

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Towards Next Generation Glycoanalytics: Automatable  
Biomarker Discovery

by

Csaba Váradi

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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Supervisor: Prof. András Guttman



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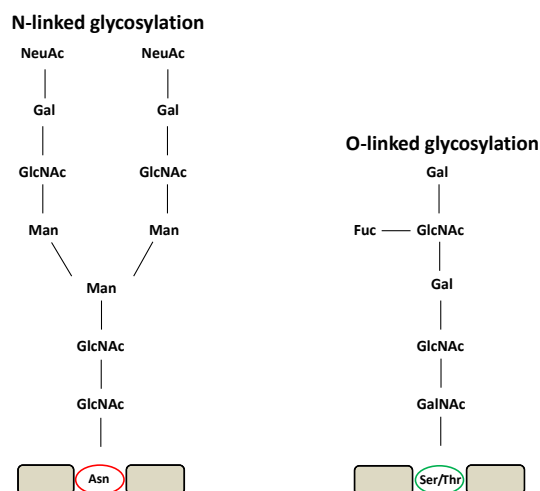
# 1. Theoretical Background

The well-known aspect of carbohydrates is their central role in energy metabolism, although in the last few years increased attention was given for oligosaccharides as post-translational modifications [1]. The process when one or more carbohydrate chains are added to proteins which are assembled in the endoplasmic reticulum called glycosylation, the attached sugars are referred to as glycans [2]. Glycosylation is a principal chemical modification of proteins, responsible for their physical, chemical and effector functions [3]. The attached glycans are composed of monosaccharide units which can be combined in many different ways due to different linkage types and positions [4]. The main aspect of glycobiology is to provide structural information of carbohydrates attached to proteins and reveal how these oligosaccharides can mediate biological processes [5]. The function of the attached glycans can be very diverse usually separated into two groups:

1. Intrinsic functions performed by glycans:
  - a. Structural components of cell walls and extracellular matrix
  - b. Modifying solubility and stability of proteins
2. Extrinsic functions by lectin-glycan interactions:
  - a. Mediating and modulating cell-cell and cell-matrix interactions
  - b. Intra- and extracellular trafficking of glycoconjugates
  - c. Intra- and extracellular signaling

There are two main glycosylation types based on the linkage of the glycan to the parent protein [6]. N-linked glycans are attached through a nitrogen atom to an asparagine while O-linked glycans are connected through oxygen to a serine or threonine. O-linked glycans are usually smaller structures, generally consisting less monosaccharide units while N-links are more complex containing several branches (Figure1) [7]. Both types of glycosylation can be found on membrane proteins and also on glycoproteins which are secreted in biological fluids [8]. The lack of specific enzymes for most O-type glycan release resulted that N-linked glycosylation is more widely studied although their biological importance is considered to be equal [9]. Both glycosylation types are essential in proper folding, stability and the effector functions of the parent protein [10]. The primary polypeptide sequence of proteins is directly encoded in the

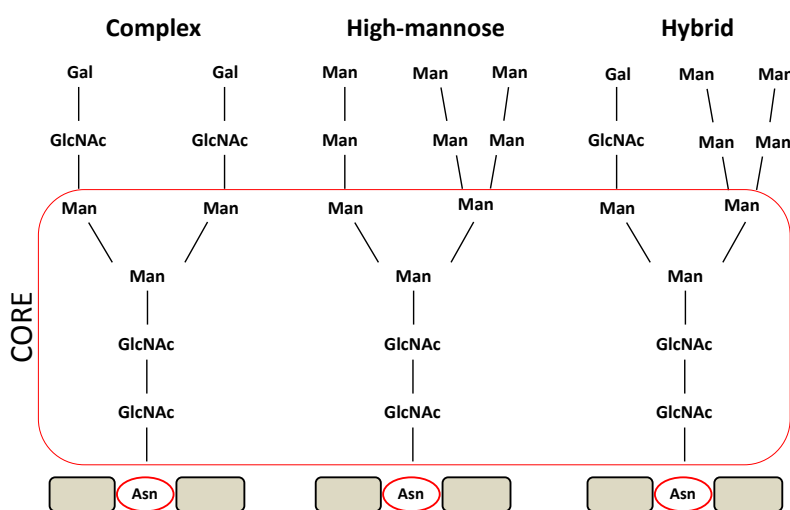
genes; however, the glycan structures are built up by monosaccharide- and linkage-specific glycosyl-transferases, thus glycans are considered as secondary gene products [1]. Although the origin of glycans is non-template driven, the structures of the specific enzymes, which are responsible for construction of the attached carbohydrates are coded in the genome [11]. This was first proven by knock-out mice based techniques showing that the elimination of glycoconjugates can lead to health disorders [12]. The carbohydrate content of glycoconjugates is variable, as the number of glycosylation sites and the occupancy at particular sites can vary within a protein and also from proteins to proteins [13]. For example in antibodies it is relatively low (<5% of total mass), while other glycoproteins can be highly glycosylated such as haptoglobin (19%), erythropoietin (40%),  $\alpha$ 1-acid glycoprotein (45%), mucins (40-80%) [14]. Glycoproteins can have multiple glycosylation sites with different glycoforms on each site resulting broad site-specific structural diversity referred to as microheterogeneity of protein glycosylation [15]. The vast structural heterogeneity of glycans is based on the expression level and activity of glycosidases and glycosyl-transferases, which can be altered in pathological conditions therefore, comprehensive glycosylation analysis offers a new avenue in biomarker discovery as glycosylation changes can be sensitive indicators of the actual state of the underlying biochemical mechanism [16]. With the help of efficient glyco-markers, diseases may be diagnosed at early stages, also saving patients from sometimes complicated and invasive diagnostic procedures [17]. The recognition of clinical relevance of glycosylation changes resulted the recent golden age of Glycomics [18].



**Figure 1.** Representative structures of N- and O-linked glycosylation

## 1.1. N-linked Glycosylation

N-glycosylation is one of the most common post-translational modification of secretory and membrane proteins in eukaryotic cells [19]. The three N-linked glycosylation types are shown in Figure 2, all comprising the common trimannosyl-chitobiose core. The complex type can be highly branched (multi-antennary structures) with different monosaccharide units added to the core, while the high mannose types usually consist of mannose units on both arm as addition to the core [20]. The hybrid structures are the mixtures of complex and high-mannose types where one of the arms is complex and the other is high-mannose type.



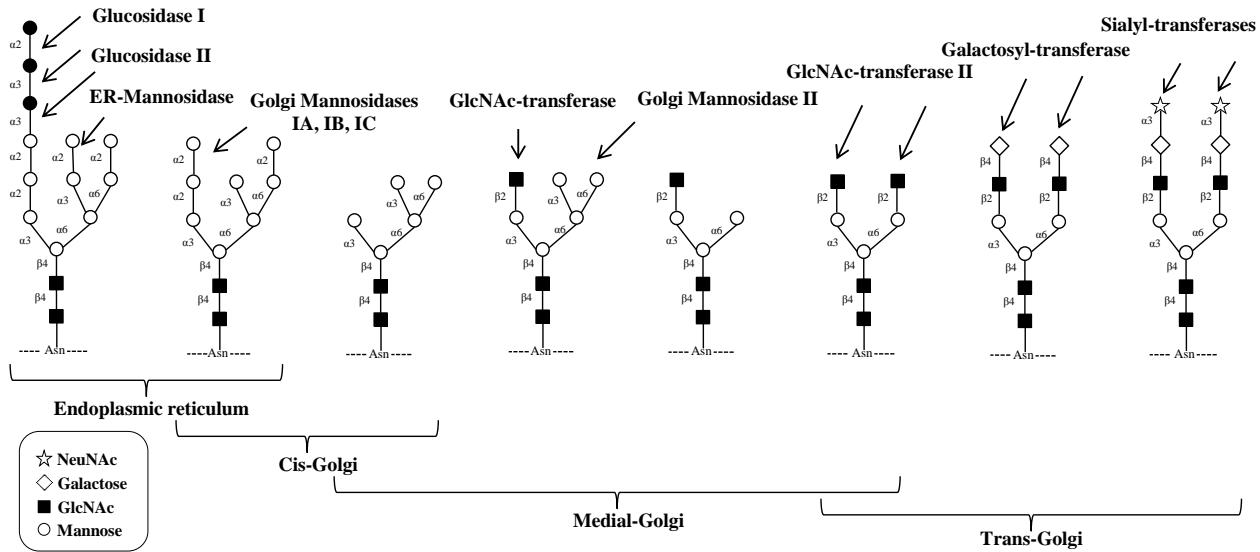
**Figure 2.** Different types of N-linked glycans

The assembly of N-glycans can be separated into 3 stages:

1. Formation of the lipid linked precursor oligosaccharide, which is a triglucosylated mannose 9 structure (Figure 3)
2. Transfer of the precursor oligosaccharide to the consensus sequence containing Asn on polypeptide chain
3. Processing to form the final structure of the oligosaccharide

The generation of the lipid-linked precursor oligosaccharide and its transfer to the polypeptide occur in the rough ER, while the processing of the oligosaccharide is mostly in the Golgi apparatus [21]. The processing step consists of sequential exoglycosidase digestions followed by rebuilding the glycan structure via monosaccharide and linkage specific exoglycosidases and

glycosyl-transferases [22]. Glucosidase I and II are part of the calnexin-calreticulin cycle, which is a quality control system that mediates protein folding [23]. These exoglycosidases are responsible for the removal of terminal glucose units from the precursor oligosaccharides, which are the last parts in the mediation of proper folding [24]. When Glucosidase II removes the remaining terminal glucose, the glycoprotein is capable to leave the ER, unless recognized by UDP-Glc glycoprotein glucosyl-transferase (UGGT), due to the misfolding of the protein [25]. UGGT reglucosylates incompletely folded proteins thus serve as a folding sensor [26]. The remaining oligosaccharide structure is further truncated by  $\alpha$ 1,2 mannosidases which process starts in the ER and finishes in the cis-Golgi. The glycan structure is digested down to a pentamannosylated core structure by the Golgi mannosidases [27]. The rebuilding of complex glycans is starting in the medial-Golgi by GlcNAc-, galactosyl- and sialyl-transferases resulting in galactosylated and sialylated structures (Figure 3) [28].



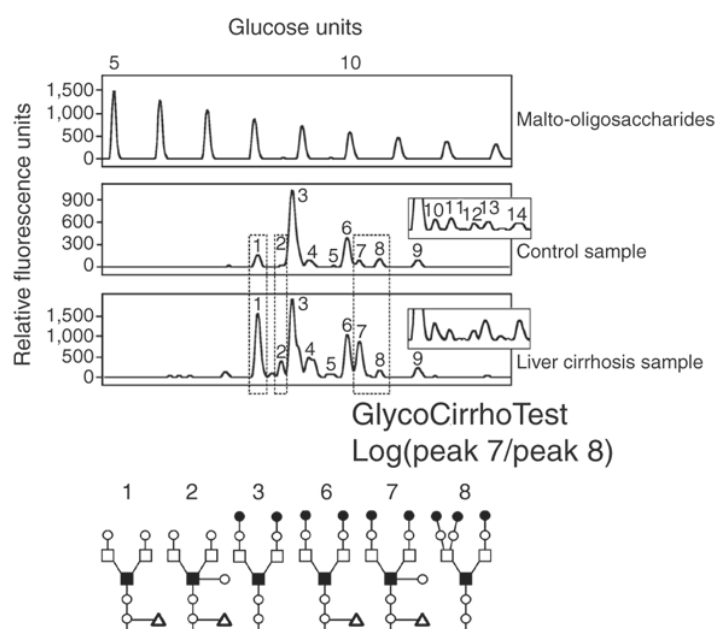
**Figure 3.** Biosynthetic pathway of N-linked glycans

N-glycans have an essential role in the process of protein folding based on lectin interactions in the ER where the glycans serve as sorting signals [29]. It has been proven that blocking the biosynthetic pathway of N-glycosylation can result in incomplete folding of many polypeptides as they are not able to reach the native conformation thus, cannot pass the ER quality control [30].



## 1.2 Glycosylation in Biomarker Discovery

Glycosylation is a result of complicated biochemical processes involving a plethora of specific enzymes, thus any alterations in the biosynthetic pathway can be expressed in the structure of glycans. Glycosylation changes are well-known in many pathological conditions such as genetic disorders, inflammatory diseases and cancer. The use of these alterations as disease-specific biomarkers has a huge potential as with the help of efficient glyco-markers, diseases might be diagnosed at early stages, also saving patients from sometimes complicated diagnostic procedures such as biopsy. The release of N-glycans from total serum results a glycosylation pattern of all glycoproteins in the blood, which can be also used in biomarker research. This was used the first time by Callewaert et al for non-invasive diagnosis of liver cirrhosis as shown in Figure 4. Using this marker in combination with clinical data, non-cirrhotic and cirrhotic patients were readily distinguished with 100% specificity and 75% sensitivity [31].

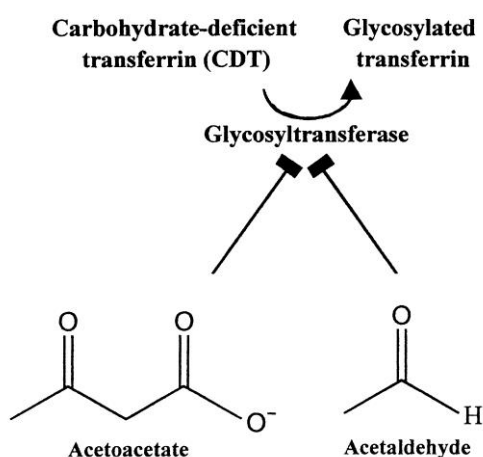


**Figure 4.** GlycoCirrho Test for non-invasive diagnosis of liver cirrhosis

Recently, two glycosylation-based biomarkers have been introduced in clinical diagnostics, namely carbohydrate-deficient transferrin as a marker of alcohol abuse and fucosylated serum alpha-fetoprotein for early recognition of hepatocellular carcinoma.

### 1.2.1. Carbohydrate-deficient transferrin as a marker of alcohol abuse

Transferrin is a glycoprotein synthesized in the liver with the main function of iron transport. Similarly to other glycoproteins increased branching and fucosylation of transferrin glycans were detected in many malignant diseases such as ovarian, breast and colon cancer. Another common alteration of transferrin glycosylation is the lack of carbohydrates in alcoholism. Glycosyltransferases, which are responsible for the glycosylation of transferrin, can be inhibited by the ethanol metabolite acetaldehyde, resulting decreased synthesis of fully glycosylated transferrin, thus higher level of carbohydrate-deficient transferrin (Figure 5) (CDT). This increased level of CDT is used in clinical diagnostics as a marker of alcohol abuse [32, 33].



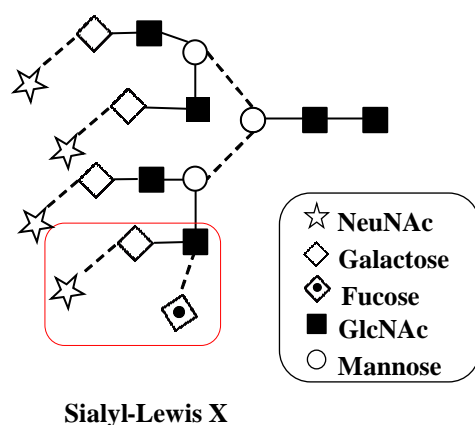
**Figure 5.** Theory for increased level of CDT in alcohol abuse

### 1.2.2. Fucosylated alpha-fetoprotein (AFP) in hepatocellular carcinoma

AFP is a glycoprotein normally produced by the yolk sac and the liver during fetal development and by regeneration of hepatocytes. Increased fucosylation of AFP is usually detected by Lectin-affinity using *Lens culinaris*, which has special affinity to fucosylated proteins. The fucosylated fraction of AFP, called as AFP-L3, is increased in hepatocellular carcinoma (HCC) and the ratio of AFP/AFP-L3 can serve as a biological marker. Patients with AFP-L3 level of 10% or higher are at potential risk for development of HCC. The *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) in combination with des-gamma-carboxy prothrombin (DCP is an immature form of prothrombin, HCC cells secrete non-carboxylated DCP in contrast to normal liver cells) and ultrasound provides ~85% sensitivity and ~95% specificity in early stage detection of HCC [34].

### 1.3. Haptoglobin glycosylation

Haptoglobin (Hp), is a positive acute phase glycoprotein (i.e., its level is increased in inflammation), synthesized in the liver and secreted into the plasma with the main function to capture and transport intravascular free hemoglobin (Hb) to macrophages [35]. The normal concentration in human plasma is between 0.3- 3 mg/ml, which is increased in the occurrence of inflammation. In lower concentrations, Hp can also be produced by other tissues, such as lung, skin, spleen, brain, intestine, arterial vessels and kidney. Increased production of haptoglobin is the result of transcriptional activation of the Hp gene by pro-inflammatory cytokines, such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF). The structure of haptoglobin consists of two heavy ( $\beta$ ) and two light ( $\alpha$ ) chains or the oligomers of these, with four N-glycosylation sites on each beta-chain (Asn184, Asn207, Asn211 and Asn241) [36]. Glycosylation is reportedly essential for its effector function; if glycans are removed the ability to capture hemoglobin can be lost [37]. Due to the iron content of free Hb, reactive hydroxyl radicals can be generated in the presence of hydrogen-peroxide, which can damage lipids, proteins and DNA thus Hp prevents oxidative stress. Also free Hb can capture free nitrogen-monoxide, which is an important regulator of vascular homeostasis. Besides the anti-oxidative effects, Hp reportedly has immunomodulatory, anti-inflammatory and bacteriostatic effects [38]. Increased Hp production has been reported in many diseases, such as atherosclerosis, myasthenia gravis, arthritis, diabetes, psoriasis, inflammatory bowel disease and different types of cancer. Besides the increased Hp production, altered glycosylation of Hp has been found in different diseases, most frequently in cancer. [49]. The standard haptoglobin N-glycans usually consist of bi- but also some tri- and tetra-antennary structures, which can be core or/and arm fucosylated [39]. The majority of reported glycosylation changes of the Hp N-glycome in cancer are increased branching and SLe<sup>x</sup> levels (Sialyl Lewis X, Figure 6), although similar markers have also been identified in chronic inflammation.



**Figure 6.** The Sialyl Lewis X ( $Sle^x$ ) epitope consists of a sialic acid residue  $\alpha 2,3$ -linked to galactose with outer-arm fucose ( $\alpha 1,3$ ) to GlcNAc

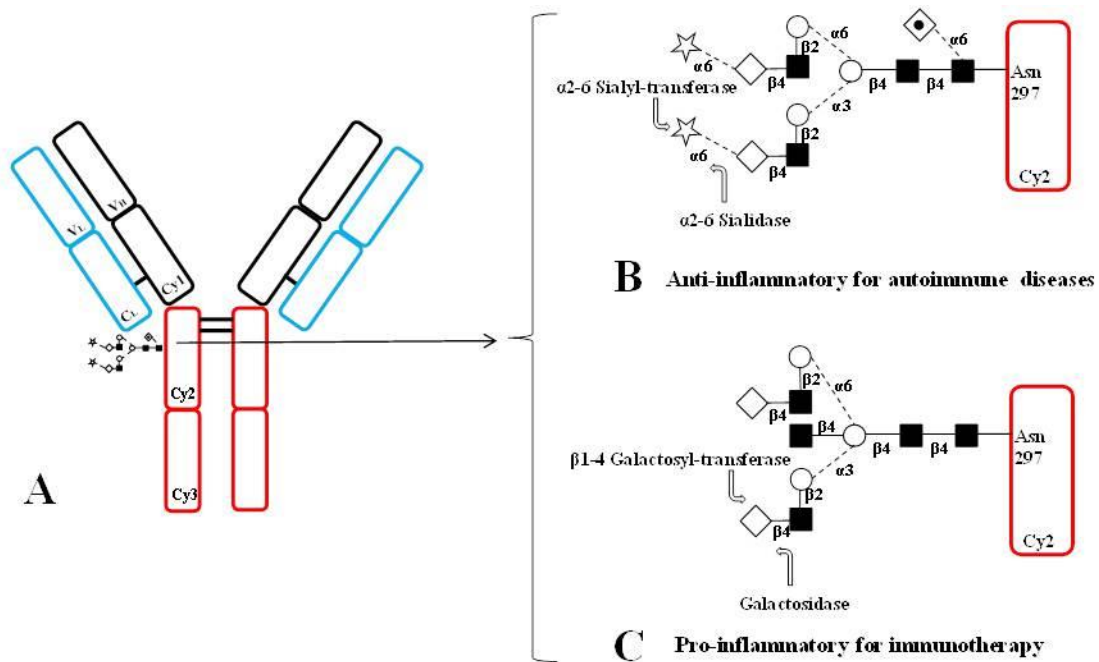
#### 1.4. IgG Glycosylation

Immunoglobulins are one of the main secretory products of the adaptive immune system with the main function to protect against foreign pathogens [40]. Five subclasses of immunoglobulins are identified in humans, such as IgG, IgM, IgA, IgE and IgD [41]. Each of them is glycosylated on their individual glycosylation sites, which can be on the Fc (crystalizable fragment), on Fab (antigen-binding fragment) domains or on both [42]. Fc and Fab are linked by a flexible hinge region in IgG, IgA, and IgD while in IgM and IgE, this is replaced by a more rigid immunoglobulin domain [43]. Each immunoglobulin has two identical 50-77 kDa class-specific heavy chains ( $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\epsilon$ , and  $\delta$ ), connected by one or more disulphide bridges [44]. Each heavy chain is linked by a disulphide bond to a 25-kDa light chain, which can be kappa ( $\kappa$ ) or lambda ( $\lambda$ ) type [45]. The glycosylation of the heavy chains are usually 2-3 % of the molecular weight of IgG (150 kDa) but can be 12-14 % in more heavily glycosylated immunoglobulins (IgM, IgD, IgE) [46]. There are no conserved glycosylation sites on the light chains.

Human IgG has 4 subclasses (IgG1, IgG2, IgG3, IgG4), with the main difference in their  $\gamma$  chain sequence and the distribution of disulphide bridges [47]. IgG1 is the most abundant serum immunoglobulin, with 10-15 mg/ml average concentration. All IgG molecules contain a conserved N-glycosylation site in the Fc region and may have variable glycosylation (either O- or N-linked) sites in the Fab region [48]. Due to the efficacy of monoclonal antibody-based therapeutics and intravenous immunoglobulins, the glycosylation of IgGs is widely studied [49].

Different glycoforms can be present in the conserved C<sub>H</sub>2 domain of the Fc fragment [50]. The large heterogeneity of the glycovariants is given by the different combination of terminal monosaccharides. The glycans linked to the Fc fragment are mostly complex bi-antennary structures with or without  $\alpha$ 1-6 linked core-fucose. The antennas can be terminated in sialic acids, galactoses or N-acetyl-glucosamines resulting 32 different structures [51]. IgG Fc glycans are required for optimal Fc $\gamma$  receptor binding [52]. Fc $\gamma$  receptors are expressed in leukocytes, including macrophages, eosinophils, neutrophils, natural killer cells and lymphocytes with distinct classes of Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32), and Fc $\gamma$ RIII (CD16) [53]. IgG Fc glycans are required for optimal binding of the antibody to all classes of receptors and for the effector functions that eliminate pathogens and control the several properties of therapeutic antibodies [54]. The glycans at the conserved Asn-297 also maintain the quaternary structure and the thermodynamic stability of the Fc [55].

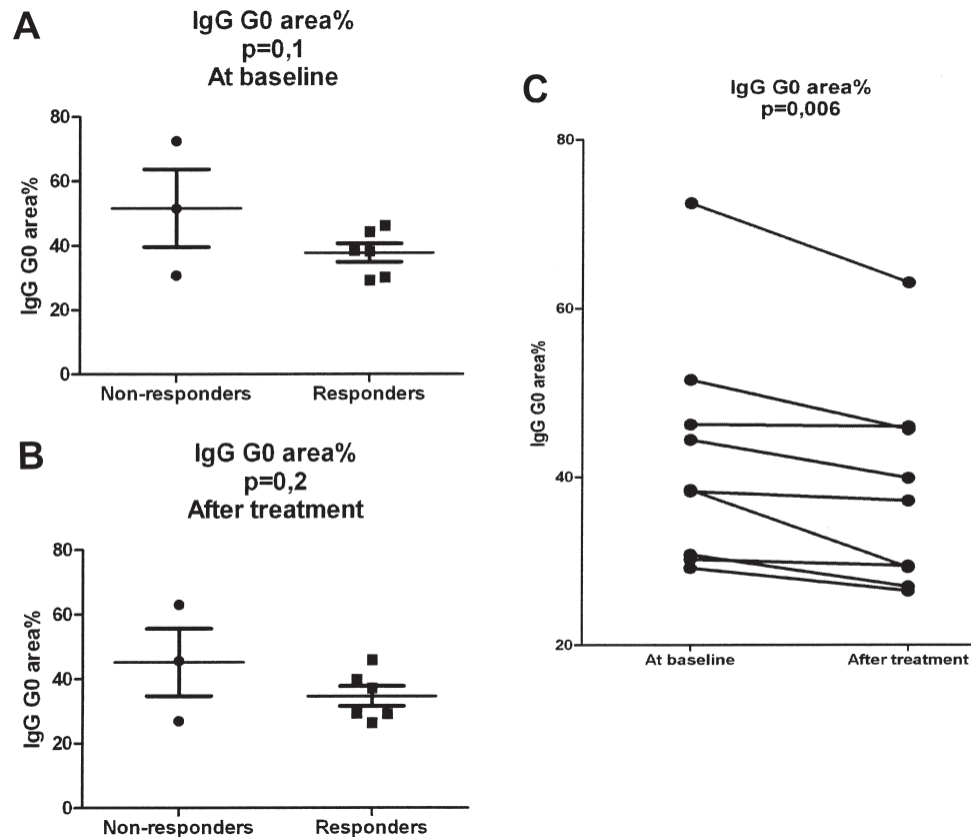
Depending on the terminal monosaccharide unit, the glycans influence essential functions of the parent protein such as half-life, complement-dependent cytotoxicity (CDC), antigen-dependent cellular cytotoxicity (ADCC) and anti-inflammatory activity (Figure 7) [56-59]. The terminal galactose content is reportedly responsible for CDC activity of IgG due to the increased binding to C1q and activates the classical pathway of the complement cascade. Terminal GlcNAc content is dependent on the terminal galactose content as the higher the terminal galactose content, the lower the terminal GlcNAc, thus increased terminal GlcNAc reduce CDC activity. Increased sialylation of Fc glycans results in decreased ADCC activity of IgG as terminal sialylation negatively affects antibody binding to the Fc $\gamma$ RIIIa receptor. Fc sialylation with  $\alpha$ 2,6-linked sialic acid residues has been suggested to effect the anti-inflammatory activity of IgG.  $\alpha$ 1,6-linked fucose at the core GlcNAc residue decreases ADCC as non-core-fucosylated IgGs have higher affinity to bind to Fc $\gamma$ RIIIa. To improve the ADCC activity of therapeutic IgGs several strategies have been developed to reduce core-fucose content of antibodies, such as silencing the  $\alpha$ 1,6 fucosyl-transferase gene or overexpression of GnTIII, which transfers bisecting GlcNAc (addition of bisecting GlcNAc has been shown to result in a reduction of core fucose content) [60].



**Figure 7.** Terminal monosaccharides are responsible for IgG effector functions [61]

Altered glycosylation of human serum IgG has been found in various conditions such as ageing, pregnancy, inflammatory diseases and cancer. One of the most common alterations of IgG glycans is reduced terminal galactosylation, thus increased terminal GlcNAc exposure in inflammatory diseases. Recently, several studies reported that the analysis of glycosylation changes in malignant and inflammatory diseases provide useful information to understand their pathogenesis [62]. Human serum IgG glycosylation was also found to be a useful marker for monitoring treatment efficacy in Rheumatoid arthritis (RA) and Crohn's disease (CD) [63-66]. RA and CD are both autoimmune diseases with similar molecular background, manifested in altered inflammatory response [67]. Blocking tumor necrosis factor alpha (TNF $\alpha$ ) has been reported as an effective treatment in chronic inflammatory diseases (RA, CD, psoriasis, asthma) [68]. As a result, several anti-TNF $\alpha$  therapeutic antibodies (infliximab, adalimumab, and certolizumab pegol) have been developed for clinical evaluation [69]. However, the efficacy of biological therapies can vary in different diseases, and the responsiveness for such therapy is usually unpredictable in different patient cohorts [70]. IgG glycosylation pattern as disease remission marker was studied earlier in RA patients, where elevated IgG galactosylation level was found after infliximab therapy [64]. In another study, RA patients were treated with anti-IL-

6 receptor (interleukin-6) antibody (tocilizumab) resulting decreased amount of agalactosylated bi-antennary N-glycans (G0) 4 weeks after the treatment in correlation with the responsiveness for the therapy (Figure 8) [71]. Serum IgG galactosylation level was also found to be a reversible alteration in RA and psoriatic arthritis (PsA) after anti-TNF $\alpha$  treatment [63]. These findings suggest the biomarker potential of IgG glycosylation in before-after-treatment studies to follow disease progression in response to the administration of the biotherapeutics agent.



**Figure 8.** IgG galactosylation to distinguish responders/non-responders before and after anti-IL-6 therapy [71]

## 1.5. Sample Preparation for Glycosylation Analysis

Ever since the importance of protein glycosylation was recognized, development of new glycoanalytical tools have been emerging [72]. Understanding the alterations in protein glycosylation provides essential information for several important aspects of biomedical sciences, the biopharmaceutical field and regulatory bodies, such as in biomarker discovery for the former and the assessment of the efficacy and safety of glycoprotein therapeutics for the latter ones [73]. This challenging task requires high-throughput and highly reproducible screening methods for protein glycosylation [74]. To fulfill this need, automated sample preparation platforms are in great demand to replace the currently used complicated, not easily automatable and time-consuming manual processes [75]. Examples include shorter incubation times during glycan release and labeling [76], the option to use of liquid handling robots for sample preparation or even multicapillary methods to increase throughput [77]. One of the major handicaps of currently used sample preparation protocols for N-glycosylation analysis (including such steps as enzymatic N-glycan release, carbohydrate purification, fluorophore labeling and sample clean-up) for subsequent capillary electrophoresis (CE) or liquid chromatography (LC) analysis is the lack of easy automation options, not requiring high end robotic systems with centrifugation capabilities.

Due to its stability, specificity and simple sample preparation conditions, the most commonly used enzyme for N-glycan release from glycoproteins is Peptide-N-glycosidase F (PNGase F) [78]. Enzymatic deglycosylation by PNGase F is conventionally accomplished at 37°C with overnight incubation, but can be accelerated by means of microwave irradiation [79], pressure cycling technology [80] and with the use of immobilized PNGase F micro-reactors [81]. It has been reported that complete deglycosylation can be obtained in 20 minutes using a domestic microwave device, where the radiation resulted in the increase of temperature up to 70°C [82]. Pressure cycling and enzyme immobilization are relatively expensive and complicated technologies, also calling for alternative methods to speed-up the digestion reaction. The next problematic step after the glycan release is to separate the remaining polypeptide chain from the released glycans. This is traditionally done by ethanol precipitation or spinfilters, both requiring centrifugation steps, thus also hard to automate. Analysis of the liberated carbohydrates by CE or LC requires chemical derivatization of the sugars in order to provide them with adequate charge



and fluorescent characteristics. This is usually accomplished by via reductive amination [83], most commonly with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) for CE and 2-amino-benzamide (2-AB) for LC [84, 85]. After completion of the labeling reaction a pre-concentration step is usually accomplished by vacuum-centrifugation. Several methods have been reported for APTS-labeling of carbohydrate structures using different reducing agents, catalysts and various amounts the labeling dyes [86]. Sodium-cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) is one of the most frequently used reducing agents, but 2-picoline-borane ( $\text{pic-BH}_3$ ) has also been reported as an equally efficient but non-toxic option [87]. The efficiency of different acid catalysts for the reductive amination reaction is well-investigated, although with the use of strong acids sialic acid loss was observed [88]. After labeling, a clean-up step is required as CE-LIF is very sensitive for salts and other charged impurities in the sample. This results another vacuum-centrifugation step as the elution volume of the clean-up is relatively high ( $>100\ \mu\text{L}$ ). The need of several centrifugation and vacuum-centrifugation steps make glycan sample preparation hard to automate, thus other techniques were investigated to support full automation. Magnetic bead based technology is one of the commonly used approaches to alleviate centrifugation requirement and widely used in the genomics [89] and proteomics [90] fields for many years ranging from simple sample clean-up to affinity based capture. Carboxyl-coated magnetic microparticles are reportedly used to facilitate the isolation and purification of nucleic acid molecules using solid phase reversible immobilization (SPRI) technology [91]. The fact that a plethora of special functional groups can be linked to the surface of magnetic particles resulted in increased attention in their use for the enrichment of biologically important molecules [92-94]. Indeed, magnetic bead based technology is one of the readily applicable options for full automation of glycan sample preparation.

## 1.6 Analytical Glycomics

Due to the complexity and heterogeneity of protein glycosylation high resolution analytical techniques are required to provide detailed structural information such as monosaccharide sequence and linkage specificity. Structural analysis of glycosylation is a very challenging task since the sugar units within a carbohydrate can bind with different linkages at different positions, resulting very high structural diversity. The most frequently used techniques for glycan analysis are NMR, lectin-based methods, high performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS) and the combination of them [95].

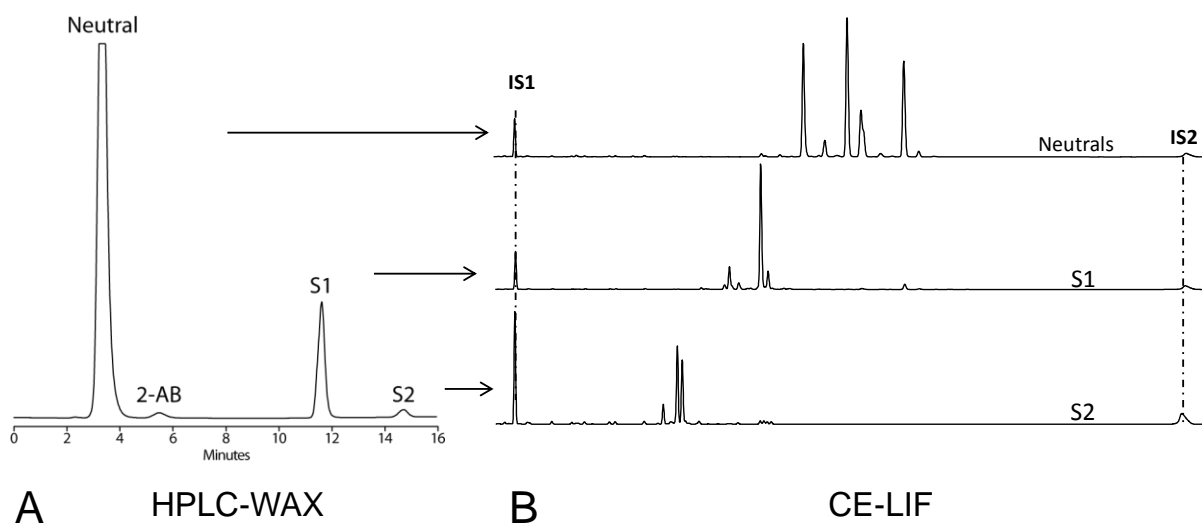
### 1.6.1. Lectins for glycan analysis

Lectin-based methods are one of the oldest techniques used in glycoanalytics [96]. Lectins are carbohydrate-binding proteins with high specificity to certain sugar epitopes [97]. Different anomeric forms ( $\alpha$ ,  $\beta$ ) can be recognized by individual lectins, which can be used for the enrichment of glycoproteins from serum, saliva, tissue, cells, just to mention a few [98, 99]. The pH dependence of lectin-carbohydrate interactions is used in lectin affinity chromatography, where after a short incubation of the sample with specific lectins, the captured glycoproteins can be eluted by changing the pH [100]. This method is reportedly useful for screening of glycosylation changes in malignant and inflammatory diseases [101]. One of the most widely used lectin based technique in clinical studies is crossed-affino-immunoelectrophoresis which is basically a 2D agarose gel-electrophoresis [102]. In the first dimension specific lectins, in the second dimension specific antibodies are added into the gel respectively, thus glycoproteins recognized by lectins in the first dimension and further partitioned in the second dimension based on their antibody specificity. This technique can provide valuable information about the antibody specificity of the enriched glycoprotein and the attached glycan structure. The conjugation of lectins with horse-radish-peroxidase allowed the development of lectin-based sandwich ELISA and lectin blotting techniques to detect and quantify one or more glycoproteins in the sample [103, 104]. One of the most sensitive and high-throughput techniques is lectin microarray, which enables the analysis of multiple lectin-glycan interactions in one experiment [105]. In this technique the lectins are immobilized on a microplate, where the different lectin-binding intensities provide the structural information of glycoproteins. One of the greatest benefits of this

technique is that glycan structures of glycoproteins can be analyzed without any purification step.

### 1.6.2. High-performance liquid chromatography (HPLC)

Glycoproteins in biological fluids are often present at low concentrations, calling for high sensitivity analytical techniques. Regulatory agencies also require reliable and detailed structural analysis of therapeutic glycoproteins (e.g., monoclonal antibodies) as glycans can affect their physical, chemical and effector functions. One of the most widely used analytical techniques for glycan analysis is high-performance liquid chromatography (HPLC) which was co-developed by a Hungarian scientist Professor Csaba Horváth [106]. HPLC can be used in different dimensions as several separation columns can be applied in the same instrument. The most common HPLC techniques in analytical glycomics are reverse-phase, anion-exchange chromatography, graphitized carbon and hydrophilic-interaction-liquid chromatography [107]. The lack of chromophore or fluorophore groups on glycans poses the need of chemical tagging of released sugars. 2-aminobenzamide labeling has been adopted in most laboratories that supports high sensitivity detection of the labeled carbohydrates [108]. One of the main advantages of HPLC compared to other techniques is that it can be used in preparative (2D) mode, for example glycans can be anion-exchange fractionated and the different fractions can be further analyzed by e.g., HILIC-HPLC, capillary electrophoresis or any other analytical techniques (Figure 9) [51].

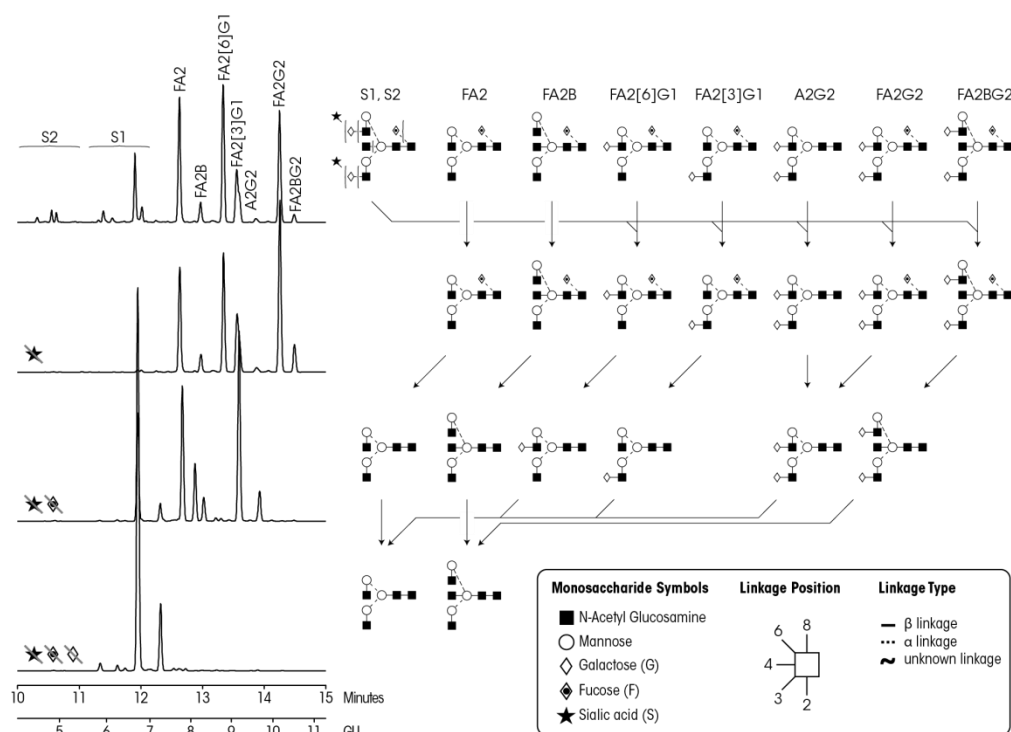


**Figure 9.** Combination of HPLC-WAX and CE-LIF for sensitive glycan analysis [51]

*CE conditions: capillary: N-CHO neutral coated capillary (effective length: 50 cm; total length: 60 cm); separation buffer: N-CHO carbohydrate separation buffer, E= 500 V/cm (reversed polarity), pressure injection: 6.89 kPa for 5 s. LC conditions: Buffer A, 500 mM ammonium formate, Buffer B, 10% methanol in water, flow-rate 0.75mL/min*

### 1.6.3. Capillary gel electrophoresis (CGE)

The migration of the injected sample in CE is electric field-mediated, resulting in separation by the hydrodynamic volume to charge ratio, thus capable of separating positional and linkage isomers with high efficiency [109]. Similarly to HPLC, CE requires chemical derivatization of glycans, however in this instance with a charged fluorophore to support electromigration. Amino-pyrene-trisulfonic acid is an excellent labeling reagent for high-sensitivity detection by laser-induced fluorescence detection (LIF) [110]. CGE-LIF is a robust tool offering rapid analysis with high resolution and excellent reproducibility [111]. N-CHO Carbohydrate Separation Buffer (SCIEX) in combination with N-CHO coated capillary columns (SCIEX) is a reportedly excellent strategy to separate APTS-labeled glycan structures based on their hydrodynamic volume to charge ratio [112]. The recombinant protein technology resulted that recently most of the monosaccharide- and linkage-specific exoglycosidases are available which gives the possibility of sequential digestion of unknown carbohydrates [113] for full structural elucidation. Targeted exoglycosidase digestions in combination with CGE-LIF offer a powerful method to reveal both structural and linkage information, thus monitoring highly detailed glycosylation changes (Figure 10) [114]. N-glycan nomenclature and symbolic representations used in this thesis as previously described by Harvey et al [115]



**Figure 10.** Exoglycosidase array based sequencing of IgG N-glycans [51]

#### 1.6.4. Mass-spectrometry (MS)

MS applied with different separation techniques provides highly detailed and reliable information on glycan structures [5]. Introduction of LC-MS and CE-MS techniques resulted in highly reliable methods providing accurate mass to each separated peak [116, 117]. Although the quantitation of these methods is not perfectly solved yet, as the ionization of carbohydrates is not unique thus the generated ion-chromatogram can provide false distribution of structures. The most commonly used ionization methods for glycan analysis are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The different ions can be analyzed in positive or negative mode, depending on the charge of the sample. Both ionization modes can be used for underivatized and derivatized glycans. Methylation is one of the most commonly used methods for sample derivatization where hydroxyl, amino, and carboxyl groups are replaced with a methyl group resulting unique ionization for both acidic and basic carbohydrates. The main disadvantage of mass-spectrometric based detection in glycan analysis is that different isomers thus isobaric structures cannot be distinguished. To solve this issue multiple detection methods [117, 118] or tandem mass-spectrometry (MS/MS) are recommended [119].

## 2. Aims and Motivation

Altered haptoglobin glycosylation was reported in previous studies as candidate marker in different lung diseases. Our aim was the full structural annotation of haptoglobin glycans by capillary electrophoresis in combination with exoglycosidase digestions. The second goal was to relatively quantify the identified structures in normal, acute inflammatory, chronic inflammatory and malignant conditions focusing on branching- and fucosylation-degree and examine the biomarker potential of haptoglobin glycosylation.

Another widely studied potential biomarker is the glycosylation of immunoglobulin G. Glycosylation variations of human serum IgG have been found in conditions such as ageing, pregnancy, inflammatory diseases and cancer. One of the most common alterations in IgG glycosylation is reduced terminal galactosylation, and the concomitantly amount of GlcNAc terminating structures in inflammatory diseases. One of our goals was to examine if IgG glycosylation was a useful marker for monitoring treatment efficacy and identifying responders. The reportedly decreased galactosylation of IgG in RA and CD was expected to be altered towards to the normal profile after treatment in correlation with the responder status of the patients.

The increasing use of glyco-markers and the huge market of therapeutic monoclonal antibodies necessitate high-throughput and automatable methods for precise glycan annotation. Currently used methods require days for sample preparation using expensive laboratory equipment and the use of centrifugation technique that is hard to automate. A new method was aimed to be developed and implemented to alleviate the need of vacuum-centrifugation and overnight incubations, thus easily automatable and needs only hours instead of days for sample preparation. This would give the opportunity of automated sample preparation for biomedical and biopharmaceutical research and development.

### 3. Materials and Methods

#### 3.1. Chemicals and Reagents

Water and acetonitrile were Chromasolv HPLC grade from Sigma Aldrich (St. Louis, MO, USA). Haptoglobin standard, dithiothreitol, iodoacetamide, acetic acid, and all other chemicals were also purchased from Sigma Aldrich. High purity 8-aminopyrene-1,3,6-trisulfonate (APTS), Agencourt Cleanseq magnetic beads were from SCIEX (Brea, CA, USA). Purified N-linked carbohydrate standards, including bi-, tri-, tetra-antennary glycans (A2, A3, A4), their analogues carrying terminal galactose residues (A2G2, A3G3, A4G4) and the core fucosylated bi-antennary galactosylated glycan (FA2G2), CU clean-up cartridges were obtained from Prozyme (Hayward, CA, USA). 96 Protein A deep-well plate was purchased from Thermo-Fisher (Waltham, MA, USA). The PNGase F deglycosylation kit was purchased from New England Biolabs (Ipswich, MA, USA) and the digestion reaction mixtures were prepared following the manufacturer's protocol.

#### 3.2. Haptoglobin Purification from Plasma Samples

Protein-G chromatography (GE-Healthcare, Bucks, UK) was used for purification of the haptoglobin specific mAbs from Biosystems International (Debrecen, Hungary). Antibody BSI 0071 was raised against the immunoprecipitate from MARS Human 7 (Agilent, Santa Clara, CA, USA) column depleted pooled control plasma using a haptoglobin specific antibody and was selected by ELISA assay. BSI 2908 antibody was raised against multi-lectin affinity column depleted lung cancer plasma and selected as described earlier [10]. 10 mg of haptoglobin specific mAb were coupled to 0.5 g CNBr-activated Sepharose 4B affinity beads according to the manufacturer's protocol (GE-Healthcare). Plasma from age and gender matched human control (2 males, average age 61.0), pneumonia (3 males, average age 60.3), COPD (3 males, average age 61.6) and lung cancer (3 males, average age 61.3) groups were collected in the Department of Pulmonology at the University of Debrecen (Hungary) under clinical protocol RKEB /IKEB:2422-2005. The study was approved by the Hungarian Research Ethics Committee and all participants signed informed consent forms. 200  $\mu$ L of individual patient plasma samples were diluted 5 times with 50 mM potassium hydrogen phosphate (pH 7.0) binding buffer. HSA was depleted by incubation with 250  $\mu$ L Blue Sepharose 6 fast flow slurry (GE-Healthcare) for 30 min at room temperature. Following centrifugation, the albumin depleted flow-through was

incubated with 250  $\mu$ L Protein-G4 fast flow slurry (GE-Healthcare) for 30 min at room temperature to deplete the IgG content. HSA and IgG depleted plasma was loaded onto an in-house made mAb affinity column (1 ml bed-volume) and incubated for 1 hour at room temperature. The column was washed with 7 column volumes of phosphate-buffered saline (pH 7.2) and haptoglobin was eluted with 2 x 1 ml 0.2 M glycine - HCl elution buffer (pH 2.7). Eluted haptoglobin was immediately neutralized by the addition of 0.2 ml of 1 M Tris-HCl (pH 9.0) buffer. Protein concentration was measured by bicinchoninic acid (BCA) kit according to the manufacturer's protocol (Pierce, Rockford, IL, USA). SDS-PAGE on 4-20% Tris-Glycine precast gradient gels (Invitrogen, Carlsbad, CA, USA) were used to confirm the purity of haptoglobin and the concentration was calculated by densitometry analysis using the Gel Logic 2200 PRO equipment and Molecular Imaging software (Invitrogen).

### 3.3. Glycan Release and Derivatization of Haptoglobin Samples

Haptoglobin samples (approximately 50  $\mu$ g/sample) were reduced with dithiothreitol, alkylated with iodoacetamide and dissolved in 50  $\mu$ L of 50 mM sodium-bicarbonate (pH 7.0). Release of the N-linked glycans was accomplished by the addition of 2 U of recombinant PNGase F (Prozyme) and incubation at 37°C overnight. The released glycans were separated from the remaining polypeptide chains by means of 10 kDa cut-off spin filters (Nanosep 10 kDa, Sigma-Aldrich) and dried in a centrifugal vacuum evaporator (Thermo Scientific, Asheville, USA). The dried sugars were fluorescently labeled via reductive amination by the addition of 5.5  $\mu$ L of 20 mM APTS in 15% v/v acetic acid and 1.5  $\mu$ L of 1 M sodium cyanoborohydride in tetrahydrofuran, overnight at 37°C. The reaction was stopped by the addition of 93  $\mu$ L of water and the unreacted fluorescent dye was subsequently removed by normal phase resin containing pipette tips (PhyNexus, San Jose, CA, USA).

### 3.4. Patient Samples for IgG glycosylation and Transcriptome Analysis

The Institutional Review Board of University of Debrecen Medical and Health Science Center approved the clinical protocol and the study in compliance with the Helsinki Declaration. Signed informed consent was obtained from all individuals providing blood sample. RA patients were on maximal-tolerable methotrexate treatment (5 to 30 mg per week), which was stable for at least 4 weeks before baseline. Prednisone therapy ( $\leq$ 10 mg per day) was allowed for CD patients, which had to be stable for at least 2 months before infliximab therapy.



### 3.5. Sample Preparation and Fluorophore Labeling of IgG Glycans

50  $\mu$ L of serum samples were diluted with 250  $\mu$ L PBS buffer (pH 7). The diluted samples were loaded into the wells of a Protein A microwell plate (Thermo Scientific), washed with 300  $\mu$ L PBS and the captured proteins were eluted by 200  $\mu$ L of 10% acetic acid. 10 kDa Nanosep Spinfilters (Sigma) were used for buffer exchange. N-glycan release was performed on the 10 kDa spinfilters after the proteins were reconstituted by 50  $\mu$ L of 50 mM ammonium-bicarbonate. For PNGase F digestion the Prozyme deglycosylation kit was used according to the manufacturer's protocol. The released glycans were dried in a vacuum-centrifuge and then fluorescently derivatized by the addition of 6  $\mu$ L of 20 mM APTS in 15 % acetic acid and 2  $\mu$ L of 1 M sodium-cyanoborohydride using overnight incubation at 37 °C. The labeled samples were purified using Prozyme CU cartridges.

### 3.6. Capillary Electrophoresis

Capillary electrophoresis profiling of the APTS labeled N-glycans was performed in a PA800 *Plus* automated capillary electrophoresis instrument (SCIEX) equipped with a solid state laser based fluorescent detector (excitation: 488 nm, emission: 520 nm). The separations were accomplished in 50 cm effective capillary length (60 cm total) N-CHO coated 50  $\mu$ m i.d. capillary columns (SCIEX) filled with the N-CHO Carbohydrate Separation Buffer (SCIEX). The applied electric field strength was 500 V/cm, with the cathode at the injection side and the anode at the detection side (reversed polarity). Samples were injected by pressure: 1 psi (6.89 kPa) for 5 sec. For migration time correction and relative quantification purposes, APTS labeled maltose (G2) was co-injected with each sample as internal standard. The Karat 32 version 7.0 software package (SCIEX) was used for data acquisition and analysis.

### 3.7. Gene Expression Analysis

Gene expression data was derived from global transcriptome analysis of the patient cohorts, (published in [70]). Microarray data are available in Gene Expression Omnibus database (accession number: GSE42296).

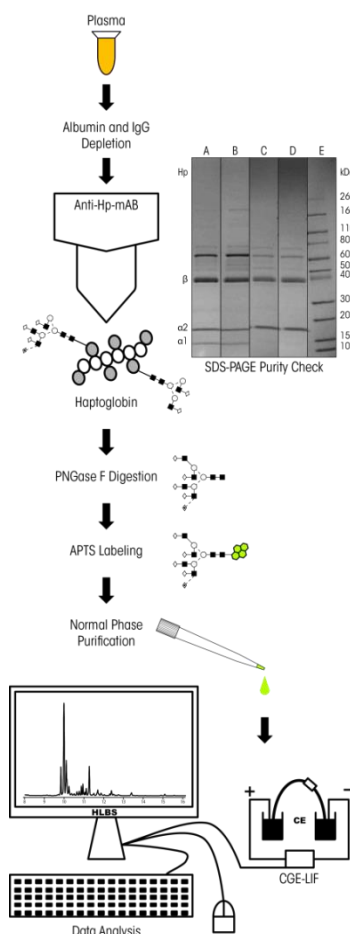
### 3.8. Statistical Analysis

IBM SPSS 20 software (New York, US) was used to perform Mann-Whitney pairwise comparison for all statistics. P values of <0.05 were considered to be significant.

## 4. Results

### 4.1. Increased Branching- and Fucosylation-Degree of Haptoglobin N-Glycosylation in Inflammatory and Malignant Lung Diseases

The analytical workflow is presented in Scheme 1. Briefly, after albumin and IgG depletion the haptoglobin was partitioned from the plasma samples of patients by affinity purification. The purity of the partitioned haptoglobin was checked by SDS-PAGE. N-glycans were then released by PNGase F, fluorescently labeled with APTS and analyzed by CE-LIF after the removal of the excess dye.



**Scheme 1.** Schematic representation of the sample preparation and analysis route of haptoglobin N-glycosylation analysis

Using the relative percentage area values of the desialylated glycan structures of haptoglobin (Table 1), fucosylation and branching degrees were determined in the control, acute and chronic inflammation as well as cancer patient samples [120]. Core (FDc) and arm (FDa) fucosylation degrees were evaluated separately. Alterations in size of the glycans were examined based on branching degree (BD) i.e., upon the level of various bi- (A2G2, A2FG2, FA2G2), tri- (A3G3, A3FG3, FA3G3) and tetra- (A4G4, A4FG4, FA4G4) antennary structures.

#### 4.1.1. Branching degree

Altered branching of the glycosylation of serum glycoproteins has been observed in pathologic conditions [121], thus, branching degree (BD) was investigated to identify the changes in the antennary structures between the different groups.

$$\text{Branching Degree (BD)} = (\text{Tri+tetra-antennary glycans}) / (\text{Total glycans}) \quad \text{Eq 1.}$$

Table 1B reveals elevated branching in all three disease groups with COPD showing the highest value compared to the control. As shown in Table 1A, a significant decrease was found in the amount of A2G2 glycan and an increase in A3G3 glycan contributing to the elevated branching degree of this group.

#### 4.1.2. Fucosylation degree

The two types of fucosylation on human haptoglobin are core-fucosylation, where the fucose unit is linked ( $\alpha$ 1-6) to the reducing end GlcNAc residue that is directly connected to the polypeptide backbone, and antennary-fucosylation where the fucose unit is linked to any of the antennary GlcNAc residues via  $\alpha$ 1-3 and/or 4 linkages. The degree of fucosylation (FD) can be expressed as the amount of fucosylated glycans relative to the total amount of glycans:

$$\text{Fucosylation degree (FD)} = (\text{Fucosylated glycans}) / (\text{Total glycans}) \quad \text{Eq 2.}$$

Total fucosylation degree was calculated for the control, pneumonia, COPD and lung cancer patient groups using equation 2 and the values are shown in Table 1C. The different fucosylation type containing structures (i.e., antennary and core) were also analyzed separately and referred to as core (FDc) and arm fucosylation (FDa) degrees. In total fucosylation, a slight decrease was observed in pneumonia and COPD and a strong increase in lung cancer. The core and arm fucosylation ratio revealed that the decrease in total fucosylation degree in pneumonia and COPD was the results of the interplay between the significant decrease in antennary-fucosylation and the slight increase in core-fucosylation, i.e., the elevated core fucosylation degree could not

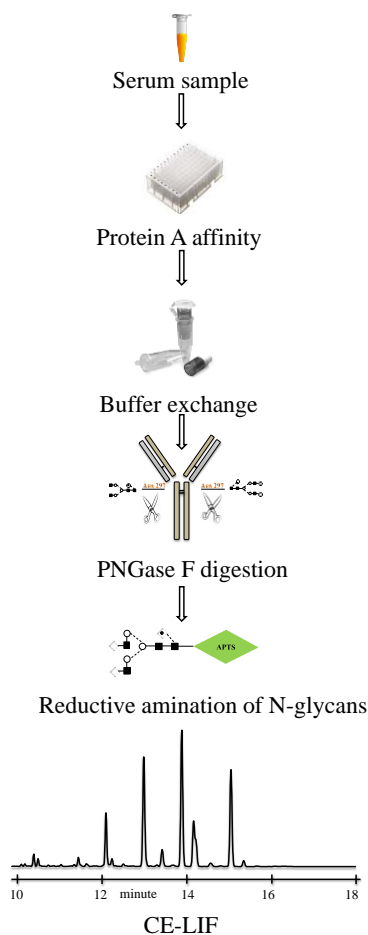
compensate for the arm fucosylation change. This observation suggested that it is critical to measure both the core and arm fucosylation degrees individually because it is possible that while the total fucosylation degree is apparently unchanged, the subgroups of FDc and FDa are potentially altered due to different expression of the fucosyl-transferases transporting the  $\alpha$ 1-6 core and  $\alpha$ 1-3/4 arm fucose units. Please note that the highest degree of total fucosylation was observed in the lung cancer group, however a closer look revealed that the arm-fucosylation was highly elevated in lung cancer, suggesting that the increase of the  $\alpha$ 1-3/4 linked arm fucosylation was the reason of the higher total fucose content in that group.

<b>Table 1A: Relative glycan peak areas</b>				
	<b>Control</b>	<b>Pneumonia</b>	<b>COPD</b>	<b>Lung cancer</b>
A2G2	65.63	61.07	58.52	61.28
A2FG2	1.55	1.00	1.18	1.77
FA2G2	6.05	7.07	5.85	5.68
A3G3	11.79	16.08	19.90	13.43
A3FG3	8.93	7.24	9.10	11.41
FA3G3	0.93	1.62	1.69	1.33
A4G4	2.93	3.82	2.36	2.51
A4FG4	1.78	1.62	1.13	2.07
FA4G4	0.39	0.44	0.25	0.48
<b>Table 1B: Branching</b>				
	<b>Control</b>	<b>Pneumonia</b>	<b>COPD</b>	<b>Lung cancer</b>
Bi-antennary	73.23	69.14	65.55	68.73
Tri-antennary	21.65	24.94	30.69	26.17
Tetra-antennary	5.10	5.88	3.74	5.06
<b>BD</b>	<b>0.267</b>	<b>0.308</b>	<b>0.344</b>	<b>0.312</b>
<b>Table 1C: Fucosylation</b>				
	<b>Control</b>	<b>Pneumonia</b>	<b>COPD</b>	<b>Lung cancer</b>
Total Fucosylated	19.63	18.99	19.2	22.74
Non-fucosylated	80.35	80.97	80.78	77.22
<b>FD</b>	<b>0.196</b>	<b>0.189</b>	<b>0.192</b>	<b>0.227</b>
<b>FDa</b>	<b>0.122</b>	<b>0.098</b>	<b>0.114</b>	<b>0.152</b>
<b>FDc</b>	<b>0.074</b>	<b>0.091</b>	<b>0.078</b>	<b>0.075</b>

**Table 1.** Measured relative peak areas (%) of the identified glycans and calculated fucosylation (FD, Eq. 1) and branching (BD, Eq. 2) degree values

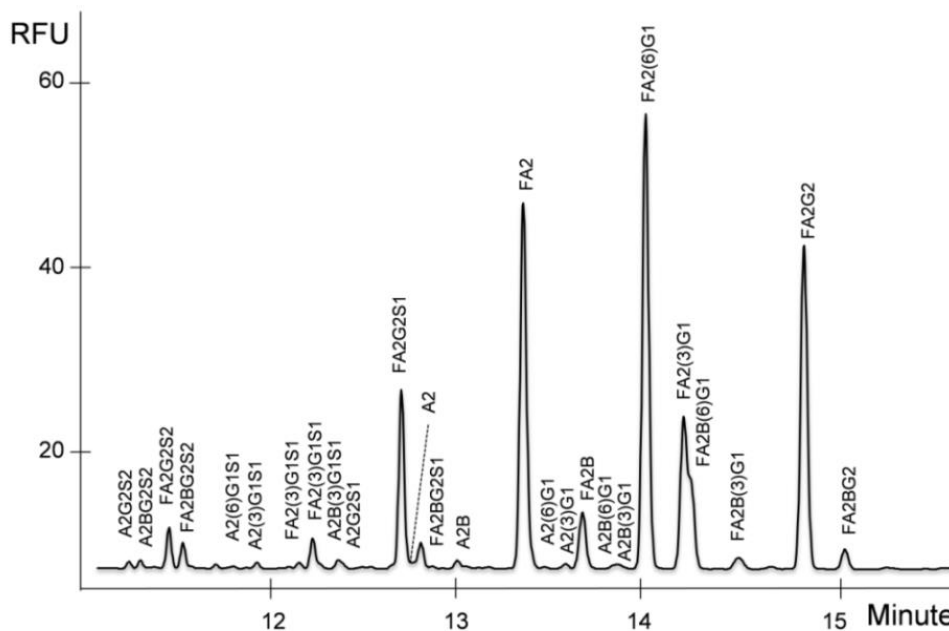
## 4.2. Combination of IgG N-Glycosylation and Corresponding Transcriptomics Data to Identify Anti-TNF $\alpha$ Treatment Responders in Inflammatory Diseases

Prediction of responsiveness in biological therapies is an important and challenging issue in different diseases. Analyzing glycosylation pattern changes of key serum glycoproteins is one of the possible avenues to follow disease remission in autoimmune diseases. The aim of this study was to investigate the changes of serum IgG glycoforms in Crohn's disease and Rheumatoid arthritis patients in response to anti-tumor necrosis factor alpha (TNF $\alpha$ ) treatment. IgG was isolated from patient serum samples using Protein A affinity pull-down, followed by the release of N-glycans with PNGase F. The released glycans were fluorescently tagged with aminopyrene-trisulfonate and analyzed by capillary gel electrophoresis with laser induced fluorescent detection (Scheme 2).



**Scheme 2.** Serum IgG glycosylation analysis workflow

Utilizing the high resolution of CE-LIF, 26 IgG glycoforms were examined in order to reveal significant changes in response to anti-TNF $\alpha$  treatment. As can see in Figure 11, most of the structures (~16) were low abundant (<2% of total area %). Our aim was to make highly detailed mapping of IgG glycoforms in order to generate a reliable and precise dataset.

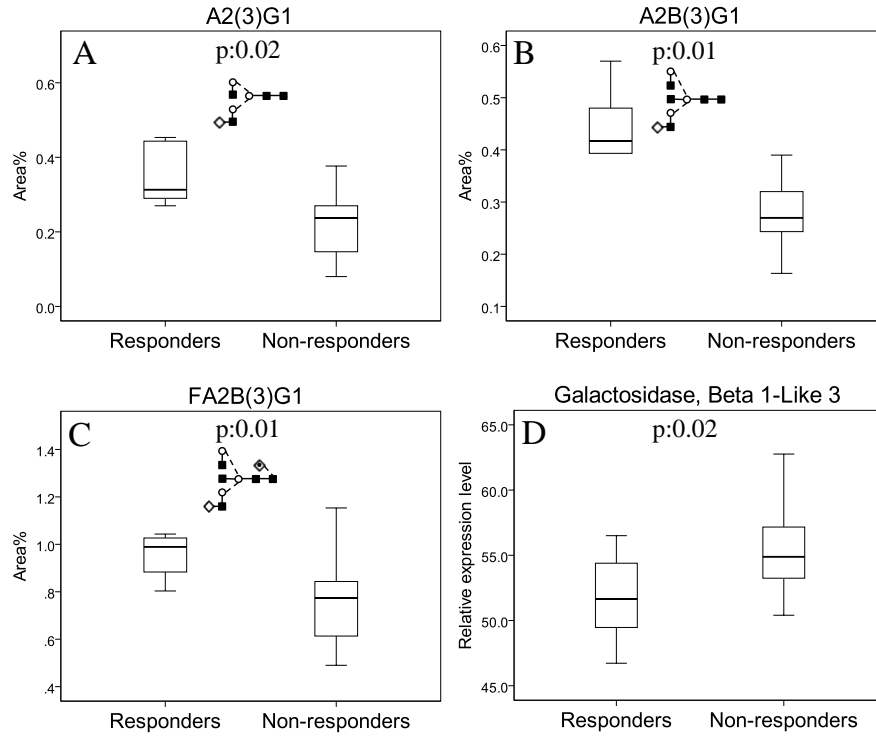


**Figure 11.** CE-LIF trace of IgG N-glycans from human serum

*CE conditions: capillary: N-CHO neutral coated capillary (effective length: 50 cm; total length: 60 cm); separation buffer: N-CHO carbohydrate separation buffer,  $E = 500$  V/cm (reversed polarity), pressure injection: 6.89 kPa for 5 s.*

#### 4.2.1. IgG N-glycosylation analysis before and after anti-TNF $\alpha$ treatment in Rheumatoid Arthritis

Special assessment was developed for RA to follow disease remission and determine patient responsiveness for the therapy. In RA, DAS28 (disease activity score examining 28 joints, which are commonly affected by RA) is the measure of disease activity in combination with measuring the level of C reactive protein (CRP) [122]. Due these long and complicated diagnostic procedures, there is a need to find non-invasive ways to determine markers that readily differentiate non-responders and responders in an early stage of the treatment. 17 serum samples from RA patients were analyzed. Six (5 females, 1 male, average age 44.3 years) were identified as responders and 11 (9 females, 2 males, average age 47 years) as non-responders by clinical evaluation based on the DAS28 scoring system. As it shown in Figure 12 (A, B, C), three low (>2%) abundant structures of A2(3)G1, A2B(3)G1 and FA2B(3)G1 were found to be significantly different between responders (R) and non-responders (NR) of RA patients before anti-TNF $\alpha$  treatment. . All of the significantly altered structures were galactosylated and in each case, responders showed higher galactosylation level. However, there were no significant changes in response to anti-TNF $\alpha$  therapy in any of the RA subgroups. It has to be noted that in both subgroups (R, NR) higher galactosylation (consequently lower agalactosylation) was detected after the therapy but this change was minimal. These findings are in agreement with previous studies where IgG galactosylation was reportedly altered in RA patients but failed to predict clinical response after anti-TNF $\alpha$  treatment [123]. To understand the molecular background of the observed glycosylation differences, the expression levels of the corresponding glycosyl-transferases and glycosidases were also investigated. In agreement with the glycosylation analysis data, there were no significant changes in the expression level of the respective enzymes after the treatment, although one reasonable difference was found between responders and non-responders at baseline (before treatment). As it shown in Figure 12D higher galactosidase activity was found in non-responders suggesting the reason of the detected lower galactosylation level compared to responders.

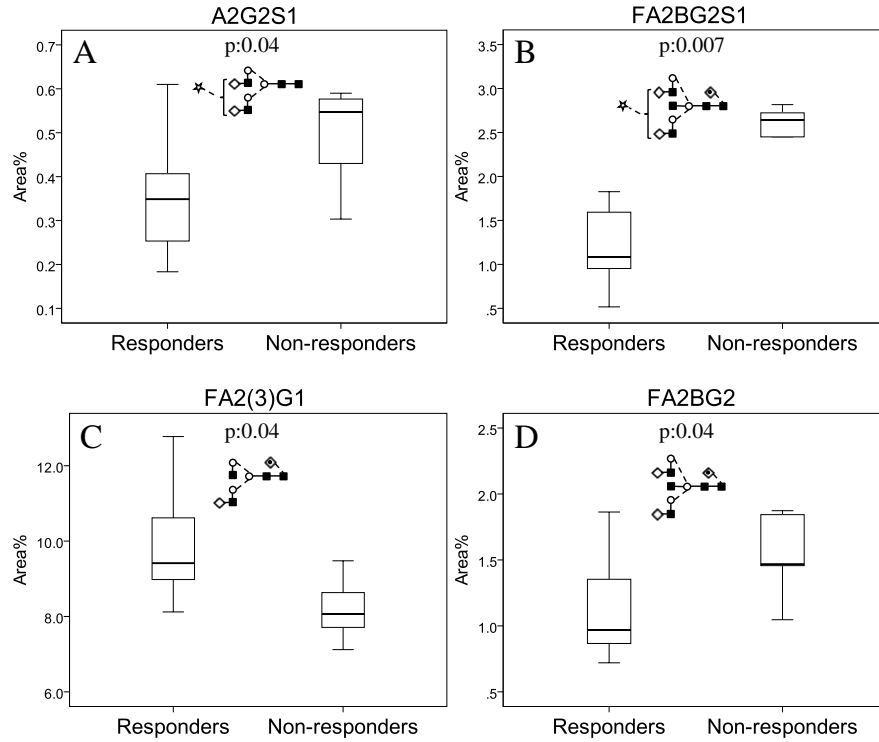


**Figure 12.** Glycosylation and gene expression based differences between responders and non-responders in RA before anti-TNF $\alpha$  treatment. A2(3)G1 (bi-antennary mono-galactosylated), A2B(3)G1 (bi-antennary mono-galactosylated with bisecting GlcNAc), FA2B(3)G1 (bi-antennary mono-galactosylated with core-fucose and bisecting GlcNAc)

#### 4.2.2. Differences of IgG glycosylation before and after anti-TNF $\alpha$ treatment in Crohn's Disease

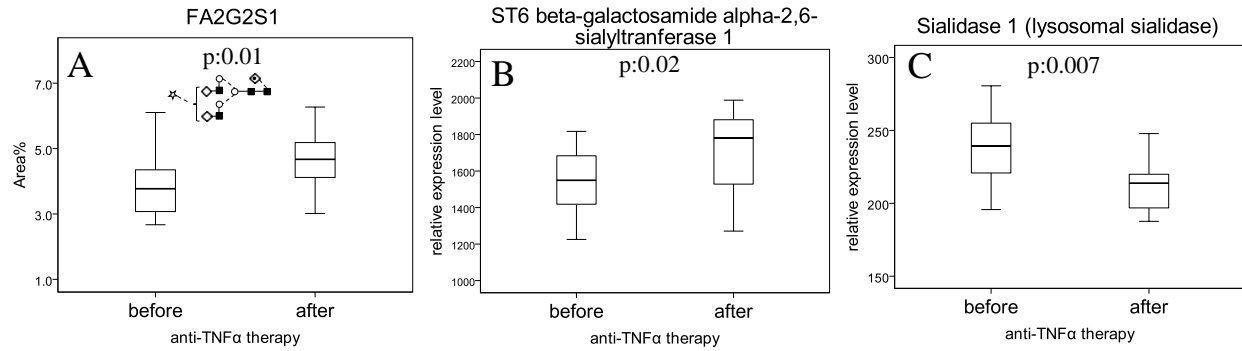
In CD, the combination of different clinical diagnostic indicators are used to quantify the symptoms, such as number of liquid stools, abdominal pain, general well-being, extraintestinal complications, need of antidiarrheal drugs, abdominal mass, hematocrit and body weight all summarized in Crohn's Disease Activity Index (CDAI) [124]. Based on the CDAI, 14 patients were identified as responders (R, 6 females, 8 males, average age 36.2 years) and 5 as non-responders (NR, 3 females, 2 males, average age 36 years). The serum samples were taken before and 2 weeks after the treatment, although the identification of responders was only possible after months by the CDAI protocol. Early recognition of status response could prevent patients from ineffective medication so our aim was to find one of the high abundant IgG glycoforms possibly providing useful information in responsiveness.





**Figure 13.** Differences of IgG glycoform levels in CD between responders and non-responders before anti-TNF $\alpha$  treatment. A2G2S1 (bi-antennary mono-sialylated), FA2BG2S1 (bi-antennary mono-sialylated with core-fucose and bisecting GlcNAc), FA2(3)G1 (bi-antennary mono-galactosylated with core-fucose) FA2BG2 (bi-antennary bi-galactosylated with core-fucose and bisecting GlcNAc)

Similarly to RA, even before the start of the treatment, significant differences were revealed between the two subgroups (R vs NR). Using Mann-Whitney pairwise comparison, A2G2S1 (Figure 13A), FA2BG2S1 (Figure 13B), FA2(3)G1 (Figure 13C) and FA2BG2 (Figure 13D) were found significantly different although it has to be noted that among these, only the FA2(3)G1 was not a low abundant structure. It has been reported earlier that serum IgG galactosylation level correlated with the disease activity in CD [125]. Our results are in agreement with these findings as non-responders showed significantly lower galactosylation (FA2(3)G1) level suggesting altered activity of galactosyl-transferases/galactosidases in responders and non-responders. However, we have not found significant differences in the expression level of the corresponding enzymes before the treatment.



**Figure 14.** Significant glycosylation and transcriptomics changes in response to anti-TNF $\alpha$  therapy. FA2G2S1 (bi-antennary bi-galactosylated with core-fucose and  $\alpha$ 2-6 linked terminal sialic acid)

The main goal of this study was to find glycosylation markers that were significantly altered in response to anti-TNF $\alpha$  treatment and thereby identify responders. We have successfully found an IgG glycoform, which level was significantly altered 2 weeks after the therapy. In the responder group of CD, FA2G2S1 was found to be significantly ( $p=0.01$ ) higher 2 weeks after anti-TNF $\alpha$  treatment (Figure 14A). Sialylation was found to be important earlier in the anti-inflammatory activity of immunoglobulins, which support our findings as the higher anti-inflammatory activity of IgGs can be helpful in inflammatory disease [126]. To confirm our findings, the expression levels were determined for the relevant sialyl-transferases and sialidases. Significant differences were found only in the responder group of CD where higher sialyl-transferase activity (Figure 14B) and lower sialidase activity (Figure 14C) was detected 2 weeks after the infliximab treatment. These results are in partial agreement with previous reports, where higher sialidase activity was found in CD and ulcerative colitis [127]. The transcriptomics data readily supported our IgG glycosylation based findings as the combination of higher sialyl-transferase and lower sialidase activity might be responsible for the higher sialylation level of the generated glycoproteins.

Prediction of patient response for any therapy is critical in inflammatory diseases such as CD and RA, as currently used scoring systems require months for responder identification. The importance of reliable biomarkers in these diseases is essential as the efficacy of biological therapies can vary between patients. In this study a highly sensitive and high resolution CE-LIF based method was applied to examine serum IgG glycosylation changes in autoimmune diseases

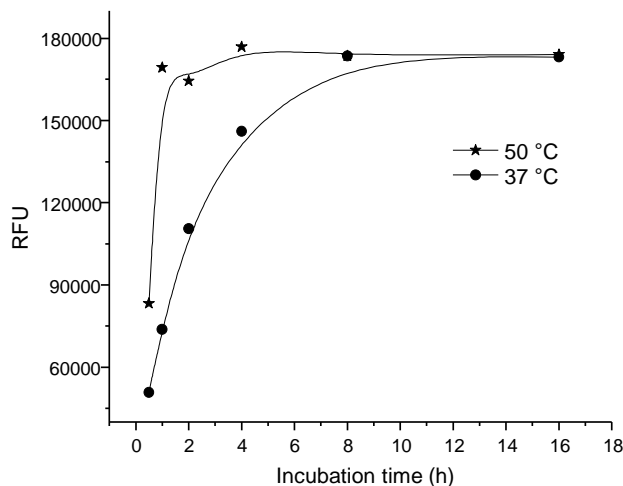
(CD and RA) before and two weeks after anti-TNF $\alpha$  therapy in order to identify responders and non-responders. Utilizing the high resolving power of CE-LIF, 26 glycoforms were separated and relatively quantified. In RA, three low abundant galactosylated structures were found to be significantly different before the treatment where in all of the cases responders showed higher galactosylation level. No significant alteration was detected in RA in response to the treatment. In CD significant differences were detected in galactosylation level between responders and non-responders before the treatment (higher in the responder group). FA2G2S1 level was significantly increased in response to anti-TNF $\alpha$  therapy, thus being a possible candidate marker for responder identification. The level of this structure was not significantly altered in any of the RA groups suggesting disease specificity for CD. Our findings were also supported by transcriptomics analysis of the corresponding glycosyl-transferase and glycosidase enzymes, as higher sialyl-transferase and lower sialidase activity were found.

### 4.3. Magnetic Bead Based Sample Preparation for Automated N-glycan analysis

In this part of our work, an optimized, rapid and high-throughput magnetic bead based sample preparation approach was developed for N-glycosylation analysis, where all preparation steps can be easily automated using simple liquid handling robots. Particular attention was given to avoid any centrifugation steps (both normal and vacuum) and overnight incubations, which are otherwise part of regular protocols of most glycan sample preparation methods. Utilization of the magnetic bead based approach alleviated the need of any centrifugation or centrifugal vacuum evaporation steps. The sample preparation protocol was demonstrated via practical examples using representative glycoprotein standards possessing high mannose, complex and highly sialylated glycans. All individual processing steps, such as glycan release, fluorescent labeling and fluorophore label cleanup were optimized to decrease processing time and increase the efficiency for the magnetic bead based method.

#### 4.3.1. Glycan release

Minimizing PNGase F mediated deglycosylation time was important, while considering easy implementation of this part for a magnetic bead based sample preparation platform. Using the liquid handling robot friendly open 96 well-plate format, the effect of temperature was important to consider as  $>60^{\circ}\text{C}$  could cause protein denaturation and buffer evaporation, this latter especially in low volumes (10-50  $\mu\text{L}$ ).



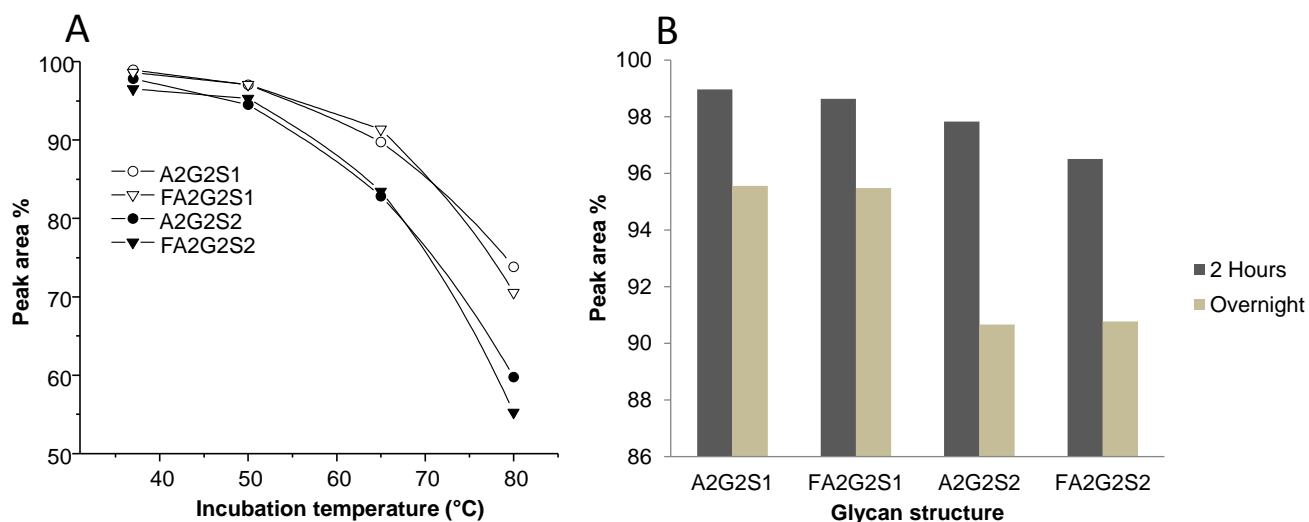
**Figure 15.** Average fluorescent intensity as a function of PNGase F digestion time at 37 °C and 50 °C.

Digestion efficiency was compared at 50°C and 37°C for the deglycosylation of IgG, fetuin and RNase B glycoprotein standards using 0.5, 1, 2, 4, 8 and 16 hours of incubation with the goal to find the shortest time that results in full deglycosylation. The released glycans were APTS labeled and analyzed by CE-LIF. Three releases were made with each digestion strategies and three analyses were made with each release, generating nine data points per digestion time and temperature. While peak distribution (measured as peak area percentages) exhibited no differences between the two incubation temperatures, the RFU values represented the changes in the amount of the released glycans. As shown in Figure 15, peak intensities increased significantly slower at 37°C comparing to 50°C, where the maximum level was already reached after 1 hour of incubation time. In addition, no differences were found in peak area percentages comparing the various digestion times, including the overnight reaction, suggesting that the same glycosylation pattern was obtained using shorter incubations with the main difference in the amount of the released sugars. As expected, accelerated glycan release was observed at higher temperature, thus 1 hour/50°C PNGase F digestion was used in all consequent experiments.

#### 4.3.2. APTS labeling

The goal of the labeling optimization part was to identify conditions that would support magnetic bead based automation using simple liquid handling robots, while providing the same labeling efficiency as conventional centrifugation based methods, in special respect to peak intensity and peak area distribution. Another important aspect of this part of the work was to achieve high labeling efficiency without the need of overnight incubation and vacuum-centrifugation based sample concentration. First, mono- and di-sialo glycan standards of A2G2S1, FA2G2S1, A2G2S2 and FA2G2S2 were labeled in duplicates with 20 mM APTS in 15 % acetic acid for 2 hours at 37, 50, 65 and 80°C. The non-sialylated counterparts of these glycans (A2G2 and FA2G2) were also labeled and used for spiking the higher temperature reaction mixtures to reveal possible temperature induced desialylation. As one can see in Figure 16A, the increase in the reaction temperature significantly elevated the desialylation process for all sialylated glycan standards. Interesting to note that di-sialo standards exhibited more enhanced sialic acid loss. On average, 2% sialic acid loss was observed at 50°C, 11% at 65°C and 33% at 80°C, suggesting that carefully chosen derivatization temperature is crucial during glycan labeling when sialylated structures are expected in the sample. The effect of incubation time on fluorophore labeling efficiency was examined at 37°C. As Figure 16B depicts, significant differences were observed

between overnight and 2 hour long incubation. The mono- and di-sialo structures showed on average 2-3% and 9-10% sialic acid loss, respectively, using overnight incubation, signifying the importance of incubation time length in sialylation loss. While shorter APTS labeling times (2 h) resulted in lower signal intensity, longer (overnight) incubation caused noticeable sialic acid loss. Considering the fact that reliable sample preparation and data generation is more important than signal intensity, a 2 hour labeling at 37°C was chosen as a good compromise and was used in all consequent experiments.



**Figure 16.** Optimization of fluorophore labeling conditions

In an effort to compensate for the lower signal intensity at shorter incubation times, the effect of catalyst (acetic acid) and fluorophore (APTS) concentration was also revisited (Table 2, Section A, B). Using 20 mM APTS in 15, 20 and 25% acetic acid, mono- and di-sialo glycan standards (A2G2S1, FA2G2S1, A2G2S2, and FA2G2S2) were labeled to assess the labeling efficiency, while trying to minimize sialylation loss. With the use of 20% acetic acid, the resulted peak pattern and area percentages showed no detectable sialic acid loss, while significantly higher peak intensities were observed (Table 2, Section A). Table 2, Section B shows the effect of APTS concentration on the labeling efficiency of the maltooligosaccharide ladder standard using 20, 40 and 80 mM APTS in 20% acetic acid at 37°C for 2 hours (triplicates). As one can see, the use of higher APTS concentration lead to increased labeling efficiency. In spite of the fact that the use of 80 mM APTS resulted in the highest intensity, a compromise was suggested here too

to use 40 mM APTS in the higher volume labeling reaction that was required by automation consideration (minimum of 20  $\mu$ L volume). Using the above suggested dye and catalyst concentrations, released glycans from 100  $\mu$ g IgG, fetuin and RNase B glycoprotein standards were labeled in duplicates using 40 mM APTS in 20% acetic acid at 37°C for 2 hours and compared to the traditionally used 20 mM APTS in 15% acetic acid labeling strategy with 2 hour and overnight incubation (Table 2, Section C). The combination of higher dye and catalyst concentrations resulted in approximately 20% higher labeling efficiency compared to the original 2 hour labeling strategy without any apparent sialic acid loss. Please note that signal intensity was still >50% lower than with the overnight reaction where on the other hand noticeable sialic acid loss was detected. As a first approximation we suggest APTS labeling at 37°C for 2 hours with 40 mM APTS in 20% acetic acid obtain adequate labeling efficiency and still minimizing sialic acid loss.

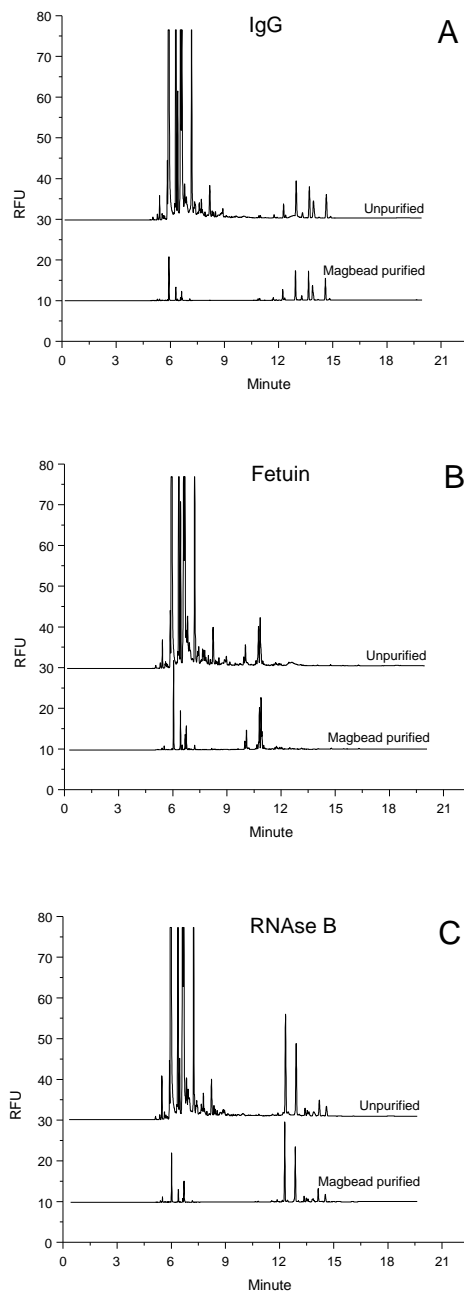
<b>Section A</b>			
AcOH concentration	15%	20%	25%
A2G2S1	1.90	10.04	10.95
A2G2S2	1.14	5.25	5.88
FA2G2S1	6.61	14.94	15.52
FA2G2S2	3.20	13.04	13.52
<b>Section B</b>			
APTS concentration	20 mM	40 mM	80 mM
3 Ladders average	14.64	39.33	46.03
<b>Section C</b>			
Labeling strategy (37°C)	2h, 15% AcOH, 20 mM APTS	2h, 20% AcOH, 40 mM APTS	O/N, 15% AcOH 20 mM APTS
IgG	9.47	24.22	52.91
Fetuin	18.93	28.17	66.31
RNase B	13.16	23.50	63.34

**Table 2.** APTS labeling efficiency study. The numbers in the table represent the average signal intensity of 3 labeling reactions.

#### 4.3.3. Magnetic bead based sample preparation

As emphasized earlier, the key aspect of this part of the work was to find optimal PNGase F based glycan release and APTS labeling parameters for easy downstream automation, with the key aspect to avoid any centrifugation steps during the sample preparation process, including vacuum centrifugation. Carboxyl coated magnetic beads were apparently capable of capturing complex carbohydrates both in their native (after glycan release) and fluorophore labeled (after APTS labeling) forms. First, magnetic beads were applied to clean-up the APTS reaction mixture, i.e., to remove excess APTS, not conjugated to the sugar structures. APTS labeled hIgG, fetuin and RNase B glycans were purified in triplicates after the labeling reaction using 200  $\mu\text{L}$  carboxyl coated magnetic bead suspension. Binding and washing steps were accomplished by using 150  $\mu\text{L}$  of 87.5% acetonitrile. The elution step was accomplished with the use of 25  $\mu\text{L}$  of water. Please note that  $\geq 20$   $\mu\text{L}$  volumes of magnetic bead suspension and binding/elution solutions were readily handled by automatic pipettors, suggesting the utilization of the same volumes in simple liquid handling robots with regular pipette tips or syringes. The eluate was directly analyzed by CE-LIF without any further processing. Second and third elution fractions were also analyzed to assess the efficiency of the first elution. When the cleanup mixture was suspended properly and 25  $\mu\text{L}$  of water was used for elution, no detectable sample remained on the beads, i.e., the second and third elution gave negative results. On the other hand, when only 15  $\mu\text{L}$  of water was used in the first elution, traces of remaining APTS labeled glycans were detected. No differences were found; however, in peak area distribution using the magnetic bead based cleanup protocol, i.e. the resulting profile was identical to the one that was obtained with conventional sample cleanup methods, suggesting no apparent bias for the different glycan structures (neutral, sialylated, high mannose) towards the beads, while most of the free APTS was removed as shown in Figure 17 panels A, B and C for IgG, RNase B and fetuin glycans, respectively.





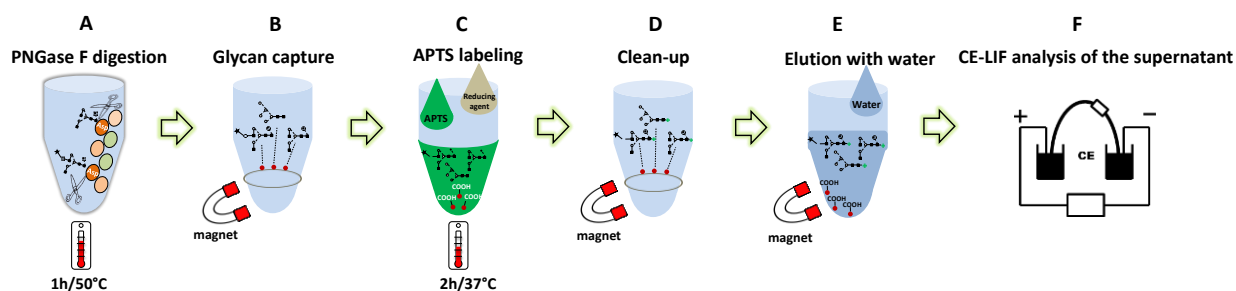
**Figure 17.** Comparative electropherograms of unpurified (upper traces) and magnetic bead purified APTS labeled glycans from IgG (A), fetuin (B) and ribonuclease B (C).

*CE conditions: capillary: N-CHO neutral coated capillary (effective length: 50 cm; total length: 60 cm); separation buffer: N-CHO carbohydrate separation buffer,  $E = 500$  V/cm (reversed polarity), pressure injection: 6.89 kPa for 5 s.*

Based on the successful implementation of centrifugation free APTS cleanup protocol with the use of a carboxyl coated magnetic beads, a similar approach was evaluated to capture the released glycans after PNGase F digestion. Magnetic beads were added to the PNGase F reaction mixture after the incubation step, first to bind the released glycans in 87.5% acetonitrile solution. In this case, however, instead of water, the free glycans were eluted by aqueous APTS solution (40 mM in 20% acetic acid) followed by the addition of the reducing agent (1 M pic-BH3 in MeCN) to immediately initiate the labeling reaction without any interim pre-concentration steps. Please note that this approach, while very effective, did not require any vacuum centrifugation based sample pre-concentration or any other purification steps to remove the remaining polypeptide chain and PNGase F enzyme from the digestion reaction mixture.

#### 4.3.4. The fully extended and optimized sample preparation protocol

The fully extended and optimized magnetic bead based glycan sample preparation protocol comprising five individual steps were compared to conventional centrifugation based and overnight digestion/labeling protocols. As Scheme 3 depicts, the first step was a one hour PNGase F digestion at 50°C (Step A). This was followed by magnetic bead based partitioning of the released glycans from the remaining polypeptide chains and the releasing enzyme using 200  $\mu$ L magnetic bead suspension in 87.5% final acetonitrile concentration. After thorough mixing, the tube was placed on the magnet (Step B) for partitioning. The supernatant was discarded and the captured glycans were eluted from the beads into the same tube by 40 mM APTS in 20 % acetic acid (21  $\mu$ L). After the elution step, the reductive amination reaction was immediately started with the addition of pic-BH3 (Step C, 7  $\mu$ L, 1 M). The reaction mixture was incubated at 37°C for 2 hours, then the excess labeling dye was removed (Step D) by the same beads which were used after the digestion in 87.5% final acetonitrile concentration (similar to Step B). After discarding the supernatant, the captured APTS-labeled glycans were eluted from the beads by water (25  $\mu$ L) and partitioned by placing the tube on a magnet (Step E). The supernatant was then analyzed by CE-LIF (Step F). Using this magnetic bead based sample preparation protocol, the full workflow of glycoprotein sample preparation takes approximately 4 hours without the need of any centrifugation and or vacuum centrifugation steps, enabling full automation with simple liquid handling robots.



**Scheme 3.** The full magnetic bead based sample preparation workflow for N-glycosylation analysis

The reliability and reproducibility of the method was evaluated by preparing six IgG, RNase B and fetuin samples with the magnetic bead based protocol and compared to conventional overnight incubation and centrifugation based protocol. Three repetitions of each release generated 54 dataset per sample preparation. The excellent reproducibility of the approach is depicted in Table 3, showing <5% and <10% RSD for major and minor glycan structures, respectively. The samples analyzed represented of neutral and slightly sialylated (Panel A), high mannose (Panel B) and highly sialylated (Panel C) glycans. Mann-Whitney pairwise comparison was applied to explore the differences in peak area percentages. Integrating 28 peaks, the significance (p) level was examined between the two methods where only 4 peaks showed significant differences ( $p < 0.05$ ). All of the different peaks were highly sialylated fetuin glycans and similarly to our previous findings (labeling optimization), the overnight method produced decreased sialylation levels suggesting the importance of incubation time during the reductive amination step. The significantly higher area % of peaks 1 and 2 with the use of the magnetic bead based protocol correlates with the lower peak area values of peaks 5 and 7, suggesting that desialylation of the highly sialylated species (tetra- and tri-sialylated) increased the amount lower sialylated species (di- and mono-sialylated).

Panel A	Magbead protocol			Overnight protocol			
IgG	Average Area %	STDEV	RSD%	Average Area %	STDEV	RSD%	significance (p)
FA2G2S2	1.19	0.04	3.72	1.21	0.09	7.01	0.937
FA2BG2S2	1.23	0.03	2.24	1.20	0.03	2.55	0.132
FA2(3)G1S1	1.71	0.06	3.78	1.73	0.10	5.77	0.818
FA2G2S1	7.45	0.25	3.34	7.48	0.31	4.12	0.937
FA2BG2S1	1.74	0.16	8.98	1.65	0.17	10.41	0.485
FA2	22.12	0.47	2.11	22.23	0.15	0.68	0.699
FA2B	3.97	0.11	2.83	3.97	0.04	1.12	0.589
FA2(6)G1	22.93	0.40	1.76	23.01	0.60	2.61	1.000
FA2(3)G1	11.59	0.06	0.51	11.57	0.17	1.49	0.589
FA2B(6)G1	4.86	0.41	8.52	4.69	0.14	3.08	0.818
FA2B(3)G1	1.02	0.09	9.12	1.07	0.06	5.39	0.132
FA2G2	18.13	0.47	2.61	18.30	0.12	0.64	0.589
FA2BG2	1.46	0.04	3.01	1.40	0.04	2.56	0.065
Panel B	Magbead protocol			Overnight protocol			
RNase B	Average Area %	STDEV	RSD%	Average Area %	STDEV	RSD%	significance (p)
Man5	43.45	0.62	1.43	43.90	0.37	0.85	0.132
Man6	33.39	0.36	1.09	33.50	0.25	0.74	0.699
Man7*	3.56	0.13	3.59	3.51	0.04	1.00	0.394
Man7**	2.58	0.08	3.17	2.52	0.07	2.69	0.240
Man7***	2.18	0.03	1.17	2.22	0.11	5.10	0.699
Man8	8.60	0.30	3.51	8.22	0.13	1.63	0.065
Man9	6.00	0.35	5.81	6.04	0.23	3.80	0.699
Panel C	Magbead protocol			Overnight protocol			
Fetuin	Average Area %	STDEV	RSD%	Average Area %	STDEV	RSD%	significance (p)
Peak 1	6.95	0.28	3.97	5.08	0.25	4.92	0.002
Peak 2	15.46	0.58	3.72	11.43	0.47	4.09	0.002
Peak 3	3.19	0.07	2.13	3.24	0.10	3.13	0.485
Peak 4	3.97	0.11	2.82	4.11	0.18	4.34	0.065
Peak 5	25.93	0.59	2.27	27.90	0.32	1.16	0.002
Peak 6	32.57	0.30	0.92	32.67	0.21	0.65	0.589
Peak 7	8.45	0.14	1.61	12.73	0.67	5.27	0.002
Peak 8	3.32	0.12	3.49	3.17	0.14	4.50	0.093

**Table 3.** Measured differences between the magnetic bead and traditional overnight protocols examining the peak area% of 28 standard N-glycans

To conclude this section, a magnetic bead based protocol was developed for N-glycosylation analysis of glycoproteins not requiring hard-to-automate centrifugation and vacuum-centrifugation steps. Glycan release, APTS-labeling and clean-up were optimized resulting in 4 hours magnetic bead based process with excellent yield and high reproducibility. The next step of this work is to apply this optimized magnetic bead based protocol with all steps from PNGase F digestion, through optimized fluorophore labeling and clean-up to high throughput sample processing in 96 well plates format with simple liquid handling robots allowing full automation.

## 5. Discussion

### 5.1. Preliminary Findings on Haptoglobin N-glycome Alterations

In our experiments disease associated changes of core and antennary fucosylation were identified by targeted exoglycosidase digestions and their levels were compared in the different patient groups. Terms such as of core- and arm-fucosylation degree, as well as branching-degree were introduced for easier characterization of the changes. Increased level of  $\alpha$ 1-6 fucosylated tri-antennary glycans was found in all disease groups compared to the control and also elevated amounts of core- and arm fucosylation on tetra-antennary glycans were detected in the lung cancer compared to the COPD. An increased level of tri- and tetra-antennary glycans exhibiting the  $\text{Sle}^x$  epitope on haptoglobin in chronic inflammatory conditions, reportedly suggested a pro-inflammatory impact on the mechanism of glycosylation [128]. Ang et al. found increased serum concentration of  $\alpha$ 1-6 fucosylated haptoglobins in hepatocellular carcinoma compared to chronic liver disease [129] while Sarrats et al. reported increased arm-fucosylation on haptoglobin in chronic pancreatitis, while in pancreatic cancer it was just slightly elevated. It has been also described that core-fucosylation on haptoglobin was increased in stage IV pancreatic cancer. Based on these results they concluded that  $\text{Sle}^x$  and increased branching were probably associated with the inflammatory response, while the increased core-fucosylation on acute phase proteins might be cancer specific [130]. These results suggest the importance of the analysis of different fucosylation types in various diseases which is in agreement with our findings.

### 5.2. IgG Glycosylation Changes in Response to Anti-TNF $\alpha$ Treatment

IgG glycosylation is reportedly a reversible alteration in inflammatory diseases [131]. During biological therapies, decreased galactosylation was found to be altered towards to the normal healthy profiles [63]. Ercan et al reported that serum hypogalactosylation fails to predict patient responsiveness in RA 3 months after the therapy although galactosylation level was improved with the treatment [123]. These findings are in agreement with our results as there was no significant glycosylation alteration in response to the treatment in RA but an increased galactosylation was detected in both responder and non-responder group. Similar results have been published in CD where the agalactosyl IgG level was correlated with the disease activity, suggesting high predictability of therapeutic outcome [132]. Our results are in partial agreement with these findings, as lower galactosylation was detected in CD although similarly to RA it

failed to predict patient response. Using intravenous immunoglobulins, sialylation was found to be crucial in anti-inflammatory activity of IgG-s [133], while terminal galactosylation was associated with CDC activation [61]. These observations suggest that in inflammatory diseases, IgG sialylation might have higher biomarker potential than galactosylation which supports our results as higher IgG sialylation was found in CD after anti-TNF $\alpha$  therapy, confirmed by transcriptomics data analysis of the corresponding glycosyl-transferases and glycosidases

### 5.3. Novel Magnetic Bead Based Glycan Preparation

Full automation to enable high throughput N-glycosylation profiling and sequencing with good reproducibility is critical to fulfill contemporary needs of the biopharmaceutical industry and requirements of national regulatory agencies. The most prevalently used glycoanalytical methods are capillary electrophoresis and hydrophilic interaction liquid chromatography, while very efficient, both necessitate extensive sample preparation and cleanup, including glycoprotein capture, N-glycan release, fluorescent derivatization, purification and pre-concentration steps during the process [75, 86]. Currently used protocols to fulfill these tasks require multiple centrifugation and vacuum-centrifugation steps making liquid handling robot mediated automated sample preparation difficult and expensive. We reported a rapid magnetic bead based sample preparation approach that enables full automation including all the process phases just in 4 hours without requiring any centrifugation and/or vacuum concentration steps. Please note that the applied carboxylated magnetic beads were never used in glycan preparation before. This novel magnetic bead based protocol has been compared to conventional centrifugation based sample preparation strategies using standard glycoproteins (IgG, fetuin and RNase B) and featured rapid processing time, high release and labeling efficiency, good reproducibility and the potential of easy automation.

## 6. Summary

The biomarker potential of glycosylation is presented in this work focusing on haptoglobin and IgG. Haptoglobin glycosylation was reportedly altered in inflammatory and malignant conditions. Fucosylation- and branching-degrees were examined and the importance of linkage-specific fucosylation-degrees was pointed out. The preliminary results were in agreement with the literature as increased branching and fucosylation was found in various pathological conditions.

Prediction of patient response for any therapy is critical in inflammatory diseases such as CD and RA, as currently used scoring systems require months for responder identification. The importance of reliable biomarkers in these diseases is essential as the efficacy of biological therapies can vary between patients. Utilizing the high resolving power of CE-LIF, 26 IgG glycoforms were examined in RA and CD before and after anti-TNF $\alpha$  treatment. In RA, three low abundant galactosylated structures were found to be significantly different before the treatment and all responders showed higher galactosylation levels. No significant alteration was detected in RA in response to the treatment. In CD FA2G2S1 level was significantly increased in response to anti-TNF $\alpha$  therapy, thus being a possible candidate marker for responder identification. This finding was also supported by transcriptomics analysis of the corresponding glycosyl-transferase and glycosidase enzymes, as higher sialyl-transferase and lower sialidase activity was found.

For reliable biomarkers huge number of samples has to be analyzed which necessitates automated high-throughput methods. A magnetic bead based protocol was developed for N-glycosylation analysis of glycoproteins not requiring hard-to-automate centrifugation and vacuum-centrifugation steps. Glycan release, APTS-labeling and clean-up were optimized resulting in a 4 hours magnetic bead based process with excellent yield and high reproducibility. The next step of this work is to apply this optimized magnetic bead based protocol with all steps from PNGase F digestion, through optimized fluorophore labeling and clean-up to high throughput sample processing in 96 well plates format with simple liquid handling robots allowing full automation.

Keywords: glycosylation, biomarkers, capillary electrophoresis



## 7. Összefoglalás

A glikoziláció biomarker potenciálja került bemutatásra ebben a tézisben, a haptoglobin és IgG glikoproteinekre fókuszálva. A haptoglobin glikozilációjának változásai már számos korábbi publikációban leírásra kerültek, mi viszont a glikánok elágazási és fukozilációs fokának változásaira fordítottunk kiemelt figyelmet. A talált eredmények megegyeznek a korábbi tanulmányokban leírtakkal minthogy emelkedett fukozilációs és elágazási fokot sikerült kimutatni a haptoglobin glikánjain a gyulladásos és daganatos tüdőbetegségeken.

A válaszadók azonosítása rendkívül fontos olyan hosszan tartó költséges kezelések esetén mint a biológiai terápiák. RA-ben és CD-ben a jelenlegi pontozós rendszerek segítségével hónapokba telik a válaszadók azonosítása. Az IgG glikoziláció köztudottan változik ezekben a betegségeken, mely magában hordozza a lehetőséget, hogy markerként hasznosítsuk. A CD pácienseken 2 héttel az anti-TNF $\alpha$  kezelés után sikerült kimutatni szignifikáns változást mely alapján megkülönböztethetők a válaszadók és nem-válaszadók. Ez az eredmény magában hordozza annak lehetőségét, hogy a jövőben az IgG glikozilációt esetleges biomarkerként használják ebben a betegségben.

A jelenlegi glikánpreparálási technológiákat több napos inkubálás, manuális pipettázás és a vákum-centrifuga nélkülözhetetlensége jellemzi. Erre jelenthet megoldást az általunk újonnan kifejlesztett mágneses gyöngy alapú mintapreparálási eljárás mely mindössze 4 óra alatt képes akár 96 mintát elkészíteni vákumcentrifuga szükségessége nélkül és teljes mértékben automatizálható. Ez nagy mértékben megkönnyítheti a kutatók dolgát a jövőben, mindamellett megnövelheti a kísérletek precizitását és reprodukálhatóságát.

Kulcsszavak: glikoziláció, kapilláris elektroforézis, biomarker kutatás

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## 9. Abbreviations

ADCC	Antigen-dependent cellular cytotoxicity
AFP	Alpha-fetoprotein
AFP-L3	Fucosylated alpha-fetoprotein
APTS	8-Aminopyrene-1,3,6-Trisulfonic Acid
Asn	Asparagine
CD	Crohn's disease
CDC	Complement dependent cytotoxicity
CDT	Carbohydrate-deficient transferrin
CE-LIF	Capillary-electrophoresis with laser-induced fluorescence detection
COPD	Chronic obstructive pulmonary disease
DCP	Des-gamma-carboxy prothrombin
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESI	Electrospray-ionization
GlcNAc	N-acetyl-glucosamine
GnTIII	Beta 1,4-N-acetylglucosaminyltransferase III
Hb	Hemoglobin
HCC	Hepatocellular carcinoma
Hp	Haptoglobin
HPLC	High performance liquid chromatography

IL-1 $\beta$	Interleukin-1- $\beta$
IL-6	Interleukin-6
LC	Liquid chromatography
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ ionization
MS	Mass spectrometry
NaBH <sub>3</sub> CN	Sodium cyanoborohydride
N-CHO	PVA coated capillary for N-linked oligosaccharide profiling
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
pic-BH <sub>3</sub>	Picoline borane
PNGase F	Peptide -N-Glycosidase F
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
IgG	Immunoglobulin G
SDS–PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sle <sup>x</sup>	Sialyl Lewis x
SPRI	Solid phase reversible immobilization
TNF	Tumor necrosis factor
UGGT	UDP-glucose: glycoprotein glucosyltransferase
WAX	Weak anion exchange

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### List of publications related to the dissertation

1. **Váradi, C.**, Holló, Z., Pólska, S., Nagy, L., Szekanecz, Z., Váncsa, A., Palatka, K., Guttman, A.:  
Combination of IgG N-glycomics and corresponding transcriptomics data to identify anti-TNF $\alpha$  treatment responders in inflammatory diseases.  
*Electrophoresis. Epub ahead of print (2015)*  
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2. **Váradi, C.**, Lew, C., Guttman, A.: Rapid Magnetic Bead Based Sample Preparation for Automated and High Throughput N-Glycan Analysis of Therapeutic Antibodies.  
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3. **Váradi, C.**, Mittermayr, S., Szekrényes, Á., Kádas, J., Takács, L., Kurucz, I., Guttman, A.: Analysis of Haptoglobin N-glycome Alterations in Inflammatory and Malignant Lung Diseases by Capillary Electrophoresis.  
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**List of other publications**

4. Guttman, M., **Váradi, C.**, Lee, K.K., Guttman, A.: Comparative glycoprofiling of HIV gp120 immunogens by capillary electrophoresis and MALDI mass spectrometry.  
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5. Szekrényes, Á., Partyka, J., **Váradi, C.**, Krenkova, J., Foret, F., Guttman, A.: Sample preparation for N-glycosylation analysis of therapeutic monoclonal antibodies by electrophoresis.  
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