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N-glycosylation structure – function characterization of omalizumab, an anti-asthma biotherapeutic product

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ABSTRACT

Omalizumab, a glycoprotein based biotherapeutics, is one of the most frequently used targeted antibody biopharmaceutical to reduce asthma exacerbations, improve lung function and reduce oral corticosteroid use. The effector function and clearance time of such glycoprotein drugs is affected by their N-glycosylation, that defines the required administration frequency to improve the quality of life in appropriately selected patients. Therefore, the glycosylation of biologics is an important critical quality attribute (CQA). The profile of asparagine linked carbohydrates is greatly dependent on the manufacturing process. Even a small deviation may have a major effect on the structure and therefore the function of the biotherapeutic product. For this reason, comprehensive N-glycosylation analysis is of high importance during production and release. Capillary electrophoresis (CE) is one of the frequently used tools to characterize protein therapeutics and utilized by the biopharmaceutical industry for protein and glycan level analysis, which are key parts both for drug development and quality control. To reveal important structure – function relationships, characterization of omalizumab is presented using capillary SDS gel electrophoresis with UV detection at the protein level and capillary gel electrophoresis with laser induced fluorescent detection at the N-linked carbohydrate level. This latter technique was also used for oligosaccharide sequencing for glycan structure validation. The results suggested no ADCC function – structure relationship due to the mostly core fucosylated biantennary glycans found. However, the presence of the high mannose structures probably affects the clearance rate of the drug.

1. Introduction

Based on their manufacturing processes, pharmaceutical drugs can be divided into the two major categories such as 1) traditional, synthetically produced small molecule drugs and 2) biopharmaceuticals, manufactured by biotechnology based processing. Albeit 95% of the pharmaceutical drugs currently on the market still belong to the traditional, small molecule class, it is apparent that this ratio will change in the future as a significant portion of contemporary pharmaceutical research gears toward biologics [1]. The share of monoclonal antibodies among these new class of drugs was more than 50% between 2015 and 2018. Importantly, biopharmaceuticals not only treat the symptoms but rather try to eliminate the source of the disease, leading to a much better outcome. These new generation therapeutics include plasma preparations, vaccines, hormones, growth factors, monoclonal antibodies as well as cell and gene therapy products. Albeit, biologics are generally more effective than their traditional small molecule drug counterparts, a drawback is that by mistake, the human body can treat them as antigens and start an elimination reaction, which can have adverse effects, sometime even

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; APCs, antigen-presenting cells; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; BST, bracketing standard; CE-LIF, capillary electrophoresis with laser-induced fluorescence; DP2, maltose; DP15, maltopentadecaose; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; FcεRI, high-affinity IgE receptor; FcεRII, low-affinity IgE receptor; GlcNAc, N-Acetylglucosamine; GU, glucose unit; HR-NCHO, high resolution separation gel buffer; IgE, immunoglobulin E; IgG, immunoglobulin G; LC, light chain; HC, heavy chain; LC/HC, light/heavy chain; HC/HC, heavy/heavy chain

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worse than the original condition itself. Currently the mostly produced biologics are protein therapeutics, mainly glycoproteins in the form of antibodies, antibody based fusion proteins and antibody drug conjugates. Quality control of these biologics are essential for the pharmaceutical companies as these drugs have many important properties, which can change their effect on the human body and small deviations may cause serious consequences. This makes it necessary to make products with the exact same quality by properly analyzing critical quality attributes (CQA), like glycosylation and other post translational modifications (PTMs). Also, it is very important to measure solubility, half-life and the ability of the active ingredient to pass through the cell membrane and become part of the metabolism [2].

Asthma is a heterogeneous chronic inflammatory disease characterized by reversible airflow obstruction. The majority of patients are well treated with inhaled steroids and long-acting beta agonists, however, the treatment of some subgroups has long posed a serious challenge to clinicians. Approximately 5% with severe asthma remains inadequately controlled despite adherence to standard controller therapy [3]. Severe uncontrolled asthmatic groups are prone to higher morbidity, mortality, emergency care and hospitalization rates, as well as poorer quality of life and systemic steroid side effects [4,5]. More than half of severe asthmatic patients have T2-type airway inflammation, with a significant portion classified as allergic asthma, in which IgE plays a prominent role in the pathogenesis. IgE is produced by B cells in response to allergen activation of cell-mediated immune response [6] and has a high-affinity receptor (FcεRI), mainly expressed on mast cells and basophils, but also present on dendritic cells and eosinophils. IgE also has a low-affinity receptor (CD23; FcεRII), that is predominantly expressed on B cells, dendritic cells and epithelial cells. The activation of mast cells requires allergen interaction with specific IgE bound to their surface receptors. The cross-link of FcεRI-bound IgE by allergen leads to mast cell activation and degranulation [7]. It has been suggested that omalizumab binds the Cε3 domain of free IgE, preventing it to bind the FcεRI by steric hindrance. It is important to note that it does not bind IgE that already bound by FcεRI or CD23 on the cell surface. By depleting IgE, omalizumab downregulates the expression of FcεRI on mast cells and basophils as well as APCs [8].

Since the effector function and the clearance of omalizumab are probably affected by its conserved Fc N-glycosylation (Asn 297), thorough examination of these carbohydrate structures is of high importance. In this paper, comprehensive N-glycosylation characterization of omalizumab, an anti-asthma biotherapeutic product, is presented using capillary SDS gel electrophoresis with UV detection at the intact and protein subunit levels and capillary gel electrophoresis with laser induced fluorescent detection at the carbohydrate level. This latter includes profiling and sequencing to reveal possible N-glycan structure – function relationships.

2. Materials and methods

2.1. Chemicals

All chemicals used in the study were analytical grade. Dithiothreitol (DDT) (1M aqueous solution), sodium dodecyl sulfate, tetrahydrofurane (THF), 2-mercaptoethanol, sodium cyanoborohydride ($NaBH_3CN$, 1M in THF), iodoacetamide (IAA) and glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and acetic acid (99–100%) was obtained from Molar Chemicals (Halasztelek, Hungary). The glycoanalysis enzymes of Peptide-N-glycosidase F, 5 U/mL neuraminidase, 25 U/mL galactosidase and 40 U/mL hexosaminidase were kindly provided by the Nanobiotechnology laboratory of University of Pannonia (Veszprem, Hungary). For protein analysis the SDS-MW Analysis Kit (Sciex, Brea, CA, USA) was used, including a 30 cm long fused silica separation capillary (50 µm ID, 20 cm effective length), the SDS-MW sample buffer (100 mM TRIS-HCl, 1% SDS, pH 9), the SDS-MW separation gel buffer, a 10 kDa internal standard, an SDS-MW protein ladder (10–225 kDa) as well as the acidic and basic rinsing solutions. For N-glycan analysis the Fast Glycan Labeling and Analysis kit (Sciex) was used including the labeling dye (8-aminopyrene-1,3,6-trisulfonate, APTS), a maltooligosaccharide ladder, the bracketing standard mixture (BST of DP 2 and 15), the magnetic beads for purification and the HR-NCHO separation gel buffer. The omalizumab (Xoliar) therapeutic monoclonal antibody was a kind gift of the Semmelweis Hospital (Miskolc, Hungary).

2.2. Sample preparation

2.2.1. Size heterogeneity and subunit integrity analysis

80 µL SDS-MW sample buffer and 2 µL of 10 kDa internal standard was added to 10 µL of 10 mg/mL omalizumab solution. In case of non-reducing sample preparation (intact level analysis), 5 µL iodoacetamide was added to the sample and incubated at 70 °C for 15 min. For reduced (subunit level) analysis, 5 µL of mercaptoethanol was also added to the sample. The denaturation step included a 0.5 °C/5 s temperature gradient to 90 °C, followed by keeping that temperature for 5 min. The reaction mixture was then cooled down in ice-water before capillary electrophoresis analysis. The accompanying protein ladder sample preparation was identical to the reducing method.

2.2.2. N-glycosylation analysis

The sample was first denatured by adding 2 µL of denaturing solution (Fast Glycan Sample Preparation and Analysis Kit) to 10 µL of 10 mg/mL omalizumab solution. The sample was mixed and subjected to temperature gradient denaturation as described above. Then 20 µL of 16.7 mM ammonium acetate and 1 µL of PNGase F (200 mU) enzyme was added. The reaction mixture was incubated for one hour at 50 °C to release the N-glycans, which were then fluorophore labeled by the addition of 1 µL of 120 mM APTS along with 50 µL of water, 50 µL of glacial acetic acid, 80 µL of tetrahydrofurane and 20 µL of sodium-cyanoborohidride (1M in THF) and followed by overnight incubation at 37 °C with open vial lid [9]. The unreacted dye was removed by the magnetic beads (Fast Glycan Sample Preparation and Analysis Kit) and washing with 87.5% of ACN four times. The purified glycans were eluted from the beads by 100 µL of HPLC grade water and the sample was stored at 4 °C until analysis.

2.3. Capillary electrophoresis

The PA 800 + capillary electrophoresis instrument (Sciex) was used with 220 nm UV detection for all protein level analyses. A 30 cm total length capillary was utilized for the measurements with 20 cm effective length in all cases. Separations were done at 25 °C at 15 kV in reverse polarity mode (cathode at the injection side). Samples were electrokinetically injected for 10 s at 5 kV. Before each run the, the capillary was conditioned by rinsing with 0.1 M NaOH for 3 min, 0.1 M HCl for 3 min, HPLC grade water for 5 min and finally with the SDS-MW gel buffer for 5 min. Every measurement was repeated three times.

For N-glycan profiling, the same CE instrument was used with a laser induced fluorescent detector (488 nm excitation laser beam and 520 nm emission filter). The separations were accomplished in a 50 µm inner diameter, 40 cm effective length (50 cm total length) bare fused silica capillary, filled with HR-NCHO separation gel buffer (Sciex). Measurements were carried out at 30 kV applied potential in reversed polarity mode at 25 °C. Sample injection was done by applying 1 psi pressure for 5 s along with the bracketing standard mixture (DP2 + DP15) to enable the determination of GU (glucose unit) values of the peaks for database mediated structural elucidation.

2.4. N-linked carbohydrate sequencing

Exoglycosidase array-based glycan sequencing reactions were performed at 37 °C overnight. The reagent array consisted of 1.0 µL neuraminidase (0.005 unit) for sialic acid removal, 1.0 µL of neuraminidase + 1.0 µL of galactosidase (0.025 unit) for sialic acid and galactose removal and 1.0 µL of neuraminidase + 1.0 µL of galactosidase + 1.0 µL of hexosaminidase (0.04 unit) for sialic acid, galactose and GlcNAc (N-acetylglucosamine) removal, as described earlier in [10]. All reaction products were subject to CE-LIF analysis to determine the peak shifts caused by the exoglycosidase array treatments.

2.5. Data analysis and interpretation

All acquired data was analyzed by the Karat32 software, version 10.1 (Sciex). Evaluation of the data collected during the separation of N-glycans was done by the Peakfit (Systat Software, Inc., San Jose, CA) program. The GU values of the peaks were calculated with the help of GUcal app (www.GUcal.hu). The bracketing internal standards (DP2 and DP15) were injected with every sample facilitating the determination of the relative migration times of the sample peaks. From these data the program calculated the GU values of the peaks and presented the suggested structures using the GUcal database. For more accurate structure determination, exoglycosidase based carbohydrate sequencing was used as described below. To determine the intact and protein subunit masses the mobility of the protein ladder peaks were plotted as a function of their logarithmic masses.

3. Results and discussion

In this work, an efficient anti-asthma monoclonal antibody drug, omalizumab was analyzed to determine the presence of the non-glycosylated heavy chain at the protein level and the N-glycosylation to understand the possible influence of this latter on the function of the molecule. Capillary gel electrophoresis was used both for protein and carbohydrate level analyses.

3.1. Intact protein and subunit integrity analysis

Sodium dodecyl sulfate capillary gel electrophoresis was used for the integrity analysis of the intact and reduced forms of omalizumab, to check the presence and level of the non-glycosylated subset of the heavy chain of the molecule. Fig. 1 compares the electropherograms of the protein MW standards (upper trace), the reduced form of omalizumab subunits with the light and heavy chains (middle trace) and the intact form of the molecule, each along with the 10 kDa internal standard. No non-glycosylated heavy chain was found. The light chain of the monoclonal antibody migrated approximately in agreement with its literature molecular weight of 24 kDa. While, the actual molecular weight of the heavy chain is 49.5 kDa, it migrated apparently slower than the 50 kDa MW standard. This electromigration discrepancy was probably due to the conserved, bulky and non-SDS binding carbohydrate moiety with decreased surface charge density at the C_H2 domain of the Fc region of the heavy chain. Again, more importantly, this trace apparently revealed no non-glycosylated heavy chain fragment (ngHC), thus, the entire mAb content possessed the glycosylated form (HC). The intact form showed even longer migration time incongruity with higher than 225 kDa apparent molecular weight instead of the actual cc 150 kDa. In addition to the glycosylation of the molecule, the longer migration time of the latter was probably caused by the bulky Y shape of the intact molecule in comparison to the linear MW size standards. Several minor peaks also appeared in the lower intact level trace, probably corresponding to the light (LC), heavy (HC), light/heavy (LC/HC) and heavy/heavy (HC/HC) chain forms, as our group reported earlier [11].

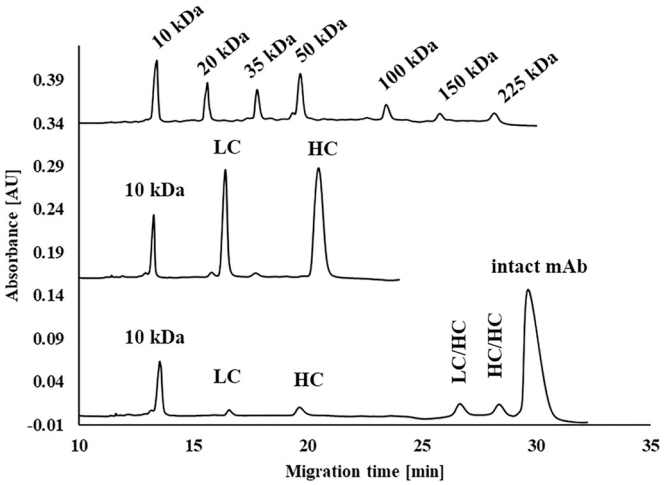

Fig. 1. Sodium dodecyl sulfate capillary gel electrophoresis analysis of the intact (lower trace) and reduced (middle trace) forms of omalizumab in comparison to the MW size standard ladder (upper trace). Peaks: 10–225 kDa MW standards, LC: light chain, HC: heavy chain, LC/HC: light/heavy chains, HC/HC: heavy/heavy chains. Conditions: SDS-MW separation gel-buffer, 50 µm diameter and 20 cm effective length (30 cm total) capillary, Applied voltage: 15 kV, separation temperature: 25 °C, Electrokinetic injection: 5 kV/10 s.

Based on the above observations, the molecular weights of the intact as well as the light and glycosylated heavy chain subunit forms of omalizumab were estimated using the MW calibration plot (Supplemental Fig. 1). The estimated mass values based on the calibration plot equation of y = −2.03E-09x + 1.32E-08 (r^2 = 0.992) were 24.2 kDa, 60.1 kDa and 298 kDa for the LC, HC and intact molecule, respectively. To determine more accurate masses either the time consuming Ferguson method [12], or mass spectrometry should be used. By all means, sodium dodecyl sulfate capillary gel electrophoresis of the intact and subunit forms of omalizumab was especially helpful as a first step towards the analysis of the subunit forms quickly providing information about the purity and integrity (glycosylated vs non-glycosylated HC) of the product. Based on the differences between the expected theoretical and measured MW, our results revealed the follows: the light chain (LC, 24 vs 24.2 kDa) probably did not possess any glycosylation, the extent of glycosylation on the heavy chain (HC, 49.5 vs 60.1 kDa), and the lack of non-glycosylated heavy chain in the product.

3.2. N-glycosylation analysis

The sodium dodecyl sulfate capillary gel electrophoresis analysis of the omalizumab subunits suggested the presence of glycosylation on the heavy chain of the molecule. Identification of these attached carbohydrates started with endoglycosidase (PNGase F) mediated removal of the oligosaccharides from the polypeptide backbone, followed by fluorophore labeling by aminopyrene trisulfonate (APTS), prior to their capillary electrophoresis analysis with laser induced fluorescent detection (CE-LIF). Fig. 2 shows the resulting N-glycan profile, featuring 8 peaks with area percentages higher than 1%, thus, included in the quantitative peak distribution profiling and the subsequent sequencing study.

All separated peaks were assigned with a glucose unit (GU) value after bracketing standard based migration time normalization as described in [13]. The GUcal software and the associated database were used for preliminary structural elucidation of the separated peaks (www.gucal.hu). To validate the software suggested carbohydrate structures in the released N-glycan pool, exoglycosidase array based oligosaccharide sequencing was performed, as shown in Fig. 3. The lower trace (a) shows the electropherogram of the released and APTS labeled untreated N-glycan pool of omalizumab. The fact that

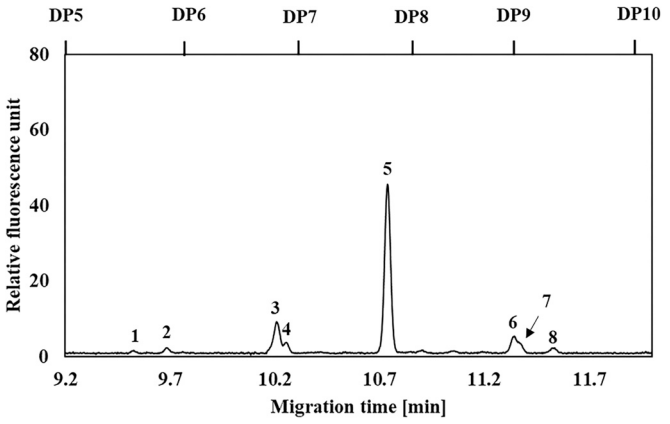

Fig. 2. CE-LIF profiling of omalizumab N-glycans. Carbohydrate structures corresponding to the numbered peaks are listed in Table 1. DP5-DP10 indicate the degree of polymerization (DP) of the APTS-labeled maltooligosaccharide ladder peaks. Conditions: HR-NCHO separation buffer, capillary: 40 cm effective length (30 cm total length, 50 um ID), applied seperation voltage: 30 kV., temperature: 25 °C. Sample injection: 2 kV for 2 s.

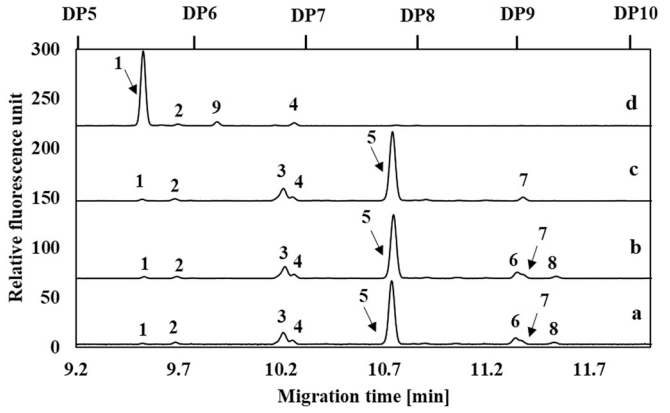

Fig. 3. Exoglycosidase digestion mediated carbohydrate sequencing of the omalizumab N-glycan pool. Traces: CE-LIF analysis of the (a) untreated sample and after (b) neuraminidase digestion, (c) neuraminidase + galactosidase digestion, and (d) neuraminidase + galactosidase + hexosaminidase digestion. DP5-DP10 indicate the degree of polymerization (DP) of the APTS-labeled maltooligosaccharide ladder peaks. Conditions were the same as in Fig. 3.

the neuraminidase treatment did not cause any peak shifts suggested the lack of sialic acid residues in the released N-glycan pool (lower middle trace, b), an important piece of information towards downstream structure – function relationship considerations. Galactosidase digestion affected peaks 6 and 8 (upper middle trace, c), consequently these were different antennary galactosylated structures as depicted in Table 1. Finally, hexosaminidase treatment removed all N-acetylglucosamine residues, causing shifts for peaks 3 and 5 into peak 1 and peak 7 to peak 9 (upper trace, d). Peak 1 was identified as the fucosylated N-linked core structure. Since peak 7 was not affected until the combined neuraminidase / galactosidase / hexosaminidase treatment, but then shifted to peak 9, a larger structure that of the fucosylated core. As a first approximation, based on earlier reports [14] it was considered as a hybrid structure, containing a GlcNAc-Gal-GlcNAc motif at the 3-arm and a mannose residue at the 6-arm (FA1GN(3)Man(6)), as depicted in Table 1. Peaks 2 and 4 were not affected by any of the three exoglycosidase array mixtures applied and were identified by their GU values as high mannose structures (Table 1), which can affect serum half-life.

Anti-IgE therapy in the treatment of severe asthma patients by omalizumab has led to a breakthrough with Th2 endotype and elevated serum IgE levels [15]. This protein therapeutic agent selectively binds to the Cε3 domain of the IgE immunoglobulin, inhibiting its ability to release inflammation mediators by binding to effector cells. Therefore, omalizumab is used to efficiently treat asthma and different allergic diseases [16]. Omalizumab can be administered every two to four weeks adjusted to body weight and baseline IgE level. Serum peek concentrations are reached about 7–8 days after subcutan administration. Clearance include complex formation with IgE and degradation in liver reticuloendothelial system and endothelial cells. The mean clearance in asthmatic patients is 2.4 mL/kg/day, which allows for adequate patient adherence and reduces the burden on the health care system [17]. It is important to note that clearance of antibodies differ from small molecule drugs as they are mainly eliminated via catabolism in the lysosomes following endocytosis. Also, humanized and fully human monoclonal antibodies can generate anti-drug antibodies, leading to formation of inactive immune complexes, triggering lysosomal degradation [18]. However, there are protective mechanism, which slow down the clearance of IgG molecules, and so monoclonal antibodies. The Fc region of mAbs protects them from lysosomal degradation by binding to FcRn (neonatal Fc receptor) in the endosomes of the endothelial cells. Modification of the Fc region of monoclonal antibodies significantly affects the

Table 1
Identified N-glycan structures (abbreviated and actual) of omalizumab with their GU values, peak area percentages and response to consecutive exoglycosidase treatments.

Peak ID	Migration time (min)	GU	% area	Response to exoglycosidase enzymes				Glycan structure
				NEU	GAL	HEX	No change	
1	9.533	5.654 ± 0.001	0.955 ± 0.090				x	**FM3**
2	9.693	5.907 ± 0.001	1.671 ± 0.089				x	**M4**
3	10.219	6.793 ± 0.001	13.146 ± 1.132			x		**FA1(3)**
4	10.261	6.865 ± 0.001	3.335 ± 0.249				x	**M5**
5	10.757	7.741 ± 0.001	68.124 ± 0.067			x		**FA2**
6	11.347	8.795 ± 0.001	5.589 ± 0.211		x			**FA2G(6)1**
7	11.373	8.842 ± 0.002	2.642 ± 0.096			x		**FA1GN(3)Man(6)**
8	11.538	9.136 ± 0.002	1.554 ± 0.146		x			**FA2G(3)1**
9	9.951	6.236 ± 0.002	4.088 ± 0.206					**FM4**

strength of the clinical effect along with the immunogenicity, pharmacokinetics and pharmacodynamics of the drug. In particular, differences in glycosylation patterns at the conserved Fc Asn 297 site play a central role in determining these parameters [18]. ADCC effector function is influenced by the presence or lack of core fucosylation of the N-linked carbohydrates at the C_H2 domain of the Fc region. In other words, core afucosylation results in enhanced ADCC due to stronger FcγR IIIa binding affinity, which also leads to accelerated clearance. In our study, only the high mannose structures were afucosylated, therefore the ADCC function was probably greatly suppressed. Galactose free IgG usually have a longer circulation time, which can be explained with a lower binding affinity to FcγRI. We found only 7% terminally monogalactosylated glycans in the released pool, supporting longer serum half-life. However, the presence of terminal N-acetylglucosamines is associated with faster clearance, so the 68% FA2 and 13% FA1 type glycans must have played a key role. The ~5% of high mannose glycans (Man4 and Man5), probably facilitated the increased clearance rate of the molecule via the mannose receptors [19,20].

4. Conclusions

As of today, the majority of biologicals are monospecific monoclonal antibodies, consisting of two identical heavy chains (HC) and two identical light chains (LC), forming a Y shape molecule with two recognition binding sites in the Fab domain and a conserved glycosylation site at the Fc domain. Omalizumab is a recombinant DNA-derived humanized and glycosylated anti-asthma immunoglobulin G1 (IgG1) monoclonal antibody produced by CHO cells [7] and consists of a human IgG1 Fc fragment and a mouse MAE 11 binding domain. The pharmacokinetics of omalizumab might be affected by its Fc glycosylation, i.e., understanding the N-glycan structure – function relationship is of importance. Examination of its asparagine linked oligosaccharide structures revealed mostly core fucosylated asialo structures, i.e., not supporting ADCC type effector function of the therapeutic protein. While the low amount of terminal galactosylation tend to increase serum half-life, the similar amount of high mannose structures in the N-glycan pool probably countered that effect. The large amount of terminal GlcNAc bearing sugars (~81%) on the other hand probably also supported faster clearance. In conclusion, our study suggested that with careful glycoengineering, the serum half-life and consequent drug administration rate can be optimized for this anti-asthma biotherapeutic product.

CRediT authorship contribution statement

Miklos Szabo: Writing – original draft, Preparation. **Csenge Filep:** Investigation, Formal analysis. **Mate Nagy:** Investigation, Formal analysis. **Daniel Sarkozy:** Investigation, Formal analysis. **Marton Szigeti:** Conceptualization, Methodology. **Edit Sperling:** Conceptualization, Methodology. **Eszter Csanky:** Resources. **Andras Guttman:** Writing – review & editing. All authors contributed to manuscript revision, read, and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114483.

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