IMMUNOGLOBULIN G GLYCOSYLATION IN COVID-19 DISEASE

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GLIKOZILACIJA IMUNOGLOBULINA G U BOLESTI COVID-19

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DOCTORAL THESIS

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The work presented in this doctoral thesis was performed at Genos Ltd., Zagreb, Croatia under the supervision of Irena Trbojević Akmačić, PhD, as a part of the postgraduate doctoral programme in Biology at the Department of Biology, Faculty of Science, University of Zagreb.

Ovaj je doktorski rad izrađen u Genos d.o.o., Zagreb, Hrvatska, pod vodstvom dr. sc. Irene Trbojević Akmačić, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

INFORMACIJE O MENTORICI

Znanstvena suradnica dr. sc. Irena Trbojević Akmačić je 2012. godine diplomirala Biokemiju i analitičku kemiju na Kemijskom odsjeku PMF-a Sveučilišta u Zagrebu. Po završetku studija, zapošljava se u Genosu, Laboratoriju za glikobiologiju kao doktorand-istraživač. U sklopu doktorata provela je četiri mjeseca usavršavajući se na Sveučilištu Johns Hopkins u SAD-u. Doktorirala je 2015. godine, a 2018. godine stekla je zvanje znanstvenog suradnika u znanstvenom području prirodnih znanosti – polje kemija. 2017. godine provela je tri mjeseca na institutu Max Planck u Magdeburgu, Njemačkoj, gdje se kao gost istraživač bavila karakterizacijom N-glikana metodom MALDI-MS.

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Autorica je preko 50 znanstvenih radova i pet poglavlja u knjigama, te urednica jedne knjige na engleskom jeziku iz užeg znanstvenog područja.

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Doctoral thesis

IMMUNOGLOBULIN G GLYCOSYLATION IN COVID-19 DISEASE

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The ongoing pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become one of the major health problems in the world. SARS-CoV-2 infection leads to a disease caused by coronavirus (COVID-19), with a spectrum of symptoms ranging from asymptomatic to life-threatening pneumonia and death. A variation in the severity of disease symptoms is one of the central questions in COVID-19 pandemics. Immunoglobulin G (IgG), the most abundant glycoprotein in human blood plasma, is one of the key molecules in immune response. Glycosylation of IgG has been poorly studied in COVID-19, but previous studies on a small sample size have shown that there is an association between glycosylation of IgG and COVID-19 severity. As a part of this doctoral thesis study, the variability of IgG N-glycome in COVID-19 will be extensively studied for the first time, in relation to the disease severity and time period after diagnosis.

(88 pages, 11 figures, 5 tables, 234 references, original in English)

Keywords: COVID-19, glycans, glycosylation, immunoglobulin G, SARS-CoV-2

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GLIKOZILACIJA IMUNOGLOBULINA G U BOLESTI COVID-19

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Pandemija koronavirusa 2 (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) koji izaziva teški akutni respiratorni sindrom postala je jedan od glavnih zdravstvenih problema u svijetu. Infekcija SARS-CoV-2 dovodi do bolesti uzrokovane koronavirusom (COVID-19) sa spektrom simptoma koji se kreću od asimptomatske bolesti do po život opasne upale pluća i smrti. Uzroci razlika u jačini simptoma kod zaraženih jedno je od glavnih pitanja u pandemiji COVID-19. Imunoglobulin G (IgG), najzastupljeniji glikoprotein u ljudskoj krvnoj plazmi, jedna je od ključnih molekula u imunosnom odgovoru. Unatoč tome, glikozilacija IgG-a je do sada slabo proučavana u bolesnika s COVID-19, a dosadašnje studije na malom broju uzoraka su pokazale da postoji povezanost glikozilacije IgG-a i težine bolesti COVID-19. U sklopu ovog doktorskog istraživanja prvi put će biti detaljno analizirana varijabilnost IgG N-glikoma u COVID-19, ovisno o jačini simptoma te vremenu trajanja bolesti.

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Ključne riječi: COVID-19, glikani, glikozilacija, immunoglobulin G, SARS-CoV-2

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1. INTRODUCTION

Glycans (oligosaccharides) are one of the most complex biological molecules found in nature (1). All cells and numerous macromolecules in nature contain covalently bonded glycans on the outermost surfaces of cellular and secreted macromolecules (1). Glycans are synthesized from monosaccharide residues by an enzyme-driven process termed glycosylation. Along with nucleic acids, proteins and lipids, glycans play a very important role in the formation of functional macromolecules, cell adhesion, cell communication, ligand binding to the receptor, and consequent activation of signaling pathways (2,3). Unlike proteins, the synthesis of glycans is not directed by a template. Rather, glycans are synthesized by complex dynamic interactions between different enzymes, metabolites, transcription factors, and other proteins, resulting in extremely high variability of glycoproteins (1). Glycans integrate these components and represent a kind of cellular memory that modulates current cellular physiology based on relatively recent events.

Almost all secretory and membrane-bound proteins produced by cells of multicellular organisms contain glycan structures (4), most of which are covalently linked to serine (Ser), threonine (Thr); or asparagine (Asn) residues of polypeptide chains. Different forms of a given glycoprotein containing specific glycans (glycoforms) depend on various factors arising from both gene expression and metabolism taking place in the cell. Glycosylation changes protein properties in a way that it alters protein solubility, availability of antigenic moieties within the glycoprotein structure, localization on the membrane, and serves as a form of protection against proteolysis (2). The alterations of glycan structures on a protein have been associated with pathophysiological processes taking place in the cell producing the protein, which ultimately leads to deviations from normal protein function (1,5–8). Protein glycosylation varies among individuals (9–11). Importantly, however, it is extremely stable in a healthy individual (12, 13) and changes significantly when the homeostasis of the organism is disturbed, whether by lifestyle changes or pathophysiological processes (5,14,15).

1.1. Glycosylation

The most predominant types of protein glycosylation result from the attachment of N-linked glycans, O-linked glycans, phosphorylated glycans, glycosaminoglycans and glycosylphosphatidylinositol (GPI) to peptide backbones (Figure 1.) (1,2,16). N-glycosylation refers to the attachment of glycan via *N*-acetylglucosamine (GlcNAc) to the nitrogen atom on the side chain of Asn in the protein backbone, while O-glycosylation refers to glycan attachment via the oxygen atom on the side chain of Ser or Thr in the protein backbone. To allow glycan attachment to Asn in a protein, Asn must be present in the triad of amino acids: Asn-X-Ser or Asn-X-Thr, where X stands for any amino acid except proline. In the endoplasmatic reticulum (ER) and Golgi apparatus (GA), glycosyltransferases add, and glycosidases cleave carbohydrate structures in a series of steps that are controlled by substrate availability, as well as enzyme concentration, location in ER and GA, and their activity. N-glycan synthesis begins with a transfer of a glycan precursor to the growing polypeptide chain and the nascent carbohydrate-protein conjugate is further processed in the ER. In this process, glucose residues are usually removed as part of a quality control process. The structure then moves to the Golgi apparatus for maturation (1), whereas O-glycosylation occurs exclusively posttranslationally in the Golgi apparatus (1). In eukaryotes, all N-linked glycans consist of GlcNAc β-1 bound to Asn, and all have the same monosaccharides in the core of the glycan: Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Ser/Thr, where Man is mannose. Other monosaccharides then bind to the basic structure resulting into three N-glycan types termed oligomannose glycans (different number of Man bound to the core structure), complex glycans (basic structure has one or more antennae starting with GlcNAc, galactose (Gal) and *N*-acetylneuraminic acid (Neu5Ac)), and hybrid glycans (combination of the previous two types in which Man extends the Manα1-6 arm of the core and one or two GlcNAc-initiated antennae extend the Manα1-3 arm) (1).

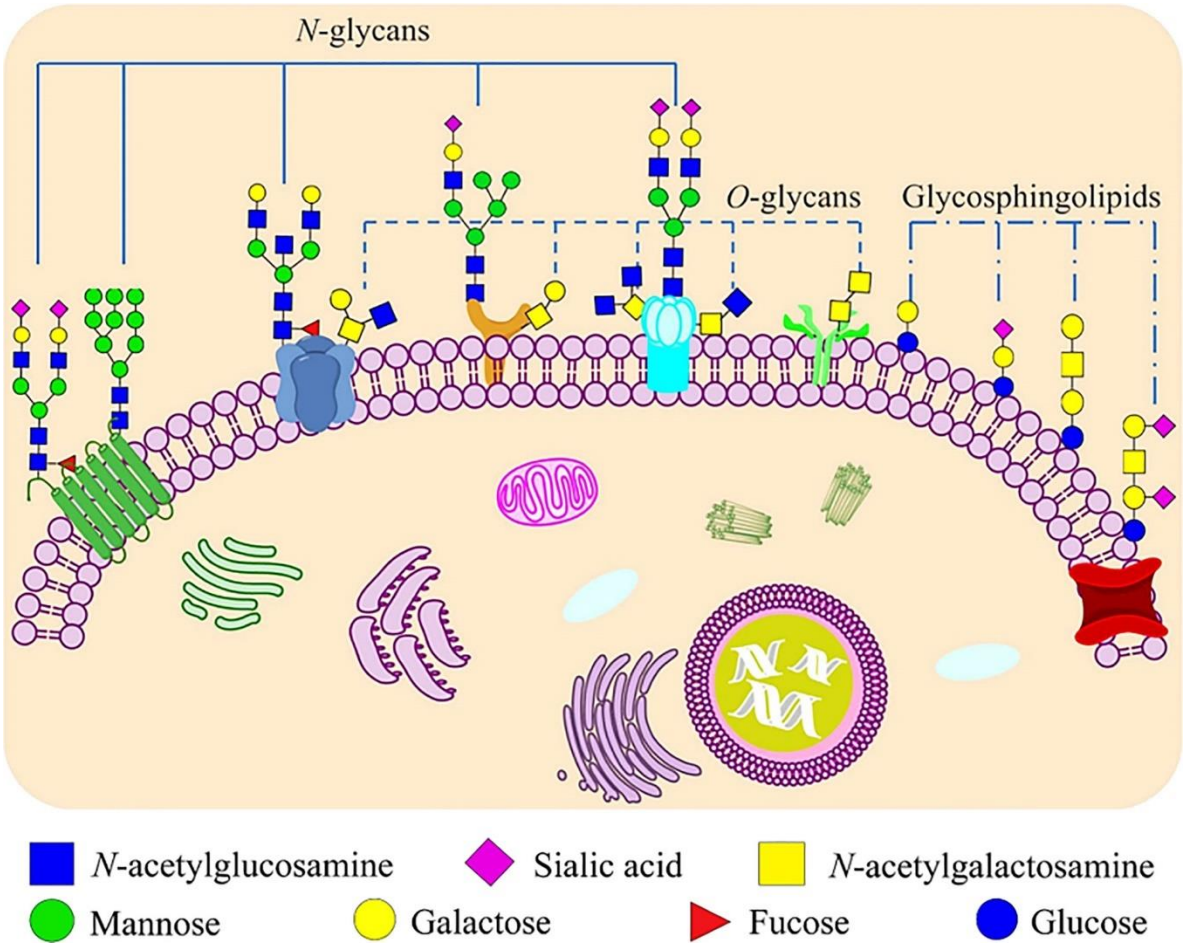

Figure 1. The most predominant types of protein glycosylation. *Glycoconjugates formed by glycans covalently attached to proteins and lipids on mammalian cell membranes. Glycoproteins consist of glycans covalently linked to a polypeptide chain via an N-glycoside linkage to Asn or via an O-glycoside linkage to Ser/Thr. N- glycans consist of GlcNAc β-1 bound to Asn at the consensus glycosylation motif Asn-X-Ser/Thr (in which X denotes any amino acid except Pro). Mucin-type O-glycosylation is the main type of O-glycosylation, consisting of N-acetylgalactosamine (GalNAc) as a common core. Glycosphingolipids are ubiquitous molecules that are formed via the covalent linkage between a glycan moiety (Gal or Glc) and cellular membrane lipids. Reprinted from* (17).

Glycans represent one of the molecular bases for interindividual differences within the human population, which serves as a tool of protection against viruses. ABO blood groups are an example of interindividual differences resulting from different glycans on the surface of erythrocytes and many epithelial or endothelial cells in tissues (18). The ABO blood group system is based on carbohydrate antigens. Blood groups A and B are determined by the presence of the homonymous antigens, blood groups AB and O, by the presence of both or neither antigen (19). Antigen A is determined by a GalNAc motif and antigen B by a Gal motif. Both are added to antigen H via an α1-3 glycosidic bond, forming the GalNAcα1-3(Fucα1-2)Gal- and Galα1-3(Fucα1-2)Gal-chains, respectively (20–22), while antigen O forms Fucα1,2-Galβ- chains (23).

Blood group antigens can influence infections, directly by acting as co-receptors or receptors for microbes and toxins, and indirectly through the anti-blood group antibodies (Abs) that can be elicited by enveloped viruses and bacteria carrying blood group-like antigens (24). It is proposed that the ABO blood group influences susceptibility to infectious diseases. It has been suspected that modification of viral glycosylation makes enveloped virions susceptible to complement lysis mediated by natural Abs, since viruses may carry ABO structures as terminal carbohydrate motifs of their envelope glycoproteins (25). Associations between ABO blood group and the risk or severity of infections have been described for human immunodeficiency virus (HIV) (26), hepatitis B (HBV) (26,27), hepatitis C (26), and West Nile virus (28). Arendrup et al. reported that anti-A Ab derived from lymphocytes from an A donor could neutralize HIV (29). Lao et al. found that HBV prevalence was higher in the blood group O compared to the blood group AB (30). These results were confirmed by Liu et al. (31). Such innate immune responses against carbohydrate structures on invading viruses may also determine the rate at which the adaptive immune response against viruses emerges. According to these results, it is not surprising that ABO blood groups have been considered as an influencing factor for the susceptibility to coronavirus disease 2019 (COVID-19). A possible association between ABO blood group and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection susceptibility has been demonstrated, although the results are contradictory. Some authors pointed out that ABO blood group has an impact on

SARS-CoV-2 infection susceptibility (32–34), severity (34,35) and mortality (34), while others did not demonstrate this association (36,37). In general, blood group A has been linked to a higher risk of SARS-CoV-2 infection, and blood group O with a lower risk, which was initially reported by Zhao et al. (23) , and later reported also by several other studies (32,38–41). Although these associations and their potential implications remain unclear; some underlying mechanisms have been hypothesized, like a protective effect of the ABO antibodies (42). Guillon et al., demonstrated that anti-A or -B Abs from O, B, and A blood group individuals can block the interaction between S glycoprotein of SARS-CoV-1 and angiotensin-converting enzyme 2 (ACE2), and consequently enable viral entry to the cell (43). Additionally, recent data by Gérard et al. suggests that the presence of anti-A immunoglobulin G (IgG) Abs in serum should be considered a more significant factor for COVID-19 susceptibility compared to the blood group itself (42).

Glycans play a major role in the immune system, particularly in modifying the inflammatory response, distinguishing "one's own" from "other's" using a cellular fingerprint, called the glycocalyx, a thick layer of glycans bound to membrane proteins and lipids covering human cells that is at least 10 and sometimes 1000 times thicker than the cell membrane itself (44). Glycan diversity represents one of the main defenses against pathogens. Also, many viral proteins are covered with a thick layer of N-glycans (45). Moreover, protein glycosylation plays a crucial role in viral pathogenesis, including mediating protein folding and stability and shaping viral tropism (46). Several viral pathogens have evolved to take advantage of glycosylation pathways, to decorate the surface of their proteins with “self” glycan moieties. On the other hand, the innate immune system has developed strategies to respond to glycosylated pathogens through various mutations that can alter the species specificity of the virus (47), and modulate its infectivity (48). In addition, Altman et al. showed that the creation of new glycosylation sites on antigens can affect the glycosylation level of viral proteins (49), which helps the virus to evade the host immune response (50).

1.2. SARS-CoV-2 structure

Coronaviruses (CoVs) belong to the order *Nidovirales*, in which all viruses are enveloped, and have non-segmented, positive-sense RNA genomes (51). The genome size of SARS-CoV-2 is approximately 30 kb (52). CoV genomes contain a 5′-cap structure along with a 3′-poly (A) tail, which allows it to function as an mRNA for the translation of replicase polyproteins. The replicase gene, which encodes 16 nonstructural proteins (Nsp1-16), occupies two-thirds of the genome, accounting for approximately 20 kb. On the other hand, genes for structural proteins, namely spike glycoprotein (S), the membrane glycoprotein (M), the envelope protein (E) and the nucleocapsid protein (N), as well as accessory genes, occupy one-third of the genome, or around 10 kb. The accessory proteins are thought to play an important role in viral pathogenesis (53). Nonstructural proteins are involved in multiple steps of the replication and viral assembly processes (54–56).

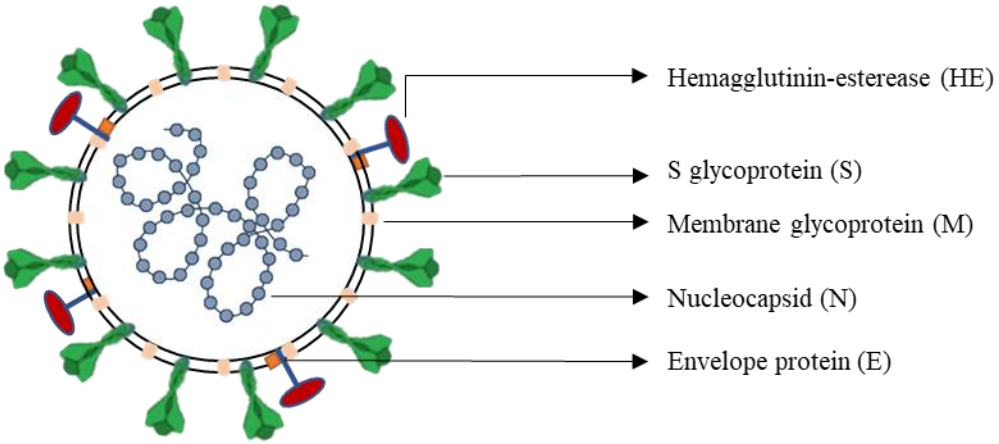

Figure 2. Schematic diagram of the SARS-CoV-2 virus. *The viral structure is primarily formed by the structural proteins such as spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins.*

M glycoprotein

The M glycoprotein (also known as E1 membrane glycoprotein) is a small (25-30 kDa) but most abundant envelope protein of SARS-CoV-2 (57). It has three N-terminal membrane-spanning domains (57,58) that are responsible for the virion shape and viral particle assembly

(59). It has also been suggested that the M glycoprotein are fused to the host cell membrane as a sugar transporter, which may influence sucrose entry into the endosome, lysosome, and/or autophagosome, aiding in viral entry release into cells (57). When the M glycoprotein is expressed together with the E protein, which is present at low levels, they form a CoV envelope (60). Studies on other CoVs show that the M protein may be N-glycosylated and/or O-glycosylated (61). N-glycosylation site is highly conserved in CoVs (62). Using in silico experiments, Dawoon et al. identified eight novel N-glycosylation sites of M protein, and six of these eight sites were found in both human SARS-CoV-2 and SARS-CoV-1 (63). The main difference is the mutation in SARS-CoV-2 resulting in an additional amino acid. Thus, although there are six common sites, the location of these sites differs by one amino acid (63).

E protein

The membrane topology of the E protein is not fully elucidated, but most data suggest that it is a transmembrane protein with an N-terminal ectodomain and a C-terminal endodomain and has an ion channel activity. The E protein is a single helix containing a short outer amino acid terminal domain, and a long inner carboxy-terminal domain (57). It is not known how the E protein helps the M glycoprotein to form the virion, but several possibilities have been proposed for the SARS-CoV-1 that could potentially be the case also with SARS-CoV-2. Weiss et al. suggest that the E protein is involved in viral lysis and subsequent release of the viral genome after SARS-CoV enters host cells. The E protein facilitates viral assembly, drives the budding process (64) and is involved in the activation of the host inflammasome, thereby enhancing the host antiviral response (65). The ion channel activity of the SARS-CoV E protein is not required for viral replication but is required for pathogenesis (66). The SARS-CoV-2 E protein may contain two potential N-glycosylation sites (67). One glycosylation site is Asn66, which could serve as a C-terminal translocation reporter (67). Another potential glycosylation site is Asn48, but, because of the proximity of this site to the membrane, it is unlikely to be occupied if the hydrophobic region is recognized by the translocon as a transmembrane (67). Although, both sites are located in C-terminal

transmembrane segment (67), further studies on the occupancy of these predicted N-glycosylation sites are needed.

N protein

Unlike the other structural proteins of SARS-CoV-2, the N protein is the only protein present in the nucleocapsid. The N protein does not pass through the secretory pathway (68) and is a critical component that protects the viral RNA genome and packages it into a ribonucleoprotein complex thus forming a nucleocapsid (69). The N protein was shown to also play a role in antagonizing the host immune response in the case of SARS-CoV-2 (51), binds the viral genome in a beads-on-a-string conformation (70), and can be considered a viral suppressors of RNA silencing. Cascarina et al. suggest that the N protein of SARS-CoV-2 may use the ability to form biomolecular condensates to dysregulate stress granules, enhance viral replication or viral protein translation, and package the viral RNA genome into new virions (71). Protein N is not expected to be glycosylated because it does not pass through the secretory pathway. In fact, the N protein of SARS-CoV-2 expressed in the human embryonic kidney (HEK293) cells is phosphorylated at Ser176 and is not glycosylated unless forced through the secretory pathway by the addition of a leader sequence during expression in HEK293 (72).

Hemagglutinin-esterase

The fifth structural protein is hemagglutinin-esterase (HE). It binds to specific receptors that have terminal Neu5Ac on surface glycoproteins, particularly on epithelial cells and mucosa of the human respiratory tract, and promotes viral spread through the mucosa (73).

S glycoprotein

The S glycoprotein (180-200 kDa) uses an N-terminal signal sequence to access the rough ER and is highly N-linked glycosylated (74,75). The S glycoprotein is a homotrimer, and each monomer can be divided into three topological domains, namely the bulbous head, the stalk, and the cytoplasmic tail (CT) (76,77), which is prominent on the surface of the virus (78,79). During viral infection, furin proteases activate the S protein by cleaving it into S1

and S2 subunits, which are necessary for attachment to host cells and membrane fusion (80,81).

The S1 subunit, consisting of the N-terminal domain (NTD), the C-terminal domain (CT), and a receptor-binding domain (RBD), is responsible for binding the S glycoprotein to cell receptors on the host cell surface. The RBD can exist in two conformations, termed open and closed, and depending on the different conformations of the S RBD domain, its corresponding function also changes (60,81). In the native state, the CoV S protein exists as an inactive precursor. The S2 subunits consist of the fusion peptide (FP), heptapeptide repeat sequence 1 (HR1), heptapeptide repeat sequence 2 (HR2), transmembrane domain (TM), and cytoplasmic domain and are responsible for viral fusion and entry into the cell (82). During binding to the host cell, FP anchors to the cell membrane when the S glycoprotein adopts the pre-hairpin conformation. Millet et al. showed that FP in case of SARS-CoV-1 plays an essential role in mediating membrane fusion by disrupting and connecting the lipid bilayers of the host cell membrane (83). HR1, located at the C-terminus of a hydrophobic FP, and HR2, located at the N-terminus of the TM domain, play essential roles in the viral-host cell membrane fusion and cell entry (82).

The first step of the SARS-CoV-2 life cycle occurs by its cellular binding initiated by the RBD and CTD region of transmembrane S glycoprotein on the outer surface of the virion. The RBD region binds to the N-terminal helix of human angiotensin-converting enzyme 2 (hACE2) in the aminopeptidase N region (76,84–86). The binding of hACE2 to the RBD can lock the RBD in the "up" conformation and initiate proteolytic cleavage of the S1 subunit by the host TMPRSS2 and cathepsin B or cathepsin L. The RBD is then bound to the N-terminal helix of hACE2. To form a six-helix bundle, three HR1 helices of S2 interact with HR2 helices (87). The result is that three HR2 helices are packed antiparallel into the hydrophobic grooves of the trimeric HR1 core. This conformational change brings the virus and host cell membranes together and facilitates subsequent membrane fusion after which the viral genomic RNA is released into the cells. The genomic RNA and structural proteins then

assemble into mature virions that are subsequently released by exocytosis to initiate another round of infection (88) (Figure 3).

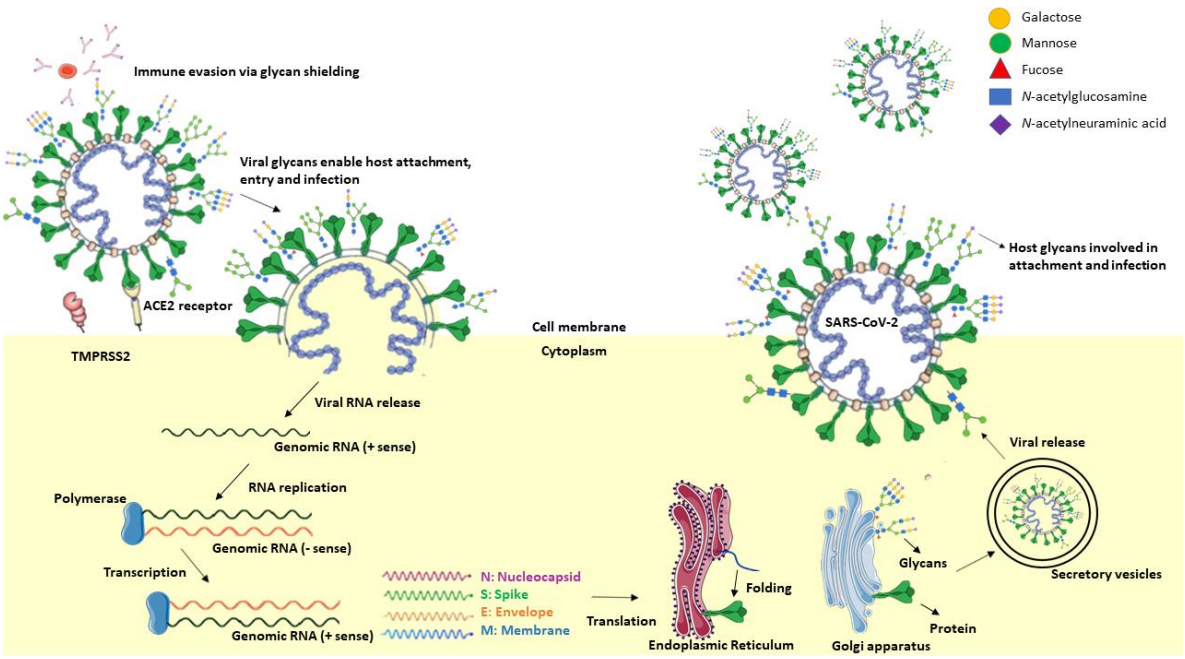

Figure 3. SARS-CoV-2 cell entry and replication. *Spike (S) glycoprotein is highly glycosylated with complex, high-mannose, or hybrid-type N-glycans. The glycan shield allows evasion from the immune system as well as efficient interaction with host cell receptors, entry into the cell, and infection. Within the infected cells, the SARS-CoV-2 S protein is post-translationally modified with host glycans before the virus is released into plasma, potentially allowing increased infectivity of viral particles. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature Switzerland AG, The Role of Glycosylation in Health and Disease by Lauc, G., Trbojević-Akmačić, I. (eds) © 2021*. (89).

1.3.SARS-CoV-2 glycosylation

Glycans on the viral surface are involved in numerous processes in viral pathobiology, including binding of the virus to host cells for entry, viral fusion, shielding of specific epitopes, and the protection of viral proteins (90–94). Glycans also mediate protein folding and stability, and influence viral tropism (95). In general, glycans near receptor-binding regions may have a negative effect on virus binding. Watanabe et al. suggest that the comparatively slender glycan shield of CoVs, in contrast to other viruses, may be advantageous for more efficient receptor binding (90). Glycosylation of viral surface proteins can prevent antibody binding by shielding surface antigens with glycan envelopes and facilitate immune evasion by blocking humoral and cellular innate immune systems (96 – 98). Envelope masking by glycosylation, mainly by increased glycan shield density and a greater amount of oligomannose N-glycans, has been observed in certain types of viral proteins such as the HA glycoprotein of influenza virus (99–102), human immunodeficiency virus-1 (HIV-1) (103–105), Ebola virus glycoprotein (106), SARS-CoV-1 S protein (107,108), and E glycoprotein of dengue, Zika, and other flaviviruses (109,110), which can evade the immune system response very effectively (91). However, the immune system responds to glycosylated pathogens in a variety of ways by either increasing or decreasing the expression of certain endogenous lectins during infection, thus combating the pathogens through lectin-mediated defense mechanisms (111,112).

Glycosylation of SARS-CoV-2 glycoproteins has been extensively studied to provide insight into its structural and functional roles during COVID-19 pathogenesis. Watanabe et al. showed that the Asn234 and Asn709 sites of SARS-CoV-2 S protein are mainly occupied by oligomannose glycans, $Man_5GlcNAc_2$ (95), with the exception of the N-glycosylation site Asn234. Previously for SARS-CoV-1 S glycoprotein, Ritchie et al. found that it is composed of 30% oligomannose glycans (113), compared to the total proportion of oligomannose glycans (mainly $Man_9GlcNAc_2$) of around 50% in other viruses such as HIV-1, influenza, and Lassa (more than 50%) (95,105,114). This suggests that Asn234 and Asn709 N-glycosylation sites are more accessible to α-1,2-mannosidase than to *N*-acetylglucosaminyltransferase I (GlcNAcT-I) (which would allow further processing to

hybrid and complex glycans in the Golgi apparatus). The role of glycosylation in camouflaging immunogenic protein epitopes has also been established for other CoVs (60,95,107), demonstrating that glycosylation helps various viruses evade the host's innate and adaptive immune response.

During viral evolution, viral protein sequences undergo mutations (antigenic drift) that result in the loss of viral species specificity (115) and modulation of the viral infectivity and antigenicity of the viral surface proteins (90,91). These mutations can alter the glycosylation of the proteins by removing existing glycosylation sites or creating new ones, as has been reported in the case of influenza viruses (49,116). Moreover, changes in glycosylation result in new viral strains that are potentially more efficient in evading the host immune response (49,117). While these observations relate primarily to the influenza virus, mutation studies on the SARS-CoV-2 S protein show that some glycosylation sites, e.g. Asn331 and Asn343, are critical for SARS-CoV-2 infectivity (118). Although a total of 9654 mutations have been detected at 400 different S glycoprotein sites, the most commonly reported mutation of the S protein is the Asp614Gly (119). Mutations in N-glycosylation sites of SARS-CoV-2 S glycoprotein have also been noticed, mainly mutation of Asn or Ser/Thr in the consensus sequence Asn-X-Ser/Thr. Mutations Ser151Ile and Ser151Gly (for Asn149 N-glycosylation site) in the S glycoprotein from Asia, as well as mutations Asn17Lys and Thr1136Ile (for Asn1134 N-glycosylation site) in the S glycoprotein from North America, have been observed (119). Most glycosylation site deletions, were less infectious, whereas deletion of both Asn331 and Asn343 glycosylation sites drastically reduced infectivity, revealing the importance of glycosylation for viral infectivity (118).

Structural analysis by cryo-electron microscopy (cryo-EM) and site-specific glycosylation analysis of SARS-CoV-2 S glycoprotein show that it is extensively glycosylated, with each protomer of the TM homotrimeric protein having 22 N-glycosylation sites and several O-glycosylation sites (46,81, 120–122). Several groups have reported occupancy of these sites, but with somewhat conflicting results, underscoring the importance of the context in which the S glycoprotein is expressed and purified before analysis, as well as analytical techniques used for the analysis (89). Watanabe et al. found that all 22 N-linked glycosylation sites were

occupied (95). On the other hand, Walls et al. observed N-glycans at 16 out of 22 potential sites in the SARS-CoV-2 S glycoprotein using cryo-EM (81). In addition, Shajahan et al. observed partial N-glycan occupancy at 17 of 22 N-glycosylation sites and five unoccupied N-glycosylation sites on recombinant SARS-CoV-2 S1 and S2 subunits expressed separately in HEK293 cells. They observed both oligomannose and complex glycans with sialylation and fucosylation and found no hybrid-type N-glycans (122). In addition, they found oligomannose glycans at site Asn234 adjacent to the RBD of the SARS-CoV-2 S protein, whereas complex N-glycans with bi- and triantennary glycans and oligomannose glycans were identified at sites Asn165, Asn331, and Asn343 (46,122), which may play a critical role in the binding of the virus to the hACE2 receptors. Moreover, a study by Brun et al. showed a high prevalence of complex N-glycans and oligomannose and/or hybrid structures on the S glycoprotein subunit S1 isolated from SARS CoV-2-infected Calu-3 lung epithelial cells (123). Additionally, they demonstrated differential expression and both N- and O-glycan processing of virions and non-stabilized S glycoproteins stressing the relevance of these aspects in vaccine design (123). Another study done by NMR on the RBD domain of the SARS-CoV-2 S glycoprotein expressed in human HEK293F cells revealed Fuc and GalNAc on the RBD domain, as well as several unexpected glycan motifs such as 4-*O*-sulfated GalNAcβ1-4GlcNAc (LacdiNAc), α2,6-sialylated LacdiNAc, LewisX (Le^X), and fucosylated terminal GalNAcβ1- 4GlcNAc (LacdiNAcFuc) along with terminal Galβ1-4GlcNAc (LacNAc), LacdiNAc, α2,3-linked sialyl (3′SLacNAc), and α2,6-linked sialyl (6′SLacNAc) (124). A recent report by Sanda et al. showed the presence of LacdiNAc structural motifs on all occupied N-glycopeptides of the recombinant full-length SARS-CoV-2 S glycoprotein expressed in HEK293 cells and polyLacNAc structures on six glycopeptides (125). Zhao et al. observed high levels of core fucosylated, bisected and LacdiNAc structures, as well as sulfated N-glycans in a soluble variant of the SARS-CoV-2 S protein from HEK293 cells (126). In this study, all 22 canonical N-glycosylation sites were shown to be utilized, confirming the findings of Watanabe et al. (95).

Except for the effects of viral protein N-glycosylation on virus infectivity and antigenicity, it has been suggested that O-glycans play a role in the biological activity of viral proteins and

that modification of furin cleavage by O-linked glycosylation may influence virus entry (127, 128). Shajahan et al. observed two occupied O-glycosylation sites at the RBD of the S1 subunit (which is expressed in HEK293 cells separately from the S2 subunit); being the first report of such glycan modification at a crucial binding site of the S glycoprotein (122). Other studies also reported the presence of O-glycosylation at Thr323 and plausible glycosylation at Ser325 (122,129). More recently, Gao et al. demonstrated O-glycosylation at residues Tyr28-Arg34, Thr678, Ser686, and Thr1160 of the SARS-CoV-2 S glycoprotein in addition to already reported Thr323 (130). Several O-linked glycans such as core 1, Tn, sialylated core, and mono- and di-sialyl core 1 are found on the S glycoprotein (122,129). It has been suggested that the O-glycans in the hinge region of RBD (Thr323 and Ser325) and near the furin cleavage site (Ser686) play a critical role in viral binding and may influence viral infectivity (122,130,131). Analysis of the glycosylation pattern of the S glycoprotein may help understand the role of glycans in immune evasion as well as in neutralizing antibodies and to develop effective vaccination strategies.

1.4. Immunoglobulin G

IgG is one of the most abundant plasma glycoproteins produced by lymphocytes and is an important component of the humoral immune response. IgG is a 150 kDa globular protein that consists of two heavy polypeptide chains (50-70 kDa) and two light polypeptide chains (25 kDa) (Figure 4.). Each heavy chain is connected to a light chain by disulfide bonds, and the heavy chains are also connected by disulfide bonds in the hinge region. The hinge region divides IgG into two domains, the Fab domain (antigen-binding fragment) and the Fc domain (crystallizable fragment). The Fab domain consists of the heavy chain CH1 (C stands for "constant" and H stands for "heavy chain") and VH (V stands for "variable") regions, and the light chain CL (L stands for " light chain") and VL regions, which bind specific antigens. The variable regions are responsible for antigen recognition and specificity. The Fc domain consists of two evolutionarily highly conserved regions, CH2 and CH3, on each heavy chain. In the CH2 regions both heavy chains contain evolutionary conserved Asn residues (Asn297) that are N-glycosylated. Moreover, 15-20% of IgG molecules contain additional glycosylation sites in the Fab domain (132–134). N-linked Fc glycans are located in a

hydrophobic pocket. They are quite rigid and are considered to hold the Fc domain in an open conformation and allow binding to the Fc gamma receptor (FcγR), whereas Fab-linked glycans are much more flexible (135–138). All IgG N-glycans are of complex-type consisting of the core structure (two GlcNAc and three Man residues branching into two antennae), which can be additionally modified with a core fucose (Fuc) and/or bisecting GlcNAc, and antennas extended with one or two GlcNAc, one or two Gal and one or two Neu5Ac residues. The exception is the oligomannose structure, Man5, which has been detected as the low-abundant IgG N-glycan structure (11). More than 30 different glycan structures are found on human plasma IgG, most of which are biantennary structures and core-fucosylated (>90%) (11). Approximately 18% of human plasma IgG glycans contain a bisecting GlcNAc and Neu5Ac is present in ~25% of IgG glycans, whereas ~30% of human IgG glycans do not contain Gal (11).

IgG represents one of the most important effector molecules of the human adaptive immune system. Through the Fab-specific antigen-binding properties, it functions as one of the major recognition molecules and can directly neutralize binding and/or cell invasion of various pathogens. Through the Fc fragment, IgG interacts with type I and type II FcγRs on the surface of diverse immune cells (including B lymphocytes, macrophages, neutrophils, natural killer cells, etc.), mediating antibody-dependent cellular cytotoxicity (ADCC), as well as triggering opsonization and phagocytosis of microorganisms (antibody-dependent cellular phagocytosis, ADCP) (139). IgG can simultaneously activate and inhibit FcγRs that are co-expressed on the surface of various innate immune cells, setting a threshold for immune cell activation (140). In addition, by interacting with other components of the immune system, such as complement component C1q, Man-binding lectin (MBL), and Man receptor, the Fc fragment can activate the complement system, cause lysis of pathogens and own damaged cells, and induce complement-dependent cytotoxicity (CDC) (141–144).

Human IgG is divided into four subclasses, designated IgG1, IgG2, IgG3, and IgG4, according to the order of amino acids and the number of disulfide bonds between heavy chains. All four IgG subclasses have a similar structure, and homology at the amino acid

level is over 90%, but there are some sequence variations in the hinge region and N-terminal CH2 domains (144). As these are the parts of IgG that interact with different effector molecules, changes in these regions affect the binding affinity of IgG subclasses to their ligands. Consequently, IgG subclasses have different biological properties such as half-life, immune complex formation, complement activation, and activation of FcγR-expressing cells (144,145). The exposure to protein antigens usually leads to a class switch to IgG1 and IgG3 (also IgG4) via T-cell-dependent mechanisms, whereas polysaccharide antigens often shift the IgG response toward the IgG2 subclass without the help of T cells (144,146). Interestingly, IgG3 has an elongated hinge region that gives the molecule increased flexibility, a short half-life, and emphasized potency in triggering proinflammatory effector functions (144), whereas IgG4 cannot activate complement (147,148). Because of its high abundance, broad spectrum of activity, and importance in the immune response, IgG is one of the most studied glycoproteins.

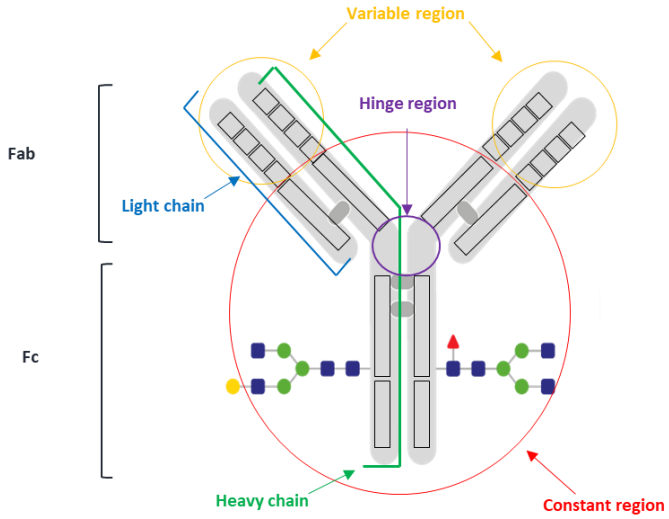

Figure 4. Schematic representation of immunoglobulin G (IgG) structure. *The IgG is composed of two heavy chains and two light chains. It can also be divided into two functional fragments: the Fab domain (antigen-binding fragment) and the Fc domain (crystallizable fragment). Each heavy chain of the Fc fragment contains a covalently attached N-glycan at the highly conserved N-glycosylation site at asparagine (Asn) 297.*

1.5. Immunoglobulin G glycosylation

Variations in N-glycan composition can affect the half-life, effector functions, structural stability, and conformation of IgG (149,150). It is known that their complete removal leads to loss of IgG proinflammatory and anti-inflammatory activity (142,151). In addition, the Fab and Fc domains are known to be differentially glycosylated. Almost all IgG Fab glycans are usually more galactosylated at both antennas, and about 40% of them have mono- and 52% di-sialylated glycans (152). Moreover, the level of glycans containing bisecting GlcNAc in the Fab region is three times higher than in the case of Fc IgG glycans, which are more core-fucosylated and mostly consist of non-sialylated (neutral) structures (152–154). In addition, changes in Fc glycans can drastically alter IgG function and are shown to depend on age, sex, and the presence or absence of disease (11,14,155).

The structural composition of Fc-linked glycans has implications for the functional properties required for binding to Fc receptors and complement factors. Differences in Fc glycosylation patterns can alter the conformation of the Fc fragment, which in turn modulates the binding affinities of IgG for FcγRs and complement factors and allows fine-tuning of the Fc-mediated immune response of IgG (156,157) (Figure 5.). Ultimately, the removal of glycans (i.e., deglycosylation) from the Fc fragment results in a complete loss of binding to the FcγRs or reduced binding to proteins involved in the complement pathways. Consequently, IgG is unable to elicit effector functions such as complement activation, ADCC, and ADCP (135, 156, 158, 159).

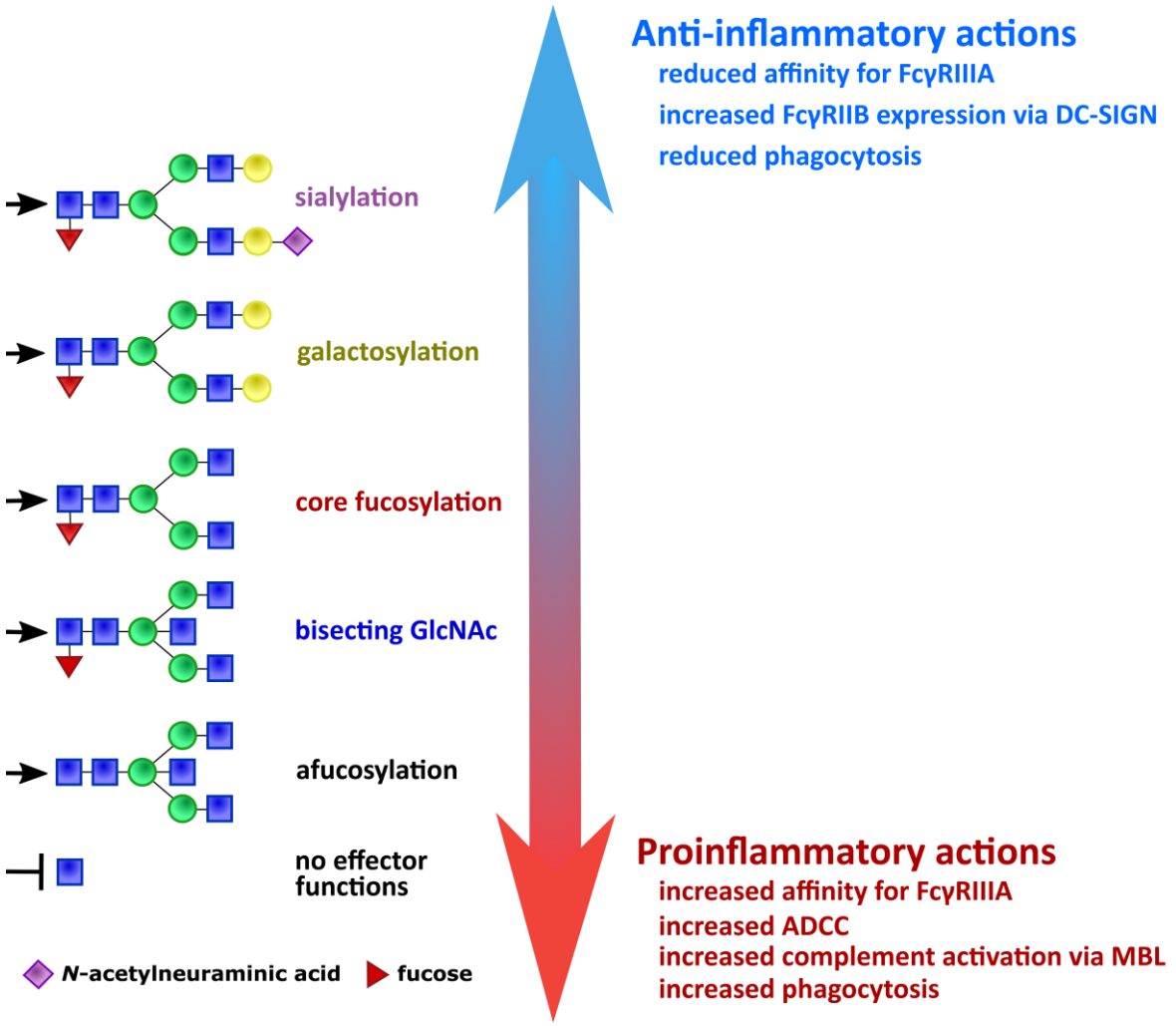

Figure 5. Immunoglobulin G (IgG) effector functions depending on its Fc N-glycosylation. *The effector functions of immunoglobulins can be very different, even opposite, such as inflammation and suppression of the immune response. IgG glycans without a core Fuc increase the binding affinity of IgG for FcγRIIIA and FcγRIIIB and result in enhanced ADCC activity. Furthermore, the addition of bisecting GlcNAc to IgG glycans increases its affinity for FcγRs and consequently enhances ADCC. IgG glycans without terminal Gal residues can interact with MBL and subsequent complement activation, demonstrating the pro-inflammatory activity of IgG. However, galactosylated IgG has an anti-inflammatory effect by inhibiting the complement pathway. IgG antibodies containing sialylated glycans exhibit anti-inflammatory properties. Removal of Asn297-bound glycans results in IgG being unable to interact with the FcγRs and elicit effector functions such as complement activation.*

Reprinted from Mol Aspects Med, N-glycans as functional effectors of genetic and epigenetic disease risk by Štambuk T, Klasić M, Zoldoš V, Lauc G. (160)

1.5.1. Galactosylation

Several studies have shown that galactosylation of IgG acts as a modulator of its inflammatory activity. Karsten et al. reported that galactosylated IgG has an anti-inflammatory effect by inhibiting the complement pathway (161). IgG glycans without terminal Gal residues (agalactosylated glycans) can interact with MBL and activate the lectin complement pathway, demonstrating the pro-inflammatory activity of agalactosylated IgG (162). A marked increase in agalactosylated structures has also been observed in some autoimmune diseases, such as rheumatoid arthritis (RA) (163,164). Additionally, several studies have shown that the terminal Gal of IgG glycans increases the affinity of IgG for the complement component, particularly CDC (165,166). Since Gal is required for sialylation to occur, agalactosylation also has an indirect pro-inflammatory effect. Terminal Gal resudies were also found to increade IgGs affinity for activating FcyRs, thus boosting ADCC (166 – 169).

1.5.2. Sialylation

Many studies have shown that sialylation serves as a switch between the anti-inflammatory and pro-inflammatory activity of IgG (170, 171). Dekkers et al. show that elevated sialylation positively stimulates complement deposition and CDC (166). IgG antibodies containing sialylated glycans exhibit anti-inflammatory properties. In addition, several studies suggest that Neu5Ac-containing N-glycan residues bound to the IgG Fc fragment are responsible for the anti-inflammatory properties of intravenous immunoglobulin (IVIG) preparations, although the exact mechanism is still unclear (171–176). On the other hand, IgG N-glycans that are not sialylated stimulate pro-inflammatory immune responses through interaction with FcγRs (156).

1.5.3. Core fucosylation

The absence of core Fuc significantly increases the binding affinity of IgG for FcγRIIIA and FcγRIIIB and results in enhanced ADCC activity, whereas the presence of core Fuc decreases the ADCC activity of IgG up to 100-fold (177–179). Ferrara et al. showed that the presence

of core Fuc on glycans bound to the Fc fragment sterically inhibits the interactions between IgG Fc glycans and glycans of the FcγRIIIa receptor, resulting in a lower binding affinity for the receptor (180).

1.5.4. Bisecting GlcNAc

It has been reported that the addition of bisecting GlcNAc to IgG glycans increases its affinity for FcγRs and consequently enhances ADCC and other effector functions of immune cells (181). However, Shinkawa et al. showed that the presence of a very high proportion of IgG glycans with bisecting GlcNAc resulted in only a small increase in ADCC activity of IgG compared to the IgG containing a high proportion of glycans without core Fuc (177). Of, note, the presence of bisecting GlcNAc inhibits the addition of core Fuc (180), so the influence of the bisecting GlcNAc level could be indirect. Additionally, Dekkers et al. generated different IgG1 glycoforms and revealed that addition of bisecting GlcNAc to IgG does not affect FcγR and C1q-binding (166).

1.6. Changes in IgG N-glycosylation associated with diseases

Glycans represent one of the most important defenses against various pathogens, and the repertoire of IgG glycans changes with a person's age and disease status (155). Many studies have reported significant changes in IgG glycosylation composition in various diseases. The first study to find an association between a disease and changes in IgG glycosylation was published by Parekh et al. in 1985 (163). They reported a higher proportion of agalactosylated IgG glycans in RA patients compared with healthy controls. Following this study, alterations in IgG glycosylation were observed in numerous other inflammatory and autoimmune diseases (182–186), infectious diseases (187–190), cancers (191–197) and in many other diseases (14), bringing IgG glycans into the focus of research as potential diagnostic and prognostic biomarkers.

Most infectious diseases that have been studied in terms of IgG glycosylation, are characterized by the different IgG glycosylation patterns in patients suffering from e.g. HIV infection (187,188), hepatitis B and C (189,190), tuberculosis (198) and others (14). In chronic hepatitis B, the aberrant IgG glycosylation has been associated with decreased IgG

opsonizing activity and severity of liver inflammation and fibrosis (189). The latter is also true for tuberculosis patients (198). The changes in total IgG glycosylation pattern in infectious disease are most likely associated with a total general pro-inflammatory status upon infection. On the other hand, changes in the glycosylation pattern of antigen-specific IgG are likely related to IgG functionality. For example, anti-Gal IgG in hepatitis B and C show a specific glycosylation profile (190). Interestingly, in dengue-infected patients, increased levels of afucosylated virus-specific IgG glycovariants correlate with disease severity (199). Similarly, antibodies against SARS-CoV-2 exhibit different glycosylation pattern in critically ill patients compared to patients overcoming infection unaided (200). This underscores the fact that IgG glycan composition should be interpreted in the context of pathological mechanisms characteristic of the disease.

1.6.1. IgG glycosylation in viral diseases

Antibody-mediated inflammatory response is the main tool in immunity against viruses. This response occurs when IgG antibodies bind pathogens and form immune complexes resulting in signaling through FcγRs on effector cells. FcγR signaling arises from interactions with Fc domains within immune complexes and the outcome of effector cell responses depends on activating or inhibiting FcγR signaling. Glycosylation of IgG Fc domains affects FcγR signaling, e.g., the absence of core fucosylation is pro-inflammatory due to the increased affinity of the Fc for the activating FcγRIIIa , whereas sialylation promotes anti-inflammatory effector responses (166, 178, 201–203). Changes in glycosylation can affect interaction with receptors, which impacts viral replication and infectivity because the virus is more recognizable by the innate factors of host immune cells. Altered patterns of Igg glycosylation have been noted in infections with any viruses that impact human health, such as HIV, influenza A and dengue, hepatitis B among others.

Previous studies have shown that chronic progressive HIV infection is associated with decreased galactosylated IgG glycans (188). Ackerman et al. have identified decreased afucosylated IgG glycoforms as a common initial glycophenotypic response characteristic of viral infections such as HIV (187). Also, decreased galactosylation and sialylation of anti-envelope IgG have been reported in addition to lowered total IgG galactosylation (187). This

IgG glycopattern is associated with increased viral clearing capacity of antibody fraction due to activation of the innate immune system (187).

Dengue virus infection in the presence of reactive, non-neutralizing IgG is the greatest risk factor for dengue hemorrhagic fever or shock syndrome. Progression to dengue hemorrhagic fever or shock syndrome is attributed to increased levels of afucosylated virus-specific IgG glycovariants correlated with disease severity. Increased afucosylation enhances the affinity of the Fc for FcγRIIIa, leading to higher viral titers and altered cytokine production during infection, explaining a role for this receptor in the pathogenesis of dengue disease. Furthermore, non-neutralizing anti-dengue virus IgGs play important role in the disease progression through antibody-dependent enhancement (ADE) mechanisms (199, 204).

A novel study by Kljaković-Gašpić Batinjan et al. reported that IgG glycosylation in influenza A patients was time-dependent. At the beginning of the disease, sialylation increased and fucosylation and bisecting GlcNAc decreased, while in the next 21 days, sialylation decreased and fucosylation increased (205). Galactosylation stayed stable in influenza infection (205).

Notably, in HBV infection altered IgG glycosylation was associated with disease severity. Specifically, decreased IgG Fc galactosylation was observed in individuals with cirrhosis related to HBV and individuals with chronic HBV infection compared with healthy controls (189). Also, decreased IgG Fc galactosylation was positively correlated with the severity of fibrosis. In the same study it was shown that antiviral therapy reversed the IgG Fc Gal deficiency in individuals with chronic HBV (189). These differences in IgG Fc galactosylation were associated with differences in antibody function, as IgG galactosylation mediates pro-inflammatory effects.

An increase in pro-inflammatory IgG glycans, marked by high levels of agalactosylated and asialylated IgG has been observed in active tuberculosis (206–208). On the other hand, specific IgG from individuals with controlled latent tuberculosis infection had increased galactosylation and sialylation as compared with IgG from individuals with active tuberculosis disease (208). These differences in pro-inflammatory glycosylation patterns are likely driven by the different inflammatory responses in each clinical group. IgG from the controlled latent tuberculosis infection group has decreased core fucosylation, which

enhances NK cell activating antibodies, and significantly elevated FcγRIIIa binding compared with the active tuberculosis disease patients group (208).

Additionally, emerging viruses such as Ebola and SARS-CoV-2 have all shown shifts in total IgG glycopatterns that are probably associated with general inflammatory status upon infection (209, 210). Also, IgG glycosylation in infectious diseases is regulated in two ways, general inflammatory cues for non-neutralizing IgG, and active tuning antibody glycosylation for antigen-specific IgG (187), suggesting that IgG glycan composition should be interpreted in the context of the disease-characteristic pathological mechanisms.

1.6.2. Previous studies on IgG glycosylation in COVID-19

Glycosylation is a key mechanism regulating the function of immunoglobulins, so it is not surprising that IgG glycosylation has been studied also as a factor contributing to disease severity in COVID-19. In a recent study, anti-SARS-CoV-2 IgG1 was shown to have decreased fucosylation and increased galactosylation and sialylation in patients with severe COVID-19 compared with total IgG (210). Chakraborty et al. demonstrated decreased fucosylation of IgG1 specific for the SARS-CoV-2 S glycoprotein receptor-binding domain (anti-RBD IgG1) compared with total IgG1 in individuals with COVID-19 (211). Another study by Larsen et al. showed low fucosylation levels of anti-RBD and anti-S IgG1 in patients with severe respiratory complications (210). Of note, Chakraborty et al. found no differences in anti-S IgG1 fucosylation levels between hospitalized groups (211), which is consistent with the observations of Pongracz et al. (212) and Ankerhold et al. (213). The lack of core fucose increases IgG affinity for FcγR and thereby triggers ADCC-regulated acute immune responses suggesting that the modulation of core fucosylation has a profound impact on disease severity and prognosis. In addition to decreased fucosylation, an increased galactosylation, as well as an increased levels of bisecting GlcNAc, of IgG is also known to increase the affinity for FcγRII, explaining a more pro-inflammatory effector function of IgGs (144,214). Larsen et al. showed decreased bisecting GlcNAc on anti-SARS-CoV-2 Abs glycans (210). Additionally, the bisection of anti-S IgG1 was found to be low in severe COVID-19 (210). Also, it has been shown that anti-RBD IgG1 protein from COVID-19

patients had lower core fucosylation, galactosylation, and bisecting GlcNAc (211). However, these results are opposite of what Larsen et al. observed, suggesting that these differences may be individual (210).

SARS-CoV-2 infections show different disease courses, and it has become clear that an evoked immune response, commonly considered protective, can lead to an exacerbation of immunopathology (215,216). It has been observed that disease worsening in COVID-19 is associated with adaptive immune system activity, with IgG playing an important role (215,217). Excessive FcγR activation by IgG antibodies seems to be crucial for this adverse reaction (210,211,217– 219). Moreover, depending on the glycan composition, IgG activates complement, ADCC, or even has an anti-inflammatory effect (14). Interestingly, since the early stages of the COVID-19 pandemic, it has been recognized that some individuals develop life-threatening conditions, while others control the infection with relatively mild symptoms (215). Age, excessive obesity and comorbidities are some of the predisposing factors for disease progression and mortality in people with COVID-19 (220,221). Additionally, antibody glycosylation is an important factor in inflammation and protection in infections with enveloped viruses, including SARS-CoV-2. Besides, SARS-CoV-2 is of concern to global public health and an understanding of the role of cellular processes such as glycosylation in the biology of viral infection is one step toward developing successful treatment strategies.

1.7. Research problem and scope of the thesis

Several studies have shown that there is a relationship between glycosylation and COVID-19 and that IgG glycans could be used to assess overall health. However, previous studies investigating changes in glycosylation in COVID-19 were characterized by several important limitations. Most studies had small sample sizes, meaning that the results obtained may not reflect the true effect. Only recently have high-throughput methods for glycan analysis made it possible to obtain more accurate and robust results in a shorter time. In addition, previous studies predominantly focused on afucosylation and anti-S IgG1 glycosylation, whereas total IgG glycome was not adequately investigated. Also, previous studies did not explore glycosylation changes in mild and asymptomatic COVID-19 cases and longitudinal changes

during COVID-19, missing out on the dynamic of glycosylation changes during the course of the disease and related to disease severity. There were also inconsistencies between studies with some observations reported in only one of the studies but not in others.

In this thesis and the scientific papers presented, we sought to perform a detailed analysis of IgG glycosylation changes in COVID-19 on a large number of samples. This thesis is expected to provide valuable insights into how IgG glycosylation changes in COVID-19 depending on the severity of COVID-19 symptoms and the duration of the disease. Finally, we aimed to investigate the potential of glycans as effective diagnostic and prognostic biomarkers.

2. Composition of the immunoglobulin G glycome associates with the severity of COVID-19

Glycobiology. 2021 May 3;31(4):372-377.

Petrović Tea; Alves Inês; Bugada Dario; Pascual Julio; Vučković Frano; Skelin Andrea; Gaifem Joana; Villar-Garcia Judit; Vicente Manuel M.; Fernandes Ângela; Dias Ana M.; Kurolt Ivan-Christian; Markotić Alemka; Primorac Dragan; Soares Adriana; Malheiro Luis; Trbojević-Akmačić Irena; Abreu Miguel; Sarmento e Castro Rui; Bettinelli Silvia; Callegaro Annapaola; Arosio Marco; Sangiorgio Lorena; Lorini Luca F; Castells Xavier; Horcajada Juan P.; Pinho Salomé S.; Allegri Massimo; Barrios Clara; Lauc Gordan.

3. IgG N-glycome changes during the course of severe COVID-19: An observational study

EBioMedicine. 2022 July, 81:104101.

Petrovic Tea; Vijay Amrita; Vučković Frano; Trbojević Amačić Irena; Ollivere J. Benjamin; Bego Tamer; Prnjavorac Besim; Đerek Lovorka; Markotić Alemka; Lukšić Ivica; Jurin Ivana; Valdes M. Ana; Hadžibegović I.; Lauc Gordan.

4. **Altered IgG glycosylation at COVID-19 diagnosis predicts disease severity**

Eur J Immunol. 2022 Jun;52(6):946-957.

Vicente Manuel M; Alves Inês; Gaifem Joana; Rodrigues Cláudia S.; Fernandes Ângela; Dias Ana M.; Štambuk Jerko; Petrović Tea; Oliveira Pedro; Ferreira-da-Silva Frederico; Soares Adriana; Seixas Nair; Teixeira Tiago; Malheiro Luis; Abreu Miguel M; Lauc Gordan; Sarmento e Castro Rui; Pinho Salomé S.

5. DISCUSSION

Glycosylation significantly affects the structural and functional properties of IgG, which has multiple implications for the immune system (222,223). Additionally, numerous studies have reported significant changes in IgG glycome composition in various diseases (223,224), including inflammatory and autoimmune diseases (182–186), infectious diseases (187– 190), and cancers (191–197). These results have demonstrated the importance of glycosylation in the biology of the IgG molecule and the regulation of the immune response, as well as how changes in IgG glycosylation are associated with other processes in the body in both physiological and pathological states. In different diseases, multiple glycan traits are associated with the disease development and progression, suggesting that several different molecular pathways interconnected with IgG glycosylation are involved in the pathophysiology of the disease. Likely, depending on the disease, IgG glycoprofile that differs from that of the healthy population represents a biomarker, a direct molecular effector, reflecting the immunological context of an individual. Therefore, it is not surprising that IgG glycosylation has been studied in COVID-19 infection as a factor influencing COVID-19 severity (210,211,225). However, comparison between severe and mild or asymptomatic COVID-19 cases, as well as longitudinal changes during COVID-19 needed more extensive research on larger sample sizes to create a more complete picture of IgG glycosylation in COVID-19. As this doctoral thesis will be the first extensive study to analyze IgG glycosylation in numerous samples, depending on the severity of COVID-19 symptoms and the duration of the disease, it is expected to provide valuable insight into changes in IgG glycosylation in COVID-19.

Within this doctoral thesis IgG glycosylation was analyzed in blood plasma samples collected from nearly 800 people with mild and severe COVID-19, with a subset of samples collected at multiple time points. Samples of people with COVID-19 originate from five different populations - Italy, Portugal, Spain, the United Kingdom, Bosnia and Herzegovina, and Croatia. COVID-19 cases were classified as mild if there was no evidence of pneumonia, no

invasive mechanical ventilation was required, and there was no admission to a hospital intensive care unit. Severe COVID-19 cases required invasive mechanical ventilation and had COVID-19 complications that required admission to a hospital intensive care unit or resulted in death. This thesis represents one of the most comprehensive studies of IgG N-glycome in COVID-19, which allowed us to deepen the current knowledge and better understand the relationship between IgG glycosylation and COVID-19 duration and severity.

Initially, we investigated potential differences in total IgG glycosylation depending on the disease severity in samples collected in Italy, Portugal, and Spain (226). We have found that the level of IgG glycans containing bisecting GlcNAc was statistically significantly changed between mild and severe COVID-19 cases. Galactosylation was also consistently decreased in severe COVID-19 cases in all three cohorts, but the statistical significance of this difference was found only for monogalactosylation in the Spain cohort. No consistent changes in the levels of sialylated and fucosylated IgG glycan structures were observed between mild and severe COVID-19 cases. With disease severity, these changes were most pronounced: 1) a decrease in levels of bisecting GlcNAc, 2) a decrease in levels of monogalactosylated glycans, 3) an increase in levels of agalactosylated glycans. Additionally, by analyzing IgG-Fc glycosylation of COVID-19 patients in a Portugal cohort, it was found that different glycosylation profiles (glycan variations) of Fc fragments detectable at diagnosis have the potential to predict the prognosis of COVID-19 (227).

It has been reported that a higher level of bisecting GlcNAc on IgG indirectly affects the affinity for FcγRs and enhances ADCC by inhibiting fucosylation, leading to a more proinflammatory function of IgG (228). A decrease in bisecting GlcNAc was observed also in the study by Larsen et al., but specifically on anti-SARS-CoV-2 antibodies (210), which is consistent with the changes that were here observed on the level of total IgG glycome (210, 226). In contrast, a recent study by Pongracz et al. (212) found a lower bisecting GlcNAc on anti-S IgG1 Fc fragment compared to the total IgG Fc fragment. Moreover, a rapid increase in bisecting GlcNAc on anti-S IgG1 Fc fragment within days and weeks of disease onset was observed, which is in line with the results on the level of total IgG1 obtained within this thesis

(212, 227). Furthermore, no difference was found in bisecting GlcNAc levels of anti-RBD IgG1 between ICU and non-ICU patients by Chakraborty et al. (211). It is important to note that these differences in results of individual studies may be primarily due to differences in the explored analytes, with some studies analyzing glycosylation of total plasma IgG (226) and some analyzing only anti-S and total IgG1 (212) or anti-RBD IgG1 (211). Alternatively, Larsen et al. (210) reported differences in glycosylation between anti-S and anti-N IgG1 compared to the total IgG. Additionally, Pongacz et al. (212) emphasized that days since the onset of COVID-19 is one of the most important confounding factors for anti-S IgG1 glycosylation, indicating the importance of time of sampling during the course of the disease.

The level of IgG fucosylation is related to immune responses to viral infections caused by enveloped viruses such as COVID-19 and to the severity of viral infection, whereas it is relatively stable in healthy individuals (180,210,211). Pongracz et al. reported low fucosylation of anti-S compared with total IgG1 during hospitalization but no difference in fucosylation between hospitalized ICU and non-ICU COVID-19 patients (212). Similarly, other studies showed that hospitalized patients had lower antigen-specific IgG1 fucosylation than non-hospitalized patients (210,211,218). Here it was observed that total IgG fucosylation has been increased in severe compared to mild COVID-19 patients (226), confirming previous findings (210). Additionally, total IgG fucosylation has been reported to be increased in severe COVID-19 cases compared to mild (229). Also, in the same study were reported decreased levels of total IgG fucosylation in severe COVID-19 cases compared with healthy controls (230). Lower core fucosylation of IgG glycans is associated with pro-inflammatory cytokine interferon (INF-α), which increases the Fc affinity of IgG to FcγRIIIA and FcγRIIIB, leading to activation of FcγRs and activation of natural killer (NK) cells, significantly increasing ADCC (14, 231). However, it has been reported that a higher release of pro-inflammatory cytokines was not triggered by lower IgG1 fucosylation (212). These results indicate that IgG fucosylation is probably driven by different molecular mechanisms involved in the systematic immune response to the virus.

Different levels of IgG galactosylation are one of the most striking glycosylation features observed in various chronic inflammatory and autoimmune diseases (222). Indeed, galactosylation of total IgG has been described to affect immune cell activation thresholds. Galactosylation appears stable in mild COVID-19 patients, whereas it is markedly altered in severe COVID-19 infection (226). This leads to a higher number of agalactosylated IgG molecules, which explains the pro-inflammatory effect of IgG (232). A lower abundance of Gal in IgG N-glycome has already been linked to the pro-inflammatory function of IgG through activation of the complement system (141,233). Indeed, we have shown that COVID-19 patients with low galactosylation of IgG have increased NK cell activation potential (227), but the mechanism underlying NK cell activation by IgG Fc-glycosylation remains to be explored.

Other studies reported increased galactosylation and sialylation of anti-S IgG1 with increased COVID-19 severity (212) and of anti-SARS-CoV-2 IgG in severe COVID-19 compared with asymptomatic and mild cases (210). Additionally, the roles of galactosylation and sialylation as anti-inflammatory features in COVID-19 need further research, as does the question of the cause and consequences of glycosylation changes during COVID-19 in the context of inflammation (234).

The reported differences in glycosylation traits most likely reflect, to some degree the specific glycosylation profiles of the different antigen-specific or total IgG analyzed in each study, as well as the dynamics of antigen-specific IgG presence over the course of COVID-19. Also, they may be related to different molecular mechanisms involved in the immune response to SARS-CoV-2. These overall findings shed light on the complexity and dynamic nature of IgG glycosylation during COVID-19. Taken together, it seems that IgG glycans have a potential as biomarkers of COVID-19.

Taken together, the observed changes in IgG glycome indicate that IgG glycosylation is a dynamic and tightly regulated process that fine-tunes the immune response to a given pathogen. However, this fine-tuning process deteriorates with age, resulting in more pro-

inflammatory glycans. Additionally, the biological aging is highly individual, and levels of pro-inflammatory IgG can vary widely from person to person, supporting the hypothesis that interindividual variability in IgG glycosylation cannot be ignored. The results also show that changes in the total IgG glycome do not necessarily correspond to those observed on the level of different antigen-specific IgG molecules and different IgG subclasses. As noted earlier, age and obesity are risk factors for severe disease and mortality in people with SARS-CoV-2 infection, suggesting that the observed changes are individual and partly dependent on these and other factors (221).

The changes in IgG glycosylation between severe and mild COVID-19 patients, as well as during the disease progression, observed within this doctoral thesis and in earlier research suggest that changes in IgG glycosylation may be an important molecular mechanism in COVID-19 pathology. However, the question arises whether the observed changes in IgG glycosylation reflect an environmental or preexisting genetic risk factor or whether they are rapid changes in IgG glycosylation that are the result of COVID-19 itself.

6. CONCLUSIONS

This thesis provides detailed information on the variability of IgG N-glycome in COVID-19 in relation to disease severity and time after diagnosis.
By examining IgG glycosylation of nearly 800 blood plasma samples from five different populations (Italy, Portugal, Spain, United Kingdom, Bosnia and Herzegovina, and Croatia), we found that:

• The analysis of IgG glycome in severe and mild COVID-19 cases revealed statistically significant differences in IgG-N glycome composition. The most striking difference was the decrease in bisecting GlcNAc in severe patients.

• Several statistically significant changes in IgG glycome composition were observed during severe COVID-19. The most statistically significant changes included increased agalactosylation of IgG, which regulates the proinflammatory effects of IgG through activation of the complement system, and decreased abundance of bisecting GlcNAc on IgG, which indirectly affects ADCC.

• The analysis of IgG Fc-N glycopeptides showed that SARS-CoV-2 positive individuals exhibit variations in the glycan composition of circulating IgG at the time of diagnosis. Levels of Gal and Neu5Ac structures on IgG may predict the development of severe COVID-19. In addition, we have shown that the presence of pro-inflammatory immunoglobulins and higher immune activation are associated with poor disease outcomes. Therefore, IgG glycans have the potential to be used as prognostic biomarkers.

• IgG glycome dynamically changes in severe COVID-19 patients during the disease, indicating a decreased immunosuppressive effect of circulating immunoglobulins. These results suggest that aberrant IgG glycome composition and/or alterations in IgG glycosylation may be an important molecular mechanism in COVID-19.

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8. CURRICULUM VITAE

Tea Pribić, maiden name Petrović, was born in Đakovo (Croatia) on 1st September 1992. She finished elementary school and gymnasium in Đakovo. In 2016, she graduated at the Molecular Biology programme at the Department of Biology, Faculty of Science, University of Zagreb.

After finishing her Master's in Molecular Biology, she worked shortly as a sales representative, but soon after, she started to work at Genos Ltd., where she has been working for the past five years. In 2018, she started postgraduate doctoral programme at the Faculty of Science.

Tea Pribić has participated in numerous national and international conferences, meetings, and workshops, and she was invited speaker. Tea Pribić is the author of more than ten scientific papers and two book chapters. From July of 2022 she started new position in Genos as an Acting head of high-throughput glycomics laboratory.