Glycosylation analysis of immune-related molecules

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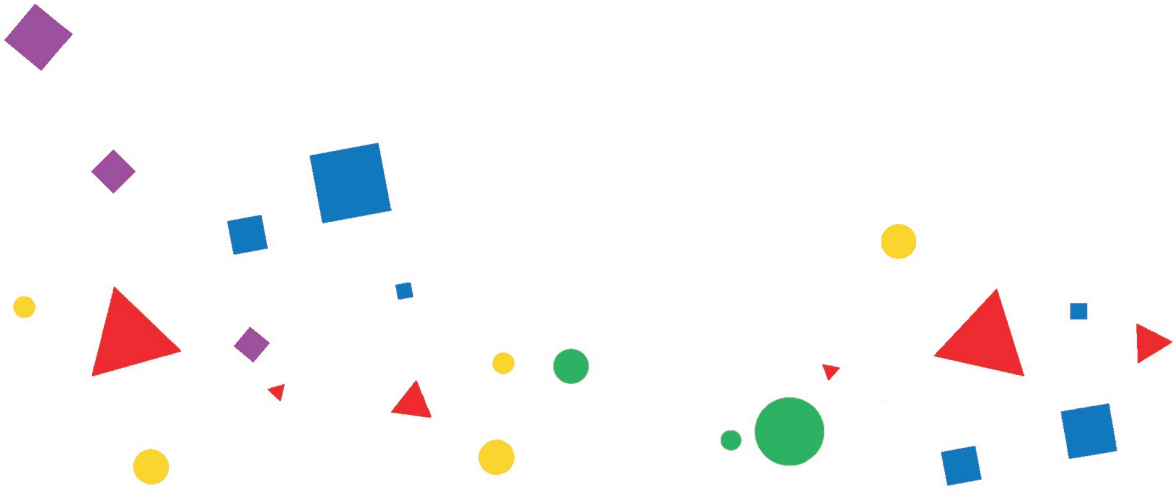

Chapter 5

High-throughput N-glycan profiling of plasma and serum using a routine and robust LC-MS analysis platform

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5.1 ABSTRACT

Alterations in the composition of plasma and serum N-glycome have been linked to many diseases, such as metabolic, autoimmune and infectious diseases. As a result, the discovery and detailed characterization of clinically relevant N-glycome signatures is a primary interest of biomedical research. While the complexity of the plasma and serum samples makes comprehensive analysis challenging, the recent advances in released N-glycan analysis have greatly simplified sample preparation and increased analytical sensitivity, specificity and throughput. Here we describe the development and optimization of a 96-well plate format method to chromatographically separate and profile plasma and serum N-glycans labeled with *Rapi*Fluor-MS by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-LC-MS) using a routine LC-MS analysis platform (BioAccord LC-MS system, Waters Corporation). The improved method for plasma and serum N-glycan profiling takes advantage of rapid protein denaturation, deglycosylation, and fluorescent derivatization with *Rapi*Fluor-MS label and offers a high sensitivity and reproducibility of the N-glycan profile. The HILIC-UPLC-MS profile of total N-glycome comprised 44 individual glycan peaks with 72 assigned N-glycan compositions. We also demonstrated its capability to reveal the biological variability of the serum N-glycome in a robust and high-throughput manner. This improved methodology provides a powerful tool in support of the rapid advancement of glycan analysis for biopharmaceutical development and biomarker discovery.

5.2 INTRODUCTION

Protein glycosylation is an enzymatic process catalyzing the addition of monosaccharides to create a variety of glycan structures on protein molecules. As an integral part of proteins, glycans modulate their physical and biological properties, including folding, solubility, stability, binding affinity and specificity, and biological activity.(1) In addition, glycosylation is recognized to play an essential role in almost every aspect of biological processes, such as cell migration, cell differentiation, inflammatory response and immune regulation.(2) In clinics, growing attention has been directed to an easily obtainable biofluid like plasma or serum (the fluid left after the plasma has clotted) since changes in plasma and serum N-glycome have been linked to many diseases, such as metabolic, autoimmune and infectious diseases, and cancer.(3, 4) All of this opens up the possibility to use plasma and serum N-glycans as potential biomarkers of major diseases. Thus, there is a great deal of interest in the development of a simple, robust and high-throughput methodology for the rapid analysis of total plasma/serum protein glycosylation.

The analysis of protein N-glycosylation can be performed at various levels of characterization, ranging from the analysis of an intact purified glycoprotein to the analysis of the total released glycome of complex biological matrices. Released N-glycan profiling has become the most prevalent strategy for high-throughput analysis of total plasma and serum N-glycome.(5) This strategy includes an enzymatic release of N-glycans, labeling of released N-glycans with a fluorescent dye, purification, and finally profiling of fluorescently labeled N-glycans using hydrophilic interaction chromatography (HILIC).(6) Chromatographic separation can be complemented with the orthogonal method, such as mass spectrometry (MS) analysis which provides rapid and confident identification of glycan structures based on accurate mass measurements.(5) Recent advances in sample preparation have driven the progression in the released N-glycan analysis. For example, the effort needed for released glycan sample preparation has been reduced by the recent introduction of the surfactant-assisted deglycosylation and the novel *Rapi*Fluor-MS (RF-MS) labeling reagent incorporated in the available GlycoWorks RF-MS N-glycan Kit (Waters Corporation).(7) With this, the standard sample preparation procedure has been simplified, sample preparation time reduced from two days to 1.5 h for 96 samples, and both fluorescence and mass spectrometric sensitivity enhanced, facilitating glycan characterization and peak identification.(8, 9) However, while the applicability of the GlycoWorks RF-MS N-glycan Kit has only been established in small and large-scale studies of isolated glycoproteins, such as Immunoglobulin G,(8, 10) it has not been validated for its applicability in the glycosylation analysis of complex sample types, such as plasma or serum.

Thus, we developed and optimized a method to chromatographically separate and profile plasma and serum N-glycans labeled with *RF*-MS by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-LC-MS) on a BioAccord LC-MS System. Using

this method, serum N-glycans from 483 individual/biological samples were profiled with a very good chromatographic resolution and repeatability enabling a robust and high-throughput detection, identification and quantification of the plasma and serum N-glycome.

5.3 EXPERIMENTAL SECTION

5.3.1 Sample description

The pooled plasma standard was used for the method development and all optimization experiments. For the method validation, 483 serum samples were obtained longitudinally from 89 patients with asymptomatic and mild COVID-19 from the Injury, Inflammation and Recovery Unit, School of Medicine, University of Nottingham, Nottingham (UK). The serum samples were collected within two months, with a one-week interval. All of the participants provided written informed consent to participate in the study. The baseline characteristics of the study participants are summarized in **Table S1**.

5.3.2 Enzymatic release and labeling of plasma and serum N-glycans

The N-glycans from total plasma or serum proteins were released, labeled, and purified with the HILIC solid phase extraction (HILIC-SPE) in a 96-well format using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit obtained from Waters Corporation (Milford, MA, USA). All steps were done in accordance with the Waters Corporation protocol ("GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use Manual"; p/n 715004793). The only modification was that all plasma and serum samples (10 µL) were aliquoted to 1 mL 96-well collection plates and diluted 70x with ultrapure water (690 µL) prior to the deglycosylation step. The dilution was made to ensure the sample's recommended protein concentration of 15 µg. Next, 10 µL of the diluted sample was loaded into a 96-well PCR plate (Eppendorf, Hamburg, Germany). GlycoWorks kit reagents including *Rapi*Gest surfactant, Rapid PNGase F enzyme, and *Rapi*Fluor-MS labeling agent were subsequently added to each sample for protein denaturation, deglycosylation, and labeling, respectively. After the glycan labeling, the HILIC-SPE step was performed using GlycoWorks µElution Plate. *Rapi*Fluor-MS labeled glycans were eluted with 90 µL of the GlycoWorks SPE Elution Buffer (200 mM ammonium acetate in 5% acetonitrile). After elution, 310 µL of the GlycoWorks Sample Diluent-DMF/ACN was added to each sample and frozen at -80 °C until LC-MS run.

5.3.3 Liquid chromatography method optimization

RF-MS labeled plasma and serum N-glycans were analyzed via HILIC-LC-MS with a BioAccord LC-MS System consisting of an ACQUITY UPLC I-Class PLUS and an ACQUITY RDa time-of-flight (TOF) mass spectrometer (Waters Corporation). The separation was carried out on an ACQUITY UPLC Glycan BEH Premier Amide chromatography column (130Å, 1.7 µm, 2.1 x 150 mm; Waters Corporation). To improve the chromatographic separation, parameters

including mobile phase ionic strength, column temperature, gradient, and flow rate were optimized through the study. The following concentrations of aqueous mobile phase A (ammonium formate in ultrapure water, pH 4.4) were evaluated: 50 mM, 100 mM and 200 mM. Mobile phase B was 100% acetonitrile (ACN, Honeywell, USA), and a number of linear gradient ranges of solvents in 35 min analytical run were explored: (I) 75-54%, (II) 73-54 %, (III) 72-54%, (IV) 71-54%, and (V) 70-54% of solvent B. Several analytical column temperatures were tested: 60, 45, and 30 °C. Two different analytical flows were used: 0.4 and 0.5 mL/min. The detailed gradient conditions of both the Universal N-Glycan Profiling Method and the optimized Plasma/Serum N-Glycome Profiling Method are listed in the **Supporting Information**.

5.3.4 Mass spectrometric analysis

The released and separated N-glycans were analyzed on-line via electrospray ionization in a positive mode using an ACQUITY RDa (TOF) mass spectrometer. The settings were as follows: range, 50–2000 *m*/*z*; capillary voltage, 1.5 kV; cone voltage, 45 V; desolvation temperature, 300 °C; and sampling rate, 2 Hz. Mass spectra were acquired under the "Full MS scan" mode. *Rapi*Fluor-MS Dextran Calibration Ladder (Waters Corporation) was injected into LC-MS to calibrate the retention time of glycan peaks. The retention times were normalized using the dextran calibration curve to Glucose Units (GU).

5.3.5 Linearity

Assuming that the protein content of the plasma/serum is 70 µg/µL,(Anderson et al., 2004; Leeman et al., 2018) dilution series of the plasma donor sample were prepared in triplicates resulting in theoretical protein amounts ranging from 0.6 to 15.3 µg (**Table S3**). To determine linearity, a curve was obtained by plotting the fluorescent signal as a function of the theoretical protein amount (**Figure S1 – S5**). The results were investigated for five different types of N-glycans by linear regression and the linear regression coefficient was determined to assess the linearity of the system.

5.3.6 Validation

The utility of the optimized Plasma/Serum N-Glycome Profiling Method was evaluated by determining the total serum protein N-glycosylation in a cohort of patients with asymptomatic and mild COVID-19. In addition to 483 biological samples, 36 in-house plasma standards and 6 blanks (ultrapure water) were included in the study to serve as positive and negative controls. All samples were randomized and distributed over six 96-well plates. Subsequently, samples were subjected to N-glycan release and RF-MS labeling followed by HILIC-LC-MS analysis utilizing the optimized LC method for human plasma/serum N-glycans.

5.3.7 Data analysis

Chromatographic and MS data were processed using UNIFI Scientific Information System (Waters Corporation). The chromatograms were integrated in a semi-automated way into 44 peaks (**Figure 3**), and the relative quantification of glycans in each peak was obtained by area-under-curve measurements and expressed as a percentage of the total integrated area (% Area). Initially, the N-glycan structural assignment was done automatically by the waters_connect software (UNIFI application) based on the obtained glucose units (GU) matched to the reference values in the relevant waters_connect library. Additionally, all N-glycan identifications were further manually verified and extended based on the recorded *m/z* values in MS1 sum spectra generated for each chromatographic peak. The manually detected *m/z* values were converted to singly charged ions and characterized with the aid of the glycobioinformatics tools GlycoMod (https://web.expasy.org/glycomod/) and according to previously published and annotated plasma and serum N-glycome profiles.(11, 12) Based on the 44 directly measured N-glycan-containing peaks, 16 derived glycosylation traits reflecting the main structural characteristics and common enzymatic activities were calculated (**Table S2**). The repeatability of the method was assessed by measuring the coefficient of variation (CV) of the pooled plasma standard technical replicates. Data were analyzed and visualized using R Statistical Software (version 4.2.1, R Core Team 2021).

5.4 RESULTS and DISCUSSION

5.4.1 Optimization of the HILIC-LC-MS separation method for plasma and serum N-glycans

To develop a reproducible and robust HILIC-LC-MS separation method specifically tailored for N-glycans released from total proteins of human plasma and serum, we used the Universal N-glycan profiling method from Waters as a starting point.(13) The universal method was established for the separation of all types of N-glycans on an ACQUITY UPLC Glycan BEH Amide columns. However, it is not optimized for any particular N-glycan sample and optimization of chromatographic conditions might be necessary. Thus, the initial fluorescence (FLR) chromatograms of *Rapi*Fluor-MS labeled plasma N-glycans, which were generated with 60 °C column temperature, 50 mM ammonium formate as a mobile phase A, and a gradient from 75% to 54% of LC-MS grade ACN (mobile phase B), showed substantial peak broadening, considerable tailing, and inadequate peak resolution (**Figure 1**).

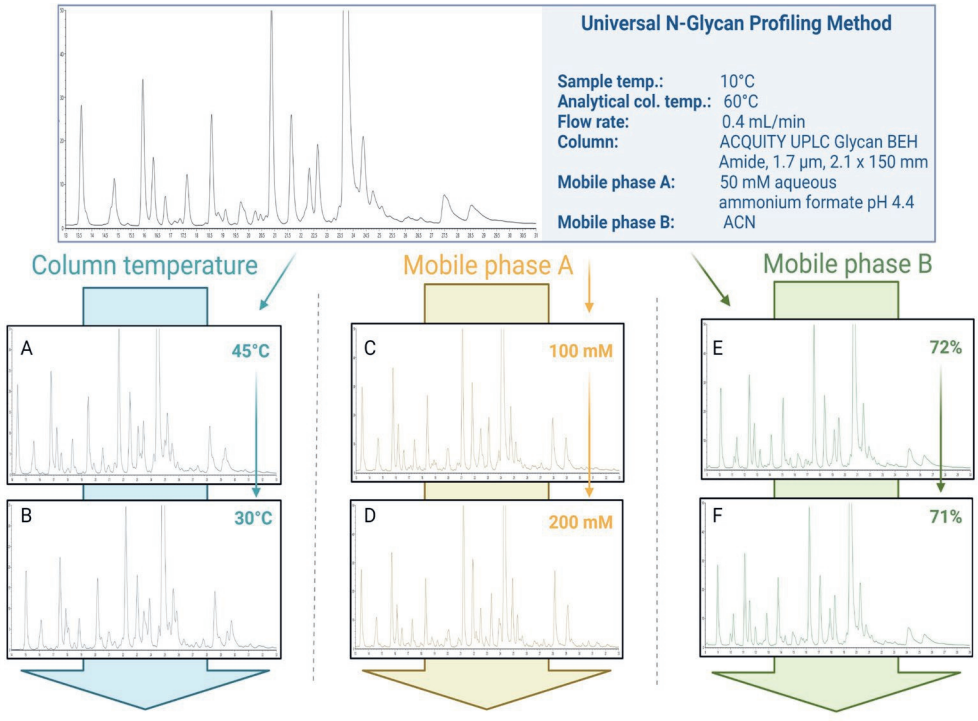

Figure 1. *Optimization of HILIC-UPLC-FLR conditions to improve peak separation of plasma N-glycans labeled with RF-MS. Initial FLR chromatograms (first row) of RF-MS labeled N-glycans were obtained at 60 °C column temperature, with 50 mM ammonium formate, pH 4.4, as a mobile phase A, and a gradient from 75% to 54% LC-MS grade ACN (mobile phase B). The following chromatograms (second and third row) present the effect of the reduced column temperature: 45 °C (**A**) and 30 °C (**B**); higher aqueous mobile phase ionic strengths: 100 mM (**C**) and 200 mM (**D**) ammonium formate; and the lowered initial concentration of mobile phase B: 72% (**E**) and 71% (**F**) on FLR plasma N-glycome separation profile.*

In order to improve the chromatographic separation and facilitate reliable quantification of plasma and serum N-linked glycans, the benchmark method was subjected to further optimization, where several factors such as temperature, mobile phase ionic strength, flow rate, and the gradient slope have been modified (**Figure 1, A-F**). All the modified factors, except for the flow rate, resulted in improved chromatographic separation. The mobile phase flow rate adjustment from initial 0.4 to 0.5 mL/min had a minimal effect on the N-glycan profile (data not shown), which is why the flow rate as a factor was excluded from further optimization. Regarding the temperature, we observed that reducing the column temperature from 60 °C to 30 °C improved resolution in the second part of the chromatogram (peak GP25 to GP44), but at the cost of peak capacity at the beginning of the chromatogram (GP6, 7 and 8). Hence, to allow for improved resolution and at the same time to preserve peak capacity we chose 45 °C as column temperature. In the context of peak shape, numerous glycan structures were shown to have sharper peaks as the column temperature decreased (GP25, GP26, GP37, GP38). Next, in order to explore the influence

of the ionic strength of aqueous mobile phase A, the concentration of ammonium formate was increased from 50 mM to 100 and 200 mM. As shown in previous studies,(14) increasing ammonium formate concentration improved both the peak shape and chromatographic resolution of sialylated glycans (GP37, GP38). However, mobile phases with high concentrations of ion paring reagent may lead to a shortened column lifetime, hence, we chose 100 mM ammonium formate as a final concentration. Next, we modified the slope of the gradient by reducing the initial % of mobile phase B from 75 to 70%. Compared to the universal method, a new, reduced gradient slope running from 71% - 54% mobile phase B resulted in significant improvement in peak resolution observed for two co-eluting glycans at the beginning of the chromatogram (GP2 and GP3).

The improvements in the HILIC-LC-MS method outlined above, such as the column temperature at 45 °C, 100 mM ammonium formate as a mobile phase A, gradient slope from 71% to 54% mobile phase B, and 0.4 mL/min flow rate resulted in a number of significant improvements in chromatographic separation of plasma N-glycans labeled with *Rapi*Fluor-MS compared to the Universal profiling method. Therefore, they were chosen as the final conditions for the HILIC-LC-MS separation. The most prominent improvements were seen for high mannose and sialylated glycan structures. For example, peaks GP3, GP9, and GP21, corresponding to high mannose (M5, M6) and disialylated glycans (FA2G2S2), co-eluted with other glycan structures when analyzed by the universal method, but they were resolved when the optimized method was used (**Figure 2**). Moreover, the adjustments of the benchmark method resulted in reduced peak broadening of sialylated structures, such as A2G2S2 (GP25), FA2G2S2 (GP26), FA2BG2S2 (GP27), as well as A3G3S3 (GP37) and A3F1G3S3 (GP38). This, in turn, facilitated the enhanced resolution of the aforementioned sialylated N-glycans.

With the use of the developed method, plasma N-glycome was integrated and separated into 44 chromatographic peaks (**Figure 3**). Next, by using the MS data simultaneously generated by the BioAccord system, we could determine N-glycan compositions that elute in each chromatographic peak. A total of 54 major and 15 minor N-glycan compositions were successfully identified (**Table S4**). To a large extent, the RF-MS plasma N-glycan profile deciphered in this study matched with the previously profiled plasma N-glycans labeled with 2-AB, with A2G2S2, A2G2S1, FA2G1, FA2, FA2G2, FA2G2S1, and A3G3S3 species being the most abundant in the spectrum.(11) However, previous studies quantified only 39 glycan peaks. This is due to the fact that the hydrophobicity of the RF-MS label is different from 2-AB resulting in the different migration patterns of several high mannose species, such as M5 (GP3), M6 (GP9), and M8 (GP19) (**Figure 3**). Notably, using optimized conditions those high mannose species appeared as individual peaks and did not co-elute with FA2B or FA2[3]BG1 as was the case in the previous study.

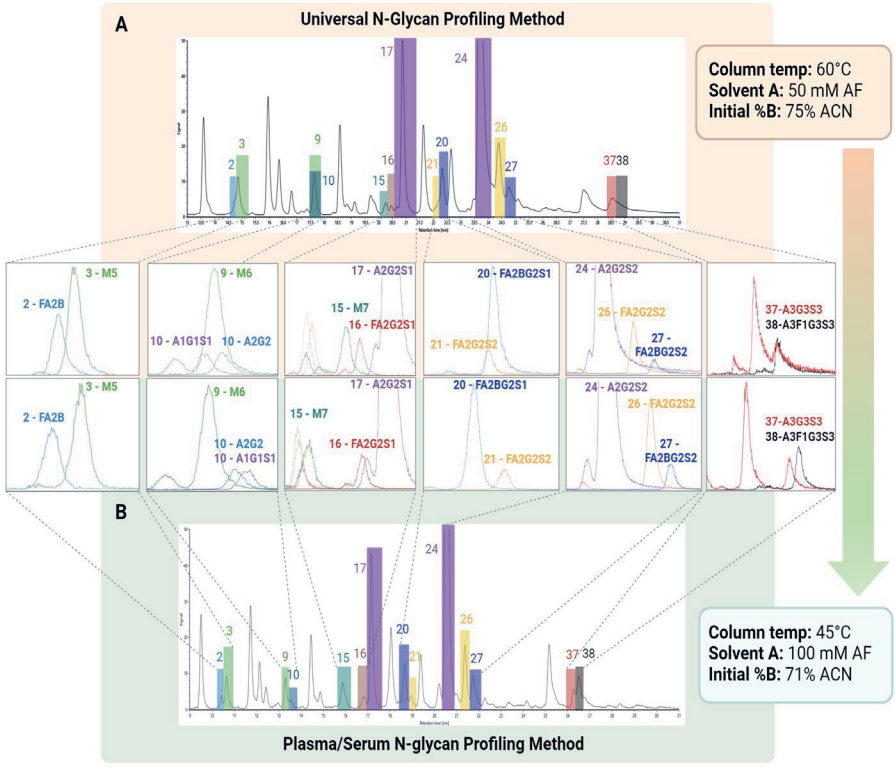

Figure 2. *Improvements in RF-MS labeled plasma N-glycan peak separation obtained using the optimized N-glycan profiling method. (A) Fluorescence (FLR) chromatogram and extracted ion chromatograms (XICs) obtained with the universal N-glycan profiling method (60 °C column temperature, 50 mM mobile phase A, gradient from 75% to 54% ACN. (**B**) FLR chromatogram and XICs obtained with the optimized method for plasma N-glycan profiling (45°C column temperature, 100 mM mobile phase A, gradient from 71% to 54% ACN). The six most prominent differences in glycan separation are shown.*

Finally, to examine potential differences in HILIC-LC-MS profiles between plasma and serum RF-MS labeled N-glycans, we compared the profiles of a pooled plasma standard and three randomly chosen serum samples from the validation cohort (data not shown). Our analysis revealed no difference in N-glycome composition (the same structures were observed in both types of samples). There were only minor differences in the abundances of certain N-glycan structures within the same peak, suggesting that the developed separation method is suitable for both plasma and serum N-glycome profiling.

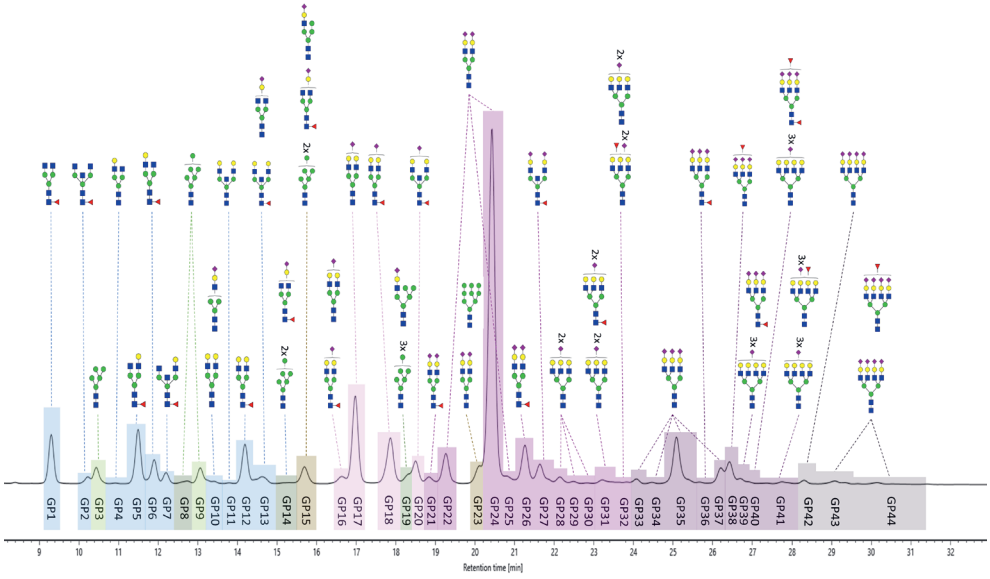

***Figure 3**. HILIC-UPLC-FLR separation of total N-glycans released from human plasma glycoproteins and labeled with RF-MS. The chromatograms were separated into 44 chromatographic glycan peaks (GP1-GP44). The N-glycans in each chromatographic peak were assigned based on respective MS1 sum spectra and established literature on plasma N-glycosylation. The structures of the most abundant glycans are shown for each peak. For the complete assignment of the glycan peaks, see **Supplementary Table S4**.*

5.4.2 Linearity of the HILIC-LC-MS separation method

Equally important as HILIC separation conditions, is the amount of plasma and serum proteins used for the sample preparation step. The recommended amount of glycoproteins for the enzymatic release and RF-MS labeling of N-glycans using Waters GlycoWorks RF-MS N-Glycan Kit is 15 µg. The protein concentration in plasma and serum is approximately 60-80 µg/µL.(15, 16) Hence, according to the theoretical protein concentration, we determined a range of the starting volume of plasma and serum which results in robust release and labeling of N-glycans. The linear range of detection for the FLR signal was determined for various N-glycan structures in the standard sample (**Figure S1-S5**). The linear response of the selected glycan peaks ($R \geq 0.9879$) demonstrated the applicability of the optimized method for profiling plasma and serum N-glycans labeled with RF-MS. The linearity was already achieved with the theoretical amount of 0.6 µg of proteins. However, to facilitate the robustness of the method, the protein amount of 9.5 µg in the middle of the linear range was chosen for further experiments.

5.4.3 Validation of the HILIC-LC-MS separation method

To elucidate the applicability of the developed Plasma/Serum N-glycan Profiling Method and especially its ability to reveal clinically relevant glycosylation changes, the total serum

N-glycome from 483 biological samples was analyzed. Next to the cohort samples, 36 replicates of pooled plasma standard samples were distributed throughout six sample plates.

For all samples, the analysis was performed in a 96-well based-format and 44 glycan chromatographic peaks were quantified. Furthermore, the individual peaks were systematized into 16 glycosylation traits reflecting a common biosynthetic pathway: high-mannose (HM), low branching (LB), high branching (HB), agalactosylation (G0), monogalactosylation (G1), digalactosylation (G2), trigalactosylation (G3), tetragalactosylation (G4), core fucosylation (CF), antennae fucosylation (AF), bisection (B), and asialylation (S0), monosialylation (S1), disialylation (S2), trisialylation (S3), and tetrasialylation (S4) (**Table S2**). The repeatability of the method was assessed by measuring the coefficient of variation (CV) for 36 replicates of pooled plasma standard samples. The average coefficient of variation for all 44 glycan peaks was only 4.7% indicating a very good analytical precision (**Figure S6**).

In order to demonstrate the potential of the method to detect biological variability among individuals in the cohort, we calculated relative abundances for each glycosylation trait in total serum N-glycome for each analyzed sample (**Figure 4**). We observed that the biological variability of all the glycosylation traits among the study population is higher than the technical variation of the standard samples. Thus, the optimized HILIC profiling method would be suitable to capture potential changes in serum and plasma N-glycome composition.

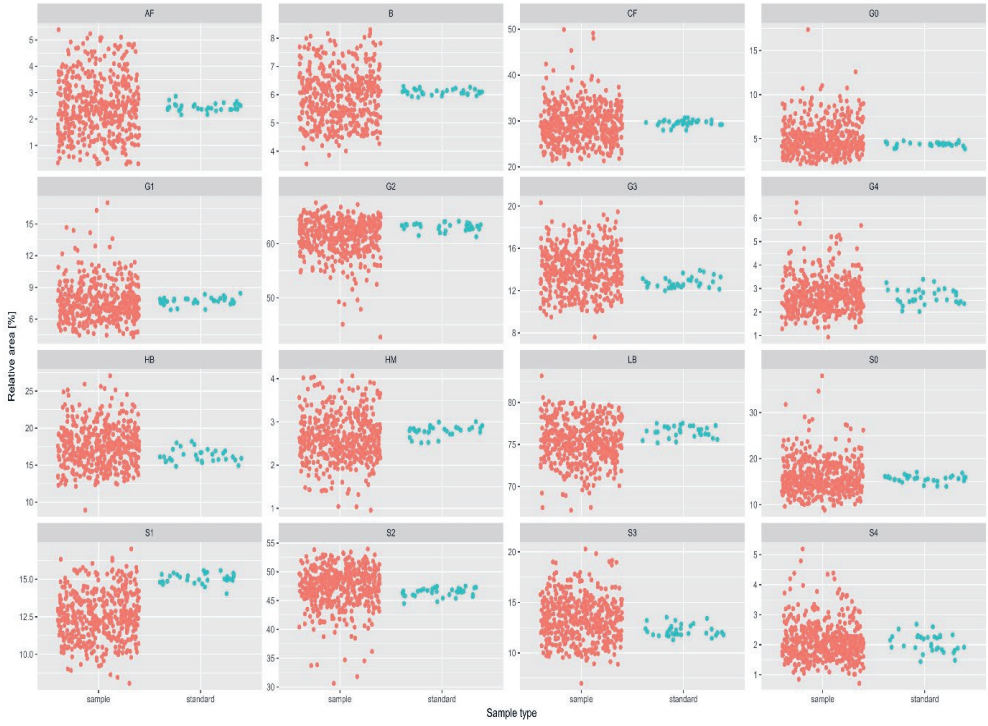

Figure 4. *Biological and technical variability of studied individuals (red) and standard samples (blue), respectively, for major traits of serum N-glycans (antennary fucosylation (AF), bisection (B), core fucosylation (CF), agalactosylation (G0), monogalactosylation (G1), digalactosylation (G2), trigalactosylation (G3), tetragalactosylation (G4), high branching (HB), high mannosylation (HM), low branching (LB), asialylation (S0), monosialylation (S1), disialylation (S2), trisialylation (S3) and tetrasialylation (S4)). Relative abundances of derived glycan traits in total serum N-glycome are shown.*

5.5 CONCLUSION

We have optimized the HILIC-LC-MS method to analyze N-glycans released from total human plasma and serum proteins and labeled with *Rapi*Fluor-MS. The developed approach combining the advantages of GlycoWorks N-glycan sample preparation with the separation capabilities of HILIC-UPLC and an integrated and routine high resolution mass spectrometer like the BioAccord LC-MS system allows for identifying clinically relevant changes in plasma and serum N-glycome in a simple, robust and high-throughput manner.

5.6 ACKNOWLEDGMENTS

We thank Marleen van Wingerden, Dr. Guillaume Bechade, Nick Pittman, Evelien Dejaegere, and WatersCorporation for their support in this work. We also thank Prof Ana M. Valdes from the School of Medicine, University of Nottingham, for obtaining biological samples from COVID-19 patients.

5.7 SUPPLEMENTARY MATERIAL

LC conditions – Universal N-Glycan Profiling Method

LC system: ACQUITY UPLC I-Class
Sample temp.: 10 °C
Column temp.: 60 °C
Injection volume: 25 µL
Fluorescence detection: Ex 265 nm/Em 425 nm, 2 Hz
Mobile phase A: 50 mM ammonium formate, pH 4.4 (LC-MS grade water)
Mobile phase B: ACN (LC-MS grade)
Gradient used for the chromatographic separation:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.00	0.400	25.0	75.0	Initial
35.00	0.400	46.0	54.0	6
36.50	0.200	100.0	0.0	6
39.50	0.200	100.0	0.0	6
43.10	0.200	25.0	75.0	6
46.60	0.400	25.0	75.0	6
55.00	0.400	25.0	75.0	6

LC conditions – Plasma and Serum N-Glycan Profiling Method

LC system: ACQUITY UPLC I-Class
Sample temp.: 10 °C
Column temp.: 45 °C
Injection volume: 25 µL
Fluorescence detection: Ex 265 nm/Em 425 nm, 2 Hz
Mobile phase A: 100 mM ammonium formate, pH 4.4 (LC-MS grade water)
Mobile phase B: ACN (LC-MS grade)
Gradient used for the chromatographic separation:

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.00	0.400	29.0	71.0	Initial
35.00	0.400	46.0	54.0	6
36.50	0.200	100.0	0.0	6
39.50	0.200	100.0	0.0	6
43.10	0.200	29.0	71.0	6
46.60	0.400	29.0	71.0	6
55.00	0.400	29.0	71.0	6

Table S1. *Overview of the baseline characteristics of the study cohort.*

Characteristics	Observation
Longitudinal samples, N	483
Individuals, N	89
Age, years (mean ± SD)	42 ± 11
Sex, N (%)	
Male	20 (22)
Female	69 (78)

Table S3. *The theoretical amount and concentration of plasma proteins in serially diluted samples. The calculation was based on the value of 70 mg/mL, the theoretical total protein concentration in human plasma.*

Sample	Theoretical protein amount [µg]	Theoretical protein concentration [µg/µL]
Tpng_1	0.6	0.05
Tpng_2	0.9	0.08
Tpng_3	1.5	0.13
Tpng_4	2.3	0.22
Tpng_5	3.7	0.34
Tpng_6	6	0.55
Tpng_7	9.5	0.88
Tpng_8	15.3	1.41

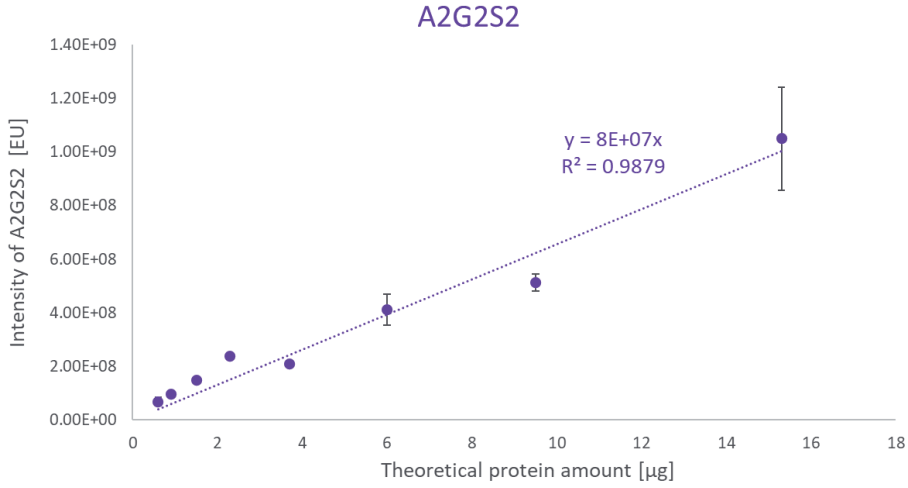

Figure S1. *The linear range of detection for the A2G2S2 glycan labeled with RapiFluor-MS. FLR signal intensities for the series dilutions of plasma proteins are shown. Standards plasma samples were analyzed in triplicate. Error bars represent the standard deviation of the triplicates.*

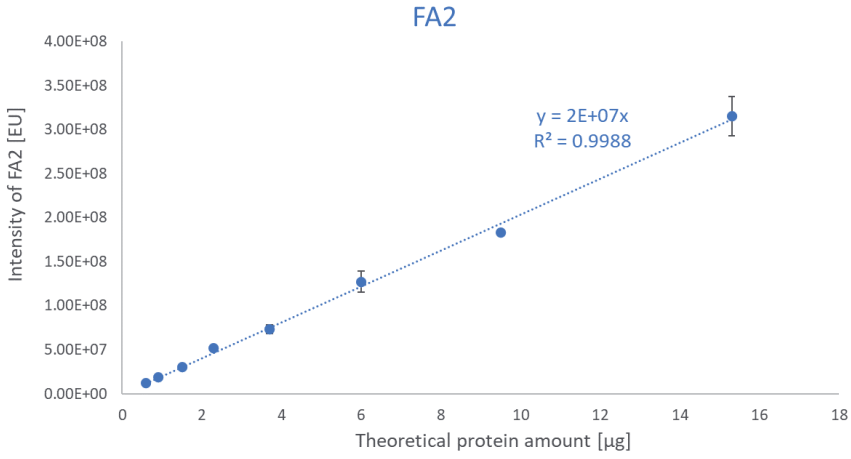

Figure S2. *The linear range of detection for the FA2 glycan labeled with RapiFluor-MS. FLR signal intensities for the series dilutions plasma proteins are shown. Standards plasma samples were analyzed in triplicate. Error bars represent the standard deviation of the triplicates.*

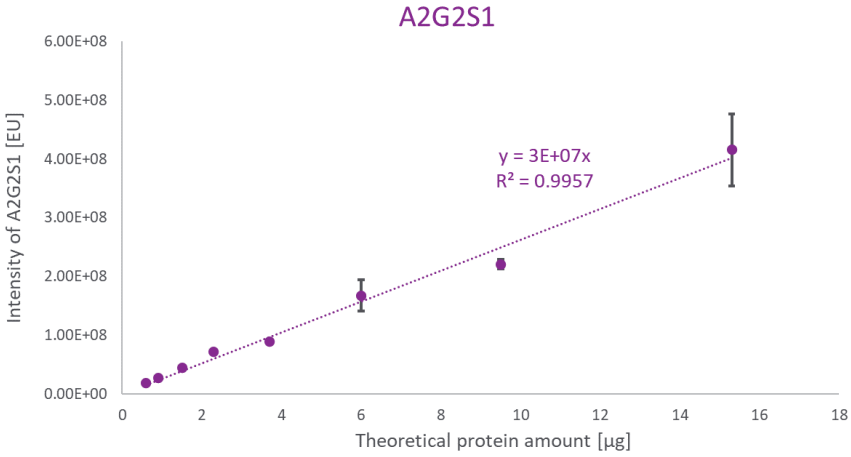

Figure S3. *The linear range of detection for the A2G2S1 glycan labeled with RapiFluor-MS. FLR signal intensities for the series dilutions of plasma proteins are shown. Standards plasma samples were analyzed in triplicate. Error bars represent the standard deviation of the triplicates.*

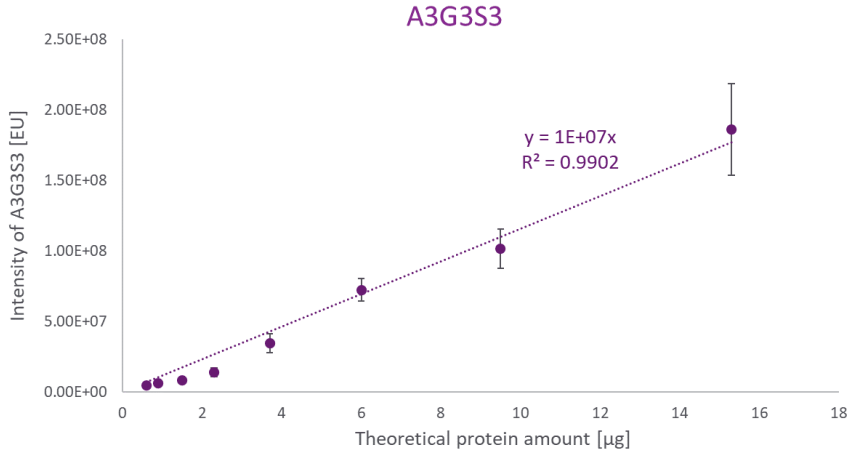

Figure S4. *The linear range of detection for the A3G3S3 glycan labeled with RapiFluor-MS. FLR signal intensities for the series dilutions of plasma proteins are shown. Standards plasma samples were analyzed in triplicate. Error bars represent the standard deviation of the triplicates.*

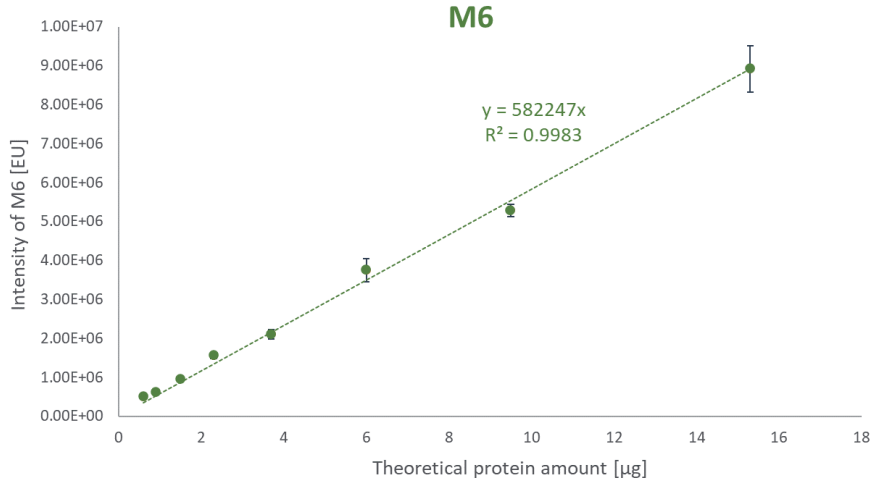

Figure S5. *The linear range of detection for the M6 glycan labeled with RapiFluor-MS. FLR signal intensities for the series dilutions of plasma proteins are shown. Standards plasma samples were analyzed in triplicate. Error bars represent the standard deviation of the triplicates.*

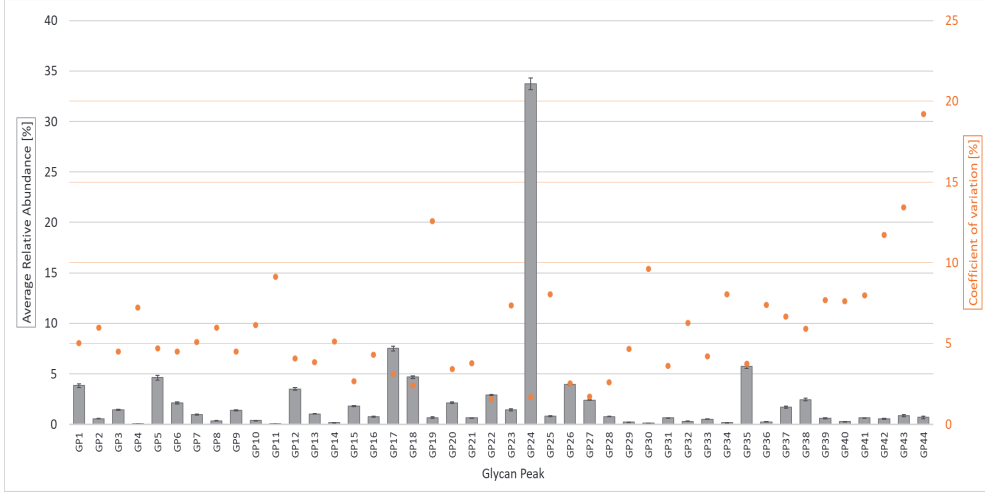

Figure S6. *Technical variability. Average relative abundances (primary y axis on the left) and coefficients of variation (secondary y axis on the right) of RF-MS labeled N-glycans from 36 replicates of a standard plasma sample. The error bars represent the standard deviation of the mean while the coefficients of variation for each glycan peak are shown as orange dots.*

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