Capillary electrophoresis analysis of N-glycosylation changes of serum paraproteins in multiple myeloma

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Keywords: Multiple myeloma, N-glycans, CE-LIF

Received: MONTH DD, YYY; Revised: MONTH DD, YYY; Accepted: MONTH DD, YYY

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201700006.

Abbreviations: MM – Multiple myeloma; IgG – immunoglobulin; Fc - fragment crystallizable region; Fab - fragment antigen-binding; CE – Capillary Electrophoresis; LIF – Laser Induced Fluorescence; APTS – 8-aminopyrene-1,3,6-trisulfonic acid; ISS – International Staging System; SF/NF – sialoform to neutral carbohydrate ratio

Abstract

Multiple myeloma (MM) is an immedicable malignancy of the human plasma cells producing abnormal antibodies (also referred to as paraproteins) leading to kidney problems and hyperviscosity syndrome. In this paper we report on the N-glycosylation analysis of paraproteins from total human serum as well as the Fc and Fab κ/λ light chain fractions of papain digested immunoglobulins from multiple myeloma patients. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF) was used for the analysis of the N-glycans after endoglycosidase (PNGase F) mediated sugar release and fluorophore labeling (APTS). While characteristic N-glycosylation pattern differences were found between normal control and untreated, treated and remission stage multiple myeloma patient samples at the global serum level, less distinctive changes were observed at the immunoglobulin level. Principal component analysis adequately differentiated the four groups (control and three patient groups) on the basis of total serum N-glycosylation analysis. 12 N-glycan features showed statistically significant differences ($p<0.05$) among various stages of the disease in comparison to the control at the serum level, while only 6 features were identified with similar significance at the immunoglobulin level, including the analysis of the partitioned F_c fragment as well as the $F_{ab}\kappa$ and $F_{ab}\lambda$ chains.

1 Introduction

Multiple myeloma (MM) is characterized as the clonal proliferation of malignant plasma cells and practically incurable even in the 21st century. Its diagnosis can be established upon the latest IMWG criteria (Supplementary Table 1)that can be time-consuming because of the deceptive clinical symptoms [1]. The most frequently used processes to diagnose multiple myeloma utilize particular combinations of laboratory, imaging, and bone marrow biopsy test results as criteria [2]. To widen

the diagnostic toolsets and to help finding proper remedies, all modern omics methods have been recently attempted [3]. As a result, several new and unexpected oncogenic mechanisms were discovered influencing the mutations of the genes involved in the translation mechanisms, histone methylation, blood coagulation and paraprotein formation [4]. These latter ones are abnormal immunoglobulin fragments or immunoglobulin light chains (both kappa and lambda) and their appearance in the serum indicates the presence of a proliferating clone of immunoglobulin-producing cells [5][6]. In the serum of healthy individuals, the albumin to immunoglobulin ratio is >2 and the total kappa (κ) to lambda (λ) chain ratio is approximately 3:1 [7]. Any changes of this ratio can be an indication of blood cell dyscrasia or multiple myeloma [8]. Indeed, in MM the albumin to immunoglobulin ratio can be <1 as a result of the extra paraproteins in the bloodstream. More importantly in respect to this study, paraprotein glycosylation can be indicative of the progress of the disease [9].

N-linked (asparagine-linked) glycosylation is involved in a wide variety of processes in living organisms representing a very important covalent co/post translational protein modification in eukaryotic cells [10]. Changes in N-glycosylation may have a significant role in a variety of human malignant diseases including multiple myeloma [11, 12], where paraproteins reportedly exhibit specific glycosylation profiles [13]. Under normal physiological conditions, N-glycosylation of IgG 1 molecules is mostly located at the highly conserved Asn^{297} site of the C_H2 domain in the F_c region. In addition to this conserved N-glycosylation site in the Fc fragment, about 20-30% of circulating human IgG can be glycosylated in the F_{ab} portion as well [14, 15]. The total glycosylation of IgG 1 type immunoglobulins accounts for only 2-3% of its mass [16] with complex biantennary type structures having a trimannosyl chitobiose core ($Man_3GlcNAc_2$) and variable outer arms with sugar residues (e.g., N-acetylglucosamine, galactose, fucose and sialic acid) [17].

Malignant transformation may alter protein glycosylation causing increased expression of pro-inflammatory glycoforms [18]. Somatic mutation of follicular lymphoma cells in the bone marrow has been shown to result in the appearance a large number of N-glycosylation sequins on the heavy and light chains of the expressed IgG molecules suggesting their involvement in tumor pathogenesis

[19]. Such changes of IgG N-glycosylation could result in compromised regulatory functions [20]. Other studies have revealed the presence of variously sialylated bi-antennary glycans on the IgG light chain in the serum of multiple myeloma patients [15]. Although their function still is unknown, increased glycosylation in the variable region of IgG might be associated with plasma cell cancers. Glavery et al., confirmed that reduced homing and engraftment of ST3GAL6 sialyltransferase knockdown in MM cells in the bone marrow decreased tumor propagation [21]. Decreased core fucosylation, appearance of bisecting forms and increased sialylation of serum glycoproteins were observed in patients with light-chain multiple myeloma (LCMM) [22]. Also in MM, recently low galactosylation and fucosylation was found [23]. Multiple myeloma patients are classified based on the International Myeloma Working Group (IMWG) criteria (Supplementary Table 1). Furthermore, testing serum beta 2-microglobulin (ß2-M) and albumin are simple but useful methods to determine the stage and indeed, the prognosis of the disease- (Supplementary Table 2)[24] [1]. Albeit ISS was only developed in 2005, it has already been proven more efficient in discriminating among the three stages of myeloma. These stages indicate different levels of projected survival rates and point to more increasingly aggressive myeloma treatment strategies [25].

Analysis of the N-glycosylation profiles of paraproteins may have prognostic value for multiple myeloma patient treatment. The most frequently used N-glycan analysis methods are liquid chromatography (LC), capillary electrophoresis (CE), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) [13, 26-30]. While all of these methods can be utilized and have their own advantages, in this paper we used capillary electrophoresis with laser-induced fluorescent detection (CE-LIF) for rapid and high resolution N-glycan profiling of the serum samples and their partitioned immunoglobulin F_c as well as the F_{ab} κ and λ light chain subsets for comparative analysis of healthy controls with freshly diagnosed, treated and remission stage multiple myeloma patients.

2 Materials and Methods

2.1 Chemicals and Reagents: Sodium hydrogen carbonate, ethylenediaminetetraacetic acid (EDTA), cysteine, iodoacetamide and sodium cyanoborohydride (1 M in THF) were from Sigma-Aldrich (St.

Louis, MO). PNGase F was from ProZyme (Hayward, CA) and papain from AppliChem (Darmstadt, Germany). The 8-aminopyrene-1,3,6-trisulfonate (APTS), the maltooligosaccharide ladder and the NCHO Carbohydrate Labeling and Analysis kit were from SCIEX (Brea, CA).

2.2 Serum samples: Serum samples were collected at the Clinical Centre of Internal Medicine (University of Debrecen, Hungary) and the 2nd Department of Internal Medicine (Semmelweis University, Budapest, Hungary) with all necessary Ethical Committee approvals (HBR/052/00437-2/2015) from 6 untreated, 6 treated and 6 remission multiple myeloma patients as well as 6 male and 6 female healthy controls using clot activator containing serum tubes (BD, Franklin Lakes, NJ). MM patients who had acute infectious diseases, acute or chronic inflammatory diseases, other malignant cancer history besides MM, and drug abuse were all excluded from the study. The collected blood samples were centrifuged at 7500 x g for 30 min and the serum fractions were stored at -70°C until further processing.

2.3 Papain digestion 1 ml papain solution (10 mg/ml papain in 1xPBS Sigma-Aldrich, St. Louis, MO) was mixed with 4 ml of 0.2 M EDTA and 0.2 M cysteine containing 1 x PBS buffer (pH 6.5). 100 µL of this reagent was added to 100 µL of serum sample and incubated at 37°C for 4 h. The reaction was stopped by the addition of 20 µL iodoacetamide solution (0.3 M in 1 x PBS).

2.3.1 Partitioning of the Fc fragment: 220 µL of the papain digest was used for all sample types (controls, untreated, treated and remission stage multiple myeloma patients) after purification on 20 µL bed-volume Protein A affinity microcolumns (PhyNexus, San Jose, CA). 200 µL of 1 x capture buffer (0.7 M NaH_2PO_4, 0.7 M NaCl, pH 7.4) was added to the serum samples and then the resin was washed with 200 µL of 0.7 M NaH_2PO_4 0.7 M NaCl buffer (pH 7.4). Then 200 µL of 140 mM NaCl was added to the microcolumns and the Fc fragments were eluted with 200 µL of 15% acetic acid. The samples were transferred to 10 kDa spin-filters and centrifuged at 11270 x g for 10 minutes. Then 100 µL of HPLC grade water was added and again centrifuged at 11270 x g for 10 minutes to remove any remaining acetic acid. The N-glycan moiety of the partitioned Fc fractions was PNGase F digested *in situ* on the filter and the liberated N-glycans were APTS labeled as described below.

2.3.2 κ/λ light chain partitioning: 220 µL of the digestion mixture (as described above) was used for all samples (controls, untreated, treated and remission stage multiple myeloma patients) and purified on CaptureSelect LC-kappa (Hu) and CaptureSelect LC-lambda (Hu) filled affinity

microcolumns (20 µL bedvolume, PhyNexus). 200 µL of capture buffer (1 x PBS) was added to the serum samples and then the resin was washed with 200 µL of 1 x wash buffer I (0.7 M NaH_2PO_4 0.7 M NaCl pH 7.4). Then 200 µL of 1 x wash buffer II (140 mM NaCl) was added to the microcolumns and the IgG kappa/lambda light chains were eluted with 200 µL of 0.1 M glycine-HCl (pH 2.0). The samples were then transferred to 10 kDa centrifugal filters, centrifuged at 11270 x g for 10 minutes followed by the addition of 100 µL of HPLC grade water and centrifugation again 11270 x g for 10 minutes. The prepared light chain fractions were PNGase F treated *in situ* on the filter and APTS labeled as described below.

2.4 N-Glycan release and Fluorophore labeling: 1 µl of serum from each patient (healthy controls as well as untreated, treated and remission stage multiple myeloma patients) was diluted by 10 µL of HPLC grade water (Millipore, Darmstadt, Germany) then 1 µL of denaturing buffer (400 mM DTT, 5% SDS) was added followed by incubation at 65˚C for 10 minutes. The reaction mixture was filtered through 10 kDa spin-filters (VWR, Radnor, PA) at 11270 x g for 10 minutes and washed by 100 µL of HPLC grade water. Both the denatured serum samples as well as the Fc and κ/λ light chain fractions were digested *in situ* on the filters by the addition of 29 µL of $NaHCO_3$ buffer (20 mM, pH 7.0) and 1 µL of PNGase F (200 mU, 2.5 U/ml, Prozyme), followed by incubation at 37˚C overnight. The released N-glycans were centrifuged though 10 kDa spinfilters at 7500 x g for 10 minutes and dried in SpeedVac (Thermo Scientific, Schaumburg, IL) prior to the fluorophore labeling step.

6 µL of 20 mM 8-aminopyrene-1,3,6-trisulfonic acid in 15% acetic acid and 2 µL of $NaCNBH_3$ (1 M in THF) were added to the dry pellet and incubated at 37˚C overnight. The labeled samples were purified with CleanSeq magnetic beads (Beckman Coulter, Indianapolis, IN) following the method reported in [31] and immediately used for CE-LIF analysis or stored at -20˚C for later analysis.

2.5 Capillary electrophoresis: A P/ACE MDQ System (SCIEX) was used to perform all capillary electrophoresis analyses. The separations were monitored by laser induced fluorescence (LIF) detection using a 488 nm Ar-ion laser with a 520 nm emission filter. 50 cm effective length (60 cm total) 50 µm i.d. NCHO capillaries were employed with the NCHO separation gel buffer system (both from SCIEX) for the analysis. The separations were accomplished in reversed polarity mode by applying 30 kV electric field strength. The samples were injected by 1 psi for 5 seconds. The 32 Karat software (SCIEX) was used for data acquisition and processing.

2.6 Statistical analysis: Principal component analysis (PCA) and one-way analysis of variance (ANOVA) were performed with SPSS 22 (IBM Corp.) using PeakAreas% as input derived from 32 Karat software (SCIEX). Tukey *post hoc* test was used to compare peak intensities between experimental groups. Differences between means at $P<0.05$ were considered as significant.

3 Results and discussion

The multiple myeloma patients participated in the study (all in ISS stage 2) were assigned into the three major groups of 1) untreated - every newly diagnosed MM patients who had not yet received any treatment; 2) treated - who were undergoing treatment at the time of sampling; and 3) remission stage - asymptomatic period patients. The control group was selected from healthy middle-aged individuals with appropriate gender balance.

3.1 Comparison of the serum N-glycome profile between the healthy and MM groups

First we analyzed the global serum N-glycan profile of the healthy control samples. A representative electropherogram is shown in Figure 1, Trace A, featuring 14 peaks of interest (peak area ≥1%) including multiisialo (1-4), monosialo (5-8) and neutral glycans (9-14). Structural elucidation of these carbohydrates were based on their calculated GU values following the triple internal standard approach of Jarvas et al. [32] in conjunction with searching the NIBRT database (https://glycobase.nibrt.ie) and also on previously published literature data [33]. In cases of any ambiguities, exoglycosidase based carbohydrate sequencing was applied (data not shown). The suggested structures are listed in Table 1 along with their % distribution in the serum samples of the healthy control, untreated, treated and remission stage patients.

After specifying the main peaks of interest in the healthy sample, the global serum N-glycan profiles of the three patient groups of untreated freshly diagnosed (trace B), treated (trace C) and remission stage (trace D) patients were analyzed and a representative set of results are compared in the upper traces in Figure 1. As one can observe, the traces revealed major differences in peak distribution.

Trace B (freshly diagnosed untreated patient group) show significant changes in the sialoform (peaks 1-8) to neutral (peaks 9-14) carbohydrate ratio, referred to as SF/NF. The SF/NF value in the control total serum glycome level was 2.97 (74.78%/25.22%), while in the untreated patient sample it was only 0.39 (28.16%/71.84%), thus practically the opposite as of in the control serum, demonstrating a striking change.

3.3 N-glycosylation profiling of the F_c and F_{ab} κ/λ light chain fractions

Next we narrowed our focus to study the N-glycosylation of the F_c and F_{ab} κ/λ light chain fractions that were partitioned by Protein A and κ/λ light chain specific microcolumn affinity pulldowns after papain digestion. The panels in Figure 2 compare the CE-LIF analysis of the N-glycans released from the immunoglobulin F_c fractions (panel A) and form the F_{ab} κ/λ light chains (panel B) for the four sample types as discussed above.

It is well known that in the serum N-glycome, most neutral species are originated from the approximately 25% immunoglobulin fraction. Please note that the total amount of immunoglobulins in ISS stage 2 patients was in the range of 5-7 g/dL compared to the normal range of 0.7-1.6 g/dL with the associated albumin level in both around 3-5 g/dL [25, 34]. Interestingly, as panel A in Figure 2 shows, the changes in the N-linked glycan profile of the immunoglobulin F_c fractions were not as substantial as was observed in the global serum N-glycan profiles (Figure 1), except the peak ratio changes in comparison to the sialoforms (peaks 1-8) in which case all three patient sample traces showed significantly increased neutral glycan species (peaks 9-14). The SF/NF ratios were as follows: control (trace a) IgG SF_{Fc}/NF_{Fc}= 0.22 (18.26%/81.74%), while in the untreated patient samples (trace b) SF_{Fc}/NF_{Fc}= 0.08 (7.49%/92.51%); treated patients (trace c) SF_{Fc}/NF_{Fc}= 0.11 (10.19%/89.81%) and in the remission stage (trace d) SF_{Fc}/NF_{Fc}= 0.14 (12.55%/87.45). Figure 2B compares the released and APTS labeled N-linked glycan profiles from the F_{ab} kappa (κ) and lambda (λ) light chains. Here no sialoforms were detected in the control group sample (trace a), i.e., the NF_{Fab}= 100%. The freshly diagnosed untreated patient group sample showed a few sialylated glycans (peaks 6, 15 and 16) in trace b: SF_{Fab}/NF_{Fab}= 6.96 (87.44%/12.56%). The N-glycan profile of the treated and remission stage

groups showed no and one sialylated structure on the lambda and kappa fragments as depicted in trace c and d, NF_{Fab}= 100% and SF_{Fab}= 100%, respectively.

3.5 Statistical analysis

During the statistical analysis, first the total serum N-glycosylation data was evaluated. In this case the first two principal component axes accounted for 47.3% and 17.2% data variance respectively, representing 64.5% of data variance cumulatively, which was sufficient to resolve the data into four distinct groups as shown in Figure 3A (control, untreated, treated and remission). In case of the papain digested IgG fragments (Figure 3B), the first two principal component axes accounted for 45.6% and 16.1% data variance respectively, representing 61.7% of data variance cumulatively, which was sufficient only to distinguish the control group from the MM patients, but not within the latter ones. The box-plots in Figure 4A and 4B delineate all structures, which showed changes of significance in the total serum and partitioned IgG fragment level among the four study groups, respectively. One-way analysis of variance (ANOVA) revealed statistically significant differences between the studied groups in mean PeakArea% as summarized in Supplementary Table 3 and 4 in case of IgG and total serum, respectively.

4 Conclusions

Structural characterization of the N-glycan profiles of healthy control and multiple myeloma patients of freshly diagnosed untreated, treated and remission stage has been reported. The study included the N-glycosylation analysis of human serum plasma proteins at the global level as well as the F_c and F_{ab} κ/λ chain fractions of papain digested paraproteins. Principal component analysis clearly differentiated the four patient groups on the basis of total serum N-glycosylation analysis. The N-glycan patterns of the papain digested immunoglobulin fragments on the other hand, did not show such distinctive differences, and only discriminated between the healthy control and the patients. The changes in the sialoform to neutral glycan ratio (SF/NF) were also very substantial at the serum

N-glycome level. One-way analysis of variance (ANOVA) analysis revealed statistically significant changes ($p<0.05$) in 12 features at the total plasma N-glycan level, while only 6 features at the immunoglobulin level. Based on our results, we suggest that in addition to the higher information content of the total serum level N-glycosylation pattern analysis for multiple myeloma patients, it is also much easier to accomplish, i.e., does not require additional digestion and partitioning steps, thus easier to implement in clinical settings.

Acknowledgment

The authors gratefully acknowledge the support of the Momentum (Lendulet) grant # 97101 of the Hungarian Academy of Sciences (MTA-PE Translational Glycomics) and the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government. This work was also supported by the NTP-NFTÖ-16 project by the Human Capacities Grant Management Office and the Hungarian Ministry of Human Capacities and the BIONANO_GINOP-2.3.2-15-2016-00017 project.

FIGURES AND TABLES

Figure 1. Comparison of the capillary electrophoresis (CE-LIF) traces of APTS labeled serum N-glycomes of healthy control (A), freshly diagnosed untreated (B), treated (C) and remission stage (D) multiple myeloma patients. Conditions: NCHO capillary with 50 cm effective length (total length 60 cm, 50 µm ID); NCHO gel buffer; Separation temperature: 25˚C; Voltage: 30.0 kV (0.17 min ramp) reversed polarity; Injection:1.0 psi, 0.5 sec.

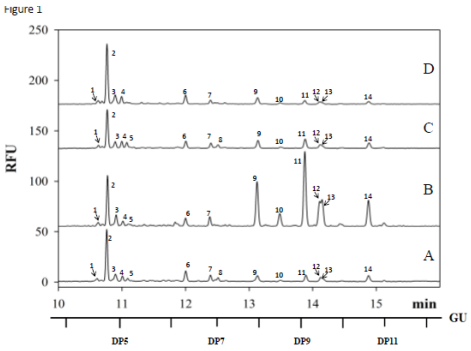


Figure 2. N-linked carbohydrate profiles of the IgG F_c (panel A) and F_{ab} κ/λ light chain (panel B) fractions from healthy control (A), freshly diagnosed untreated (B), treated (C) and remission stage (D) multiple myeloma patients. Conditions were the same as in Figure 1.

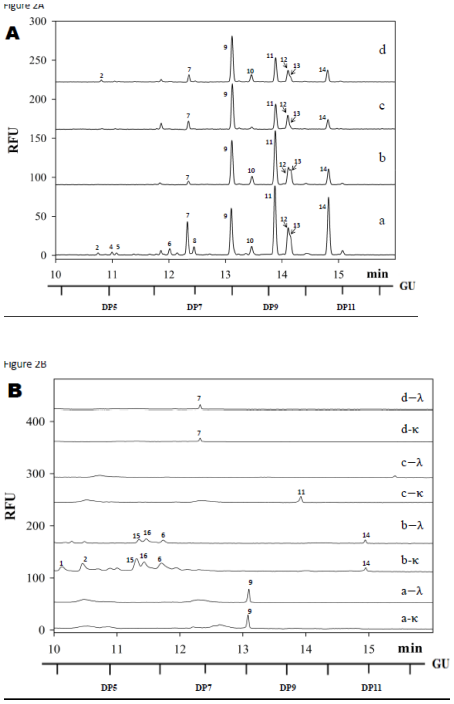
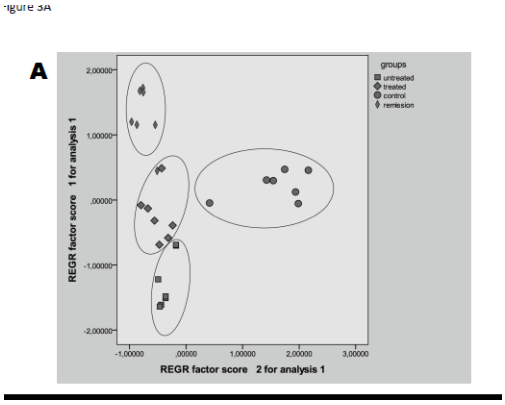


Figure 3. Principal component analysis of the N-glycosylation data obtained from the analysis for the total serum (A) and papain partitioned IgG chains (B).



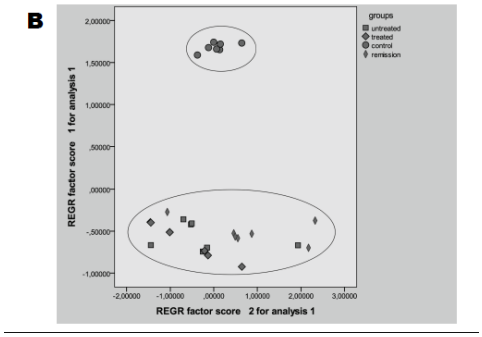


Figure 4. Box-plots showing the trends of significance of the glycoforms in total serum (A) and papain partitioned IgG chains (B) of the four study groups.

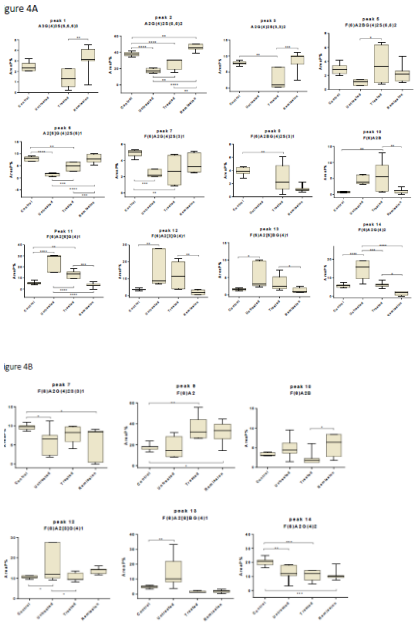
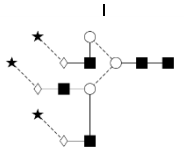
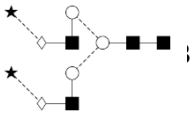
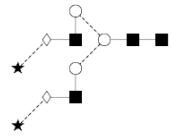
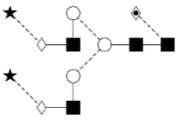
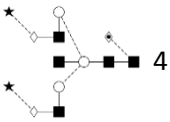
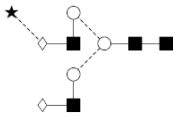
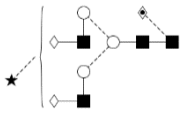
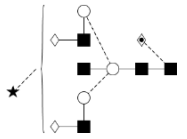
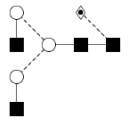
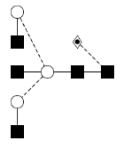
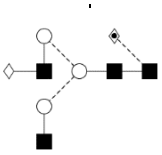
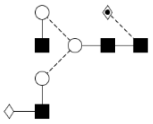
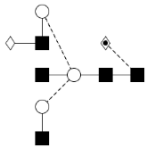
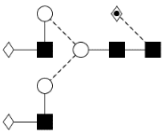
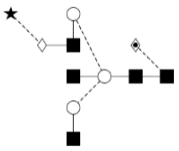
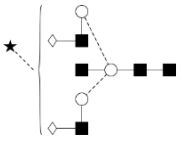


Table 1. Structural interpretation of the N-glycans analyzed in the study. Numbers are corresponding to the peak numbers in Figures 1 and 2.

Peak Number	Total Serum Glycans	Sialylation level	GU_{CE}-unit	Glycan Structures	Control Area %	Untreated Area %	Treated Area %	Remission Area %

1	A3G(4)3S(6,6,6)3	trisialo glycan	4.50		1	2.78	3.84
2	A2G(4)2S(6,6)2	disialo glycan	4.57		20.72	16.38	45.16
3	A2G(4)2S(3,3)2	disialo glycan	4.76		2.3	1.79	10.36
4	F(6)A2[6]G(4)2S(6,6)2	disialo glycan	5.83		1.04	1.38	7.17
5	F(6)A2BG(4)2S(6,6)2	disialo glycan	5.89		-	0.81	1.39
6	A2[6]G(4)2S(6)1	monosialo glycan	6.65		1.4	5.14	8.61
7	F(6)A2G(4)2S(3)1	monosialo glycan	7.07		1.7	5.85	5.93
8	F(6)A2BG(4)2S(3)1	monosialo glycan	7.12		-	1.96	-
9	F(6)A2	neutral glycans	7.67		7.08	13.93	1.01
10	F(6)A2B	neutral glycans	8.13		6.11	-	-

11	F(6)A2[6]G(4)1	neutral glycans	8.67		29.65	9.93	7.59
12	F(6)A2[3]G(4)1	neutral glycans	9.00		6.79	14.22	1.93
13	F(6)A2[6]BG(4)1	neutral glycans	9.09		3.08	22.68	3.93
14	F(6)A2G(4)2	neutral glycans	10.17		19.13	3.16	3.08
15	F(6)A2[6]BG(4)1S(6)1	monosialo glycan	6.01		21.78	-	-
16	A2BG(4)2S(6)1	monosialo glycan	6.41		17.05	-	-

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