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Experimental Procedures

All chemicals and solvents were of the highest purity available. Purified water used for sample preparation was freshly prepared using a Milli-Q water purification system (referred to as "Milli-Q water", 18.2 MΩ·cm^{-1} at 25°C, total organic carbon <3 ppb; Merck Millipore, Darmstadt, Germany). For preparation of LC-MS solvents ultrapure water was used, which was freshly prepared using the same system but equipped with an additional filter (referred to as "Milli-Q water MS"; LC-Pak Polisher, Merck Millipore). Aqueous solutions are indicated with (aq).

Proteolytic Digestion via Filter-Aided Sample Preparation (FASP)

Commercially available standard glycoproteins of different origin were proteolytically digested using a modified version of the filter-aided sample preparation (FASP) method introduced by Wisniewski et al. (25). Fibrinogen (Fib), immunoglobulin gamma (IgG) – both derived from human plasma, Lactotransferrin (LTF) – derived from human milk, and ribonuclease B (RNase B) – derived from bovine pancreas, were purchased from Sigma-Aldrich (# F3879; # I4506-10MG; # L4894-5MG; R7884-100MG; Steinheim, Germany). Briefly, 50 µg of each protein were dissolved in phosphate-buffered saline (PBS) pH 7.4, transferred to a filter unit (Nanosep® Omega™ with polyethersulfone membrane, molecular weight cut-off 10 kDa; # OD010c34, PALL Life Sciences, Ann Arbor, MI, USA), and centrifuged for 10 min at 14,000 x g at room temperature (RT) (holds true for all subsequent centrifugation steps, Heraeus™ Fresco™ 17 Microcentrifuge, 24 x 1.5/2.0 mL rotor, Thermo Scientific, Waltham, MA, USA). The flow-through was discarded. Subsequently, samples were re-dissolved in 200 µL of urea $buffer_{(Tris\text{-}HCl)}$ (8 M urea in 0.1 M Tris-$HCl_{(aq)}$ pH 8.5; # A1049, # A3452, AppliChem, Darmstadt, Germany), incubated at RT for 5 min while constantly shaking at 600 rpm on a Thermomixer comfort (Eppendorf, Hamburg, Germany), and centrifuged again. Cysteine disulfide bonds were reduced with DL-dithiothreitol (DTT; # D5545-5G, Sigma-Aldrich), and subsequent carbamidomethylation of -SH groups (thiol) was carried out using iodoacetamide (IAA; # I1149-25G, Sigma-Aldrich). DTT (24.68 mg) and IAA (40.7 mg) were dissolved in 50 mM ammonium $bicarbonate_{(aq)}$ (ABC buffer; # 09830-500G, Sigma-Aldrich) and diluted ten times with urea $buffer_{(Tris\text{-}HCl)}$ to a final concentration of 40 mM DTT and 55 mM IAA, respectively. To each filter unit, 100 µL of 40 mM DTT were added followed by shaking at RT for 1 min and 600 rpm. After incubation at 56°C for 20 min with gentle agitation (300 rpm), the samples were centrifuged and the flow-through was discarded. Subsequently, 100 µL of 55 mM IAA were added to each filter. Protected from light, samples were shaken at RT for 1 min and 600 rpm, and allowed to incubate for 20 min at RT. After centrifugation, the flow-through was discarded. Each filter unit was washed three times with urea $buffer_{(Tris\text{-}HCl)}$ and then three times with ABC $buffer_{(aq)}$ according to the following scheme: addition of 100 µL of buffer, incubation for 5 min at RT while constantly shaking at 600 rpm, centrifugation, discarding the flow-through. Finally, the filter units were transferred into new 2 mL microcentrifuge tubes. Proteins were proteolytically digested either with trypsin (Sequencing Grade Modified Trypsin; # V5111, Promega, Madison, WI, USA) using an enzyme:protein ratio of 1:30 (w/w, 0.033 µg enzyme per µg protein, in total 1.67 µg enzyme), or proteinase K (# A4392, AppliChem) using an enzyme:protein ratio of 1:10 (w/w corresponding to 0.1 µg enzyme per µg protein, in total 5 µg enzyme). Before addition to the filter units, both enzymes were brought to a final volume of 100 µL by dilution with ABC $buffer_{(aq)}$ + 1 mM calcium chloride ($CaCl_{2(ABC\ buffer)}$) + 5% (v/v) $acetonitrile_{(aq)}$ (ACN)

($CaCl_2$, # A4689, AppliChem; ACN, LC-MS CHROMASOLV®, # 34967-1L, Sigma-Aldrich). Samples were shaken at RT for 1 min and 600 rpm before incubation overnight at 37°C and 350 rpm using a temperature-controlled incubator (Titramax 1000 + Inkubator 1000, both Heidolph, Schwabach, Germany). Digests were collected by centrifugation. Filter units were washed twice, first using 50 µL ABC $buffer_{(aq)}$ + 5% (v/v) $acetonitrile_{(aq)}$, then using 50 µL Milli-Q water; in between samples were centrifuged. The flow through was kept along with the digest for subsequent drying by vacuum centrifugation (RVC 2–33 CDplus, ALPHA 2–4 LDplus, Martin Christ GmbH, Osterode am Harz, Germany). The dried digests were reconstituted in 50 µL 0.1% (v/v) $trifluoroacetic\ acid_{(aq)}$ (TFA; # 28904, Thermo Fisher Scientific, Waltham, MA, USA) to a final concentration of 1 µg/µL. Samples were shaken at RT for 10 min and 1,000 rpm, and finally stored at -20°C.

Glycopeptide Enrichment via Spin-Cotton-HILIC-SPE

Glycopeptides were enriched using to a modified version of the cotton-HILIC-SPE protocol introduced by Selman et al. (26). In brief, cotton-HILIC microtips were prepared by filling 20 µL pipette tips to the 10 µL marking with cotton wool derived from commercially available cotton pads (100% cotton) (supplemental figure 1). In contrast to the original protocol, centrifugation – instead of pipetting up and down – was used during washing, loading, and elution steps; i.e. liquid was pipetted on top of the cotton wool stationary phase, the cotton-HILIC microtip was placed in an 1.5 mL microcentrifuge tube, and was centrifuged for 1 min at 2,400 x g (Heraeus™ Fresco™ 17 Microcentrifuge, 24 x 1.5/2.0 mL rotor, Thermo Scientific). Cotton-HILIC microtips were washed and equilibrated three times with 20 µL Milli-Q water and 20 µL 83% (v/v) $ACN_{(aq)}$ + 0.1% (v/v) $TFA_{(aq)}$, respectively. Each cotton-HILIC microtip was loaded with 10 µg of protein digest (dried by vacuum centrifugation and resuspended in 10 µL 83% (v/v) $ACN_{(aq)}$ + 0.1% (v/v) $TFA_{(aq)}$). For proper adsorption, the protein digest was loaded three times. The final flow through was collected and referred to as “glycopeptide-depleted fraction”. Cotton-HILIC microtips were washed three times with 20 µL 83% (v/v) $ACN_{(aq)}$ + 0.1% (v/v) $TFA_{(aq)}$. The flow-through was collected and referred to as “wash fraction”. Glycopeptides were eluted three times with 20 µL of Milli-Q water each. Along with the other two fractions, the eluate – referred to as “glycopeptide-enriched fraction” – was dried, and resuspended in 20 µL of 0.1% (v/v) $TFA_{(aq)}$ while shaking at RT for 10 min and 1,000 rpm.

Nano-RP-LC-ESI-OT-OT-MS/MS (HCD)

HILIC-enriched (glyco)peptides were analyzed by nano-reversed-phase liquid chromatography using an UltiMate 3000 RSLCnano system (Thermo Scientific) coupled online to a linear ion trap-orbitrap hybrid mass spectrometer (nano-RP-LC-ESI-OT-OT-MS/MS; LTQ Orbitrap Elite hybrid mass spectrometer, Thermo Scientific). For each measurement an equivalent of 4 µg protein digest were injected. Within the first four minutes after sample injection, (glyco)peptides were loaded isocratically on a C18 nano pre-column (Nano Trap Column, packed with Acclaim PepMap100 C18, 5 µm, 100Å, 100 µm i.d. x 2 cm, # 164564, Thermo Scientific). During this pre-concentration and desalting step, 98% (v/v) Milli-Q water MS, 2% (v/v) ACN, 0.05% (v/v) TFA (“loading pump solvent”) was used at a flow rate of 7 µL/min. Subsequently, the C18 nano pre-column was switched in line with the C18 nano separation column (Acclaim PepMap RSLC C18, 2 µm, 100Å, 75 µm i.d. x 25 cm, # 164536, Thermo Scientific) for gradient elution. Here, the following solvents were used at a constant flow rate of 300 nL/min: “A” (98% (v/v)

Milli-Q water MS, 2% (v/v) ACN, 0.1% (v/v) formic acid [FA, # 56302-10X1ML-F, Sigma Aldrich]); "B" (10% (v/v) Milli-Q water MS, 10% (v/v) 2,2,2-trifluoroethanol [TFE, # 808259, Merck, Darmstadt, Germany], 80% (v/v) ACN, 0.1% (v/v) FA). A binary gradient was applied as follows: 4% B for 4 min, linear gradient to 30% B for another 29 min, linear gradient to 34% B within 1 min, isocratic washing at 90% B for 4 min, finally 22 min re-equilibration at 4% B. After 42 min the pre-column was switched back into loading-pump flow to be re-equilibrated for 18 min at 100% "loading pump solvent" at 7 µL/min flow rate. The column oven temperature was kept constant at 40°C.

The mass spectrometer was interfaced with a nanoelectrospray source (Nanospray Flex™ ion source, NSI, Thermo Scientific) operated in positive ion mode. For electrospray ionization the following parameters were used: source voltage (2.7 kV); capillary temperature (275°C); sheath gas flow, aux gas flow, and sweep gas flow were set to zero arbitrary units. Full scan spectra (MS^1) were acquired in the orbitrap mass analyzer (OT): ions between m/z 300-2,000 were recorded at a resolution of 30,000 (defined at m/z 400) using profile mode; automatic gain control (AGC) target was set to $1x10^6$; and maximum injection time was set to 500 ms. For enhanced mass accuracy internal real-time mass calibration was enabled using a background polysiloxane peak at m/z 371.1012 ($[M+H]^+$) as lock mass. Fragment ion spectra (MS^2) were obtained by data-dependent acquisition: the five most intense precursor ions with a charge state ≥2 and signal intensity ≥500 counts were subjected to higher-energy collisional dissociation (HCD, data-dependent acquisition). HCD spectra were acquired in the OT using profile mode: mass range was set to "normal"; AGC target was set to $1x10^6$, and maximum injection time was set to 500 ms. Three HCD fragmentation regimes were used, differing in applied normalized collisional energies (NCE): HCD.low with NCE of 20, HCD.high with NCE of 50, and HCD.step with stepped NCE of 35 (width: 15%, steps: 2). An isolation width of 4 m/z units was used; charge state screening as well as monoisotopic precursor selection was enabled; target ions selected for MS^2 were dynamically excluded using the following settings: exclusion size list (500), exclusion duration (5 s), repeat count (1), and repeat duration (30 s). The number of micro scans was set to one.

Glycopeptide Data Analysis Using glyXtool^{MS}

glyXtool^{MS} is an OpenMS- and python™-based software pipeline, that was developed in-house for the semi-automated analysis of glycopeptide mass spectrometry data (Pioch et al. 2018, submitted, 2018). Briefly, acquired spectra were converted to *.mzML format, and subsequently processed using a dedicated analysis workflow in OpenMS. For each glycoprotein, the respective protein sequence (UniProtKB/Swiss-Prot database; Swiss-Prot 51.6; 257964 sequences; 93947433 residues; downloaded February, 2013) along with appropriate *N*- or *O*-glycan composition databases (in-house; *N*-glycan compositions: 378; core 1 & 2 mucin-type *O*-glycan compositions: 8; for mucin-type *O*-glycans only the major compositions relevant for human plasma proteins were considered: non-, mono- and disialylated glycoforms, no further modifications/elongations (27)) were implemented in the workflow. The following parameters were used for the in silico proteolytic digest: number of missed cleavages (2; only relevant for tryptic digests); cysteine carbamidomethylation as fixed modification; methionine oxidation and asparagine/glutamine deamidation as variable modifications. Fragment ion spectra were automatically classified as glycopeptide spectra based on the presence of oxonium ions (B-ions) and glycan-derived neutral loss fragment ions (Y-ions) by glyXtool^{MS}. Automatic annotation of the glycan and

peptide moiety was manually validated using the glyXtool^{MS} Evaluator. A mass tolerance of ±10 ppm and ±20 ppm was accepted for precursor ions and fragment ions, respectively. For the annotation of the glycan moiety the following abbreviations/symbols are used: N-acetylglucosamine (GlcNAc, HexNAc, N, "blue square"), mannose (Man, Hex, H, "green circle"), fucose (Fuc, DHex, F, "red triangle"), N-acetylneuraminic acid (NeuAc, NANA, Sa, "purple diamond"), N-glycolylneuraminic acid (NeuGc, NGNA, Ng, "light blue diamond"), peptide (Pep). Further details on glyXtool^{MS} are given in Pioch et al. (Pioch et al. 2018, submitted, 2018). In addition to the glycopeptide-focused analysis via glyXtool^{MS}, conventional protein identification was also performed. To this end the fragment ion spectra of the untreated proteolytic digests (without HILIC enrichment) were searched against the UniProtKB/Swiss-Prot database using MASCOT 2.5.1.0 (Matrix Science, London, UK). The applied search parameters and results can be found in supplemental table 1. To pave the way for standardized analyses, the MIRAGE consortium (minimum information required for a glycomics experiment) has specified reporting guidelines for collecting, sharing, integrating, and interpreting mass spectrometry-based glycomics and glycoproteomics data (28). Data reported in this manuscript is in agreement with these guidelines. All N- and O-glycopeptide mass spectrometry raw data (HILIC-SPE fractions) have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the MassIVE repository (ftp://massive.ucsd.edu/MSV000082137 | password: Glycoproteomics).

Site-Specific Relative Quantitation of N-glycoforms

To determine the site-specific relative abundance of IgG, Fib, and RNase B N-glycoforms (microheterogeneity), the summed peak intensities of the corresponding glycopeptide precursor ion isotopic pattern along the peak elution window were used (referred to as peak intensity). Thereby, peak integration boundaries of the respective extracted ion chromatograms were set automatically by glyXtool^{MS}. For glycopeptides registered with different precursor charge states, the respective peak intensities were summed. Generally, peak intensities were only considered for manually validated HCD.low or HCD.step fragment glycopeptide ion spectra; in case glycopeptides were identified using both methods an average value was used. The relative site-specific glycoform abundance was calculated by dividing the individual peak intensities by the sum of all peak intensities covering the same glycosylation site.

Relative Quantitation of N- and O-Glycopeptide Oxonium Ions

The relative oxonium ion abundance was calculated by dividing the individual oxonium ion peak intensities (monoisotopic peak) by the sum of all oxonium ion peak intensities detected in the fragment spectrum (relative abundance). For glycopeptides with more than one corresponding fragment ion spectrum (scans) the average relative abundance is given. Error bars indicate the standard deviation of the relative oxonium ion abundance for glycopeptides with more than two scans.

1.1. Further Details on Differences and Characteristics of *HCD.low*, *HCD.step*, and *HCD.high*

A general caveat of *HCD.step* compared to *HCD.low* is, that due to the acquisition with two different collisional energies, the duty cycle of *HCD.step* is slightly longer. Depending on the complexity of the sample, this can lead to a decrease in the number of acquired fragment ion spectra. For tryptic IgG glycopeptides, for example, 40% less fragment ion spectra were acquired by *HCD.step* compared to *HCD.low* (HCD.low: 3690; HCD.step: 2281 spectra). Still, for the majority of glycopeptides identified in our study we were able to acquire an *HCD.step* as well as *HCD.low* fragment ion spectrum (63 %). Another aspect that needs to be considered when using *HCD.step* fragmentation is that internal glycopeptide fragment ions can occur for peptide sequences which contain proline: for a tryptic *N*-glycopeptide ($_{23}$CANLVPVPIT**N**ATLDR$_{38}$ +HexNAc$_4$Hex$_5$NeuAc$_2$) derived from alpha-1-acid glycoprotein 2 we have noticed internal glycopeptide cleavages N-terminal to proline (Supplemental Figure 2, pp. 101-110). Accordingly, two truncated *N*-glycopeptides ("y-ions", $_{28}$PVPIT**N**ATLDR$_{38}$ +HexNAc$_2$, $_{30}$PIT**N**ATLDR$_{38}$ +HexNAc$_2$) were detected by the corresponding conserved *N*-glycopeptide fragmentation patterns, including the respective peptide masses and peptide fragment ions. This cleavage pattern might be explained by the cyclic structure of proline and the ability to introduce a kink in alpha helices (1). Awareness of this fragmentation behavior is important to avoid spectra misinterpretation.

Of note, using solely high-energy collisional fragmentation (*HCD.high*) on *N*- and *O*-glycopeptides results in fragment ion spectra of low intensity that predominantly feature oxonium and immonium ions. Thereby only a very few to none peptide backbone fragment ions (water-loss b- and y-ions), and no glycan-derived Y-ions, can be registered (Supplemental Figure 2, pp. 9-11).

1.2. Reproducibility and Glycopeptide Enrichment Efficiency of the Spin-Cotton-HILIC-SPE

Comparing the base peak chromatograms (BPCs MS^1) of three LTF glycopeptide spin-cotton-HILIC-SPE preparations shows a high reproducibility between the individual replicates. The glycopeptide enrichment efficiency was evaluated by analyzing the *glycopeptide-depleted fraction* (loading), the *wash fraction*, and the *glycopeptide-enriched fraction* (elution) of spin-cotton-HILIC-SPE treated IgG glycopeptides. To this end, the *HCD.low* fragment ion spectra of the aforementioned HILIC fractions were screened for the presence of glycopeptides, both manually and software-assisted (glyXtool^{MS}). Consistently, both analyses revealed a lossless enrichment of glycopeptides in the *glycopeptide-enriched fraction* (elution). I.e., no glycopeptides were detected in the *glycopeptide-depleted fraction* or the *wash fraction*. Compared to the unenriched IgG sample, the number of identified *N*-glycopeptides could be doubled, further highlighting the efficiency of the enrichment step (number of identified *N*-glycopeptides using glyXtool^{MS}: 25 (unenriched), 51 (spin-cotton-HILIC-SPE enriched)). Analyses of the glycopeptide enrichment efficiency of all other proteins investigated in this study also resulted in an efficient and lossless enrichment of *N*-and *O*-glycopeptides (data not shown).

1.3. Criteria Applied for the Validation of Glycopeptide Identifications

The following twelve criteria were applied for accepting a glycopeptide identification: (I) EIC: no overlapping or split peak, (II) isotopic pattern: correctly assigned and non-overlapping, (III) presence of ions corresponding to the peptide mass and/or peptide +HexNAc, (IV) presence of the conserved *N*-glycopeptide fragmentation pattern (applies only to *N*-glycopeptides; fragmentation pattern needs to be present with *HCD.step*), (V) unambiguous identification of the glycan moiety, based on Y-ions, (VI) glycan features deduced from Y-ions, such as sialylation, need to agree with detected oxonium ions, (VII) unambiguous identification of the peptide moiety, based on a-, b-, and y-ions (b- and y-ions also with loss of water or ammonia), (VIII) presence of immonium ions to support peptide identification, (IX) accepted mass error: precursor ion (±10 ppm), fragment ions (±20 ppm), (X) check retention time for plausibility: retention times of different glycoforms attached to the very same peptide should not differ too much (<10 min) - sialylated glycopeptides have significantly longer retention times compared to nonsialylated glycopeptides, (XI) different charge states of the same glycopeptide should have nearly the same retention time, and (XII) if present, identifications, derived from both *HCD.low* and *HCD.step*, should be in line with each other. In rare cases, peptide identifications suggested by the software, were inconclusive. In this case manual *de novo* sequencing was performed to account for potential amino acid modifications or protein sequences not considered during the initial protein identification search. During every glycopeptide validation step, special attention was paid on ambiguous and conflicting masses (Supplemental Figure 4, bottom). One prominent example in this context, is the mass of a carbamidomethylation (e.g. on cysteine), which equals the mass of glycine (2). Awareness of this ambiguity is particularly relevant for the analysis of (glyco)peptides generated by broad- or non-specific proteolysis. Examples for ambiguous identifications of IgG *N*-glycopeptides are given in Supplemental Figure 5.

1.4. Human Immunoglobulin Gamma *N*-Glycoproteomics (Fc-glycosylation)

Human IgG comprises four subclasses (IgG 1, 2, 3, and 4). Our analysis allowed to identify 87 *N*-glycopeptides derived from the constant CH2 region of the heavy chain of IgG (Fc part, fragment crystallizable part; *N*-glycosylation sites: IgG 1 | N_{180}, IgG 2 | N_{176}, IgG 3 | N_{227}, N_{322}, IgG 4 | N_{177}) (Supplemental Figure 2, pp. 3-26). In agreement with literature, the majority of identified *N*-glycopeptides [84] is derived from IgG 1 and 2 – in human adult serum both have a relative abundance of 60% and 32%, respectively (total concentration of IgG about 10 g/L) (3). A profiling of these IgG 1 and 2 *N*-glycopeptides, revealed predominantly core-fucosylated diantennary complex-type *N*-glycans with different degrees of galactosylation, with and without a terminal NeuAc and/or a bisecting-GlcNAc (Supplemental Figure 6). The three main glycoforms, for both IgG 1 and IgG 2, were $HexNAc_4Hex_3Fuc_1$, $HexNAc_4Hex_4Fuc_1$, and $HexNAc_4Hex_5Fuc_1NeuAc_1$ - also known as G0F, G1F, G2FS (IgG 1|2: 23|27%; 32|23%; 11|11%). This is in good agreement with previously reported data (4). For IgG 4 only three *N*-glycopeptides corresponding to two glycoforms (G0F, G1F; 72|28%) were identified (relative abundance of IgG 4 in human adult serum about 4% (3)). For IgG 3, no *N*-glycopeptides were identified. In total, glycan compositions corresponding to 24 different *N*-glycoforms were registered for IgG 1, 2, and 4

(Supplemental Table 2). Since the tryptic digest already generated *N*-glycopeptides that could be readily analyzed by LC-MS/MS, meaning the generated peptide moieties were neither too short nor too long, the proteinase K digest was not considered for the glycoproteomic analysis here.

1.5. Human Fibrinogen *N*- and *O*-Glycoproteomics

Human fibrinogen is a hexamer comprising two identical heterotrimers connected by a central nodule. Each heterotrimer comprises an α-, β-, and γ-chain. The protein is known to be *N*- and *O*-glycosylated (5-8). The α-chain is reported to solely carry mono- and disialylated mucin-type-1 *O*-glycans (T-antigen), while the γ-chain is reported to carry solely diantennary non-, mono- and disialylated complex-type *N*-glycans (7). The β-chain, in contrast, is known to carry both types of glycosylation (7). Fibrinogen features several heavily *O*-glycosylated regions that cannot be analyzed by trypsin alone due to the lack of sufficient tryptic cleavages sites. Previous studies have shown, that the broad, yet reproducible, cleavage specificity of proteinase K yields complementary results to trypsin - making heavily *O*-glycosylated regions analytically amenable, too (6, 7). In the present study, both the tryptic and the proteinase K digest allowed a reliable identification and characterization of *N*-glycopeptides covering the already known *N*-glycosylation sites present on the β-, and γ-chain (β-chain: N_{394}, γ-chain: N_{78}). Additionally, some lower-abundant *N*-glycopeptides derived from the *N*-glycosylation site N_{686} present on the α-chain were detected. This *N*-glycosylation site has been reported to be occupied before (9), but, to the best of our knowledge, the attached *N*-glycans have not been described so far (Supplemental Figure 2, pp. 27-31; Supplemental Figure 7; Supplemental Table 3). For the tryptic digest, a total of 38 *N*-glycopeptides were identified. Analysis of these *N*-glycopeptides revealed for the α- and γ-chain exclusively diantennary mono- and disialylated complex-type *N*-glycans without a core-fucose ($HexNAc_4Hex_5Fuc_1NeuAc_{1/2}$ (G2FS1/2)). The β-chain, in contrast, also features non-sialylated, core-fucosylated, and glycoforms with a bisecting GlcNAc - though at much lower relative abundance. Using proteinase K, a total of 15 *N*-glycopeptides were registered. In contrast to the tryptic digest, only the two major glycoforms $HexNAc_4Hex_4Fuc_1NeuAc_{1/2}$ (G1FS1/2) were detected (α-chain: only monosialylated; γ-chain: only disialylated; β-chain: both). It is worth mentioning that also some *N*-glycopeptides of human alpha-1-antitrypsin were detected in the fibrinogen sample, pointing to a potential contamination of this supposedly pure protein sample. The tryptic digest allowed identification of 20 fibrinogen *O*-glycopeptides. Slightly more, 26 O-glycopeptides, were identified using proteinase K (Supplemental Figure 2, pp. 32-60; Supplemental Figure 7; Supplemental Table 3). In both cases, non-, mono- and disialylated mucin-type core-1 *O*-glycans (T-antigen) were detected for these glycopeptides. The fibrinogen alpha chain appears to be heavily *O*-glycosylated: using trypsin, 18 *O*-glycopeptides were detected - covering six different *O*-glycosylation regions including one newly discovered region (S_{609}, S_{616}, S_{618}, S_{619}). The proteinase K digest allowed to identify 18 α-chain *O*-glycopeptides, and yielded, for the most part, complementary results with respect to the covered *O*-glycosylation sites/regions. In total eight *O*-glycosylation sites/regions were detected, of which three regions (I: S_{325}, S_{326}; II: S_{356}, S_{357}, S_{359}; III: T_{499}, T_{501}) and three sites (I: T_{499}, II: T_{522}; III: S_{534}) have not been reported so far. For the β-chain, both trypsin and proteinase K generated *O*-glycopeptides covering the already known O-glycosylation region

(S_{58}, S_{67}). In agreement with previous reports, no *O*-glycopeptides were detected on the fibrinogen γ-chain. As a side note, two contaminant *O*-glycopeptides derived from von Willebrand factor (disialylated mucin-type core-1 O-glycan, S_{1263}) were also detected in the fibrinogen sample. Those two *O*-glycopeptides cover an *O*-glycosylation site that has recently been reported by Solecka *et al*. (10).

1.6. Human Lactotransferrin *N*-Glycoproteomics

Human lactotransferrin is a globular glycoprotein that is reported to be solely *N*-glycosylated. The protein harbors three potential *N*-glycosylation sites (N_{156}, N_{497}, N_{642}), of which only the first and the second are reported to be glycosylated (11, 12). In this study, a total of 73 tryptic lactotransferrin *N*-glycopeptides were detected – for the first time, covering all three potential *N*-glycosylation sites (Supplemental Figure 2, pp. 61-90; Supplemental Table 4). The first *N*-glycosylation site, N_{156}, features mono- and disialylated core-fucosylated complex- and hybrid-type *N*-glycans that can also have additional antenna fucoses and/or LacNAc extensions (LacNAc = $GlcNAc_1Gal_1$). The two major glycoforms are $HexNAc_4Hex_5Fuc_1NeuAc_{1/2}$ (G2FS1/2). Discrimination between a LacNAc extended antenna and the presence of an additional antenna is based on a diagnostic oxonium ion that corresponds to two LacNac units plus a fucose (LacNac extended antenna plus antenna fucose, $HexNAc_2Hex_2Fuc_1$). Detected by *HCD.low*, this oxonium ion was only present in lactotransferrin *N*-glycopeptides presumed to feature a LacNAc extension with an antenna fucose. Moreover, this oxonium ion is absent in *HCD.low N*-glycopeptide fragment ion spectra of IgG, Fib, and RNase B – all of which are reported to not feature any LacNAc extensions with an antenna fucose (the $HexNAc_2Hex_2Fuc_1$ fragment ion is also not present in fragment ion spectra of multi-antennary glycoproteins, like alpha-1-acid glycoprotein 1). Unfortunately, LacNAc extensions that lack an antenna fucose could not be linked to a diagnostic oxonium ion: a potential candidate would have been an oxonium ion with two LacNAc units ($HexNAc_2Hex_2$); this oxonium ion, however, is also present in *N*-glycopeptide fragment ion spectra featuring diantennary *N*-glycans that lack a LacNAc extension. The second *N*-glycosylation site, N_{497}, features non-, mono-, and disialylated complex-type N-glycans,that are mainly core-fucosylated, but also non-fucosylated. The two major glycoforms are $HexNAc_4Hex_5Fuc_1NeuAc_{1/2}$ (G2FS1/2). As for site N_{156}, glycoforms with additional antenna fucoses were also detected. Interestingly, though, no LacNAc extended glycoforms were registered. Surprisingly, we also detected *N*-glycans on the third *N*-glycosylation site, N_{642} (not described before). This site features mono- and disialylated core-fucosylated complex-type *N*-glycans, that can also have additional antenna fucoses and/or LacNAc extensions. All identified glycoforms exhibited similar abundance; hence, there is no single predominant glycoform on this *N*-glycosylation site. For LTF, no *O*-glycopeptides were detected, which is in agreement with common knowledge.

1.7. Quantification of Oxonium Ions, Complex-type *N*-glycopeptides: General Observations

In the following, characteristics of oxonium ions produced by fragmentation of complex-type *N*-glycopeptides via *HCD.low* and *HCD.step* will be described. The first noticeable characteristic is that relative oxonium ion abundance do not differ between different glycoproteins or *N*-glycosylation sites when considering the same *N*-glycoform and the same collisional energy, as evidenced by comparison of IgG 1 and 2 as well as LTF and IgG 2 (Supplemental Figure 9: Parts A and B). The produced oxonium ion patterns thus seem to be conserved for specific complex-type *N*-glycoforms independent of the peptide backbone. In most cases, the charge state of the *N*-glycopeptide precursor ion also seems to have only a minor influence on the produced oxonium ions. Using *HCD.low*, a slight increase in the relative abundance of HexNAc, HexNAc -H_2O, NeuAc, and NeuAc -H_2O oxonium ions was observed at higher charge states for some glycoforms (Supplemental Figure 9: Part C**,** NeuAc/NeuAc -H_2O not shown); with *HCD.step*, the influence of the precursor ion charge state seems negligible. In general, relative abundance of oxonium ions produced by *HCD.low* and *HCD.step* differ significantly from each other when comparing the very same complex-type *N*-glycopeptides (Supplemental Figure 9: Part D). With increasing collisional energy, the relative abundance of di- and trisaccharide oxonium ions, such as $HexNAc_1Hex_1$, $HexNAc_1Hex_2$ or $HexNAc_1Hex_1NeuAc_1$ decreases – some di- and trisaccharide oxonium ions even disappear due to decomposition into mono- and disaccharide oxonium ions.

The comparison of the relative oxonium ion abundance detected for seven IgG 1 and 2 *N*-glycopeptides exemplifies this effect (Supplemental Figure 9: Part J (I-II)): while the relative abundance of the $HexNAc_1Hex_1NeuAc_1$ oxonium ion is about 10% with *HCD.low*, the relative abundance drops to about 1% with *HCD.step*. The same holds true for the $HexNAc_1Hex_2$ oxonium ion (average relative abundance: *HCD.low* 6%, *HCD.step* 0.9%). A second example is given in Supplemental Figure 9: Part E (II, V): while *HCD.low* fragmentation of the lactotransferrin *N*-glycopeptide $HexNAc_4Hex_5Fuc_2NeuAc_1$ (G2F2S1) produces a strong $HexNAc_1Hex_1Fuc_1$ oxonium ion signal of about 8%, the relative abundance with *HCD.step* is only about 1%. Another striking characteristic that differentiates *HCD.step* from *HCD.low* is that *HCD.step* fragmentation of complex-type *N*-glycopeptides always results in the HexNAc oxonium ion peak being the dominant peak among the oxonium ions – independent of the present complex-type *N*-glycoform (relative abundance always >38%) Supplemental Figure 9: (Parts A (VI-XIII), D, E (IV-VI), J (II)). In addition, at higher collisional energy, the relative abundance of corresponding water-loss species is also increased compared to lower energy fragmentation (e.g. average relative abundance for HexNAc -H_2O: *HCD.low* 6%, *HCD.step* 19%) (Supplemental Figure 9: Part J (I-II)). At lower collisional energy, in contrast, either the HexNAc or $HexNAc_1Hex_1$ oxonium ion dominates, depending on the present glycoforms (Supplemental Figure 9: Part J (I)). The $HexNAc_1Hex_1$ oxonium ion dominates whenever at least one galactose is present in the *N*-glycopeptide glycoform (Supplemental Figure 9: Part D (II-IV)); in case there is no galactose the HexNAc oxonium ion dominates (Supplemental Figure 9: Parts A (III), D (I)). An exception seems to be the presence of a single galactose as part of a di- or more-antennary *N*-glycan (with/without bisecting GlcNAc): in this case either the $HexNAc_1Hex_1$ or the HexNAc oxonium ion dominates depending on the precursor ion charge state (Supplemental Figure 9: Part A (I, II)). Another exception is the presence of a single GlcNAc attached to the trimannosyl-chitobiose core

without an additional galactose and without being a bisecting GlcNAc (single antenna GlcNAc without additional galactose): in this case, the $HexNAc_1Hex_1$ oxonium ion dominates (Supplemental Figure 9: Part D(I)).

1.8. Quantification of Oxonium Ions, Complex-type *N*-glycopeptides: antenna GlcNAc vs bisecting GlcNAc

Comparing the relative oxonium ion abundance of individual fragment ion scans acquired for a particular *N*-glycopeptide with each other, for both *HCD.low* and *HCD.step*, only marginal differences can be detected in most cases, as indicted by the standard deviation (<0.5; Supplemental Figure 9: Parts A, D). Supplemental Figure 10 (Part A) exemplarily shows the individual *HCD.low* fragment ion scans acquired for the IgG 1 *N*-glycopeptide $HexNAc_5Hex_3Fuc_1$ (G0FN) over time. This *N*-glycopeptide features a non-galactosylated *N*-glycan with a bisecting GlcNAc. In agreement with our general observations, the low-energy fragmentation of a non-galactosylated complex-type *N*-glycopeptide results in the HexNAc oxonium ion being the dominant peak among the oxonium ions. For the IgG 1 *N*-glycopeptide $HexNAc_5Hex_3Fuc_1$ this was consistently observed across all acquired scans. The presence of a bisecting GlcNAc gives rise to two characteristic fragment ions when using low-energy fragmentation: (I) peptide+$HexNAc_3Hex_1$ (Y-ion, $[M+H]^+$, $[M+2H]^{2+}$) and (II) $HexNAc_2$ (oxonium ion, B-ion, $[M+H]^+$). These fragment ions were consistently found in all *HCD.low* scans of the IgG 1 *N*-glycopeptide $HexNAc_5Hex_3Fuc_1$ (Supplemental Figure 10: Part A). With *HCD.step* these fragment ions are not present or only at very low intensity (not shown). The same fragmentation behavior was also observed for all acquired *HCD.low* scans for the IgG 2 *N*-glycopeptide $HexNAc_5Hex_3Fuc_1$ (G0FN) (Supplemental Figure 10: Part B) and for the IgG 1 *N*-glycopeptide $HexNAc_3Hex_2Fuc_1$ (Supplemental Figure 10: Part C). Again, the relative abundance of the oxonium ions and the presence of the peptide+$HexNAc_3Hex_1$ and the $HexNAc_2$ fragment ions suggest a non-galactosylated *N*-glycan with a bisecting GlcNAc being attached to the peptide. The two diagnostic fragment ions (I) peptide+$HexNAc_3Hex_1$ and (II) $HexNAc_2$ were absent in *N*-glycopeptides derived from glycoproteins largely lacking *N*-glycans with a bisecting GlcNAc, such as Fib and LTF (both have almost exclusively complex-type *N*-glycans without bisecting GlcNAc), or RNase B (only high-mannose-type *N*-glycans). Interestingly, *HCD.low* fragmentation of the IgG 1 *N*-glycopeptide $HexNAc_3Hex_3Fuc_1$ (Supplemental Figure 10: Part D) showed an inconsistent oxonium ion pattern across the acquired scans (which also explains the high standard deviation in Supplemental Figure 10: Part D(I)). In the first four scans the $HexNAc_1Hex_1$ oxonium ion dominates, which along with the absence of a peptide+$HexNAc_3Hex_1$ fragment ion suggests a non-galactosylated *N*-glycan with an antenna GlcNAc attached to the peptide (Supplemental Figure 10: Part D, scan #1-4). In scans #5 and #6, however, the $HexNAc_1$ oxonium ion dominates, which along with the presence of the peptide+$HexNAc_3Hex_1$ and the $HexNAc_2$ fragment ion in scan #6, suggests a non-galactosylated *N*-glycan with a bisecting GlcNAc attached (Supplemental Figure 10: Part D, scan #5-6). The same fragmentation behavior could also be observed for the IgG 2 *N*-glycopeptide $HexNAc_3Hex_3Fuc_1$ (Supplemental Figure 10: Part F). Again, the earlier eluting non-galactosylated *N*-glycopeptide with an antenna GlcNAc shows a different oxonium ion pattern (Supplemental Figure 10: Part F, scan #1) and no peptide+$HexNAc_3Hex_1$ fragment ion,

compared to the later eluting non-galactosylated *N*-glycopeptide with a bisecting GlcNAc (Supplemental Figure 10: Part F, scan #2-3). Thus, this finding might enable discrimination between isobaric *N*-glycopeptides featuring either an antenna GlcNAc or a bisecting GlcNAc, based on differences in the retention time, characteristic *HCD.low* oxonium ion pattern, and diagnostic fragment ions. With *HCD.step* the observed change in the oxonium ion pattern between antenna GlcNAc and bisecting GlcNAc could not be observed (Supplemental Figure 10: E and G).

1.9. Quantification of Oxonium Ions, Complex-type N-glycopeptides: type of sialic acid, degree of sialylation

N-acetylneuraminic acid (NeuAc, NANA) and *N*-glycolylneuraminic (NeuGc, NGNA) are the two most commonly found types of sialic acids in vertebrates (13). High- as well as low-energy fragmentation of NeuAc or NeuGc containing *N*-glycopeptides produces intense and distinct oxonium ions allowing a clear distinction of these two types of sialic acid: NeuAc oxonium ions (NeuAc; NeuAc $-H_2O$; $HexNAc_1Hex_1NeuAc_1$), NeuGc oxonium ions (NeuGc; NeuGc $-H_2O$; $HexNAc_1Hex_1NeuGc_1$) (NeuGc not shown in this study). To assess the degree of NeuAc sialylation the relative abundance of oxonium ions derived from mono- and disialylated glycoforms of LTF glycopeptides were compared (Supplemental Figure 9: Part G). With *HCD.low* relative abundance of the NeuAc and NeuAc $-H_2O$ oxonium ions did not differ significantly between mono- and disialylated LTF *N*-glycopeptides. The relative abundance of the $HexNAc_1Hex_1NeuAc_1$ oxonium ion, though, was found to be higher with disialylated LTF *N*-glycopeptides in most, yet not all, cases (relative abundance $HexNAc_1Hex_1NeuAc_1$: monosialylated, between 10-18%; disialylated, between 22-28%). With *HCD.step* relative abundance of the NeuAc and NeuAc $-H_2O$ oxonium ions were slightly higher with disialylated LTF *N*-glycopeptides (NeuAc: monosialylated, about 5%; disialylated, about 10%. NeuAc $-H_2O$: monosialylated, about 12%; disialylated, about 20%). The relative abundance of $HexNAc_1Hex_1NeuAc_1$ oxonium ion, in contrast, was very low (<2%), and did not differ significantly between mono- and disialylated LTF *N*-glycopeptides when applying higher collisional energy ($HexNAc_1Hex_1NeuAc_1$: monosialylated, about 1%; disialylated, about 2%). Overall, predicting the degree of NeuAc sialylation based on the relative abundance of NeuAc, NeuAc $-H_2O$, and $HexNAc_1Hex_1NeuAc_1$ oxonium ions seems promising, but needs to be further investigated, as for instance the influence of different sialic acid linkages needs to be evaluated, and might have caused the observed inconsistencies. Also of note, and in agreement with reports by Halim *et al.* (14), along with NeuAc and NeuAc $-H_2O$ six lower abundant oxonium ions corresponding to $[NeuAc -2xH_2O]^+$ *m/z* 256.0821, $[NeuAc -3xH_2O]^+$ *m/z* 238.0715, $[NeuAc -2\times H_2O -NH_2C(O)CH_3]^+$ *m/z* 197.043, $[NeuAc -2\times H_2O -COH -NH_2C(O)CH_3]^+$ *m/z* 167.0375, $[NeuAc -4xH_2O]^+$ *m/z* 220.061, and $[NeuAc -2\times H_2O -CHO -NH_2C(O)CH_3 -COOH]^+$ *m/z* 121.032 were consistently detected; the last two were only detected with *HCD.step*, though.

1.10. Quantification of Oxonium Ions, Mucin-type O-glycopeptides

Apart from various *N*-glycopeptide also *O*-glycopeptide fragment ion spectra have been investigated with respect to relative oxonium ion abundance. To this end, core-1 mucin-type *O*-glycopeptides derived from human fibrinogen (Fib) were fragmented by *HCD.low* or *HCD.step* and the resulting oxonium ion abundance were analyzed. As for *N*-glycopeptides, also for core-1 mucin-type *O*-glycopeptides differences in the relative abundance of the oxonium ions depending on the applied collisional energy were observed. Again, the increased collisional energy accompanied by *HCD.step* leads to a decrease or absence of signals corresponding to di- and trisaccharide oxonium ions, as can be seen for the relative abundance of $HexNAc_1Hex_1$ and $HexNAc_1Hex_1NeuAc_1$ oxonium ions (Supplemental Figure 9: Part K (I, II)). While there were striking differences between *HCD.low* and *HCD.step* for the oxonium ion abundance of non-sialylated *O*-glycopeptides (Supplemental Figure 9: Part K (I)), differences were less prominent for sialylated *O*-glycopeptides (Supplemental Figure 9: Part K (II)). The latter showed differences primarily in the relative abundance of NeuAc (NeuAc, NeuAc $-H_2O$, $HexNAc_1Hex_1NeuAc_1$) and HexNAc related oxonium ions (HexNAc, HexNAc $-H_2O$); the overall distribution, with NeuAc $-H_2O$ being the dominant oxonium ion, remained unaffected, though. With non-sialylated *O*-glycopeptides, in contrast, HexNAc and $HexNAc_1Hex_1$ oxonium ions dominated at *HCD.low*, while HexNAc and HexNAc $-H_2O$ oxonium ions dominated at *HCD.step*. Comparing the relative oxonium ion abundance of the very same *O*-glycoform between different Fib *O*-glycosylation sites/regions (α and β chain), differences between mono- and disialylated *O*-glycoforms become apparent (Supplemental Figure 9: PartL). For disialylated core-1 mucin-type *O*-glycopeptides only slight differences between different *O*-glycosylation sites/regions were observed, independent of the applied collisional energy (Supplemental Figure 9: Part L (II, IV)). Thus, it seems that the relative oxonium ion abundance of disialylated *O*-glycopeptides are conserved, and independent of the peptide backbone. On the contrary, for monosialylated core-1 mucin-type *O*-glycopeptides, and particularly for *HCD.low*, significant differences between the relative oxonium ion abundance of the fibrinogen α and β chain were detected (Supplemental Figure 9: Part L (I, III)). This suggests that the relative oxonium ion abundance of monosialylated *O*-glycopeptides are not conserved, and not independent of the peptide backbone. Surprisingly, a direct comparison, between mono- and disialylated *O*-glycoforms present on the very same peptide, revealed no significant differences in the relative oxonium ion abundance – neither for *HCD.low* nor for *HCD.step*. Hence, differentiating mono- and disialylated core-1 mucin-type *O*-glycoforms based on their relative oxonium ion abundance seems to be not possible (Supplemental Figure 9: Part M (I, II)). However, a general comparison of the relative oxonium ion abundance acquired for core-1 mucin-type *O*-glycopeptides with those acquired for *N*-glycopeptides, revealed significant differences that enable differentiation of these two forms of protein glycosylation. The most striking difference is the lack of the $HexNAc_1Hex_2$ oxonium ion for core-1 mucin-type *O*-glycopeptides. This oxonium ion was found consistently across all analyzed *N*-glycopeptides, independent of the applied collision energy (Supplemental Figure 9: Parts A-J). It appears to represent a characteristic oxonium ion that occurs upon fragmentation of (I) the chitobiose core – a main fragmentation event during *N*-glycopeptide fragmentation – and (II), optionally, further antenna-directed fragmentation steps, to ultimately generate a fragment ion corresponding to 2nd chitobiose GlcNAc with two attached mannoses ($HexNAc_1Hex_2$).

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