Glycomic markers of inflammatory bowel disease and IgA nephropathy

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Chapter 1

INTRODUCTION

Introduction

1.1. Definition

Glycosylation, a word formed from the Greek *glyco-*, meaning sweet/sugar, *-yl-* meaning "wood/material/from which something is made of", and from the Latin suffix *-ation*, a process, the action of doing something. The process of adding a derivative of sugar (the glycosyl) to something.

1.2. Background

Glycosylation is a post translational modification (PTM) of proteins, lipids or other molecules where a sugar moiety composed of building blocks called carbohydrates, monosaccharides, or glycans, are enzymatically attached to a peptide backbone *i.e.* of a human protein, thus creating a glycoprotein[1]. Glycosylation is not to be confused with glycation, which is a non-enzymatic direct chemical reaction[2]. Glycosylation is the most abundant protein PTM and more than half of the human proteins are glycosylated[3, 4]. The glycan part of a glycoprotein can be composed of only a few monosaccharides and be small in comparison to the size of the naked protein, but it can also be much larger and become a big part of the total mass of the protein in the case where multiple glycans are attached at different places of the molecule[5-7].

Glycosylation can affect the physical properties and biological activities of proteins and has been associated with health and disease[8, 9]. It is also becoming of growing importance for medical diagnosis and biopharmaceutical treatments[10-14].

The research presented in this thesis aims to expand the knowledge about human protein glycosylation in health and disease as the understanding of glycosylation patterns in disease can help elucidate pathological mechanisms and provide new basis for the development of techniques using glycans as biomarkers for early disease detection, stratification of patients and for better targeted medication.

1.3. Monosaccharides

There are seven major types of monosaccharides found in the glycosylation of human proteins: Glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and *N*-acetylneuraminic acid[15]. Glucoses, galactoses and mannoses are hexose sugars with different stereochemical configuration of the alcohol groups on carbon two and four, while *N*- acetylglucosamine and *N*-acetylgalactosamine are their homologues where an *N*-acetyl group is replacing the alcohol on the second carbon of their respective hexose base, creating an amide bond (**Figure 1**). Fucose is based on a hexose sugar but the alcohol group of the 6th carbon of hexoses is replaced by a hydrogen atom. *N*-Acetylneuraminic acid is a complex sugar with a 9-carbon atom backbone and belongs to the family of sialic acids (SA). Other monosaccharides like xylose are

present in glycan structures of plants while *N*-glycolylneuraminic acid is mostly found in non-human mammals (not shown). Human ancestors were able to produce *N*-glycolylneuraminic acid until a mutation of the gene encoding the CMP-*N*-acetylneuraminic acid hydroxylase occurred[16].

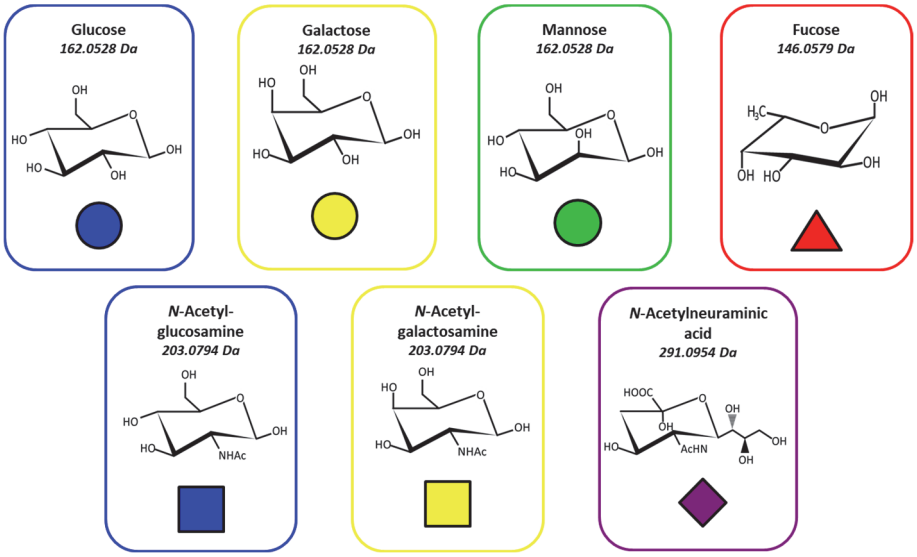

Figure 1: Monosaccharides. *The structure and graphical representation of the monosaccharides presents in human plasma, according to definition by the Consortium of Functional Glycomics*[17]

To simplify the nomenclature of these sugars, they are often referred to with H for hexoses, N for *N*-acetylhexosamines, F for fucoses and S for sialic acids. In this thesis, the sialic acids are sometimes differentiated on the basis of their linkage reflected in differential chemical derivatization are then abbreviated with E and L if they are respectively α2,3- or α2,6- linked[18].

1.4. Biosynthetic pathways

The monosaccharides are used as building blocks and can be added and removed during the synthesis of complex glycan structures by the various enzymes found in the endoplasmic reticulum (ER) and the Golgi apparatus[19, 20]. These carbohydrates are most often arranged in complex branched structures, unlike DNA and amino acids sequences which are built linearly.

This biosynthetic pathway of glycosylation does not follow an exact template but more depends on genetic regulation, on the availability of the nucleotide sugars, the time spent in the endoplasmic reticulum (ER) and in the Golgi apparatus[21]. It also highly depends on the activity of the specific glycosidases and glycosyltransferases responsible for the cutting and transfer of building blocks during the synthesis. This biosynthetic pathway can also be affected by diseases and some environmental factors which will be discussed in a later part of this thesis. By having an extensive knowledge of the synthetic pathway, hypotheses can be formed about the root cause of certain types of defects in glycosylation patterns depending on which step of the trimming or building shows abnormal activity[22, 23].

1.5. *N*-glycosylation

A prevalent type of glycosylation is called "*N*-" glycosylation, because the carbohydrate chain is linked to the amide nitrogen of an Asparagine - abbreviated Asn or "N" - in the amino acid sequence Asparagine-X-Serine/Threonine (Asn-X-Ser/Thr). As exception, the X in this sequence cannot be a Proline (Pro) because of the steric hindering of its sidechain which would block enzymes from getting close enough to the glycosylation site to attach glycans on the neighboring Asparagine. In rare cases, *N*-glycosylation has also been reported in other sequence *i.e.* Asn-X-Cys[24]. With the help of protein sequencing tools, databases and advances in computational biochemistry, it is possible to predict the potential glycosylation sites of a protein[25-28]. These glycosylation sites must also be accessible by being at the surface of the protein, otherwise the glycosyltransferases are not able to reach the suitable location to transfer the precursor oligosaccharide. On the glycan moiety, the glycosylation bond is created on the *N*-acetylglucosamine (GlcNAc) reducing end on carbon one of the precursor oligosaccharide.

The biosynthetic pathway of such branched glycan structures can be divided in three major steps. The first step begins in the endoplasmic reticulum (ER) where a precursor of glycan structure is formed on a lipid donor called dolichol which is a polymeric chain composed of 14 to 21 open isoprene units[29, 30]. At first, the hydrophobic part of the dolichol donor is fixed in the membrane of the ER while the hydrophilic part with the glycan precursor is facing the cytoplasm side of the membrane. Glycosyltransferase enzymes will attach GlcNAc and mannose sugars to the dolichol. When the precursor glycan reaches a size of 7 sugars ($Man_5GlcNAc_2$), the whole precursor flips in the membrane and the glycan part switches to the lumen side of the membrane where additional sugars are added until the complete precursor glycan reaches a size of fourteen sugars ($Glc_3Man_9GlcNAc_2$)[20].

Once the precursor glycan has reached its final size, the second part of the process takes place where the precursor glycan is transferred *en-bloc*, meaning at once, from

the dolichol donor to the glycosylation site of the receiving protein by an oligosaccharyltransferase in the rough ER. The released dolichol donor will then flip back in the membrane where it will be able to start a new cycle[20, 30].

The third part of the glycan synthesis starts when the newly created glycoprotein undergoes folding quality control and migrates from the ER to the Golgi apparatus while a succession of glycosidases (glucosidases and mannosidases) start to trim down the precursor glycan. Additionally, a succession of various glycosyltransferases like GlcNAc transferase I and II, galactosyl transferases and sialyl-transferases start to attach new monosaccharides on the non-reducing termini side of the terminal sugars to build elaborate glycan branches. In some cases, the trimming process will not happen or stay incomplete, leading to what is called oligomannose (M) glycoforms, with the composition of the branched glycan remaining with nine to five mannoses[20].

In humans, there are three *N*-glycan types called high-mannose (M), hybrid (Hy) or of the complex (C) type. They share a common core composed of two successive GlcNAcs, three mannoses and the later composition of the antennas determines the type of glycan category (**Figure 2**). The antennas of high-mannose glycans are composed exclusively of mannoses, complex-type antennas contain GlcNAc, galactoses or sialic acids bound in specific order and hybrid glycans contain a mix of high-mannose and complex antennas. Complex glycans can have up to four antennas. The two branching mannoses are either α1-3- or α1-6-linked to the GlcNAc-linked mannose and give the names to the respective 1-3 arm and 1-6 arm of the glycan. Fucoses can be found in different places on the glycan linked to a GlcNAc either on the antenna or on the first core GlcNAc and are respectively called antenna and core fucoses.

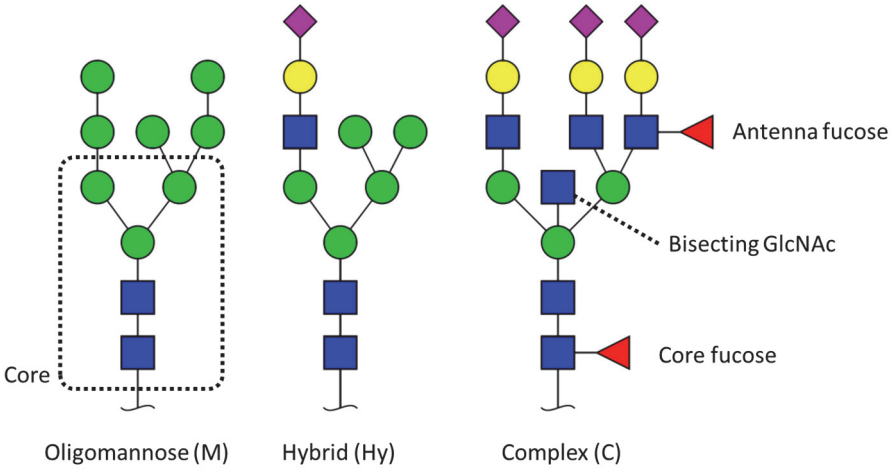

Figure 2: Schematic representation of *N*-glycans types. *The common part to oligomannose-, hybrid- and complex-type glycans called the core is composed of two N-acetylglucosamine (GlcNAc) and three mannoses. Fucoses are differentiated whether they are linked to a GlcNAc in the core of the glycan or on the side of an antenna and are respectively called core and antenna fucoses. In complex type glycans, there is the possibility to have GlcNAc linked β1,4 to the first core mannose which is called bisecting GlcNAc.*

It is not uncommon to have variations of the glycan present, for example when a third GlcNAc is added on the fourth position of the innermost core mannose sugar which is called a bisecting GlcNAc[29].

In other organisms like yeasts including *Saccharomyces cerevisiae*, much bigger structures can be found with elongated hyper mannosylated antennae[31]. Insects also have glycosylated proteins with different fucosylation and elongated structures[32]. Plants also have glycosylation that differs from humans by having a monosaccharide not present in humans, xylose, in the sidechain of some glycan structures[33].

Knowing about glycans biosynthetic pathways can lead to educated hypotheses about the root cause of misformed glycans[22, 23]. For example, observing a high percentage of oligomannose glycoforms with 8 or 9 mannoses on a protein (or in the total plasma *N*-glycome) could indicate a deficiency in the activity or in the genes coding for mannosidases while an elevated percentage of glycoforms with five mannoses could make one think about a lack of activity of the GlcNAc I transferase. Because the building process of the glycan branches happens in a quite ordered sequence, if one specific step fails, the following steps are unlikely to occur. There are also intermediate situations where the trimming of one arm of the precursor is

incomplete while the building of the other arm follows a normal path which leads to hybrid structures.

1.6. *O*-glycosylation

Another type of protein glycosylation present in humans is called "*O*-"glycosylation because the glycans are attached to the -OH residue of amino-acids serine or threonine (Ser/Thr)[34]. The *O*-glycans are generally smaller and of simpler structure (less branching but sometimes elongated repetitive sequences of glycans) compared to the *N*-glycans. The building blocks of the *O*-glycans are the same as in the *N*-glycans, however their biosynthetic pathway differs on multiple points. The *O*-glycosylation doesn't require a specific amino-acid sequence pattern like the *N*-glycans (Asn-X-Ser/Thr) to be possible and the post-translational transfer of the glycan precursor occurs in the Golgi apparatus instead of in the ER for the *N*-glycans. While the start of the *N*-glycosylation process depends on a single oligosaccharyltransferase, *O*-glycosylation on a serine or threonine can be carried out by numerous different enzymes. This multiplicity of enzymes that can be part of the *O*-glycosylation leads to less specificity needs for the glycosylation sites, and the reaction is less likely to be blocked by the steric hindrance of the glycosylation sites than for the *N*-glycans, meaning that *O*-glycans can be added even in parts of the protein that are not directly at its surface. The most common type of O-glycosylation is mucine-type *O*-glycosylation, featuring a GalNAc as the innermost monosaccharide.

O-glycans are built based on core structures like the *N*-glycans and their glycan chains can be elongated with repetitive sequence and can contain fucoses and sialic acids (**Figure 3**). Due to their relatively small size, it is not uncommon to find multiple *O*-glycosylation sites in close vicinity. This close vicinity of the sites complicates the analysis of the specific site's glycosylation and often, only a total glycosylation of a glycopeptide can be determined without the micro-heterogeneity information (cf **§ 1.7**). The relative high content of terminal sialic acids in the *O*-glycans makes that portion of the protein quite hydrophilic and creates charges that can attract water[34].

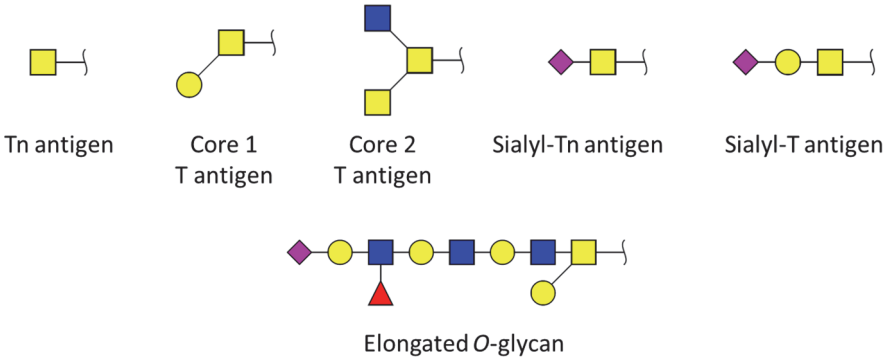

Figure 3: Examples of *O*-glycans cores. *The Tn antigen core O-glycan is composed of one GalNAc, the core 1 (also called T antigen) is composed of one GalNAc and one galactose, the core 2 (T antigen) contains two GalNAc and one GlcNAc, the sialyl-Tn antigen is composed of one sialic acid and one GalNAc and the Sialyl-T antigen is a core 1 with an additional sialic acid.*

One of the most well known biological effect of *O*-glycosylation in human immune response is the different type of blood types (A,B, AB, O and others) where different *O*-glycans are present at the surface of red blood cells (RBC) **Figure 4**. The immune response between the donor's blood and the acceptor's plasma antibodies during the transfusion will depend on the antigens present at the surface of their RBCs[35]. A mismatch of blood compatibility between the donor and acceptor can have fatal outcomes and the discovery of the compatibility matrix between blood types for transfusion is probably the first personalized treatment involving glycosylation.

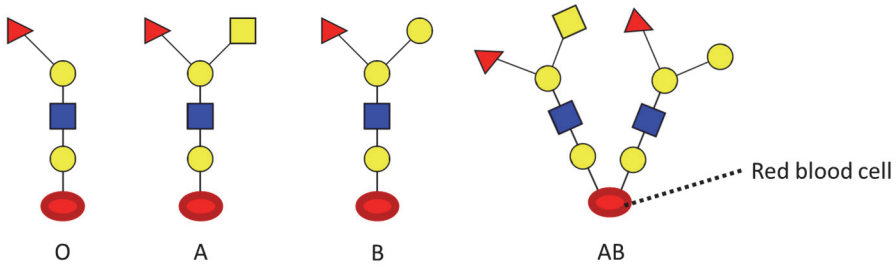

Figure 4: Example of blood type and *O*-glycan structures. *The different O-glycan structures of the blood types A, B, AB and O are linked to the surface of red blood cells.*

Like mammalians, insects have GalNAc *O*-glycans linked to serine and threonine. Plants also possess GalNAc *O*-glycans on threonine and serine residues. These *O*-glycan structures are abundantly found in the cell walls and contain arabinose or

galactose residues attached to hydroxyproline. Yeasts often contain mannose-based *O*-glycosylation.

In humans, O-glycosylation is highly abundant in large mucus proteins known as mucins. Mucins are widespread at the surface of body parts and secreted body fluids that are exposed to the environment like the digestive system, in the lungs and in genital organs. These proteins are found in many body fluids and are of interest for targeted analysis because of their readily availability and presence on body surfaces that can be damaged by diseases. The affinity for water created by the presence of the negatively charged sialic acids in mucins confers them their viscous and sticky aspect and helps them stay at the surface e.g. of the intestinal tract. The stickiness of the mucus is a first barrier against microbes that either get physically trapped in it or that are recognized by the antibodies in the mucus layer as part of the immune response mechanism.

Overall, *O*-glycosylation has received less research attention than *N*-glycosylation, primarily due to analytical challenges such as the complex prediction of glycosylation sites resulting from diverse initiating enzymes, the presence of clustered *O*-glycosylation sites on large and difficult-to-digest peptides, and the lack of efficient enzymatic labelling or release methods. However, recent advances in glycoproteomic platforms and analytical technologies now allow the simultaneous characterization of both *O*- and *N*-glycosylation within a single workflow, renewing scientific interest in the analysis of *O*-glycans in large patient groups[36, 37].

1.7. Macro- and micro-heterogeneity

In both *N*- and *O*-glycosylation, when a protein contains one or several potential glycosylation sites predicted from its amino acid sequence, the extent to which these sites are occupied is referred to as macro-heterogeneity. On a site-specific level, the variety of glycan structures that can be attached to this site is called micro-heterogeneity (**Figure 5**).

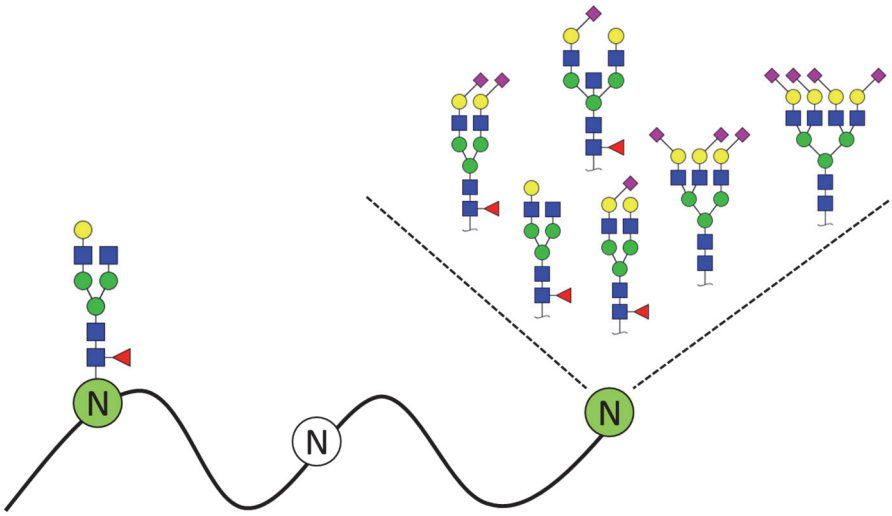

Figure 5: Micro- and Macro-heterogeneity of *N*-glycosylation sites on protein backbone. *The rate of site occupancy, the macroheterogeneity, is represented by the green and white balls for the occupied and respectively unoccupied predicted N-glycosylation sites while the microheterogeneity is represented on the right glycosylation site where different glycans can occupy this site.*

1.8. Biological roles of glycosylation

Glycans can have a great variety of effects on their conjugate. They can influence physical properties such as thermodynamic stability by preventing protein aggregation during thermal stress[38-40]. Glycans may also protect against freezing damage by forming a barrier at ice-nucleation points, thereby isolating them from the surrounding environment[41]. In addition, glycosylation can affect protein folding by promoting disulfide-bond formation, preventing aggregation of unfolded intermediates, and marking misfolded proteins for degradation[42, 43].

Glycans can also influence protein structure by protecting or exposing hydrophobic parts of the protein surface[44, 45], influence their secretion process by modulating their binding with transport proteins[46-48], and also modify the half-life of proteins by protecting them from clearance[40, 49, 50]. Sometimes, the glycosylation has an impact on the protein even before it is released in the circulation by acting as a quality control agent *e.g.* by ensuring proper protein folding during the synthesis and blocking the secretion of malformed proteins and tagging them for elimination[51, 52]. The glycans can also influence the functions of the protein, the recognition with its ligand, a binding site or pathogens[7, 53-55].

Considering all the possible effects of glycosylation on protein properties and interactions with its receptors, it becomes clear that glycans can play a major role in shaping protein behavior in healthy individuals and it is easy to imagine that any deviations from the intended function of one or multiple proteins can lead to severe diseases and/or be affected and may reflect such changes. The knowledge of whether the changes in glycosylation are due to mutations in the genes encoding the proteins and enzymes involved in the synthesis of the glycoproteins or during the refining of the glycans post transfer can give very specific information about the failure mode, and help elucidate unknown disease mechanisms.

1.9. Glycosylation in healthy individuals

In healthy individuals, glycosylation traits have been associated with age, sex and lifestyle. The most common change of glycosylation that has been associated with age is the decrease of terminal galactosylation and sialylation in immunoglobulins such as IgG and an increase in bisecting GlcNAc[56-58]. There are even some researches specialized on individual with exceptional longevity trying to reveal the secret of long life[59]. Glycosylation patterns have also been shown to differ significantly between males and females, for example in the fucosylation of triantennary glycans. In addition, interactions between age and sex have been reported, with certain glycosylation changes occurring exclusively in females and coinciding with hormone-related transitions such as menopause[60].

There is a high variability in the glycosylation of healthy individuals and there is a genetic and hereditary component to human glycosylation which has been demonstrated by analyzing the plasma of isolated populations, for example living on islands[61]. Certain protein polymorphisms, e.g. in haptoglobin, have been shown to vary across different ethnic groups, and distinct glycosylation patterns have also been observed between these polymorphic forms[3, 62-64]. In healthy individuals, a change in the protein sequence causing an allotype can occur exactly at the position of a glycosylation site, and cause the addition or the deletion of a glycoside, like in the allotype A2M of IgA2, without necessarily causing a disease[65].

Sometime, lifestyle can be traceable by its effect on the glycosylation. The serotransferrin glycosylation pattern has been reported to be affected by alcohol usage while smoking was also reported to have an effect on protein levels and glycosylation patterns[66-69]. Protein glycosylation has also been associated with medication in healthy individuals, for example with the intake of contraceptive pills[70].

1.10. Glycosylation changes in diseases

It is known that glycosylation significantly influences protein folding, stability, and function, affecting processes like cell-cell interaction, immune response, and molecular recognition. Glycosylation is involved in the quality control of protein folding within the endoplasmic reticulum[71]. Misfolded glycoproteins are often degraded, preventing the accumulation of dysfunctional proteins or other deviations of the intended function. Glycosylation of antibodies influences their efficacy and interactions with immune cells, affecting immune responses and therapeutic antibody design. Glycans are involved in biomolecular interactions, such as those between pathogens and host cells[72]. Glycosylation modulates receptor activity on cell surfaces, impacting signal transduction pathways[73]. This regulation is critical in processes like growth, differentiation, and apoptosis. Glycans mediate cell adhesion processes, crucial for tissue formation and repair, as well as metastasis in cancer biology.

Understanding protein glycosylation helps explain disease pathogenesis. The research reported in this thesis helps researchers understand disease mechanisms and identify new intervention points for early diagnosis and monitoring disease progression. One step further than explaining disease pathogenesis, understanding glycosylation pathways can lead to the development of new therapeutic strategies. For instance, targeting specific glycan structures can modulate immune responses in diseases like cancer and chronic inflammation[74, 75]. In the future, glycomic data will be used to design personalized treatment plans based on individual glycosylation patterns, leading to more effective and tailored therapies with personalized medicine[76].

Glycosylation patterns on viral proteins are key in vaccine design. For example, the efficacy of influenza and HIV vaccines is influenced by glycosylation, affecting antigen recognition by the immune system[77]. Biopharmaceuticals, such as monoclonal antibodies, require specific glycosylation patterns for optimal efficacy and reduced immunogenicity. Ensuring correct glycosylation is vital in biopharmaceutical manufacturing[78]. Understanding protein glycosylation thus provides comprehensive insights into health, disease, and therapeutic development, highlighting its critical role in modern biomedical research.

Considering the complexity of the glycosylation pathway, the number of genes, enzymes and environmental factors that can have an impact on the glycans and the proteins they are bound to, it is likely that at some point, on top of the changes occurring in healthy individuals, something deviates from the intended path and leads to harmful outcomes. As the glycosylation is already varied in healthy individuals due to heredity, sex, age and environmental factors, additional changes

in glycosylation are observed in diseases. Studying these interactions can for example lead to new antimicrobial strategies[79, 80].

Altered glycosylation patterns are associated with various diseases, including cancer, diabetes, and autoimmune disorders. Using glycan profiling in diagnostic tests for various conditions, including liver diseases and congenital disorders of glycosylation, constantly improves diagnostic accuracy and patient care. Mapping glycosylation changes in various diseases leads to the discovery of novel biomarkers, aids in detection and treatment[81].

Certain glycosylation abnormalities can be grouped in two categories called congenital disorders of glycosylation (CDG) I and II[23]. Type I CDGs are caused by malfunctions during the synthesis or transfer of the precursor glycan to its target protein and result in unoccupied glycosylation sites. Type II CDGs are the result of disruptions in the genes, proteins or enzymes involved in the trimming and/or building phase of the glycan already bound to its glycosylation site on the protein. In type II CDGs, the glycosylation sites are occupied by abnormal glycans[23, 82].

Prevalent diseases such as rheumatoid arthritis (RA), diabetes, cancers and other inflammatory diseases have been reported to be associated with changes in glycosylation[83-85]. The most observed changes in glycosylation with diseases are the levels of galactosylation, bisection and fucosylation, the antennarity/branching of *N*-glycans and some recent methods also reported changes in the type of terminal sialic acids linkages. Although not always seen as a disease, aging is also reported to be associated with similar glycosylation changes as inflammation and led to the phenomenon called inflammaging[56]. Changes in glycosylation have also been reported with the menopause transition[60].

In this thesis, the glycosylation changes of the plasma protein are investigated and put into perspective with the theoretical knowledge about the total human glycosylation. Acute phase proteins and immunoglobulins are the proteins impacted the most in the immune response to diseases and inflammation, it is natural that not only their levels but also their glycosylation changes strongly with disease. Immunoglobulin G and A are often found to be the main contributors to the global glycosylation changes, likely due to their high abundance (IgG) and exposure to pathogen due to their presence in many mucosal layers (IgA). While IgG and its glycosylation is already broadly investigated in clinical studies due to its relatively high abundance and its recognized marker of inflammation role, IgA receives less attention, also due to the higher complexity of its analysis. This prompted further investigation of IgA glycosylation in the disease of focus of this thesis (IBD) and in a study where IgA is known to be the pivotal point of the condition in IgA Nephropathy (IgAN) patients.

1.11. Diseases approached in this thesis

1.11.1. Inflammatory bowel diseases (IBD)

The two main diseases of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC). They are both chronic inflammation of the intestinal tract and are considered autoimmune diseases. IBD is not to be confused with IBS, which stands for irritable bowel syndrome, in which the patients suffer from abdominal pain and abnormal bowel movements. About one percent of the world population is affected by IBD and yet there is no definitive way to cure these diseases[86]. Medication and surgery have beneficial effects for the quality of life of the patients but relapses can occur in many cases[87-89]. The exact causes of IBD are still unknown but several risk factors have been identified[90].

The differential diagnosis of CD and UC is not an easy task as the symptoms are very similar. There is a need to segregate patients suffering from CD or UC as early as possible because the medication and eventual surgical interventions can differ. When IBD onset occurs in young patients, the final diagnosis is often confirmed years after the first medical visit.

One of the differences between the CD and UC is that UC is affecting more localized portions of the intestinal tract while CD can spread in multiple locations (**Figure 6**). Because of this, UC patients can more often undergo successful surgical treatment to remove the inflamed portion of the intestine while CD patients do not benefit as much from such interventions.

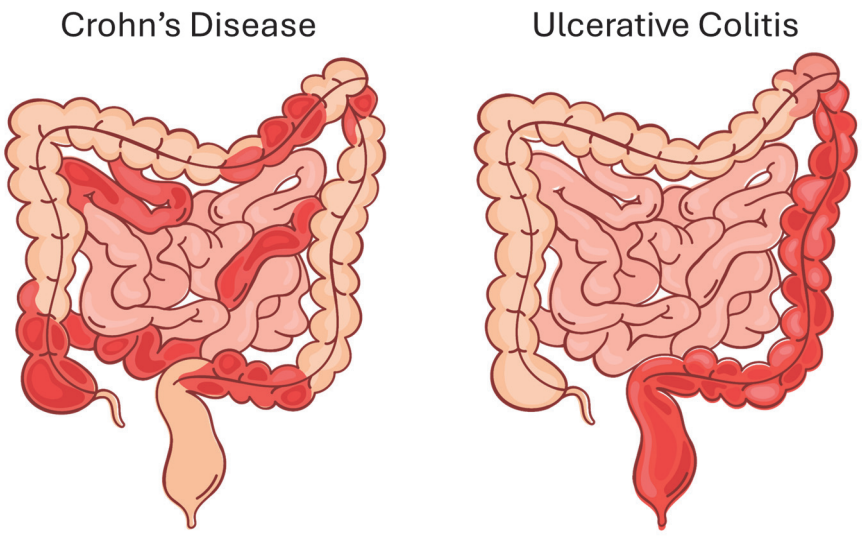

Figure 6: Schematic representation of Crohn's Disease and Ulcerative Colitis [91]**.** *In Crohn's Disease, the inflammation appears in a patchy pattern while in Ulcerative Colitis the inflammation tends to be localized in a single portion of the intestinal tract.*

1.11.2. Glycosylation changes with IBD

Glycosylation has early been identified to play a role in inflammatory bowel disease. Long before mass-spectrometric techniques, lectin based assays have revealed a high content of oligomannose in the colonic mucus UC patients and changes in sialylation, fucosylation, galactosylation and total carbohydrate content in the glycoproteins of both CD and UC patients[92-96].

Advances in derivatization and chromatographic techniques allowed new approaches to the analysis of glycans and helped to remove some of the bias that can be introduced by lectin-based assays. These new techniques also allow distinguishing the protein specific changes *i.e.* subclasses of IgG, and offer more powerful analysis tools to investigate micro- and macro-heterogeneity aspects and linkage specific information of glycoproteins.

Lower galactosylation of IgG is reported to be the strongest glycan marker of inflammation and has widely been associated with UC and CD[97-101]. IgG contributes for the major part of the total plasma protein glycosylation of diantennary glycans and the changes in IgG glycosylation are also visible in non-targeted total plasma glycome analysis[102].

A recent paper[103] on the total serum of IBD patients reports lower galactosylation of diantennary glycans in CD and UC, higher bisection, higher fucosylation of triantennary glycans in IBD and tetraantennary glycans in CD, and was able to associate glycosylation with clinical markers and treatment escalation relating to the findings of this thesis reported in **chapter 3**.

IgA levels, and the glycosylation of its *O*-glycans, mainly the decreased number of N-acetylgalactosamines per hinge glycopeptide, have been reported to be associated with CD with limited clinical diagnostic power[96, 104]. **Chapter 5** of this thesis builds on this and investigates further the changes of IgA glycosylation and reports novel associations in *N*- and *O*-glycosylation in IBD[105].

Several genome studies have found associations between the genes encoding glycosylation and glycosyltransferase enzymes with IBD[106, 107]. Among others, they report alterations in the genes regulating IgG glycosylation or other glycosylation pathways in patients with IBD. Altered *N*-glycosylation of proteins like lower branching and lower overall glycosylation has been associated to elevated T Cell signaling and increased immune response in UC patients[108]. In other proteins such as α1-acid glycoprotein, antitrypsin and haptoglobin, glycosylation changes have been reported and some have been proposed as markers of IBD[109, 110]. Increased branching and increased sialylation of transferrin has been reported in UC patients[111, 112].

In **chapter 3** of this thesis, novel information about the terminal sialic acids of the total plasma *N*-glycome linkage is reported and comes to further increase our knowledge and understanding of IBD mechanisms[3].

1.11.3. IgA Nephropathy

IgA Nephropathy, also known as Berger's disease, is a kidney damaging disease characterized by the deposition of the immunoglobulin A (IgA) antibody in the glomeruli in the kidney[113, 114]. Some patients can live for several years without complications due to the slow progression of the disease. However, once the condition reaches an advanced stage and kidney function deteriorates significantly, patients begin to experience pain and require intensive medical support, such as frequent dialysis sessions or a full kidney transplant.

The exact cause of the disease remains unknown, but it is known that during the immune response, the immune system produces antibodies recognizing abnormally glycosylated IgA. The antibodies bind to galactosyl-deficient IgA (gd-IgA) and this complex forms a deposit in the glomerular mesangium and causes damage and inflammation to the kidney which can progress to total renal failure[115].

Tests like protein in urine and blood in the urine can indicate the first signs of potential kidney malfunction. The main current biomarker to detect IgAN is the measure of Gd-IgA or autoantibodies in kidney tissue biopsies. There is currently no treatment that can prevent IgAN or repair the failing glycosylation process causing the abnormally glycosylated IgA. Like for IBD, there is no permanent cure for nephropathy although some medications exist to mitigate and slow down disease progression by either trying to reduce the production of Gd-IgA or with immunomodulatory treatments to reduce the formation of the immune complexes deposits. Patients can take some medications like angiotensin-converting enzyme to reduce blood pressure or corticosteroids to minimize kidney inflammation but none of these actually treat the disease.

As the disease can go unnoticed during early stages, the possibility to have an easily accessible biomarker becomes of interest for clinicians.

1.11.4. Glycosylation changes with IgA nephropathy

To this day, much less is known about the glycosylation changes in IgA Nephropathy than in other diseases such as RA, diabetes, cancers or even IBD. The primary glycosylation change in IgA Nephropathy is the under-galactosylation of *O*-glycans in the hinge region of IgA1, resulting in the formation of galactose-deficient IgA1(Gd-IgA1) and polymeric IgA complexes which influences the interactions with mesangial cells and inflammatory response[116]. The level of Gd-IgA1 is negatively correlated with the estimated glomerular filtration rate (eGFR) which is an indicator of disease severity[117]. Mice models with gene knockout of galactosyltransferase *b4GalT* have shown kidney lesions similar to human IgAN[118]. Many hypotheses have been made regarding the origin of the galactose deficient IgA and its potential implication in the cascade of immune response and activation of the complement system.

Unlike IgA1 that possesses nine potential sites of glycosylation in its hinge region, IgA2 is not glycosylated in the hinge region and is then believed to not play a major role in the pathology. On average, three to six of the potential *O*-glycosylation sites of IgA1 are occupied with short core-1 glycans (GalNAc-Gal) or Tn antigen (GalNAc) which can have an additional one or two sialic acids.

Several lectin-based assays using HAA or jacalin have identified Gd-IgA to associate with disease severity, however their selective affinity for certain types of terminal glycans can induce bias in the binding of certain glycoforms of IgA, thus not representing the complete picture of IgAN glycosylation changes[119-121]. In **chapter 4** of this thesis, our investigation approach based on capturing affinities for the peptide portion of IgA and analysis of digested glycopeptides by LC-MS remove this bias inherent to HAA and jacalin based assays.

Most of the effect of IgA glycosylation is believed to be due to the changes in the *O*-glycans, but nephropathy also impacts the *N*-glycosylation of IgA1, where elevated oligomannose and sialylated diantennary structures have been reported[122, 123]. There were no reports of changes of IgA2 glycosylation with IgAN, probably due to its tenfold lower abundance in serum and less explored role in inflammation, although it is reported to have a stronger proinflammatory effect than IgA1[124].

In **chapter 4,** glycosylation changes of IgA1 and IgA2 are reported in IgAN patients with a better prediction power for IgAN and glomerular function than the currently available eGFR ELISA techniques.

1.12. Methodology

The study of glycosylation in humans began in the mid-20th century when researchers first recognized that proteins and lipids often carry carbohydrate modifications that are often essential for the biological function[125, 126]. Early methods relied on labor-intensive techniques like paper chromatography and lectin agglutination to detect glycan structures[127]. The development of enzymatic tools, such as glycosidases, allowed for the first structural analyses of *N*- and *O*-linked glycans[128]. Initial studies focused on understanding congenital disorders of glycosylation (CDGs), highlighting the clinical importance of glycan modifications[129]. Mass spectrometry and HPLC technologies, introduced in the 1980s and 1990s, revolutionized glycan analysis by allowing high-resolution profiling[130-132]. Early glycomic studies revealed the diversity and complexity of human glycans, particularly in plasma glycoproteins like immunoglobulins[133]. These foundational works established glycosylation as a critical post-translational modification influencing immunity, development, and disease. The field has since expanded rapidly with advances in analytical techniques and bioinformatics[134-136].

The research presented in this thesis relies on two main analytical workflows. The first one is based on MALDI-TOF-MS for the analysis of plasma-released glycans[102], and the second relies on LC-ESI-MS for the targeted analysis of IgA glycopeptides[105, 137].

For plasma *N*-glycan analysis, a method developed within the research group was applied. This method enhances the robustness of sialic acid analysis in plasma-derived glycans using MALDI-TOF-MS[138]. This approach for the analysis of human plasma glycans released by PNGase F employs a simple and efficient chemical derivatization to stabilize sialic acids in a linkage-specific manner. It also allows for high-throughput analysis, supporting the acquisition of up to 384 samples per MS run, with excellent suitability for automation and large-scale studies[139].

The second major analytical workflow employed in this thesis focuses on the analysis of IgA glycopeptides. In this thesis, IgA antibodies are captured from plasma samples with camelid antibody domains immobilized on agarose beads and protein digestion is performed with TPCK-treated trypsin generating IgA glycopeptides. IgA glycoprotein purification and digestion is performed in 96-well filtration and microtitration plates, and the mass spectrometric analysis is executed by reverse-phase (RP)-LC-ESI-MS with optimized LC-MS conditions by adding low concentrations of trifluoroacetic acid (TFA) to the eluents for the chromatographic separation part, resulting in co-elution of glycopeptides sharing a specific peptide portion, and enabling robust, high-throughput data processing, as described in two chapters of this thesis [105, 137].

Both the optimized sample preparation and data acquisition strategies resulted in large datasets that exceeded the processing capabilities of standard instrument software. To manage this, two in-house developed software were employed for efficient processing of both MALDI-MS and LC-MS data [140, 141]. These tools allowed simultaneous data alignment, calibration, and analyte extraction directly from open-format (LC-)MS data files, supporting the high-throughput nature of the research presented in this thesis.

1.13. Scope of the thesis

This thesis explores the glycomic signatures of two immune-mediated diseases, inflammatory bowel disease and IgA nephropathy. For this, both protein-specific glycosylation and global glycosylation of total plasma and serum proteins are studied from patient cohorts.

Chapter 1 provides some biological and biomedical background on protein glycosylation as well as the diseases studied in this thesis.

Chapter 2, entitled “Human plasma protein *N*-glycosylation” conveys knowledge about the 25 most abundant glycosylated human plasma proteins in health and disease with a review article. The goal of this chapter is to put together the pieces of scattered knowledge of the most abundant plasma glycoproteins and to provide a global representation of human glycosylation in a single picture. To evaluate the pertinence of this theoretically built plasma *N*-glycosylation spectrum, it is compared with some averaged measurements of the total plasma *N*-glycome. When comparing total glycosylation profiles of healthy individuals and patients, this theoretical spectrum can help researchers make informed hypotheses about potential target proteins based on observations of the total plasma glycans.

Chapter 3 of the thesis titled “Plasma *N*-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases” is focused on the analysis of the total

plasma *N*-glycosylation (TPNG) in two cohorts of patients with IBD. As the mechanisms of the diseases are still unknown, mapping the glycosylation changes in IBD can help with early detection and to predict patient outcomes. The large-scale robotized sample preparation platform coupled to a MALDI-TOF analyzer proves to be a robust platform to analyze more than 3500 samples and report a multitude of associations of the TPNG with CD and UC and to give new leads for further research on targeted glycoproteins and personalized medication.

In **chapter 4** of this thesis, a new large-scale sample preparation platform coupled with reverse-phase LC-MS detection is used to analyze plasma samples of patients suffering from IgA Nephropathy. In the context of this disease, deficient IgA glycosylation is already recognized and used as biomarker of disease severity, but the previous studies using lectin-based methods for capturing IgA introduce some bias due to the specific affinities of Jacalin and HAA for the glycosylated part of IgA. The newly developed workflow used here captures IgA with affinities for the peptide part of the protein, removing this bias of certain glycoforms, and allows to focus better on the newly reported glycosylation changes and lead to new findings in IgA Nephropathy.

Chapter 5 of this thesis reports the findings of the analysis of captured IgA in a subset of the IBD samples from the discovery cohort described in **chapter 3**, with the same LCMS method as developed in **chapter 4**. This study is the largest study of IgA in the context of IBD and provides new insights into glycosylation changes with the disease.

Chapter 6 of this thesis places the work in a broader perspective and provides some recommendation for further research.

1.14. Notes for reading the thesis

Due to the format of the supplementary information for each respective chapter and the substantial dimensions of the tables, the supplementary material is not suitable for printing in a readable manner. Consequently, the reader is invited to visit the journal websites of each article to access the supplementary material. To facilitate this process, direct links to the online versions of the articles are included at the end of each chapter.

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