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Quantifying Protein-Specific N-glycome Profiles by Focused Protein- and Immunoprecipitation-Glycomics

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ABSTRACT: Serum N-glycans have been reported to be potential diagnostic and therapeutic biomarkers for many diseases and conditions, such as inflammation, fibrosis, and cancer progression. We previously described the focused protein glycomic analysis (FPG) from gel-separated serum proteins. With this methodology, we sought novel glycan biomarkers for non-alcoholic steatohepatitis (NASH) and successfully identified some N-glycans which were significantly elevated in NASH patients compared to non-alcoholic fatty liver patients. Among them, tri-sialylated mono-fucosylated tri-antennary glycan (A3F) of alpha-1 antitrypsin showed the most dynamic change. For rapid identification of N-glycans on the focused proteins, we constructed a simplified method called an immunoprecipitation-glycomics (IPG), where the target proteins were immuno-precipitated with affinity beads and subsequently subjected to glycomic analysis by MALDI-TOF MS. Focusing on alpha-1 antitrypsin and ceruloplasmin as the target proteins, we compared the values of N-glycans determined by FPG and IPG. The quantified values of each N-glycan by these two methods showed a statistically significant correlation, indicating that high throughput and quantitative N-glycomics of targeted proteins can be achieved by the

simplified IPG method. Thus, an analytical strategy combining FPG and IPG can be adapted to general biomarker discovery and validation in appropriate disease areas.

KEYWORDS: biomarker; glycomics; fucosylation; alpha-1 antitrypsin; Ceruloplasmin; NASH; MALDI-TOF MS

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome which is frequently associated with obesity, diabetes mellitus and dyslipidemia. NAFLD develops in patients who do not consume significant amounts of alcohol and shares liver histological similarity with alcoholic liver diseases.[1] NAFLD is divided into non-alcoholic fatty liver (NAFL), which is a non-progressive form of NAFLD, and non-alcoholic steatohepatitis (NASH), which is a progressive form of NAFLD that can lead to cirrhosis and the development of hepatocellular carcinoma (HCC).[2,3] To date, the diagnosis and staging of NAFLD rely heavily on liver biopsy. However, liver biopsy is an invasive procedure that may lead to undesirable complications[4,5] and sampling bias. Several noninvasive biomarkers and scoring systems have been reported,[6] but no established diagnostic tests or biomarkers are available at present. Thus, identifying and validating novel non-invasive biomarkers would be valuable.

Glycosylation is one of the most common post-translational modifications occurring in proteins. Glycosylation can affect the biological activity of proteins, their transport towards the cell surface and the stabilization of their functional conformation.[7] Glycans are the determinants of some clinically-used cancer biomarkers, such as CA19-9, CA125 and AFP-L3. Recently,

Mac-2 Binding Protein Glycosylation isomer (M2BPGi) was reported as a novel fibrosis biomarker[8] and clinically used in Japan. Miyoshi et al. reported that fucosylated haptoglobin could serve as a biomarker for pancreatic cancer[9] and also predict the presence of ballooning hepatocytes in NAFLD.[10] As represented by these molecules, the changes of glycosylation reflecting disease severity are now receiving increasing attention in various areas of disease research, including chronic liver diseases.

Because the liver is a major source of circulating serum proteins, the progression of liver diseases can be expected to be reflected in the circulating glycoproteins. Some studies have shown that changes in the N-glycosylation of total serum proteins can serve as non-invasive diagnostic markers for liver-related diseases, including hepatocellular carcinoma, cirrhosis, chronic hepatitis B and non-alcoholic steatohepatitis.[11-15] Such alterations in serum N-glycome often result from the glycomic change of a subset of serum proteins, and importantly, protein-specific alterations in glycosylation may serve as more specific biomarkers than the total serum glycomic profile.[16] In fact, many studies have documented that alterations in serum protein-specific N-glycans can be diagnostic markers for liver-related diseases.[17-19] Therefore, analyzing serum protein-specific N-glycomes should be a powerful strategy for seeking novel biomarkers of liver diseases.

We previously developed the focused protein glycomics (FPG) procedure, which allows analysis of glycan profiles of gel-separated serum proteins by MALDI-TOF MS.[20] In that study, we analyzed the N-glycan status of major serum glycoproteins from mouse models of progressive liver disease, and demonstrated that several glycans changed significantly with the progression of liver disease. In the present study, we determined the N-glycomic profile of

focused serum glycoproteins from NAFL and NASH patients by the FPG method, and successfully discovered NASH biomarker candidates.

The FPG procedure makes it possible to obtain comprehensive protein-specific N-glycomic profiles, but protein fractionation by affinity columns and protein extraction from the polyacrylamide gel are not suitable for the measurement of tens or hundreds of clinical samples, which is generally required for validation study of biomarker candidate molecules. To resolve this problem, we constructed an immunoprecipitation-glycomics (IPG) method that enables rapid purification of the target proteins from human serum samples for the subsequent analysis of N-glycosylation. By comparing N-glycomic profiles of the targeted serum glycoproteins from NAFL and NASH patients, we verified the usefulness of the IPG procedure, and validated the NASH biomarker candidate that was discovered by FPG analysis. We also investigated the sialic acid linkage of the biomarker candidate by sialic acid linkage specific alkylamidation (SALSA) technique.[21]

Experimental Section

Study populations. Five NAFL and six NASH patients, who had been biopsy-proven, from Hokkaido University Hospital were included in this study. Diagnosis of NASH was based on the fatty liver inhibition of progression (FLIP) algorithm.[22] Clinical and biochemical data of the patients are shown in Table S1. The exclusion criteria from this study included daily alcohol consumption >30 g for men and >20 g for women and a history of hepatic disease, such as hepatitis B, hepatitis C, hepatocellular carcinoma, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's

Disease and congested liver. The protocol and informed consent were approved by the institutional review board at Hokkaido University Hospital.

N-Glycosylation analysis of proteins extracted from polyacrylamide gel. Human serum proteins were fractionated using the Multiple Affinity Removal System (Agilent; Santa Clara, CA) following the manufacturer's instructions. Briefly, 50 μL of human serum was diluted with 150 μL of Buffer A (Agilent) and filtered using Cellulose Acetate Spin Filters (0.22 μm), then loaded onto a Multiple Affinity Removal Spin Cartridge HSA/IgG (Agilent) for the depletion of Human Serum Albumin and Immunoglobulin G. After washing with Buffer A, proteins bound to the column were eluted with Buffer B (Agilent) and used for subsequent analyses as the "HSA/IgG fraction". The flow-through fraction and the wash fraction of Multiple Affinity Removal Spin Cartridge HSA/IgG were combined, and one-fifth was loaded onto the Human 14 Multiple Affinity Removal System Spin Cartridge (Agilent). Proteins bound to the column were eluted with Buffer B and used for subsequent analyses as the "high-abundance protein (HAP) fraction". The flow-through fraction and the wash fraction of Human 14 Multiple Affinity Removal System Spin Cartridge were combined and used for subsequent analyses as the "low-abundance protein (LAP) fraction". The solvent of each fraction was replaced with PBS using size-exclusion spin columns (5k MWCO). The protein concentration of each fraction was determined by bicinchoninic acid assay, and 10 μg of proteins of each fraction was separated by 4-12% gradient polyacrylamide gel with MOPS buffer under non-reducing condition (for HSA/IgG fraction and HAP fraction) or reducing condition (for HAP fraction and LAP fraction). The major protein bands after CBB staining were excised from the gel and subjected to tryptic digestion with In-Gel Trypsin Digestion Kit (Pierce Biotechnology; Waltham, MA). For the analysis of N-glycosylation, trypsinized samples were subsequently treated with 1 U of N-

glycosidase F (PNGase F, Roche; Basel, Switzerland). Protein Identification was done by peptide mass fingerprinting and MS/MS analysis, as described in Supporting Information.

MALDI-TOF and TOF/TOF MS analysis of glycan profiles of serum glycoproteins. Released N-Glycans from standard glycoprotein and separated glycoproteins were subjected to glycoblotting procedure[23] and MALDI-TOF MS analysis as described in the Supporting Information. Each glycan's concentration were calculated by the following equation: quantity of each glycan (pmol) / serum volume (μL). Representative MS/MS spectra of N-glycans measured in this study are shown in Figure S1.

Immunoprecipitation of α1-antitrypsin from human serum and its N-glycomic analysis. For the N-glycomic analysis of α1-antitrypsin (AAT), 50 μL of human serum was diluted with 150 μL of 20 mM phosphate buffer (pH 7.0) and filtered with a 0.45 μm centrifugal filter unit (Ultrafree-MC HV; Millipore). For the depletion of IgG and albumin, 70 μL of Protein G Sepharose (GE Healthcare; Little Chalfont, United Kingdom; 70% slurry) and 350 μL of Affigel-Blue Gel (Bio-Rad, 70% slurry) were prepared in another 0.45 μm centrifugal filter unit and equilibrated with 20 mM phosphate buffer (pH 7.0). Diluted serum was loaded onto the filter unit containing Protein G and Affigel-Blue Gel, and incubated at room temperature for 10 minutes with horizontal shaking. The flow-through fraction after the centrifugation (2,500 x g, room temperature, 1 min) and the wash fraction after washing with 250 μL phosphate buffer (pH 7.0) were mixed, and saline was added to obtain a final concentration of 150 mM. In another filter unit, 20 μL of Alpha-1 antitrypsin Select (60% slurry; GE Healthcare), which is the packed-bed affinity chromatography resin with high selectivity for AAT, was prepared and equilibrated with phosphate-buffered saline (20 mM phosphate buffer with 150 mM NaCl). IgG/HSA-depleted serum was loaded onto the filter unit containing Alpha-1 antitrypsin Select and

incubated at room temperature for 30 minutes with horizontal shaking. After washing three times with 200 μL PBS, AAT was eluted with 2 M $MgCl_2$ in 20 mM Tris-HCl (pH 7.0). The buffer of the eluate was exchanged by repeating condensation and dilution with 10 mM ammonium bicarbonate, using Amicon Ultra 0.5 mL centrifugal filter (Millipore). The concentrations of AAT in sera and the purified fractions were determined by Human Serpin A1 DuoSet ELISA (R&D Systems) following the manufacturer's instructions.

For the N-glycomic analysis, 10 μg of purified AAT was trypsinized and N-glycans were released with 1 U PNGase F. The released glycans derived from 2 μg AAT were subjected to glycoblotting and HILIC purification, followed by MALDI-TOF MS analysis. The serum concentrations of AAT-glycans were calculated from the amount of each glycan per 1 μg AAT determined by MALDI-TOF MS and the serum concentration of AAT by ELISA.

Immunoprecipitation of Ceruloplasmin from human serum and its N-glycomic analysis. Ceruloplasmin (CP) was purified from human sera as previously described[24] with minor modifications. To obtain anti-human CP antibody beads, 7.5 μg mouse anti-human CP monoclonal antibody (clone 3B11) was crosslinked to 40 μL Pierce Protein A/G Plus Agarose, using a Pierce Crosslink Immunoprecipitation Kit (Pierce) according to the manufacturer's instructions. Human serum, 25 μL, was diluted with 25 μL of 20 mM phosphate buffer (pH 7.0) and filtered with a 0.45 μm centrifugal filter unit (Ultrafree-MC HV). In another 0.45 μm centrifugal filter unit, 17.5 μL of Protein G Sepharose, 17.5 μL of Protein A Sepharose, and 55 μL of Affigel-Blue Gel were prepared and equilibrated with 20 mM phosphate buffer (pH 7.0). Diluted serum was loaded onto the filter unit containing Protein G, Protein A and Affigel-Blue Gel, and incubated at room temperature for 20 minutes with horizontal shaking. The flow-through fraction after the centrifugation (1,000 x g, room temperature, 1 min) and the wash

fraction after washing with 75 μL 20 mM phosphate buffer (pH 7.0) were mixed and used in the next step. To completely remove IgG and IgM, albumin/IgG-depleted serum was loaded onto the new 0.45 μm centrifugal filter unit containing 25 μL Pierce Protein A/G Plus Agarose and 25 μL Capto L (GE Healthcare), which was equilibrated with 20 mM phosphate buffer (pH 7.0). After incubating at room temperature for 30 minutes with horizontal shaking, the flow-through fraction after the centrifugation (1,000 x g, room temperature, 1 min) and the wash fraction after washing with 50 μL 20 mM phosphate buffer (pH 7.0) were mixed, and saline was added to obtain a final concentration of 150 mM. The albumin/IgG/IgM-depleted serum was loaded onto anti-human CP antibody beads, and incubated overnight at 4°C. After washing twice with 200 μL PBS, CP was eluted with 2 M $MgCl_2$ in 20 mM Tris-HCl (pH 7.0). The buffer of the eluate was exchanged by repeating condensation and dilution with 10 mM ammonium bicarbonate, using Amicon Ultra 0.5 mL centrifugal filter. N-Glycans of CP were analyzed by the same method used for AAT.

RESULTS AND DISCUSSION

FPG analysis of glycoproteins from NAFL/NASH serum samples. We previously described the analytical method of N-glycosylation of focused proteins (FPG) from mouse sera.[20] We modified this procedure for analyzing N-glycans of human serum proteins. Figure 1A shows the scheme of human serum protein separation and the subsequent N-glycomic analysis by FPG. To search for the novel NASH biomarker, sera from five NAFL and six NASH patients were used in this study. Serum samples were fractionated with the Multiple Affinity Removal System into three fractions: HSA/IgG fraction, high-abundance protein (HAP) fraction, and low-abundance

protein (LAP) fraction. After SDS-PAGE separation and CBB staining (Figure 2 and S2 for entire gel images), the major protein bands of each fraction were extracted from polyacrylamide gel and identified by Mascot database search (http://www.matrixscience.com/search_form_select.html) after MALDI-TOF MS and MS/MS analysis. We chose seven proteins to perform N-glycosylation analysis in this study (Table 1).

N-glycans of glycoproteins were purified and labeled through glycoblotting procedure,[23] and quantified by MALDI-TOF MS. Despite the fact that the glycans' ionization efficiencies could be strictly different depending on the glycan structure, we performed absolute quantitation by comparing the signal intensity of each glycan with that of the known amount of spiked internal standard (NeuAc2Gal2GlcNAc2 + Man3GlcNAc1; A2GN1) based on our previous observation that the signal strengths of the various aoWR-labeled glycans were fairly similar, irrespective of their structure. The accuracy and precision of the absolute quantitation are shown[25]. In this study, we calculated the quantitative values of each glycan using A2GN1 in the same manner. Figure S3A shows the calibration curves of the various glycan amounts and good linearity with the amounts of AAT.

Table 2 shows N-Glycosylation profile of seven serum glycoproteins. MS/MS spectra of protein N-glycan were also obtained as shown in Figure S1. The level of tri-sialylated mono-fucosylated tri-antennary glycan (H3 N3 F1 S3 + core; A3F) of alpha-1 antitrypsin (AAT) significantly increased in NASH compared to NAFL (3.78 times higher in NASH). The level of H3 + core glycan (Man6) of complement C3 was significantly lower in NASH compared to NAFL. The levels of H2 N2 S1 + core (A1), H2 N2 S2 + core (A2) and H3 N3 S3 + core glycan (A3) of ceruloplasmin (CP) were significantly higher in NASH patients.

Because A3F glycan of AAT (AAT-A3F) showed the most dynamic elevation in NASH patients, we focused on AAT glycosylation in the subsequent study. As any gel band may contain a number of proteins, the abundance of AAT in the corresponding band was estimated by comparing the spectra of tryptic peptides obtained using HAP fraction of serum with those obtained from commercially available standard AAT. As shown in Figure S4A and B, MS spectra of tryptic peptides were quite similar, suggesting that AAT is predominantly major component of the band and observed N-glycans were derived mainly from AAT.

N-Glycomic analysis of AAT by the IPG method. For the high-throughput determination of AAT-A3F, we developed an analytical method using the immunoprecipitation technique (IPG) (Figure 1B). In this method, AAT was purified from sera using commercially available immuno-affinity beads and the recovery rate was more than 60% by comparing the amounts before and after immunoprecipitation (Figure S5C). The purity of the immunoprecipitated AAT was confirmed by a single band on SDS-PAGE (Figure S5A). To further confirm the degree of the purification by IPG, standard AAT was processed with IPG procedure, and the spectra of tryptic peptides were compared with those of serum-purified AAT. The spectra were quite similar (Figure S4C and D), indicating that AAT were highly purified by IPG.

To compare the quantitative values of AAT-A3F by FPG and IPG, we prepared sera containing 4.6, 9.3, 18.5, 37 and 74 μM AAT using AAT-depleted serum and commercially available AAT, and AAT-A3F was measured by both two methods. As shown in Figure 3A and B, good linearity was observed between concentrations of AAT and that of AAT-A3F determined by FPG or IPG. Importantly, AAT-A3F values obtained by FPG and IPG highly correlated (Figure 3C), demonstrating that quantification of protein specific glycans by FPG and IPG would lead to the same results. It should be noted that the values from IPG were higher than

those from FPG, which can be explained by the low recovery rate of target proteins in FPG analysis. The recovery rate of AAT-glycans extracted from SDS gel band was estimated to be about 25% (Figure S3B and C), which would lead to underestimated values in FPG. It was reported that AAT are present around 20 μM in human serum and has three N-glycosylation sites.[26] In this study, the mean serum AAT-A3F concentrations of NAFL and NASH patients were 0.53 ± 0.34 and 1.83 ± 1.09 μM, respectively (Table 3). These results indicated that A3F constituted approximately 2% of total glycans. One of the advantages of the IPG method is that more glycan structures can be detected because of the higher recovery rate of target proteins. Taken together, targeted proteins discovered by FPG method can be efficiently purified by IPG method and used for detailed glycomic analysis.

Using IPG method, we analyzed the glycomic profiles of AAT in serum samples which had been analyzed by FPG. Similarly to the results obtained by FPG, AAT-A3F showed significant and marked elevation in NASH compared to NAFL (Table 3). When the glycan amounts of each patient determined by FPG and IPG were compared, a good correlation was observed (Figure 4A) and this was statistically significant ($P<0.001$), indicating that N-glycans of targeted proteins can be quantitatively determined by the simplified IPG method.

Structural information of AAT-A3F. To obtain structural information of AAT-A3F, we employed the sialic acid linkage specific alkylamidation (SALSA) technique[21] to distinguish α2,3-/α2,6-linkage isomers. Sialic acids of AAT-A3F glycan turned out to be of two α2,6- and one α2,3-linkages (Figure S6) and other isomers were not detected. Furthermore, to clarify the position of fucosylation, AAT-glycans were treated with alpha 1-3/4 fucosidase which specifically cleaves outer-arm fucose residues. Ninety to ninety-five percent of fucose residues

on AAT-A3F from all patients were cleaved by alpha 1-3/4 fucosidase (Figure S7 and S8), suggesting that the fucose residue of AAT-A3F is mainly on the outer-arm with α2,3-sialic acid.

Reports have accumulated regarding the association of liver diseases and the glycosylation of AAT, especially fucosylation,[27,28] but whether the glycosylation of AAT changes with NAFLD progression is not known. In our study, we found that the serum level of A3F glycan on AAT was significantly higher in NASH patients compared to NAFL patients. Importantly, the serum concentrations of AAT itself were not different between these two pathological states (Figure S9A), indicating that the elevation of AAT-A3F in NASH could be attributed to alterations of N-glycosylation. It was reported that outer arm fucosylation of liver-secreted glycoproteins is mainly regulated by fucosyltransferase FUT6.[29] The expression of FUT6 was reported to be positively regulated by interleukin 6,[30] which is a possible inflammatory regulator of NASH.[31,32] Thus, elevated outer-arm fucosylation of AAT might be associated with the hepatic inflammation in NASH patients. Further studies will be needed to clarify the underlying mechanisms of glycosylational alterations in NASH.

N-Glycomic analysis of CP by IPG method. To verify that N-glycomic analysis by IPG can be applied to proteins other than AAT, we developed the IPG method for ceruloplasmin (CP), which was one of the proteins for which we carried out FPG analysis. We selected CP for the IPG analysis based on the following reasons. First, significant elevation of several N-glycans on CP was observed in the FPG analysis. The second reason was because CP was found in the LAP fraction through FPG fractionation, IPG construction of CP should allow application of this approach to other serum proteins found in the LAP fraction. The third reason was that antibody-coupled beads for the purification of CP were not commercially available, as is the case for most target proteins. We crosslinked commercially available anti-human CP monoclonal antibody to

Protein A/G Plus-Agarose, and CP was purified from sera in the same manner as AAT. The concentrations of CP in sera and the purified fractions were determined by ELISA, and the recovery rate of CP from sera was found to be more than 60%. We confirmed high purity of immunoprecipitated fractions by SDS-PAGE separation (Figure S5B). When the N-glycomic profile of purified CP was determined by MS, the quantified values of A2 correlated well with those determined by FPG, with a correlation coefficient of 0.83 (Figure 4B). Therefore, we concluded that N-glycomic analysis by IPG can be generally applied to serum glycoproteins, even if the target protein is included in the LAP fraction in FPG fractionation. The results above also indicate that the glycomic analysis of focused proteins by IPG can be achieved if antibodies against the target proteins are available.

The relationship between N-glycomic changes of CP and liver diseases is almost unknown, except for one report that core-fucosylated glycans on CP increased significantly in alcohol-related HCC samples compared to that in alcohol-related cirrhosis samples.[24] In this study, we discovered glycosylational alterations on CP in NASH patients, although the underlying mechanisms are not clear. The serum concentrations of CP remained unchanged between NAFL and NASH (Figure S9B), which showed that the glycosylation pattern changed specifically with disease progression.

The results obtained from the N-glycomic analysis of AAT and CP led us to conclude that the N-glycomic analyses of specific proteins can be achieved either by FPG or by IPG. FPG meets the demands for comprehensive analysis of protein-specific N-glycans that can be applicable for the screening of disease biomarkers, while IPG enables the high-throughput purification of targeted protein from biological samples and determination of N-glycomic profiles of purified proteins.

CONCLUSION

N-Glycomic analysis of targeted proteins by FPG is a useful method for biomarker discovery. But in order to validate biomarker candidates using tens or hundreds of clinical samples, glycomic profiling by the FPG approach is laborious and time-consuming. To overcome this, we developed a simplified method (IPG) for the N-glycomic analysis of focused proteins, using the immunoprecipitation technique. It was previously shown that immunoprecipitation enables efficient purification of target proteins and determination of protein-specific N-glycosylation when combined with MALDI-TOF MS or liquid chromatography coupled to mass spectrometry (LC/MS).[33-36] We chose two serum glycoproteins, AAT and CP, as the target proteins of IPG, because their glycosylation patterns were found to differ between NAFL and NASH by FPG analysis. When N-glycans of these two proteins were determined by FPG and IPG methods, the quantified values of each N-glycan by these two methods showed a good correlation coefficient and provided quantitative glycan information. This strongly indicates that the same results can be obtained by these two approaches: FPG, which is suitable for biomarker explorations by comprehensively analyzing N-glycans of gel-separated proteins; and IPG, which can be applicable to the measurement of the large numbers of samples needed to validate biomarker candidates. We propose IPG is a useful method for general biomarker validation studies. This method should expand the range of biomarker research for many diseases, because glycosylation may change more sensitively than its carrier proteins as presented in this study and previous reports.[16]

We demonstrated the N-glycomic alterations of various serum proteins from NAFL and NASH patients using MALDI-TOF MS after glycoblotting strategy. Among these changes, AAT-A3F exhibited the most dynamic elevation in NASH. We suggest that protein-specific glycosylation, especially AAT-A3F, can serve as potential biomarkers for NASH. A validation study of AAT-A3F with larger numbers of NAFLD patients is now in progress.

Figure 1. Scheme of N-glycomic analysis of human serum proteins: A, FPG analysis; B, IPG analysis.

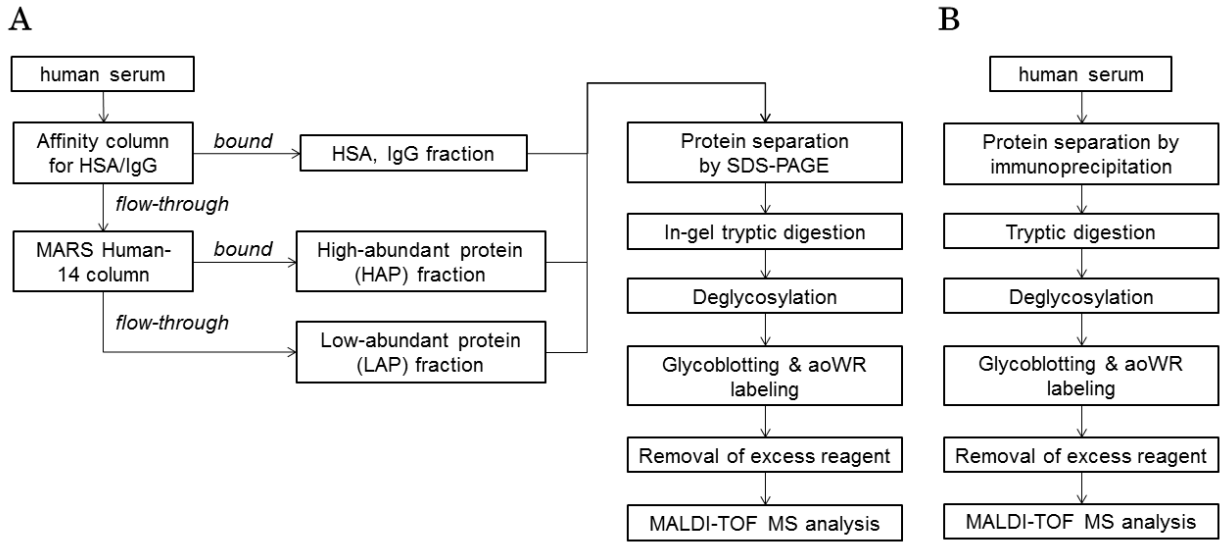


Figure 2. Representative SDS-PAGE image of human serum proteins. Proteins marked with triangles were subjected to N-glycomic analysis. The list of identified proteins is shown in Table 1.

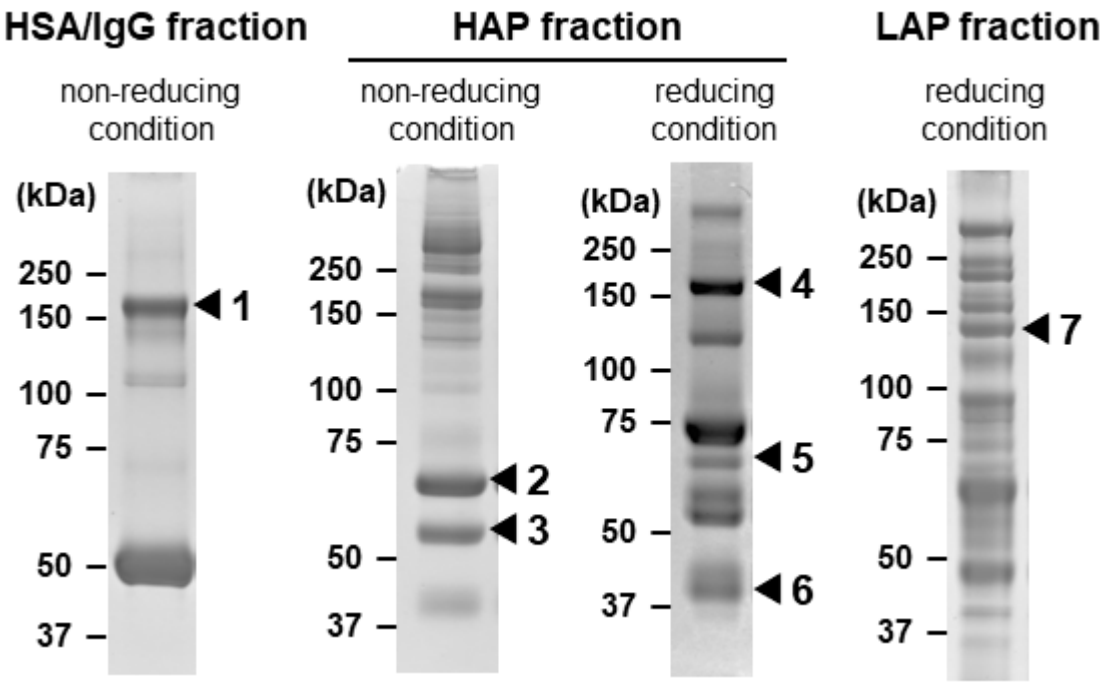


Table 1. List of serum glycoproteins analyzed by FPG.

Band No.	Protein Name	Accession No. (UniProtKB)	Protein sequence coverage (%)	Total number of peptides assigned	Matched number of peptides	Number of N-glycosylation sites
1	Immunoglobulin gamma-1 heavy chain	P0DOX5	46	89	18	2
2	Serotransferrin	P02787	61	96	51	3
3	Alpha-1-antitrypsin	P01009	50	82	23	3
4	Alpha-2-macroglobulin	P01023	42	100	53	8
5	Complement C3 (β chain)	P01024	26	90	44	1
6	Haptoglobin (β chain)	P00738	52	85	20	4
7	Ceruloplasmin	P00450	60	61	44	4

Table 2. N-Glycosylation profile of serum glycoproteins determined by FPG analysis.

Protein	Structure	Serum Conc. (Average ± SD)		ratio	*p*-value
		NAFL (N=5)	NASH (N=6)		
Immunoglobulin G	N2 F1 + core	19.73 ± 9.74	25.31 ± 15.66	1.28	
	H1 N2 + core	0.27 ± 0.17	0.53 ± 0.33	1.99	
	H1 N2 F1 + core	22.78 ± 5.90	26.03 ± 10.64	1.14	
	H2 N2 + core	0.13 ± 0.07	0.25 ± 0.11	1.92	
	H2 N2 F1 + core	6.49 ± 1.79	7.30 ± 4.42	1.12	
	N3 F1 + core	1.72 ± 0.85	1.94 ± 0.71	1.13	
	H1 N3 F1 + core	1.74 ± 0.39	1.69 ± 0.46	0.97	
	H2 N3 F1 + core	0.11 ± 0.02	0.12 ± 0.08	1.09	
	H1 N2 F1 S1 + core	0.33 ± 0.16	0.54 ± 0.33	1.63	
	H2 N2 F1 S1 + core	2.41 ± 0.94	3.55 ± 3.49	1.47	
	H2 N2 F1 S2 + core	0.15 ± 0.14	0.24 ± 0.21	1.57	
	H2 N3 F1 S1 + core	0.19 ± 0.12	0.22 ± 0.15	1.14	
Transferrin	H2 N2 S1 + core	2.91 ± 0.75	2.48 ± 0.33	0.85	
	H2 N2 F1 S1 + core	0.04 ± 0.02	0.06 ± 0.01	1.54	
	H2 N2 S2 + core	38.44 ± 5.88	35.4 ± 4.28	0.92	
	H2 N2 F1 S2 + core	0.37 ± 0.17	0.50 ± 0.31	1.37	
	H3 N3 S2 + core	0.07 ± 0.03	0.06 ± 0.01	0.83	
	H3 N3 S3 + core	0.15 ± 0.05	0.10 ± 0.03	0.67	
Alpha-1 antitrypsin	H2 N2 S1 + core	3.59 ± 0.88	4.05 ± 1.22	1.13	
	H2 N2 F1 S1 + core	0.10 ± 0.05	0.24 ± 0.16	2.40	

	H2 N2 S2 + core	44.73 ± 9.32	45.57 ± 13.76	1.02	
	H2 N2 F1 S2 + core	0.66 ± 0.32	1.38 ± 0.83	2.08	
	H3 N3 S2 + core	0.16 ± 0.05	0.16 ± 0.04	0.99	
	H3 N3 S3 + core	1.34 ± 0.36	1.19 ± 0.32	0.89	
	H3 N3 F1 S3 + core	0.07 ± 0.03	0.25 ± 0.11	3.78	**<0.01**
Alpha-2 macroglobulin	H2 + core	0.78 ± 0.36	0.56 ± 0.47	0.71	
	H3 + core	0.05 ± 0.02	0.07 ± 0.10	1.28	
	H2 N2 + core	0.12 ± 0.06	0.10 ± 0.09	0.86	
	H2 N2 F1 + core	0.10 ± 0.05	0.07 ± 0.07	0.74	
	H2 N1 S1 + core	0.31 ± 0.21	0.30 ± 0.31	0.96	
	H3 N1 S1 + core	0.32 ± 0.22	0.29 ± 0.32	0.91	
	H1 N2 S1 + core	0.14 ± 0.09	0.19 ± 0.24	1.37	
	H2 N2 S1 + core	4.51 ± 2.99	4.18 ± 3.80	0.93	
	H2 N2 F1 S1 + core	0.81 ± 0.36	0.70 ± 0.60	0.86	
	H2 N2 S2 + core	12.60 ± 8.64	11.82 ± 9.92	0.94	
	H2 N2 F1 S2 + core	0.20 ± 0.08	0.26 ± 0.27	1.30	
Complement C3 (β chain)	H2 + core	0.08 ± 0.05	0.04 ± 0.02	0.51	
	H3 + core	0.74 ± 0.37	0.19 ± 0.04	0.25	**<0.05**
	H2 N2 S1 + core	0.03 ± 0.02	0.04 ± 0.02	1.12	
	H2 N2 S2 + core	0.26 ± 0.23	0.25 ± 0.18	0.97	
Haptoglobin	H2 N2 S1 + core	6.46 ± 5.82	4.25 ± 3.49	0.66	
	H2 N2 S2 + core	39.27 ± 33.41	22.25 ± 15.29	0.57	
	H2 N2 F1 S2 + core	0.11 ± 0.11	0.07 ± 0.05	0.62	

	H3 N3 S2 + core	1.20 ± 1.17	0.58 ± 0.53	0.49	
	H3 N3 S3 + core	7.67 ± 7.01	3.49 ± 2.86	0.45	
	H3 N3 F1 S3 + core	0.22 ± 0.19	0.25 ± 0.17	1.14	
Ceruloplasmin	H2 N2 S1 + core	0.04 ± 0.01	0.09 ± 0.04	2.16	**<0.05**
	H2 N2 S2 + core	0.86 ± 0.16	1.68 ± 0.69	1.96	**<0.05**
	H2 N2 F1 S2 + core	0.04 ± 0.02	0.17 ± 0.19	4.15	
	H3 N3 S3 + core	0.02 ± 0.01	0.05 ± 0.02	2.18	**<0.05**

Detailed N-glycan informations derived from human serum proteins are summarized in Table S2 in the Supporting Information. H, Hexose; N, N-acetylhexosamine; F, Fucose; A, N-acetylneuraminic acid; core, (Man)3(GlcNAc)2.

Table 3. N-Glycosylation profile of serum alpha-1 antitrypsin from individual serum samples determined by IPG analysis.

Structure	Serum Conc. (Average ± SD)		ratio	*p*-value
	NAFL (N=5)	NASH (N=6)		
H2 N2 S1 + core	3.06 ± 0.51	3.3 ± 0.58	1.08	
H2 N2 F1 S1 + core	0.13 ± 0.04	0.24 ± 0.06	1.87	**<0.01**
H2 N2 S2 + core	79.42 ± 13.12	100.92 ± 21.8	1.27	
H2 N2 F1 S2 + core	4.09 ± 1.41	6.47 ± 2.33	1.58	
H3 N3 S2 + core	0.27 ± 0.08	0.27 ± 0.03	1.02	
H3 N3 S3 + core	5.75 ± 2.11	6.14 ± 0.8	1.07	
H3 N3 F1 S3 + core	0.53 ± 0.34	1.83 ± 1.09	3.45	**<0.05**

Figure 3. AAT-A3F measured by FPG (A) or IPG (B) using human serum containing 4.6 – 74.1 μM AAT. Correlation between AAT-A3F determined by FPG and IPG (C)

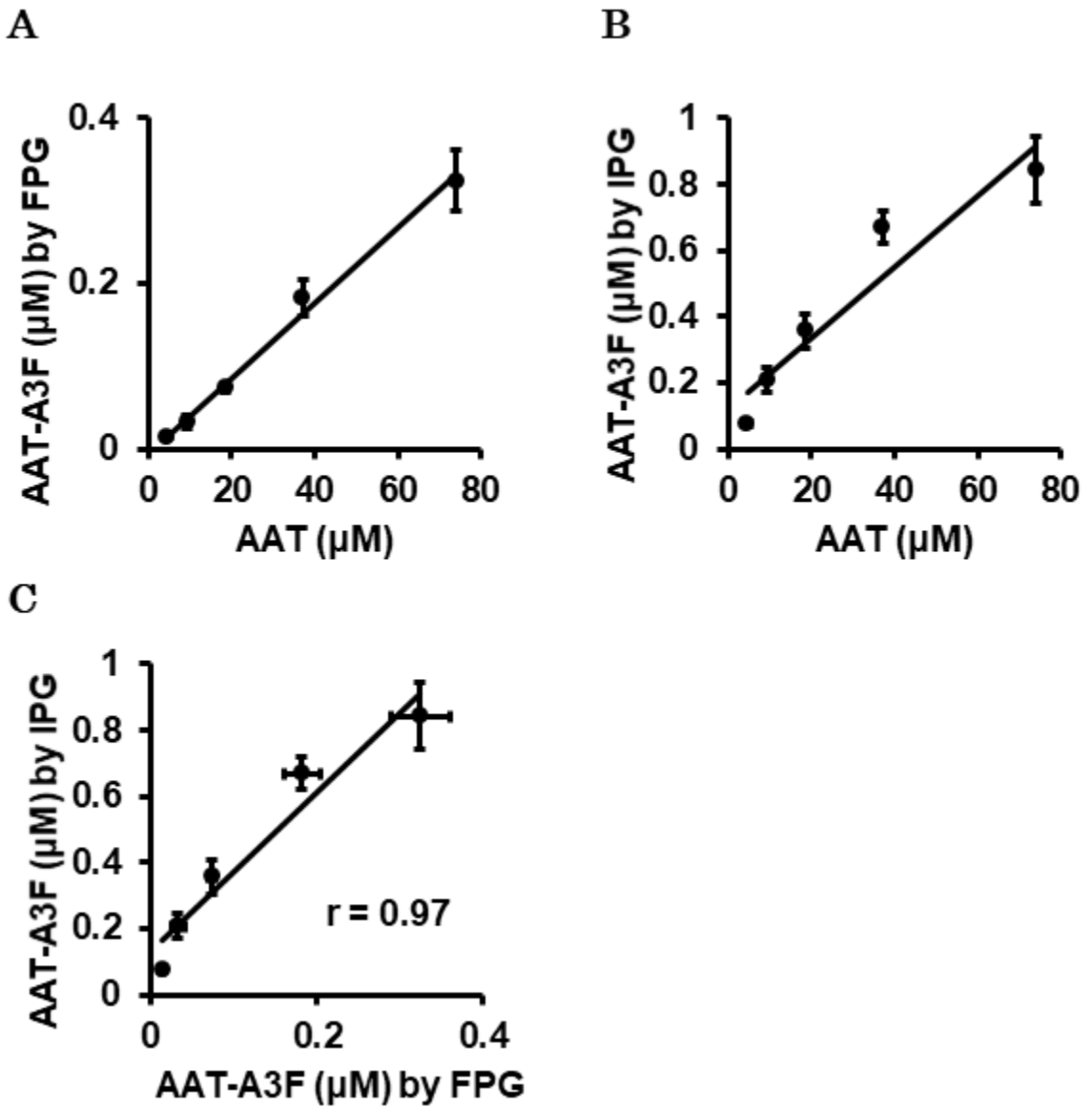
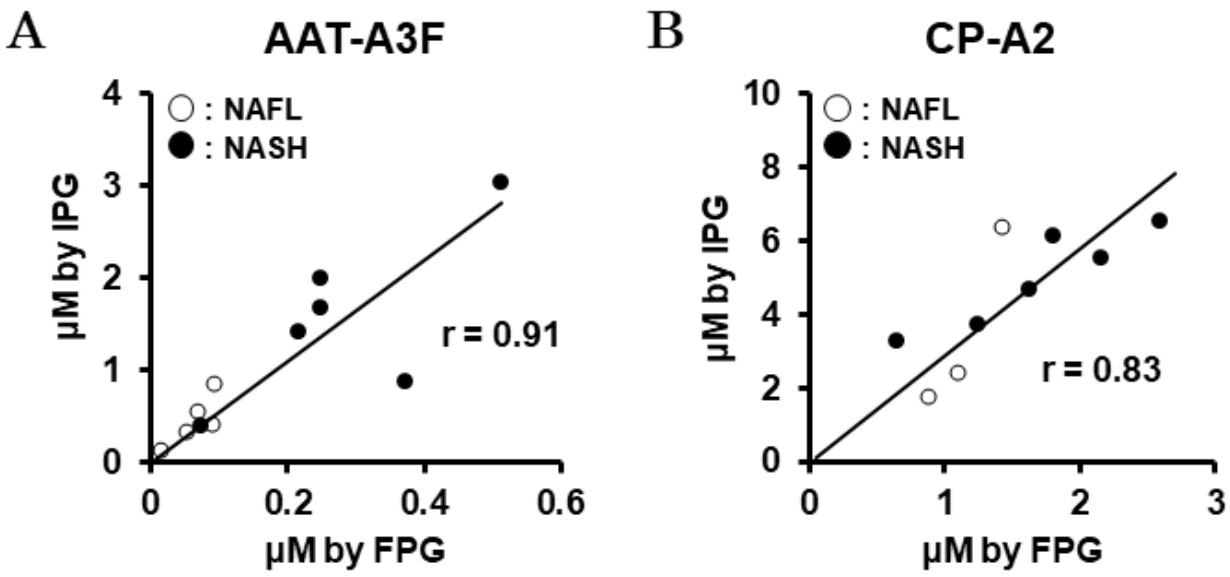


Figure 4. Correlation between glycan amount obtained by FPG and by IPG. A, A3F of AAT; B, A2 of CP



ASSOCIATED CONTENT

SUPPORTING INFORMATION:

The following supporting information is available free of charge at ACS website http://pubs.acs.org

Table S1: Clinical characteristics of NAFL and NASH patients

Table S2: N-glycans derived from human serum proteins analyzed in this study

Figure S1: Representative MS/MS spectra of N-glycans measured in this study

Figure S2: Entire gel images of SDS-PAGE analysis of human serum proteins shown in Figure 2

Figure S3: N-glycan amounts of AAT measured by the FPG method or after direct tryptic digestion

Figure S4: Representative MALDI-TOF MS spectra of tryptic peptides of AAT

Figure S5: SDS-PAGE analysis of AAT and CP immunoprecipitated from NAFLD patient sera

Figure S6: MALDI-TOF MS spectra of SALSA-derivatized N-glycans released from AAT of pooled human serum samples

Figure S7: Representative MALDI-TOF MS spectra of N-glycans released from AAT after exoglycosidase digestion

Figure S8: MS/MS spectra of AAT-A3F after sialidase digestion

Figure S9: Serum concentrations of IPG target proteins

Supplementary Methods

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ABBREVIATIONS

AAT, alpha-1 antitrypsin; CP, ceruloplasmin; FPG, focused protein glycomics; IPG, immunoprecipitation-glycomics; A3F, tri-sialylated mono-fucosylated tri-antennary glycan; MALDI-TOF MS, Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis

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