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Characterization of Fibrinogen Glycosylation and Its Importance for Serum/Plasma *N*-Glycome Analysis

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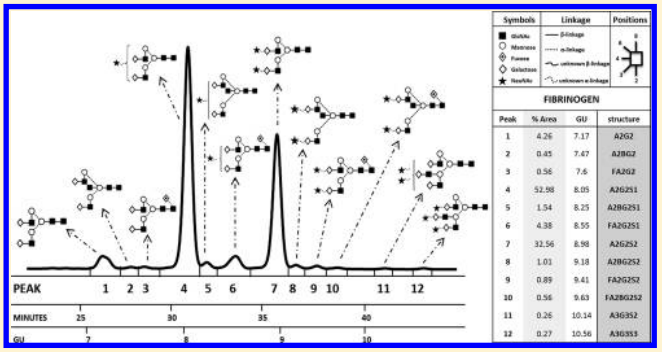
ABSTRACT: The majority of proteins present in human serum/plasma are glycoproteins, validating this fluid as an ideal starting material for *N*-glycan analysis and discovery of potential biomarkers. The glycoprotein content for both serum and plasma is very similar, except for proteins removed in the coagulation process, including fibrinogen. Our aim was to characterize fibrinogen glycosylation in order to determine its contribution to differences between serum and plasma *N*-glycomes. *N*-Glycans from human fibrinogen were released, labeled, and analyzed by HILIC−HPLC and MS. Structural characterization of fibrinogen subunits revealed that the α chain was not *N*-glycosylated, whereas β and γ contained identical oligosaccharide structures, mainly biantennary digalactosylated monosialylated structures (A2G2S1) and biantennary digalactosylated disialylated structures (A2G2S2). Blood was collected from five healthy volunteers into four testing tubes: silicone-coated glass for serum and EDTA, Na-heparin, and Li-heparin glass tubes for plasma. *N*-Glycans were analyzed using the high-throughput HILIC−HPLC method. *N*-Glycan profiles from serum and plasma samples differed largely in glycans identified in fibrinogen, suggesting that this glycoprotein represents a major factor distinguishing these body fluids. This result emphasizes the important of consistent body fluid collection practices in biomarker discovery studies.



FIBRINOGEN

Peak	% Area	GU	structure
1	4.26	7.17	A2G2
2	0.45	7.47	A2BG2
3	0.56	7.6	FA2G2
4	52.98	8.05	A2G2S1
5	1.54	8.25	A2BG2S1
6	4.38	8.55	FA2G2S1
7	32.56	8.98	A2G2S2
8	1.01	9.18	A2BG2S2
9	0.89	9.41	FA2G2S2
10	0.56	9.63	FA2BG2S2
11	0.26	10.14	A3G3S2
12	0.27	10.56	A3G3S3

KEYWORDS: *fibrinogen, glycan analysis, serum, plasma, HILIC−HPLC*

INTRODUCTION

Body fluids, in particular serum and plasma, are the most useful biological samples as a result of their accessibility and representative complexity. This complexity is due, in part, to the wide dynamic range of protein/glycoprotein concentrations. It is estimated that there are more than 10^6 different proteins in human serum, but the great majority are present at very low concentrations.[1] The number of high-abundance proteins is relatively small, and the most abundant proteins (albumin, immunoglobulins IgG and IgA, transferrin, haptoglobin, α1-antitrypsin)[2] represent together approximately 85% of the total protein mass content in serum.[3] Albumin represents the most abundant nonglycosylated protein in the serum.[4] The other high abundance proteins and the majority of low-level proteins present in human are glycoproteins, validating this fluid as an ideal starting material for *N*-glycan analysis and for discovery of potential biomarkers.

There has been an overwhelming interest in trying to decipher differences between serum and plasma.[5−8] The process of coagulation makes serum qualitatively different from plasma, so it comes as no surprise that most of the differences are attributable to proteins related to coagulation.[9] The removal of a large portion of the fibrinogen content from plasma in the form of the fibrin clot results in serum having a protein concentration lower than that of plasma.[10] While the primary effect of the coagulation process is the formation of the fibrin clot, platelets, erythrocytes, and leukocytes also release certain proteins during the same process.[11] Multiple studies have reported minor differences, such as total cholesterol and triglyceride values 3% higher in serum than in plasma[12,13] and higher concentrations of platelet-derived vascular endothelial growth factor (VEGF) in serum.[14]

Nevertheless, when the composition of the glycoproteomes for the plasma and serum samples was compared, there was a close correlation between the samples, except for the absence of fibrinogen in serum.[15] Fibrinogen is a soluble 340 kDa glycoprotein that is synthesized in the liver by hepatocytes and plays a key role in blood clotting.[16] Fibrinogen is a hexamer containing two sets of three different polypeptide chains (α, β, and γ) linked to each other by disulfide bonds. It is cleaved by thrombin into fibrin, an essential structural component of the thrombus, thereby preventing the loss of blood after injury.[17]

Currently there are many tubes commercially available for blood sample collection. Most of them are vacutainer types that offer a safe method of blood collection compared to old-fashioned glass vessels. Using an evacuated blood collection system has many advantages including high quality of samples and analysis results,

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along with minimized risk of infection to staff. There is broad range of tubes available that includes appropriate additives, various color codes, and different dimensions to meet all clinical requirements.

Since one of the main differences between serum and plasma is fibrinogen content, it is critical to consider fibrinogen glycans when performing glycomic studies using serum or plasma. Altered *N*-glycan profiles of particular glycoproteins have been investigated with the aim of increasing the overall sensitivity and specificity of biomarkers for cancer or other diseases.[18−21] In the classical study, sample collection procedure is identical for all the samples. Inconsistency problem may appear when samples are collected from a number of separate sites using different protocols for collection, handling, and storage. Thus, it is very important to ensure consistency of samples at an early stage. Any discrepancies due to collecting conditions and sample type may introduce false conclusions in regards to *N*-glycome alterations.

In this paper, we describe for the first time *N*-glycan structures present on fibrinogen subunits. Characterization of fibrinogen glycosylation is of interest because this glycoprotein is absent in serum and present in plasma. In addition, we have evaluated different tubes for blood collection to ensure high quality and comparability of *N*-glycome analyses.

MATERIALS AND METHODS

Human Samples and Collecting Conditions

Blood samples were taken from 5 healthy adult donors, 4 males and 1 female. Their average age was 41.4 ± 3.2 (SD) years (range 36−44). The blood was drawn into four different testing tubes: one for serum collection, glass vacutainer plain serum blood collection tube (silicone-coated, Catalogue no. 366430, BD Biosciences), and three for plasma collection, EDTA tube (367861, BD Biosciences), Na-heparin tube (367871, BD Biosciences), and Li-heparin plasma separator tube (PST) (367960, BD Biosciences). Plain glass tubes were allowed to clot for 2 h at RT followed by centrifugation at 1300*g* for 10 min at 4 °C to separate serum. Plasma was separated from the anticoagulant either by immediate centrifugation (0 d) or after RT storage for 24 h (1 d) or 48 h (2 d). The separated samples were aliquoted and stored at −80 °C until further analysis.

This study was performed in conformance to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethics committee of Brigham and Women's Hospital, Boston, MA. All participants gave signed informed consent for participation in the study.

Chemicals and Reagents

Water used throughout this study was obtained from a Milli-Q Gradient A10 Elix system (Millipore, Bedford, MA) and was 18.2 MΩ or greater with a total organic carbon (TOC) content less than 5 parts per billion (ppb). Acetonitrile was Fisher Far UV gradient grade (Fisher Scientific, Dublin, Ireland). All other chemicals used were purchased from Sigma Aldrich (Dublin, Ireland) and were of the highest available quality.

Fibrinogen from Pooled Human Plasma

The purity of purchased fibrinogen (Catalog no. 341576, Merck, Darmstadt, Germany) was assessed using 17% reducing SDS-PAGE in an Xcell SureLock Mini-Cell (Invitrogen, Carlsbad, CA) according to the manufacturer. Precision Plus Protein All Blue Standards (BioRad, Hercules, CA) was used as the molecular weight marker. The gel was run at 50 mA for 180 min and stained with GelCode Blue staining reagent (Pierce, Rockford, IL).

Glycan Release and Labeling

Serum/plasma (5 μL) and purchased fibrinogen (100 μg) samples were immobilized in a block of SDS-polyacrylamide gel, and *N*-glycans were enzymatically released by digestion with recombinant *N*-glycosidase F (Prozyme, Hayward, CA). The enzyme was used at 10% of the final digestion volume. This was done in a 96-well plate to achieve the best throughput of sample preparation. After extraction, glycans were fluorescently derivatized via reductive amination with 2-aminobenzamide (2-AB) with sodium cyanoborohydride in 30% v/v acetic acid in DMSO at 65 °C for 2 h. The labeling reaction was quenched by the addition of water to yield a final volume of 100 μL. Nine hundred microliters of acetonitrile was then added to the reaction vial with subsequent excess fluorophore removal by HILIC phase pipet tips (PhyNexus, San Jose, CA) using an adaptation of the method of Olajos et al.[22] *N*-Glycan nomenclature and symbolic representations used throughout is as previously described by Harvey et al.[23]

Hydrophilic Interaction Liquid Chromatography (HILIC)

2-AB-derivatized *N*-glycans from purchased fibrinogen and serum/plasma samples were separated using HPLC with fluorescence detection on a 2795 Waters Alliance separation module equipped with a Waters temperature control module and a Waters 2475 fluorescence detector (Waters, Milford, MA). Separations were performed using a TSK-Gel Amide-80 5 μm 4.6 m × 250 mm or 3 μm 4.6 mm × 150 mm column (Tosoh Bioscience, Stuttgart, Germany). Solvent A was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was acetonitrile. Samples were maintained at 5 °C prior to injection and the column temperature was set to 30 °C. Gradient conditions were as follows: 60 min method, a linear gradient of 35−47% solvent A over 48 min at a flow rate of 0.8 mL/min, followed by 1 min at 47−100% A and 4 min at 100% A, returning to 35% A over 1 min and then finishing with 35% A for 6 min. Samples were injected in 80% acetonitrile. The fluorescence detection excitation/emission wavelengths were ex = 330 nm and em = 420 nm, respectively. Retention times were converted into glucose unit (GU) values by time-based standardization against a dextran hydrosylate ladder, as described previously.[24]

Weak Anion Exchange High Performance Liquid Chromatography (WAX-HPLC)

WAX-HPLC was performed using Prozyme GlycoSep C 7.5 mm × 75 mm column (Prozyme, Leandro, CA) on a 2795 Alliance Separation module with a 2475 fluorescence detector (Waters, Milford, MA). Solvent A was 20% (v/v) acetonitrile in water, and solvent B was 0.1 M acetic acid adjusted to pH 7.0 with ammonia solution in 20% (v/v) acetonitrile. Gradient conditions were as follows: 100% A for 5 min, then a linear gradient of 100% to 0% A for 15 min at a flow rate of 0.75 mL/min, followed by 0% A for 2.5 min, returning to 100% A for 1.5 min and then finishing with 100% A for 7 min. Samples were injected in water. A fetuin *N*-glycan standard was used for calibration.[25]

Exoglycosidase Digestions

All enzymes were purchased from Prozyme, San Leandro, CA, USA. 2-AB-labeled glycans were digested in 10 μL of 50 mM sodium acetate buffer, pH 5.5 for 18 h at 37 °C, using arrays of the following enzymes: ABS, *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18, releases α2-3,6,8 linked non-reducing terminal sialic acid); NAN1, recombinant sialidase (EC 3.2.1.18, releases α2-3 linked non-reducing terminal sialic acids); BKF, bovine kidney α-fucosidase (EC 3.2.1.51, releases α1-2,6 linked non-reducing

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terminal fucose residues more efficiently than α1-3,4 linked fucose, digests core α1-6 fucose); BTG, bovine testes β-galactosidase (EC 3.2.1.23, hydrolyses non-reducing terminal β1-4 and β1-3 linked galactose); and GUH, hexosaminidase cloned from *Streptococcus pneumoniae* expressed in *E. coli* (EC 3.2.1.30, releases GlcNAc residues but not a bisecting GlcNAc linked to Man). After incubation, enzymes were removed by filtration using Pall spin filters 10 kDa (Pall Corporation). *N*-Glycans were then analyzed by HILIC−HPLC.

Mass Spectrometry

LC−fluorescence mass spectrometry analysis was performed using a Waters Xevo G2 QTof with Acquity UPLC and BEH Glycan column (2.1 mm × 150 mm, 1.7 μm particle size). Data was acquired in negative mode with the following conditions: 2000 V capillary voltage, 40 V cone voltage, 120 °C source temperature, 450 °C desolvation temperature, 50 L/h cone gas flow, and 800 L/h desolvation gas flow. The fluorescence data rate was 1 pts/s and a PMT gain = 10 with 330 nm excitation and 420 nm emission wavelengths. The flow rate was 150 μL/min. Solvent A was 50 mM formic acid adjusted to pH 4.4, and solvent B was acetonitrile. A 30 min linear gradient was used and was as follows: 35−47% A for 48 min, 47−100% A for 1 min, 4 min 100% solvent A, 35−100% solvent A for 1 min, and 35% solvent A for 6 min. Samples were diluted in 65% acetonitrile prior to analysis.

RESULTS

Glycomic Characterization of Fibrinogen

The glycosylation of purchased fibrinogen was investigated to determine whether plasma/serum *N*-glycome differences might be due to presence or absence of this glycoprotein in a given body fluid.

The purity of fibrinogen was examined by separation on SDS-PAGE gel, revealing three clearly visible bands corresponding to the molecular masses of α (~63.5 kDa), β (~56 kDa), and γ (~47 kDa) chains. Therefore, the sample was confirmed to be highly pure and suitable for structural analysis of oligosaccharide structures. Following separation, individual bands were excised from gels and further processed (Figure 1), as well as total fibrinogen

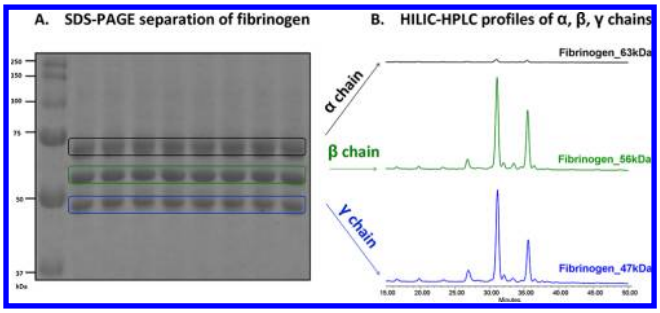

Figure 1. (A) SDS-PAGE of a purified fibrinogen, three bands representing α, β, and γ chains are clearly visible. (B) HILIC−HPLC profiles of individual fibrinogen chains; structures of these oligosaccharides are described in results section HILIC−HPLC of *N*-Glycans Present on Fibrinogen.

in order to ensure removal of *N*-glycans from either of the chains. Eight aliquots of 100 μg of purified fibrinogen were reduced and alkylated; *N*-glycans were released, labeled, and subjected to chromatographic separations (Figure 2).

HILIC−HPLC of *N*-Glycans Present on Fibrinogen

N-Glycans released from fibrinogen (bands and total molecule) via overnight incubation with PNGaseF (*N*-glycosidase F) were labeled with 2-aminobenzamide (2-AB) and profiled using HILIC−HPLC as previously described.[24] The resulting chromatographic data confirmed that the α chain was not glycosylated, whereas β and γ contained identical oligosaccharide structures. A representative HILIC−HPLC profile of fibrinogen (presented in Figure 3) was annotated with GU values by comparison with a dextran hydrosylate ladder. Initial structural assignment of the glycans present in the peaks was performed by comparison of the experimental data with GlycoBase (http://glycobase.nibrt.ie/).[26,27]

Table 1 lists the initial assignments for the glycans present in each peak, along with the relative proportions of each glycan present. The experimental data presented in Table 1 suggest that biantennary digalactosylated monosialylated (A2G2S1) and biantennary digalactosylated disialylated (A2G2S2) glycans are the most prevalent glycans present in fibrinogen, accounting for over 85% of the total profile. Modifications of A2G2S1 and A2G2S2 with either bisecting GlcNAc or core fucose residues were also found to be present, although in considerably lower quantities. In order to confirm structures, WAX separation as well as a full panel of exoglycosidase digestions has been performed and separated again using HILIC−HPLC (Figure 5). These structures were further confirmed by LC−fluorescence−MS (Figure 4).

Weak-Anion Exchange of *N*-Glycans Present on Fibrinogen

Using WAX-HPLC, fibrinogen *N*-glycans were separated according to the number of sialic acids, revealing that mono-, di-, and trisialylated glycans are present (Figure 5). The most abundant peak in the profile represented monosialylated structures at 57% of the total profile, whereas disialylated structures accounted for 41% and only 2% were trisialylated glycans.

Structural Annotation of Fibrinogen *N*-Glycans Using Exoglycosidase Digestions

A series of exoglycosidase digestions were performed to confirm and assign fibrinogen structures to particular chromatographic peaks obtained by sample separation using HILIC−HPLC. A full panel of digestions for fibrinogen *N*-glycans that included most commonly used exoglycosidase enzymes is shown in Figure 6.

The digested glycans separated by HILIC have a logical movement in glycans, whereby each sugar can be accounted for by a constant value, depending on linkage. On the basis of the GU shifts we can track structures that are moving upon addition of certain enzyme.

The undigested profile consisted of 12 chromatographic peaks, where two peaks were most abundant. After ABS digestion, A2G2S2 structure with GU 8.98 and A2G2S1 structure with 8.05 digest back to an A2G2 structure with a GU value of 7.17. The digestion with NAN1 enzyme (recombinant sialidase that removes α2-3 linked non-reducing terminal sialic acids) confirms that both structures A2G2S2 and A2G2S1 contain sialic acids in both α2-3 and α2-6 linkages. Further digestion with BKF releases all structures containing core fucose (linked to core GlcNAc by α1-6 linkage) and all peaks shift to one that represents A2G2 glycan. On addition of BTG enzyme all galactose residues are hydrolyzed, and peak A2G2 moves to the A2 structure. Subsequent digestion with GUH removes two β-linked GlcNAc's leaving the *N*-glycan core structure (M3).

Initial profile of undigested fibrinogen sample consisted of 12 peaks. After sequential digestion with exoglycosidases we were able to assign glycan structures to each peak, including the minor

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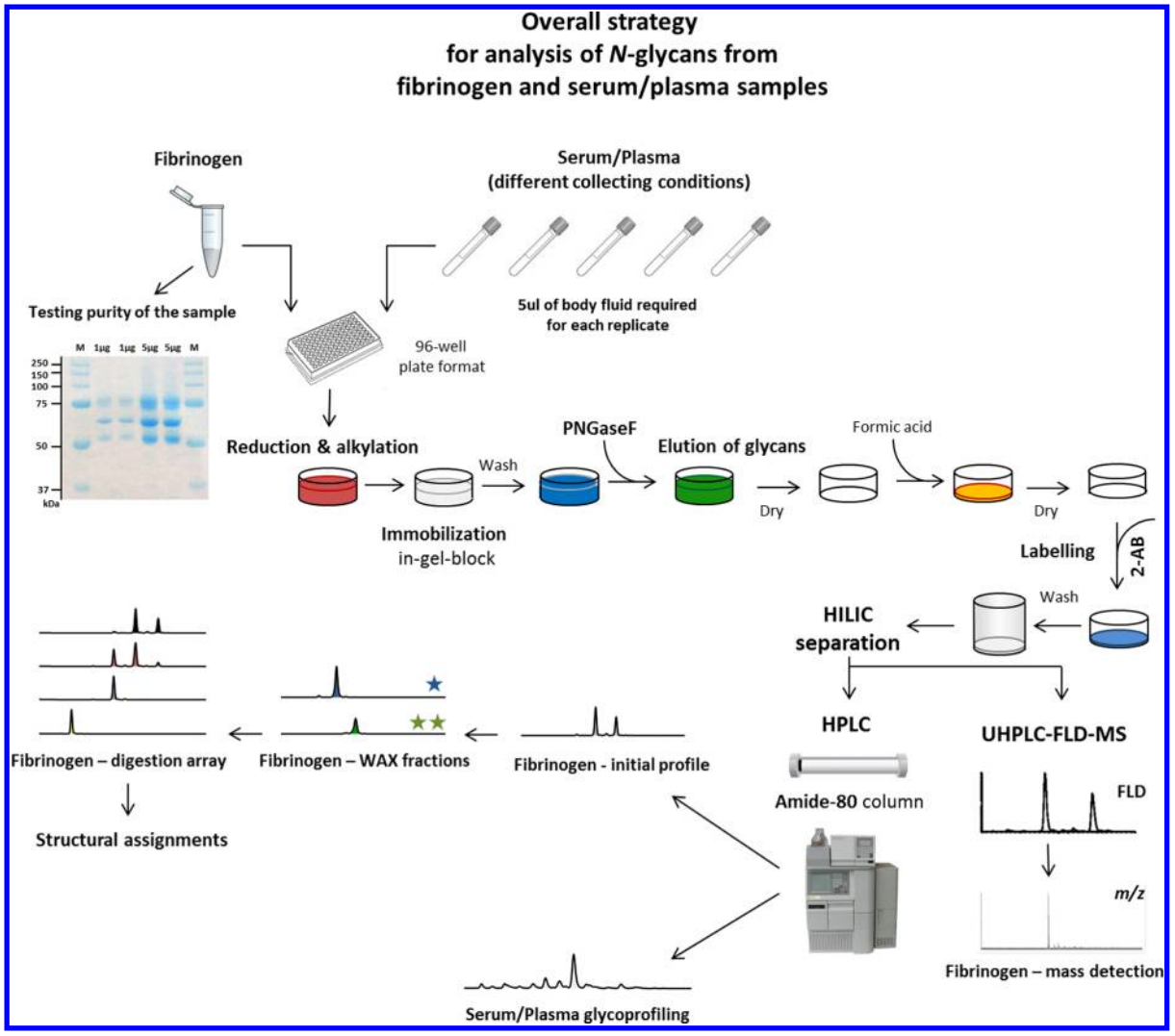

Figure 2. Overall strategy for *N*-glycan analysis using an HPLC-based approach.

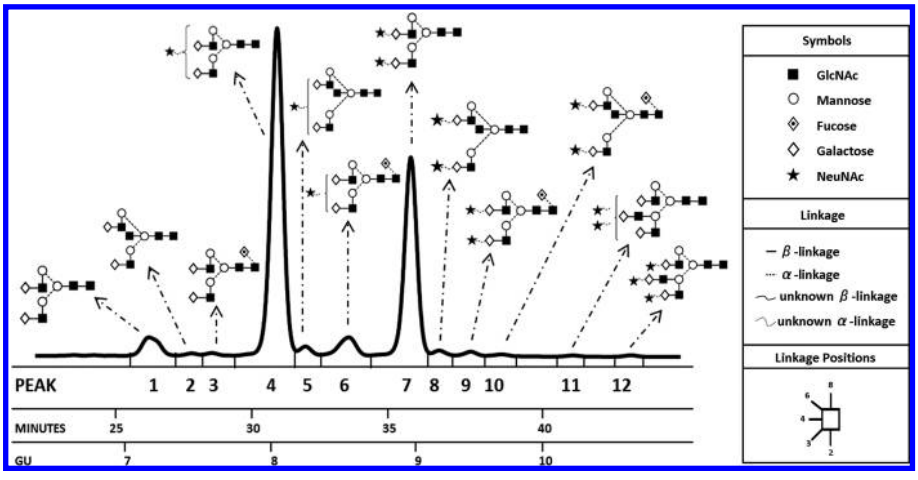

Figure 3. HILIC–HPLC chromatogram of *N*-glycans released from fibrinogen divided into 12 peaks with corresponding glucose units values and structural assignments. The Oxford glycan notation was used to represent *N*-linked glycan composition and structure.[23]

peaks. The most predominant structure present on fibrinogen molecule is A2G2S1 (52.98%), and the second most abundant is A2G2S2 (32.56%). A2G2 and FA2G2S1 were accounted for at about 4% presence each. The percent area of the remaining peaks was less than 2%.

Serum and Plasma *N*-Glycome Analysis

In order to evaluate blood collecting conditions for their impact upon *N*-glycan analysis, we tested four different blood sampling tubes in current clinical use. One of them was for serum collection (anticoagulant-free), and the other three for plasma

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Table 1. Structure Details of Fibrinogen Glycans[a]

peak	% area	GU	structure
1	4.26	7.17	A2G2
2	0.45	7.47	A2BG2
3	0.56	7.6	FA2G2
4	52.98	8.05	A2G2S1
5	1.54	8.25	A2BG2S1
6	4.38	8.55	FA2G2S1
7	32.56	8.98	A2G2S2
8	1.01	9.18	A2BG2S2
9	0.89	9.41	FA2G2S2
10	0.56	9.63	FA2BG2S2
11	0.26	10.14	A3G3S2
12	0.27	10.56	A3G3S3

[a]Percentage area (the amount of glycans expressed in percentage of the total glycans measured by HPLC) and glucose unit (GU) from the HILIC−HPLC data.

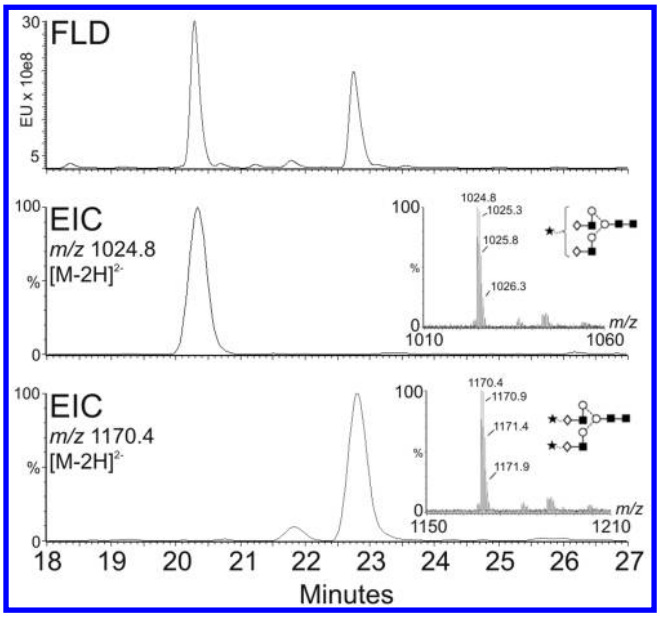

Figure 4. LC−FLD−MS of 2-AB labeled fibrinogen *N*-glycans. The extracted ion chromatograms (EIC) of the monosialylated biantennary (*m*/*z* 1024.8) and disialylated biantennary (*m*/*z* 1170.4) *N*-glycans confirm the two major peaks in the fluorescence chromatogram (FLD).

collection included EDTA, Na-heparin, and Li-heparin. Tubes of each type were drawn from 5 healthy volunteers. All samples were analyzed in two replicates to ensure reproducibility and consistency of results.

The *N*-glycomes from serum and plasma samples were analyzed by HILIC−HPLC. Profiles were separated into 16 peaks, as previously reported.[28] The area under each peak was expressed as percent of the total profile, and results were compared across categories of classification (different collecting conditions including silicone-coated glass for serum, EDTA, Na-heparin, and Li-heparin tubes for plasma samples).

There was no difference seen between the percentage areas across all peaks for all plasma conditions (EDTA, Na-heparin, Li-heparin). In addition, the delay in centrifugation of plasma samples (immediate separation, 0 d; after 24 h, 1 d; and after 48 h, 2 d) did not change the relative percentages of *N*-glycans and ratios (Table 2). However, comparison between plasma and serum data revealed variance. Further examination showed that the relative percentage areas of peak 7 (representing the A2G2S1 structure) was significantly altered in serum versus plasma samples, and in all subjects the difference was around 2% for given peak. The average percent value for peak 7 for all plasma samples was 8.95%, whereas for serum it was 6.99% (Table 2 and Figure 7).

Figure 8 represents plasma and fibrinogen profiles to denote peaks potentially contributing to serum/plasma differences. These data confirmed that most likely fibrinogen structures contributed to the around 22% increase of peak 7 in plasma samples. Glycan peak 9 is another peak that could potentially be influenced by fibrinogen presence. However, when comparing the abundance of A2G2S2 present in whole serum *N*-glycome (∼36%) that originates from many glycoproteins, increase in plasma samples due to fibrinogen was minimal.

DISCUSSION

In recent years, many reports describing *N*-glycans as potential biomarkers for various diseases have been published.[29,30,21] Different sources of clinical samples were investigated including specific glycoproteins,[31,20] cells,[32,33] seminal plasma,[34] synovial fluid,[35] and cerebrospinal fluid,[36] but the majority utilized blood fluids such as serum and plasma. These are the most popular sources as the samples may be easily and readily obtained. The use of body fluids for *N*-glycan biomarker discovery has substantial relevance to the identification of circulating disease biomarkers and has been supported by extensive research employing plasma[37] or serum samples.[38]

We decided to investigate *N*-glycan structures present on fibrinogen because this glycoprotein could play a very important role when comparing serum and plasma samples. Variation caused by presence or absence of fibrinogen in the sample being analyzed could influence analysis of *N*-glycome and discovery of potential biomarkers. A combination of complementary chromatography and mass spectrometry based approaches was used in this study to characterize fibrinogen glycosylation. All samples were processed using a newly developed method for high-throughput glycan analysis and characterized by LC and MS. The different modes of separations included three dimensions: HILIC−HPLC with exoglycosidase sequencing (one dimension), WAX-HPLC followed by HILIC−HPLC (two dimension separation), and mass spectrometry (third dimension). Each of the methods is complementary as it delivers unique and specific kinds of information that enables confident glycan structural characterization.

Fibrinogen, as an essential part of one of the most important physiological defense systems, has been intensively studied. It has been demonstrated that there are extensive post-translational modifications of the protein backbone including glycosylation, hydroxylation, phosphorylation, and sulphation.[39] Analyses of fibrinogen structural variants by Henschen-Edman et al. successfully characterized the N-terminal α chain fragment as containing a phosphorylated part, and the N-terminal γ chain fragment revealed disialo-, monosialo-, and asialo-glycan forms.[40] Topfer-Petersen et al. found that the fibrinogen β chain also featured *N*-glycosylation of fibrinogen.[41] The observation that β and γ chains are *N*-glycosylated is in agreement with our data. In addition, we confirmed that the α chain is free of *N*-glycosylation, whereas both β and γ chains contain *N*-glycans in a biantennary configuration that differs in the degree of sialylation at the end of each antennae.

Thus, our findings are consistent with existing reports of fibrinogen structure. Extending these previous studies, we describe in

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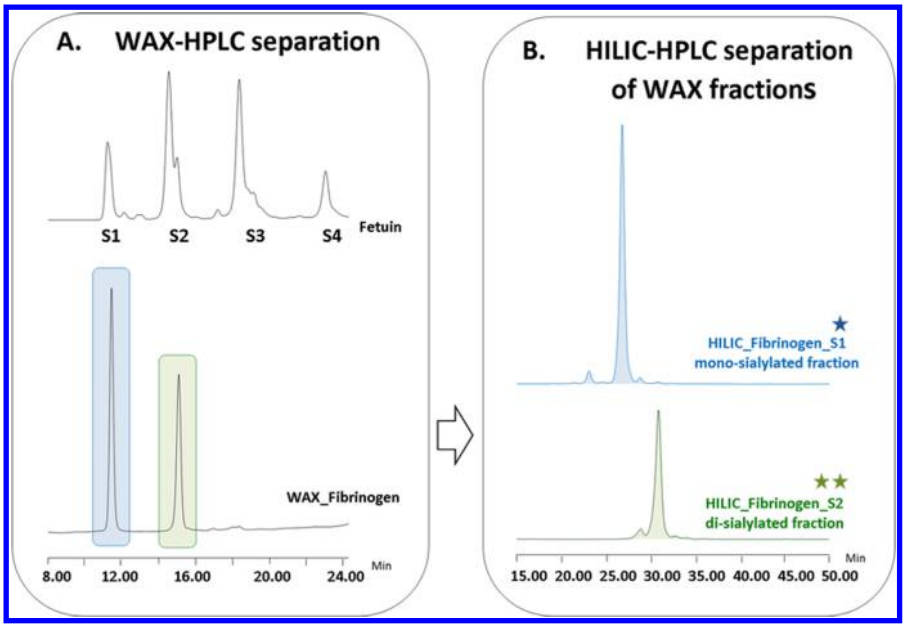

Figure 5. (A) Sialylated fractions of the fibrinogen separated by WAX-HPLC. Fetuin was used as a control. (B) Profiling of the individual fractions mono- and disialylated using HILIC−HPLC.

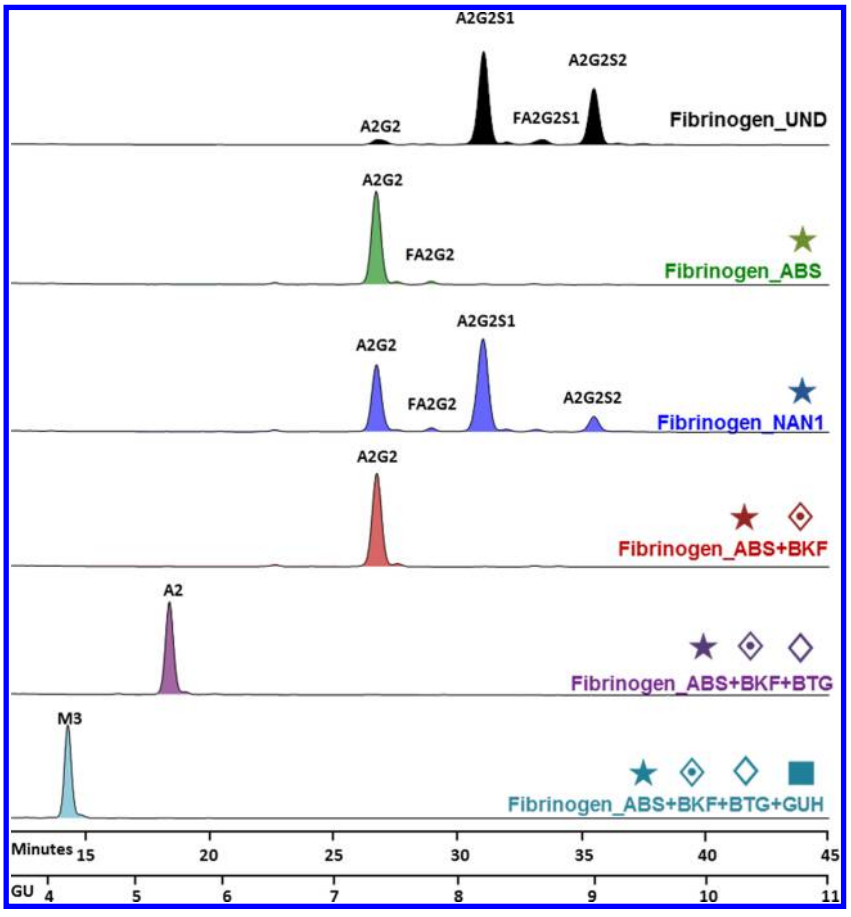

Figure 6. HILIC−HPLC profiles of fibrinogen *N*-glycans pool subjected to exoglycosidase digestions in order to assign structures. UND represents the undigested pool of glycans; this pool was digested with combinations of sialidases (ABS and NAN1), α-fucosidase (BKF), β-galactosidase (BTG), and hexosaminidase (GUH). The composition of each large peak is described alongside.

detail the fine structure and abundance of each fibrinogen-associated *N*-glycan using a combination of the chromatographic and mass spectrometry methods. Small differences in the collection, handling, or processing of samples can have dramatic effects

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Table 2. Relative Percentage Area for Peak 7 for Five Healthy Volunteers (Subjects A–E) and All Tested Conditions[a]

		peak number (major structures)															
		GP1 (A2)	GP2 (FA2)	GP3 (FA2B, A2G1, M5)	GP4 (FA2G1, FA2BG1)	GP5 (A2G2, A2BG2, A2G1S1)	GP6 (FA2G2, FA2BG2, FA2G1S1)	GP7 (A2G2S1)	GP8 (FA2G2S1, FA2BG2S1, A3G3)	GP9 (A2G2S2)	GP10 (FA2G2S2)	GP11 (FA2BG2S2)	GP12 (A3G3S2, A3BG3S2, A2F1G2S2)	GP13 (A3G3S3)	GP14 (A3F1G3S3)	GP15 (A4G4S4)	GP16 (A4F1G4S4, A4G4LacS4, A4F2G4S4)
subject A	A_EDTA_0	0.15	4.29	1.78	7.45	2.38	4.63	9.52	11.68	35.15	8.82	2.05	1.39	4.91	4.41	0.56	0.82
	A_EDTA_1	0.15	4.69	1.81	8.08	2.24	4.88	9.14	11.47	34.56	8.50	2.07	1.43	4.85	4.49	0.60	1.04
	A_EDTA_2	0.15	4.56	1.81	7.98	2.29	4.84	9.17	11.36	34.65	8.50	2.14	1.42	4.89	4.53	0.64	1.10
	A_Na_Hep_0	0.13	4.34	1.78	7.46	2.23	4.65	9.22	11.41	34.92	8.72	2.07	1.45	5.10	4.69	0.67	1.16
	A_Na_Hep_1	0.14	4.66	1.92	8.16	2.29	5.01	9.37	11.49	34.35	8.49	2.10	1.44	4.69	4.30	0.59	1.00
	A_Na_Hep_2	0.15	4.66	1.86	8.08	2.20	4.83	9.23	11.23	34.71	8.60	1.99	1.41	4.84	4.51	0.62	1.08
	A_Li_Hep_0	0.14	4.54	1.77	7.78	2.21	4.82	9.23	11.47	34.64	8.58	2.13	1.43	4.98	4.58	0.63	1.07
	A_Li_Hep_1	0.13	4.38	1.81	7.68	2.24	4.73	9.23	11.49	34.80	8.84	2.03	1.45	4.94	4.59	0.61	1.03
	A_Li_Hep_2	0.14	4.51	1.91	7.92	2.29	4.87	9.45	11.62	34.56	8.79	2.09	1.38	4.70	4.27	0.56	0.93
	A_Serum	0.13	4.48	1.64	7.65	2.27	4.74	7.46	11.89	34.63	9.46	2.23	1.42	5.35	4.87	0.66	1.11
subject B	B_EDTA_0	0.16	3.29	2.09	7.86	2.53	5.90	7.78	12.87	37.63	6.57	2.00	1.38	4.97	2.97	0.85	1.16
	B_EDTA_1	0.16	3.04	1.98	7.35	2.50	5.55	7.67	12.86	38.40	6.92	2.03	1.39	5.19	3.13	0.77	1.06
	B_EDTA_2	0.18	3.26	2.13	7.79	2.73	5.82	8.12	13.25	38.29	6.86	1.93	1.27	4.48	2.55	0.57	0.76
	B_Na_Hep_0	0.15	2.95	1.96	7.16	2.53	5.46	7.95	12.91	38.71	6.85	2.06	1.46	5.09	3.00	0.75	1.00
	B_Na_Hep_l	0.08	2.80	1.88	6.99	2.45	5.38	7.90	12.95	38.92	6.97	2.10	1.36	5.34	3.06	0.73	1.06
	B_Na_Hep_2	0.14	2.97	2.06	7.08	2.61	5.43	8.02	13.08	38.54	7.10	2.06	1.41	5.00	2.95	0.68	0.89
	B_Li_Hep_0	0.16	3.08	1.98	7.38	2.56	5.65	7.88	13.00	38.47	G.S2	2.00	1.35	5.02	2.92	0.75	0.97
	B_Li_Hep_l	0.16	3.09	2.00	7.39	2.55	5.65	7.90	12.99	38.27	6.94	1.97	1.37	5.09	2.95	0.72	0.95
	B_Li_Hep_2	0.14	2.89	1.93	6.99	2.49	5.35	7.74	12.92	38.62	7.01	2.03	1.42	5.32	3.17	0.82	1.16
	B_Serum	0.11	2.46	1.62	6.01	2.68	4.88	5.86	13.30	39.50	8.28	2.10	1.52	6.02	3.58	0.87	1.19
subject C	C_EDTA_0	0.12	2.21	1.95	8.14	2.98	8.61	9.97	14.99	32.89	7.71	1.66	1.14	3.19	3.66	0.23	0.54
	C_EDTA_1	0.11	2.32	1.88	8.20	2.89	8.61	9.73	14.79	33.23	7.69	1.63	1.18	3.32	3.81	0.26	0.33
	C_EDTA_2	0.14	2.09	1.79	7.42	2.76	7.93	9.31	14.56	33.13	8.14	1.69	1.29	3.85	4.80	0.37	0.73
	C_Na_Hep 0	0.12	2.37	1.97	8.59	2.98	8.98	9.94	15.20	32.64	7.55	1.75	1.10	3.07	3.31	0.17	0.26
	C_Na_Hep_l	0.12	2.13	1.83	7.86	2.79	8.36	9.57	14.74	33.56	7.87	1.71	1.21	3.47	4.06	0.28	0.43
	C_Na_Hep_2	0.10	2.01	1.77	7.46	2.63	8.00	9.39	14.46	33.54	8.08	1.56	1.26	3.91	4.86	0.34	0.62
	C_Li_Hep_0	0.11	2.09	1.84	7.76	2.77	8.29	9.46	14.72	33.03	7.84	1.79	1.29	3.62	4.41	0.34	0.65
	C_Li_Hep_1	0.14	2.77	2.36	9.95	3.30	9.90	10.35	15.34	30.93	7.01	1.59	0.92	2.44	2.62	0.16	0.21
	C_Li_Hep_2	0.10	2.01	1.83	7.49	2.72	8.10	9.36	14.69	33.13	7.99	1.72	1.32	3.78	4.65	0.36	0.74
	C_Serum	0.08	1.75	1.46	6.55	2.80	7.41	7.54	15.54	33.99	9.54	1.86	1.32	4.07	5.05	0.36	0.66
subject D	D_EDTA_0	0.14	5.36	2.27	7.80	2.02	4.75	8.40	11.07	36.09	7.12	2.03	1.33	4.79	5.23	0.59	1.01
	D_EDTA_1	0.18	6.71	2.91	9.56	2.46	5.5G	9.16	11.34	34.27	6.75	1.84	1.09	3.46	3.64	0.38	0.69
	D_EDTA_2	0.15	4.80	2.27	7.02	2.11	4.48	8.44	11.34	36.55	7.53	2.08	1.38	4.96	5.42	0.59	0.87

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Table 2. continued

		peak number (major structures)															
		GP1 (A2)	GP2 (FA2)	GP3 (FA2B, A2G1, M5)	GP4 (FA2G1, FA2BG1)	GP5 (A2G2, A2BG2, A2G1S1)	GP6 (FA2G2, FA2BG2, FA2G1S1)	GP7 (A2G2S1)	GP8 (FA2G2S1, FA2BG2S1, A3G3)	GP9 (A2G2S2)	GP10 (FA2G2S2)	GP11 (FA2BG2S2)	GP12 (A3G3S2, A3BG3S2, A2F1G2S2)	GP13 (A3G3S3)	GP14 (A3F1G3S3)	GP15 (A4G4S4)	GP16 (A4F1G4S4, A4G4LacS4, A4F2G4S4)
	D_Na_Hep_0	0.14	5.28	2.25	7.55	1.90	4.65	8.28	11.10	36.20	7.40	2.00	1.37	4.83	5.28	0.62	1.14
	D_Na_Hep_1	0.16	5.34	2.35	7.84	2.05	4.79	8.47	11.02	36.00	7.52	1.94	1.35	4.75	5.13	0.55	0.74
	D_Na_Hep_2	0.16	5.28	2.32	7.69	2.02	4.74	8.29	11.03	36.15	7.25	1.96	1.36	4.89	5.32	0.60	0.95
	D_Li_Hep_0	0.13	5.25	2.34	7.66	2.12	4.78	8.61	11.14	35.61	7.28	2.14	1.41	4.68	5.15	0.60	1.09
	D_Li_Hep_1	0.19	7.08	3.04	9.86	2.62	5.69	9.36	11.64	34.20	6.55	1.81	0.95	3.23	3.08	0.31	0.40
	D_Li_Hep_2	0.14	5.26	2.31	7.69	1.96	4.77	8.17	11.26	36.50	7.18	1.95	1.28	4.89	5.25	0.57	0.80
	D_Serum	0.13	5.03	2.15	7.38	2.14	4.65	6.54	11.65	36.61	8.13	2.19	1.39	5.06	5.42	0.57	0.96
subject E	E_EDTA_0	0.13	3.05	1.53	5.57	2.00	3.97	8.89	10.61	37.09	7.91	2.55	1.69	6.37	6.68	0.71	1.26
	E_EDTA_1	0.15	3.51	1.71	6.41	2.12	4.56	9.34	10.83	37.04	7.87	2.29	1.58	5.54	5.51	0.58	0.95
	E_EDTA_2	0.13	3.02	1.62	5.65	2.33	4.13	9.45	11.09	36.55	8.59	2.59	1.64	5.85	5.87	0.58	0.93
	E_Na_Hep_0	0.13	3.05	1.61	5.63	2.07	4.07	9.09	10.64	36.65	8.17	2.49	1.74	6.24	6.50	0.71	1.22
	E_Na_Hep_1	0.14	3.35	1.80	6.22	2.22	4.36	9.43	10.95	37.02	8.04	2.45	1.59	5.56	5.52	0.56	0.81
	E_Na_Hep_2	0.10	2.67	1.55	5.14	2.18	3.90	9.21	11.17	36.31	9.14	2.61	1.71	6.26	6.38	0.64	1.01
	E_Li_Hep_0	0.07	3.06	1.55	5.72	1.97	4.09	9.31	10.86	37.72	8.21	2.44	1.54	6.14	6.17	0.51	0.64
	E_Li_Hep_1	0.15	3.53	1.88	6.63	2.27	4.57	9.67	11.22	36.99	8.03	2.46	1.48	5.10	4.89	0.46	0.68
	E_Li_Hep_2	0.13	2.90	1.58	5.30	2.05	3.90	8.92	10.96	37.27	8.38	2.54	1.58	6.33	6.41	0.67	1.07
	E_Serum	0.13	2.95	1.56	5.47	2.08	3.98	7.58	11.00	36.99	8.75	2.62	1.70	6.60	6.79	0.69	1.11

[a]The table represents average values for replicates from each of the condition.

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in analytical reliability and reproducibility. The routine use of either plasma or serum may introduce biased results due to variation in its content. It is particularly important in glycomics studies to clearly distinguish these two body fluids that differ in glycoprotein content. To our best knowledge, this is the first report that investigated *N*-glycans from both types of samples. Our results showed that there is significant difference in the relative percentage area for biantennary digalactosylated monosialylated (A2G2S1) and biantennary digalactosylated disialylated (A2G2S2) structures between the plasma and serum samples. This difference appears to be largely attributable to the presence of fibrinogen in plasma samples and lack of this glycoprotein in serum samples. The results gained through this research confirm that samples from different sources should not be directly compared in glycomics projects.

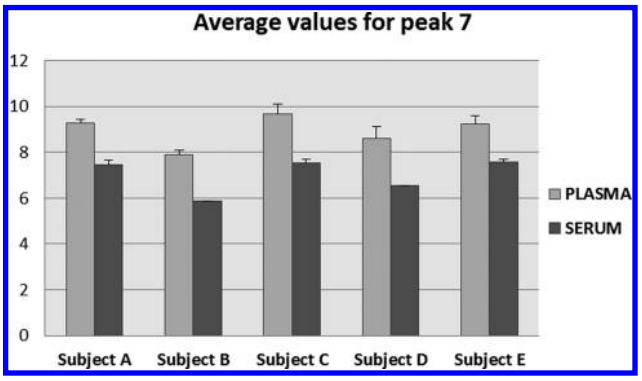

Figure 7. Average relative percentage areas for peak 7 for five healthy volunteers (subjects A–E) for both plasma and serum. Each column represents the average value for a given peak with error bars representing SD.

In order to improve and ensure the quality of blood samples to carry out an *N*-glycan analysis, we tested four different blood sampling tubes for both serum and plasma. By comparison of testing tubes, we examined how different collecting conditions might influence glycoprofiling results. Furthermore, we also aimed to verify whether delay between plasma collection and centrifugation showed any effect on *N*-glycosylation. We have discovered that only the presence or absence of anticoagulant agent influenced the outcome of analysis, while the specific anticoagulant factor (EDTA, Na-heparin, and Li-heparin) was found not to influence results. Whatever the anticoagulant used for plasma preparation, a difference for *N*-glycosylation analysis was not observed. This finding confirms that comparisons of serum versus plasma whole *N*-glycan profiles is problematic, while supporting the general comparability of plasma samples irrespective of the particular anticoagulant used.

Glycoprotein biomarker discovery and development is still a challenging task. However, with the advances in glycomics analyses, detection of glycoproteins and glycans for biomarker discovery will become more accessible for the broader scientific community. The latest glycoproteomic technologies will be applied to clinical studies more often providing more evidence that glycans play an important role in many biological processes. Glycan heterogeneity was shown to be associated with multiple factors such as age, gender, smoking, and body mass index, to name a few, as well as with numerous diseases.[28−30,42] Therefore, it is very critical to implement strict sample collection procedures to ensure high quality data and consistent results. The key variable will be case

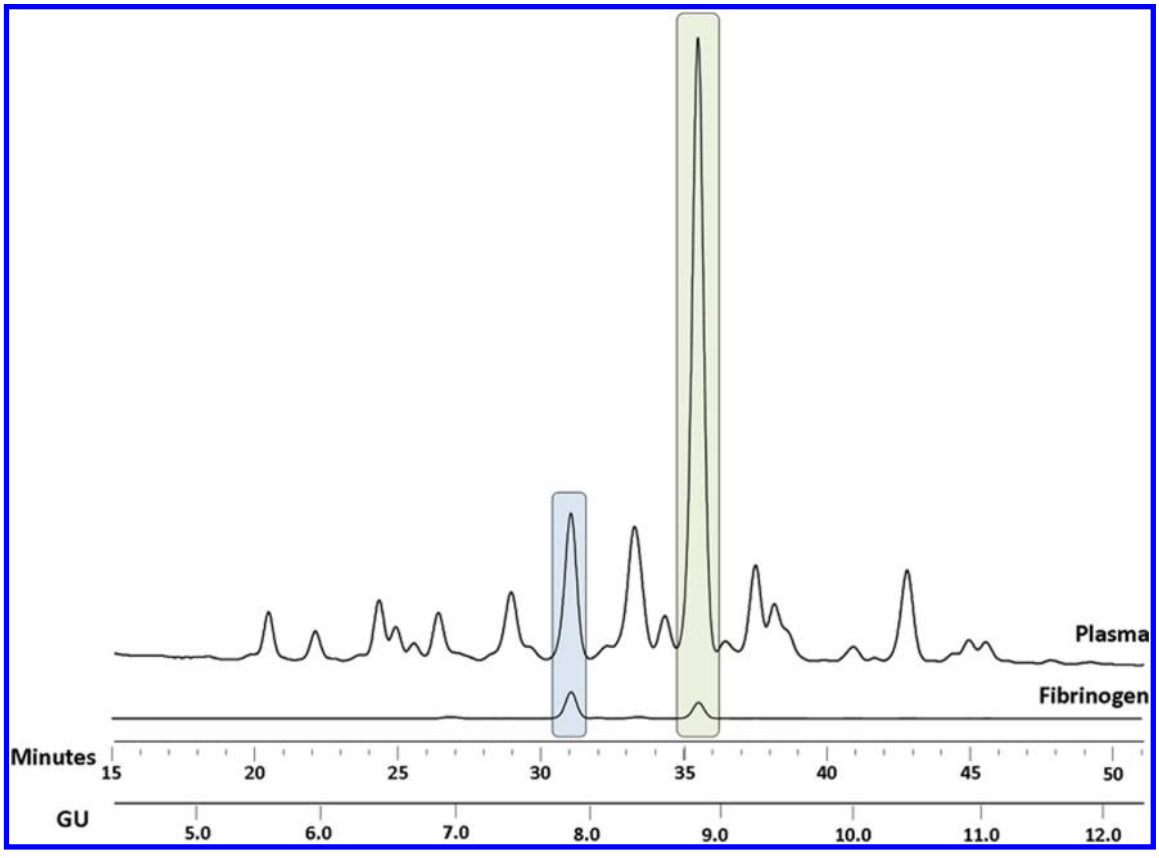

Figure 8. HILIC–HPLC chromatograms of plasma *N*-glycome and fibrinogen profile with two overlapping peaks indicated in color (A2G2S1 in blue and A2G2S2 in green).

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and control samples that should be handled in the exact same manner throughout the entire analytical process.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

2-AB, Two -aminobenzamide; ABS, *Arthrobacter ureafaciens* sialidase; BKF, bovine kidney α-fucosidase; BTG, bovine testes β-galactosidase; GU, glucose unit; GUH, hexosaminidase cloned from *Streptococcus pneumoniae* expressed in *E. coli*,; HILIC, hydrophilic liquid chromatography; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; MS, mass spectrometry; PNGaseF, peptide-*N*-glycosidase-F; PST, plasma separator tube; VEGF, vascular endothelial growth factor; WAX, weak anion exchange

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