

N-glycosylation site occupancy in serum glycoproteins using multiple reaction monitoring liquid chromatography mass spectrometry

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Abbreviations

CDG	congenital disorders of glycosylation
cps	counts per second
HFI	hereditary fructose intolerance
MRM	multiple reaction monitoring
MRM-LC-MS/MS	multiple reaction monitoring liquid chromatography tandem mass spectrometry
PNGaseF	peptide N-glycanase F
QTRAP	quadrupole ion trap mass spectrometer
XIC	extracted ion chromatogram

Summary

Congenital disorders of glycosylation (CDG) are a family of N-linked glycosylation defects associated with severe clinical manifestations. In CDG type-I, deficiency of lipid-linked oligosaccharide assembly lead to the under-occupancy of N-glycosylation sites on glycoproteins. Although the level of residual glycosylation activity is known to correlate with the clinical phenotype linked to individual CDG mutations, it is not known whether the degree of N-glycosylation site occupancy by itself correlates with the severity of the disease. To quantify the extent of underglycosylation in healthy control and in CDG samples, we developed a quantitative method of N-glycosylation site occupancy based on multiple reaction monitoring LC- MS/MS. Using isotopically labeled standard peptides, we directly quantified the level of N-glycosylation site occupancy on selected serum proteins. In healthy control samples, we determined 98-100% occupancy for all N-glycosylation sites of transferrin and α 1-antitrypsin. In CDG type-I samples, we observed a reduction in N-glycosylation site occupancy, which correlated with the severity of the disease. In addition, we noticed a selective underglycosylation of N-glycosylation sites, indicating preferential glycosylation of acceptor sequons of a given glycoprotein. In transferrin, a preferred occupancy for the first N-glycosylation site was observed and a decreasing preference for the first, third and second N-glycosylation site was observed in α 1-antitrypsin. This multiple reaction monitoring LC-MS/MS method can be extended to multiple glycoproteins, thereby enabling a glyco-proteomic survey of N-glycosylation site occupancies in biological samples.

Introduction

In human, disturbances in the biosynthesis of glycoconjugates lead to diseases with heterogeneous biochemical and clinical characteristics(1). Deficiencies in the biosynthesis of serine- or threonine-linked (O-linked), asparagines-linked (N-linked), glycosylphosphatidylinositol and glycosphingolipid glycans have been described(2-5). Inherited deficiencies in the N-glycosylation biosynthetic pathway are referred to as congenital disorders of glycosylation (CDG). CDGs represent multi-organ diseases involving central and peripheral nervous defects and are often associated with endocrine and coagulation disorders(6). The heterogeneous symptoms associated to CDG and the structural diversity of glycoconjugate render the identification of glycosylation defects a difficult task. After initial clinical suspicion of CDG, the serum glycoprotein transferrin serves as a biomarker for possible defects of N-glycosylation. The integrity of transferrin N-glycosylation is tested by isoelectric focusing electrophoresis, Western blot analysis and by MS measurements of the purified glycoprotein(7-9). Deficiencies of N-glycosylation site occupancy are classified as CDG type-I (CDG-I), whereas defects of N-glycan trimming and elongation are classified as CDG type-II (CDG-II)(10).

To date, 12 distinct genetic defects, which affect the N-glycosylation output to different levels, have been identified as cause of CDG-I. Mutations in the phosphomannomutase-2 gene represent the most frequent form of CDG-I. The level of residual enzymatic activity associated to individual mutations in the phosphomannomutase-2 gene has been correlated with the severity of the clinical presentations(11). However, the exact degree of protein underglycosylation in CDG-I has not been determined yet, and it is unclear whether the degree of underglycosylation directly

relates to the severity of the disease. Also, the degree of N-glycosylation site occupancy found in healthy individuals has not been defined yet.

To better understand the functional significance of N-glycosylation in humans, a precise measure for the glycosylation site occupancy in healthy and in CDG-related conditions is necessary. In proteomics, the most widely applied experiment for the determination of peptide sequences relies on product ion scanning mass spectrometry (MS/MS). Precursor ions of selected peptides are selected in a first mass analyzer, fragmented in a collision cell and the generated product ion fragments are scanned in a second mass analyzer. Triple quadrupole mass spectrometers are particularly suitable for quantitative determinations of changes in serum protein levels. These instruments allow multiple reaction monitoring (MRM) experiments, a sequential selection of peptide specific precursor ions in the first mass analyzer together with a characteristic fragment ion in the second mass analyzer upon fragmentation in the collision cell. Transitions of precursor to fragment ions lead to signals in the detector and can be recorded as a function of time during chromatographic elution. Additionally, the sequence of eluting peptides can be confirmed by the acquisition of corresponding product ion spectra. The selectivity of the MRM transitions together with short scan times for each transition allow for sensitive, quantitative analyses of complex samples(12, 13).

In the present study, we describe a MRM LC-MS/MS approach to quantify the N-glycosylation site occupancy of the serum glycoproteins transferrin and α 1-antitrypsin. We analyzed 28 serum samples and quantified glycosylation site occupancies in healthy and in disease related samples. For the first time, we found a clear correlation in CDG-I between the degree of N-glycosylation site occupancy and the severity of the disease.

Experimental Procedures

Isoelectric focusing gel electrophoresis

Serum transferrin was saturated for 30 min with ferric citrate (0.4 mM) in the presence of sodium hydrogen carbonate (20 mM) and separated on a rehydrated Immobiline DryPlate gel (GE Healthcare) with a pH range of 4.0-7.0 using the Phast-System (Amersham Biosciences). Transferrin was visualized by gel immunoprecipitation using polyclonal rabbit anti-human transferrin antibodies (DakoCytomation). The antibody solution was directly applied onto the gel and incubated for 1h. To remove excess of antibodies and non-bound proteins, the gel was washed over night in 0.9% NaCl, followed by fixation and staining with PhastGel Blue R (GE Healthcare) according to the manufacture protocol.

Immuno-affinity enrichment of glycoproteins from human serum

Serum glycoproteins were purified with a custom made HU-6 multiple affinity removal system column (MARS column, 4.6 x 50 mm, Agilent Technologies), targeting transferrin, IgG and α 1-antitrypsin. The purification was performed from 5 μ l human serum using the buffers and protocol recommended by the manufacturer. The glycoproteins were eluted into 1 ml fractions and the elution was monitored by UV absorption at 280 nm.

Proteolytic digestion and glycopeptide labeling

The proteins in the collected fractions were precipitated with TCA, pooled with reduction buffer (0.57 M Tris-HCl, pH 8.5, 50 mM DTT) to a final volume of 250 μ l. The proteins were reduced at 80 °C for 5 min, cooled to room temperature and 63 μ l of 1 M iodoacetamide in water were added. The samples were incubated for 40 min at room temperature in the dark and the alkylated

proteins were desalted by TCA precipitation. The reduced and alkylated samples were re-dissolved in 50 μ l of 20 mM ammonium bicarbonate, 10% ACN containing Asp-N and trypsin (0.5 μ g each, Roche) and digested for 16 h at 37 °C. The digests were heat inactivated at 80 °C for 5 min. Then, 0.5 μ mol EDTA, 100 μ g aprotinin, 8 pmol of the standard peptides corresponding to the transferrin N-glycosylation sites (TFP1NK, TFP1NKS, TFP1DK, TFP1DKS, TFP2NVT, TFP2DVT, see Table 1) and 2 pmol each of the standard peptides corresponding to the α 1-antitrypsin N-glycosylation sites (ATP1N, ATP1D, ATP2N, ATP2D, ATP3N, ATP3D, see Table 1) were added. The samples were lyophilized for at least 16 h. The samples were re-dissolved in 20 mM ammonium bicarbonate in 95 atom% H_2^{18}O (Sigma) and 1.5 units peptide N-glycanase F (PNGaseF, Roche) reconstituted with 95 atom% H_2^{18}O were added. The samples were digested for 8 h at 37 °C, heat inactivated at 80 °C for 5 min and stored at -20 °C until further use.

Mass spectrometry

An Applied Biosystems QTRAP 3200 LC-MS/MS system, equipped with a NanoSpray II ion source and an Eksigent nanoLC-2D system was used. The sample reaction mixture (2 to 10 μ l) was filtered with 0.22 μ m centrifugal filter devices, adjusted to 20 μ l 0.1% formic acid and 2% ACN and loaded on an LC Packings C18 PepMap 100 trap column (300 μ m i.d. x 5 mm, Dionex). The trap column was washed with 60 μ l loading buffer and switched in line to a ProteoPep2 column (75 μ m i.d. x 15 cm, C18, IntegraFrit, New Objective). A binary gradient at 250 nl/min was applied. Buffer A was 0.1% formic acid and buffer B was 80% ACN containing 0.1% formic acid. Buffer B was held at 14% for 3 min, increased to 54% over 102 min, to 100%

over 8 min, held at 100% for 12 min, decreased to 14% over 12 min and the column was re-equilibrated at 14% buffer B for 16 min. MRM transitions and product ion scans were acquired sequentially and the LC gradient was divided into three acquisition periods. The first 23 min MRM transitions and product ion scans corresponding to the second, C-terminal transferrin N-glycosylation site were recorded (TFP2NVT, TFP2DVT and corresponding sample peptides). During the second period data corresponding to the first N-terminal transferrin N-glycosylation site were acquired for 25 min (TFP1NK, TFP1NKS, TFP1DK, TFP1DKS and corresponding sample peptides). During the third period data corresponding to the α 1-antitrypsin N-glycosylation sites were acquired until completion of the LC run (ATP1N, ATP1D, ATP2N, ATP2D, ATP3N, ATP3D and corresponding sample peptides). Fragment ions and fragmentation settings for the sample derived peptides were set according to the MRM transitions established for the standard peptides (Table 1). The average counts per second (cps) of the sample peptide peaks were divided by the average cps of the standard peptide and multiplied with the amount of standard peptides added to the sample preparation. The sum of the amounts of previously glycosylated and unglycosylated peptides was set to 100%. The percentage of site occupancy was calculated as the part of previously glycosylated, i.e. PNGaseF sensitive peptides.

Results

The serum glycoprotein transferrin is the most widely used biomarker for the clinical diagnosis of CDG-I and -II. Normal serum transferrin typically contains two sialylated, biantennary complex type N-glycans attached to the glycosylation sites at Asp413 and Asp611(14). Serum samples from CDG-I, CDG-II, hereditary fructose intolerance (HFI) and healthy individuals were analyzed by transferrin isoelectric focusing gel electrophoresis and the typical deviations of the band patterning of CDG sera from the healthy controls could be observed (Fig. 1). A major

band corresponding to tetrasialylated, fully glycosylated transferrin was observed in control samples. In CDG sera, undersialylation was detected with more intense bands corresponding to tri-, di-, mono- and asialylated transferrin. In CDG-II, this pattern can be explained by altered trimming and elongation of transferrin N-glycan structures, which leads to decreased transfer of terminal sialic acid. In CDG-I, decreased N-glycosylation site occupancy results in the undersialylation as observed in the transferrin isoelectric focusing gel (Fig. 1). In HFI the metabolite fructose-1-phosphate accumulates in the liver, which leads to inhibitory effects in early steps of N-glycosylation biosynthesis. Therefore, HFI can be considered as a secondary CDG syndrome and CDG-I typical patterns are observed in transferrin isoelectric focusing gel electrophoresis(15). Polypeptide polymorphisms of transferrin also lead to altered gel patterning, which can be revealed by sialidase treatment prior isoelectric focusing electrophoresis.

Figure 1

To precisely measure the N-glycosylation site occupancy in healthy as well as in pathological conditions, sera from 17 CDG patients, 1 alcohol abuse patient, 1 HFI patient and 9 sera from healthy individuals were selected for analysis. The serum of an alcohol abuse patient was included as a positive control. Alcohol abuse leads to reduced N-glycosylation site occupancy of transferrin, thus mimicking CDG-I(16). The samples were subjected to glycoprotein purification using a custom made multiple affinity column directed against human transferrin, α 1-antitrypsin and IgG. The reduced and alkylated proteins were digested with a combination of trypsin and Asp-N and isotopically labeled standard peptides were added(17) (Table 1).

Table 1

Table 2

An isotope label was introduced into N-glycosylated sample peptides with PNGaseF in H₂¹⁸O buffer. PNGaseF deglycosylates Asn-linked glycopeptides and converts the corresponding Asn to Asp. In the presence of H₂¹⁸O buffer ¹⁸O is incorporated into the carboxyl-group of the newly formed Asp(18, 19). The added standard peptides were synthesized according to the transferrin and α 1-antitrypsin N-glycosylation site sequences with either Asn or Asp introduced into the N-glycosylation sequon. The precursor and fragment ion pairs used for each MRM transition were selected empirically based on maximal signal intensities and signal specificities as judged from the chromatography of extracted ion pairs (Table 2). For the sample peptides, the corresponding precursor ion and fragment ion types as selected for the standard peptides were used. The inclusion of the standard peptides facilitated the identification and quantitation of N-glycosylation site occupancy with LC-MS/MS. The peptide mixture was separated on a reverse-phase nanoLC column and introduced to the mass spectrometer via a nanoelectrospray ion source. The chromatography was monitored from the total ion chromatogram (Fig. 2). MRM transitions for each target peptide and the fragment ion spectra of the corresponding sample peptides were recorded sequentially. The acquisition was grouped into three time periods according to the elution positions of the second and the first transferrin target peptides and the elution positions of the α 1-antitrypsin target peptides, respectively (Fig. 2).

Figure 2

The MRM transitions together with the co-elutions of the sample peptides with the labeled standard peptides served as primary criteria for peptide identification. In addition, the enhanced product ion spectra of the sample peptides were acquired and the peptide sequences were deduced from the fragment spectra by comparison with previously recorded fragment ion spectra of the corresponding standard peptides (Fig. 3).

Figure 3

Figure 4

The average signal intensities of the sample and standard peptides were used to calculate the site occupancies of each glycosylation site (Fig. 4). Overall, the sensitivity for detection of each peptide was depending on their ionization characteristics, and was partially accounted for in adjusted dwell times of the respective MRM transition (between 20 and 200 ms, Table 2). The results are summarized in Table 3. The site occupancy for all detected N-glycosylation sites in the healthy control samples ranged between 98 and 100%. The transferrin glycosylation sites are equally occupied under normal physiological conditions. In α 1-antitrypsin, the first and the third N-glycosylation sites were always occupied to 100%, whereas the second N-glycosylation site appeared slightly less stringently occupied with an average of $99 \pm 1.4\%$ under normal physiological conditions.

The level of site occupancy in CDG-I samples was decreased to variable extents depending on the of N-glycosylation sites analyzed. In transferrin, the first N-glycosylation site was occupied

from 70% to 96%, whereas the occupancy of second N-glycosylation site was reduced down to 41% in CDG-I samples (Table 3). Generally, the second transferrin glycosylation site was less occupied than the first site, indicating a preferred glycosylation of the first site in this glycoprotein. The underglycosylation level of α 1-antitrypsin was less pronounced than that of transferrin in CDG-I. In α 1-antitrypsin, the second N-glycosylation site showed the strongest reduction, down to 65% occupancy, whereas the first and third sites were hardly altered, showing occupancies between 94-100% and 89-100%, respectively (Table 3). The site occupancy of the first α 1-antitrypsin N-glycosylation site could not be determined in every sample, possibly due to low ionization efficiencies of the triply charged peptide in the ion source. Additionally, this peptide contains N-terminal glutamine, which can circularize to pyroglutamic acid and a methionine residue which can oxidize to methionine sulfoxide, thus evading detection via the MRM transitions applied. The occupancy of transferrin N-glycosylation sites in HFI and in alcohol abuse samples were not as pronounced as in CDG-I samples, yet like in CDG-I, the second site was less glycosylated than the first one. The N-glycosylation of α 1-antitrypsin was hardly reduced in the HFI and alcohol abuse samples investigated (Table 3). As expected, N-glycosylation site occupancy was unchanged in CDG-II samples, which are characterized by defects of glycan trimming and elongation. The N-glycosylation site occupancies of transferrin and α -1-antitrypsin were determined with high precision as illustrated by the standard deviations obtained. The maximum deviation reached 10.8 % in one out of the 28 serum samples analysed. The standard deviations calculated for the other samples ranged from 0.0 to 7.8 % per glycosylation site. The highest accuracy was obtained in the CDG-II, HFI, alcohol abuse and healthy control samples. In these samples the maximum standard deviation was calculated to be 2.5 % (Table 3). The reasons for the higher deviations in the measurements of the N-

glycosylation site occupancies of the CDG-I sera are not clear. Possibly, lower serum protein levels as observed in CDG-I patients contribute to this observation.

Table 3

From all N-glycosylation sites investigated the second transferrin site appeared to respond most sensitively to defects of glycosylation. Clinical information was available for 11 CDG-I cases investigated here and we grouped these cases into three groups of patients displaying severe, moderate and mild clinical manifestation(11). A clear correlation between the occupancy of the second transferrin N-glycosylation site and the severity of CDG-I condition could be observed (Fig. 5). The lowest level of site occupancy detected in CDG-I samples never reached less than 40%. A minor reduction of N-glycosylation site occupancy to about 85% of normal levels as seen in transferrin was already compatible with a disease state, indicating that N-glycosylation site occupancy is stringently required for proper physiological function of glycoproteins.

Figure 5

Discussion

Routinely, isoelectric focusing gel electrophoresis of transferrin is applied for the diagnosis of CDG. Individual transferrin glycoforms are separated according to charge as mediated by terminal sialic acid residues attached to the complex type N-glycans. In combination with

Western blotting analysis of transferrin, CDG-I and -II can be easily discriminated. Potential transferrin peptide polymorphisms can be revealed by sialidase treatment prior to IEF.

Additional molecular weight determinations of transferrin by ESI-MS or SELDI-MS can further corroborate the diagnosis(7, 8). However, these indirect approaches do not discriminate between individual N-glycosylation sites and quantitative information relies on densitometric measurements of glycoprotein bands in IEF or SDS-PAGE. In this study we developed a method for directly determining the presence and absence of N-glycosylation site occupancy and fine deviations from normal N-glycosylation site occupancy could be determined.

In a small survey we analyzed sera from healthy individuals and authentic serum samples from patients suffering from disease conditions associated with reduced N-glycosylation site occupancy. We used MRM LC-MS/MS, which allows for sensitive and specific detection of target peptides by taking advantage of the dynamic quantitative range capabilities inherent of quadrupole mass analyzers(13, 20). Target peptides can be detected and quantified reliably even in complex mixtures. Additional confidence in peptide sequence assignment is provided by acquisition of enhanced product ion spectra in the ion trapping mode of the quadrupole ion trap mass spectrometry (QTRAP) system used. For the first time using this approach, we quantitatively mapped N-linked glycosylation sites of selected serum glycoproteins. A striking feature of the glycoproteins analyzed, transferrin and α 1-antitrypsin, is the narrow range of N-glycosylation site occupancy under physiological conditions. To warrant normal physiological function N-glycosylation site occupancy of 99% and 100% is preserved. Symptomatically mild CDG cases show only a slight reduction in N-glycosylation site occupancy, indicating a small threshold from healthy to pathological conditions.

Our data show a tendency of preferential glycosylation of the first N-linked glycosylation sites in transferrin and α 1-antitrypsin under conditions of limited dolichol-linked oligosaccharide substrate availability. In addition, transferrin was more prone to underglycosylation than α 1-antitrypsin. The reasons for this observation are not clear. Transferrin is about four times more abundant in human serum and almost two fold larger than α 1-antitrypsin, which might require different subsets of the translation and glycosylation machinery in the cell(21).

Of all N-glycosylation sites analyzed the second transferrin site showed the greatest dynamics in site occupancy in pathological conditions. This feature could be exploited for the development of an assay targeting the second transferrin site and might be applied in a postnatal screen upon suspicion of CDG-I. Information about the glycosylation site occupancy at this stage would be beneficial in providing estimates about the developmental prospects of the affected individuals.

With the clinical information available, we classified CDG-I samples according to previously suggested criteria for mild clinical expressions of this disease(11). The remaining samples were grouped into moderate and severe clinical expressions. Most samples analyzed were derived from patients with a moderate pathology, comprising the classical CDG-I symptoms like failure to thrive, seizures, developmental delay, hypotonia, ataxia, or mental regression. The severe cases comprised conditions such as death in early childhood in addition to the moderate CDG-I pathology. When plotted against the N-glycosylation site occupancy, we found a clear correlation between the presence of glycosylation and clinical expression of the disease, confirming the causative coherency of the absence of N-glycosylation and clinical expression in CDG-I. These findings underline the significance of N-glycosylation in health and development of the human organism. Overall, our data validate transferrin as a sensitive marker protein for CDG-I and alcohol abuse conditions. The method developed in this study will be useful for

unambiguously mapping the sensitivity of proteins to defects of glycosylation. Furthermore, this method can be extended by including additional serum glycoproteins, which will contribute to better understand the significance of N-glycosylation in human physiology.

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Figure legends

Figure 1. Isoelectric focusing of selected serum transferrin samples.

The profile of a serum sample derived from a patient with a severe form of CDG-Ia is shown in lane 11. Profiles of moderate CDG-Ia samples are shown in lanes 2, 3, 8, 9, 10. Profiles of mild CDG-Ia samples are shown in lanes 7 and 12. Lane 1 shows the profile of a moderate CDG-Ic serum. Lane 4 shows a profile from serum of a patient with hereditary fructose intolerance (HFI), lane 5 shows the profile of a CDG-IIx serum and lane 6 shows the serum profile of a transferrin polymorphism. Samples from healthy control serum are shown in lanes HC. The numbers C0 to C6 indicate the number of charges as mediated by sialic acid residues of the transferrin bi- and tri-antennary N-glycans.

Figure 2. Total ion chromatogram of a typical MRM-LC-MS/MS run.

The total ion chromatogram acquisition is divided into three time periods. Peptides corresponding to the second transferrin glycosylation site (TFP2) elute within the first period and peptides corresponding to the first transferrin glycosylation site (TFP1) elute during the second period. MRM transitions and fragment ion spectra corresponding to the α 1-antitrypsin N-glycosylation sites (ATP1-3) are recorded during the third period starting at 50 min.

Figure 3. Enhanced product ion spectra (EPI) of a standard and a sample peptide.

The upper panel shows the EPI spectrum of the synthetic standard peptide TFP1DK corresponding to the first, previously occupied transferrin glycosylation site. The lower panel shows the EPI spectrum of the corresponding sample peptide sequence as recorded during a LC-MS run. Major fragment ions referring to the a-, b- and y-series are assigned and the isotopically labeled amino acids underlined in the depicted peptide sequences. The isotopic label in the sample peptide results from the N-glycanase digestion in H₂¹⁸O containing buffer. For this peptide pair the N-terminal fragment ions b₃, a₄ and b₄ are identical whereas the recorded y-type fragment ions are mass shifted according to the introduced amino acid isotope differences. The major y₉-fragment ion was used for the MRM transition for this peptide pair.

Figure 4. Extracted ion chromatogram (XIC) of the MRM transitions of two peptide pairs recorded during the second period.

The standard peptide TFP1DKS (solid line) together with the corresponding sample peptide (dotted line) and the standard peptide TFP1NKS (dashed line) are shown in the upper panel. Average signal intensities are depicted in histogram of the lower panel and were used for calculating N-glycosylation site occupancy. The average MRM signal intensities of the eluting peaks at 29 min are shown. The peptide pair referring to the TFP1DK peptide co-elutes with the TFP1DKS pair in the chromatography, resulting in the MRM signal bars Sample/TFP1DK at the same elution time.

Figure 5. Transferrin glycosylation site occupancy versus clinical presentation.

Clinical information was available for nine CDG-Ia patients, one CDG-Ic patient and one patient of unknown CDG-I defect. The samples were classified according to the clinical expression of individual patients into severe, moderate and mild forms of CDG-I. The occupancy of the second transferrin N-glycosylation site is plotted for each patient in the allocated group. The control group contains the values obtained from nine healthy individuals.

Table 1. Custom synthesized standard peptides.

Isotopically labeled amino acids are bold, underlined, F: +10Da, P: +6Da, L: +7Da, A: +4Da.

Designation	Sequence	Q3 ion
ATP1N	QLAHQSNSTNIFFSPVSIATA <u>F</u> AMLSLGTK	y ₉
ATP1D	QLAHQSDSTNIFFSPVSIATA <u>F</u> AMLSLGTK	y ₁₂
ATP2N	DEILEGLNFNLT <u>E</u> IP <u>P</u> EAQIHEGFQELLR	y ²⁺ ₁₄
ATP2D	DEILEGLN <u>E</u> DLTEIP <u>E</u> EAQIHEGFQELLR	y ²⁺ ₁₄
ATP3N	YLGDATAIFF <u>L</u> P	y ₁
ATP3D	YLGDATAIF <u>E</u> LP	b ₁₁
TFP1NK	CGLVPV <u>L</u> AENYNK	y ₉
TFP1NKS	CGLVPV <u>L</u> AENYNKS	y ₁₀
TFP1DK	CGLVPV <u>L</u> AENYDK	y ₉
TFP1DKS	CGLVPV <u>L</u> AENYDKS	y ₁₀
TFP2NVT	QQQH <u>L</u> FGSNVT	b ₅ -NH ₃
TFP2DVT	QQQH <u>L</u> <u>E</u> GSDVT	b ₉

Table 2. Triple quadrupole settings for the target peptides.

Q1 and Q3 represent the m/z values of the precursor ions with the corresponding fragment ions selected for the MRM transitions. The fragmentation conditions for each standard peptide were optimized empirically for maximum signal intensities. Dwell = time per transition, DP = declustering potential, EP = entrance potential, CEP = collision cell entrance potential, CE = collision energy, CXP = collision cell exit potential

Designation	Q1	Q3	Dwell (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
ATP1N	1064.56	977.56	200	35	9.5	55	44	7
ATP1D	1066.89	1220.68	200	35	9.5	60	50	49
ATP2N	1091.89	836.94	200	46	7.5	55	45	6
ATP2D	1093.56	833.93	200	43	9.0	60	45	6
ATP3N	667.37	116.07	50	21	7.0	37	27	50
ATP3D	1337.72	1222.66	20	85	9.5	40	45	50
TFP1NK	742.39	1054.56	100	40	9.0	40	41	45
TFP1NKS	785.90	1141.60	100	41	9.5	43	44	48
TFP1DK	744.88	1059.56	100	41	9.0	40	41	45
TFP1DKS	788.40	1146.59	100	41	9.5	43	44	48
TFP2NVT	633.32	625.29	150	40	9.0	35	37	43
TFP2DVT	635.32	1051.50	150	40	9.0	35	37	44

Table 3. N-glycosylation site occupancy of transferrin and α 1-antitrypsin.

Values for each glycosylation site are averaged and given in percentage site occupancy. Standard deviations are given for sites where three or more values were averaged (SD with $n \geq 3$). The values for the healthy control are averaged from MRM-LC-MS/MS runs of nine individual serum samples.

Group	Gene defect	OMIM entry		TFP1D ¹	TFP2D ¹	ATP1D ¹	ATP2D ¹	ATP3D ¹
Healthy controls			average:	99	99	100	99	100
			SD (n=3-14)	1.1	0.7	0.0	1.4	0.2
CDG-Ia	<i>PMM2</i>	601785	average:	96	94	100	97	100
			SD (n=3)	3.3				0.0
CDG-Ia	<i>PMM2</i>	601785	average:	89	75	100	100	98
			SD (n=3-6)	3.3	0.9	0.0	0.0	1.2
CDG-Ia	<i>PMM2</i>	601785	average:	70	59	100	94	96
			SD (n=3-6)	4.2	3.7			2.0
CDG-Ia	<i>PMM2</i>	601785	average:	72	41	94	65	94
			SD (n=3-5)	10.8	6.2	10.8	5.4	4.0
CDG-Ia	<i>PMM2</i>	601785	average:	88	56	100	92	98
			SD (n=3-4)	5.2	4.5	0.0	2.3	2.2
CDG-Ia	<i>PMM2</i>	601785	average:	72	41	98	82	90
			SD (n=3-4)	4.2	2.1			3.8
CDG-Ia	<i>PMM2</i>	601785	average:	95	89	100	99	99
			SD (n=3)	3.3				0.6
CDG-Ia	<i>PMM2</i>	601785	average:	79	67	100	100	95
			SD (n=3-4)	2.0	2.4			2.3
CDG-Ia	<i>PMM2</i>	601785	average:	91	76		94	98
			SD (n=4)	2.9	2.3			0.5
CDG-Ib	<i>MPI</i>	154550	average:	93	91	100	97	99
			SD (n=3-4)	2.8			0.4	0.3
CDG-Ic	<i>ALG6</i>	604566	average:	90	62	100	100	93
			SD (n=4)	1.8	2.5			2.2
CDG-Ic	<i>ALG6</i>	604566	average:	83	57	99	79	89
			SD (n=3-4)	4.4	7.8			2.3
CDG-Ie	<i>DPM1</i>	603503	average:	91	85	100	99	99
			SD (n=3)	2.8				0.2
CDG-If	<i>MPDU1</i>	604041	average:	92	85		92	98
			SD (n=4-5)	1.1	1.7			0.7
CDG-Ix	unknown		average:	86	79	98	95	98
			SD (n=3)	9.3				2.0
CDG-IIa	<i>MGAT2</i>	602616	average:	99	97	100	99	100
			SD (n=4-5)	0.2	1.7	0.2	0.2	0.3
CDG-IIx	unknown		average:	100	98		99	100
			SD (n=3-4)	0.0	0.3		0.1	0.1
HFI	<i>ALDOB</i>	229600	average:	96	91	100	98	99
			SD (n=3-4)	0.9	2.5	0.1	1.8	0.2
Alcohol abuse	aquired		average:	96	91	100	97	99
			SD (n=4-5)	1.3	1.4	0.0	0.9	0.2

¹ TFP1D, first transferrin N-glycosylation site; TFP2D, second transferrin N-glycosylation site; ATP1D, first α 1-antitrypsin N-glycosylation site; ATP2D, second α 1-antitrypsin N-glycosylation site; ATP3D, third α 1-antitrypsin N-glycosylation site.

Figures

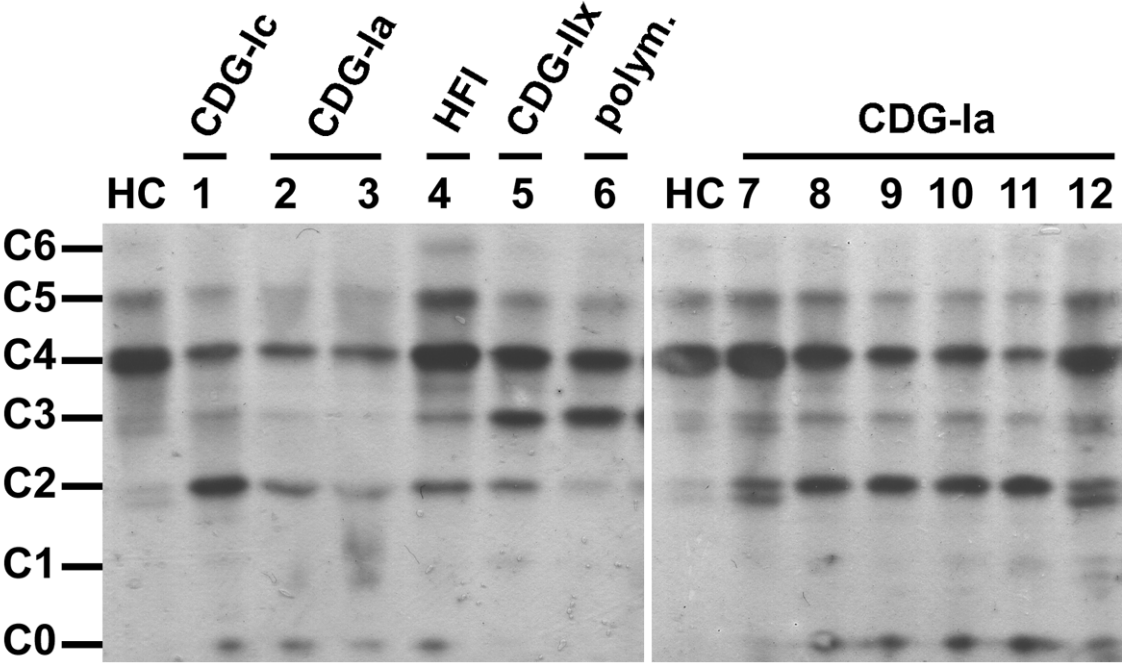


Figure 1. Isoelectric focusing of selected serum transferrin samples.

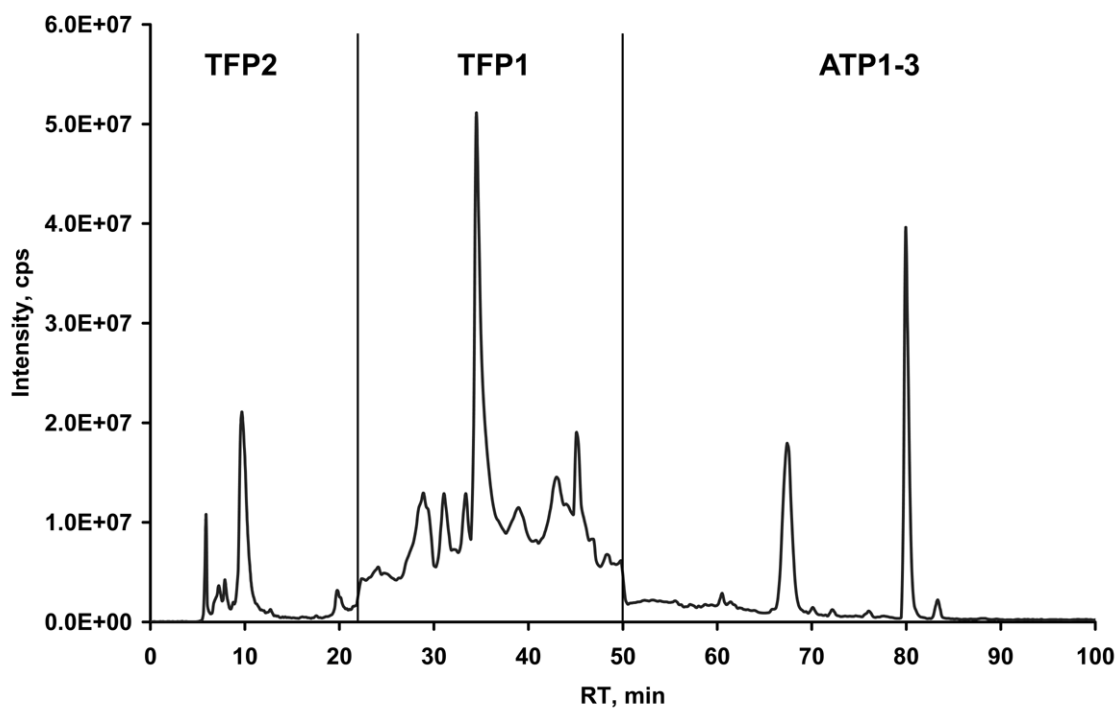


Figure 2. Total ion chromatogram of a typical MRM-LC-MS/MS run.

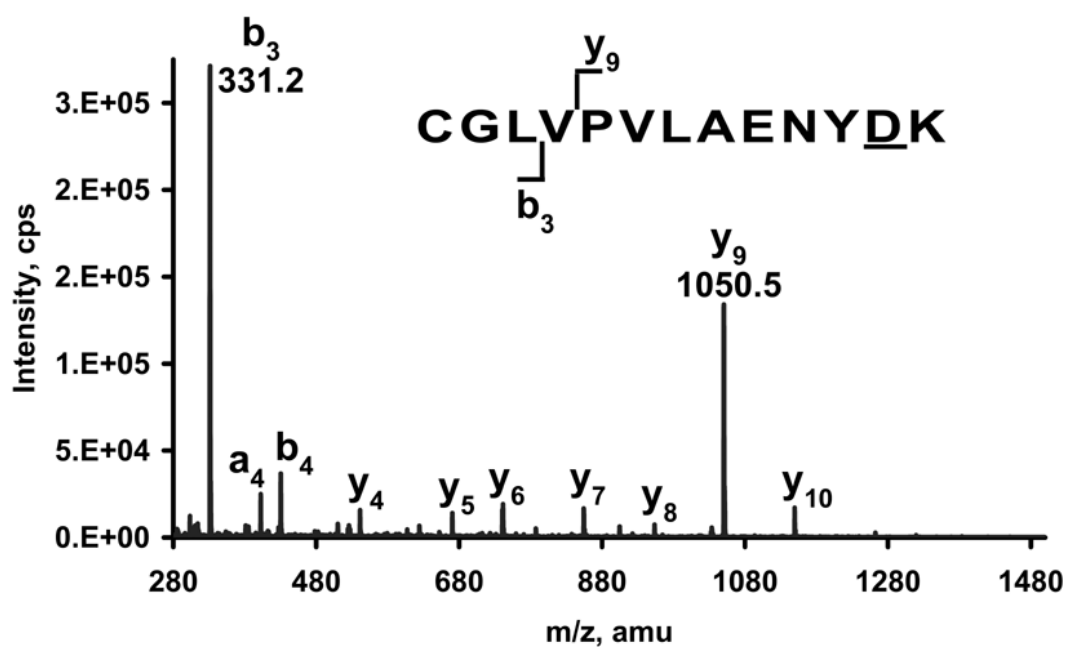
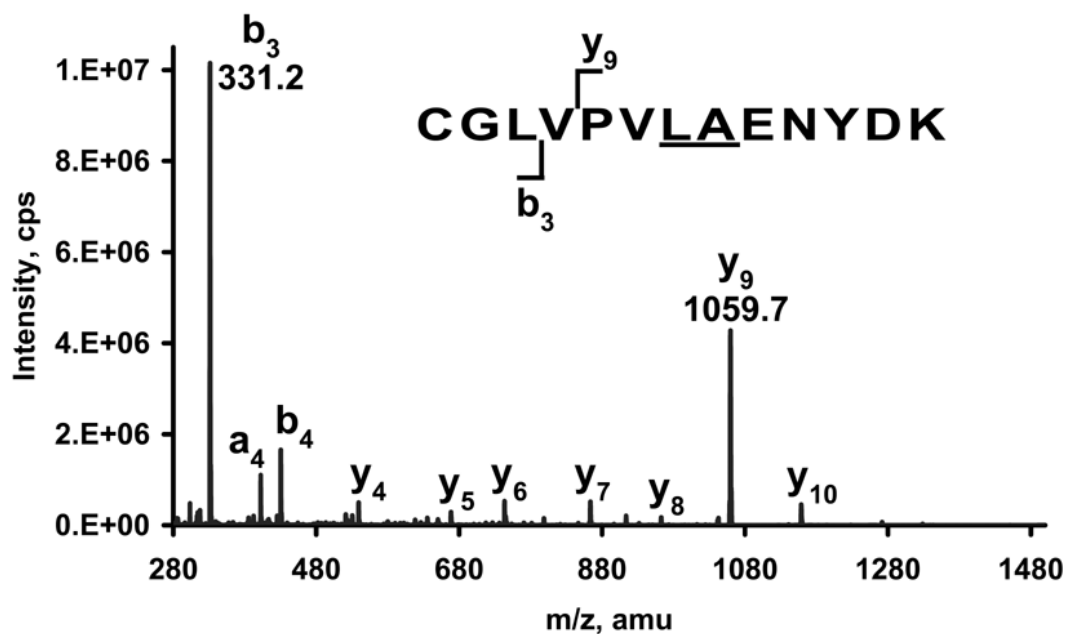


Figure 3. Enhanced product ion spectra (EPI) of a standard and a sample peptide.

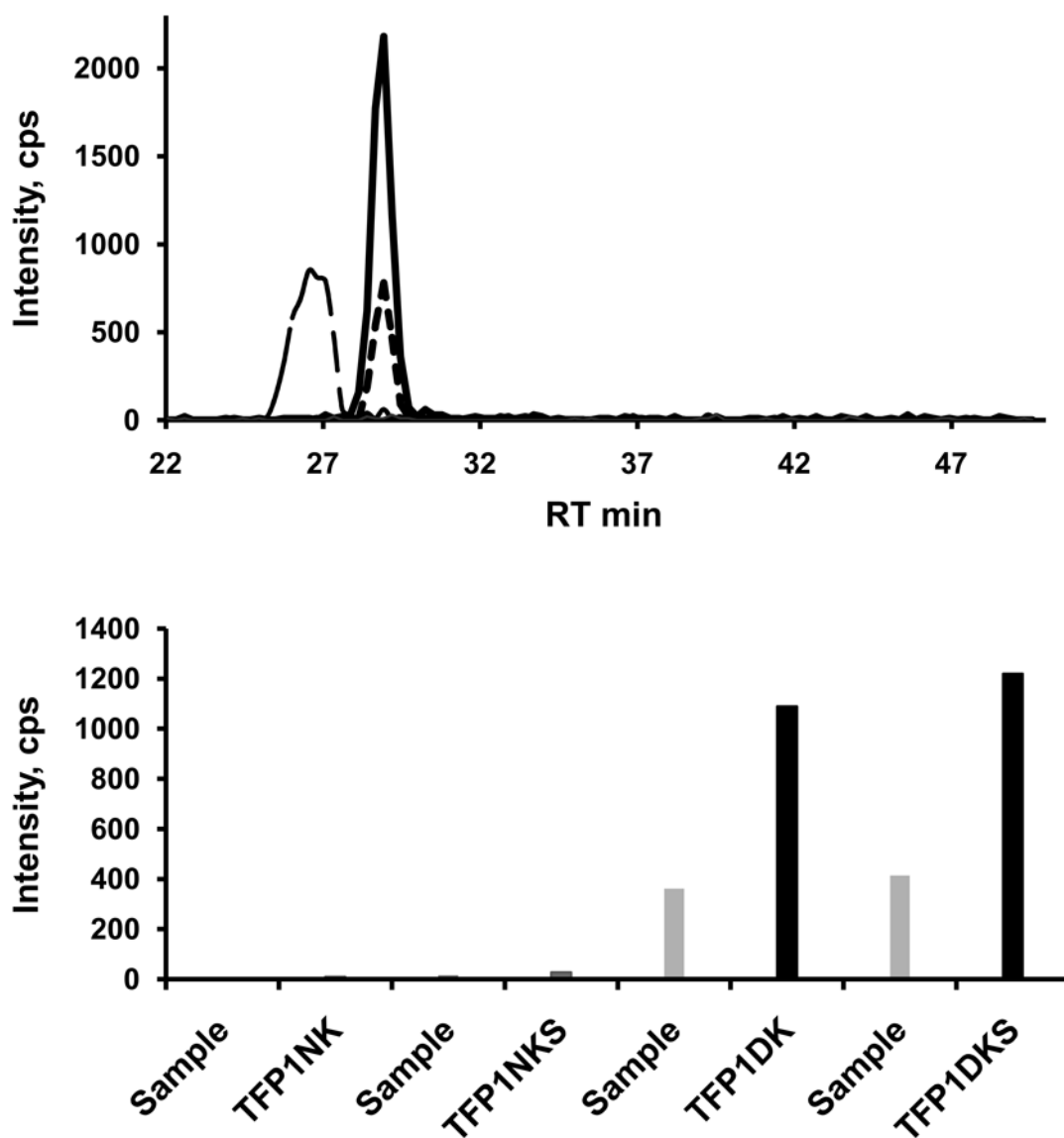


Figure 4. Extracted ion chromatogram (XIC) of the MRM transitions of two peptide pairs recorded during the second period.

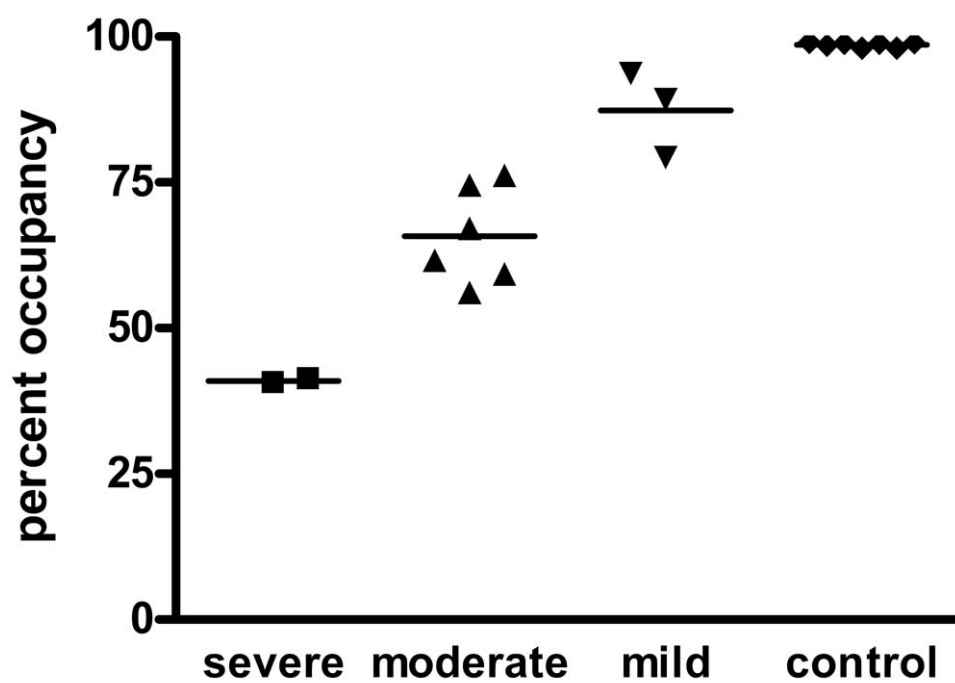


Figure 5. Transferrin glycosylation site occupancy versus clinical presentation.