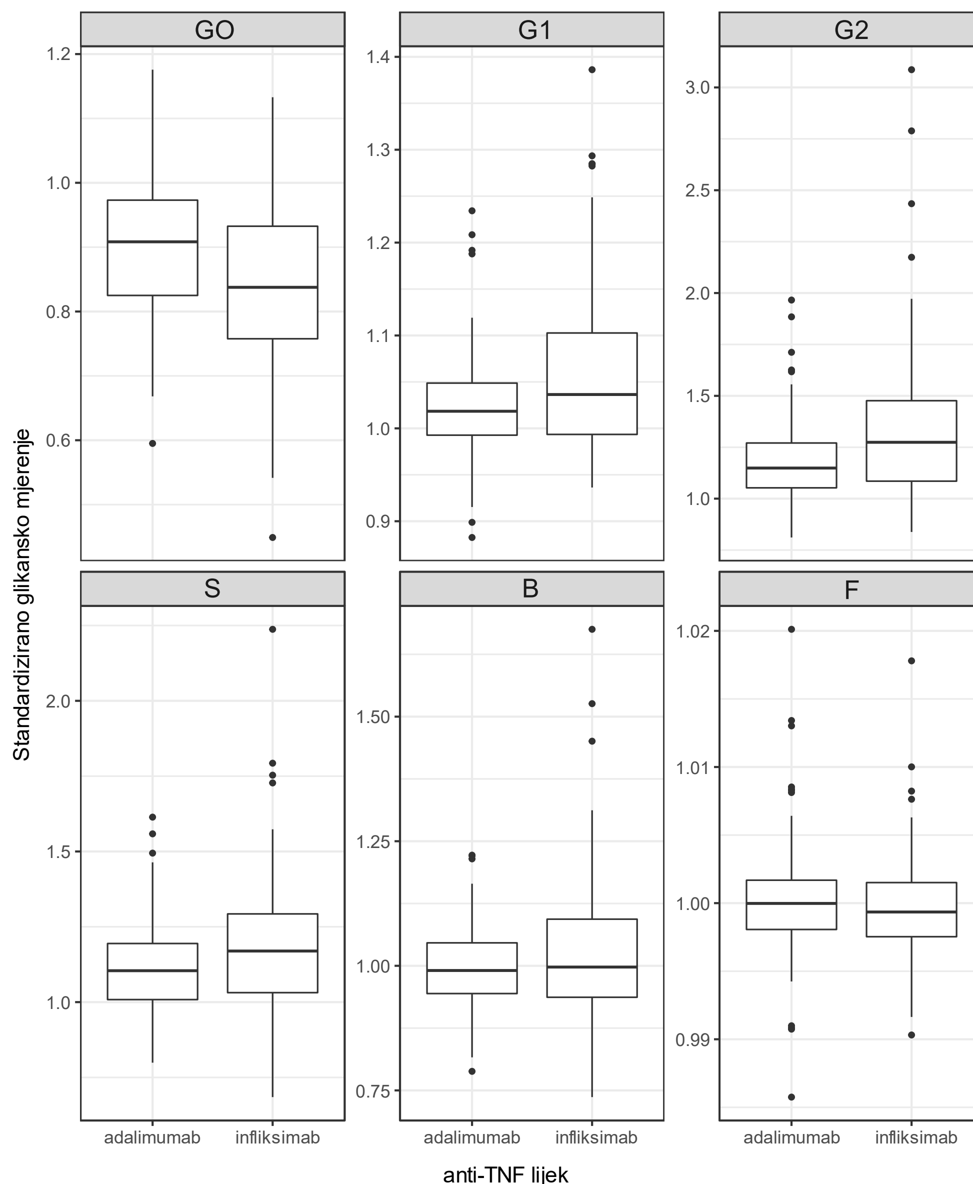
mjerenje; T1 - druga vremenska točka, 14. tjedan); Ordinata - relativna zastupljenost *N*-glikana serumskog IgG-a izražena u postocima. Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).

Tablica 6. Utjecaj anti-TNF lijekova na izravno mjerena svojstva *N*-glikozilacije serumskog IgG-a (GP1 - GP24) kod bolesnika s CB-om u periodu od 14 tjedana. Za analizu je korišten linearni model s mješovitim učincima. P-vrijednost korigirana je na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05 (podebljano).

Izravno mjereno glikansko svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
GP1	**-0,56**	0,05	**8.87×10^{-23}**	**5.32×10^{-22}**
GP2	**-0,11**	0,04	**1.02×10^{-2}**	**1.71×10^{-2}**
GP3	**-0,49**	0,05	**1.53×10^{-18}**	**7.33×10^{-18}**
GP4	**-0,55**	0,04	**1.09×10^{-28}**	**8.68×10^{-28}**
GP5	0,01	0,08	8.64×10^{-1}	9.70×10^{-1}
GP6	**-0,21**	0,03	**4.21×10^{-10}**	**1.26×10^{-9}**
GP7	0,04	0,04	3.31×10^{-1}	4.62×10^{-1}
GP8	**0,31**	0,04	**7.28×10^{-13}**	**2.50×10^{-12}**
GP9	**0,13**	0,04	**3.25×10^{-4}**	**6.50×10^{-4}**
GP10	**0,23**	0,04	**4.53×10^{-8}**	**1.21×10^{-7}**
GP11	-0,05	0,05	3.46×10^{-1}	4.62×10^{-1}
GP12	**0,22**	0,04	**2.49×10^{-6}**	**5.43×10^{-6}**
GP13	**0,15**	0,06	**1.07×10^{-2}**	**1.71×10^{-2}**
GP14	**0,61**	0,04	**4.42×10^{-33}**	**9.04×10^{-32}**
GP15	**0,35**	0,04	**1.79×10^{-15}**	**7.16×10^{-15}**
GP16	**0,10**	0,03	**2.95×10^{-3}**	**5.45×10^{-3}**
GP17	0,00	0,05	9.97×10^{-1}	9.97×10^{-1}
GP18	**0,58**	0,04	**7.54×10^{-33}**	**9.04×10^{-32}**
GP19	-0,03	0,06	6.09×10^{-1}	7.69×10^{-1}
GP20	0,01	0,07	8.89×10^{-1}	9.70×10^{-1}
GP21	-0,09	0,07	1.79×10^{-1}	2.69×10^{-1}
GP22	-0,02	0,07	8.27×10^{-1}	9.70×10^{-1}
GP23	0,28	0,05	1.06×10^{-7}	2.54×10^{-7}
GP24	0,00	0,05	9.40×10^{-1}	9.81×10^{-1}

Učinak – promjena u izravno mjerenom svojstvu *N*-glikozilacije serumskog IgG-a (izražena u jedinicama standardne devijacije) između dvije vremenske točke (T0 i T1); SE – standardna pogreška.

Linearnim modelom s mješovitim učincima također je ispitan utjecaj infliksimaba i adalimumaba zasebno na promjenu sastava *N*-glikoma serumskog IgG-a bolesnika s CB-om tijekom 14 tjedana terapije. Pritom su kod svakog lijeka primijećeni isti trendovi u promjeni sastava glikoma kao i kad su lijekovi promatrani skupno, odnosno dolazi do značajnog smanjenja agalaktozilacije te povećanja razine monogalaktozilacije, digalaktozilacije i sijalinizacije *N*-glikana IgG-a (Slika 8). Nadalje, pokazano je da terapija infliksimabom nakon 14 tjedana uzrokuje izraženiju promjenu sastava *N*-glikoma serumskog IgG-a, a pad razine agalaktozilacije odnosno povećanje mono- i digalaktozilacije te sijalinizacije je značajno veće u odnosu na promjenu uzrokovanu adalimumabom (Tablica 7).



Slika 8. Utjecaj infliksimaba i adalimumaba na promjenu deriviranih svojstava *N*-glikozilacije serumskog IgG-a kod bolesnika s CB-om u periodu od 14 tjedana. Dobivene vrijednosti na ordinati izračunate su dijeljenjem vrijednosti pojedinog deriviranog svojstva u T1 i vrijednosti istog svojstva u

T0, te su prikazane u obliku kutijastog dijagrama (engl. *box plot*). Kutija predstavlja 25. i 75. percentil, odnosno interkvartilni raspon (IQR, engl. *interquartile range*). Vodoravna linija unutar kutije predstavlja medijan mjerenja. Okomite linije prikazuju minimalnu i maksimalnu vrijednost mjerenja unutar ± 1,5 IQR, dok su vrijednosti koje odskaču prikazane kao točkice (> 1.5 IQR). G0 – agalaktozilacija, G1 – monogalaktozilacija, G2 – digalaktozilacija, S – sijalinizacija, F – sržna fukozilacija, B - učestalost račvajućeg *N*-acetilglukozamina (GlcNAc). Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).

Tablica 7. Promjena u deriviranim svojstvima između skupine bolesnika s CB-om liječenih infliksimabom i skupine bolesnika s CB-om liječenih adalimumabom tijekom 14 tjedana. Podaci su analizirani linearnim modelom s mješovitim učincima. P-vrijednost korigirana je na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05 (podebljano)..

Derivirano svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
G0	**-0,24**	0,08	**$1,73 \times 10^{-3}$**	**$3,46 \times 10^{-3}$**
G1	**0,28**	0,09	**$1,08 \times 10^{-3}$**	**$3,23 \times 10^{-3}$**
G2	**0,26**	0,08	**$8,85 \times 10^{-4}$**	**$3,23 \times 10^{-3}$**
S	**0,18**	0,08	**$2,34 \times 10^{-2}$**	**$3,51 \times 10^{-2}$**
B	0,05	0,07	0,49	0,49
F	-0,06	0,08	0,47	0,49

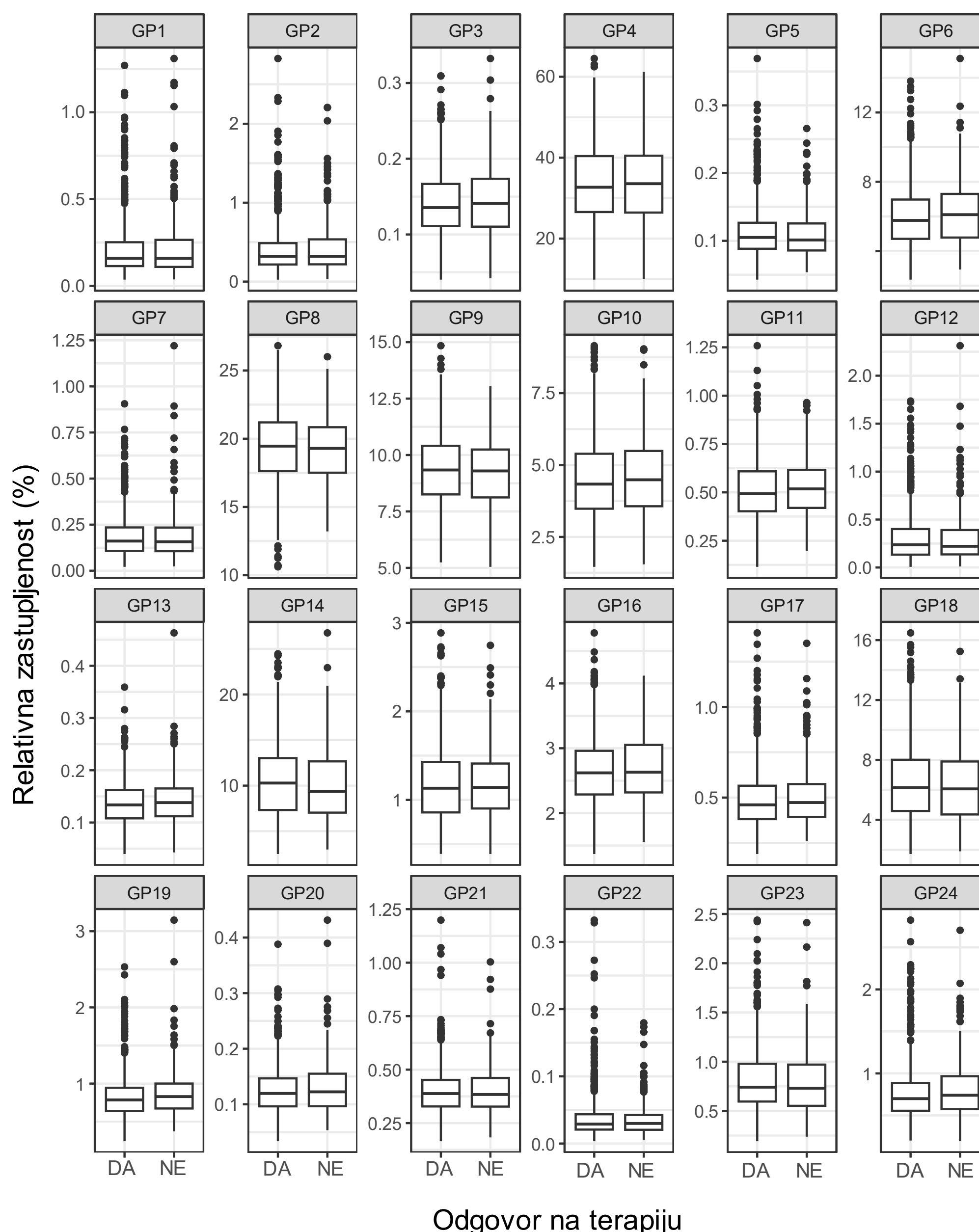
Učinak - razlika između dva koeficijenta modela, gdje svaki koeficijent prikazuje promjenu deriviranih svojstava specifičnu za tretman između dvije vremenske točke (T0 i T1); SE – standardna pogreška. G0 – agalaktozilacija, G1 – monogalaktozilacija, G2 – digalaktozilacija, S – sijalinizacija, F – sržna fukozilacija, B - učestalost račvajućeg *N*-acetilglukozamina (GlcNAc). Preuzeto i prilagođeno iz Hanić i sur., 2023. (95).

3.3. Povezanost baznog sastava N-glikoma serumskog IgG-a i terapijskog odgovora na anti-TNF lijekove kod bolesnika s Crohnovom bolesti

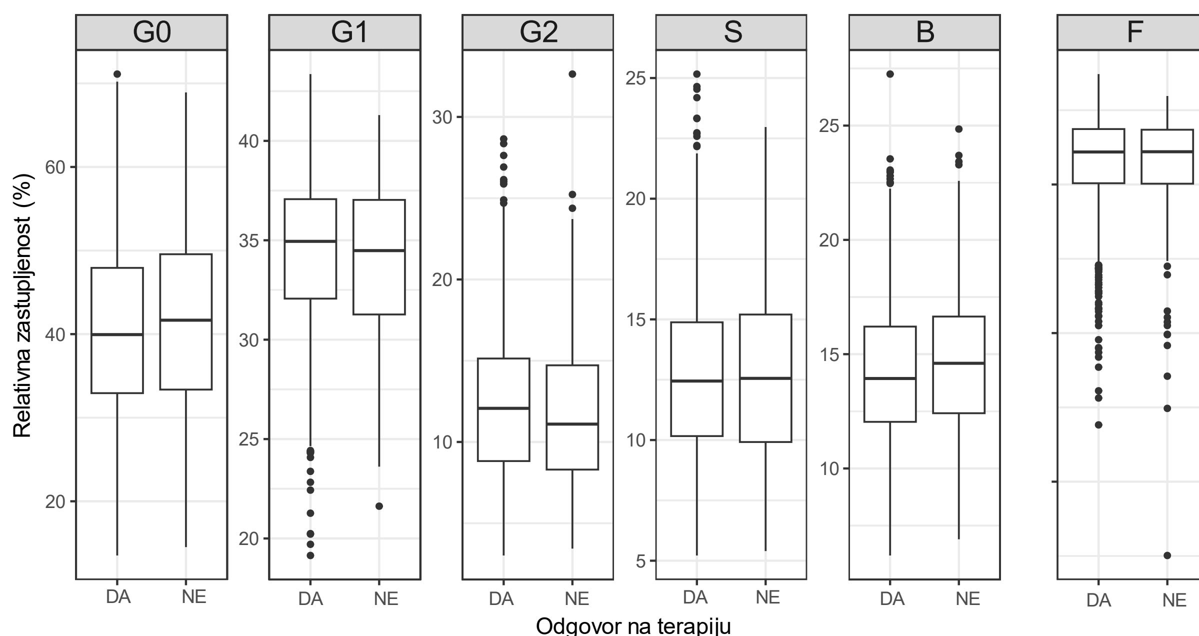
Povezanost odgovora na terapiju anti-TNF lijekovima utvrđenog u 14. tjednu liječenja te sastava *N*-glikoma serumskog IgG-a pri baznom mjerenju (vremenska točka T0) kod ukupno 1315 bolesnika provedena je generalnim linearnim modelom. Od navedenog broja, PNR je ustanovljen kod 258 bolesnika. Analiza je provedena na razini izravno mjerenih glikanskih svojstava serumskog IgG-a (Slika 9) te na razini deriviranih svojstava *N*-glikozilacije serumskog IgG-a (Slika 10).

Dobiveni rezultati pokazuju da sastav *N*-glikoma serumskog IgG-a u vremenskoj točki T0, dakle prije započinjanja terapije anti-TNF lijekovima, nije povezan s terapijskim odgovorom na istu terapiju, a koji je naknadno utvrđen u 14. tjednu liječenja. Drugim riječima, ne postoji statistički značajna povezanost razine pojedinih *N*-glikana serumskog IgG-a (Tablica

8) i deriviranih svojstava *N*-glikozilacije serumskog IgG-a (Tablica 9) s odgovorom bolesnika s CB-om na anti-TNF terapiju infliksimabom i adalimumabom.



Slika 9. Usporedba relativne zastupljenosti pojedinog izravnog glikanskog svojstva serumskog IgG-a (GP1 - GP24) prije početka terapije anti-TNF lijekovima kod bolesnika s CB-om u odnosu na terapijski odgovor utvrđen u 14. tjednu liječenja. Podaci su prikazani u obliku kutijastog dijagrama (engl. *box plot*). Kutija predstavlja 25. i 75. percentil, odnosno interkvartilni raspon (IQR, engl. *interquartile range*). Vodoravna linija unutar kutije predstavlja medijan mjerenja. Okomite linije prikazuju minimalnu i maksimalnu vrijednost mjerenja unutar ± 1,5 IQR, dok su vrijednosti koje odskaču prikazane kao točkice (> 1.5 IQR).



Slika 10. Usporedba relativne zastupljenosti pojedinog deriviranog svojstva *N*-glikozilacije serumskog IgG-a prije početka anti-TNF terapije kod bolesnika s CB-om u odnosu na terapijski odgovor utvrđen u 14. tjednu liječenja. Podaci su prikazani u obliku kutijastog dijagrama (engl. *box plot*). Kutija predstavlja 25. i 75. percentil, odnnosno interkvartilni raspon (IQR, engl. *interquartile range*). Vodoravna linija unutar kutije predstavlja medijan mjerenja. Okomite linije prikazuju minimalnu i maksimalnu vrijednost mjerenja unutar ± 1,5 IQR, dok su vrijednosti koje odskaču prikazane kao točkice (> 1.5 IQR). G0 – agalaktozilacija, G1 – monogalaktozilacija, G2 – digalaktozilacija, S – sijalinizacija, F – sržna fukozilacija, B - učestalost račvajućeg *N*-acetilglukozamina (GlcNAc).

Tablica 8. Povezanost terapijskog odgovora i izravno mjerenih svojstava *N*-glikozilacije serumskog IgG-a (GP1 - GP24) prije započinjanja terapije anti-TNF lijekovima. P-vrijednost je korigirana na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05.

Izravno mjereno glikansko svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
GP1	0,07	0,07	0,28	0,88
GP2	-0,06	0,06	0,34	0,88
GP3	0,08	0,06	0,24	0,88
GP4	0,04	0,06	0,51	0,88
GP5	-0,07	0,07	0,30	0,88
GP6	0,00	0,06	0,98	0,98
GP7	-0,12	0,06	0,07	0,88
GP8	0,00	0,07	0,96	0,98
GP9	-0,12	0,07	0,07	0,88
GP10	-0,03	0,06	0,60	0,88

GP11	-0,04	0,06	0,50	0,88
GP12	-0,10	0,06	0,11	0,88
GP13	0,03	0,06	0,59	0,88
GP14	-0,05	0,06	0,42	0,88
GP15	-0,03	0,06	0,67	0,88
GP16	0,03	0,07	0,68	0,88
GP17	0,04	0,07	0,60	0,88
GP18	-0,02	0,06	0,70	0,88
GP19	0,09	0,07	0,18	0,88
GP20	0,08	0,07	0,27	0,88
GP21	0,01	0,07	0,91	0,98
GP22	-0,04	0,07	0,53	0,88
GP23	-0,01	0,07	0,88	0,98
GP24	0,10	0,07	0,13	0,88

Učinak – razlika u izravno mjerenim glikanskim svojstvima (izražena u jedinicama standardne devijacije) između srednjih vrijednosti skupina bolesnika koji odgovaraju odnosno ne odgovaraju na terapiju; SE – standardna pogreška

Tablica 9. Povezanost terapijskog odgovora i deriviranih svojstava *N*-glikozilacije serumskog IgG-a prije započinjanja anti-TNF terapije infliksimabom i adalimumabom. P-vrijednost je korigirana na višestruka testiranja i smatrana značajnom ukoliko je manja od 0,05.

Derivirano svojstvo	Učinak	SE	P-vrijednost	Korigirana P-vrijednost
G0	0,03	0,06	0,62	0,88
G1	-0,07	0,07	0,27	0,88
G2	-0,05	0,06	0,39	0,88
S	0,01	0,07	0,94	0,98
B	0,01	0,06	0,91	0,98
F	0,04	0,06	0,53	0,88

Učinak – razlika u izravno mjerenim glikanskim svojstvima (izražena u jedinicama standardne devijacije) između srednjih vrijednosti skupina bolesnika koji odgovaraju odnosno ne odgovaraju na terapiju; SE – standardna pogreška.

4. RASPRAVA

U današnjem svijetu suvremene medicine, personalizirana skrb o bolesniku zauzima sve značajnije mjesto. Pritom je važno pronaći alate koji bi omogućili prevenciju, ranu dijagnozu i prognozu bolesti ili potpomogli odabir odgovarajuće terapije za pojedinog bolesnika. Istraživanja u području glikozilacije IgG-a pokazala su da kod različitih patofizioloških stanja dolazi do značajne promjene u sastavu njegovog *N*-glikoma, a da neke od tih promjena imaju izrazit potencijal u razvoju dijagnostičkih i prognostičkih biomarkera (96). Jedan od primjera je povišen omjer agalaktozilirane frakcije fukoziliranih *N*-glikana serumskog IgG-a u odnosu na digalaktoziliranu frakciju (G0F/G2F), kojim bi se bolesnici s CB-om mogli razlikovati od zdravih pojedinaca ili osoba oboljelih od UK-a, ali kojim bi se mogla pratiti i aktivnost bolesti (62). Dosadašnja saznanja o povezanosti glikozilacije proteina i IBD-a općenito, bila su poticaj za ovu doktorsku disertaciju kako bi se istražilo sadrži li sastav *N*-glikoma serumskog IgG-a biomarkerski potencijal u predviđanju terapijskog odgovora i ispitalo mijenja li se sastav *N*-glikoma serumskog IgG-a kod bolesnika s CB-om tijekom terapije anti-TNF lijekovima.

4.1. Promjena N-glikozilacije serumskog IgG-a kod bolesnika s Crohnovom bolesti liječenih anti-TNF terapijom

N-glikozilacija serumskog IgG-a analizirana je kod 198 bolesnika oboljelih od CB-a i to u dvije vremenske točke, neposredno prije započinjanja anti-TNF terapije infliksimabom ili adalimumabom (T0) te 14 tjedana nakon prve doze, a neposredno prije sljedeće infuzije/injekcije lijeka (T1). Prema sadašnjim saznanjima, ovo je najveće istraživanje promjene sastava *N*-glikoma serumskog IgG-a bolesnika s aktivnim luminalnim CB-om tijekom terapije infliksimabom ili adalimumabom, a pritom su uočene i statistički značajne razlike na razini deriviranih *N*-glikozilacijskih svojstava serumskog IgG-a između dvije vremenske točke. Preciznije, pokazano je da dolazi do statistički značajnog smanjenja agalaktozilacije te povećanja razine monogalaktozilacije, digalaktozilacije i sijalinizacije u navedenom periodu kod bolesnika liječenih infliksimabom i adalimumabom te da takva promjena nije ovisna o terapijskom odgovoru. Nedavno objavljena longitudinalna studija proučavala je *N*-glikozilaciju svake od potklasa serumskog IgG-a, točnije glikozilaciju pripadajuće Fc regije kod bolesnika oboljelih od kroničnih upalnih bolesti (65). Studija je između ostalog sadržavala tri kohorte u koje je bilo uključeno ukupno 34 bolesnika s UK-om i 113 bolesnika s CB-om. Meta-analizom potvrđeno je da tijekom liječenja navedenih bolesnika infliksimabom, vedolizumabom, tocilizumabom ili azatioprinom, razina galaktozilacije i

sijalinizacije Fc regije serumskog IgG-a raste bez obzira na terapijski odgovor te da su promjene istog smjera vidljive i kod drugih upalnih bolesti poput reumatoidnog artritisa i ankiloznog spondilitisa. Takvi rezultati upućuju na zaključak da opažena promjena glikozilaciji IgG-a tijekom terapije ovim lijekovima nije specifična za tip bolesti ili vrstu lijeka (65). Rezultati ove doktorske disertacije u skladu su sa spomenutom studijom, odnosno promjena opažena u sklopu ove disertacije na cjelokupnoj molekuli IgG-a (regije Fc i Fab) analogna je promjeni u *N*-glikozilaciji IgG-a opaženoj samo na Fc regiji. To je i za očekivati budući da je samo 20% molekula IgG-a nosi *N*-glikane u Fab regiji te možemo reći da je Fc regija u najvećoj mjeri odgovorna za opažene promjene. Važno je također, naglasiti da *N*-glikani u Fc i Fab regiji IgG-a imaju različitu zastupljenost monosaharidnih jedinica te da, primjerice, većina sijaliniziranih glikana potječe upravo s Fab regije (48).

Rezultati ove disertacije pokazuju da je primjenom anti-TNF terapije najizraženija promjena pad razine agalaktoziliranih glikana serumskog IgG-a kod bolesnika s CB-om, što može upućivati na zaključak da se primjenom infliksimaba smanjuje količina serumskog IgG-a koja potiče upalne procese. Kao što je već rečeno, povišena razina agalaktoziliranih *N*-glikana serumskog IgG-a često se dovodi u vezu s mnogim upalnim i autoimunim stanjima. Prije gotovo 40 godina objavljeni su rezultati studije koja je uspoređivala bolesnike oboljele od osteoartritisa i reumatoidnog artritisa sa zdravim pojedincima na razini glikozilacije serumskog IgG-a (97). Obje bolesti u svojoj patofiziološkoj pozadini imaju snažnu upalnu i autoimunu komponentu. Pritom je pokazano da između skupina postoje značajne razlike u *N*-glikozilaciji serumskog IgG-a te da kod oboljelih osoba postoji sustavni pomak u sastavu *N*-glikana serumskog IgG-a prema povećanoj razini agalaktoziliranih glikana (97). Nešto kasnije pokazano je da postoji snažna korelacija između prevalencije agalaktoziliranog serumskog IgG-a i aktivnosti reumatoidnog artritisa kod mladih i odraslih (98). Danas znamo da je smanjena razina agalaktoziliranog IgG-a značajna karakteristika mnogih upalnih, autoimunih, infektivnih bolesti i karcinoma te da je povezana s progresijom i rasplamsavanjem bolesti, primjerice kod multiple skleroze, mijastenije gravis, psorijaznog artritisa, ankiloznog spondilitisa, primarnog Sjögrenovog sindroma te već spomenutog IBD-a i reumatoidnog artritisa (99). Predloženi mehanizmi koji objašnjavaju kako agalaktozilirani IgG dovodi do proupalnog odgovora uključuju aktivaciju alternativnog puta sustava komplementa fiksiranjem C3b komponente (100) te aktivaciju puta preko lektina koji veže manozu (engl. *mannose-binding lectin*) (101), koji ipak nema značajnu ulogu u patofiziologiji IBD-ja (102). Zanimljivo je da pod utjecajem estrogena u trudnoći kod bolesnica s reumatoidnim artirtisom dolazi do smanjenja razine

agalaktoziranog IgG-a te značajnog poboljšanja kliničke slike (103), dok se u periodu nakon poroda razine agalaktoziliranog IgG-a i posljedično pogoršanje bolesti vraća na razine prije trudnoće (104).

Nadalje, rezultati ove doktorske disertacije pokazuju porast razine mono- i di-galaktoziliranih *N*-glikana serumskog IgG-a tijekom terapije što također govori u prilog smanjenju upalne aktivnosti. Kako se agalaktozilirani IgG prvenstveno povezuje s upalom u organizmu, tako se prisutnost galaktoze u strukturi *N*-glikana serumskog IgG-a povezuje s protuupalnim učincima, iako se često spominje dvojaka uloga takvog IgG-a. Smatra se da visokogalaktozilirane IgG1 molekule promoviraju povezivanje između inhibicijskog FcγRIIB i dektin-1 receptora posredovanu galektinom-3, lektinom koji veže beta-galaktozilne ostatke te suprimiraju proupalnu funkciju C5a komponente sustava komplementa (54,105). No postoje saznanja da galaktozilirani IgG potiče heksamerizaciju IgG-a, pospješuje vezanje C1q komponente i posljedično aktivira klasični put sustava komplementa i CDC (55,106), a može dovesti i do povećanja aktivnosti ADCC-a interakcijom s FcγR niskog afiniteta (107). Ipak, to samo podupire tezu o kompleksnosti *N*-glikozilacije serumskog IgG-a, pripadajućih biosinteznih puteva i utjecaja na nizvodnu kaskadu, gdje najvjerojatnije mora postojati prag u razini pojedine glikoforme IgG-a nakon kojeg će se primarno ispoljavati pro- ili protuupalni učinak. Iz tog razloga opažene promjene ne smiju se interpretirati na temelju samo jednog izravno mjerenog ili deriviranog svojstva već sagledati kao cjelina.

Razina sijalinizacije IgG-a također raste tijekom longitudinalne primjene infliksimaba ili adalimumaba kod bolesnika s CB-om, što također sugerira smanjenje mogućnosti takvog IgG-a da potencira upalu. U uvjetima homeostaze, sijalinizacija IgG-a odgovorna je za održavanje stanja smanjene upale, a u slučaju poremećaja homeostaze i prisutnosti patogena, odnosno antigena, antigen specifični IgG može prijeći u populaciju snižene sijalinizacije i posredovati u zaštitnom upalnom odgovoru interakcijom s FcγR (108,109). Sijalinizirani IgG potiče ekspresiju inhibicijskog FcγRIIB na efektorskim stanicama (109), smanjuje afinitet IgG-a za aktivirajući FcγRIIIA i aktivnost ADCC-a (56), smanjuje vezanje C1q komponente sustava komplementa, a time i aktivnost CDC-a (57). Upravo su prisutnost terminalnih α2-6 sijalinskih kiselina u strukturi glikana intravenoznog imunoglobulina (IVIg, engl. *intravenous immunoglobulin*) i inhibicijski FcγRIIB nužni za njegov terapijski učinak, a koji je kao lijek izuzetno učinkovit u liječenju raznih autoimunih i upalnih bolesti (110).

S obzirom da je poznato da je kod bolesnika s CB-om smanjena galaktozilacija i sijalinizacija *N*-glikana serumskog IgG-a u odnosu na zdrave osobe (62–65), rezultati ovog doktorskog rada upućuju na to da tijekom terapije dolazi do pomaka serumskog IgG-a iz populacije s povećanom proupalnom aktivnošću ka populaciji sa smanjenom proupalnom aktivnošću te da ta promjena nije povezana sa odgovorom na terapiju. Drugim riječima, primjena anti-TNF terapije ima suprotan učinak na sastav *N*-glikoma serumskog IgG-a od učinka samog CB-a kada se on uspoređuje sa *N*-glikomom serumskog IgG-a zdravih pojedinca (65). Premda su neke studije kod bolesnika s CB-om pokazale promjenu u razini fukozilacije IgG-a i razini glikana s račvajućim GlcNAc-om u odnosu na zdrave osobe (63,64), rezultati ovog istraživanja pokazuju da ne dolazi do značajne promjene u razini ovih svojstava tijekom primjene anti-TNF terapije.

Nije poznato na koji način primjena anti-TNF terapije modulira promjenu *N*-glikozilacije serumskog IgG-a. Iako su infliksimab i adalimumab po svojoj molekularnoj strukturi IgG1, ne očekujemo da se njihova intravenska/supkutana primjena odrazila na zastupljenost pojedinog glikana ili deriviranog svojstva serumskog IgG-a. To potvrđuje i činjenica da je najzastupljeniji glikan u oba lijeka fukozilirani glikan bez galaktoze (FA2) (78,79), a koji je uključen u izračun deriviranog svojstva agalakotozilacije IgG-a, koja opada s primjenom anti-TNF terapije u ovom istraživanju. Infliksimab se intravenski primjenjuje u dozi od 5 mg/kg u nultom, drugom i šestom tjednu za vrijeme faze indukcije, a kasnije svakih osam tjedana, dok se adalimumab primjenjuje supkutano u dozi od 160 mg u fazi indukcije, 80 mg dva tjedna nakon i potom 40 mg svaka dva tjedna (111). Značajnija promjena i utjecaj na glikozilacijski profil serumskog IgG-a mogao bi se očekivati u slučaju primjene IVIg-a, koji se primjenjuje u dozi od 400-800 mg/kg, a koje se smatraju niskim dozama, pa sve do 3000 mg/kg ovisno o indikaciji (112). Moguće je da je opažena promjena u glikozilaciji serumskog IgG-a tijekom primjene anti-TNF terapije odraz smanjenja ne samo crijevne upale nego i upale na razini cijelog organizma (82). S obzirom da limfociti B eksprimiraju TNFR2 preko kojeg TNF u homeostaznim uvjetima djeluje kao autokrini faktor rasta limfocita B, za očekivati je da navedena terapije ima utjecaj na populaciju limfocita B u perifernoj krvi (35,113). Jedno istraživanje pokazalo je da u CB-u dolazi do specifičnih promjena u sastavu i sazrijevanju limfocita B u perifernoj krvi, a koja se u potpunosti normalizira primjenom infliksimaba (34) te da je obnavljanje populacije limfocita B povezano s odgovorom na terapiju (114). Broj limfocita B u krvi približava se normalnim vrijednostima zdravih pojedinaca kod bolesnika s reumatoidnim artritisom nakon primjene anti-TNF terapije (115,116). Glikozilacija serumskih

proteina kod bolesnika s upalnim artritisom također se značajno mijenja s primjenom anti-TNF terapije, dolazi do povećanja galaktozilacije IgG-a, a opažene promjene snažno koreliraju s padom CRP-a što sugerira utjecaj anti-TNF lijekova na imunosni sustav, bez obzira na terapijski odgovor (117).

Usporedbom skupina bolesnika liječenih ili infliksimabom ili adalimumabom, opaženo je da se promjene u glikozilaciji serumskog IgG-a kreću su istom smjeru, odnosno ka profilu koji se povezuje sa smanjenjem upalnih procesa u organizmu. Međutim, kod skupine bolesnika liječene infliksimabom ustanovljeno je da dolazi do značajnijeg pada u razini agalaktoziliranih IgG glikana, odnosno snažnijeg rasta galaktoziliranih i sijaliniziranih *N*-glikana serumskog IgG-a u odnosu na skupinu bolesnika liječenu adalimumabom. Postoje saznanja da je infliksimab učinkovitiji u indukciji remisije kod bolesnika s CB-om u odnosu na adalimumab (118,119) te da je učinkovitiji kod postizanja terapijskog odgovora, zacjeljivanja sluznice crijeva i remisije kod bolesnika s UK-om (120). S druge strane, objavljene su i studije koje govore o podjednakoj učinkovitosti ova dva lijeka u postizanju terapijskog odgovora (121,122). Pritom se često naglašava važnost dodatne uporabe imunomodulatora u kombinaciji s infliksimabom radi postizanja zadovoljavajućeg odgovora i remisije, dok se adalimumab koristi u monoterapiji i povezan je s manje nuspojava (121,122). S obzirom na to da za ovo istraživanje nije bila dostupna informacija u uporabi imunomodulatora u kombinaciji s infliksimabom, moguće je da, ukoliko su korišteni, pridonose značajnijoj promjeni sastava *N*-glikoma serumskog IgG-a u odnosu na adalimumab u monoterapiji, no takva tvrdnja trebala bi biti predmet nekog narednog istraživanja.

4.2. Sastav N-glikoma serumskog IgG-a i predviđanje odgovora na terapiju anti-TNF lijekovima u Crohnovoj bolesti

Kako bi se utvrdilo je li u *N*-glikomu serumskog IgG-a sadržana informacija o budućem odgovoru na terapiju infliksimabom i adalimumabom, analiziran je *N*-glikom IgG-a pročišćenog iz krvnog seruma 1315 bolesnika s aktivnim luminalnim CB-om, koji je izuzet prije davanja prve doze lijeka. Od tog broja, 926 bolesnika adekvatno je odgovorilo na primijenjenu terapiju, što je utvrđeno u 14. tjednu iste, dok je PNR ustanovljen kod njih 258.

Rezultati su pokazali da između ove dvije skupine ne postoji značajna razlika u razini niti jednog izravno mjerenog niti deriviranog svojstva *N*-glikozilacije serumskog IgG-a prije

započinjanja terapije, odnosno da sastav *N*-glikoma serumskog IgG-a pri baznom mjerenju nije povezan s budućim odgovorom na terapiju infliksimabom ili adalimumabom. Dobiveni rezultati u skladu su sa već spomenutom studijom koja je na manjem broju ispitanika koji pate od kroničnih upalnih bolesti, meta-analizom utvrdila da pri baznom mjerenju ne postoji razlika u sastavu *N*-glikoma serumskog IgG-a bolesnika koji su ušli u remisiju u odnosu na one koji nisu. No, u istoj studiji pokazano je da između longitudinalnih mjerenja postoje razlike u *N*-glikomu serumskog IgG-a između bolesnika s aktivnim oblikom bolesti i onih koji su ušli u remisiju. Bolesnici koji su ušli u remisiju pokazuju snažniji rast u digalaktozilaciji potklasa IgG2 i IgG3 od početka terapije u odnosu na bolesnike s aktivnim oblikom bolesti (65). Kod bolesnika s reumatoidnim artritisom također ne postoji povezanost između odgovora na anti-TNF terapiju i sastava *N*-glikoma proteina seruma pri baznom mjerenju (117,123). Premda *N*-glikani serumskog IgG-a pri baznom mjerenju ne pružaju informaciju u mogućem PNR-u, rezultati ukazuju na činjenicu da bazni sastav *N*-glikoma serumskog IgG-a nema značajnijeg utjecaja na djelovanje lijeka, odnosno da endogeni IgG sa svojim pojedinim glikoformama ne maskira terapijski učinak anti-TNF lijekova, posebice mehanizam djelovanja koji je posredovan Fc regijom lijeka i posljedične efektorske funkcije molekule lijeka.

5. ZAKLJUČCI

U ovom doktorskom radu analizirana je *N*-glikozilacija serumskog IgG-a izoliranog iz ukupno 1513 uzoraka krvnog seruma bolesnika s aktivnim luminalnim CB-om. Cilj je bio ispitati utjecaj anti-TNF lijekova infliksimaba i adalimumaba na sastav *N*-glikoma serumskog IgG-a u vremenskom periodu od 14 tjedana te razlikuje li se on u odnosu na terapijski odgovor. Nadalje, cilj je bio i ispitati mogućnost predviđanja terapijskog odgovora odnosno utvrđivanje PNR-a na temelju sastava *N*-glikana serumskog IgG-a prije početka terapije anti-TNF lijekovima, odnosno infliksimabom i adalimumabom. Pritom su ostvareni postavljeni ciljevi te je ovaj doktorski rad pridonio proširenju znanja o *N*-glikozilaciji serumskog IgG-a bolesnika s CB-om, pružila nova saznanja o utjecaju anti-TNF lijekova, infliksimaba i adalimumaba, na *N*-glikom serumskog IgG-a u CB-u te opovrgnula mogućnost korištenja sastava *N*-glikoma serumskog IgG-a u svrhu predviđanja terapijskog odgovora kod bolesnika s CB-om prije početka terapije anti-TNF lijekovima.

Na temelju dobivenih rezultata doneseni su sljedeći zaključci:

- Primjena anti-TNF terapije kod bolesnika s aktivnim luminalnim CB-om statistički značajno mijenja sastav *N*-glikoma serumskog IgG-a bolesnika nakon 14 tjedana terapije. Najizraženija promjena je pad razine agalaktoziliranih te rast galaktoziliranih i sijaliniziranih *N*-glikana serumskog IgG-a što govori u prilog smanjenoj mogućnosti IgG-a da sudjeluje u poticanju upalnog odgovora u organizmu.

- Opažena promjena u sastavu *N*-glikoma serumskog IgG-a nakon 14 tjedana terapije anti-TNF lijekovima nije povezana s povoljnim ili nepovoljnim terapijskim odgovorom bolesnika s CB-om. To govori u prilog pretpostavci da *N*-glikozilacija endogenog serumskog IgG-a ne utječe na terapijski učinak anti-TNF lijekova.

- U odnosu na adalimumab, infliksimab uzrokuje izraženije promjene u sastavu *N*-glikoma serumskog IgG-a kod bolesnika s CB-om nakon 14 tjedana terapije.

- Na temelju sastava *N*-glikoma serumskog IgG-a prije započinjanja terapije anti-TNF lijekovima, nije moguće predvidjeti nepovoljan terapijski odgovor utvrđen u 14. tjednu kod bolesnika s CB-om.

6. POPIS LITERATURE

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7. PRILOZI

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Anti-TNF Biologicals Enhance the Anti-Inflammatory Properties of IgG N-Glycome in Crohn's Disease

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Abstract: Crohn's disease (CD) is a chronic inflammation of the digestive tract that significantly impairs patients' quality of life and well-being. Anti-TNF biologicals revolutionised the treatment of CD, yet many patients do not adequately respond to such therapy. Previous studies have demonstrated a pro-inflammatory pattern in the composition of CD patients' immunoglobulin G (IgG) N-glycome compared to healthy individuals. Here, we utilised the high-throughput UHPLC method for N-glycan analysis to explore the longitudinal effect of the anti-TNF drugs infliximab and adalimumab on N-glycome composition of total serum IgG in 198 patients, as well as the predictive potential of IgG N-glycans at baseline to detect primary non-responders to anti-TNF therapy in 1315 patients. We discovered a significant decrease in IgG agalactosylation and an increase in monogalactosylation, digalactosylation and sialylation during the 14 weeks of anti-TNF treatment, regardless of therapy response, all of which suggested a diminished inflammatory environment in CD patients treated with anti-TNF therapy. Furthermore, we observed that IgG N-glycome might contain certain information regarding the anti-TNF therapy outcome before initiating the treatment. However, it is impossible to predict future primary non-responders to anti-TNF therapy based solely on IgG N-glycome composition at baseline.

Keywords: Crohn's disease; IgG glycosylation; infliximab; adalimumab; PANTS study

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic immune-mediated inflammatory disease of the gastrointestinal tract that can be classified as Crohn's disease (CD) or ulcerative colitis (UC) using available diagnostic tools. In 2017, nearly 3.9 million women and 3.0 million men worldwide were affected by IBD [1]. The prevalence of IBD continues to increase globally, and it significantly burdens healthcare systems and economies, particularly in developing countries [1,2]. Although the aetiology of IBD is still not fully understood, it is generally considered that aberrant mucosal innate and adaptive immune responses [3] and environmental risk factors, such as early exposure to antibiotics, poor diet, air pollution, psychological stress and altered composition of gut microbiota [4,5], significantly contribute to the pathogenesis of IBD in genetically susceptible individuals [6]. However, protein glycosylation is another critical component associated with the development and progression of IBD [7,8]. Glycans are a vast group of complex oligosaccharides found on proteins and lipids in our bodies. Their monosaccharide composition is tailored by genetic and environmental influences [9], making them an excellent reflection of the body's current state with exquisite diagnostic, prognostic and biomarker potential [9]. Alterations in the

glycome (set of glycans) of immunoglobulin G (IgG), the central molecule of humoral immunity, has been extensively studied in ageing, carcinomas and inflammatory diseases such as autoimmune diseases and chronic inflammatory states (e.g., low back pain) [10]. Biantennary N-glycans of IgG are responsible for fine-tuning its effector functions by modulating the interaction of the fragment crystallisable (Fc) part of the IgG molecule with various Fcγ receptors on effector cells and, consequently, orchestrating the activity of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) [11–13]. In addition, roughly 20% of IgG molecules are glycosylated in the variable region of the fragment antigen-binding (Fab) domain, which is essential for the interaction of antibodies with specific antigens and its immunomodulatory effect through interaction with lectins [14]. Decreased galactosylation of IgG is a hallmark of many inflammatory diseases [10], and IBD is no exception. In fact, the IgG N-glycome shows increased pro-inflammatory potential in CD patients compared to healthy individuals. The IgG N-glycan repertoire in CD contains fewer galactosylated and sialylated N-glycans and more glycans with bisecting *N*-acetylglucosamine (GlcNAc) [15,16], and it can also distinguish CD patients from healthy controls and UC patients [16].

Inflammation in CD is promoted by increased intestinal tissue levels of tumour necrosis factor (TNF), along with interferon-gamma and interleukin (IL)-12, all of which are potent pro-inflammatory cytokines [17,18]. Early and effective control of inflammation in CD patients is of utmost importance. The treatment of CD reached a significant milestone when the first anti-TNF drug, the chimeric IgG1 monoclonal antibody infliximab (IFX), was introduced into practice in 1998 [19]. Both IFX and the fully human IgG1 monoclonal antibody adalimumab (ADA) are now widely used for CD. Their mechanism of action involves Fab-mediated neutralisation of transmembrane and soluble TNF and destruction of TNF-producing cells through CDC and ADCC by the interaction of the IgG Fc region with receptors on effector cells [20]. This interaction is strongly influenced by N-glycans bound to the IgG Fc region [21,22].

There are several reports of anti-TNF therapy changing the serum and IgG glycome composition in chronic inflammatory diseases (CID) [16,23,24]. However, knowledge about the longitudinal influence of anti-TNF treatment on IgG glycome in CD patients is relatively scarce.

Up to one-third of patients do not respond to anti-TNF therapy induction regimen (primary non-response, PNR), and a further 30–50% of patients who initially respond to therapy lose their response during the maintenance regimen (secondary loss of response, LOR) [25,26]. Prediction of therapy response to anti-TNF is one of the ultimate goals in the personalised approach to treating IBD. However, all the predictive biomarkers reported to date lack clinical utility [25]. In patients with UC, low levels of branched glycans in intestinal T cells associate with failure of standard therapy independent of other clinical parameters. The predictive accuracy is further increased when the C-reactive protein (CRP) level is accounted [27].

Therefore, this study aims to investigate (A) longitudinal changes in IgG N-glycome in CD patients treated with anti-TNF monoclonal antibodies IFX and ADA, and (B) baseline IgG N-glycome patterns that predict primary non-response to IFX and ADA.

2. Materials and Methods

2.1. Clinical Samples and Ethical Considerations

Patients with CD were recruited as a part of the Personalized Anti-TNF Therapy in Crohn's Disease (PANTS) study conducted in hospitals across the United Kingdom. The PANTS study was a prospective uncontrolled observational cohort study investigating the mechanism of PNR, LOR and adverse drug reactions (ADR) to IFX and ADA in anti–TNF-naïve patients with severe active luminal CD. To enter the study, subjects had to meet several inclusion criteria: age of 6 years and over, presence of active luminal CD involving the colon and/or small intestine (Montreal classification L1, L2 or L3) supported with raised CRP and/or faecal calprotectin levels, and no prior exposure to anti-TNFα

medication. A total of 1513 CD patients' serum samples were collected at two time points, before the first dose of anti-TNF therapy (week 0/baseline, N = 1315) and for subset of patients, immediately before the next scheduled anti-TNF injection/infusion (week 14, N = 198). PNR was determined at week 14 by the following: ongoing use of corticosteroids, cessation of anti-TNF drug for non-response, or failure of both the Harvey-Bradshaw Index (HBI) to fall by 3 or more points from week 0 baseline or to 4 or below, and the CRP to fall to within normal range (≤3 mg/L) or by 50% from week 0. Grey zone, as an intermediate response between PNR and response, was defined as a decrease in CRP level to 3 mg/L or less, or by 50% or more from baseline; or as a decrease in HBI score to 4 points or less, or by 3 points or more from baseline; but not both. Treatment response was defined as a decrease in CRP to 3 mg/L or less, or by 50% or more from baseline and a decrease in HBI score to 4 points or less, or by 3 points or more from baseline for adults; or a decrease in sPCDAI to 15 points or less, or by 12.5 points from baseline for children. Remission was defined as CRP of 3 mg/L or less and HBI score of 4 points or less (sPCDAI score ≤ 15 points), no ongoing steroid therapy, and no exit due to treatment failure. The patient's gastroenterologist decided to continue or suspend anti-TNF therapy between weeks 12 and 14. Samples were collected with the approval of The South West Research Ethics Committee (Research Ethics Committee reference: 12/SW/0323) and informed written consent was obtained from all participants.

2.2. IgG N-Glycan Sample Preparation

Prior to IgG isolation, block randomisation was performed to define the position of samples across 96-well plates, and replication standard samples were included as well. Sample preparation was based on previously described protocols [28,29] for hydrophilic-interaction-based ultra-high performance hydrophilic liquid chromatography with fluorescence detection (HILIC-UHPLC-FLD) for IgG N-glycan analysis in a high-throughput manner. Briefly, 100 µL of serum was diluted with 700 µL 1 × PBS and applied to a Protein G monolithic plate (BIA Separations, Ajdovscina, Slovenia). After three 1 × PBS washes, IgG was eluted in 1 mL 0.1 M formic acid (Merck, Darmstadt, Germany) and neutralised with 170 µL 1M ammonium bicarbonate (Merck). An equal volume of each IgG eluate containing an equivalent of 100–300 µg of IgG was dried in a vacuum concentrator and denatured with 30 µL of 1.33% (*w*/*v*) SDS (Invitrogen, Carlsbad, CA, USA) by incubation at 65 °C for 10 min. Denatured IgG was incubated with 10 µL of 4% (*v*/*v*) Igepal-CA630 (Sigma-Aldrich, St. Louis, MO, USA) and deglycosylated by addition of 1.2 U of PNGase F (Promega, Madison, WI, USA) in 10 µL of 5 × PBS for 18 h at 37 °C. Released N-glycans were labelled with a labelling mixture. The labelling mixture was freshly prepared by dissolving 0.48 mg 2-aminobenzamide (2-AB, Sigma-Aldrich) and 1.12 mg 2-picoline borane (2-PB, Sigma-Aldrich) in 25 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and glacial acetic acid (Sigma-Aldrich) mixture (70:30, *v*/*v*) per sample. After a 10 min shake, a labelling reaction was conducted for two hours at 65 °C. The free label and reducing agent excess were removed by hydrophilic interaction liquid chromatography-solid phase extraction (HILIC-SPE). For that purpose, 2-AB labelled IgG N-glycans (total volume of 75 µL) were diluted with 700 µL of acetonitrile (ACN) and applied to an AcroPrep GHP filter plate with 0.2 µm pore diameter (Pall Corporation, Ann Arbor, MI, USA). After five washes with 96% (*v*/*v*) ACN using a vacuum manifold, 2-AB labelled IgG glycans were collected in two fractions of 90 µL ultra-pure water (total volume of 180 µL).

2.3. HILIC-UHPLC-FLD Analysis of 2-AB Labelled IgG N-Glycans

Fluorescently labelled N-glycans were separated by hydrophilic interaction liquid chromatography on a Waters Acquity Ultra performance liquid chromatography (UPLC) H-class instrument (Waters Corporation, Milford, MA, USA) consisting of a quaternary solvent manager, sample manager and a fluorescence detector set with excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was under the control of Empower 3 software, build 3471 (Waters Corporation). Labelled N-glycans

were separated on a Waters bridged ethylene hybrid (BEH) 100 mm × 2.1 mm glycan chromatography column filled with 1.7 μm BEH particles. The mobile phase consisted of 100 mM ammonium formate (pH 4.40) as solvent A and ACN as solvent B. The separation method used a linear gradient of 75% to 62% ACN (v/v) at a flow rate of 0.4 mL/min in a 28 min analytical run. Samples were maintained at 10 °C before injection, and the column temperature was set at 60 °C. In order to calibrate UHPLC runs against day-to-day and system-to-system changes, an external standard of hydrolysed and 2-AB labelled glucose oligomers (dextran ladder) was used as a reference from which the retention times for the individual glycans were converted to glucose units (GU). Obtained chromatograms were all separated in the same manner into 24 glycan peaks (GP1–GP24) via the automatic chromatogram extraction (ACE) method [30]. The amount of glycans in each peak was expressed as a percentage (%) of the total integrated area.

2.4. Statistical Analysis

In order to remove experimental variation from measurements, normalisation and batch correction were performed on UHPLC IgG N-glycan data. To make measurements across samples comparable, normalisation by total area was performed, where the peak area of each of 24 glycan structures was divided by the total integrated area of the corresponding chromatogram. Before the batch correction, normalised glycan measurements were log-transformed due to right-skewness of their distributions and the multiplicative nature of batch effects. Batch correction was performed on log-transformed measurements using ComBat method (R package sva), where the technical source of variation (which sample was analysed on which plate) was modeled as a batch covariate. Estimated batch effects were subtracted from log-transformed measurements to get measurements corrected for experimental noise. An additional six derived traits were calculated from 24 directly measured and normalised glycan traits (GP1-GP24) and defined as: the percentage of agalactosylated glycans in total IgG glycans–G0 = SUM(GP1 + GP2 + GP3 +GP4 + GP6)/GP × 100; the percentage of monogalactosylated glycans in total IgG glycans–G1 = SUM(GP7 + GP8 + GP9 +GP10 + GP11)/GP × 100; the percentage of digalactosylated glycans in total IgG glycans–G2 = SUM(GP12 + GP13 + GP14 + GP15)/GP × 100; the percentage of mono- and disialylated glycans in total IgG glycans–S = SUM(GP16 + GP17 + GP18 + GP19 + GP21 + GP22 + GP23 + GP24)/GP × 100; the percentage of glycans with core fucose in total IgG glycans–F = SUM(GP1 + GP4 + GP6 + GP8 + GP9 + GP10 + GP11 + GP14 + GP15 + GP16 + GP18 + GP19 + GP23 + GP24)/GP × 100; the percentage of glycans with bisecting *N*-acetylglucosamine (GlcNAc) in total IgG glycans–B = SUM(GP3 + GP6 + GP10 + GP11 + GP13 + GP15 + GP19 + GP22 + GP24)/GP × 100. These derived traits average particular glycosylation features across different individual glycan structures and therefore are more closely related to individual enzymatic activities and underlying genetic polymorphisms.

Longitudinal analysis of patient samples through their observation period was performed by implementing a linear mixed effects model where glycan measurement was the dependent variable, time was modeled as a fixed effect while individual ID was included in a model as a random intercept, with age and gender included as additional covariates. Treatment effect on N-glycome change through time was analysed using a linear mixed effects model where time was modelled as a fixed effect, the interaction between time and treatment was modelled as a fixed effect, while individual sample ID was modeled as a random intercept, with age and gender included as additional covariates. Association analyses between therapy response/remission status and baseline glycomic measurements were performed using a regression model with age, gender, BMI, disease duration, disease location and behaviour included as additional covariates. Prior to analyses, glycan variables were all transformed to a standard normal distribution (mean = 0, standard deviation = 1) by inverse transformation of ranks to normality (R package "GenABEL", function rntransform). Using rank-transformed variables in analyses makes estimated effects of different glycans comparable as transformed glycan variables have the same standardised variance. False discovery rate was controlled using Benjamini-Hochberg

procedure (function p.adjust(method = "BH")). Data were analysed and visualised using R programming language (version 4.0.2).

3. Results

Using HILIC-UHPLC-FLD as a high-throughput method for IgG N-glycan analysis, we successfully glycoprofiled and quantified a total of 1513 IgG samples isolated from the serum of CD patients treated with IFX or ADA monoclonal antibodies from the PANTS cohort. More detailed demographic characteristics of included CD patients are given in Table 1. The IgG N-glycoprofiles obtained from each patient were separated into 24 glycan peaks (GP1-GP24), the composition of which has been reported previously (Figure S1) [31]. Relative glycan abundance in a particular glycan peak was expressed as a percentage of the total integrated area. Furthermore, glycans with shared structural features were summarised into six derived traits and included in the statistical analysis. We compared the IgG N-glycan profiles of 198 patients whose serum samples were longitudinally collected at baseline (week 0) and at week 14 to assess the effect of anti-TNF therapy on the composition of IgG N-glycome of CD patients. Next, we searched for patterns in the IgG N-glycome of 1315 CD patients whose serum samples were collected before anti-TNF induction to identify biomarkers of primary non-response.

Table 1. Demographics of the studied cohort.

	Week 0 (Baseline)	Week 14
Number of samples (N)	1315	198
Median age at first dose [IQR], yr	33 [23–47]	33 [24–46]
Number of females (%)	673 (51.2)	86 (43.4)
Median disease duration at first dose [IQR], yr	2.5 [0.7–9.0]	2.0 [0.6–10.1]
Anti-TNFα treatment (n)		
Infliximab (IFX)	820	100
Adalimumab (ADA)	495	98
Therapy outcome (n)		
Primary non-response (PNR)	258	33
Response	180	165
Grey zone	231	/
Remission	515	/
N/A	131	/

Week 0: first time point/baseline data collected before the first dose of anti-TNF therapy; Week 14–second time point data collected for the subset of patients immediately before the next scheduled anti-TNF injection/infusion.

3.1. *Anti-TNF Therapy Changes the IgG N-Glycome Composition of Crohn's Disease Patients*

Overall, we identified several significant changes in the levels of derived IgG N-glycan traits between baseline and week 14 of treatment with both IFX and ADA (Figure 1). These changes were observed in CD patients regardless of their response to treatment. The most pronounced change discovered was the decreased abundance of IgG N-glycans lacking galactose (agalactosylation, G0). This change was followed by an increase in the levels of monogalactosylated (G1), digalactosylated (G2) and sialylated glycans (S). However, no statistically significant change was discovered in glycans with core-fucose (F) or bisecting GlcNAc (B) (Table 2). Results for the individual directly measured glycan traits are given in Figure S2.

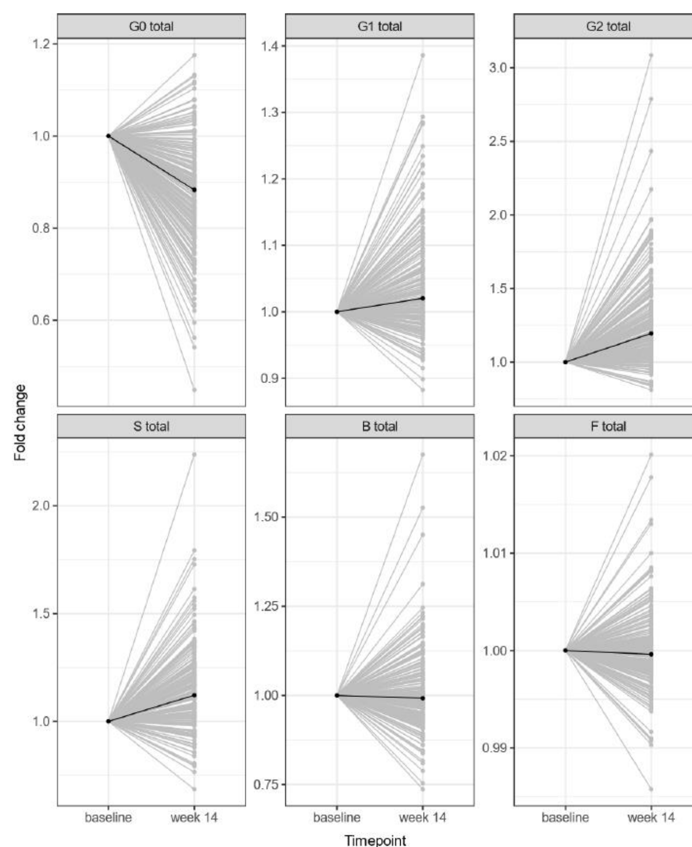

Figure 1. The change in derived IgG N-glycan traits during 14 weeks of anti-TNF treatment (both IFX/ADA). Median glycan values for each time point are bolded. *Y* axis: relative change in glycan value normalised to baseline; *X*-axis: time point (baseline, week 14). Abbreviations: G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

Table 2. Change in derived IgG traits during the 14-week anti-TNF treatment (both IFX/ADA included) of CD patients. Analysis was performed by implementing a linear mixed-effects model, with time as a fixed effect and the individual sample measurement as a random effect. *p*-values were adjusted for multiple testing and considered significant if <0.05 (bold). Effect: model coefficient (slope) represents the change of a derived trait between two time points (expressed in standard deviation units).

Derived Trait	Effect	Standard Error	*p*-Value	Adjusted *p*-Value
G0	**−0.55**	0.04	1.34×10^{-29}	**4.03×10^{-29}**
G1	**0.35**	0.05	1.74×10^{-12}	**2.61×10^{-12}**
G2	**0.60**	0.04	1.51×10^{-32}	**9.07×10^{-32}**
S	**0.46**	0.04	1.88×10^{-21}	**3.75×10^{-21}**
B	0.02	0.04	6.25×10^{-1}	6.25×10^{-1}
F	−0.02	0.04	5.77×10^{-1}	6.25×10^{-1}

Abbreviations: G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

3.2. *Infliximab Causes More Pronounced Changes in IgG N-Glycome than Adalimumab in Crohn's Disease Patients*

We compared the group of patients treated with ADA (n = 98) with a group treated with IFX (n = 100). Both drugs change the composition of IgG N-glycome in the same direction. In other words, the abundance of G0 glycans decreased, and G1, G2 and S glycans increased in their level in the course of 14 weeks, while B and F derived traits remained the same in that particular period (Figure 2). However, IFX caused a significantly greater decrease in the G0 trait and a greater increase in G1, G2 and S derived traits in CD patients compared to the group treated with ADA (Table 3).

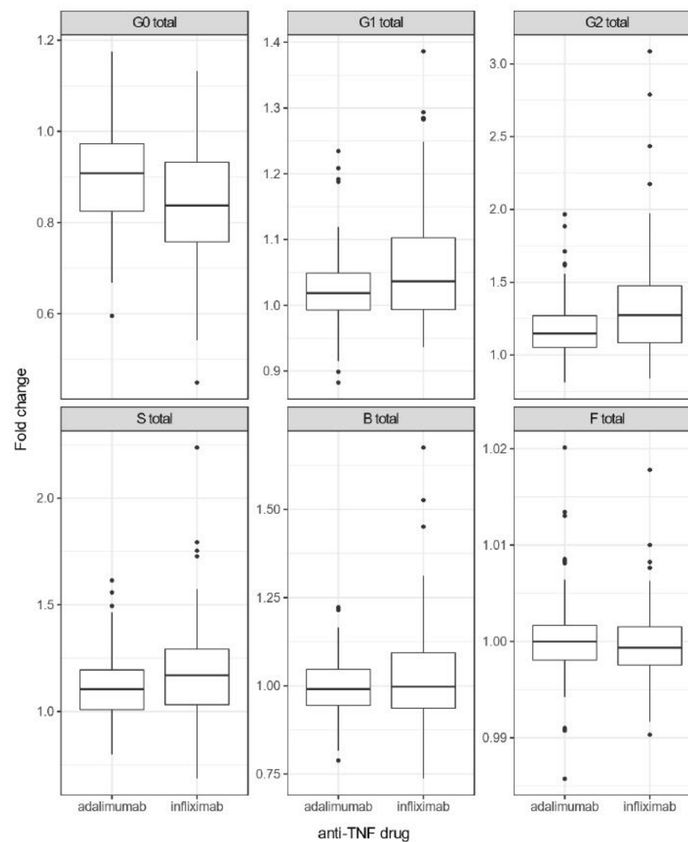

Figure 2. Change in derived IgG N-glycan traits in CD patients during the 14-week anti-TNF treatment with IFX or ADA. Each box represents the 25th to 75th percentiles (interquartile range-IQR). Lines inside boxes stand for the median. The whiskers are the lowest and highest values within boxes $\pm$ 1.5 $\times$ the IQR. Dots are outliers (>1.5 $\times$ IQR). Abbreviations: G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

Table 3. Change in derived IgG N-glycan traits during the 14-week anti-TNF treatment between the groups of CD patients treated with ADA or IFX. Analysis was performed by implementing a linear mixed-effect model. Effect: the difference between two model coefficients (slopes), where each coefficient represents a treatment-specific change of a derived trait between two time points (expressed in standard deviation units). *p*-values were adjusted for multiple testing and considered significant if <0.05 (bold).

Derived Trait	Effect	Standard Error	*p*-Value	Adjusted *p*-Value
G0	**−0.24**	0.08	1.73×10^{-3}	$\mathbf{3.46 \times 10^{-3}}$
G1	**0.28**	0.09	1.08×10^{-3}	$\mathbf{3.23 \times 10^{-3}}$
G2	**0.26**	0.08	8.85×10^{-4}	$\mathbf{3.23 \times 10^{-3}}$
S	**0.18**	0.08	2.34×10^{-2}	$\mathbf{3.51 \times 10^{-2}}$
B	0.05	0.07	4.88×10^{-1}	4.88×10^{-1}
F	−0.06	0.08	4.73×10^{-1}	4.88×10^{-1}

Abbreviations: G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

3.3. Composition of IgG N-Glycome Differs between Groups with Different Anti-TNF Therapy Outcomes

Baseline measurements of IgG N-glycans expressed through derived traits were compared between the four groups with different therapy outcomes established at week 14 (PNR, response; grey zone, remission) (Figure 3). A statistically significant association was observed between therapy outcomes and G0, G1, G2 and S derived IgG N-glycan traits in general (Table 4). However, it is impossible to clearly distinguish the potential predictors of therapy non-response (PNR) based only on the composition of IgG N-glycome at baseline nor observe any particular change trend between the four groups. Pairwise associations of derived IgG N-glycan traits at baseline with therapy outcome are given in Table S1.

Table 4. Associations between derived IgG N-glycan traits at baseline and therapy outcomes established at week 14 in PANTS cohort. *p*-values were adjusted for multiple testing and considered significant if <0.05 (bold).

Derived Trait	*p*-Value	Adjusted *p*-Value
G0	$\mathbf{9.81 \times 10^{-8}}$	$\mathbf{1.96 \times 10^{-7}}$
G1	$\mathbf{2.95 \times 10^{-8}}$	$\mathbf{1.77 \times 10^{-7}}$
G2	$\mathbf{7.98 \times 10^{-8}}$	$\mathbf{1.96 \times 10^{-7}}$
S	$\mathbf{1.31 \times 10^{-3}}$	$\mathbf{1.97 \times 10^{-3}}$
B	9.16×10^{-2}	9.16×10^{-2}
F	7.41×10^{-2}	8.89×10^{-2}

Abbreviations: G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

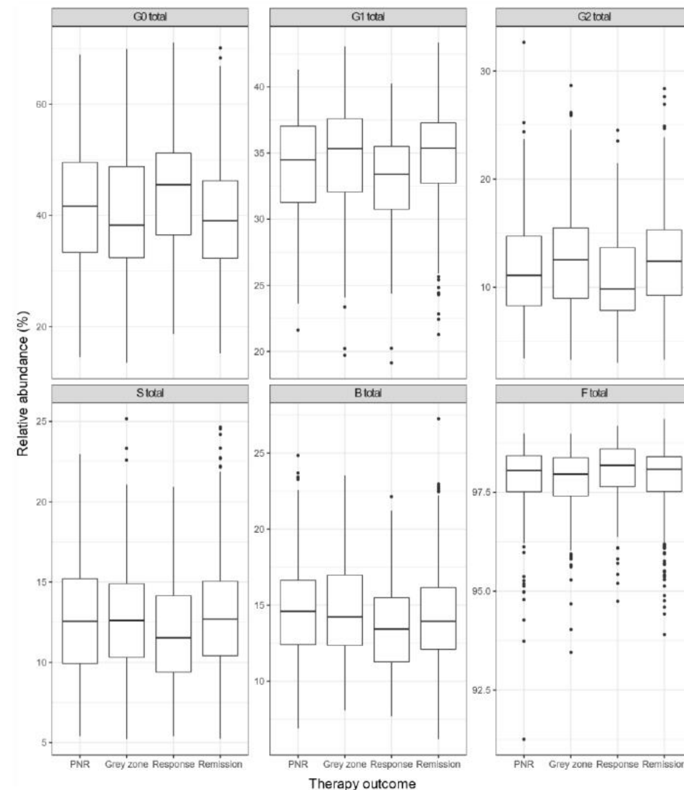

Figure 3. The difference in the relative abundance of derived IgG N-glycan traits at baseline between the groups of Crohn's disease patients with different therapy outcomes (established at week 14). Each box represents the 25th to 75th percentiles (interquartile range—IQR). Lines inside boxes stand for the median. The whiskers are the lowest and highest values within boxes $\pm$ 1.5 $\times$ the IQR. Dots are outliers (>1.5 IQR). Abbreviations: PNR–primary non-responders to IFX/ADA therapy, Response–responders to IFX/ADA therapy, Grey zone–CD patients with an intermediate response between primary non-response and response, Remission–CD patients in remission. G0–agalactosylated glycans, G1–monogalactosylated glycans, G2–digalactosylated glycans, S–mono- and disialylated glycans, B–glycans with bisecting GlcNAc, F–core-fucosylated glycans.

4. Discussion

Crohn's disease is a type of IBD that can affect individuals of any age and may result in severe disability and morbidity since most individuals will develop complications or require surgery within ten years of diagnosis [32]. Therefore, early diagnosis, proper disease control, and, most importantly, induction and remission maintenance are crucial in managing CD. Anti-TNF drugs revolutionised the treatment of CD. However, up to 40% of patients do not respond to therapy (PNRs), almost half of the patients may have a secondary LOR, and roughly 10% may develop adverse drug reactions [33]. Due to its integral role in humoral immune processes, IgG is one of the most studied glycoproteins in health and disease, and its glycan part plays a vital role in the modulation of IgG effector functions [10]. Several studies reported lower overall galactosylation [15,16,23,34] and a decrease in sialylation [15,16,23] of IgG glycome in CD patients compared to the healthy controls, indicating an enhanced pro-inflammatory potential of serum IgG in CD. Here, we

have successfully glycoprofiled the total serum IgG of 198 CD patients to examine the effect of anti-TNF monoclonal antibodies IFX and ADA on the IgG N-glycome during the first 14 weeks of the treatment. We discovered a significant shift in IgG N-glycome composition towards less inflammatory glycosylation patterns regardless of therapy response to IFX and ADA. To elaborate, the relative abundance of agalactosylated glycans was significantly lower 14 weeks after the initiation of anti-TNF therapy. The observed decrease in agalactosylated glycans was accompanied by an increase in monogalactosylated and digalactosylated glycans and an increase in sialylated glycans. At the same time, no significant changes were observed for fucosylated glycans nor glycans with bisecting GlcNAc. These results are accordant to the recently reported longitudinal changes in patients with various chronic inflammatory diseases such as CD, UC, systemic lupus erythematosus (SLE) and arthritic patients treated with anti-TNF therapy as well [23].

Our subsequent finding demonstrated an increased capability of IFX to change the IgG N-glycome towards a less inflammatory pattern compared to ADA. Several previous findings suggested that IFX is more effective in the induction of remission in CD patients than ADA [35,36] and in providing a clinical response, mucosal healing and clinical remission in UC patients [37]. It has also been reported that IFX effectively reduced the concentration of agalactosylated IgG in RA [38] and inflammatory arthritis [24], and which was also associated with clinical improvement for those patients.

It is still not fully understood how anti-TNF therapy affects the concentration of primarily agalactosylated IgG glycoforms and dampens systemic inflammation. However, it is evident that both IFX and ADA have an opposite effect on the IgG N-glycome composition compared to the effect of CD, and it should be further investigated in more detail.

Many studies report that galactosylated N-glycans enhance the anti-inflammatory properties of total serum IgG, as seen in RA patients and pregnancy-induced improvement, where enhanced galactosylation accompanied by lower agalactosylation of total serum IgG was associated with clinical improvement [39], independent of sialylation [40]. Even though the role of sialylation as a switch between pro- and anti-inflammatory properties of IgG is still a matter of debate, it is considered that the presence of sialylated N-glycans contributes to the anti-inflammatory properties of IgG by diminishing IgG affinity for activating receptor FcγRIIIA, reducing ADCC [10] and impairing CDC by decreasing C1q binding [41].

Since therapy response prediction is one of the ultimate requirements in the era of personalised medicine, we searched for differences in the IgG N-glycome composition of 1315 CD patients at baseline that might identify PNR. Patients were divided into four groups (PNR, response, grey zone and remission) based on the therapy outcome established at week 14. We discovered that information about therapy response/outcome at week 14 might be contained in the composition of IgG glycome at baseline. However, even though a statistically significant association between the derived IgG N-glycan traits and different therapy outcomes was detected, we could not specify which group significantly differed from another, nor could we observe any trend of change when focusing on transitioning from one therapy outcome to another. The obtained results suggest that IgG N-glycan composition at baseline does not have a predictive potential to detect future PNRs or other anti-TNF therapy outcomes. Similarly, a recently published paper from our group discovered that it is not possible to provide information about future disease activity based solely on IgG Fc glycan composition at baseline. However, the distinction between the patients in remission and those with active CD was instead contained in the rate of change of IgG Fc N-glycome [23].

In conclusion, we replicated and expanded the knowledge about the effect of longitudinal use of anti-TNF therapy IFX and ADA on the composition of total serum IgG N-glycome in patients with active luminal CD in a large, well-described, prospective, uncontrolled observational PANTS cohort. Even though we detected significant differences in the relative abundance of derived IgG N-glycan traits between the groups with different therapy outcomes, the composition of IgG N-glycome at baseline does not hold a signifi-

cant predictive power to detect PNRs among CD patients. Therefore, further studies are warranted in this direction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom13060954/s1, Figure S1: Representative HILIC-UHPLC-FLD N-glycoprofile of total serum IgG; Figure S2: Changes in the relative abundance of directly measured IgG N-glycans in CD patients during the anti-TNF treatment (both IFX and ADA included); Table S1: Pairwise associations of derived IgG N-glycan traits at baseline with therapy outcome; Table S2: PANTS Consortium.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by South West-Cornwall & Plymouth Research Ethics Committee (Research Ethics Committee reference: 12/SW/0323, date of approval: 23 January 2013) in January 2013.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding authors.

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Conflicts of Interest: G.L. is the founder and CEO of Genos Ltd., a private research organization specializing in high-throughput glycomic analysis, and has several patents in this field. M.H., F.V., H.D. and I.T.A. are employees of Genos Ltd., Osijek, Croatia, G.L., is the founder and owner of Genos Glycoscience Ltd., Osijek, Croatia —a spin-off of Genos Ltd. that commercializes its scientific discoveries. F.V. and I.T.A. are employees of Genos Glycoscience Ltd. G.L. is the founder and CSO of GlycanAge Ltd.—the company offering the first glycan-based test for biological age. S.L. reports non-financial support from Pfizer outside the submitted work. J.R.G. reports grants from F. Hoffmann-La Roche AG, grants from Biogen Inc, grants from Celltrion Healthcare, grants from Galapagos NV and non-financial support from Immundiagnostik outside the conduct of the study. T.A. reports grants and non-financial support from F. Hoffmann-La Roche AG, grants from Biogen Inc, grants from Celltrion Healthcare, grants from Galapagos NV and non-financial support from Immundiagnostik; personal fees from Biogen inc, grants and personal fees from Celltrion Healthcare, personal fees and non-financial support from Immundiagnostik, personal fees from Takeda, personal fees from ARENA, personal fees from Gilead, personal fees from Adcock Ingram Healthcare, personal fees from Pfizer, personal fees from Genentech and non-financial support from Tillotts, outside the submitted work.

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7.2. ZNANSTVENI RAD 2

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Article

Distinct Longitudinal Changes in Immunoglobulin G N-Glycosylation Associate with Therapy Response in Chronic Inflammatory Diseases

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Abstract: Immunosuppressants and biologicals are widely used therapeutics for various chronic inflammatory diseases (CID). To gain more detailed insight into their downstream effects, we examined their impact on serum immunoglobulin G (IgG) glycosylation. We analyzed IgG subclass-specific fragment crystallizable (Fc) N-glycosylation in patients suffering from various CID using the LC-MS approach. Firstly, we compared IgG Fc N-glycosylation between 128 CID patients and 204 healthy controls. Our results replicated previously observed CID-related decrease in IgG Fc galactosylation (adjusted p-value range 1.70×10^{-2}–5.95×10^{-22}) and sialylation (adjusted p-value range 1.85×10^{-2}–1.71×10^{-18}). Secondly, to assess changes in IgG Fc N-glycosylation associated with therapy and remission status, we compared 139 CID patients receiving either azathioprine, infliximab, or vedolizumab therapy. We observed an increase in IgG Fc galactosylation (adjusted p-value range 1.98×10^{-2}–1.30×10^{-15}) and sialylation (adjusted p-value range 3.28×10^{-6}–4.34×10^{-18}) during the treatment. Furthermore, patients who reached remission displayed increased Fc galactosylation levels (p-value range 2.25×10^{-2}–5.44×10^{-3}) in comparison to patients with active disease. In conclusion, the alterations in IgG Fc glycosylation and the fact these changes are even more pronounced in patients who achieved remission, suggest modulation of IgG inflammatory potential associated with CID therapy.

Keywords: chronic inflammatory diseases; inflammatory bowel disease; IgG glycosylation; response; personalized medicine; autoimmune diseases

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1. Introduction

Chronic inflammatory diseases (CID) affect various organs such as the intestinal tract, skin, or joints and represent a common disease group of the immune system with rising incidence in Western industrialized countries. CID arise at the interplay of genetic risk, individual lifestyle, and environmental factors. Chronicity, long-term debilitating consequences due to anatomical destruction of the affected organ systems (mucosal atrophy, joint destruction, scar formation, intestinal failure), and the lack of causative treatments are among the factors that explain the high individual burden of disease.

Since there is no curative therapy available for CID, treatments are mostly focused on reducing symptoms and inflammation, and have to be given lifelong [1]. The current mainstay in the therapy of moderate to severe Crohn's disease (CD) and ulcerative colitis (UC) is the use of targeted therapies (anti-TNF, anti-IL-12/23, anti-α4β7 integrin), so-called biologics, to intercept the chronic perpetuation of mucosal inflammation. However, the long-term efficacy of biologics is restricted due to primary and secondary loss of response in a large proportion of patients [2]. Whereas primary non-response describes a refractory state to a given drug at treatment induction, secondary loss of response develops in patients over time after having initially undergone therapy-induced amelioration of bowel inflammation. Despite growing evidence of host and microbial factors involved in the development of primary non-response to, e.g., anti-TNF treatment, a thorough understanding of the underlying mechanisms of developing secondary loss of response is scarce [3–5]. With respect to anti-TNF therapy, the development of neutralizing anti-drug antibodies against anti-TNF has shown to substantially contribute to secondary loss of response, leading to proactive therapeutic drug monitoring as a key concept in anti-TNF therapy across several CID [6,7]. However, with regard to different treatment modalities, such as anti-α4β7 integrin or anti-IL12/23 treatment, the understanding of underlying molecular mechanisms contributing to primary or secondary loss of response is limited [8].

The majority of cell surface and secreted proteins are N-glycosylated, while the absence of N-glycosylation results in embryonic death in murine models [9]. Glycosylation affects protein structural and functional features and is thus involved in the regulation of numerous biological processes [10,11]. There is no genetic template for glycosylation. Instead, genetic and environmental factors influence glycan synthesis in complex biosynthetic pathways. Consequently, the structure of N-glycans depends, among other things, on the expression and activity of enzymes and the availability of their substrates. Changes in glycan levels can be seen in different physiological and pathological states. Numerous diseases such as inflammatory diseases, cancers, and autoimmune diseases display extensive changes in protein N-glycosylation patterns [12,13].

Immunoglobulin G (IgG) is the most abundant antibody in plasma and is a key molecule of the humoral immune response, linking innate and adaptive immunity through its multiple roles. IgG can be divided into four subclasses: IgG1, IgG2, IgG3, and IgG4 with different affinities towards Fc and other receptors, thus distinctive effector functions [14]. Each IgG molecule contains a conserved N-glycosylation site on a fragment crystallizable (Fc); and a sporadic N-glycosylation site in the Fab region resulting from somatic hypermutation events [14]. The most complex IgG Fc glycan contains 12 monosaccharide units and represents a biantennary digalactosylated and monosialylated structure with bisecting β(1,4) *N*-acetylglucosamine (GlcNAc) and an α(1,6) fucose attached to the core GlcNAc. The remaining IgG glycans correspond to this structure with the lack of one or more sugar units.

Changes in Fc glycans alter IgG conformation and interactions with different receptors, which define specific downstream immune responses [15,16]. Sweeping changes in IgG inflammatory functions can be observed with various IgG glycan patterns [17,18]. For example, low levels of galactosylation and core fucosylation are associated with higher inflammatory potential, while the presence of core fucose and sialic acids fosters anti-inflammatory functions of IgG [19,20]. Pro-inflammatory IgG glycan patterns—the most striking effect being a lowered proportion of galactosylated structures—were observed in a wide range of chronic inflammatory diseases (CID), including various autoimmune diseases such as Crohn's disease (CD), ulcerative colitis (UC), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [20–23]. Moreover, IgG glycan patterns also associate with UC, CD, and SLE severity and differ between CD and UC patients [22,24]. There are several mechanisms for how deficiency of terminal galactoses on IgG can act in a pro-inflammatory manner. Proposed mechanisms include activation of complement via the alternative pathway, or via the lectin pathway after binding to mannose-binding lectin

[25,26]. However, the exact mechanism of action, through which the lack of terminal galactoses activates pro-inflammatory IgG properties, is still a subject of debate [20,27]. When it comes to the effects of high IgG Fc galactosylation levels, immune complexes can more effectively activate the anti-inflammatory cascade, through binding to the FcγRIIB receptor, if galactoses are present on IgG Fc glycan [28]. In a similar way, a high level of IgG galactosylation is required for immune complexes to inhibit the pro-inflammatory activity of the C5a complement component [29]. On the other hand, a lack of galactose can also act in a pro-inflammatory way. The presence of galactose on IgG Fc increases complement-dependent cytotoxicity (CDC) through the classical pathway of complement activation, while elevated IgG galactosylation levels also activate antibody-dependent cellular cytotoxicity (ADCC) through enhanced binding of galactosylated IgG to activating FcγRs [30,31].

The aims of this study were 1) to replicate patterns in IgG glycosylation previously reported in patients suffering from chronic inflammatory diseases compared to control subjects, 2) to examine the impact of targeted therapies on IgG Fc glycan patterns over time, and 3) to identify potential patterns of IgG Fc glycosylation that are associated with clinical response to targeted therapies in CID.

2. Results

The first goal of this study was to replicate previously reported changes in IgG glycosylation patterns observed in CID patients. For that reason, we compared IgG Fc glycosylation of CID patients with healthy controls. Secondly, we examined the impact of immunosuppressive and biological therapies on IgG Fc glycosylation in patients with CID. To answer this question, we analyzed IgG Fc N-glycan profiles in CID patients on azathioprine and biological therapies: anti-TNF and anti-$\alpha 4\beta 7$ integrin monoclonal antibodies—infliximab and vedolizumab, respectively. Patients originated from three independent cohorts and were followed up for 104 (Cohort 1), 30 (Cohort 2), or 14 (Cohort 3) weeks. Thirdly, we compared the extent of modification of IgG glycan profiles between patients in remission and patients with active disease to evaluate the potential of IgG glycans as predictors of disease activity.

2.1. IgG Fc Galactosylation and Sialylation Are Lower in Chronic Inflammatory Diseases Patients Compared to Controls

In order to replicate previously reported changes in IgG glycosylation in chronic inflammatory diseases, we compared CID patients to a control group. At baseline (prior to initiation of therapy), patients from Cohort 1 suffering from UC (n=12) and CD (n=32) displayed considerable shifts in IgG Fc glycan profiles compared to controls (n=204; Figure 1). The nature of observed changes in IgG Fc glycans with the disease was similar for all measured IgG subclasses (Table S1). Agalactosylated glycan species (represented by G0F glycoform) increased, while mono- and di-galactosylated glycans (represented by G1F and G2F glycoforms, respectively) decreased in all subclasses. Changes in IgG4 galactosylation traits showed smaller effect sizes compared to the other two subclass clusters, and the difference in IgG4 G0F abundance in CD patients compared to controls did not reach statistical significance. IgG Fc sialylation levels (G2FS) showed a significant decrease with comparable effect size across all subclasses in both UC and CD patients. Therefore, overall galactosylation and sialylation levels decreased in CID patients compared to the control cohort.

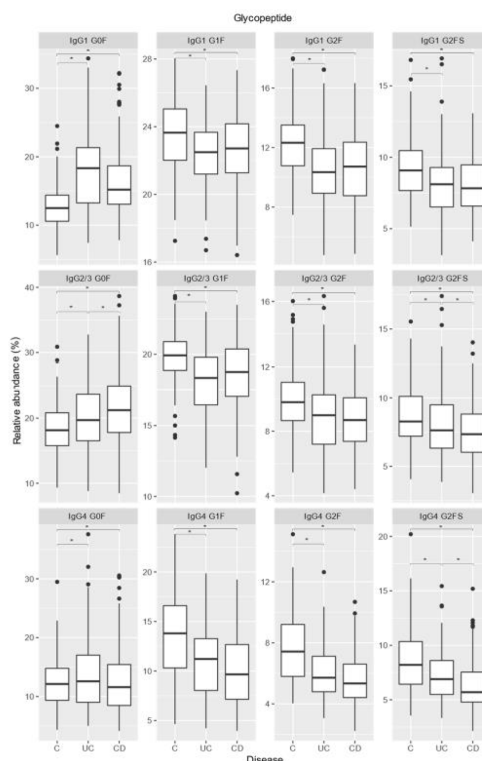

Figure 1. IgG Fc-glycan patterns in controls, patients suffering from ulcerous colitis, and patients suffering from Crohn's disease (Cohort 1 at baseline) for all IgG subclasses. Each box represents the 25th to 75th percentiles (interquartile range—IQR). Lines inside boxes stand for the median. The whiskers are the lowest and highest values within boxes ±1.5 × the IQR. Dots are outliers (>1.5 × IQR). Significant differences ($p<0.05$) are marked with an asterisk *. Normalized size effect and *p*-values are given in Table S1. C—controls; UC—ulcerous colitis; CD—Crohn's disease.

Differences in IgG Fc glycan profiles were also observed between the two disease entities: Compared to UC patients' glycan profiles, CD patients in the IgG2/3 subclass showed an increase in agalactosylation levels (G0F) and decreased presence of terminal sialic acid (G2FS). CD patients also showed a decrease in terminal sialic acid (G2FS) in the IgG4 subclass compared to UC patients (Figure 1, Table S1).

In Cohort 1, similar differences in IgG glycosylation, compared to controls, were observed when, besides UC (n=12) and CD (n=32), patients suffering from other chronic inflammatory diseases were taken into account. Namely, patients with several types of arthritis (psoriatic arthritis—PsA, n=22; seropositive rheumatoid arthritis—RA+, n=37; seronegative rheumatoid arthritis—RA-, n=19; and systemic lupus erythematosus—SLE, n=6) also showed lower IgG Fc galactosylation and sialylation when compared to controls (Table S2, Table S3, Table S4).

2.2. IgG Fc Galactosylation and Sialylation Levels Increase during Therapy

Having shown that CID patients display a significantly altered IgG glycosylation profile in comparison to controls, we next aimed to understand whether a targeted biologic therapy dynamically affects IgG Fc glycosylation patterns over time. The statistical

analysis has shown that during therapy (Cohort 1—26 weeks, Cohort 2—30 weeks, and Cohort 3—14 weeks) IgG glycan profiles of 34 UC and 113 CD patients changed over time regardless of their clinical response (Figure 2, Table S5).

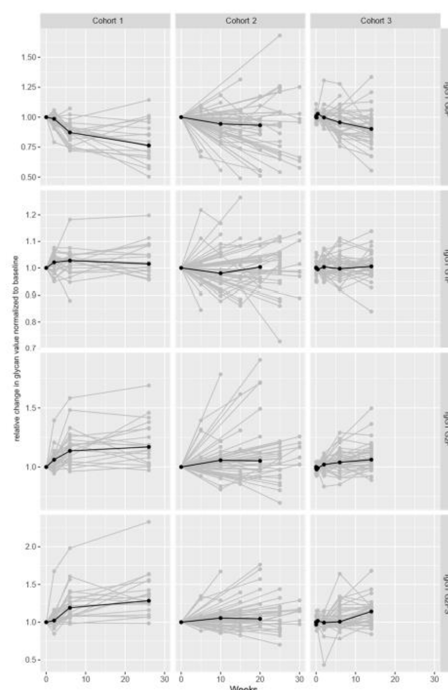

Figure 2. Changes in IgG1 Fc glycan profiles during treatment in three cohorts of UC and CD patients. Median glycan values for each time point are bolded. Y-axis: relative change in glycan value normalized to baseline (1st timepoint); X-axis: duration of follow-up (weeks). Results of meta-analysis are shown in Table 1, while the results of statistical analysis for individual cohorts are shown in Table S5.

A significant change was detected for most of the observed 12 glycan traits, with all three cohorts showing very similar patterns of change. In all cohorts, galactosylation and sialylation levels increased (decrease in G0F accompanied by an increase in G2F and G2FS) for IgG1 and IgG2/3. For the IgG4 subclass, this pattern was observed only in Cohort 1. In the other two cohorts, only some changes reached statistical significance: a decrease in G0F in Cohort 2, and a decrease in G0F accompanied by an increase in G2FS in Cohort 3.

Meta-analysis of all three cohorts confirmed the change of IgG Fc glycan pattern over time on therapy. IgG Fc galactosylation and sialylation levels of all three IgG subclasses showed a significant increase in patients on therapy. The increase in galactosylation was represented by a decrease in G0F accompanied by an increase in G2F structures, while an increase in G2FS structure represented increased sialylation (Table 1).

To further verify whether longitudinal changes in IgG glycosylation patterns are a unifying feature of targeted therapies in all chronic inflammatory disorders, we validated our findings on patients with psoriatic arthritis (PsA, *n*=22), seronegative rheumatoid arthritis (RA-, *n*=19), seropositive rheumatoid arthritis (RA+, *n*=37), ankylosing spondylitis

(AS, *n*=17), UC (*n*=12), and CD (*n*=32) that were monitored after therapy initiation (Cohort 1) (Figure S1). Within 26 weeks of therapy, CD patients and all arthritis patients showed a significant change in IgG1 and IgG2/3 Fc glycosylation (Table S6, Table S7). In addition, several Fc glycans of the IgG4 subclass significantly changed with therapy for AS, CD, RA-, and RA+ patients (Table S8). Interestingly, in ulcerative colitis patients, Fc glycosylation patterns did not significantly change in any IgG subclass.

Table 1. Changes in IgG Fc glycan profiles during therapy in three cohorts of UC and CD patients: meta-analysis (Cohort 1—26 weeks, Cohort 2—30 weeks, and Cohort 3—14 weeks on therapy). *p*-values were adjusted for multiple testing and considered significant if <0.05 (bold). Effect: model coefficient (slope) representing the weekly change of a glycan trait (expressed in standard deviation units). Changes in IgG1 Fc glycan patterns during treatment for individual cohorts are visualized in Figure 2 and results of statistical analysis for individual cohorts are shown in Table S5.

IgG subclass	Glycan	Effect	Standard error	*p*-value	Adjusted *p*-value
IgG1	G0F	**-2.93×10^{-2}**	4.00×10^{-3}	2.41×10^{-13}	**7.24×10^{-13}**
	G1F	7.98×10^{-4}	3.41×10^{-3}	8.15×10^{-1}	8.15×10^{-1}
	G2F	**2.25×10^{-2}**	3.12×10^{-3}	5.64×10^{-13}	**1.35×10^{-12}**
	G2FS1	**2.89×10^{-2}**	3.23×10^{-3}	3.62×10^{-19}	**4.34×10^{-18}**
IgG2/3	G0F	**-2.10×10^{-2}**	3.06×10^{-3}	7.74×10^{-12}	**1.55×10^{-11}**
	G1F	4.25×10^{-3}	3.33×10^{-3}	2.02×10^{-1}	2.20×10^{-1}
	G2F	**2.09×10^{-2}**	2.56×10^{-3}	3.25×10^{-16}	**1.30×10^{-15}**
	G2FS1	**2.16×10^{-2}**	2.48×10^{-3}	2.76×10^{-18}	**1.66×10^{-17}**
IgG4	G0F	**-1.95×10^{-2}**	3.13×10^{-3}	5.03×10^{-10}	**8.62×10^{-10}**
	G1F	**-6.43×10^{-3}**	2.68×10^{-3}	1.65×10^{-2}	**1.98×10^{-2}**
	G2F	**1.19×10^{-2}**	3.59×10^{-3}	9.35×10^{-4}	**1.25×10^{-3}**
	G2FS1	**1.48×10^{-2}**	3.12×10^{-3}	2.19×10^{-6}	**3.28×10^{-6}**

2.3. Pronounced IgG Fc Galactosylation Associates with Clinical Remission in CID Therapy

Having shown that targeted therapy changes the IgG Fc glycosylation patterns across all CID, and irrespectively of the therapeutic outcome, we next aimed to identify specific IgG glycosylation patterns that are indicative of therapy response in CID. For that purpose, we further analyzed glycosylation patterns in 34 UC and 113 CD patients based on their clinical remission status. We observed that patients in Cohort 2 who reached clinical remission displayed a starker increase in IgG2/3 subclass digalactosylation (G2F) since therapy initiation compared to patients with active disease. A similar, but even more pronounced change was independently validated in Cohort 3, where patients in remission showed a starker increase in digalactosylation and sialylation accompanied by a starker decrease in agalactosylation (G0F) of the IgG2/3 subclass (Figure 3, Figure S2, Figure S3, Table S9).

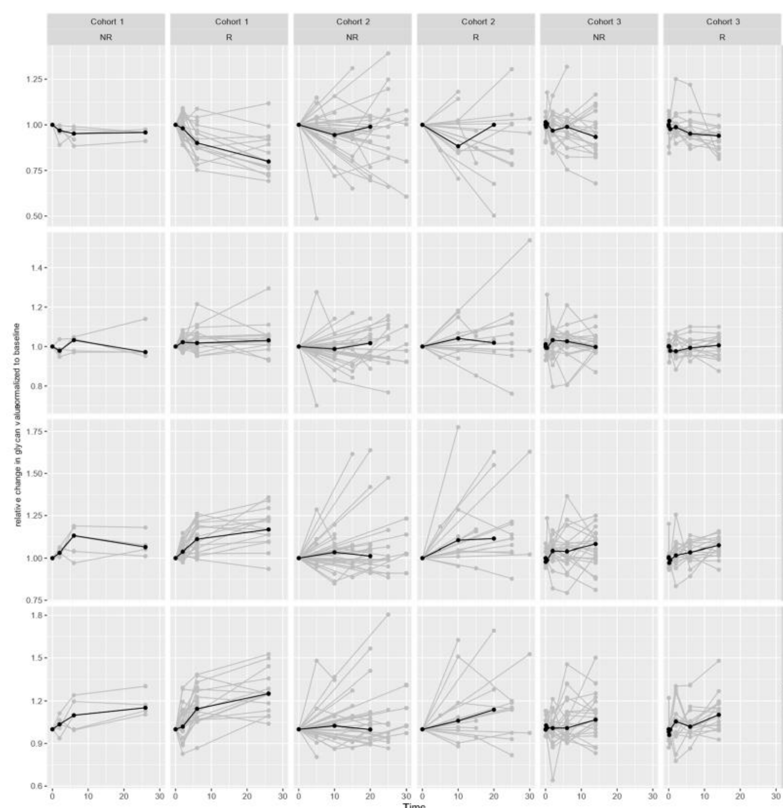

Figure 3. Changes in IgG2/3 Fc glycan profiles during treatment in CID (UC and CD) patients that entered remission (R) vs. patients with active disease (NR) in three cohorts. Median glycan values for each time point are bolded. Y-axis: relative change in glycan value normalized to baseline (1st timepoint); X-axis: duration of follow-up (weeks). Associations of IgG Fc glycan profiles with disease activity during treatment, for each patient cohort separately, are shown in Table S9, while the results of meta-analysis of all three cohorts are presented in Table 2.

In Cohort 1, there were no significant differences in the rate at which IgG Fc glycan patterns change between patients that achieved remission and patients with active disease. A meta-analysis of all three cohorts revealed two substantial differences in the rate of IgG Fc glycan profiles changes between patients who did vs. patients who did not achieve remission: patients who entered remission displayed a more pronounced increase in digalactosylation of IgG2/3 and a more pronounced decrease in agalactosylation of IgG4 (Table 2, Table S9).

We also found that IgG glycosylation levels measured at baseline do not provide information on future disease activity, i.e., response to therapy. Namely, there is no statistically significant difference between patients who enter vs. patients who do not enter remission in their IgG Fc glycan profiles measured at therapy initiation (Table S10).

Table 2. Associations between the rate of change of IgG Fc glycan profiles and disease activity during UC and CD treatment—meta-analysis of three cohorts (Cohort 1—26 weeks, Cohort 2—30 weeks, and Cohort 3—14 weeks). Effect: the difference between two model coefficients (slopes), where each coefficient represents a group-specific weekly change of glycan trait (expressed in standard deviation units). p-values were adjusted for multiple testing and considered significant if <0.05

(bold). Changes in IgG glycan patterns with time on therapy in patients with active vs. inactive disease are visualized in Figure 3, Figure S2, Figure S3, and results of statistical analysis for individual cohorts are shown in Table S9.

IgG subclass	Glycan	Effect	Standard error	*p*-value	Adjusted *p*-value
IgG1	G0F	-1.45×10^{-2}	7.61×10^{-3}	5.61×10^{-2}	1.35×10^{-1}
	G1F	-3.86×10^{-3}	6.50×10^{-3}	5.52×10^{-1}	6.02×10^{-1}
	G2F	8.27×10^{-3}	6.25×10^{-3}	1.85×10^{-1}	3.18×10^{-1}
	G2FS1	7.89×10^{-3}	6.38×10^{-3}	2.16×10^{-1}	3.24×10^{-1}
IgG2/3	G0F	-1.36×10^{-2}	5.58×10^{-3}	1.50×10^{-2}	6.00×10^{-2}
	G1F	1.24×10^{-2}	5.75×10^{-3}	3.08×10^{-2}	9.23×10^{-2}
	G2F	**1.81×10^{-2}**	5.16×10^{-3}	4.54×10^{-4}	**5.44×10^{-3}**
	G2FS1	8.14×10^{-3}	5.34×10^{-3}	1.28×10^{-1}	2.55×10^{-1}
IgG4	G0F	**-1.69×10^{-2}**	5.85×10^{-3}	3.75×10^{-3}	**2.25×10^{-2}**
	G1F	-5.56×10^{-3}	5.75×10^{-3}	3.34×10^{-1}	4.00×10^{-1}
	G2F	6.82×10^{-4}	7.43×10^{-3}	9.27×10^{-1}	9.27×10^{-1}
	G2FS1	7.06×10^{-3}	6.61×10^{-3}	2.85×10^{-1}	3.80×10^{-1}

3. Discussion

In this study, we analyzed subclass-specific IgG Fc glycosylation profiles in CID patients treated with immunosuppressive and biological therapies. We replicated previously published alterations of IgG glycan profiles in UC, CD, arthritis, and SLE patients when compared to controls [24,32]. Furthermore, in UC, CD, and arthritis patients on azathioprine and biological therapy, we observed changes in IgG Fc glycosylation which reflect a less inflammatory IgG glycopattern: an increase in the proportion of galactosylated and sialylated structures. Moreover, UC and CD patients undergoing remission showed a more pronounced increase in anti-inflammatory IgG Fc glycan levels when compared to non-remission patients.

The most pronounced change we saw in IgG Fc glycan patterns, which occurred in CID patients at baseline, was a decrease in IgG galactosylation levels. Such a shift in IgG glycan profiles towards lower IgG galactosylation levels, which is generally considered a pro-inflammatory property, can be observed in various inflammatory and autoimmune diseases and conditions [20,22,33]. There are several proposed mechanisms through which the absence of galactose on IgG glycans can act in a pro-inflammatory manner; however, these are still subject to debate [26,28,34]. Besides changes in galactosylation levels, we also detected a decrease in sialylation in all IgG subclasses of CID patients. Sialylation of IgG glycans is considered a switch between pro- and anti-inflammatory activity of the IgG molecule through modulation of binding to activating FcγRs, lectin (type II) receptors, and the C1q complement component [34,35]. However, these reports remain controversial and exact mechanisms remain elusive [20,36]. In general, a decrease in IgG galactosylation and sialylation when compared to control subjects is considered a potential modulator of the immune activation threshold, reflecting a general pro-inflammatory environment in CID patients, which was observed in numerous studies (reviewed in [20,37]).

The effect of immunosuppressive and biological therapy on IgG Fc glycan patterns in CID patients was examined by monitoring changes in Fc glycan levels during treatment. The most pronounced change was in the galactosylation level, which significantly increased during treatment in all cohorts. Interestingly, the direction of this change was irrespective of the remission status of patients. We observed the same trends in various arthritis patients on anti-TNF therapy, indicating that this change is neither disease nor drug specific. Similar shifts in glycan patterns were observed in several published longitudinal studies showing recovery of serum IgG galactosylation levels in rheumatoid and

psoriatic arthritis patients treated with immunosuppressive and biological therapy [38–41]. Recently published studies demonstrated changes in glycan profiles of IgG or total plasma proteins in CD and UC patients and attributed glycan profiles to medication or disease course [24,32,42].

There are several lines of evidence showing that the observed glycosylation profiles could be attributed to the suppressed systemic inflammation resulting from biological or azathioprine therapy. Although the mechanisms of changes in IgG Fc glycosylation following CID therapy are still unclear, the majority of treatments included in this study show a profound impact on the fate of B cell lineage. Azathioprine in high doses suppresses B cell activity and differentiation [43,44]. Of the used biologicals, anti-TNF therapy was found to restore the number of circulating B cells in patients on therapy close to levels observed in healthy controls [45]. Furthermore, it has been observed that anti-TNF treatment changed the expression of glycosyltransferases in bovine synovitis [46]. On the other hand, blocking of the $\alpha4\beta7$ integrin pathway with vedolizumab did not affect the level of plasma cells [8,47].

The link between IgG glycosylation and inflammation is demonstrated by dynamic changes in IgG galactosylation levels in female RA patients during and after pregnancy. During pregnancy, RA patients experience hormone-related remission of disease and an increase in IgG galactosylation to near-normal levels. However, in the postpartum period, the disease relapses accompanied by a decrease in IgG galactosylation to the levels before pregnancy [48,49].

Apart from changes in galactosylation, we have also detected an increase in sialylation levels in patients on therapy, which is thought to have a protective effect against inflammation. This observation matches IgG glycan changes on native self-targeting autoantibody complexes observed in RA patients showing the reduced inflammatory potential of IgG [50]. Therefore, observed changes of IgG Fc glycan profiles during therapy and regardless of patients' clinical response status suggest a reduced inflammatory potential of IgG, likely as a consequence of suppressed therapy-associated systemic inflammation [20].

We also showed changes in IgG glycan profiles of UC and CD patients in remission induced by azathioprine and biological drugs. Although this change was not detected in Cohort 1, the metanalysis of all three cohorts together showed that patients in remission displayed a more pronounced increase in IgG2/3 digalactosylation levels and a more pronounced decrease in IgG4 agalactosylation levels when compared to patients with active disease. Our findings are supported by a smaller previous study of 19 CD patients monitored before and two weeks after initiation of anti-TNF therapy, where a significant increase in galactosylation was found in responders even at baseline [51]. The observed increase in galactosylation suggests a further reversal of IgG glycosylation towards a healthy non-inflammatory IgG glycan profile in patients in remission. Based on the presented data, longitudinal monitoring of IgG Fc glycosylation can serve as an additional indicator of the remission status.

Since the early prediction of therapy response and disease outcome is an essential therapeutic goal, we assessed the predictive power of the IgG glycosylation pattern at baseline. Based on IgG Fc glycan levels at baseline, we were not able to segregate patients based on their future remission status.

We acknowledge several limitations of our study: The three included patient cohorts are not completely matched in terms of therapy and nosology. However, these are longitudinal cohorts that were analyzed independently of each other, and the initial status of a single cohort was used as its baseline. Furthermore, we did not analyze every specific diagnosis and therapeutic modality separately, as a small number of patients in these groups and an increased number of tests would have led to inadequate statistical power to detect relevant effects.

In conclusion, we replicated differences in IgG glycosylation patterns between CID patients and control subjects. Moreover, we further expanded the knowledge about the

CID therapy effect on IgG glycome composition, which implies that therapy-driven shifts in IgG Fc glycan patterns are neither disease nor drug specific. Finally, we discovered differences in the rate of IgG Fc glycome change between patients who eventually entered remission vs. patients with active CID. Altogether, our findings show extensive modulation of IgG inflammatory potential driven by the applied medications.

4. Materials and Methods

4.1. Patient Samples

Three longitudinal cohorts of CID patients were used all naïve to therapy (**Table S11**). Patients were fully informed as to the purpose of the study and signed a written consent in accordance with the Helsinki declaration. The ethics committee of the Christian-Albrechts-Universität zu Kiel approved the study (A 124/14).

Cohort 1 comprised 146 patients with CD, UC, SLE, or various arthritic diseases: psoriatic arthritis (PsA), seronegative rheumatoid arthritis (RA-), seropositive rheumatoid arthritis (RA+), ankylosing spondylitis (AS). Patients were on anti-TNF therapy (infliximab), and blood samples were collected at multiple time points after initiation of therapy (up to 104 weeks). For easier comparison with other cohorts in this study, only the four earliest time points (baseline, 2, 6, and 26 weeks) were used for statistical analysis. AS samples were not used in baseline comparison due to missing baseline timepoints. SLE patients were not longitudinally analyzed through their observation period due to a small patient number.

Two hundred and four healthy participants served as controls for Cohort 1 patients at baseline.

Cohort 2 comprised 61 patients treated with azathioprine and anti-TNF. Blood samples were collected at baseline and two of the four additional time points during follow-up: 2 weeks, 6 weeks, 14 weeks, and 30 weeks.

Cohort 3 included 42 patients who were prescribed infliximab or vedolizumab. Blood samples were collected by venipuncture at baseline, 2 weeks, 6 weeks, and 14 weeks after commencement of therapy.

4.2. Immunoglobulin G Isolation

IgG was isolated from plasma samples using protein G affinity chromatography as described previously [52]. In short, 100 μL plasma from each sample was centrifuged at 1620× *g* and filtered through a 0.45 μm pore filter plate to remove aggregated lipids. Filtered plasma was then diluted eight times with an in-house prepared 1× phosphate-buffered saline (PBS) and loaded onto a 96-well protein G monolithic plate (BIA Separations, Ajdovščina, Slovenia). Each well of the monolithic plate was washed three times with 2 mL of 1× PBS and IgG was eluted from protein G using 1 mL of 0.1M formic acid (Merck, Darmstadt, Germany). The eluate was immediately neutralized by adding 170 μL of 1M ammonium bicarbonate (Across Organics, Pittsburgh, PA, USA). IgG concentrations were measured using NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA).

4.3. IgG Trypsin Digestion and Solid-Phase Extraction of Glycopeptides

IgG was digested with trypsin and the obtained glycopeptides were purified as described before with slight changes [53]. In brief, 0.1 μg of sequencing grade trypsin (Promega, Fitchburg, WI, USA) was added to 40 μL (on average ~20 μg) of isolated IgG and incubated overnight at 37°C. The digest was then diluted ten times using 0.1 % (v/v) trifluoroacetic acid (TFA) and loaded to C18 ec sorbent (Macherey-Nagel, Düren, Germany). The samples were washed three times with 200 μL of 0.1% TFA and eluted from the phase with 20% liquid chromatography-mass spectrometry (LC-MS) grade acetonitrile (Honeywell, Morris Plains, NJ, USA). The eluted glycopeptides were vacuum-dried and redissolved in 80 μL of ultrapure water.

4.4. Liquid Chromatography-Mass Spectrometry Analysis of IgG Fc Glycopeptides

IgG Fc glycopeptides were separated on an Acquity M-class chromatographic system (Waters, Milford, MA, USA), which was coupled to a Compact mass spectrometer (Bruker, Bremen, Germany) using a CaptiveSpray source equipped with a nanoBooster. Glycopeptides (6 μL) were loaded onto PepMap 100 C8 (5 mm × 300 μm i.d; Thermo Fisher Scientific, Waltham, MA, USA) in a mobile phase A (0.1 % TFA) at a flow rate of 40 μL/min. IgG subclasses were separated on a C18 analytical column (150 mm × 100 μm i.d., 100 Å; Advanced Materials Technology, Wilmington, DE) in a 3.5-min-long gradient from 16% to 25% of mobile phase B (80% can in 20% solvent A) at a flow rate of 1 μL/min. The column temperature was maintained at 30°C. Acetonitrile vapors were introduced directly into the source using a nanoBooster to increase the ionization of glycopeptides. Mass spectra were recorded using an *m/z* range of 600–1900 with 0.5 Hz and averaging two sequential scans. The collision and quadrupole energies were set to 4 eV, while transfer time and pre-pulse storage were set to 110 μs and 10 μs, respectively. The nanoACQUITY UPLC system was controlled by MassLynx software version 4.1 (Waters), while the mass spectrometer was controlled by HyStar software version 4.1.2 (Bruker).

4.5. Data Processing

Data were extracted using LacyTools v1.0.1 software [54]. The peak intensity of exact mass was adjusted to a predefined value for each of the separated subclasses for spectra alignment. The three most intensive glycoforms (G0F, G1F, and G2F) were used for alignment. The time window to search for maximal peak intensity was set to 55 s and the mass window was 0.2 *m/z*. Only samples with a minimum of five analytes per sample with signal-to-noise (S/N) higher than nine were aligned. In Caucasian populations, the IgG2 and IgG3 glycopeptides have the same peptide sequence, resulting in the ions of the same *m/z*. Therefore, these two subclasses cannot be separated by LC-MS [55]. Fc glycopeptides of the four subclasses were therefore chromatographically separated into only three clusters: IgG1, IgG2/3, and IgG4. In the time window of 30 s around the defined time, spectra were summed, and a sum spectrum was created for each of the separated IgG subclasses. Three calibrants with S/N larger than nine were used for the calibration of sum spectra with a calibration window of 0.2 Da. Integration of areas containing 99% of the isotopic pattern was performed, followed by summing of doubly and triply charged species originating from a single analyte. Signals corresponding to agalactosylated (G0F), monogalactosylated (G1F), digalactosylated (G2F), and sialylated (G2FS) Fc glycans carrying core fucose were extracted.

4.6. Statistical Analysis

Normalization and batch correction were performed on the LC-MS glycopeptide data to remove experimental variation from the measurements. Normalization by total area was performed to make measurements across samples comparable. Prior to the batch correction, normalized glycan measurements were log-transformed because of the right-skewness of their distributions and the multiplicative nature of batch effects. The batch correction was performed on log-transformed measurements using the ComBat method (R package sva), where the plate designation was modeled as a batch covariate for each sample. Estimated batch effects were subtracted from log-transformed measurements to correct measurements for experimental noise.

Longitudinal analysis of samples through their observation period was performed by implementing a linear mixed-effects model where time was modeled both as a fixed effect and random slope, the interaction between time and therapy response was modeled as a fixed effect, while individual sample ID was modeled as a random intercept. Analyses were first performed for each cohort separately and then combined using the inverse-variance weighted meta-analysis approach (R package metafor). Prior to the analyses, glycan

variables were all transformed to a standard normal distribution by the inverse transformation of ranks to normality (R package "GenABEL"; function rntransform). Using rank transformed variables makes estimated effects of different glycans comparable as these will have the same standardized variance. The Benjamini–Hochberg procedure was used to control the false discovery rate (FDR) at the specified level of 0.05. Data were analyzed and visualized using the R programming language (version 3.5.2).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23158473/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: M.P., P.R., K.A. and G.L. designed the study; S.N., F.T., S.S. and A.F. recruited participants and provided plasma samples; J.Š., S.H., M.H. and M.N. prepared samples and conducted LC-MS analysis; F.V. performed statistical analysis; J.Š. and M.P. prepared the manuscript. All authors have read, edited as needed, and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Christian-Albrechts-Universität zu Kiel (A 124/14).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from authors upon reasonable request.

Conflicts of Interest: G.L. is the founder and CEO of Genos Ltd., a private research organization specializing in high-throughput glycomic analysis, and has several patents in this field. J.Š., F.V., S.H., M.N., and M.P. are employees of Genos Ltd. G.L. is the founder and owner of Genos Glycoscience Ltd.—a spin-off of Genos Ltd. that commercializes its scientific discoveries. M.P. and F.V. are employees of Genos Glycoscience Ltd. G.L. is the founder and CSO of GlycanAge Ltd.—the company offering the first glycan-based test for biological age. The other authors declare no conflict of interest.

Abbreviations:

ADCC	antibody-dependent cellular cytotoxicity
AS	ankylosing spondylitis
CD	Crohn's disease
CDC	complement-dependent cytotoxicity
CID	chronic inflammatory diseases
Fc	fragment crystallizable
GlcNAc	*N*-acetylglucosamine
IBD	inflammatory bowel disease
IgG	immunoglobulin G
G	galactose
F	fucose
S	*N*-acetylneuraminic acid (sialic acid)
IL-6	interleukin 6
LC-MS	liquid chromatography-mass spectrometry

PBS	phosphate buffered saline
PsA	psoriatic arthritis
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
S/N	signal-to-noise
TNF	tumor necrosis factor
UC	ulcerative colitis

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8. ŽIVOTOPIS AUTORA S POPISOM OBJAVLJENIH RADOVA

Maja Hanić rođena je u Puli 22. kolovoza 1991. godine. Integrirani preddiplomski i diplomski studij farmacije na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu upisala je 2010. godine, tijekom kojeg je nagrađena Rektorovom nagradom 2013. godine. Pod mentorstvom prof. dr. sc. Gordana Lauca diplomirala je u travnju 2015. godine, a nakon završenog fakultetskog obrazovanja obavila je stručnu praksu u ljekarni te stekla odobrenje za samostalni rad magistra farmacije. U prosincu 2016. zaposlila se na mjesto analitičara u Laboratoriju za glikobiologiju tvrtke Genos d.o.o., a 2017. godine upisala je doktorski studij Farmaceutsko-biokemijske znanosti na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu. Kroz svoj rad stekla je iskustvo u dizajnu studija i eksperimenta te u njihovom provođenju, analizi podataka i interpretaciji rezultata, prvenstveno kroz analizu glikozilacije različitih proteina kod ljudi i životinja upotrebom metoda tekućinske kromatografije i kapilarne elektroforeze. U srpnju 2022. godine unaprijeđena je na radno mjesto voditeljice Laboratorija za kapilarnu elektroforezu. Do danas, koautorica je 12 znanstvenih radova, a svoja znanja i vještine usavršavala je dvomjesečnim boravkom i radom na institutu Max Planck u Magdeburgu u Njemačkoj te sudjelovanjem na konferencijama u Hrvatskoj i inozemstvu.

Objavljeni znanstveni radovi:

1. **Hanić M**, Vučković F, Deriš H, Bewshea C, Lin S, Goodhand JR, Ahmad T, Trbojević-Akmačić I, Kennedy NA, Lauc G, Pants Consortium. Anti-TNF Biologicals Enhance the Anti-Inflammatory Properties of IgG N-Glycome in Crohn's Disease. Biomolecules 2023;13:954.

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Doktorski rad

Glikozilacija imunoglobulina G u predviđanju terapijskoga odgovora i tijekom anti-TNF terapije u Crohnovoj bolesti

Maja Hanić

SAŽETAK

Crohnova bolest (CB) jedan je od oblika upalne bolesti crijeva koja može zahvatiti stijenku čitavog gastrointestinalnog sustava, od usta do rektuma, no najčešće je ograničena na distalni dio tankog crijeva (ileum) i debelo crijevo. Za takvu upalu kažemo da je kroničnog, diskontinuiranog i transmuralnog karaktera. Smatra se da je CB, odnosno upalna bolest crijeva općenito, posljedica pretjeranog imunosnog odgovora u probavnom traktu zbog narušene crijevne barijere, promijenjenog sastava crijevne mikrobiote i utjecaja okolišnih čimbenika kod osoba koja imaju genetičku predispoziciju za razvoj bolesti. Glavni ciljevi u liječenju CB-a jesu kontrola upale, postizanje remisije i njezino održavanje. U tu svrhu koriste se i biološki lijekovi poput antagonista faktora nekroze tumora, odnosno anti-TNF lijekovi. Ipak, takva je terapija izrazito skupa, ne mora biti prikladna za svakog bolesnika, a često dio bolesnika ne odgovora zadovoljavajuće na terapiju ili kasnije tijekom režima gubi odgovor na istu. Isto tako, mogu se razviti i nuspojave ili protutijela na sam lijek. U središtu ovog doktorskog rada jest imunoglobulin G (IgG). Istraživanja u području glikobiologije su pokazala da složeni oligosaharidi, odnosno glikani vezani na IgG imaju izravan učinak na njegovu izvršnu funkciju te da osobe oboljele od CB-a imaju drugačiji sastav ukupnih *N*-glikana serumskog IgG-a u odnosu na zdrave osobe. Stoga ova doktorska disertacija ima za cilj ispitati mogućnost predviđanja terapijskog odgovora na anti-TNF terapiju temeljem analize sastava *N*-glikoma serumskog IgG-a prije započinjanja terapije te istražiti longitudinalnu promjenu u sastavu *N*-glikoma serumskog IgG-a tijekom te iste terapije. Pritom je za istraživanje korišteno ukupno 1513 uzoraka krvnog seruma bolesnika s CB-om regrutiranih u sklopu studije PANTS (engl. *Personalised Anti-TNF therapy in Crohn's disease*). U svrhu predviđanja terapijskog odgovora na anti-TNF terapiju korišteno je 1315 uzoraka krvnog seruma bolesnika s od CB-om, a dodatno prikupljenih 198 uzoraka krvnog seruma korišteno je kao druga vremenska točka zbog ispitivanja longitudinalne promjene u sastavu *N*-glikoma serumskog IgG-a kod istih bolesnika tijekom terapije. Iz uzoraka krvnog seruma pročišćen je IgG te je njegova *N*-glikozilacija analizirana metodom tekućinske kromatografije. Dobiveni rezultati pokazali su da tijekom primjene anti-TNF terapije dolazi do promjene u sastavu *N*-glikoma serumskog IgG-a bez obzira na terapijski odgovor. Preciznije, dolazi do smanjenja zastupljenosti agalaktoziliranih, a povećanja mono- i digalaktoziliranih te sijaliniziranih *N*-glikana IgG-a, što upućuje na smanjenje proupalnog potencijala serumskog IgG-a tijekom terapije. Nadalje, rezultati upućuju da sastav *N*-glikoma serumskog IgG-a prije započinjanja anti-TNF terapije ne sadrži informaciju o budućem terapijskom odgovoru te da se ne može koristiti u svrhu identificiranja bolesnika s CB-om koji neće primjereno odgovoriti na anti-TNF terapiju lijekovima.

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Basic documentation card

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Doctoral dissertation

Immunoglobulin G glycosylation in the prediction of therapeutic response and during anti-TNF therapy in Crohn's disease

Maja Hanić

SUMMARY

Crohn's disease is a type of inflammatory bowel disease that can affect the entire gastrointestinal system, from the mouth to the rectum. However, it most commonly affects the lower part of the small intestine (ileum) and the colon. Crohn's disease is represented by discontinued, chronic transmural inflammation of the gut. It is primarily a consequence of an exaggerated intestinal immune response, changes in the gut microbiota composition and exposure to certain environmental factors in individuals with a genetic predisposition to the disease. The main goals in the treatment of Crohn's disease are to control inflammation and to achieve and maintain remission. For this purpose, there are several therapeutic approaches available, including tumour necrosis factor antagonists (anti-TNF). However, this treatment can be quite expensive and may not be suitable for every patient. Some patients do not adequately respond to the anti-TNF therapy, lose their response over time, experience side effects or develop antibodies against the medication. The primary focus of this doctoral dissertation is on the total serum immunoglobulin G (IgG) *N*-glycosylation in Crohn's disease. Research in the field of glycobiology has shown that complex oligosaccharides called glycans have a direct impact on IgG effector function. Interestingly, patients with Crohn's disease have been found to have an altered composition of serum IgG *N*-glycans compared to healthy individuals. Therefore, this doctoral dissertation aims to study how the composition of total serum IgG *N*-glycans changes over time during anti-TNF therapy with infliximab and adalimumab. Additionally, it aims to explore the predictive potential of total serum IgG *N*-glycans in therapy response to anti-TNF drugs before initiating treatment. For those purposes, a total of 1513 serum samples were collected from CD patients as a part of the PANTS (Personalised Anti-TNF therapy in Crohn's disease) study. From that number, 1315 serum samples were used to assess the prediction of therapy response to anti-TNF drugs. Additional 198 serum samples were collected at the second time point to explore the longitudinal change in the composition of total serum IgG *N*-glycome. The IgG was extracted from serum samples and its *N*-glycosylation pattern was analyzed using liquid chromatography. The results showed that the anti-TNF treatment in patients with Crohn's disease is associated with significant changes in the composition of total serum IgG *N*-glycans, regardless of therapy response. Specifically, the level of agalactosylated IgG *N*-glycans significantly decreased, while mono- and digalactosylated and sialylated glycans increased in their abundance, all of which suggest the reduced proinflammatory potential of serum IgG in patients with Crohn's disease during therapy. Furthermore, the results indicated that the composition of total serum IgG *N*-glycome at baseline does not hold predictive power to detect future non-responders to anti-TNF therapy.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 98 pages, 10 figures, 9 tables and 123 references. Original is in Croatian language.

Keywords: Crohn's disease, immunoglobulin G, *N*-glycosylation, anti-TNF therapy, HILIC-UHPLC-FLD

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