Journal of proteome research

Article

Cite This: J. Proteome Res. 2018, 17, 222−233

pubs.acs.org/jpr

Multiple Reaction Monitoring for the Quantitation of Serum Protein Glycosylation Profiles: Application to Ovarian Cancer

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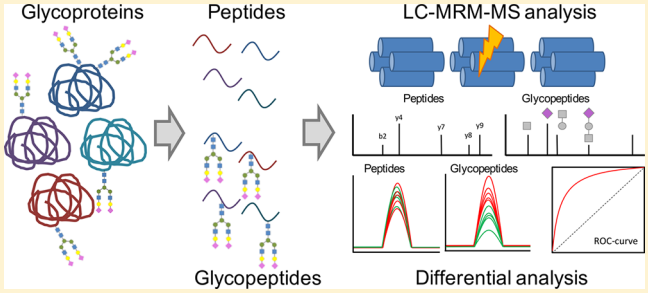
Supporting Information

ABSTRACT: Protein glycosylation fingerprints are widely recognized as potential markers for disease states, and indeed differential glycosylation has been identified in multiple types of autoimmune diseases and several types of cancer. However, releasing the glycans leave the glycoproteins unknown; therefore, there exists a need for high-throughput methods that allow quantification of site- and protein-specific glycosylation patterns from complex biological mixtures. In this study, a targeted multiple reaction monitoring (MRM)-based method for the protein- and site-specific quantitation involving serum proteins immunoglobulins A, G and M, alpha-1-antitrypsin, transferrin, alpha-2-macroglobulin, haptoglobin, alpha-1-acid glycoprotein and complement C3 was developed. The method is based on tryptic digestion of serum glycoproteins, followed by immediate reverse phase UPLC-QQQ-MS analysis of glycopeptides. To quantitate protein glycosylation independent of the protein serum concentration, a nonglycosylated peptide was also monitored. Using this strategy, 178 glycopeptides and 18 peptides from serum glycoproteins are analyzed with good repeatability (interday CVs of 3.65−21−92%) in a single 17 min run. To assess the potential of the method, protein glycosylation was analyzed in serum samples from ovarian cancer patients and controls. A training set consisting of 40 cases and 40 controls was analyzed, and differential analyses were performed to identify aberrant glycopeptide levels. All findings were validated in an independent test set (n = 44 cases and n = 44 controls). In addition to the differential glycosylation on the immunoglobulins, which was reported previously, aberrant glycosylation was also observed on each of the glycoproteins, which could be corroborated in the test set. This report shows the development of a method for targeted protein- and site-specific glycosylation analysis and the potential of such methods in biomarker development.



KEYWORDS: *protein glycosylation, glycopeptides, LC-MRM-MS, biomarker discovery, serum, protein quantitation, epithelial ovarian cancer*

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INTRODUCTION

Protein glycosylation is a common post-translational modification, and differential protein glycosylation has been identified as a characteristic of many common diseases.[1,2] Hence, there is active interest in the development and application of analytical techniques aimed to monitor protein glycosylation. Glycan analysis at the site- and protein-specific level remains difficult, because identification of both the peptide moiety and the glycan is necessary.[3] This is even more prominent for analyses in a high-throughput manner, which are often necessary in biomedical research involving large numbers of patient samples. Common analytical strategies involve either release of the glycans directly from all proteins in the tissue (e.g., refs[4,5]) or glycan- or glycopeptide analysis of individual proteins purified from biological samples (e.g., refs[6,7]). In the first approach, all glycan attachment information is lost, while the second approach requires protein purification at sufficiently high levels for analysis. The latter may further result in glycosylation bias as capturing methods may have glycan specificity.

Numerous studies have employed total glycan analysis of serum proteins to examine glycan changes associated with aging,[8,9] pregnancy,[10] autoimmune diseases[11,12] and several types of cancer.[1,13] Due to the high abundance of a few glycoproteins in serum, differential glycosylation that is observed between cancer and control may involve primarily the immunoglobulins and other abundant proteins.

Received: August 2, 2017
Published: December 5, 2017

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DOI: 10.1021/acs.jproteome.7b00541
J. Proteome Res. 2018, 17, 222−233

Protein specific glycosylation has been performed primarily with serum immunoglobulins. The glycosylation profile of the immunoglobulins, particularly IgG,[14] is by far the best characterized in disease as compared to other serum proteins. Studies involving the glycan characterization of even highly abundant serum glycoproteins, which are mostly acute-phase proteins such as haptoglobin, transferrin, alpha-1-antitrypsin, alpha-2-macroglobulin, alpha-1-acid glycoprotein and complement C3 are far more limited.

An alternative to both approaches is to analyze glycopeptides using multiple reaction monitoring mass spectrometry (MRM MS), which allows for the detection of tryptic glycopeptides from the immunoglobulins IgG, IgA and IgM directly from serum samples without enrichment.[15,16] The high sensitivity and specificity of MRM on triple quadrupole (QQQ) mass spectrometers facilitates accurate quantitation of proteins from biological specimens. MRM is currently applied for the targeted quantitation of proteins and metabolites in biofluids,[17] and it is being evaluated for its potential in clinical settings.[18] We recently optimized the MRM technique for the relative quantitation of the glycosylation patterns of IgG, IgA and IgM[15,16] and applied this method to evaluate protein-specific differential glycosylation in serum samples from ovarian cancer patients compared to matched controls.[19]

In this study, we expand our MRM repertoire with additional glycoproteins haptoglobin (HP), transferrin (TF), alpha-1-antitrypsin (A1AT), alpha-2-macroglobulin (A2MG), alpha-1-acid glycoprotein (AGP) and complement C3 (C3). MRM transitions are developed and optimized for peptides and glycopeptides. The method is then applied to biomarker research for ovarian cancer where biomarkers with great sensitivity and specificity are desperately needed for ovarian cancer detection. We evaluate the potential of glycopeptide profiles to differentiate ovarian cancer cases from controls.

EXPERIMENTAL SECTION

Chemicals and Reagents

Human IgG, IgA, IgM, α-2-macroglobulin, haptoglobin, α-1-antitrypsin, α-1-acid glycoprotein and transferrin were purchased from Sigma-Aldrich (St. Louis, MO). Human complement C3 was purchased from Athens Research & Technology (Athens, GA). Sequencing grade modified trypsin and dithiothreitol (DTT) were purchased from Promega (Madison, WI). Iodoacetamide (IAA) was purchased from Sigma-Aldrich (St. Louis, MO).

Patient Information

Institutional Review Board (IRB) approval was obtained to use serum samples obtained from the GOG tissue-banking repository in this project through University of California, Davis Medical Center (IRB #251975). The GOG collected whole blood specimens from patients with epithelial ovarian cancer (EOC) and healthy female controls from multiple participating institutions as described by the GOG #136 protocol (revised August 2003), along with clinical information that included demographics and tumor characteristics including stage, grade, and histology. Controls were healthy female volunteers without a history of malignancy and no family history of breast or ovarian cancer. Control samples were not obtained in conjunction with surgery. All serum samples including controls were uniformly prepared from the whole blood samples by the GOG per their protocol. The subjects selected for our study included healthy female volunteers (controls) and epithelial ovarian cancers. Serum samples of stage III and stage IV patients were age-matched to control samples in 5-year age blocks. Preoperative, nonfasting blood samples were collected and deidentified prior to release to UC Davis.

Two separate sets of serum samples were subjected to glycoproteomics analysis independently at different times. The first set served as a training set (OC1) for discovery, which contained 40 cases and 40 matched controls. The second set (OC2) was used as a test set for validation and comprised 44 cases and 44 matched controls; this set was selected and analyzed independently and did not include any samples from the training set (OC1). Patient characteristics for both sample sets are summarized in Table 1.

Table 1. Patient Characteristics of the Samples Used

variable	OC1 (discovery set)	OC2 (testing set)
Total sample size, *n*	80	88
Healthy controls, *n* (%)	40 (50%)	44 (50%)
Cancer cases, *n* (%)	40 (50%)	44 (50%)
By stage		
EOC stage III	35 (87.5%)	35 (79.5%)
EOC stage IV	5 (12.5%)	9 (20.5%)
Age (year), Mean ± SD		
Healthy controls	51.83 ± 5.84	51.77 ± 6.67
Cancer cases	52.0 ± 5.91	53.09 ± 6.72
CA125, mean ± SD		
Healthy controls	19.73 ± 4.37	16.82 ± 11.49
Cancer cases	512.46 ± 673.23	591.73 ± 763.32

Trypsin Digestion

Protein standards composed of 100 μg of IgG, 50 μg IgA, IgM, A2MG, HP, A1AT, AGP, TF and C3 were each reconstituted in 50 mM NH_4HCO_3 (freshly made) to a total volume of 100 μL. A 87 μL aliquot of 50 mM NH_4HCO_3 was added to 2 μL of serum samples and shaken for 5 min. Proteins were reduced using 2 μL of 550 mM DTT in a 60 °C water bath for 50 min, and alkylated using 4 μL of 450 mM IAA at room temperature in the dark for 1 h. Proteins were then digested using 1 μg of trypsin in a 37 °C water bath for 18 h. The tryptic reaction was quenched by cold deactivation of trypsin; the samples were frozen at −20 °C, and the resulting peptide mixture was injected directly for mass spectrometric (MS) analysis without further sample cleanup or dilution. The autosampler was kept at 6 °C to ensure sample stability. To assess the stability of the sample preparation during the experiment, two standard serum samples (Sigma-Aldrich) were included prior and after the patient samples, in the training set, while standard serum samples were included every ten samples in the testing set.

Calibration Curves for Absolute Quantitation

Tryptic digested glycoprotein standards were used to determine the protein concentration of the individual proteins in a standard serum sample (Sigma-Aldrich). A serial dilution with dilution factors 1:2:5:10:50:100:500:1000 was performed on each of the glycoprotein standards to generate 8 concentrations for absolute quantitation.

UPLC-ESI-QqQ Analysis

The peptide samples were analyzed similarly to the previously developed method for IgG[15] using an Agilent 1290 infinity LC system coupled to an Agilent 6490 triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA).

DOI: 10.1021/acs.jproteome.7b00541
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Table 2. Transitions Monitored for Protein and Protein Subclass Quantitation

protein	peptide sequence	precursorion (m/z)	production (m/z)	collision energy (eV)	retention time (min)
IgG1234	DTLMISR	418.2	506.3	9	4.62
A1AT	^{360}AVLTIDEK^{367}	444.8	718.4	8	4.6
IgA1	^{213}TPLTATLSK^{221}	466.3	415.8	15	4.65
J chain	^{62}IIVPLNNR^{69}	469.8	613.3	12	5.35
IgG3	^{207}WYVDGVEVHNAK^{218}	472.9	534.3	9	5.16
TF	^{216}DGAGDVAFVK^{225}	489.8	735.4	11	4.85
HP	^{278}VGYVSGWGR^{286}	490.8	562.3	9	5.1
AGP12	^{74}TEDTIFLR^{81}	497.8	764.4	14	5.9
AGP1	^{171}SDVVYTDWK^{179}	556.8	712.3	14	5.35
IgM	^{301}FTCTVTHTDLPSPLKQ^{315}	573.0	734.9	15	5.8
AGP2	^{139}NWGLSFYADKPETTK^{153}	586.3	728.9	12	6.6
IgA12	^{264}WLQGSQELPR^{273}	607.4	914.5	18	5.4
A2MG	^{1004}AIGYLNTGYQR^{1014}	628.3	1071.5	21	5.2
IgG4	^{273}TTPPVLDSDGSFFLYSR^{289}	634.7	425.2	9	8.8
C3	^{892}SSLSVPYVIVPLK^{904}	701.6	928.6	16	9.02
IgA2	^{141}DASGATFTWTPSSGK^{155}	756.9	475.3	25	5.85
IgG1	^{158}FNWYVDGVEVHNAK^{171}	839.4	968.5	30	6.4
IgG2	^{102}CCVECPPCPAPPVAGPSVFLFPPKPK^{127}	970.1	1100.6	28	8.5

An Agilent Eclipse plus C18 (RRHD 1.8 μm, 2.1 × 100 mm) column, coupled with an Agilent Eclipse plus C18 precolumn (RRHD 1.8 μm, 2.1 × 5 mm) was used for UPLC separation.

Tryptic digested samples (1 μL) were injected and analyzed using a 17 min binary gradient consisting of solvent A of 3% acetonitrile, 0.1% formic acid, solvent B of 90% acetonitrile, 0.1% formic acid in nanopure water (v/v) at a flow rate of 0.6 mL/min. The LC gradient used was as follows: 0−5.5 min, 0−20% B; 5.5−13.5 min, 20−40% B; 13.5−14 min, 40−44% B; 14−14.1 min, 44−100% B; flush at 100% B for 1.1 min and equilibrate for 1.4 min at 0% B (see Supplementary Figure S1).

We previously optimized the MS conditions for MRM of oligosaccharides,[15,20] and the optimized conditions were used here. Briefly, the MS was operated in positive mode using unit resolution and the dynamic MRM mode was used to reduce the duty cycle. The cycle time was fixed at 500 ms.

Data Analysis

MRM results were analyzed using Agilent MassHunter Quantitative Analysis B.5.0 software. Data was exported as.csv files and normalized glycopeptide ratios relative to the relevant protein was determined prior to statistical analysis. The limit of detection (LOD) and limit of quantitation (LOQ) were defined as S/N ≥ 3 and 6, respectively.

Statistical Analysis

Prior to statistical analysis, glycopeptides observed in fewer than 70% of the patient samples were excluded from analysis and any remaining missing values were imputed with one-half the glycopeptide-specific minimum observed value. To meet underlying assumptions of statistical procedures, intensity values for glycopeptides from AGP and C3, and the glycoprotein concentrations were $\log_2$ transformed.

Differential analysis was conducted to identify glycoprotein concentrations and glycopeptides ratios significantly differentially regulated between cancer and control samples. Each set of glycoproteins and glycopeptides was analyzed individually. We adjusted for age by regressing glycoprotein and glycopeptide values on age and used the residuals in the differential analysis. Differences in age-adjusted means between cancer and control groups were evaluated with t-statistics. A permutation null distribution consisting of 100 000 permutations was used to determine raw p-values. A False discovery rate (FDR)—the expected proportion of errors among the rejected hypotheses—was then calculated for each glycopeptide or glycoprotein to account for multiple testing.[21] Compounds with FDR < 0.05 were considered to differ significantly between cancer and control subjects. For the individual glycoproteins and glycopeptides that significantly differed between the diagnostic groups in OC1 set, the Area Under the Receiver Operating Characteristic (ROC) curve (AUC), sensitivity, and specificity were calculated to evaluate whether they had diagnostic capabilities as potential biomarkers and then validated in an independent set of OC2 samples. All statistical analyses were conducted in R 2.14.0 language and environment.

RESULTS AND DISCUSSION

In this work we developed a method for concurrent protein- and site-specific determination of glycosylation profiles in serum samples from a subset of serum glycoproteins. No specific protein enrichment methods were used, allowing for a fast and robust sample preparation and analysis method. The method was then applied to two independent sample sets of ovarian cancer patients and matched controls to determine protein- and site-specific differential glycosylation profiles associated with ovarian cancer.

Development of Transitions for Protein Quantification

We previously reported the use of glycopeptide quantitation relative to the protein response for IgG, IgA, IgM and the J-chain[15] and provided MRM transition lists. In this study, we therefore focus on the transitions developed for six other abundant glycoproteins, HP, TF, A1AT, A2MG, AGP and C3, while using nonglycosylated peptides for protein quantitation. Because the aim of the peptide quantitation was primarily to allow for normalization of glycopeptides to the content of the respective glycoprotein and not to absolutely quantify proteins, we did not include stable isotope labeled peptides in this study. Transitions for nonglycopeptides were developed based on previous literature as well as nLC-Q-TOF runs of tryptic digests of glycoprotein standards.[16] The Global Protein Machine[22] was

DOI: 10.1021/acs.jproteome.7b00541
J. Proteome Res. 2018, 17, 222−233

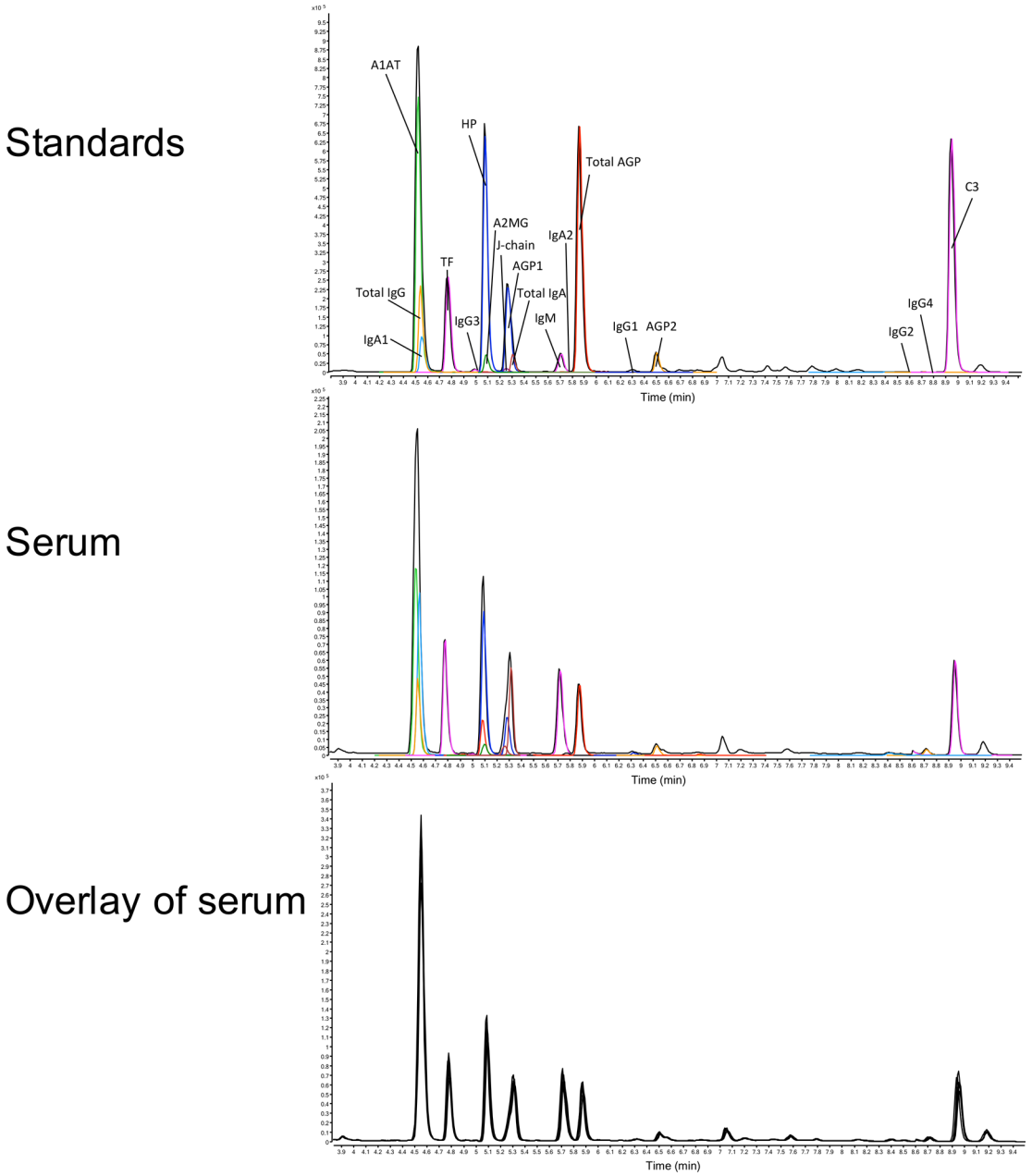

Figure 1. MRM chromatograms for the quantitation of nine high abundant proteins and their glycopeptides. Chromatograms are shown for a mixture of nine protein standards (top) and a standard serum sample (middle). The total MRM chromatogram is shown in black, while the extracted MRM chromatograms are indicated in color. Peptides used for protein quantitation have been assigned with their relative protein ID in the top graph, but the same color indicates the same protein ID in the serum sample. Instrument repeatability of the method is observed in the overlay of ten repeated injections of a standard serum sample (bottom). Here total MRM chromatograms are shown.

used to analyze peptide profiles and identify unique tryptic peptides. To further select peptides for protein quantitation the same criteria were used as previously discussed in our IgG method development study.[15] Briefly, peptides were selected with few or no modifications and missed cleavages to improve reproducibility across digestions. Because AGP is a mixture of the two subclasses AGP1 and AGP2, a peptide common to both subclasses (^{74}TEDTIFLR^{81}) as well as peptides specific for the individual subclasses (^{171}SDVVYTDWK^{179} and ^{139}NWGLSFYADKPETTK^{153} for AGP1 and AGP2, respectively) were selected. As specifically peptides that are common or not common for the subclasses needed to be monitored, some of these peptides—particularly the peptides for AGP total (AGP12) and AGP2—were not ideal. The lack of pure AGP1 and AGP2 standards prohibits their individual quantitation; however, variations in concentration can be studied using the absolute ion abundance as well as the ion abundance relative to the common AGP12 peptide. Fragmentation behavior of the selected peptides from all proteins was studied, and the most abundant fragments, typically *b*- or *y*- ions, were chosen for detection to provide maximum MRM sensitivity. The MRM transitions used in this study for protein quantitation and their optimized conditions are listed in Table 2. Chromatograms showing the transitions for peptides from the different proteins in a standard protein mixture and a serum sample are shown in Figure 1.

DOI: 10.1021/acs.jproteome.7b00541
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Table 3. Sites of Glycosylation from the Glycoproteins Monitored and the Number of Glycopeptides Monitored Per Site

protein	site(s)	AA range	# MC	peptide	M (Da)	RT (min)	# GPs monitored
A1AT	70	64−93	0	QLAHQSN^{70}STNIFFSPVSIATAFAMLSLGTK	3180.6	14.6	2
A1AT	107	94−125	0	ADTHDEILEGLNFN^{107}LTEIPEAQIHEGFQELLR	3690.8	12	5
A1AT	271	268−283	0	YLGN^{271}ATAIFFLPDEGK	1754.9	9.2	2
A1AT	271 w/ MC	268−298	1	YLGN^{271}ATA1FFLPDEGK_LQHLENELTHDITK	3539.8	11.7	2
A2MG	55	47−68	0	GCVLLSYLN^{55}ETVTVSASLESVR	2396.2	11.2	3
A2MG	70	69−71	0	GN^{70}R	345.2	0.5	6
A2MG	247	238−270	0	IITILEEEMN^{247}VSVCG LYTYGKPVPGHVTVSICRK	3733.9	9.6	3
A2MG	396, 410	388−422	0	GNEANYYSN^{396}ATTDEHGLVQFSIN^{422}TTR...fWMGTSLIR...NVMGTSLTVR	3803.8	N/A	0
A2MG	869	864−895	0	SLGNVN^{869}FTVSAEALESQEICGTEVPSVPEHGR	3412.6	7.9	5
A2MG	991	946−1001	0	ASVSVIGDLGSAMQNTQNLLQMPYGCGE...QNMVLFAPIYVLDYLN^{991}ETQQLTPEIK	6228.1	14.7	1
A2MG	1424	1422−1440	0	VSN^{1424}QTLSIFFTVLQCVPVR	2162.2	12.4	6
AG P1	33	19−42	0	QIPLCANLVPVPITN^{33}ATLDQITGK	2575.4	N/A	0
AG P1	93	87−101	0	QDQCIYN^{93}TTYINVQR	1914.9	5.8	13
AG P1	103	102−108	0	EN^{103}GTISR	775.4	1.8	12
AGP1/ AGP2	56	52−57	0	NEEYN^{56}K	795.3	1.5	4
AGP1/ AGP2	72	58−73	0	SVQEIQATFFYFTPN^{72}K	1918.9	N/A	0
AGP1/ AGP2	72 w/ MC	58−81	0	SVQEIQATFFYFTPN^{72}K_TEDTIFLR	2895.5	10.7	7
AGP2	33	19−38	0	QIPLCANLVPVPITN^{33}ATLDR	2204.2	N/A	0
AGP2	93	87−101	0	QNQCFYN^{93}SSYLNVQR	1919.9	N/A	0
AGP2	103	102−108	0	EN^{103}GTVSR	761.4	N/A	0
C3	85	74−94	0	TVLTPATNHMGN^{85}VTFTIPANR	2254.2	6.4	3
C3	939	938−940	0	MN^{939}K	391.2	N/A	0
C3	1617	1616−1624	0	PN^{1617}LSYIIGK	1003.6	N/A	0
HP	184	179−202	0	MVSHHN^{184}LTTGATLINEQWLLTTAK	2678.4	7.9	9
HP	241	236−251	0	VVLHPN^{241}YSQVDIGLIK	1794.0	7.1	12
HP	207, 211	203-215	0	NLFLN^{207}HSEN^{211}ATAK	1457.7	4	8
IgAl (+IgA2)	144	127−153	0	LSLHRPALEDLLLGSEAN^{144}LTCTLTGLR	2962.6	10.7	10
IgAl	340	332−353	0	LAGKPTHVN^{340}VSWMAEVDGTCY	2346.1	N/A	0
IgA2	47	Aug-51	0	VFPLSLDSTPQDGNVVVACLVQGFFPQEPLSVTWSESGQN^{47}VTAR	4775.4	N/A	0
IgA2	92	89−113	0	HYTN^{92}PSQQVTVPCPVPPPPPCCHPR	2908.3	N/A	0
IgA2 (+IgAl)	131	114−140	0	LSLHRPALEDLLLGSEAN^{131}LTCTLTGIR	2962.6	10.7	10
IgA2	205	200−208	0	TPLTAN^{205}ITK	957.6	4	7
IgA2	327	319−340	0	MAGKPTHVN^{327}VSVVMAEVDGTCY	2364.1	N/A	0
IgGl	180	176−184	0	EEQYN^{180}STYR	1188.5	2.5	11
IgG2	176	172−180	0	EEQFN^{176}STFR	1156.5	4	10
IgG3	227	223−231	0	EEQYN^{227}STFR	1172.5	3.1	5
IgG4	177						
IgM	46	44−54	1	YKN^{46}NSDISSTR	1283.6	2	8
IgM	209	203−223	0	GLTFQQN^{209}ASSMCVPDQDTAIR	2338.1	6.1	5
IgM	439	431−452	0	STGKPTLYN^{439}VSLVMSDTAGTCY	2364.1	7.3	5
IgM	272, 279	269−300	0	THTN^{272}ISESHPN^{279}ATFSAVGEASICEDDWNSGER	3517.5	N/A	0
J chain	71	70−80	0	EN^{71}ISDPTSPLR	1227.6	4.5	1
J chain	71 w/ MC	62−80	1	IIVPLNNR_EN^{71}ISDPTSPLR	2147.2	6.4	2
TF	432	421−433	0	CGLVPVLAENYN^{432}K	1475.7	6.6	5
TF	630	622−642	0	QQQHLEGSN^{630}VTDCSGNFCLFR	2514.1	7.7	6

Development of Transitions for Relative Quantification of Site-Specific Glycosylation Patterns

In order to allow for the development of targeted MRM transitions toward glycopeptides, literature study was used in combination with our own previous experience in determining the site-specific glycosylation profiles. Transitions for glycopeptides from IgG, IgA, IgM and the J-chain were previously established in our laboratory,[15,16] and were included in this study. Transitions for the six glycoproteins A1AT, AGP, A2MG, TF, HP and C3 were developed based on previous literature[7,23−32] in combination with site-specific glycosylation profiles obtained using Q-TOF analysis of commercially available protein standards with subsequent database search using an in-house built software tool GPFinder.[16,33] For each of the glycopeptides reported, the theoretical mass and the theoretical m/z at charges ranging from 1 through 5 were

DOI: 10.1021/acs.jproteome.7b00541
J. Proteome Res. 2018, 17, 222−233

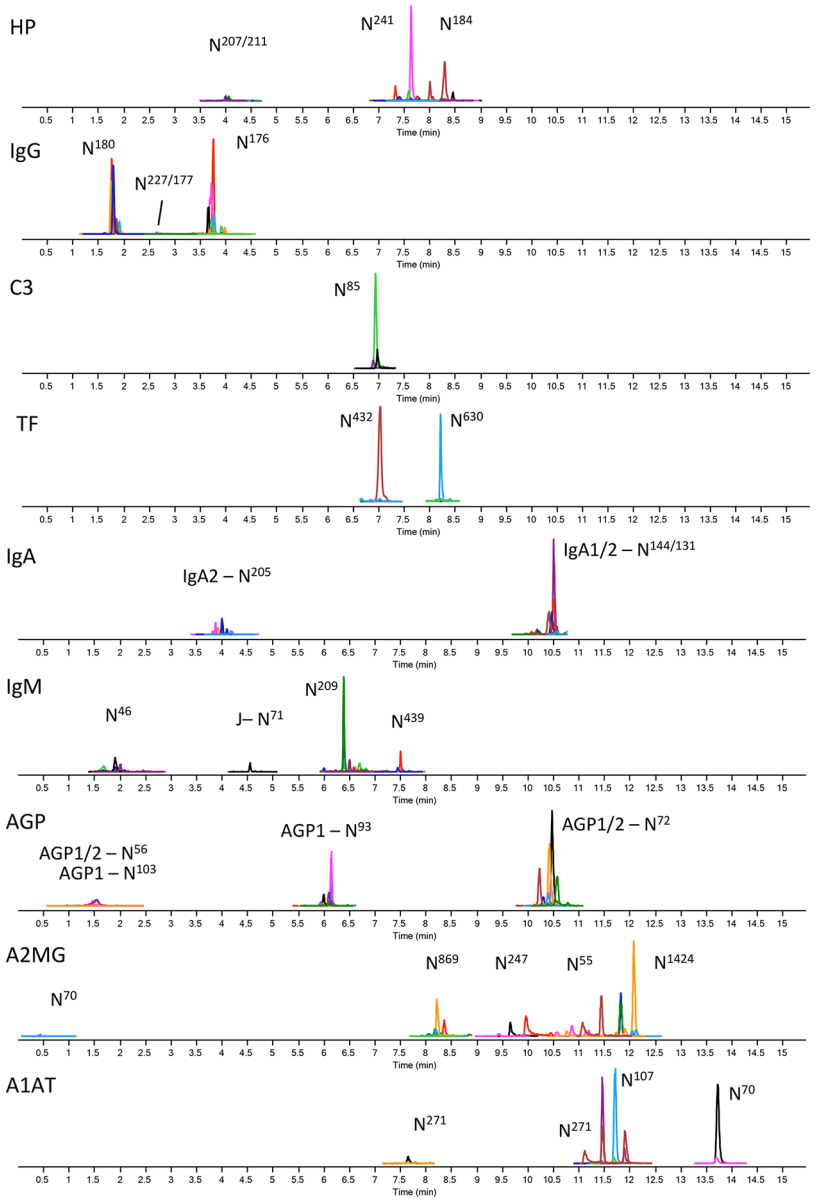

Figure 2. Analysis of glycopeptides for relative quantitation using MRM. Extracted ion chromatograms are shown for the nine glycoproteins measured, with the *y*-axis representing relative ion abundance. Because the UPLC separation is performed on a reverse phase stationary phase, retention of the glycopeptides can mostly be attributed to the peptide moiety. Thus, the chromatograms are assigned by glycopeptides groups originating from the same site of glycosylation. Different colors indicate different glycopeptides.

calculated. When no site-specific glycosylation profiles were available, theoretical glycopeptides were calculated for each of the glycans at each of the sites of glycosylation. Transitions for each of the theoretical m/z between 500 and 1400 were then checked using a digest of standard proteins as well as a standard serum sample. Fragments that were monitored were the oxonium ions 204.1, 366.1 and, for sialic acid containing glycopeptides, 657.2. An overview of the transitions that were checked is shown in Supplementary Table S1, while all transitions observed are reported in Supplementary Table S2. A summary of the glycopeptides monitored in this study is included in Table 3 and chromatograms of each of the glycopeptides, separated by protein of origin is shown in Figure 2.

Method Stability and Repeatability

To assess the intraday stability of the method for protein quantitation, samples of each of the standard proteins and three standard serum samples were digested using trypsin. Serial dilutions were made from the tryptic digest of the standard proteins to concentrations of 9.00×10^{-2} μg/mL, 9.00×10^{-1} μg/mL, 9.00 μg/mL, 18.0 μg/mL 90.0 μg/mL, 1.80×10^{2} μg/mL, 4.50×10^{2} μg/mL, and 9.00×10^{2} μg/mL to generate calibration curves. The calibration curves for all proteins show good linearity over 3 orders of magnitude with fitted R^2 between 0.994 and 0.998 (Supplementary Figure S2). The limits of detection (LOD) were defined as the lowest concentration measured at which signal was obtained with a signal-to-noise (S/N) ratio of at least 3. LODs were determined to be 9.0×10^{-4} mg/mL for AGP, 9.0×10^{-4} mg/mL for A1AT, 1.8×10^{-2} mg/mL for A2MG, 9.0×10^{-3} for C3, $9.0 \times$

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Table 4. Repeatability of the Protein Quantitation Based on Glycoprotein Standard Digests

		AGP (mg/mL)	A1AT (mg/mL)	A2MG (mg/mL)	C3 (mg/mL)	HP (mg/mL)	TF (mg/mL)
Day 1		0.66	3.27	1.82	1.54	2.27	4.63
		0.51	3.34	1.83	1.52	2.17	4.23
		0.64	3.13	1.94	1.54	2.14	3.80
Day 2		0.41	2.96	2.03	1.57	2.29	4.34
Day 3		0.49	2.73	2.09	1.43	2.28	4.46
Intraday	Avg	0.60	3.24	1.86	1.54	2.19	4.22
	Std dev	0.08	0.11	0.07	0.01	0.07	0.41
	RSD (%)	13.5	3.25	3.71	0.66	3.11	9.75
Interday	Avg	0.51	2.94	2.02	1.51	2.24	4.20
	Std dev	0.11	0.20	0.07	0.08	0.09	0.35
	RSD (%)	21.92	6.79	3.65	4.99	3.89	8.32
Previously reported		0.39−1.15	1.0−1.9	1.0−2.8	0.7−1.75	0.3−2.0	2.0−3.6

10^{-4} mg/mL for HP and 9.0×10^{-4} mg/mL for TF. The limits of quantitation (LOQ) were defined as the lowest concentration at which signal was obtained with a S/N ratio of at least 6. Because very low noise was observed, the same values were obtained for the LOQ and the LOD.

The calibration curves were then used for protein quantitation of each of the standard serum samples. This process was repeated on three consecutive days to determine the interday stability of the method. Using the repeated samples run over 3 days, the intra- and interday variation of the protein quantitation from the serum sample was determined. The peptides selected for protein quantitation are reported in Table 4 and each shows CV < 10% for the inter- and intraday repeatability, with the exception of AGP12, which shows an interday variability of 22%. However, no better performing peptides common for AGP1 and AGP2 subclasses could be identified. The concentrations of the proteins determined in the standard serum samples using the standard protein-based calibration are 4.2 mg/mL, 2.9 mg/mL, 2.0 mg/mL, 2.1 mg/mL, 1.5 and 0.5 mg/mL for TF, A1AT, A2MG, HP, C3 and AGP, respectively. These levels are slightly higher or within limits compared to what has been previously reported (see Table 4).

Site-Specific Differential Glycosylation in Serum Samples from Ovarian Cancer Patients

To evaluate whether the developed method for the relative quantitation of glycopeptides from serum can be used for the development of biomarkers for epithelial ovarian cancer, the described MRM-based method was first applied to a cohort of 80 serum samples. Serum samples were obtained from 40 ovarian cancer patients (stage III and IV) and 40 age matched controls and hereafter referred to as OC1. The patient characteristics of OC1 are shown in Table 1.

In addition to the patient samples, two standard serum samples were run as sample preparation controls at the beginning and the end of the run. Furthermore, nine replicate injections of a standard serum sample were run in between every ten patient samples as instrument controls. The serum samples showed good concordance (Supplementary Figure S3), indicating little technical variation during the analytical process.

To determine whether the expression of proteins and glycopeptides was associated with ovarian cancer, a differential analysis was performed. First, the levels of glycoproteins and their isoforms were assessed. Of the eight protein variables assessed (A1AT, A2MG, AGP, AGP1, AGP2, C3, HP and TF), seven were statistically significantly (FDR < 0.05) differentially expressed in the serum of OC patients compared to controls. Levels of A1AT, AGP and HP glycoproteins were increased in serum of cancer patients, while the levels of A2MG and TF were decreased (see Figure 3). The average serum concentration in controls were 2.63 mg/mL (SD 0.62), 1.97 mg/mL (SD 0.46), 0.95 mg/mL (SD 0.14), 1.54 mg/mL (SD 0.29), 2.09 mg/mL (SD 0.94) and 4.39 (SD 0.61) for A1AT, A2MG, AGP, C3, HP and TF, respectively. The average concentrations in cases were 4.41 mg/mL (SD 1.60), 1.39 mg/mL (SD 0.56), 0.88 mg/mL (SD 0.31), 1.56 mg/mL (SD 0.41), 4.67 mg/mL (SD 1.90) and 2.64 (SD 0.80) for these same proteins. Interestingly, the relative levels to total AGP of AGP1 were increased, while the relative levels of AGP2 were decreased, thus indicating that the increased levels of the overall AGP are solely due to increased levels of AGP1. This was further confirmed by the similar responses (non-normalized) of AGP2 between cancer cases and controls.

We then assessed the differential expression of the glycopeptides from the six additional glycoproteins (not including IgG, IgA and IgM, which have been reported previously[19]). Of the 114 glycopeptides monitored, 73 glycopeptides were observed in at least 70% of the samples and these glycopeptides were included for further statistical analysis to assess differential protein glycosylation in the serum of OC patients and controls. The differential glycosylation patterns of the six glycoproteins studied here will be discussed subsequently, with an emphasis on the glycoproteins A1AT, A2MG and AGP, for which most significantly altered glycopeptides were observed (Figure 4).

Levels of three nonfucosylated glycopeptides from A1AT were significantly (FDR < 0.05) decreased in OC patients compared to the controls: the bi- and triantennary glycans $H_5N_4S_2$ and $H_6N_5S_3$ attached to site N^{107}, and the biantennary glycan $H_5N_4S_2$ on site N^{271} (indicated by a blue colored dot in Figure 4). Interestingly, levels of all fucosylated glycopeptides were increased, although not statistically significantly at FDR < 0.05 (Supplementary Table S3). This trend holds true for all three sites of glycosylation (N^{70}, N^{107} and N^{271}) on A1AT monitored in this study.

Expression levels of ten glycopeptides from A2MG were significantly altered in serum of OC patients (Figure 4, Supplementary Table S3). We observed that site N^{1424} in A2MG had the largest number of glycopeptides, reflecting a large microheterogeneity, and at this site four glycopeptides were significantly altered. Interestingly, on this site occupancy by monosialylated biantennary glycans $H_5N_4S_1$ and $H_5N_4F_1S_1$ was decreased, while the bisialylated biantennary glycan $H_5N_4S_2$ was significantly increased and the fucosylated bisialylated

DOI: 10.1021/acs.jproteome.7b00541
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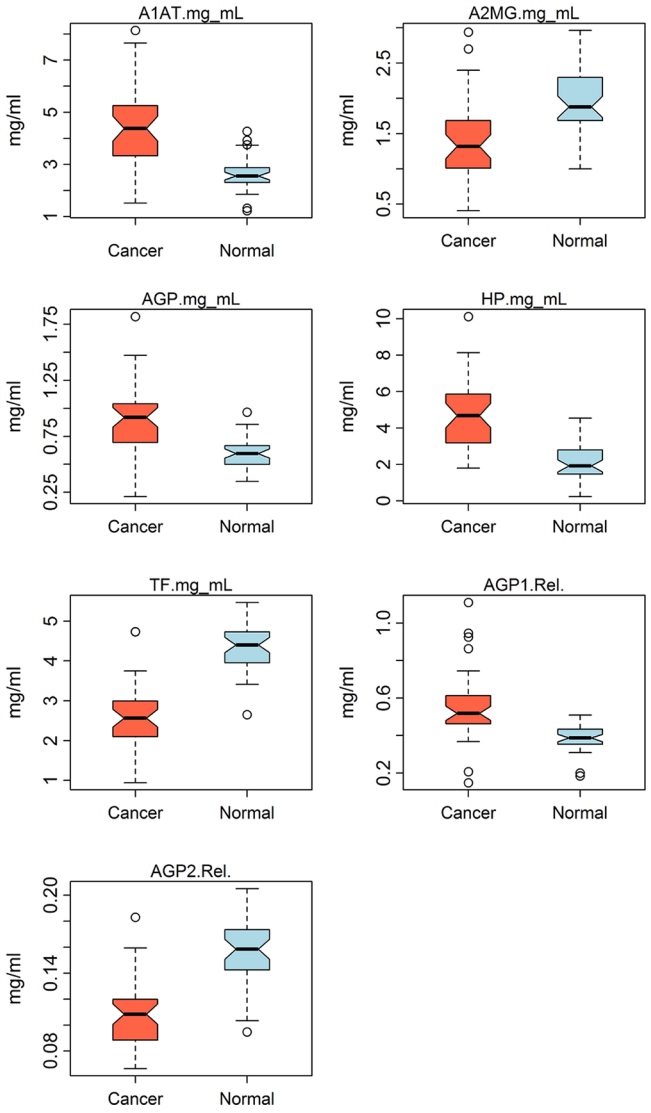


Figure 3. Differential glycoprotein concentrations in OC patients compared to controls. Box-whisker plots are depicted for the protein concentrations that were observed to be significantly different in the OC1 sample set. For A1AT, A2MG, AGP, HP and TF concentrations are plotted, while for AGP1 and AGP2 the ion abundance relative to the total AGP protein concentration is shown.

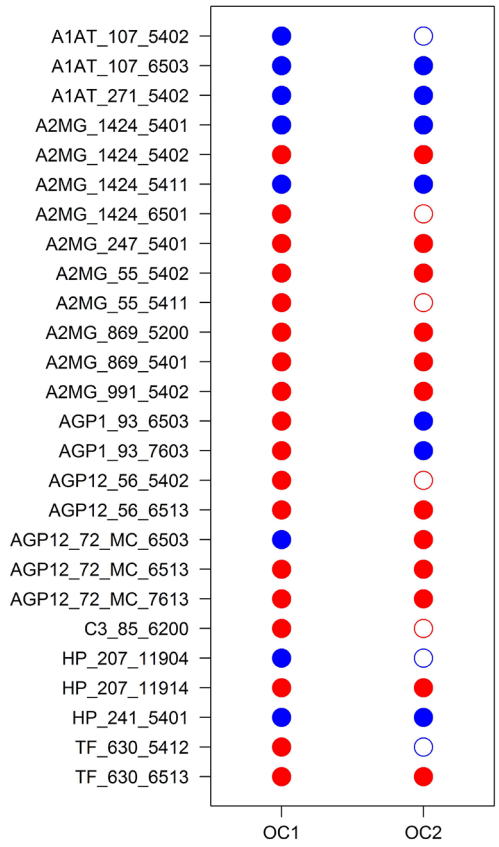


Figure 4. Differential expression of glycopeptides in serum of OC patients relative to controls. Dot plots are shown for all glycopeptides with significantly differential expression in the OC1 sample set. Increased levels (red dots) as well as decreased levels (blue dot) are indicated, and significant results are represented by a filled dot, while nonsignificant results are represented by an open dot in the OC2 sample set. The nomenclature used for the glycopeptide follows the format [protein abbreviation]_[site of glycosylation]_[glycan abbreviation], where the glycan abbreviation shows # of hexoses, # of hexosamines, # of fucoses and # of *N*-acetylneuraminic acids (or sialic acids), respectively.

$H_5N_4F_1S_2$ showed an increased trend, albeit not significant. This indicates a role for increased sialylation on site N^{1424}. Remarkably, this trend was not observed for the triantennary glycans.

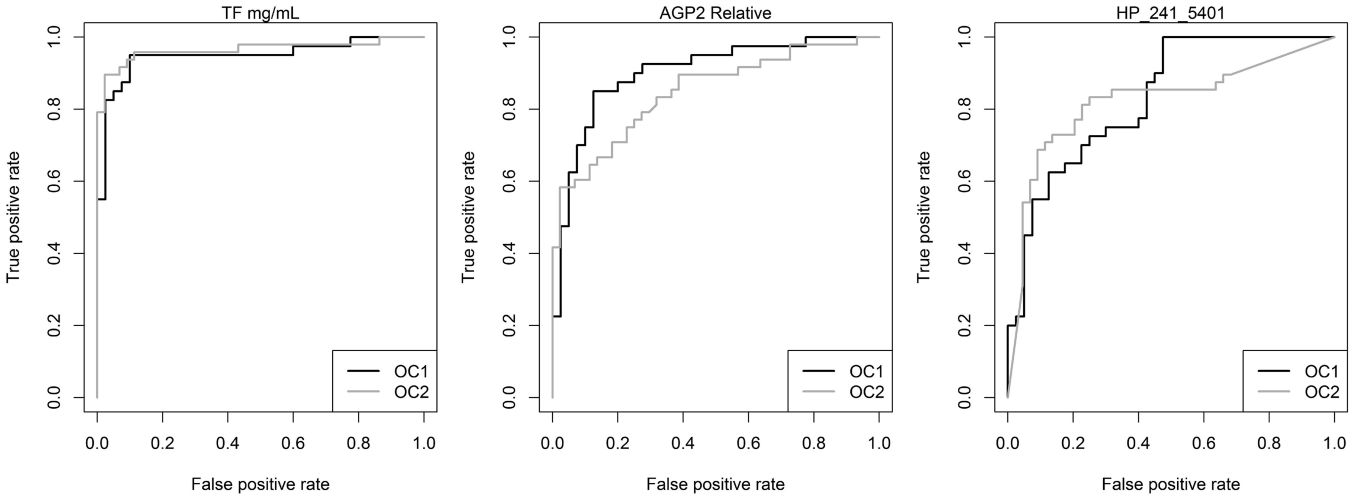
The glycosylation pattern of AGP (both isoforms 1 and 2) was dominated by tri- and tetra-antennary glycans, with or without fucose (Figure 4, Supplementary Table S3). Three glycopeptides from AGP 1 and 2 site N^{72} showed significant differential expression in OC patients. While the levels of tri- and tetra antennary fucosylated glycopeptides with glycan moieties $H_6N_5F_1S_3$ and $H_7N_6F_1S_3$ were increased in OC patients, the levels of the nonfucosylated glycopeptide with moiety $H_6N_5S_3$ were decreased, indicating increased fucosylation of AGP at site N^{72} in OC patients. Increased fucosylation was not immediately apparent at the other sites; the fucosylated glycopeptide $H_6N_5F_1S_3$ was increased at N^{56}, but increased levels of nonfucosylated glycopeptides were also observed.

For the three other glycoproteins monitored (C3, TF and HP), fewer glycopeptides were significantly differentially expressed (Supplementary Table S3). While three high mannose type glycopeptides of N^{85} were monitored for C3, only one of them containing the glycan moiety H_6N_2 was observed consistently. Levels of this glycopeptide were significantly increased in the serum samples from OC patients. For TF, six glycopeptides were observed consistently, two at site N^{432} and four at site N^{360}. The two fucosylated glycopeptides at site N^{630} were elevated in OC cases compared to control, while levels of the nonfucosylated glycopeptides remained constant. Three glycopeptides showed significant differential expression on HP. At site N^{207}/N^{211} levels of overall glycan moiety $N_{11}H_9S_4$ were decreased, while levels of $N_{11}H_9F_1S_4$ were increased. Both these results again indicate increased levels of fucosylation on selected sites of glycosylation in serum samples from OC cases (Supplementary Table S3).

Validation of Differential Glycan Profiles in an Independent Test Set

To further validate reproducibility of these differential findings, glycopeptide levels were determined in a second, independent test set. The set consisted of 88 serum samples obtained from

DOI: 10.1021/acs.jproteome.7b00541
J. Proteome Res. 2018, 17, 222−233



Variable	OC1			OC2		
	AUC	Specificity	Sensitivity	AUC	Specificity	Sensitivity
TF mg/ml	0.948	0.90	0.88	0.965	0.79	0.98
AGP2 relative	0.901	0.85	0.85	0.848	0.69	0.82
HP_241_5401	0.831	0.70	0.75	0.821	0.81	0.77

Figure 5. Prediction of OC by the three best performing glycopeptides. ROC curves are depicted (top) for both OC1 (black) and OC2 (gray). AUC, sensitivity and specificity as determined for these three glycopeptides are indicated (bottom).

44 ovarian cancer patients (EOC stage III and IV) and 44 age matched controls and is hereafter referred to as OC2. The patient characteristics of OC2 are shown in Table 1. In addition to the patient samples, standard serum samples were run as sample preparation controls every ten samples and nine replicate injections of a standard serum sample were run every ten patient samples as instrument controls. Similar to the OC1 run, the OC2 serum samples showed good concordance, indicating limited technical variability during the analytical process.

Similar to the OC1 set, the levels of glycoproteins and their isoforms were assessed. Of the seven glycoproteins whose expression was significantly differentiating by cancer in the OC1 set, six glycoproteins were observed to be differentially expressed in the OC2 as well (A1AT, A2MG, AGP1, AGP2, HP and TF, see Figure 3). This indicates good reproducibility between the OC1 and OC2 sample sets in terms of their expression levels associated with ovarian cancer.

Differential analysis was also performed for the glycopeptides and the results are shown in Figure 4 and Supplementary Table S3. Of the three glycopeptides from A1AT that were observed to be decreased in the OC patients in the OC1 set, two were also significantly decreased in the OC2 test set. Eight of the ten glycopeptides from A2MG that were differentially expressed in OC1 were also significantly different in serum of cancer patients compared to controls in OC2. Less concordance of results was observed for AGP1, for which two glycopeptides were observed to be increased in serum of cancer patients of the OC1 set, but decreased in the OC2 set. Of the other five glycopeptides that are shared between AGP1 and AGP2, three were significantly differentially expressed in OC2 as well. Six glycopeptides were differentially expressed from HP, C3 and TF in OC1. Of these glycopeptides, three were also significant in OC2. Overall, from the 26 glycopeptides that were observed to be differentially expressed in the OC1 set, 16 were equally statistically differentially expressed in the OC2 set at FDR < 0.05 level (Figure 4), indicating that these glycopeptides with great reproducibility in two independent sample sets might be good candidates for potential use as a blood based biomarker.

Predictive Potential of Glycopeptides for OC in Serum Samples

To assess the predictive potential of the consistently differentially expressed peptides and glycopeptides in both OC1 and OC2 samples as diagnostic biomarkers that can discriminate cancer patients from controls, AUC, specificity and sensitivity were calculated (Supplementary Table S3). Of the 24 peptides and glycopeptides that showed the significant differential expression in serum of OC patients in the OC1 and OC2 set, 11 had AUC values > 0.75 in both independent sets. Of these 11 variables, three showed AUC values 82% or greater, showing the potential of its utility in biomarker discovery for ovarian cancer. ROC curves for three variables with highest AUC values in OC1 and OC2 are shown in Figure 5.

CONCLUSIONS

This study shows that it is feasible to monitor a large number of glycopeptides from higher abundant proteins in a 17 min LC-MRM-MS run, and that differential glycosylation patterns in serum samples from ovarian cancer patients can be determined and corroborated. Both glycosylation site occupancy (macroheterogeneity, reflected by the analysis of the "unglycosylated" peptide from haptoglobin at Asn_{241}) and site-specific glycosylation patterns (microheterogeneity, reflected, e.g., by the 13 glycopeptides with different glycan moieties of AGP1 at Asn_{93}) could be monitored. Furthermore, to obtain glycopeptide responses irrespective of the glycoprotein concentration, peptides from the glycoproteins were quantified. Within this study 7 glycoprotein variables (concentrations of A1AT, A2MG, HP, AGP and TF and relative abundances of AGP1 and AGP2) were differentially expressed in serum samples of OC patients in a discovery set. Of these, 6 were corroborated in the independent test set. Similarly, 26 glycopeptides from all 6 glycoproteins were shown to be differentially expressed in the

DOI: 10.1021/acs.jproteome.7b00541
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discovery set, of which 16 could be corroborated in the test set (Figure 3). Moreover, 11 variables showed AUC values > 0.75 in both the discovery and test sets, indicating that these variables have great potential as candidates for diagnostic markers for the detection of OC in blood.

These results show the potential for targeted mass spectrometry based approaches for the quantification of glycopeptides, and potentially other post-translational modifications. However, it should be noted that the protein quantitation obtained here is by no means an accurate protein quantitation, the concentration was calculated based on an external calibration curve of standard protein in buffer and we did not include stable isotope labeled peptides in our samples, which would have facilitated more accurate quantitation. This strategy was chosen because we aimed to use the protein concentration primarily for normalization of the obtained glycopeptides responses. Furthermore, we only observed N-glycopeptides, which seem to be easier to address than O-glycopeptides. To a high extent this is caused by the relatively few numbers of O-glycosylation sites in the proteins studied, but O-glycosylation has been reported on IgA,[34] which we did not observe in this study. The use of trypsin as the protease of choice for the method developed also has implications. As we and others observed, two N-glycosylation sites of HP were not cleaved into distinct glycopeptides by trypsin, but rather resulted in one glycopeptide with a two glycans attached, due to the lack of an arginine or lysine residue in the amino acid sequence. Similarly, this is likely the main reason why no IgA O-glycosylation was observed, as all 5 O-glycans are attached to one single glycopeptides, resulting in large heterogeneity of these glycopeptides. It is highly likely that such instances would also occur in other lower abundance proteins, and the choice of protease, or combination thereof, is therefore of high importance during the development of MS-based bottom-up glycopeptides assays.

Interestingly, the current knowledge on the glycosylation profiles of high abundance plasma glycoproteins was recently reviewed.[35] It has to be acknowledged that not all glycopeptides and sites of glycosylation could be monitored in this study likely in part due to it being a highly complex mixture in which ion suppression cannot be avoided. This, together with the glycan heterogeneity and reduced signal strength observed for glycosylated peptides,[36] results in limited sensitivity for the analysis of all possible glycopeptides from serum. It is likely that additional glycopeptides can be monitored upon sample purification using a glycan-specific enrichment method such as HILIC,[37,38] but this strategy has the disadvantage that nonglycosylated peptides are removed, and thus protein concentration information is lost.

The results presented in this preliminary study show the potential of glycopeptides from high abundance glycoproteins to serve as markers for ovarian cancer in the two sample sets studied. However, the sample sets used in this study only comprised samples from healthy controls and patients of stage III–IV. While it is common to use healthy controls in an initial evaluation, this design is a drawback, particularly for this study, in which the glycosylation patterns of immunoglobulins and acute phase proteins is studied. These proteins are generally considered markers of inflammation, and the specificity of the observed changes for OC or even cancer in general can thus not be established. Furthermore, only samples from patients with late-stage disease were included and thus we cannot draw any conclusions regarding the usefulness of these glycopeptides for detection of early stage disease. Therefore, further investigation in early stage samples as well as samples from a broad range of control patients with other inflammatory diseases, benign gynecologic conditions, and chronic medical conditions is warranted in future studies to assess the potential utility of glycan-based biomarkers for cancer detection in high-risk populations.

In this study quantitation of site- and protein-specific glycosylation patterns is achieved in a relative manner, with the observed ion intensities per glycopeptide being normalized to the ion intensities of a nonglycosylated peptide. While this allows for the evaluation of the glycosylation pattern independent of the glycoprotein concentration, this does not allow for absolute quantitation, for which stable isotope labeled glycopeptide standards would be necessary. Currently, such standards are not readily available. An initial attempt at the synthesis of glycopeptides was made to investigate the effects of glycan micro heterogeneity on signal intensity in mass spectrometry using solid-phase peptide synthesis with modified Asn residues,[36] but this strategy is currently not suitable for the production of large enough quantities. This is, together with several other aspects, including but not limited to our restricted knowledge of the enzyme kinetics of proteases for the production of glycopeptides, the influences of specific glycans on glycopeptides formation, the lowered ionization efficiency of glycopeptides relative to peptides, which results in lower sensitivity, and the specialized knowledge currently needed for glycopeptides analysis, a major limitation for the implementation of glycopeptides as markers in a clinical chemistry laboratory, where absolute quantitation under well-controlled conditions is strived for.[39,40]

We therefore believe that the results presented here show the great potential of the targeted, mass spectrometry based method as an analytical tool for biomarker development for ovarian cancer and other disease diagnosis, but further work is needed, including the validation of the glycopeptides markers in additional, prospective sample sets, and technological advancements to allow for absolute quantitation of glycopeptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00541.

Figures S1–S3; Tables S1–S2 (PDF)
Table S3 (XLSX)
Table S4 (XLSX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the receipt of serum samples from the GOG tissue-banking repository. This work was supported by the

DOI: 10.1021/acs.jproteome.7b00541
J. Proteome Res. 2018, 17, 222–233

Ovarian Cancer Research Fund (to G.L.) and NIH grant R01GM049077 (to CBL).

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