Optimized *N*-glycome analysis of human IgG- and total serum by capillary electrophoresis laser-induced fluorescence: applications for epithelial ovarian cancer and rheumatoid arthritis

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Zusammenfassung

Die Glykosylierung von Proteinen ist eine wichtige co- und posttranslationale Modifikation mit fundamentalem Einfluss auf die Funktionen von Proteinen und vielen biologischen Prozessen. Im Gegensatz zur DNA-Synthese ist die Biosynthese der Glykane ein Prozess ohne Matrize, was zur strukturellen Glykan-Heterogenität beiträgt. Änderungen im Glykom korrelieren häufig mit der Entstehung von Erkrankungen, wie Krebs und verschiedene Entzündungskrankheiten. Folglich könnten sich diese Änderungen als diagnostisch relevant erweisen. Immunglobulin G (IgG) ist das Serumglykoprotein mit der höchsten Konzentration. IgG nimmt eine wesentliche Rolle in der humoralen Immunantwort ein. Es bindet und entfernt gesundheitsschädliche Antigene und Pathogene. Während pathologischer oder unter veränderten physiologischen Bedingungen kommt es zu Modifizierungen in der *N*-Glykosylierung am IgG Fc-Fragment. Dies führt zur Modulation der Effektorfunktionen des Antikörpers.

Im ersten Abschnitt dieser Arbeit wurde das humane *N*-Glykom mittels Kapillarelektrophorese mit Laser-induzierter Fluoreszenzdetektion (CE-LIF) detailliert untersucht, um das Spektrum der bekannten *N*-Glykan-Isomere zu erweitern. Bisherige Forschungen begrenzten sich auf maximal zwölf Glykanstrukturen für Untersuchungen des sich verändernden *N*-Glykoms im Verlauf von Erkrankungen.

Für die Identifizierung von Isomeren desialylierter *N*-Glykane aus dem humanen Serum wurden Glykoproteinstandards sowie Exoglykosidaseverdaus verwendet. Insgesamt konnten 34 *N*-Glykan-Isomere aus dem Serum von Patientinnen mit epithelialem Ovarialkarzinom (EOC) zugordnet und quantifiziert werden. Im Vergleich zu früheren Untersuchungen konnte eine Konzentrationsabnahme von biantennären Strukturen sowie dem mannosereichen

N-Glykan (Man5) im Serum von Patientinnen mit EOC gegenüber gesunden Kontrollen bestätigt werden. Charakteristische und tumorassoziierte Veränderungen des *N*-Glykoms, wie ein erhöhter Verzweigungsgrad der komplexen *N*-Glykane und eine generelle Zunahme der antennären Fukosylierung, konnten ebenfalls nachgewiesen werden. Die Untersuchungen führten außerdem zur Identifizierung von Kern-fukosylierten tri- und tetraantennären *N*-Glykanstrukturen mit relativ seltenem Vorkommen. Trotzdem konnte deren biologische Signifikanz nachgewiesen werden, sodass grundlegende Verbesserungen der *N*-Glykomanalytik im Vergleich zu früheren Studien erzielt wurden.

Ein weiteres Ziel dieser Arbeit war die Optimierung der *N*-Glykananalytik mittels CE-LIF. Gegenwärtig ist die CE-LIF eine der meist verwendeten Methoden für die Trennung von IgG *N*-Glykan-Isomeren. Jedoch sind Messungen von sialylierten *N*-Glykanen mit diesem Gerät grundsätzlich problematisch. Die Ursache liegt in deren negative Ladung, welche kurze Migrationszeiten bedingt und sowohl zur Komigration von Peaks als auch zu verringerter Auflösung führt.

In diesem Zusammenhang sollte ein Protokoll zur verbesserten Auflösung der *N*-Glykane entwickelt werden. Dies wurde durch Methylierung der terminalen Sialinsäuren und damit einhergehender Neutralisierung der negativen Ladung von Carboxylgruppen umgesetzt. Das Entfernen der Eigenladung führte zu verzögerten Migrationszeiten, was in einer verbesserten Auflösung und präziserer Quantifizierung resultierte. Für die Evaluierung dieser Methode wurde die IgG Fc-Glykosylierung in Serumproben von Patientinnen mit epithelialem Ovarialkarzinom mit der konventionellen und der Methylierungsmethode vergleichend analysiert. In beiden Methoden konnte eine Zunahme von degalaktosylierten Strukturen nachgewiesen werden. Aufgrund der verbesserten Auflösung der Methylierungsmethode konnte zusätzlich die Zunahme einer defukosylierten und trunkierten Glykanstruktur detektiert werden.

Es wurde bereits mehrfach bestätigt, dass die Hypogalaktosylierung von IgG mit der Entzündungsaktivität in der rheumatoiden Arthritis korreliert. Des Weiteren zeigt die IgG-Glykosylierung eine starke Abhängigkeit zur Aktivierung der humoralen Immunantwort. Basierend auf diesen Grundlagen wurde im dritten Abschnitt der Arbeit untersucht, ob der Zusammenhang zwischen IgG-Degalaktosylierung und Entzündungsaktivität von der Anwesenheit von Antikörpern gegen citrullinierte

Proteine (ACPA), Rheumafaktor (RF) oder HLA-DRB1 "shared epitope" (SE) abhängig ist. Zusätzlich wurde getestet, ob es einen Zusammenhang zwischen IgG-Glykosylierung und Entzündung in der axialen Spondyloarthritis (axSpA) gibt.

Die IgG-Fc *N*-Glykane aus Serum von Patienten mit RA und axSpA sowie gesunden Kontrollen wurden mittels CE-LIF nach der in dieser Arbeit entwickelten Methode analysiert. Es stellte sich heraus, dass eine Abnahme der Galaktosylierung von IgG für die RA spezifisch ist und dies in Abhängigkeit zur Entzündungsaktivität steht. Dieses Phänomen konnte bei den Patienten mit axSpA aufgrund ausbleibender humoraler Autoimmunprozesse nicht nachgewiesen werden. Nach Unterteilung der RA-Kohorte hinsichtlich ACPA- und SE-Status konnte im Serum der Patienten ohne SE und ACPA eine vergleichbare Galaktosylierung wie bei Patienten mit axSpA und gesunden Kontrollen detektiert werden. Zusammenhänge zwischen IgG-Degalaktosylierung und den Krankheitsaktivitätsmarkern (C-reaktives Protein, Blutsenkungsgeschwindigkeit, disease activity score DAS28) wurden hinsichtlich des ACPA-, RF- und SE-Status getestet. Signifikante Unterschiede wurde zwischen den Untergruppen sowohl des 'shared epitope' (SE+/-) als auch des Rheumafaktors (RF+/-) gefunden. Im Gegensatz dazu konnte zwischen den ACPA-Untergruppen (+/-) kein Unterschied beobachtet werden.

Abstract

Protein glycosylation is an important co- and post-translational modification that is critical for many protein functions and biological processes. Glycan biosynthesis is a non-template driven cellular process, which gives rise to glycan structural heterogeneity. Glycome modulations coincide with the development of a number of diseases including cancer as well as inflammatory diseases. Therefore, such alterations are of diagnostic relevance to monitor remission and relapse. Immunoglobulin G (IgG), the most abundant serum glycoprotein, plays a vital role in the humoral immune response by recognizing and eliminating harmful antigens as well as pathogens. *N*-Glycosylation of the constant IgG Fc portion is altered during pathological or changed physiological conditions that modulates antibody effector functions.

In a first article, the human *N*-glycome was investigated in detail by CE-LIF. Glycome modulations of serum proteins in the course of diseases had so far only been identified for the 12 most abundant glycan isomers.

Reference glycoproteins as well as an exoglycosidase digestion array were used to identify linkage and positional isomers of desialylated *N*-glycans from human serum by CE-LIF. It was possible to assign and quantify 34 *N*-glycan isomers in the serum of epithelial ovarian cancer (EOC) patients. It could be confirmed that the levels of diantennary structures and of high-mannose 5 were significantly lower in patients with EOC than in healthy controls. The hallmark of cancer modulation, namely elevated branching as well as increased antennary fucosylation, was also detected in EOC patients. In addition, low abundant but biologically significant core-fucosylated tri- and tetraantennary *N*-glycan structures were identified, which resulted

in an improvement of *N*-glycome analysis when compared with earlier studies. This could also lead to novel glycan biomarkers for EOC.

The second aim of this work was the optimization of *N*-glycan analysis using capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF). CE-LIF is currently one of the most popular methods for separating IgG *N*-glycan isomers. To date, the measurement of sialylated *N*-glycans using this instrument was challenging. Due to their negative charge sialic acids have short migration times, which results in peak co-migration and reduced resolution.

A protocol was developed in this work to enhance *N*-glycan resolution by methylating the negative charge of carboxylic groups of sialic acids. The migration times of sialylated *N*-glycans are then delayed, which results in improved resolution and better quantification of *N*-glycan isomers by CE-LIF. To evaluate this new method, Fc-glycosylation of IgG isolated from EOC patients and healthy controls was analyzed using the conventional and the methylation method. With both methods, an increase of agalactosylated structures was measured. However, due to the superior resolution of the methylation method, an increase of an afucosylated truncated structure could be also detected.

IgG hypogalactosylation has been widely recognized for its association with inflammatory disease activity in rheumatoid arthritis (RA) and its dependence on activation of humoral immunity. It was therefore examined in a third article whether the correlation between IgG agalactosylation and inflammation depends on the presence of anti-citrullinated protein antibodies (ACPA), rheumatoid factor (RF), or HLA-DRB1 shared epitope (SE). Furthermore, it was investigated whether IgG glycosylation is related to inflammation in axial spondyloarthritis (axSpA).

IgG-Fc *N*-glycosylation analysis was carried out using CE-LIF to compare the serum of RA patients, axSpA patients, and healthy controls. Differences in IgG-Fc galactosylation were assessed in relation to C-reactive protein (CRP) concentrations. The relation between IgG hypogalactosylation and disease activity markers (CRP, erythrocyte sedimentation rate, DAS28) was studied in RA stratified by HLA-DRB1 SE, ACPA, or RF. Aberrant galactosylation of IgG was found to be strongly dependent on inflammatory activity in RA, but was missing in non-humoral autoimmune processes like axSpA. In RA, IgG galactosylation was present in SE-

negative/ACPA-negative individuals and was comparable to galacosylation levels of axSpA patients and healthy controls, indicating humoral activation with a different specificity in this subset. Assessing the correlation between IgG hypogalactosylation and disease activity markers, a significant heterogeneity between strata defined by SE as well as RF but no difference between both ACPA-strata was found. This finding sheds novel light on the relation between SE, ACPA and RF in RA.

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1. INTRODUCTION

1.1 Glycosylation

The covalent attachment of complex oligosaccharides to proteins and lipids is called glycosylation [1, 2]. It is an important and strictly regulated post-translational modification (PTM) giving rise to a large group of glycoconjugates. More than half of all human proteins possess at least one potential *N*-glycosylation site and therefore [3], glycoproteins can be found in all compartments of the cell and also in extracellular space, either as membrane proteins or secreted proteins. Glycans play a decisive role in numerous mechanisms such as modulation of protein folding, secretion and degradation, cell signaling, immune functions, signal transduction and targeting processes [4, 5].

The attachment of glycans to proteins results in *N*-, *O*-, and *C*-linked glycans [6]. *N*-Linked glycans are attached co-translationally through an *N*-acetylglucosamine (GlcNAc) to an asparagine residue of a polypeptide chain. An *O*-linked glycan is usually α-linked via *N*-acetylgalactosamine (GalNAc) to a serine or threonine residue of the polypeptide chain and is often very heterogeneous, with different structural classes [7-10]. *C*-linked glycans represent another form of glycosylation, in which an α-mannose is added to the indole ring of the first tryptophan (Trp) of the consensus sequence Trp-X-X-Trp [11, 12].

Glycosylation is a non-template driven cellular process, which gives rise to the large structural diversity of glycans. Glycan biosynthesis is influenced by several factors, including expression levels and activity of the glycosylation enzymes (e.g. glycosyltransferases and glycosidases) [13], quantity of the glycoprotein that is being glycosylated and the availability and concentration of the activated sugar nucleotides. Furthermore, genetic and environmental factors affect glycan processing. Above all though, the monosaccharide types, the addition or the loss of monosaccharides, the different linkage types between monosaccharides as well as the degree of branching are mainly responsible for the heterogeneity of glycans [14]. The amino acid sequence of the protein forms the basis for putative glycosylation sites, in which glycome macroheterogeneity (variable glycosylation site occupancy) and microheterogeneity (multiple glycoforms attached to a particular glycosylation site)

results in an increase of different proteomic species [15]. A growing number of studies demonstrated that structural glycome alterations are associated with the development and the severity of inflammatory diseases. Examples are autoimmune diseases such as systemic lupus and rheumatoid arthritis. In cancer, a couple of glycoforms represent markers for tumor progression [16-19].

1.2 *N*-Glycosylation

1.2.1 Structure and Functions of *N*-Glycans

All *N*-linked glycans, which are derived from a common oligosaccharide precursor, are covalently linked to a polypeptide chain through a β-glycosidic bond to the nitrogen atom of an asparagine residue in the consensus sequence Asn-X-serine/threonine, where X can be any amino acid except of proline [6, 11, 20]. *N*-Glycans share a common pentasaccharide core consisting of two GlcNAcs and three mannose (Man) residues [21], and are synthesized and further processed in the endoplasmic reticulum (ER) and the Golgi apparatus, which results in the generation of three types of *N*-glycans. High-mannose-type *N*-glycans result from the trimming of the oligosaccharide precursor, whereas in complex-type *N*-glycans two or more antennae are attached to the core by a GlcNAc, resulting in bi-, tri- and tetraantennary glycans. Complex *N*-glycans may be extended with additional monosaccharides: the antennary GlcNAc residues can bind β-linked galactose (Gal) residues and these in turn predominantly α2,3- and α2,6-linked *N*-acetylneuraminic acid (Neu5Ac) residues. Moreover, reducing- and antennary GlcNAc residues as well as Gal residues can be coupled with α1,6- or α1,3-linked fucose (Fuc), respectively. Additionally, bisecting GlcNAc, which is β1,4-linked innermost mannose of the pentasaccharide core, may be attached to the glycan [22]. The hybrid-type is characterized by a combination of high-mannose- and complex-type *N*-glycans, in which mannose residues are attached to the Man α(1,6) arm and with one or two (complex) antennae being attached to the Man α(1,3) arm (Figure 1) [6, 23, 24].

Glycans have central function in many pathological and physiological processes. The external orientation of glycans on glycoproteins shields them from proteolytic degradation and can increase their solubility. Moreover, glycans take part in proper folding of synthesized polypeptides in the ER and function as quality control

for the subsequent transport to the Golgi apparatus. In addition, the attached glycan moieties on proteins help maintaining their structural folding and conformation for correct interaction processes (see section 1.4.4) [25]. In IgG for instance, oligosaccharides directly affect immunoglobulin-functions in the immune response, including cytotoxicity-induced cell death and phagocytosis. In this context, specific IgG glycan types are associated with a higher or lower degree of inflammation, which effect is explained by influencing binding of the IgG to its Fcγ receptors (FcγRs). Moreover, *N*-glycans play a fundamental part in key mechanisms of cellular regulation, which includes cell-cell adhesion and interaction, cell migration and invasion, as well as inter- and intracellular signaling and recognition features (see section 1.5.6) [25, 26]. In addition to congenital disorders of glycosylation [6], pathological events, such as inflammation, tumor and disease progression, as well as metastasis can change the structure of *N*-linked oligosaccharides attached to proteins. It has been shown that tumor cells often display different glycan compositions when compared to normal cells. In addition, aberrations of IgG *N*-glycosylation have been shown to be associated with rheumatoid arthritis [27]. Previous studies indicate that the characterization of glycans will further the understanding of disease mechanisms and clinical diagnosis [28].

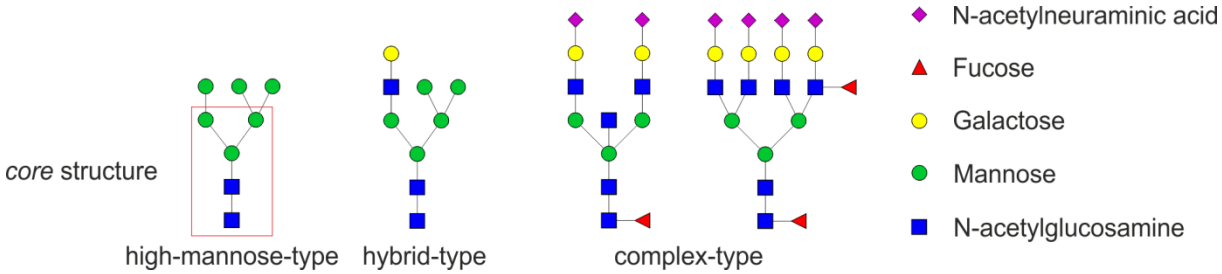


Figure 1 The three types of *N*-glycans.

1.2.2 *N*-Glycan Biosynthesis

The biosynthesis of N-glycans starts on the cytosolic site of the ER. The addition of GlcNAc-P from uridine diphosphat-GlcNAc (UDP-GlcNAc) to the membrane-bound dolicholphosphate (Dol-P) leads to the formation of dolicholpyrophosphate-GlcNAc (Dol-PP-GlcNAc). Subsequently, one further GlcNAc and five mannoses (Man) are added one by one from UDP-GlcNAc and guanidine diphosphate-Man (GDP-Man) to Dol-PP-GlcNAc to generate Dol-PP-$GlcNAc_2Man_5$

[29]. The single transfer reaction steps are catalyzed by specific glycosyltransferases. The oligosaccharide precursor is translocated to the luminal side of the ER membrane [30, 31], and four additional mannoses and three glucoses (Glc) are transferred from the lipid precursors (Dol-P-Man and Dol-P-Glc) to Dol-PP-$GlcNAc_2Man_5$. The completed lipid-linked oligosaccharide precursor (Dol-PP)-$GlcNAc_2Man_9Glc_3$ is cleaved, and then transferred 'en bloc' from the dolicholpyrophosphate carrier to the asparagine residue of the nascent protein by the oligosaccharyltransferase (OST) enzyme complex (Figure 2) [32-35]. Subsequently, the three glucoses are trimmed in the ER lumen by α-glucosidase I and II, and the terminal α1,2-linked mannoses are trimmed by ER α-mannosidase I. These first trimming processes are important for the correct folding of the glycoprotein, in which a deglucosylation-reglucosylation cycle consisting of the lectins calnexin and calreticulin are involved [36-40].

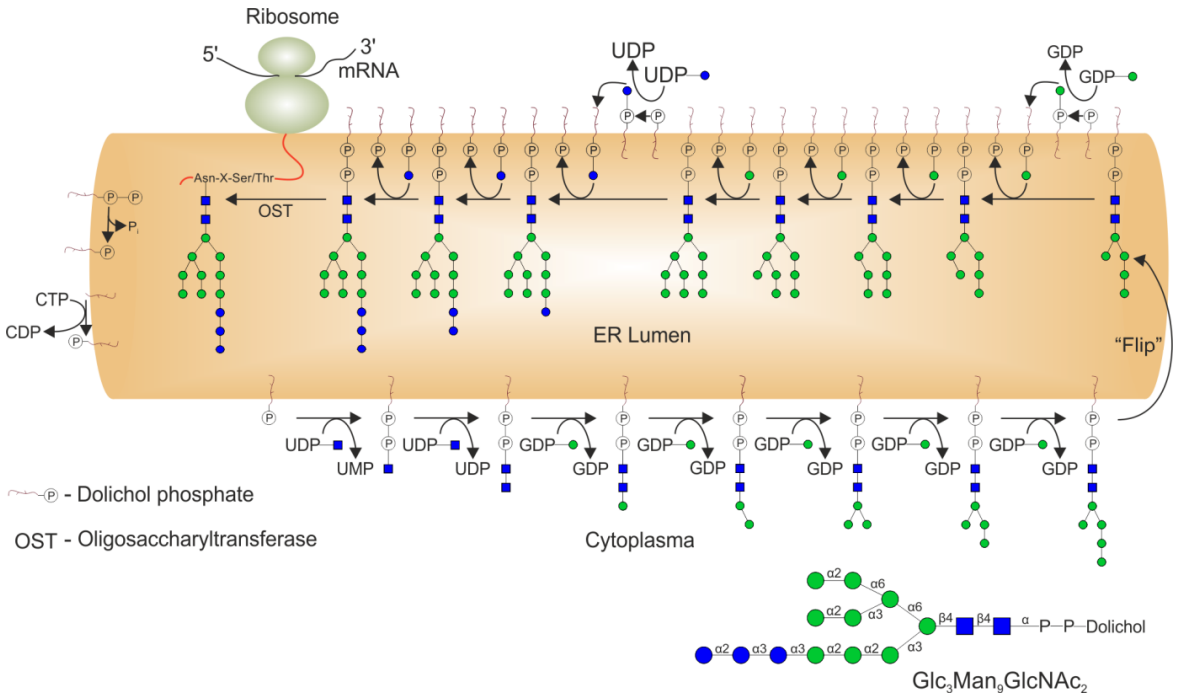


Figure 2 Synthesis of the lipid-linked oligosaccharide precursor in the ER. Transfer of the lipid-linked glycan to an asparagine moiety of an Asn-X-Ser/Thr sequon of a polypeptide [6].

When a glycoprotein is folded correctly and has reached the Golgi apparatus, further mannoses are trimmed by α-mannosidases IA - IC, resulting in protein-bound $GlcNAc_2Man_5$ [21]. *N*-Glycans that are not completely trimmed result in high-mannose *N*-glycans. The addition of a GlcNAc residue to the α1,3-linked Man of the

core structure by *N*-acetylglucosaminyltransferase I (GlcNAcT I) leads to the formation of hybrid-type and complex-type *N*-glycans [41]. Complex *N*-glycans are generated by trimming of two additional terminal α1,3- and α1,6-linked mannoses by α-mannosidase II and a subsequent addition of another GlcNAc residue of the α1,6-linked Man to the core structure by GlcNAcT II. The branching of biantennary glycans can be increased by the action of GlcNAcT IV and V, resulting in up to four antennae. Another modification is the addition of a bisecting GlcNAc to the β-mannose of the core structure. The truncated *N*-glycans can be elongated through the addition of Fuc, Gal and Neu5Ac residues by specific glycosyltransferases in the trans-Golgi (Figure 3). The resulting complex-type glycoproteins are transported to the cell surface or secreted.

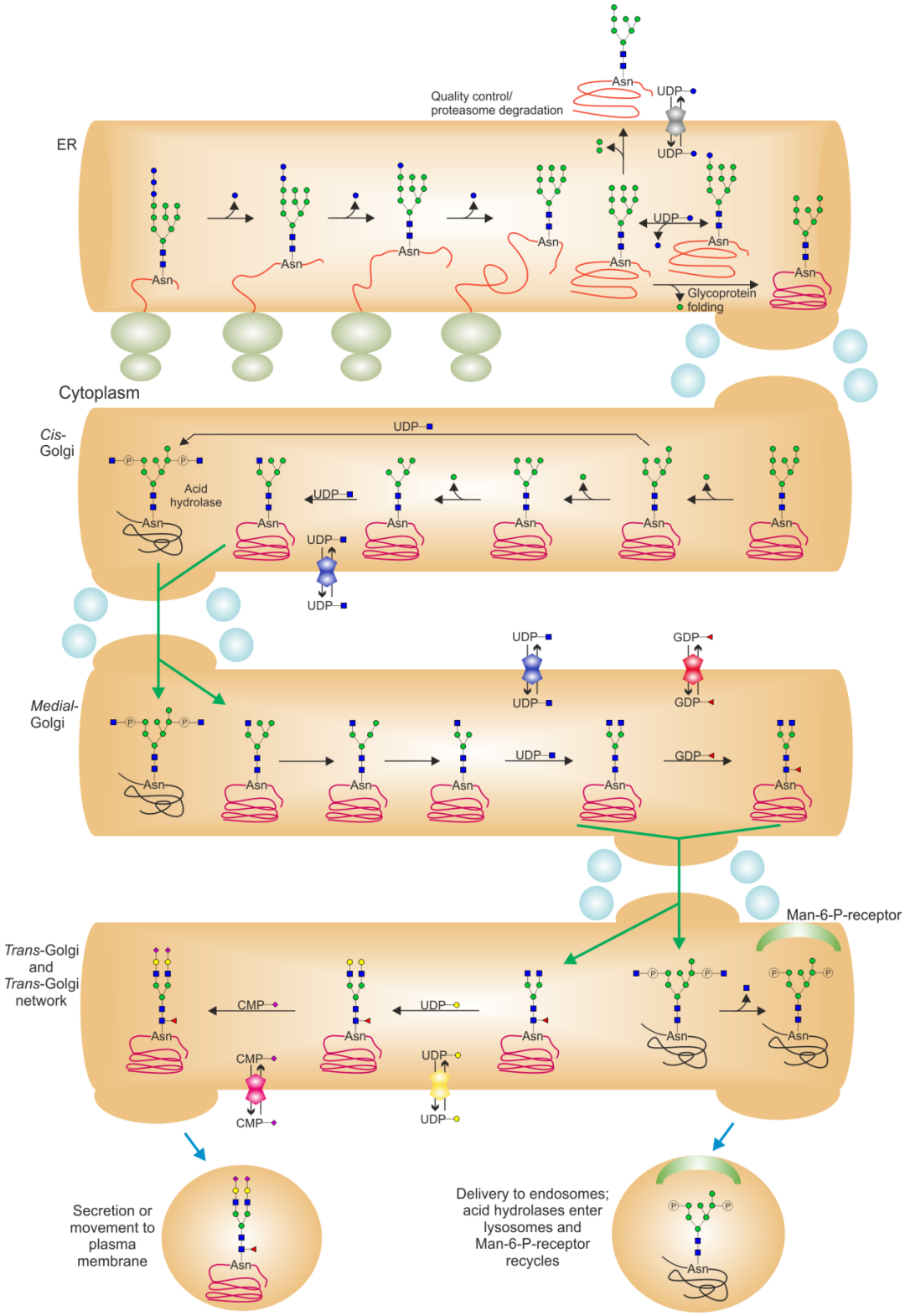


Figure 3 Biosynthesis of *N*-glycans. Trimming and processing of *N*-linked oligosaccharides in the ER and Golgi apparatus. In the ER, protein glycosylation functions as the quality control of protein biosynthesis. Properly folded proteins are transported to the Golgi, where a set of glycosyltransferases diversifies the various antennae of the glycans, which results in a multitude of complex structures. The resulting complex-type glycoproteins are transported to the cell surface or secreted [6].

1.3 The Immune System

The immune system is a defense mechanism that protects the body from harmful substances and pathogens. This is accomplished by recognizing and removing those antigens [42]. The first line of defense are physical barriers like the skin and the mucosa, which trap small particles. Enzymes in tear drops and skin oils as well as the cough reflex hinder harmful molecules from entering the body [43]. If these external anatomic barriers are breached, for instance by wounds in the skin or inhalation of particles through the respiratory system, the body has two types of immune defense, the innate and the adaptive immune system.

The innate immune system is the initial response of the body. After exposure to a harmful molecule or infection of cells, immune cells such as phagocytic leukocytes and natural killer (NK) cells [44-46] are activated and eliminate the foreign pathogens. The activation of the complement system results in a catalytic cascade of twenty different proteins. This supports the innate immune response by marking pathogens for destruction and increasing the permeability of their cell membrane (see section 1.4.2) [47, 48]. This type of immune response is quite swift, but nonspecific to anything that is identified as foreign or non-self.

The adaptive immune system is based on antigen-specific defense mechanisms that take time to establish their protective effect and to remove the invading specific antigen [49]. The adaptive immune system develops throughout life and its corresponding response is highly effective due to its precision. There are two modes of adaptive response, the antibody response and the cell-mediated immune response, and both are carried out by B- and T-lymphocytes. For the antibody response, the antigen is transported to lymphoid organs where it is recognized as foreign. This happens when epitopes of that antigen bind to naive B- and T-lymphocytes by means of epitope-specific receptor molecules. In the cell-mediated immune response, the epitope is bound to the T-cell receptors (TCRs) of the T-lymphocytes. TCRs recognize the epitope, and T-lymphocytes are activated. They then react directly against the foreign antigen that is presented to them by antigen presenting proteins known as Major Histocompatibility Complex (MHC) molecules, which are found on the surface of host cells [50]. While T-lymphocytes are able to destroy and to remove whole cells and pathogens [51], B-lymphocytes make use of

the antibody response. The epitope receptors on the surface of B-lymphocytes are antibodies, which recognize and bind to foreign antigens [52]. The B-lymphocytes are activated and subsequently proliferate, differentiate, and start secreting antibodies also called immunoglobulins. Antibodies circulate in the bloodstream and bind specifically to the foreign antigen that stimulated their production. This inactivates the foreign molecules' ability to bind to receptors on host cells. Antibodies also mark invading pathogens for destruction, making it easier for phagocytic cells. When a B-lymphocyte recognizes an antigen, the structural features of the pathogen are presented on its surface and stored by memory cells in B-lymphocyte differentiation [53]. If this pathogen infects the body again, the memory function leads to a timely and rapid production of the specific antibody.

1.4 Immunoglobulin G

1.4.1 IgG Structure

Immunoglobulins (Igs) play a decisive role in the humoral immune response by recognizing and binding to particular antigens [54]. The human Igs are the most abundant serum glycoproteins and can be divided into five classes: IgG, IgA, IgD, IgE and IgM. They differ in effector functions and antigen specificity as well as responses primarily due to their structural variability. IgG, the most abundant antibody class, is present in circulating blood at a concentration of approximately 10 mg/mL. It is an important molecule of the humoral immune system as it is able to activate a variety of effector functions to remove pathogens [55]. IgG antibodies can be divided into four subclasses (IgG1, IgG2, IgG3, and IgG4). This subdivision is based on the differences in the amino acid sequences and the length of the hinge region as well as the quantity of disulfide bonds [56]. IgG consists of two heavy chains and two light chains linked together by disulfide bonds. Each heavy chain is composed of one variable region (V_H) and a constant region containing three domains (CH1, CH2, CH3). Each light chain has constant (CL) and variable regions (VL). The two light chains, together with two domains (CH1 and VH) of the heavy chains, form the two fragment antigen binding (Fab) moieties. These are linked through the hinge region to the effector fragment crystallizable (Fc) part formed by the remaining domains (CH2 and CH3) of the heavy chains (Figure 4). The two variable domains (the VH

and VL domain) of the Fab (fragment antigen binding) region represent the variable fragment (Fv). The Fv is responsible for the antigen specificity of the antibody. The constant domains provide a structural framework. Each Fv is composed of six hypervariable loops, which are known as the complementarity-determining regions (CDRs). IgG can interact through its Fc region with Fcγ receptors (FcγRs). The effector function requires the presence of a glycan in the Fc-region and is crucially influenced by its structure. The *N*-linked glycan is attached to the conserved asparagine (Asn) residue at the position 297. Additional *N*-glycosylation sites may occur in the Fab hypervariable regions.

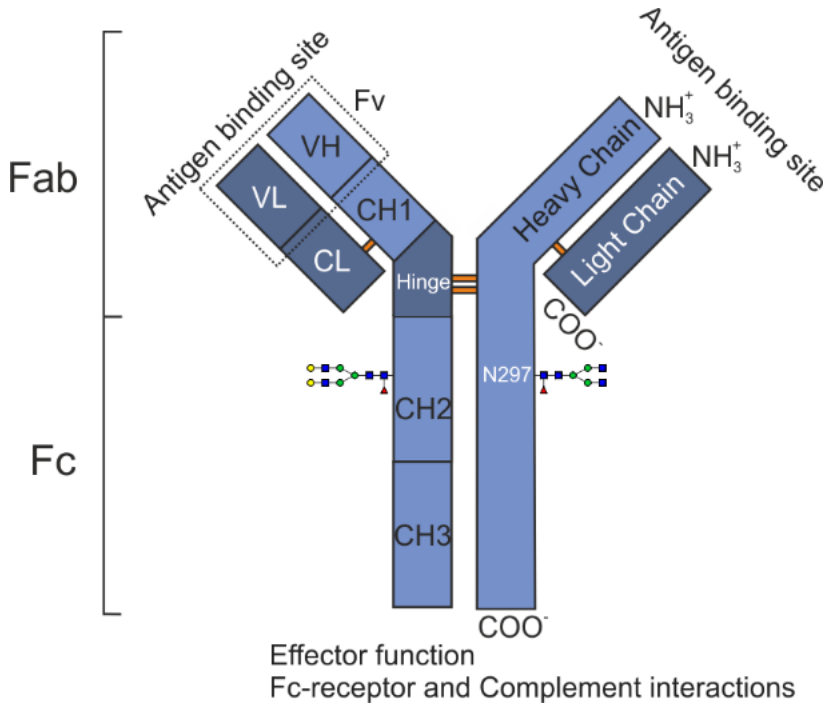


Figure 4 Schematic representation of an immunoglobulin G (IgG) molecule. An IgG consists of two heavy (H) and two light (L) chains linked by disulphide bonds. Each heavy chain contains a variable (V) domain (the VH domain) and three constant (C) domains (the CH1, CH2 and CH3 domain). Each light chain contains a V domain (the VL domain) and a single C domain (the CL domain). The two variable domains (the VH and VL domain) of the Fab (fragment antigen binding) region constitute the variable fragment (Fv) [57].

1.4.2 IgG Functions

IgG can interact through its Fc fragment with FcγRs expressed by effector cells or with complement component 1q (C1q). This potentially supports the destruction of target cells through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) as well as phagocytosis.

ADCC is a mechanism of the humoral immune response in which antibodies cover the antigen by binding with their Fab portion while the Fc region binds to FcγRs, causing a lytic attack [58]. ADCC is mediated by activating the FcγRs, which are expressed on leukocytes including macrophages, eosinophils, neutrophils, and natural killer (NK) cells [59, 60]. When ADCC is triggered by NK cells, the FcγRIIIa receptor (CD16) binds to antibody-coated target cells and causes their apoptosis by releasing cytotoxic granules and cytokines like interferon γ (IFN-γ) [61].

The complement system is an important part of the innate immune response that clears microbes and damaged cells. The classical pathway, sometimes named the antibody-dependent pathway, is a process triggered by an immune complex formed by the binding of the Fab portion of IgM or IgG antibodies to the surface of an antigen. This activates the complement through the binding of IgG to C1q. The first step of complement cascade splits C2 and C4, producing C3 convertase C4b2a, which promotes the cleavage of C3 [62]. The amplifying cascade of further cleavages leads to the formation of the membrane attack complex and ultimately to the destruction of the target cells [63]. The lectin pathway is triggered by opsonin, either mannose-binding lectins (MBL) or ficolins that bind to mannose residues on the pathogen surface [64-67]. It activates the complement cascade by immune complex formation with MBL-associated serine proteases (MASPs) [66]. MASPs split C4 and C2 and then form the C3 convertase as in the classical pathway. The alternative pathway is continuously activated at a low level by spontaneous C3 hydrolysis in plasma [68, 69]. The hydrolysed product can bind to factor D, forming the C3 convertase as in the classical pathway.

When the Fab region of the IgG binds to the antigen-coated target cell and the Fc region binds to a FcγR on a phagocyte, the phagocytosis of the pathogen is enhanced. The antibody that induces phagocytosis by binding a pathogen to induce

an immune response, being named opsonin, the process is named opsonophagocytosis [70].

As noted above in paragraph 1 and 2, FcγRs binding to antibodies are important for inducing the IgG effector functions, such as ADCC and phagocytosis [54]. In humans, six different Fc-receptors that bind to IgG are involved, FcγRI (CD64), FcγRIIa (CD32A), FcγRIIb (CD32B), FcγRIIc (CD32C), FcγRIIIa (CD16A) and FcγR (CD16B), which differ in their affinity for the IgG-Fc portion due to their different structure [54, 71]. The FcγRI is a high affinity receptor, while FcγRII and FcγRIII display a low to moderate affinity [72]. With regard to initiation of the immune response, the FcγRs can be further subdivided into being inhibitory or activating [73]. FcγRIIb is the only inhibitory FcγR, which is involved in regulation of cellular activation. It suppresses the activation of activating receptors [74]. IgG does not only binds to the canonical FcγRs, but also to the neonatal FcR (FcRn), whose interaction is responsible for the half-life of circulating IgG by protection from degradation [75].

It has been shown that sialylated IgG Fc domains have anti-inflammatory properties, this activity is clearly restricted to α2,6-sialic acid residues though. The mechanism of sialylated IgG-mediated anti-inflammatory activity is largely unknown, but the C-type lectin dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN (CD209)) is involved [76, 77]. It is currently assumed that the binding of sialylated IgG to DC-SIGN causes an anti-inflammatory effect by reducing the binding affinity to canonical FcγRs [78].

1.4.3 IgG *N*-Glycosylation

The IgG-Fc region contains one highly conserved *N*-linked glycosylation site at asparagine 297 in the C_H2 domain of each heavy polypeptide chain [79] and this site is exclusively occupied by complex-type biantennary *N*-glycans [79-81]. IgG *N*-linked glycans share a common pentasaccharide core, which can be modified with additional monosaccharides by expression-dependent glycosyltransferases during glycan biological processing. The final product carries two antennae formed by GlcNAc-Gal-Neu5Ac. IgG-Fc *N*-glycans are frequently core-fucosylated (around 80% of total human serum IgG). Bisecting GlcNAc and the fact that only a small portion of these glycans carry the complete set of monosaccharides on both antennae contributes to the high degree of complexity [55, 82]. Since IgG exhibits

hypogalactosylation in rheumatoid arthritis [27, 83, 84], the occurring biantennary glycans can be divided into three glycoforms with either both arms or one arm carrying galactose residues (G2 and G1, respectively), or with neither of the arms bearing a galactose (G0) (Figure 5) [85-88]. *N*-Glycans are known to be present on approximately 20% of the IgG-Fab portions of polyclonal antibodies [89-91]. In contrast to IgG-Fc *N*-glycans, the Fab region showed the presence of highly sialylated as well as bisected complex *N*-glycans, and a non-negligible portion of high-mannose *N*-glycans [92-94].

While the Fab *N*-glycans of an antibody are primarily responsible for antigen binding and specificity [95-97], Fc *N*-glycans have many important functions including the recruitment of effector mechanisms, maintaining the structure and increasing the half-life [98].

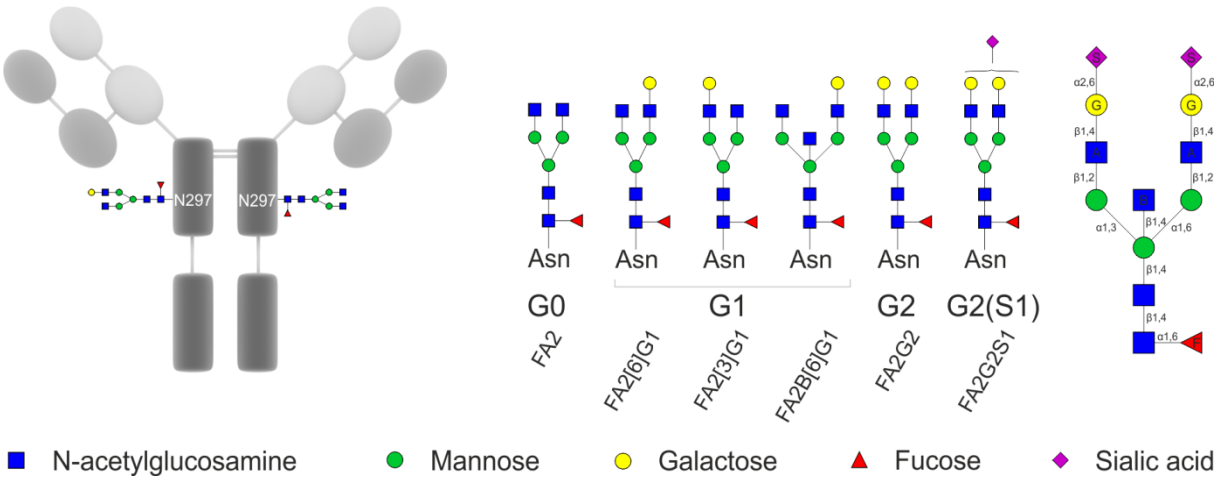


Figure 5 Composition of biantennary *N*-glycan structures attached to IgG Asn297. Major structures being present in agalactosylated (G0; FA2), monogalactosylated (G1; FA2[3]G1, FA2[6]G1 and FA2B[6]G1), digalactosylated (G2; FA2G2), and sialylated (G2S1; FA2G2S1) forms. *N*-Glycans share a common pentasaccharide core consisting of two *N*-acetylglucosamines (GlcNAc) and three mannose (Man) residues; F indicates a core fucose α1-6 linked to the inner GlcNAc; Ax, number of antennary GlcNAc attached to the trimannosyl core; B, bisecting GlcNAc linked β1-4 to β1-3 mannose; Gx, number of β1-4 linked galactose (G); [3]G1 and [6]G1 indicates that the galactose is on the antenna of the α1-3 or α1-6 mannose; Sx, number of *N*-acetylneuraminic acids linked to galactose.

1.4.4 IgG *N*-glycan Functions

The interaction of IgG with Fc gamma receptors (FcγRs), complement C1q protein (C1q) and C-type lectin receptors (CLRs) is not only dependent on the IgG subclass, but also modulated by variable Fc glycosylation. IgG-Fc carbohydrates are largely enclosed between two C_H2 domains and help maintaining the three-dimensional structure of the IgG Fc regions by non-covalent interactions with the protein surface [79, 99]. This enables an open conformation between the two heavy chains and facilitates interaction with FcγRs and C1q. Deglycosylated or truncated Fc glycans cause a breakdown (closed conformation) of the Fc region [99]. Binding and consequently ADCC and CDC are hindered [100, 101].

1.4.4.1 Fucosylation

The diversity of IgG-Fc *N*-glycans has functional consequences. Glycosylation of IgG modulates the interaction with FcγRs or CLRs, thereby influencing antibody-dependent cell cytotoxicity and pro-inflammatory activity [102]. Several expression systems with an α1,6-fucosyltransferase (FUT8) deficient variant show that afucosylated IgG increases the interaction with activating FcγRIIIa (CD16), which is associated with an enhancement of ADCC [58, 103-106]. An elevated bisecting GlcNAc level resulting from a co-overexpression of the recombinant IgG1 and β1,4-N-acetylglucosaminyltransferase III (GnTIII) in CHO cells also results in an increased binding affinity to FcγRIIIa. In comparison to afucosylated glycoforms with an up to 50-fold increase in affinity [103, 107], bisecting IgG exhibits a moderate 10-fold increase in affinity [58, 108, 109]. Increased ADCC activity can also be observed in the presence of predominantly agalactosylation (G0) IgG Fc-linked carbohydrates, especially with a lack of fucose residues. While ADCC activity is highly dependent on the glycosylation profile of the IgG-Fc portion, afucosylated monoclonal antibodies (as well as the addition or the removal of other monosaccharide residues) show a high degree of therapeutic potential for treating tumors as well as inflammatory diseases [109-112].

1.4.4.2 Galactosylation

In general, truncated IgG *N*-glycans decrease CDC [113-115]. Agalactosylated glycoforms however give way to a new epitope which triggers the production of autoantibodies and facilitates the interaction with mannose-binding lectin (MBL) [115,

116], which activates the complement cascade by immune complex formation with serine proteases (MASPs). The presence of terminal galactose as well as sialic acid residues reduces the binding to activated FcγRs and is associated with enhanced anti-inflammatory effects of IgG [66, 117]. Terminal galactose residues on IgG Fc glycans mediate an anti-inflammatory activity by facilitating the association of the inhibitory FcγRIIB and C-type lectin-like receptor dectin-1 (Figure 6) [118]. The formation of IgG-immune complexes and the associated anti-inflammatory activity is subclass specific, for example, galactosylated IgG2a fails to trigger such inhibition [118].

1.4.4.3 Sialylation

As mentioned above, sialylated IgG Fc domains have anti-inflammatory properties. This activity is specific to α2,6-sialic acid residues [119, 120]. The presence of terminal sialic acid residues reduces the binding to FcγRs and is associated with enhanced anti-inflammatory effects resulting from conformational changes initiated by binding to the human C-type lectin receptor DC-SIGN [77, 121]. Therapeutic intravenous gamma globulin (IVIG) is a purified IgG fraction prepared from the pooled serum of healthy donors. It is used to treat various autoimmune diseases such as rheumatoid arthritis (RA), immune thrombocytopenic purpura, multiple sclerosis and systemic lupus erythematosus (SLE), administered at high doses (1-2 g/kg) [119, 120, 122-126]. Deglycosylation of IVIG Fc *N*-linked glycans by PNGase F leads to an inactivation of anti-inflammatory activity *in vivo*. Completely desialylated IVIG shares the same effect, whereas no differences is observed when α2,3-sialic acids are removed using specific α2,3-sialidase, indicating that α2,6-sialylated IgG plays a key role in the induction of anti-inflammatory activity [119, 120, 126]. This mechanism shows that certain IgG-Fc glycoforms are associated with inflammatory states. During an inflammatory condition, the levels of sialylation, galactosylation, and at best fucosylation of the IgG-Fc glycans should be reduced, which cause ADCC and trigger inflammation. In contrast, the *N*-glycans of anti-inflammatory IgG should be highly sialylated. This regulation system of pro- and anti-inflammatory IgG could be a mechanism for homeostatic balance [77].

While specific glycan types on the IgG-Fc portion directly contribute to the effector function of IgG, antibodies are used for therapeutic treatment. For this

purpose, recombinant antibodies with targeted effector functions are produced for patient-related therapies.

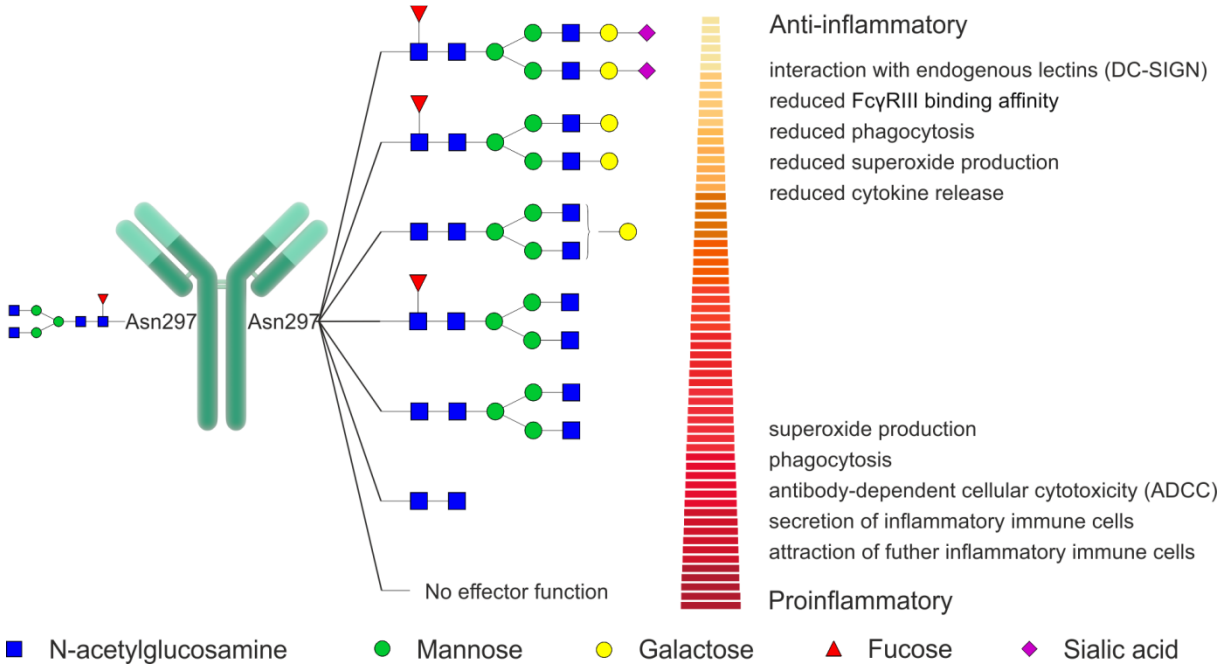


Figure 6 IgG-Fc glycoforms and their inflammatory properties. The IgG-Fc *N*-linked glycan can be classified broadly as being either G0, G1, or G2, as described in Figure 5. The composition of IgG-Fc glycan influences IgG-mediated cellular inflammatory responses. G0 glycans terminate with GlcNAc residues and have a higher affinity for FcγRIII. Glycans can also be sialylated and fucosylated, which can lead to anti-inflammatory properties of IgG caused by decrease binding affinity for FcγRIII [127].

1.5 Glycosylation in Health and Diseases

The glycosylation of serum proteins varies distinctly and gives insights into the physiological and biochemical conditions of an individual. As different glycoforms may be attached to each glycosylation site, the resulting heterogeneity is the most important feature of glycoproteins. Their glycosylation pattern is cell-specific and generally highly reproducible in a given physiological state. However, several studies demonstrate that changes in glycosylation are associated with many diseases. The accompanying cellular alterations affect glycan processing and are depending on various factors. These include the altered expression levels of glycosyltransferases and glycosidases, which, owing to dysregulation at the transcriptional level [128-131],

causes changes in the availability of sugar nucleotide donors and localization of the relevant glycosyltransferases in the Golgi apparatus. Ultimately, the tertiary conformation of peptide backbones is influenced [132-135]. Altered IgG glycosylation is not only associated with pathological conditions [136-138], such as inflammation [27, 136, 139] and disease progression [140-142], but it can be also found physiologically in healthy individuals, for instance during the aging process or during pregnancy [143-146]. Also gender, physical activity and lifestyle (e.g. smoking) play a role.

1.5.1 Impact of Age, Gender and Pregnancy on IgG *N*-Glycans

Several studies have shown that IgG glycosylation in a healthy population is age- and sex-dependent. Almost three decades ago, Parekh *et al*. were the first to describe the decrease of IgG galactosylation during aging [143]. These results were repeatedly confirmed and complemented by subsequent works, which show that the decrease of the galactosylation level on IgG does not begin before the age of 25 [143]. It was further found that IgG sialylation also decreases upon aging and that the decrease of IgG galactosylation and sialylation is more common in women [143, 145]. Kristic *et al*. proposed a combined index comprised of three IgG *N*-glycans (FA2B, FA2G2, and FA2BG2), which are considerably altered during aging. These three account for up to 58% of the variance in aging [147].

Furthermore, pregnancy is accompanied by an increase in IgG galactosylation and sialylation as well as a decrease postpartum. These glycosylation changes were found for both healthy individuals as well as RA patients [137, 138, 148-151].

It has been shown that several diseases, observed for the first time in rheumatoid arthritis and osteoarthritis, are associated with decreased galactosylation of IgG *N*-glycans [27]. This was also found in different pathological conditions such as autoimmune diseases (Crohn's disease [83, 149, 152], juvenile onset chronic arthritis [149], psoriatic arthritis [153], rheumatoid arthritis [27, 83, 149, 152-154], systemic lupus erythematosus [149, 152, 153, 155]), infectious diseases (hepatitis C virus infection [156] and human immunodeficiency virus (HIV) infection [157]), and tumors (gastric cancer [158-160], lung cancer [160], ovarian cancer [140, 161, 162], prostate cancer [141], thyroid cancer [163]). It is currently unknown whether this

phenomenon is related solely to aberrant *N*-glycan formation during IgG synthesis or whether altered *N*-glycan degradation plays a role as well.

1.5.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common chronic inflammatory disease of unknown etiology [164]. As many patients develop joint damage at an early stage after disease onset, it is of special clinical importance to identify and characterize the pathomechanisms in those patients who then need an early and aggressive therapy [165, 166]. In approximately 75% of RA patients, the clinical course is significantly influenced by the B-cell involvement in the production of rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPAs). This results in more severe symptoms and joint destruction in general, but also in more cardiovascular complications and an increased mortality [164, 167, 168]. Therefore, it is assumed that pathogenetically and clinically distinct RA subsets are defined by the presence or absence of these antibodies [169]. A genetic background for their production as response to different environmental triggers is primarily given by the HLA-DRB1 shared epitope (SE) [170-174].

Rheumatoid arthritis most commonly afflicts hand and feet joints, but also other joints like the shoulder and knee, which involves an inflammation of the synovial tissue [175]. Symptoms of RA are joint inflammation as well as swelling and pain in the affected joints. Without medical treatment this leads to erosion, deformity, and loss of function of the joint. The most prominent feature of RA is the formation of immune complexes by autoantibodies, predominantly RF and ACPA of IgG and IgM classes, which can lead to abundant complement activation [175-178]. Eventually, the abnormal immune response results in increased concentrations and an epitope diversity of ACPAs, leading to an increased T cell activation. Together with synovial macrophages, monocytes and dendritic cells function as antigen-presenting cells. This enhances the binding of autoantibodies to the Fc-receptors and antigens, resulting in an inflammation of the synovium and a local immune reaction in the tissue [127, 179, 180]. In this context, the production of pro-inflammatory cytokines (e.g. interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF)-alpha) as well as certain enzymes is involved causing chronic inflammation [181-185]. This leads to joint destruction and systemic complications, with increased comorbidity [169].

1.5.3 RA Diagnosis

Up to date, no diagnostic criteria for rheumatoid arthritis exists. The classification criteria for RA diagnosis have been compiled by the American College of Rheumatology (1987) [186]. These comprise the number of tender and swollen joints, morning stiffness, and routine laboratory tests including C-reactive protein measurement (elevated) and erythrocyte sedimentation rate. Nevertheless, this data is not specific for RA, particularly with regard to the differential diagnosis of arthritis. In order to better distinguish between RA and arthritis [175], new classification criteria for RA (2010) have been established by examining patients with early arthritis [187, 188]. A 21% higher sensitivity was achieved through the utilization of ultrasound, radiographic examinations (MRI), the assessment with the disease activity score 28 (DAS28) including joint counts and the serological markers RF and ACPA [189].

However, classification is not tantamount to diagnosis. Based on serological ELISA tests, one can say that the sensitivity and selectivity of RF- and ACPA in RA diagnosis is limited [190, 191]. Thus, additional diagnostic approaches to predict RA or monitor the development and progression of RA are necessary. Novel approaches rely increasingly on the determination of the ratio between agalactosylated and monogalactosylated *N*-glycans (G0/G1 ratio) of IgG, because this value is characteristic for alterations in IgG glycosylation [27, 154]. Aberrant galactosylation precedes disease onset of several years and correlates with the severity of RA [154, 192]. In patients showing severe alterations in radiological imaging and an elevated amount of the inflammation marker CRP, increased IgG-G0 levels were observed, as well as an association between decreased sialylation of IgG and bone loss [154, 193-196].

Although both ACPA and serum IgG have exclusive specificity for RA, the glycosylation patterns of both types significantly differ, lower levels of galactosylation and sialylation were found for ACPA [154]. Additional studies have shown that ACPA glycosylation also differs between serum and inflamed synovial fluid. ACPA in synovial fluid show reduced levels of galactosylation and sialylation and therefore can be distinguished from ACPA in serum [88, 139]. However, the relationship between aberrant IgG galactosylation and specific processes involved in humoral autoimmune pathogenesis of RA is currently less understood. Ercan *et al*.

hypothesized that IgG is the source of G0/G1 aberrancy, which is synthesized as a response to autoimmune stimulation [154].

1.5.4 IgG Glycosylation in RA

ACPA and RF detection procedures predominantly focus on IgG. Galactosylated *N*-glycans represent the most common IgG glycoforms in total IgG glycosylation. These types best serve for the analysis of alterations in the glycan composition. The nomenclature of the glycoform (G0, G1, G2) is dependent on the number of *N*-glycan antennae occupied by galactose [197]. It has been shown that the amount of agalactosylated *N*-glycans of IgG (IgG-G0) are increased in RA patients when compared to healthy individuals [27, 83]. While other variations of IgG-Fc glycans directly affect the effector functions, agalactosylation of IgG changes in immunogenicity and that causes an altered immune response of these antibodies. In truncated IgG *N*-glycans, a new epitope is presented, which triggers the production of autoantibodies and facilitates the interaction with mannose-binding lectin (MBL). This activates the complement cascade, leading to an inflammatory response [66, 198, 199]. Furthermore, agalactosylation of IgG increases the tendency to be bound by RF, and this is associated with an increased immune complex formation [200-203]. It is worth noting that remission of rheumatoid arthritis during pregnancy and upon effective treatment is associated with a normalized glycosylation [138, 139, 204-209].

It is presumed that the increase of IgG agalactosylation in RA is directly associated with a reduced galactosyltransferase (β1,4GalT I, GTase) activity in lymphocytes [210]. Previous studies have shown a possible mechanism for post-translational regulation of GTase activity in RA. In RA, either qualitative and quantitative changes of GTase expression could result in an altered enzymatic activity, or the activity of specific B cell and plasma cell subsets with a preference for agalactosylated IgG is increased in RA [211].

1.5.5 IgG Glycosylation in Cancer

Studies on IgG glycosylation as potential marker for cancer showed that the mono- (G1) and digalatosyl (G2) IgG N-glycans decrease, while agalactosyl (G0) IgG N-glycans increase during prostate cancer (PCa) progression. The corresponding G0/(G1+G2) ratio in PCa patients is significantly higher than in healthy individuals and correlates with serum prostate specific antigen (PSA) levels, the best current

marker for PCa [141]. As mentioned above, increased levels of core-fucosylated agalactosyl biantennary glycans can be found in various types of cancers including gastric, lung, ovarian, prostate and thyroid cancer [140, 141, 158, 160, 162, 163]. This glycan is almost exclusively present on IgG (IgG G0 glycoform).

1.5.6 *N*-Glycome Modulation and Function in Cancer

Cellular changes in cancer influence the glycosylation process, leading to altered glycans when compared to normal individuals. Analysis of the serum glycome and specific glycoproteins may pose as a useful tool for diagnostics and determination of the correct therapeutic strategy to fight cancer.

The most characterized cancer-associated changes in *N*-linked serum glycome are the sialylation, including the antigens sialyl Lewis^a (SLe^a) and sialyl Lewis^x (SLe^x), the degree of glycan branching, and α1,6 fucosylation [135, 197, 212].

1.5.6.1 Sialylation, Fucosylation and Lewis Epitopes

An increase in overall sialylation, predominantely in α2,3- and α2,6-linked sialic acids, results from an altered α2,3- (ST3Gal IV) and α2,6-sialyl transferase (ST6Gal I) expression [16]. Polysialic acid (polySia), which comprises repeating sialic acid units connected via α2,8-linkages, can be found in neuroblastoma and lung cancer. Polysialylation occurs relatively seldom. The neural cell adhesion molecule (NCAM, CD56) is the most common acceptor protein to receive polysialylation [213, 214].

An elevated sialylation of integrins influences cell-extracellular matrix (ECM) interactions and stimulates cell migration in colon cancer progression. In colon carcinoma, sialylation of β1 integrins blocks the cell adhesion to galectin-3 (gal-3) and protects tumor cells against induced apoptosis effects of gal-3 [215, 216].

The SLe^x epitope is another sialylated structure associated with cancer, assembled from an α2,3-linked sialic acid residue to galactose and an α1,3-linked fucose to GlcNAc. Apart from α2,3-sialylation added by the ST3Gal IV sialyltransferase, the fucosyl transferase (FUT) VI is the major enzyme modulating the SLe^x biosynthesis in cancer by attaching α1,3-fucose residues [217]. The attachment of antennary fucoses may also be performed by fucosyl transferase III. Elevated expression levels of the SLe^x epitope have been found in many cancer

forms, for example in colorectal cancer (CRC) and is associated with poor patient survival [218]. These glycan alterations can also be found in acute phase proteins such as haptoglobin and α1-acid glycoprotein during chronic inflammation [217, 219-221]. In stage four ovarian cancer patients, elevations in serum glycoproteins containing SLe^x, correlate with the levels of the serum inflammatory marker C-reactive protein (CRP) [140].

Due to the ligand function for endothelial selectin (E-selectin), SLe^x plays a key role during the process of leukocyte extravasation. During inflammation, SLe^x binds to selectins, which mediate the attachment of endothelial cells and leukocytes and initiate rolling. In cancer, sialyl Lewis epitopes serve as ligands for endothelial cells (E-selectin), leukocytes (L-selectin), and platelets (P-selectin), favoring cancer cell adhesion and metastasis [222-226].

Compared with SLe^x, the SLe^a epitope shows other linkages between the monosaccharides. It is composed of a galactose β1,3-linked to GlcNAc and a fucose α1,4-linked to GlcNAc. The detection of SLe^a as cancer-associated epitope is the principle used for clinical diagnostic in the CA19-9 serum assay. It is typically used to detect colon and pancreatic cancer, but also for various types of gastrointestinal cancer, such as colorectal cancer, esophageal cancer, and hepatocellular carcinoma [227-230].

Antennary fucosylation can be found predominantly in sialyl Lewis antigen. In lung and breast cancer however, also the core often occurs [231, 232]. The fucosyl transferase FUT VIII catalyzes the addition of α1,6-fucose to the innermost GlcNAc residue of *N*-glycans (see section 1.5.8).

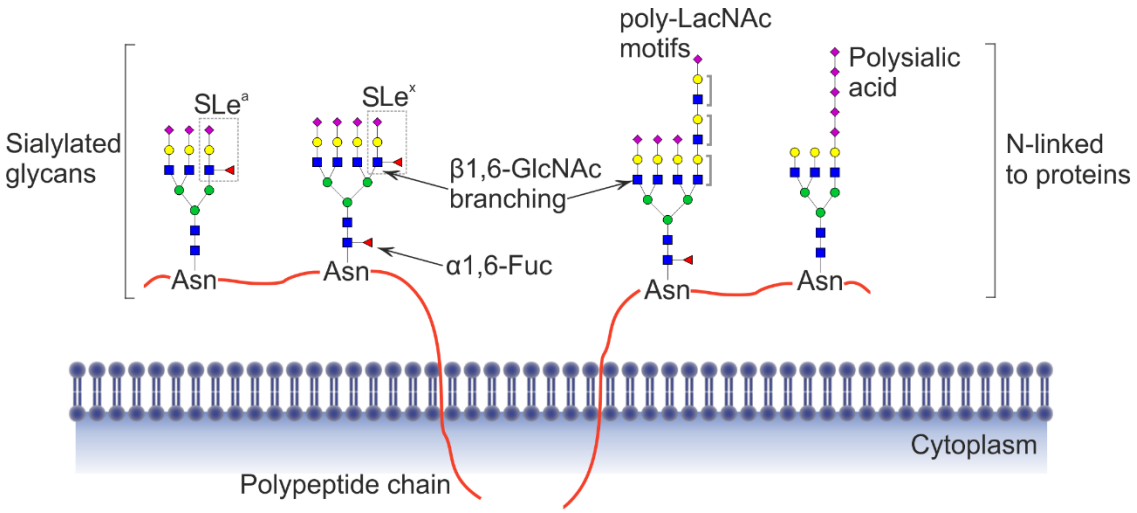


Figure 7 Tumor-associated *N*-glycans. Glycome modulations associated with cancer include an increase in sialylation, SLe^x and SLe^a antigens as well as α2,8-linked polysialic acid. Furthermore, this also applies for the degree of glycan branching and the addition of α1,6-fucose to the innermost GlcNAc ('core' fucosylation) [135].

1.5.6.2 Branching

Increased *N*-glycan branching is an essential characteristic of cancer-associated glycosylation changes. The biosynthesis of complex tri- (β1,4-linked GlcNAc) and tetraantennary (β1,6 linked GlcNAc) *N*-glycan structures is performed by the GlcNAc tranferases (GlcNAcTs) IV and V [233, 234]. Increased expression levels of β1,6-branched oligosaccharide structures are directly associated, firstly, with increased activity of GlcNAcT V in cancer cells, and secondly, with the metastatic potential of cells. It has been shown that GlcNAcT V expression in mink lung epithelial cells, Mv1Lu, promoted characteristics of transformation, including reduced adhesion to fibronectin- and collagen-coated surfaces, contact-inhibition of cell growth, motility and increased tumorigenicity [197, 235]. This can be attributed to increased β1,6-branched *N*-glycans on epithelial cadherin (E-cadherin) and α5β1 integrin, which are responsible for cell-cell adhesion as well as cell migration and cell invasion [236]. The modification of E-cadherin impairs cell adhesion and promotes tumor cell invasion through incorrectly assembled and non-functional adherens junctions. Changes in integrin *N*-glycans in cancer induce an increased cell migration and invasion because of an interrupted receptor function for fibronectin [237]. After *N*-glycan branching elongation, the structure is capped first by galactose and, where appropriate, extended with poly-*N*-acetyllactosamine (repeating Galβ1,4GlcNAcβ1,3).

N-Glycan processing is completed by fucose and sialic acid residues to produce Lewis antigens. The poly-*N*-acetyllactosamine epitope is a ligand for most galectins [238]. These play an important role in tumor cell survival and angiogenesis [239]. The GlcNAcT III catalyzes the attachment of bisecting GlcNAc to biantennary *N*-glycans and that results in the suppression of the action of GlcNAcTs IV and V. This leads to a considerable reduction of the formation of branched *N*-glycan structures and it has been shown that GlcNAcT III is involved in suppression of tumor metastasis [240].

1.5.7 *N*-Glycome Modulations of Serum Glycoproteins in Cancer

The previously mentioned *N*-glycosylation structures can be found in most cancer forms and inflammation processes. They are mainly related to IgG as well as to acute phase proteins (APPs), predominantly haptoglobin (HPT), α1-antitrypsin (A1AT), α1-acid glycoprotein (A1AG) as well as α1-antichymotrypsin (AACT) [220, 221, 241, 242]. During inflammation, liver hepatocytes upregulate the expression of APPs in response to inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor alpha (TNF-α) [243-245]. In the acute-phase response, inflammatory cytokines not only alter the expression levels of APPs, but also influence the expression of glycosyltransferases that are responsible for glycosylation. Stimulation of IL-1β-induced expression of ST3Gal IV and FUT VI in a hepatic cell line (HuH-7) results in an increased SLe^x expression level [246]. Increased branching and SLe^x epitopes of secreted α1-acid glycoprotein have also been found in HuH-7 cells caused by IL-1β and IL-6 [247]. In the above-mentioned positive APPs, HPT, A1AT and AACT, an increase of antennarity and $Lewis^x$ motif was found in tri- and tetraantennary *N*-glycans of patients compared to the glycans of age-matched healthy controls [248-250]. Tetraantennary *N*-glycans containing three $Lewis^x$ epitopes and triantennary *N*-glycans containing a β(1-6) branch and a $Lewis^x$ epitopes were only present in epithelial ovarian cancer (EOC) patients. Furthermore, a core-fucosylated biantennary *N*-glycan on α1-acid glycoprotein was identified as potential biomarker for EOC [251]. In stage four ovarian cancer patients, the elevation of serum glycoproteins containing SLe^x correlates with the concentration of the serum inflammatory marker C-reactive protein (CRP) [140]. It must be taken into account that these glycosylation changes mainly derive from highly abundant APPs and IgG. This conceals a glycosylation modulation of low abundant glycoproteins with appropriate cancer specificity. Studies focusing on a detailed serum *N*-glycan

analysis in cancer have shown an upregulation of disialylated monofucosylated tetraantennary *N*-glycans in breast cancer patients and a decrease of high-mannose structures in ovarian cancer patients [142, 252].

1.5.8 Glycans in Cancer Diagnosis

Up to now, some of the serological biomarkers in clinical use for cancer diagnosis are glycoproteins. These biomarkers are commonly utilized for patients with ovarian cancer (carcinoma antigen 125 (MUC16), CA125) [253], breast cancer (MUC1, CA15-3) [254, 255], prostate cancer (prostate-specific antigen, PSA) [256], colon cancer, pancreatic cancer and gastric cancer (sialyl Lewis^a (SLe^a), CA19-9) [229, 230]. It should be noted that, except from PSA, they usually have low sensitivity and specificity upon detection of primary early stage malignant tumors, all these biomarkers display an aberrant glycosylation in cancer patients [236, 257-259]. Therefore, new approaches for early diagnosis, assessment of disease progression and treatment evaluation are needed not only for cancer, but also for other diseases.

As mentioned above, the addition of α1,6-linked fucose to the core structure potentially plays a role in a number of cancers. For the early detection of hepatocellular carcinoma (HCC), core-fucosylated α-fetoprotein (AFP) is an important marker, which makes it possible to discriminate between HCC patients and patients suffering from chronic liver diseases [260, 261]. Increased core-fucosylation of the epidermal growth factor receptor (EGFR) can be detected in breast cancer patients. This aberrant glycosylation is associated with increased dimerization and phosphorylation of EGFR, which affects tumor cell growth and malignancy [231, 262].

Diagnostics of aberrant glycosylation could supplement the existing routine serum markers, thereby increasing the overall sensitivity and specificity. Key challenges to achieve this goal include the automation of high-throughput glycomics, with less labor-intensive and easily practicable technologies and an accelerated transfer of relevant clinical findings into medical application [263-266].

1.6 Glycan Analytics

The heterogeneity of glycans includes macro- and microheterogeneity, as well as structural variability of oligosaccharides (monosaccharide type, position, linkage type and branching), which render glycan analysis complex. A miscellany of technical methods providing various degrees of structural information is available to analyze *N*-linked glycans. These include the capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF) [267-273], the ultra performance liquid chromatography (UPLC) coupled with pulsed amperometric detection (PAD) or with fluorescence detection (FD) [266, 274, 275], the liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) [276-278], and the matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [204, 279-283]. UPLC-FD and CE-LIF allow the quantification and rapid separation of glycan isomers, while MS-based technologies are rather used to analyze glycopeptides and to provide site-specific information, such as subclass-specific IgG-Fc glycosylation.

1.6.1 Purification of glycoproteins

In order to analyze the glycosylation of single proteins from complex protein mixtures, for instance from serum, plasma, or cell culture supernatant, the first step consists of protein purification. The most widely used protein purification techniques are the immunopreciptation (IP) using an antibody that specifically binds to that particular protein [204, 284, 285], the in-gel digestion of proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [251, 286-288] and the fast performance liquid chromatography (FPLC), based on differences in components partitioning behavior between the mobile liquid phase and the stationary phase [289]. Protein A (binding IgG1, IgG2 and IgG4) and protein G (binding all four IgG subclasses) are bacterial proteins that are commonly used for antibody purification and in applications such as IP due to the high binding affinity and specificity with the Fc region of the antibody [89, 204, 290, 291]. A separate analysis of both, the Fc and the Fab domain glycosylation requires a proteolytic digestion of IgG, either using the enzyme pepsin or IdeS (known commercially as FabRICATOR®) [92, 162, 180, 204, 292, 293]. Pepsin cleaves near the hinge region and results in an $F(ab')_2$ fragment and numerous small peptides of the Fc portion.

IgG-Fc peptides can be separated from the F(ab')$_2$ fragment by ultrafiltration using molecular weight cut-off (MWCO: 10 kDa) centrifugal membrane filters [162].

1.6.2 Release of *N*-Glycans

N-Glycans can be released from glycoproteins or serum samples using either enzymatic or chemical methods. Due to the inferior practicability of the chemical deglycosylation (hydrazinolysis) and the associated side reactions [294, 295], enzymatic methods have become widely accepted for glycan analysis. Before releasing glycans enzymatically, proteins have to be denatured in order to unfold their secondary and tertiary structure and to enhance the accessibility of the glycosylation sites to an enzyme. For this, heat and detergents such as sodium dodecyl sulfate (SDS) and NP-40 are used [296, 297]. Another possibility for destabilizing the three-dimensional structure of a protein is to break its disulfide bonds with a reducing agent such as dithioerythritol (DTE) [298]. Furthermore, proteins can be proteolyzed by endopeptidase digestion, typically using trypsin [299, 300]. This leads to a more efficient release of glycans from the peptides. Enzymatic release of all *N*-linked glycans is commonly performed using the endoglycosidase Peptide-N^4-(acetyl-β-glucosaminyl) asparagine amidase (PNGase F) (Figure 8), which cleaves *N*-glycans of high-mannose, hybrid and complex types, even when they are α1,6 core-fucosylated. The enzyme is not active on α1,3 core-fucosylated *N*-glycans found in plants and invertebrates [295, 301, 302]. In this case, PNGase A is used.

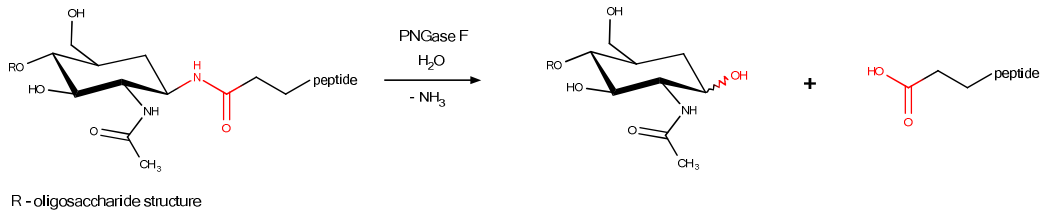


Figure 8 Reaction scheme for the enzymatic cleavage of *N*-glycans by PNGase F. The release of *N*-glycans from glycopeptides takes place when two β(1,4)-linked GlcNAc residues are attached to Asn on the peptide and the glycosylation site is accessible for the active PNGase F enzyme. PNGase F hydrolyzes the amide bond of β-aspartylglycosylamine by deamination of Asn. Subsequently, the unstable glycosylamine is hydrolyzed non-enzymatically forming the chitobiose core of *N*-glycan with free reducing end (stable).

1.6.3 Labeling and Clean-up

Released *N*-glycans can be labeled at their reducing end by reductive amination with fluorescent reagents such as: 8-aminopyrene-1,3,6-trisulfonic acid (APTS) [303-305] (Figure 9) and 2-aminobenzamide (2-AB) [306, 307]. This allows their detection in CE-LIF and UPLC-FD, respectively.

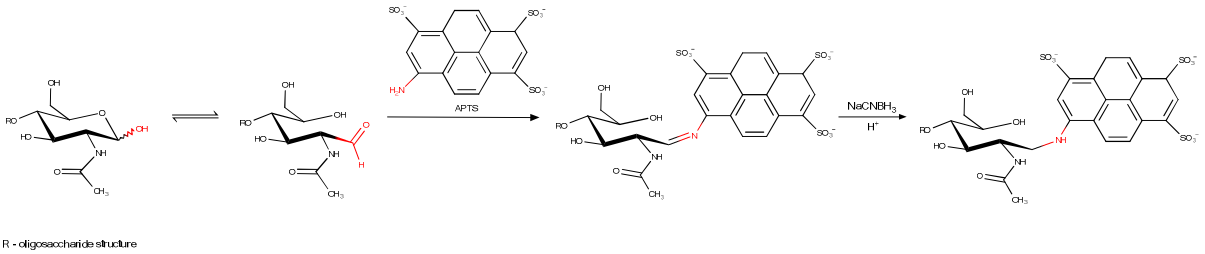


Figure 9 Reductive amination of oligosaccharides with 8-aminopyrene-1,3,6-trisulfonic acid (APTS). A condensation reaction of acyclic oligosaccharide and primary amine of APTS form a Schiff's base. Subsequent reduction of the imine with sodium cyanoborohydride results in a labeled oligosaccharide.

In order to perform either of the analyses adequately, it is necessary to remove the excess of labeling reagents from the labeled glycans. Several purification procedures for labeled glycans can be performed, such as size-exclusion chromatography (SEC; Sephadex G-10) [308] [309], solid-phase extraction (SPE) with different stationary phases, including graphitized carbon [162, 310] and hydrophilic interaction (HILIC; hydrophilic polypropylene GHP filter plate) [311, 312], liquid-liquid extraction (for instance with water and chloroform) [313, 314], and paper chromatography [286, 315]. Typical examples of purification strategies are summarized in Table 1.

Table 1 Sample purification after glycan labeling [316].

Method	Material	Label	Comments	References
SPE	RP, C18	RA, aniline Permethylation		[142, 295, 317, 318]
	PGC	RA, 2-AB RA, APTS	Sample application in water, elution using 10-50% (v/v) ACN	[271, 310]
	HILIC, cellulose HILIC, Oasis HLB HILIC, GHP HILIC, DPA-6S resin	RA, 2-AA RA, 2-AB RA, APTS	Sample application in 80-96% ACN, elution in water	[263, 312, 319-322]
Gel filtration	Sephadex G10, G15	RA, APTS RA, 2-AB RA, PA		[309, 323, 324]
Liquid-liquid extraction	Water/chloroform	Permethylation	Permethylated glycans are in the organic (lower) phase	[142, 313, 314, 325]
Paper chromatography	Whatmann filter paper	RA, 2-AB RA, DAP	Glycans are eluted using water	[286, 315, 326, 327]

2-AA 2-aminobenzoic acid, *2-AB* 2-aminobenzamide, *ACN* acetonitrile, *APTS* 8-aminopyrene-1,3,6-trisulfonic acid, *DAP* 2,6-diaminopyridine, *DPA* polyamide resin, *GHP* hydrophilic polypropylene, *HLB* hydrophilic-lipophilic balance, *HILIC* hydrophilic interaction liquid chromatography, *PA* 2-aminopyridine, *PGC* porous graphitized carbon, *RA* reductive amination, *RP* reverse phase, *SPE* solid-phase extraction

1.6.4 Separation Techniques

Next to HPLC, capillary electrophoresis (CE) is a powerful analytical technique for the analysis of glycans as it can separate structural isomers. The principle of this method is that the separation of analytes takes place in a thin capillary filled with an electrolyte solution under the influence of an electric field (Figure 10).

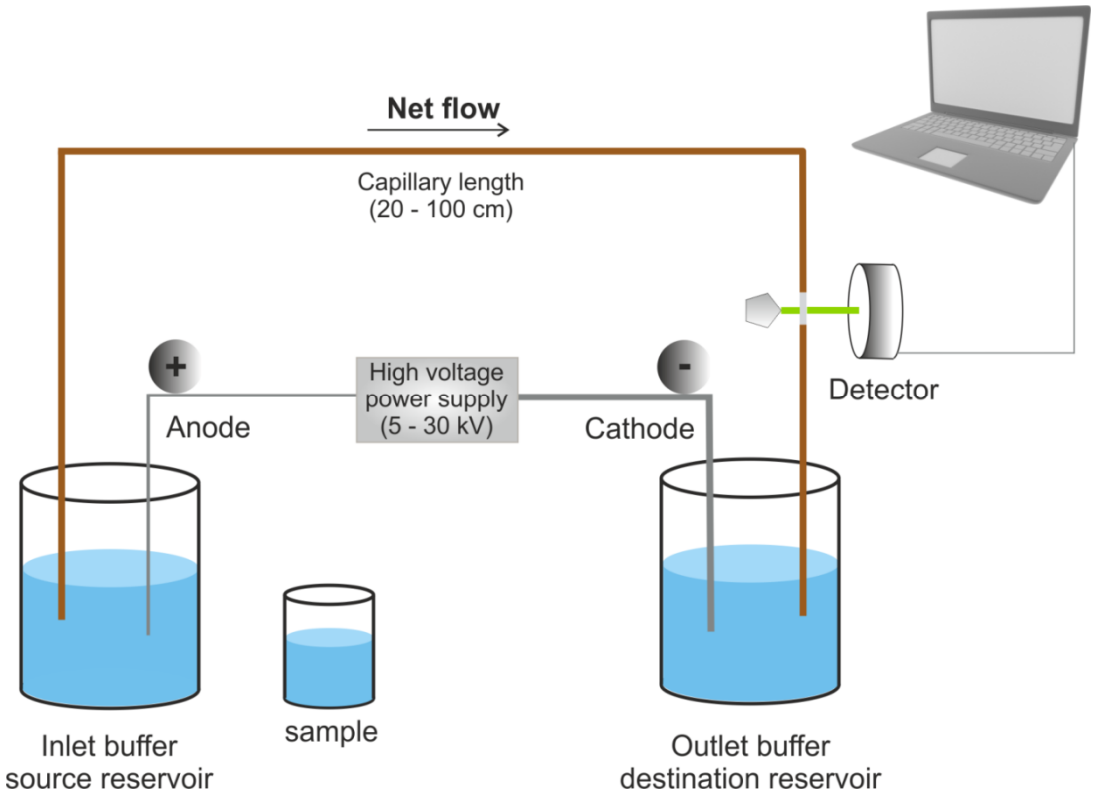


Figure 10 Instrumental setup of a capillary electrophoresis system.

Silanol groups on the bare fused-silica capillary inner-surface are ionizable contact with an electrolyte solution, and its pH value determines the degree of ionization. Negatively charged silanol groups of the capillary bind cations of the electrolyte solution, forming a charged double layer. Cations of the double layer migrate to the cathode when a current is applied, which results in an unilateral solution flow direction called electroosmotic flow (EOF). The migration rate of the ions through the capillary and detector is determined by several factors including EOF, the electrophoretic mobility and the instrumental setup of the machine. The v_{EOF} EOF is defined through the following equation, in which ε is the dielectric constant, η the viscosity of the electrolyte and ζ the zeta-potential. The EOF is dependent on the pH value, ionic strength, temperature and viscosity of the electrolyte system as well as the electrophoretic mobility.

$$v_{EOF} = \frac{\varepsilon\,\zeta}{4\,\pi\,\eta}\,E$$

The latter represents the migration speed of the ions, which is predominantly influenced by the charge, shape, and effective size (hydrodynamic volume) of the individual analytes, and the instrumental setup of the machine - through strength of the electric field, the length of the capillary, and the nature of the capillary wall (Figure 11).

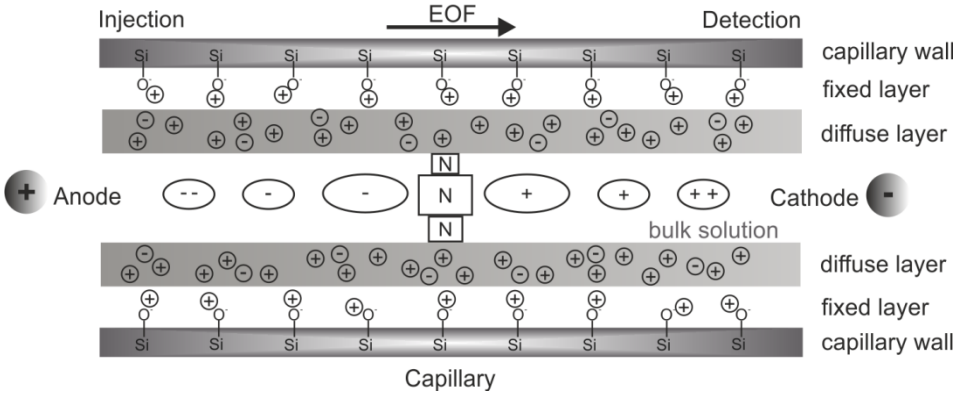


Figure 11 Schematic diagram of the electrical double layer within the capillary tube and the associated charge density distribution. Separation of charged and neutral analytes occurs according to their respective electrophoretic and electroosmotic flow mobilities. N: neutral.

The advantages of CE lie in its ease of handling, efficient separation of complex glycan mixtures with high sensitivity, short analysis time and minimal required quantities of samples and reagents. It permits a safe and reliable quantification, at high resolution of positional and linkage glycan isomers [267, 271, 328-330]. Therefore, CE is an excellent method for *N*-glycan analysis and has the potential to become the first glycomic application run at automated high-throughput in routine clinical laboratories. However, APTS-labeled glycans migrate according to their negative charge, resulting in short migration times of sialylated glycans. This is associated with a reduced resolution and the occurrence of co-migration with one another or with neutral *N*-glycans. In the last two decades, different derivatization methods for neutralizing the negative charge of α2,3- and α2,6-linked sialic acids have been developed with the aim of avoid sialic acid loss and to improve the sensitivity of MS analysis [331]. For instance, the free carboxylic groups of sialylated glycans and glycopeptides can be neutralized by methylation [332], methylamidation [273, 333] and permethylation [279, 334]. Sialic acid derivatization has been adapted

for the analysis of *N*-glycans by CE-LIF. The rate of migration is exclusively influenced by the size of the analytes, improving the resolution of glycan isomers. Prior to derivatization, released *N*-glycans are partially isolated from the protein moieties using hydrophobic stationary phase C-18 cartridges and subsequently desalted using graphitized carbon columns to enrich the product and to increase the efficiency of the subsequent reaction [335].

Despite the high-resolution separation of isomeric glycans, the glycan peak assignment, the development of a glycan database for CE peak annotation and inter-laboratory technical standards are challenges that have to be conquered for this technique in order to make it viable for routine diagnostics. Until now, several tools, such as internal standards to normalize migration time, glucose unit analysis to express the migration time of unknown oligosaccharides in terms of length of the dextran hydrolysate ladder (GU_{CE}) [264, 269, 336, 337] and exoglycosidase digestion array [267, 271], have been utilized to compile the information from different approaches. Nevertheless, a sole separation by the CE lacks structural information. Apart from the above-mentioned methods, *N*-glycan structures were also identified by correlating electrophoretic mobilities with their molecular masses determined by MALDI-TOF-MS analysis. Until recently, CE separation coupled with subsequent MS analysis was extremely difficult to achieve because of incompatible buffers and gel used for CE. Recently, Snyder *et al*. identified 77 *N*-glycan structures from human serum by direct coupling of CE to MS, which makes this method promising for future investigations [338].

This shows that none of the current analytical techniques is able to perform a complete glycan structural analysis by itself. However, a combination of available methods offers the possibility to characterize complex glycan mixtures in detail.

2. SCIENTIFIC GOALS

The aim of this thesis was to further the understanding of glycome modulations under pathological conditions. Alterations in glycosylation are associated with a variety of diseases such as cancer and autoimmune diseases and have therefore been proposed as a diagnostic basis for new diagnostics. The main goal of this work was to optimize the *N*-glycome analysis of human IgG- and total serum by capillary electrophoresis equipped with laser-induced fluorescence, and to provide an improved dataset for the use in glycan biomarker discovery. Furthermore, this work aimed at providing a deeper insight into the role of IgG glycosylation in rheumatoid arthritis and its involvement in inflammation.

The first aim was to extend the portfolio of glycan assignments by CE-LIF as research findings were limited in the past to the study of 12 glycan structures. In this thesis, assignment of linkage and positional isomers of desialylated *N*-glycans from human serum was performed using CE-LIF. For this purpose, glycoprotein standards as well as exoglycosidase digestion experiments were used to identify novel glycan structures in the *N*-glycome of human serum. In accordance with the extended structural dataset, glycan differences between EOC patients and healthy individuals were studied.

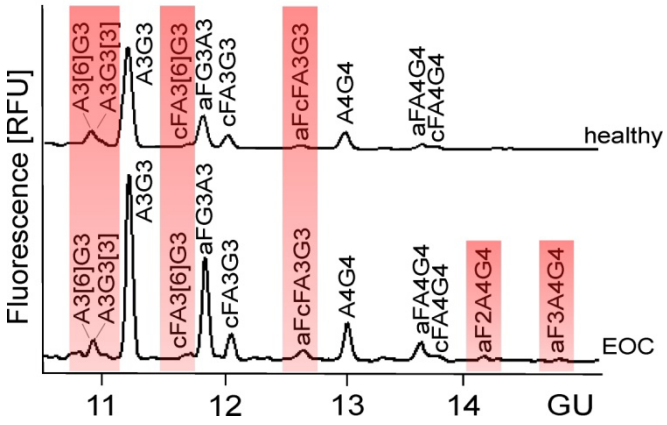
The second part of this thesis focused on the development of an IgG purification technique from human serum and the optimization of an electrophoretic method for the analysis of IgG *N*-glycan with a high resolution. For an improved separation and identification of sialylated N-glycans using CE-LIF, a derivatization method for neutralizing the negative charge of α2,3- and α2,6-linked sialic acids was developed and optimized. The resulting protocol was applied to quantify IgG-Fc *N*-glycan structures of patients suffering from epithelial ovarian cancer (EOC) and healthy controls in order to reveal new glycan biomarkers.

In the third part of the thesis, a recently developed method for IgG-Fc *N*-glycosylation analysis was applied to enable a large-scale study of the IgG-Fc *N*-glycome in a set of over 400 samples. IgG glycosylation was studied in plasma samples of rheumatoid arthritis patients, axial spondyloarthritis patients as well as healthy controls. Differences in IgG galactosylation between the cohorts were assessed by taking the inflammatory activity (C-reactive protein level, CRP) into

consideration. The relation between IgG galactosylation and disease activity markers (CRP, erythrocyte sedimentation rate, and disease activity score DAS28) was studied in RA, stratified by HLA-DRB shared epitope, ACPA, and RF.

3. PUBLICATIONS

3.1 Identification of 34 *N*-glycan isomers in human serum by capillary electrophoresis coupled with laser-induced fluorescence allows improving glycan biomarker discovery



This chapter was published in the following journal:

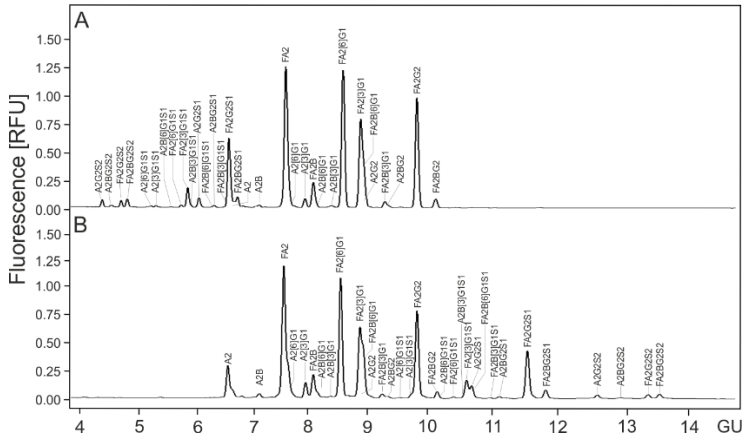
Christian Schwedler, Matthias Kaup, Stefan Weiz, Maria Hoppe, Elena I. Braicu, Jalid Sehouli, Berthold Hoppe, Rudolf Tauber, Markus Berger and Véronique Blanchard. Anal Bioanal Chem 2014 Nov; 406(28):7185-93.

The original article and supplementary data are available at:
https://dx.doi.org/10.1007/s00216-014-8168-y
DOI: 10.1007/s00216-014-8168-y

Author contribution:

- Laboratory analyses
- Data analysis and interpretation
- Drafting the manuscript

3.2 Sialic acid methylation refines capillary electrophoresis laser-induced fluorescence analyses of immunoglobulin G *N*-glycans of ovarian cancer patients



This chapter was published in the following journal:

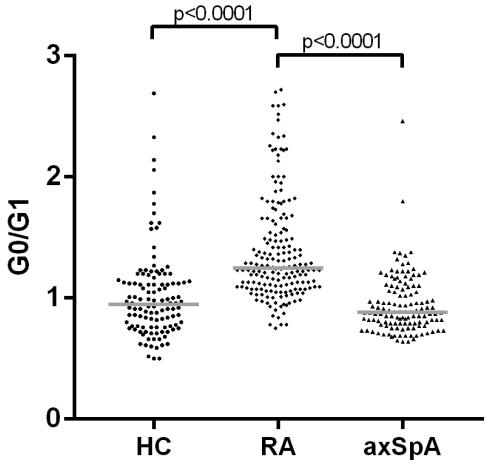
Christian Schwedler, Matthias Kaup, Dominique Petzold, Berthold Hoppe, Elena I. Braicu, Jalid Sehouli, Marc Ehlers, Markus Berger, Rudolf Tauber, and Véronique Blanchard. Electrophoresis 2014 Apr; 35(7):1025-1031.

The original article and supplementary data are available at:
https://dx.doi.org/10.1002/elps.201300414
DOI: 10.1002/elps.201300414

Author contribution:

- Development and design of the method
- Laboratory analyses
- Data analysis and interpretation
- Drafting the manuscript

3.3 Hypogalactosylation of immunoglobulin G in rheumatoid arthritis: relationship to HLA-DRB1 shared epitope, anticitrullinated protein antibodies, rheumatoid factor, and correlation with inflammatory activity



This chapter was submitted in the following journal:

Christian Schwedler, Thomas Häupl, Ulrich Kalus, Véronique Blanchard, Gerd-Rüdiger Burmester, Denis Poddubnyy, and Berthold Hoppe. Arthritis Res Ther 2018 Mar; 20(1):44.

The original article and supplementary data are available at:
https://dx.doi.org/10.1186/s13075-018-1540-0
DOI: 10.1186/s13075-018-1540-0

Author contribution:

- Development the hypotheses and design of the study
- Coordination of sample acquisition and laboratory analyses
- Data analysis and interpretation
- Drafting the manuscript
- Critical revision of the manuscript

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5. APPENDIX

5.1 Abbreviations

A1AG	alpha-1 acid glycoprotein
A1AT	alpha-1 antitrypsin
AACT	alpha-1 antichymotrypsin
Ab	antibody
ACPA	anti-citrullinated protein antibodies
ADCC	antibody-dependent cellular cytotoxicity
APP	acute phase protein
APTS	8-aminopyrene-1,3,6-trisulfonic acid
Asn	asparagine
C1q	complement component 1
CA	carcinoma antigen
CDC	complement-dependent cytotoxicity
CE-LIF	capillary electrophoresis with laser induced fluorescence
CLR	C-lectin receptor
CRP	C-reactive protein
DAS28	disease activity score in 28 joints
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin
ELISA	enzyme-linked immunosorbent assay
EOF	electroosmotic flow
EOC	epithelial ovarian cancer
ER	endoplasmic reticulum
Fab	fragment antigen binding
Fc	fragment crystallizable
FcyR	Fcy receptor
FUT8	α(1,6)-fucosyltransferase
Gal	galatose
GlcNAc	*N*-acetylglucosamine
GnT	*N*-acetylglucsaminyltransferase

HLA	human leukocyte antigen
HPT	haptoglobin
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IP	immunoprecipitation
IVIg	intravenous immunoglobulin
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
mAb	monoclonal antibody
Man	mannose
MALDI-TOF-MS	matrix-assisted laser-desorption ionization time-of-flight mass spectrometry
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MS	mass spectrometry
MUC	mucin
Neu5Ac	*N*-acetylneuraminic acid
NK cells	natural killer cells
PNGase F	Peptide-N^4-(N-acetyl-β-glucosaminyl) asparagin amidase F
RA	rheumatoid arthritis
RF	rheumatoid factor
SE	shared epitope
SLe	sialyl Lewis
TNF-α	tumor necrosis factor-alpha
UPLC	ultra performance liquid chromatography

5.2 List of Publications

Frisch E, **Schwedler C**, Kaup M, Braicu EI, Grone J, Lauscher JC, Sehouli J, Zimmermann M, Tauber R, Berger M, Blanchard V. Endo-beta-*N*-acetylglucosaminidase H de-*N*-glycosylation in a domestic microwave oven: application to biomarker discovery. Anal Biochem. 2013 Feb; 433(1):65-69.

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Schwedler C, Häupl T, Kalus U, Blanchard V, Burmester GR, Poddubnyy D, Hoppe B. Hypogalactosylation of immunoglobulin G in rheumatoid arthritis: relationship to HLA-DRB1 shared epitope, anticitrullinated protein antibodies, rheumatoid factor, and correlation with inflammatory activity. Arthritis Res Ther. 2018 Mar; 20(1):44.

5.3 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.