Fc specific IgG glycosylation profiling by robust nano-reversed phase HPLC-MS using a sheath-flow ESI sprayer interface

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Abstract

Biological activities of immunoglobulin G such as effector functions via Fcγ-receptor interactions are influenced by Fc linked N-glycans. Here we describe a fast, robust and sensitive nanoLC-ESI-MS method for detailed subclass-specific analysis of IgG Fc N-glycosylation. High capacity extraction and fast separation of tryptic (glyco) peptides from affinity chromatography purified human polyclonal plasma/serum IgG was achieved by combining a porous particle C18 trap column with a fused core particle C18 nanoLC column. A sheath-flow ESI sprayer was used as a sensitive zero dead volume plug-and-play interface for online MS coupling, generating a very constant spray and ionization over the entire LC gradient. The propionic acid containing sheath-liquid effectively suppressed TFA gas-phase ion-pairing, enabling the use of TFA containing mobile phases. The fixed position of the sheath-flow ESI sprayer, far away from the glass capillary inlet, reduced MS contamination as compared to conventional nano-ESI. The method was found to be suitable for fast and detailed subclass-specific IgG Fc N-glycosylation profiling in human plasma. The obtained subclass-specific IgG Fc N-glycosylation profiles were processed automatically using in house developed software tools. For each of the IgG subclasses the 8 major glycoforms showed an interday analytical variation below 5%. The method was used to profile the IgG Fc N-glycosylation of 26 women at several time points during pregnancy and after delivery, revealing pregnancy-associated changes in IgG galactosylation, sialylation and incidence of bisecting N-acetylglucosamine.

Introduction

In human blood IgG represents the most abundant antibody class (approximately 10 mg/ml serum).[1,2] IgGs occur in four subclasses (i.e. IgG1-4)[4] and consist of two heavy and two light polypeptide chains each having a variable and a constant region. The two light chains and parts of the two heavy chains form the fragment antigen binding (Fab) moieties, while the remainder of the two heavy chains forms the fragment crystallizable (Fc) moiety. A biantennary complex type N-glycan is present on each of the heavy polypeptide chains at asparagine 297 in the C_H2 domain of the Fc moiety. This N-glycan is often core-fucosylated and varies in the number of antenna galactose residues resulting in the prominent glycoforms G0F (no galactose), G1F (1 galactose), and G2F (2 galactoses). A small part of the Fc N-glycans may carry a bisecting N-acetylglucosamine (GlcNAc). In addition, these glycans may contain a sialic acid residue attached to an antenna galactose.

Fc N-glycans influence the biological activity of IgG by modulating Fcγ-receptor binding.[19,35,38,39,43,44,146] For example, cleavage of the chitobiose core with endoglycosidase from *Streptococcus pyogenes* (Endo S) results in a closed Fc conformation which strongly decreases binding to activated monocytes.[38,39] Even small changes in the Fc N-glycan structure may have profound effects on the Fcγ-receptor binding. The absence of a core-fucose on IgG1 Fc N-glycans was found to promote binding to Fcγ-receptor IIIa expressed on macrophages and resulted in a drastic enhancement of antibody-dependent cellular cytotoxicity (ADCC).[43,44] Bioengineered antibodies lacking a core-fucose may, therefore, represent a potent new generation of anti-cancer therapeutics.[44,46] In a mouse arthritis model it has been shown that the anti-inflammatory properties of intravenous immunoglobulin depend on α2,6-linked sialic acid on IgG Fc N-glycans which lead to Fc mediates signaling via the C-type lectin SIGN R1 (an orthologue of the human DC-SIGN) expressed on murine macrophages in the splenic marginal zone.[35,47,48] Moreover, decreased levels of IgG Fc sialylation have been observed in a murine nephritis model suggesting that the decrease in sialylation may provide a switch from the steady state innate anti-inflammatory activity to an adaptive pro-inflammatory response.[35] For a variety of autoimmune diseases, infectious diseases and cancer lowered IgG Fc galactosylation has been observed.[3,32,147] Moreover, changes in IgG Fc N-glycans of healthy individuals have been associated with age, gender and pregnancy.[8-10]

Accurate analysis of IgG Fc N-glycosylation is essential for e.g. the characterization of biotechnologically produced therapeutic monoclonal antibodies and obviously is of benefit for the analysis of glycosylation changes of polyclonal IgGs as part of humoral immune responses. Fc N-glycosylation of IgGs can be determined by mass spectrometry (MS) after enzymatically releasing the N-glycans or after preparation of (glyco)peptides. With released N-glycans subclass-specific Fc N-glycosylation profiling is only allowed when IgG subclasses are separated prior to N-glycan release. By contrast, analysis

of IgG Fc N-glycopeptides allows discrimination between subclasses on the basis of the peptide moieties and masses. Analysis of IgG glycopeptide microheterogeneity can be achieved by MALDI-MS[77,102,119,148] or LC-MS.[4,50,79,140] MALDI-MS is very well suited for high-throughput analysis and has been shown to allow subclass-specific Fc N-glycopeptide profiling of human polyclonal IgG.[148] However, the presence of isomeric glycopeptides prevents proper determination of IgG2 fucosylation and IgG4 glycosylation.[148] By performing an LC separation prior to MS detection detailed characterization of IgG subclass-specific Fc N-glycosylation can be achieved in a single analysis.[4,79]

To allow direct coupling of the chromatographic separation with the mass spectrometer, formic acid (FA) is often applied as acidic mobile phase additive.[45,71,125] Analyte retention and peak shapes can be improved by using trifluoroacetic acid (TFA) in stead of FA.[128] Moreover, ionic interactions of basic analytes with residual silanol groups on silica based RP stationary phases are strongly reduced with TFA.[129] However, TFA is known to form gas-phase ion-pairs with positively charged analyte ions resulting in ESI ion suppression. By applying a mixture of isopropanol (IPA) and propionic acid (PA) or acetic acid (TFA fix) prior to ESI this effect can be substantially reduced.[149-151]

Interfacing of TFA containing separations to MS is predominantly performed with analytical to capillary scale LC separations as conventional nano-ESI sources often lack robustness, even when run by qualified operators. Here we present a fast and robust nano-reversed phase-LC-MS method for IgG subclass-specific Fc N-glycopeptide profiling, using TFA containing mobile phases and a sheath-flow ESI sprayer for HPLC-MS interfacing. Fast separation is achieved on fused core particles at high flow rates. The sheath-flow ESI sprayer provides a plug-and-play zero dead volume interfacing and allows successful in-spray mixing of the LC gradient with TFA fix with minimal sample dilution. Moreover, MS contamination was found to be significantly reduced compared to standard nanoLC-MS interfacing. The robustness, sensitivity and reproducibility of the nanoLC-MS setup were evaluated with tryptic IgG digests. The method was applied to study pregnancy-associated IgG Fc glycosylation changes in a cohort of 26 women.

Experimental section

IgG purification and enzymatic cleavage. IgGs were affinity captured from total human serum or plasma and digested by trypsin as described previously.[148] Briefly, human polyclonal IgGs were captured from 2 µl plasma or serum by affinity chromatography with Protein A-Sepharose™ Fast Flow beads (GE Healthcare, Uppsala, Sweden), and cleaved overnight at 37˚C with 200 ng sequencing grade trypsin (Promega, Madison, WI). The entire sample preparation was performed in 96-well plate format. Monoclonal antibody samples (provided by Hoffmann la Roche, Penzberg, Germany) were diluted in PBS and subjected to trypsin cleavage.

Fast nano-reversed phase-LC-ESI-MS. Separation was achieved on a Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), consisting of a degasser unit, binary loading pump, dual binary gradient pump, a temperature controlled autosampler maintained at 5°C and two column oven compartments maintained at 30°C. The Ultimate 3000 autosampler was fitted with a 10 µl PEEK sample loop. Samples were centrifuged at 4000 rpm for 5 min and 250-5000 nl were applied to a Dionex Acclaim PepMap100 C18 (5 mm x 300 µm i.d.) solid phase extraction (SPE) trap column conditioned with 0.1% TFA (Fluka, Steinheim, Germany; mobile phase A). In an alternative set up samples were applied to a 330 nl HALO C18 StemTrap SPE trap column (Optimize Technologies, Oregon City, USA) conditioned with mobile phase A. Sample loading was performed on the SPE trap column with mobile phase A at 25 µl/min for 1 min, followed by switching the SPE in-line with the gradient and separation column for 8 min. After 8 min the SPE was washed off-line by three full loop injections containing 5 µl 5% IPA + 0.1% FA and 5 µl 50% IPA + 0.1% FA. Separation was achieved on an Ascentis Express C18 nanoLC column (50 mm x 75 µm i.d., 2.7 µm HALO fused core particles; Supelco, Bellefonte, USA) conditioned at 900 nl/min with mobile phase A after which the following gradient of mobile phase A and 95% acetonitrile (ACN; Biosolve BV, Valkenswaard, the Netherlands; mobile phase B) was applied: 0 min 3% B, 2 min 5% B, 5 min 20% B, 6 min 30% B, 8 min 30% B, 9 min 0% B, and 14 min 0% B. The analytical column was protected for particulates coming from the sample injection by a stainless steel frit (2 µm pore size) mounted between the autosampler transfer tubing and the trap column. The HPLC system was coupled to a Dionex Ultimate UV detector (recording absorbance at 214 nm) equipped with a 3 nl flow cell. The HPLC was interfaced to a quadrupole-TOF-MS mass spectrometer (micrOTOF-Q; Bruker Daltonics, Bremen, Germany) with a standard ESI source (Bruker Daltonics) and a sheath-flow ESI sprayer (Figure 1; capillary electrophoresis ESI-MS sprayer; Agilent Technologies, Santa Clara, USA) applying the UV outlet tubing (20 µm i.d., 360 µm o.d.) as sprayer needle. To reduce the gas-phase TFA ion-pairing and assist with ESI spray formation a 2 µl/min sheath-flow of 50% IPA, 20% PA and 30% water was applied by one of the binary gradient pumps. To improve mobile phase evaporation a nitrogen stream was applied as dry gas at 4 l/min with a nebulizer pressure of 0.4 bars. Alternatively the LC-MS interfacing was achieved with a nano-ESI source (Bruker Daltonics) equipped with a metal nano-ESI needle (30 µm i.d.; Proxeon Biosystems, Odense M, Denmark). Scan spectra were recorded from 300 to 2000 dalton with 2 average scans at a frequency of 1 Hz. Quadrupole ion energy and collision energy of the MS were set at 2 and 4 eV, respectively. The total analysis time per sample was 16 min. The Ultimate 3000 HPLC system and the Bruker micrOTOF-Q were operated under Chromeleon Client version 6.8 and micrOTOF control version 2.3 software, respectively.

Data processing. Liquid chromatography-mass spectrometry datasets were calibrated internally using a list of known glycopeptides and were exported to the open mzXML format by Bruker DataAnalysis 4.0 in batch mode.[152] Each dataset was then aligned to

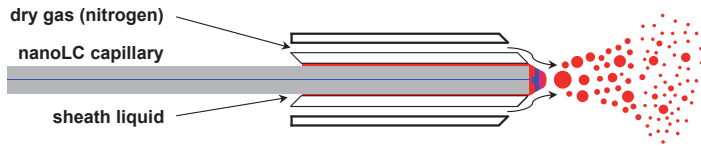

Figure 1. Schematic representation of the sheath-flow ESI sprayer.

a master dataset of a typical sample (containing many of the (glyco-)peptide species shared between multiple samples) using msalign2[153] and a simple warping script in AWK.[154] A list of 402 pre-defined features, defined as peak maximum (applied in this study) or peak area in specific mass and retention time windows, i.e. an accurate mass and time tag,[155] were then extracted from each dataset using the in-house developed software "Xtractor2D" and merged to a complete data matrix. As input, Xtractor2D takes one dataset in the mzXML format aligned to the master dataset and a reference list with pre-defined features with *m/z* windows and retention times in seconds. The retention times are on the chromatographic time scale of the master dataset used for the alignment and *m/z* values are calculated. The software and ancillary scripts are freely available at www.ms-utils.org/Xtractor2D. The complete sample-data matrix was finally evaluated using Microsoft Excel. Structural assignment of the detected glycoforms was performed on the basis of literature knowledge of IgG N-glycosylation.[3,4,9,10,79,148] Relative intensities of the glycopeptide species (Table 1) derived from IgG1 (18 glycoforms), IgG4 (10 glycoforms), and IgG2 (18 glycoforms) were obtained by integrating and summing three isotopic peaks followed by normalization to the total IgG subclass-specific glycopeptide intensities.

On the basis of the normalized intensities the level of galactosylation, sialylation, bisecting N-acetylglucosamine, fucosylation, and the number of sialic acid moieties present on the galactose moieties (SA/Gal) were calculated according to the following formulas: Galactosylation = (G1F + G1FN + G1FS + G1FNS + G1 + G1N + G1S) * 0.5 + G2F + G2FN + G2FS + G2FNS + G2 + G2N + G2S for IgG1 and IgG2, and (G1F + G1FN + G1FS + G1FNS) * 0.5 + G2F + G2FN + G2FS + G2FNS for IgG4. Sialylation = G1FS + G2FS + G1FNS + G2FNS + G1S + G2S for IgG1 and IgG2, and G1FS + G2FS + G1FNS + G2FNS for IgG4. Bisecting GlcNAc = G0FN + G1FN + G2FN + G1FNS + G2FNS + G0N + G1N + G2N for IgG1 and IgG2, and G0FN + G1FN + G2FN + G1FNS + G2FNS for IgG4. Fucosylation IgG1 and IgG2 = G0F + G1F + G2F + G0FN + G1FN + G2FN + G1FS + G2FS. No fucosylation level was determined for the IgG4 subclass as the non-fucosylated species remained below the limit of detection. The number SA/Gal is calculated by dividing the prevalence of IgG sialylation by 2 * the level of galactosylation.

Glycopeptide profiling of a pregnancy cohort. Serum from 26 healthy pregnant Caucasian women without adverse obstetric history ranging in age between 24 and 40 years was obtained at three time points during pregnancy (1st, 2nd, 3rd trimester) and at three time points postpartum (6, 12, and 28-52 weeks).[15] Human polyclonal

Table 1. Theoretical *m/z* values of human IgG Fc glycopeptides detected by nanoLC-ESI-MS.

	IgG1 P01857[b]		IgG2 P01859[b]		IgG4 P01861[b]	
Glycan species	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
No glycan	595.260	397.176	579.265	386.513	587.262	391.844
G0F[c]	1317.527	878.687	1301.532	868.024	1309.529	873.356[a1]
G1F	1398.553	932.705	1382.558	922.042	1390.556	927.373[a2]
G2F	1479.580	986.722	1463.585	976.059	1471.582	981.391
G0FN	1419.067	946.380	1403.072	935.717	1411.069	941.049[a3]
G1FN	1500.093	1000.398	1484.098	989.735	1492.096	995.066[a4]
G2FN	1581.119	1054.416	1565.124	1043.752	1573.122	1049.084
G1FS	1544.101	1029.737	1528.106	1019.073	1536.104	1024.405[a5]
G2FS	1625.127	1083.754	1609.132	1073.091	1617.130	1078.423
G1FNS	1645.641	1097.430	1629.646	1086.766	1637.643	1092.098
G2FNS	1726.667	1151.447	1710.672	1140.784	1718.670	1146.116
G0	1244.498	830.001	1228.503	819.338	-	-
G1	1325.524	884.019	1309.529	873.356[a1]	-	-
G2	1406.551	938.036	1390.556	927.373[a2]	-	-
G0N	1346.038	897.694	1330.043	887.031	-	-
G1N	1427.064	951.712	1411.069	941.049[a3]	-	-
G2N	1508.090	1005.730	1492.096	995.066[a4]	-	-
G1S	1471.072	981.051	1455.077	970.387	-	-
G2S	1552.098	1035.068	1536.104	1024.405[a5]	-	-

[a1 – a5]isomeric glycopeptide species of IgG4 and IgG2.
[b]SwissProt entry number
[c]glycan structural features are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting N-acetylglucosamine (N), and N-acetylneuraminic acid (S).

IgGs were purified with Protein G-Sepharose™ Fast Flow beads (GE Healthcare), digested overnight with trypsin and the resulting glycopeptides were analyzed by fast nano-reversed phase-LC-ESI-MS. Liquid chromatography-mass spectrometry datasets were processed automatically. Differences during pregnancy were evaluated using the Repeated Measures analysis (ANOVA) and differences between the 3rd pregnancy trimester and the last time point (28 to 52 weeks after delivery) were evaluated with the Paired-Samples T-test. *p*-Values were adjusted for multiple comparisons by Bonferroni correction, and *p*-values ≤ 0.013 were considered statistically significant. Data evaluation and statistical analysis were performed with Microsoft Excel and SPSS 16.0, respectively.

Results

Nano-reversed phase-LC-ESI-MS. In a first setup of the method, a tryptic IgG (glyco-)peptide pool prepared from plasma of a healthy donor by protein A affinity chromatography[148] was analyzed by direct injection of 250 nl onto the Ascentis Express C18 column. Separation was achieved at 500 nl/min using an ACN gradient with formic acid (FA) as acidic mobile phase additive to allow direct coupling with the mass spectrometer. The LC separation was interfaced to the mass spectrometer by a 30 µm i.d. metal nanoLC-ESI-MS sprayer. Good separation of IgG Fc N-glycopeptides from different human IgG subclasses was obtained within a window of 15 min (Figure 2A). Sialylated IgG Fc N-glycopeptides of each specific subclass eluted later than the non-sialylated counterparts showing almost baseline separation.

To simplify LC-MS coupling and improve system robustness we interfaced the nanoLC separation to the mass spectrometer by a Agilent sheath-flow ESI sprayer (Figure 1) mounted in the standard ESI source at the default, fixed position. The UV outlet tubing (20 µm i.d., 360 µm o.d.) is applied directly as sprayer tip coupling the LC separation to the MS with zero dead volume. To assist with the electrospray formation a sheath-flow of 50% IPA in water with 2% PA was applied at 2 µl/min. Furthermore, a nitrogen stream was applied as dry gas at 4 l/min with a nebulizer pressure of 0.4 bars to improve mobile phase evaporation.

The MS intensities of tryptic IgG Fc N-glycopeptides obtained with the sheath-flow sprayer (Figure 2B) were compared to those obtained with the metal nanoLC-ESI sprayer interface (Figure 2A). While IgG1 and IgG4 glycopeptides revealed similar intensities with both sprayers, the metal nanoLC-ESI sprayer provided higher IgG2 glycopeptide signals. In contrast to the metal nano-ESI sprayer the sheath-flow ESI sprayer provided a constant spray current which was independent of the LC gradient. This resulted in a more stable spray which performed equally well over the entire LC gradient. Moreover, the larger distance between the sheath-flow sprayer tip and the MS inlet resulted in a strong contamination reduction of the MS glass capillary as compared to the metal nanoLC-ESI sprayer.

To improve the separation and reduce the observed higher retention of sialylated glycopeptides FA was substituted for TFA. This resulted in sharper peaks and co-elution of sialylated IgG Fc N-glycopeptides with their non-sialylated counterparts (Figure 3). However, lower IgG glycopeptide signals were obtained (Figure 3A), which could be restored by increasing the PA concentration in the sheath-liquid to 20% (Figure 3B). Potential sample dilution and band broadening during ESI as a result of mixing with the sheath-liquid was evaluated by varying the sheath-flow rate from 1 to 10 µl/min. Best results in terms of MS signal intensities were obtained at 2 µl/min sheath-flow. Moreover, all sheath-flows revealed peaks of similar widths indicating that band broadening upon mixing is negligible. In addition to IPA, acetone, ACN and ethanol were evaluated as sheath-liquid additives at different flow rates but proved to be less effective than IPA in reducing/eliminating TFA gas-phase ion paring.

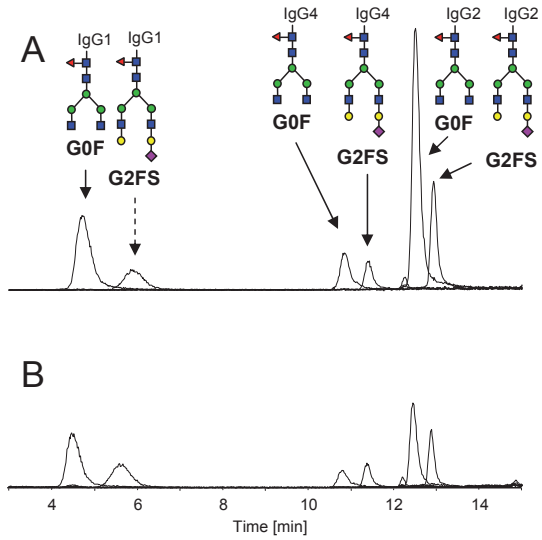

Figure 2. Comparison of the different sprayers for IgG Fc N-glycopeptide ionization. Extracted ion chromatograms obtained with a metal nano-ESI sprayer (A) and a sheath-flow ESI sprayer (B). Figures are represented with the same scaling. IgG Fc N-glycopeptides were separated at 500 nl/min using an ACN gradient with FA as mobile phase additive. The sheath-flow sprayer was applied with a 2 µl/min sheath-flow of 50% IPA containing 2% PA. Glycan code as in Figure 1 of Chapter 1.

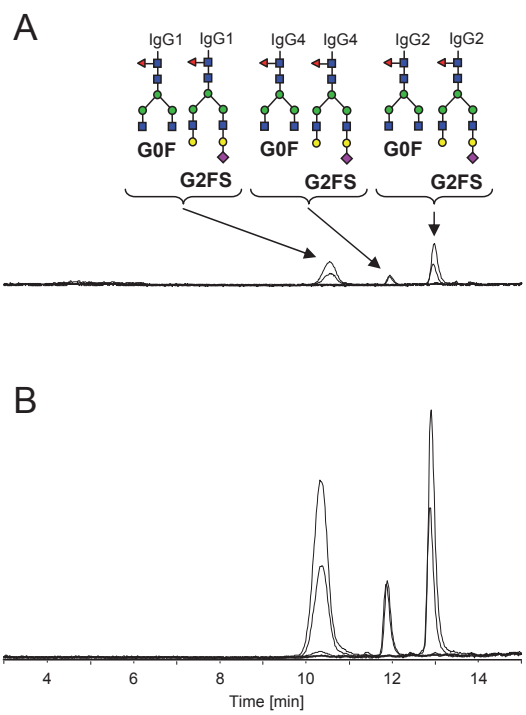

Figure 3. Effect of the PA concentration in the sheath-liquid on reduction of the TFA signal suppression. Extracted ion chromatograms of IgG glycopeptides with a 2 µl/min sheath-flow of 50% IPA containing 2% (A) or 20% (B) PA. Figures are represented with the same scaling. IgG Fc N-glycopeptides were separated at 500 nl/min using an ACN gradient with TFA as mobile phase additive. Glycan code as in Figure 1 of Chapter 1.

To shorten the total analysis time (TAT) we increased the mobile phase flow rate to 900 nl/min and implemented a 330 nl HALO C18 StemTrap trap column for fast on-line analyte extraction and purification of large sample volumes. Extraction and separation were evaluated at three different temperatures (30, 40 and 60°C) showing low recoveries for all analytes with peak tailing (data not shown). Moreover, very broad IgG1 glycopeptide peaks were observed at 40°C, and peak splitting occurred at 60°C. In an effort to improve the analyte recoveries we replaced the StemTrap with a Dionex PepMap trap column and reevaluated the separations at the three different temperatures. While analyte recoveries were significantly improved, broad peaks were observed for separations at 40 and 60°C. Separation at 30°C provided sharp and symmetrical peaks for all subclass-specific IgG Fc glycopeptides. Moreover, separation could be achieved within 7 to 10 min using a short steep ACN gradient allowing a TAT of 16 min from sample to sample (Figure 4).

IgG Fc N-glycopeptides already eluted from the trap column within the first 8 min of the gradient. For efficient washing of the trap column and in order to minimize carryover, we switched the trap column off-line at 8 min and performed full loop injections (10 µl) onto the trap column with various IPA dilutions. With three full loop injections filling the loop with 5 µl 50% IPA containing 0.1% FA and 5 µl 5% IPA containing 0.1% FA the carryover was found to be below 1.5% throughout.

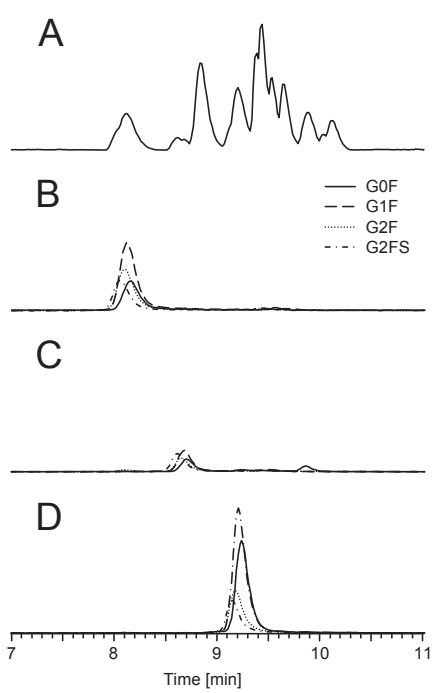

Figure 4. Fast nanoLC-ESI-MS of a tryptic IgG digest employing a sheath-flow sprayer. Base peak chromatogram of *m/z* 700-1700 (A); Extracted ion chromatogram of IgG1 (B), IgG4 (C), and IgG2 (D) Fc N-glycopeptides. Figures are represented with the same scaling. Separation was performed at 900 nl/min using an ACN gradient with TFA as mobile phase additive.

Optimization of mass spectrometry settings. In addition to the subclass-specific glycoforms (Table 1), using the standard (proteomics) MS settings IgG1, IgG4 and IgG2 glycopeptide decay products were observed as doubly charged fragment ions at *m/z* 1215.983, 1207.986 and 1199.988 Da, respectively. To minimize these decay products we evaluated various MS transfer parameters including capillary exit voltage, funnel voltages, quadrupole ion energy and collision energy. In the sheath-flow ESI setup, the capillary exit and funnel voltages hardly influenced the level of these decay products. By contrast, significant less decay products were observed when lowering the quadrupole ion energy (5 eV) and collision energy (10 eV) to 2 and 4 eV, respectively. While decreasing these energies resulted in lower total MS intensities, the signal to noise ratios remained unaffected. Tryptic IgG Fc N-glycopeptide ions were predominantly observed as $[M+3H]^{3+}$ and $[M+2H]^{2+}$ with low amounts of $[M+2H+Na]^{3+}$ (Figure 5). A total of 38 glycopeptide species derived from IgG1 (14 glycoforms), IgG4 (10 glycoforms), and IgG2 (9 glycoforms) were registered in both doubly charged and triply charged form for the healthy donor serum sample (Table 1) which is in accordance with previous reports on IgG Fc glycosylation profiling by LC-MS.[4,79]

Repeatability. To evaluate the repeatability of the nanoLC-MS analysis a tryptic IgG Fc glycopeptide pool was analyzed eight times on three different days. Compound spectra were processed automatically which involved internal calibration, LC alignment and peak picking, revealing a good intra- and interday repeatability with lower than 5% relative standard deviation (RSD) of the normalized peak heights for the 8 major glycoforms of IgG1, IgG4 and IgG2 (data not shown). The robustness and repeatability of the entire analytical method including sample preparation (protein A affinity purification and trytpic cleavage) and mass spectrometric detection was assessed by performing three independent experiments comprising eight replicates each. The intraday variability for IgG1 (Figure 6A), IgG4 (Figure 6B), and IgG2 (Figure 6C) was determined within the three experiments and the interday repeatability was determined by comparing the results of the three experiments (Figure 6). For the 8 major IgG1, IgG4 and IgG2 glycoforms the RSDs for the intra- and interday repeatability were below 5% of the normalized peak heights, indicating that the variability of the sample preparation was below the variability of the nanoLC-MS analysis.

Limit of detection. To evaluate the limit of detection (LOD) of the reverse phase nanoLC-ESI-MS setup we prepared a dilution series with tryptic digests of two monoclonal antibodies ranging from 0.024 to 100 ng/µl and analyzed 1 µl of each dilution. In the Fc portion of each antibody two N-glycans are located, which may vary in glycan composition. Upon analysis of the lowest measurable concentration three different glycoforms were observed. The LOD was estimated thereof to be 870 amol and was calculated by:

$$\frac{\text{Lowest measurable antibody concentration (0.195 ng/µl) * Number of Fc N-glycans (2)}}{\text{Molecular mass antibody (150 000) * Number of observed glycoforms (3)}}$$

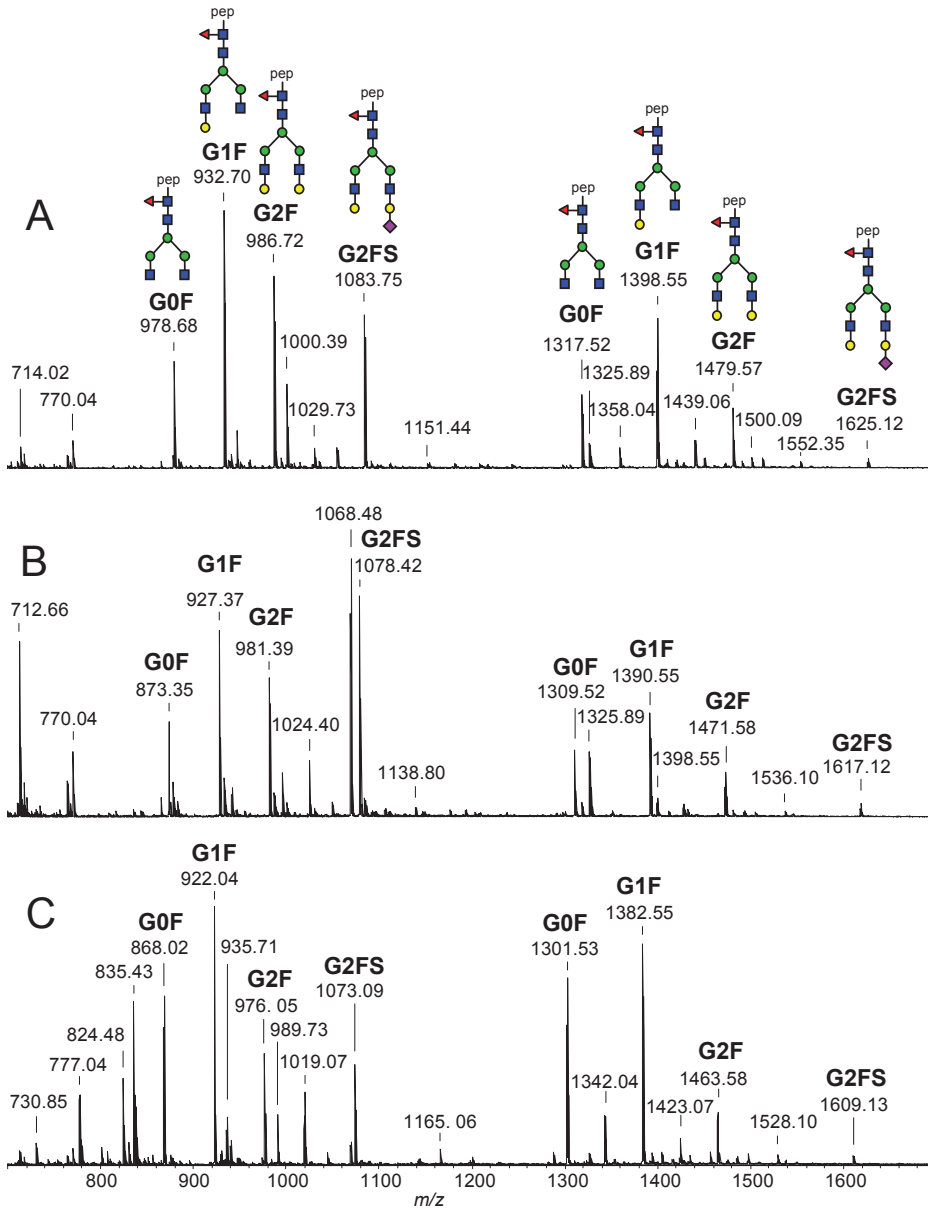

Figure 5. Tryptic glycopeptide mass spectra of IgG1 (A), IgG4 (B), and IgG2 (C) analyzed by fast nanoLC-ESI-MS with the sheath-flow ESI sprayer interface. Glycan code as in Figure 1 of Chapter 1.

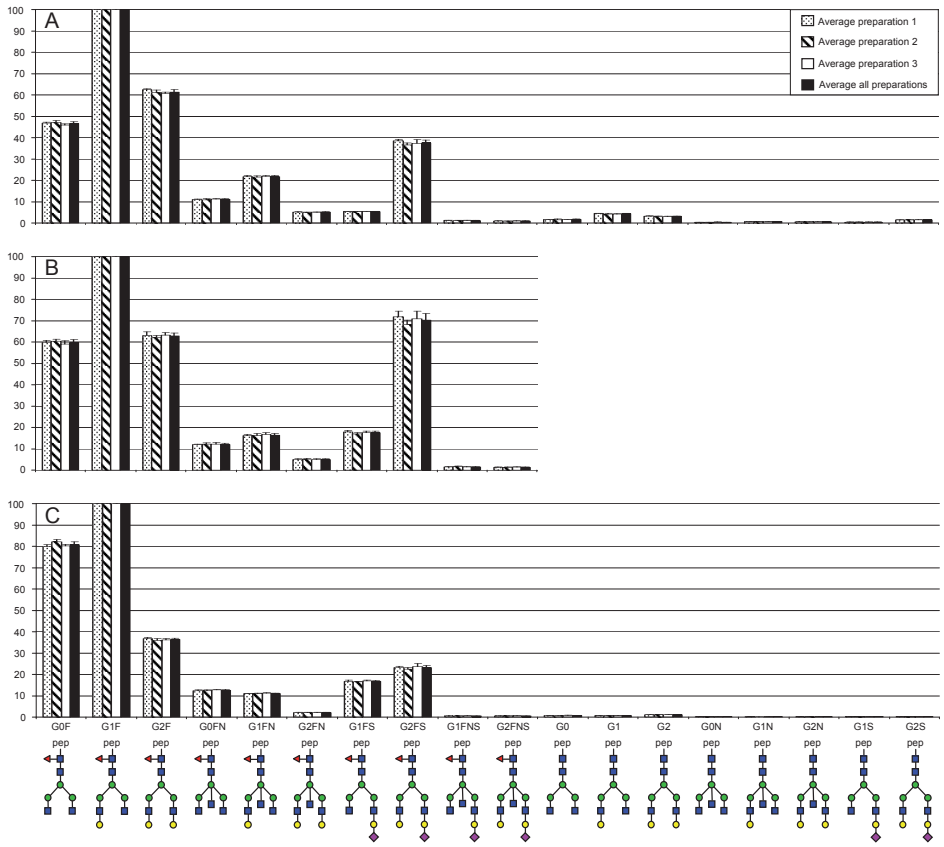

Figure 6. Repeatability of the sample preparation (protein A capturing, trypsin digestion) and analysis (fast nanoLC-ESI-MS with sheath-flow ESI sprayer interface) of IgG1 (A), IgG4 (B) and IgG2 (C). Relative intensities and standard deviations (STD) were determined from three independent experiments comprising eight replicates each. Glycan code as in Figure 1 of Chapter 1.

Pregnancy related IgG Fc N-glycosylation changes. The developed nano-reversed phase-LC-ESI-MS setup was applied to profile the IgG Fc N-glycosylation of 26 pregnant women at several time points during and after pregnancy. The obtained data revealed that the level of galactosylation, sialylation, and the number of SA/Gal increased significantly from the 1st to the 2nd pregnancy trimester for IgG1 (p-values < 0.005), IgG2/IgG3 (p-values < 0.010), and IgG4 (p-values < 0.015), while no significant increase was observed from 2nd to the 3rd pregnancy trimester (Table 2, Figure 7). During pregnancy no changes were observed for the level of fucosylation and bisecting N-acetylglucosamine of IgG1, IgG2/IgG3 and IgG4. The most prominent glycosylation changes between two consecutive time points were observed for the 3rd pregnancy trimester (time point 3) and 6 weeks *post partem* (time point 4; Table 2): all of the

analyzed glycosylation features, i.e., galactosylation, sialylation, SA/Gal, bisecting GlcNAc, and fucosylation showed significant changes (p-values < 0.001; Table 2). While galactosylation, sialylation, and SA/Gal were found to be decreased after delivery, the incidence of bisecting GlcNAc as well as fucosylation were increased for IgG1, IgG2/IgG3, and IgG4 (Table 2, Figure 7).

In order to identify pregnancy-associated glycosylation changes which are supposed to be reversed 28-52 weeks after delivery, IgG glycosylation was compared between the 3rd pregnancy trimester (time point 3) and time point 6. Again, the levels of galactosylation, sialylation, and the number of SA/Gal for IgG1, IgG2/IgG3, and IgG4 were significantly higher in the 3rd pregnancy trimester compared to 28-52 weeks after pregnancy (p-values < 0.001). By contrast, significantly higher levels of bisecting GlcNAc and fucosylation were observed for IgG1 and IgG2/IgG3 28-52 weeks after pregnancy compared to the 3rd pregnancy trimester (p-values < 0.005).

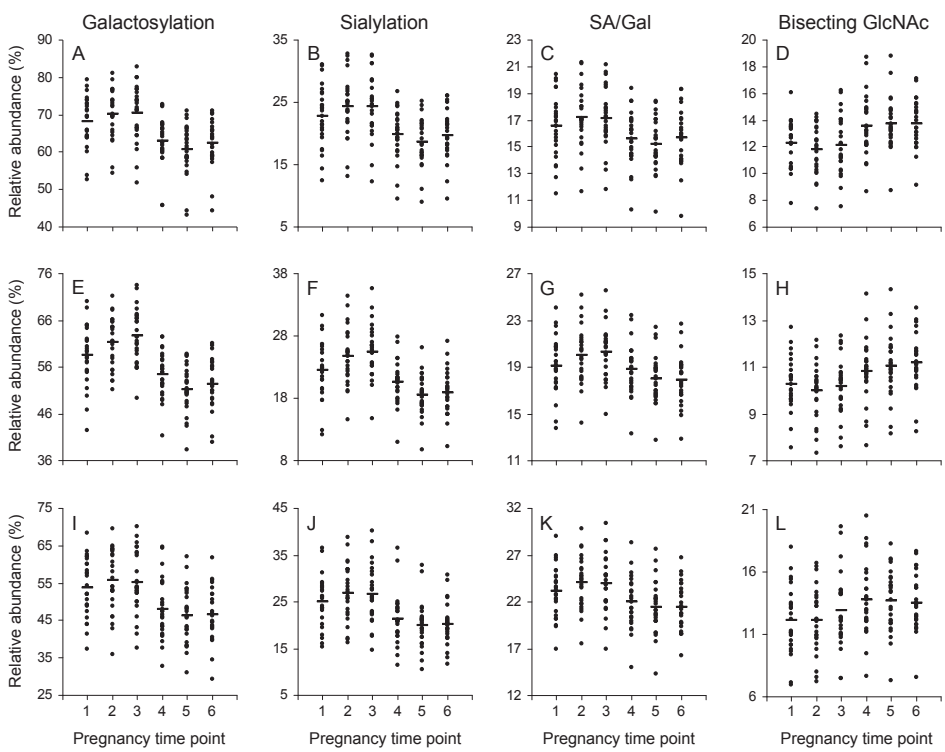

Figure 7. Pregnancy-associated IgG Fc N-glycosylation changes in 26 Caucasian women. For IgG1 (A-D), IgG2/IgG3 (E-H), and IgG4 (I-L) the level of galactosylation (A, E, I), sialylation (B, F, J), the incidence of SA/Gal (C, G, K) and the incidence of bisecting GlcNAc (D, H, L) are given for time point 1 (1st trimester), 2 (2nd trimester), 3 (3rd trimester), 4 (6 weeks after delivery), 5 (12 weeks after delivery), and 6 (28 to 52 weeks after delivery). Mean values are indicated by horizontal bars.

Table 2. Pregnancy related IgG Fc N-glycosylation changes.

Glycosylation feature	Time point	IgG1		IgG2/IgG3		IgG4	
		Mean (%)	*p*-value*	Mean (%)	*p*-value*	Mean (%)	*p*-value*
Galactosylation	1	68.34		59.02		53.24	
	2	70.16	< **0.001**	61.35	**0.009**	55.06	**0.012**
	3	70.68	0.453	63.23	0.036	54.67	0.515
	4	63.13	< **0.001**	54.68	< **0.001**	47.55	< **0.001**
	5	61.21	< **0.001**	51.65	< **0.001**	46.02	**0.006**
	6	61.92	0.140	52.07	0.338	46.44	0.469
Sialylation	1	22.98		22.76		24.72	
	2	24.22	< **0.001**	24.55	< **0.001**	26.46	< **0.001**
	3	24.47	0.449	25.65	0.020	26.35	0.728
	4	19.83	< **0.001**	20.61	< **0.001**	21.08	< **0.001**
	5	18.74	**0.001**	18.56	< **0.001**	19.85	< **0.001**
	6	19.44	0.023	18.73	0.544	19.96	0.768
SA/Gal	1	16.66		19.18		23.07	
	2	17.10	**0.001**	19.89	< **0.001**	23.86	< **0.001**
	3	17.14	0.734	20.18	0.019	23.92	0.578
	4	15.56	< **0.001**	18.76	< **0.001**	21.96	< **0.001**
	5	15.16	0.017	17.86	< **0.001**	21.37	< **0.001**
	6	15.57	0.031	17.87	0.967	21.36	0.927
Bisecting GlcNAc	1	12.44		10.37		12.41	
	2	11.98	0.148	10.10	0.167	12.37	0.937
	3	12.35	0.232	10.28	0.329	13.22	0.108
	4	13.79	< **0.001**	10.98	< **0.001**	14.03	< **0.001**
	5	13.81	0.921	11.16	0.092	13.91	0.605
	6	13.80	0.956	11.21	0.534	13.54	0.041
Fucosylation	1	92.24		97.28		n.d.	n.d.
	2	92.24	0.966	97.19	0.134	n.d.	n.d.
	3	92.03	0.362	97.00	**0.003**	n.d.	n.d.
	4	92.90	< **0.001**	97.34	< **0.001**	n.d.	n.d.
	5	93.15	0.071	97.42	0.253	n.d.	n.d.
	6	92.86	0.111	97.40	0.816	n.d.	n.d.

*Repeated Measures analysis *p*-values < 0.013 are considered to be significant and are highlighted in bold.
p-Values represent differences between time point 1 vs. 2, 2 vs. 3, 3 vs. 4, 4 vs. 5 and 5 vs. 6.
n.d., not determined.

Discussion

Here we describe a fast nanoLC-MS method for detailed Fc N-glycosylation profiling of human IgG subclasses in a single analysis. Human polyclonal IgGs were purified from plasma by protein A (captures IgG1, IgG2, and IgG4) or protein G (captures IgG1-4) affinity chromatography and digested overnight with trypsin in 96-well plate format. Aliquots of the samples containing IgG (glyco)peptides were loaded onto a C18 trap column and separated on a fused core C18 nanoLC column. In a first version of the setup, samples were injected directly onto the nanoLC column. The LC was interfaced to the MS by a metal nano-ESI sprayer and separation was performed with a gradient of ACN (mobile phase B) and FA (mobile phase A). No acid was added to the ACN to avoid potential acid-catalyzed hydrolysis of ACN to acetic acid and ammonia[156-158] which might result in the formation of ammonium adducts. The metal nano-ESI sprayer was set close to the glass capillary inlet and the position was optimized manually to obtain the best ESI current.

During the LC gradient the increase in the ACN content of the mobile phase resulted in an increase of the total ion current and higher MS intensities later in the gradient. On reverse phase LC columns IgG glycopeptides elute at a relative low percentage of ACN and thus experience less favorable ESI spray conditions with standard nano-ESI sources. By replacing the nano-ESI source and sprayer with a conventional ESI source and a sheath-flow ESI sprayer we obtained a plug-and-play zero dead volume nanoLC-MS interface. Compared with regular nano-ESI sprayers, the sheath-flow ESI sprayer is positioned considerably further away from the glass capillary inlet. This reduced contamination of the MS and allowed many sequential injections without intensity loss. The volatile sheath-liquid required for proper spraying with the sheath-flow sprayer provided a very constant spray and ionization over the entire gradient with MS intensities early in the gradient being similar to those observed with a metal nano-ESI sprayer. The sheath-liquid composition was optimized to enhance analyte signal intensities,[159] and reduce TFA gas-phase ion-pairing with basic analytes by introducing TFA fix[149,151] during spray formation without excessive sample dilution. We expect that the sheath-flow ESI sprayer will find acceptance for other applications which require robust and sensitive coupling of nanoLC to mass spectrometry.

With standard porous particle nanoLC columns separation is achieved with mobile phase flow rates between 100-300 nl/min generating pressures up to 200 bars. Current fused core particle columns offer higher efficiency due to the combination of a smaller longitudinal diffusion coefficient and lower eddy diffusion[160] and can be applied at higher mobile phase flow rates resulting in efficient separation without requiring ultra-high pressures.[161] Moreover, similar resolving power (plate height) has been reported for both low and high mobile phase flow rates. We, therefore, increased the mobile phase flow rate 1.8 times which effectively reduced the TAT to 16 min. Separation at 30°C of StemTrap extracted analytes showed peak tailing for all IgG Fc N-glycopeptides which could not be eliminated by diluting the sample or lowering the injection volume. Tailing

with HALO stationary phases has been reported for separations of basic peptides at lower temperatures which disappeared when the temperature was increased to 60°C.[162] We, therefore, explored this possibility by increasing the separation temperature up to 60°C. While recoveries were comparable at the three temperatures, tailing of the analyte peaks increased at higher temperatures with even peak splitting at 60°C, which suggests that the tailing was a result of the relatively low loading capacity of the fused core trap column (surface area ~150 m^2/g). To improve analyte recoveries and eliminate peak tailing we, therefore, replaced the fused core trap column by a porous particle trap column (surface area ~300 m^2/g). Both porous particle trap column and fused core particle analytical column revealed similar retention for the IgG Fc N-glycopeptides providing a system with high capacity sample extraction and fast analyte separation.

The soft nature of ESI allows analysis of intact glycopeptides including sialylated glycopeptides. However, at standard (proteomics) MS settings glycopeptides are prone to show in-source decay and/or fragmentation during ion-transfer.[137,139] We observed doubly charged IgG glycopeptide ions which only occur when the linkage between one of the antenna GlcNAc residues and the core mannose breaks. By lowering the default collision energy and quadrupole ion energy occurrence of these decay products could almost be reduced to noise levels. The funnel setup of the quadrupole-TOF-MS did not appear to influence the level of glycopeptide decay products observed, indicating a softer ion transfer compared to the setup with skimmer and lenses used in the TOF-MS (Bruker micrOTOF™) where lowering the voltage difference between the capillary exit and lens 1 was required to reduce these decay products (data not shown).

Simultaneous glycosylation profiling of different IgG subclasses can be achieved by direct infusion-MS,[137] MALDI-MS[102,148] and LC-MS.[4,79] Detailed glycosylation analysis of human polyclonal IgG by direct infusion-MS and MALDI-MS is limited by the presence of isomeric glycopeptide species.[148] By contrast, LC-MS provides detailed glycosylation profiles of different IgG subclasses in a single analysis.[4,79] The nanoLC-MS method described in this manuscript allows fast and detailed subclass specific analysis of IgG Fc N-glycosylation with a similar LOD but a substantially shorter TAT (16 min) compared to previously described methods.[4,79] Due to the sheath-flow ESI sprayer interface the system remains highly stable over a long period of time (more than 3 weeks) without the requirement of MS cleaning and maintenance (data not shown). The ionic interactions of sialylated glycopeptides with residual silanol groups on the silica based RP stationary phase is suppressed by the presence of TFA improving chromatographic resolution.[129] Due to the predominantly peptide-based retention the complete IgG subclass specific glycopeptide profile is obtained in a 30 s separation window (Figure 4). The here developed robust, fast and sensitive method for IgG Fc glycosylation analysis will not only be suitable to analyze the glycosylation of clinical IgG samples but will also be applicable in a biotechnological setting for the characterization of recombinantly produced therapeutic IgGs.

For a variety of diseases lowered IgG galactosylation has been reported.[147] In healthy individuals IgG Fc N-glycosylation is known to be influenced by age, gender and pregnancy.[4,8-10,13,14] During pregnancy a decreased percentage of agalactosylated IgG has been reported which after delivery increased to values similar to before conception.[14] The here developed fast nano-reversed phase HPLC-MS method was successfully applied to profile IgG Fc N-glycosylation of 26 pregnant women, confirming the previous reported increase of IgG galactosylation during pregnancy.[15] The applied technique additionally allowed sensitive and accurate determination of sialylated IgG glycopeptides, revealing increased levels of IgG1, IgG2/IgG3, and IgG4 sialylation during pregnancy. The pregnancy-associated increase in sialylation was found to be more pronounced than the increase in galactosylation. This is reflected by the fact that the ratio of SA/Gal is higher during pregnancy compared to after delivery (Figure 7C, G, and K). This finding indicates that IgG Fc galactosylation and sialylation, although they tend to show some correlation,[15] are not fully concerted in terms of regulation.

Furthermore, we observed lower levels of IgG1 and IgG2/IgG3 bisecting GlcNAc and fucosylation during pregnancy than after delivery. Differences in IgG glycosylation are postulated to be a reflection of altered N-glycan biosynthesis in B-lymphocytes.[12,57] Thus the decreasing levels of bisecting GlcNAc and fucosylation with increasing levels of galactosylation and sialylation during pregnancy may indicate altered activities of GlcNAc transferase III (catalyzes the addition of a β-1,4-linked GlcNAc to the β-linked mannose of the trimannosyl core),[163] α1,6-fucosyltransferase (catalyzes the addition of a fucose residue to the innermost GlcNAc of the chitobiose core),[164] β4-galactosyltransferase(s) (catalyzes the addition of β1,4-linked galactoses to the GlcNAc residues),[165] and α2,6/α2,3-sialyltransferase (catalyzes the addition of α2,6 or α2,3-linked sialic acids to the antennae galactose residues).[166,167] Future studies will hopefully elucidate the regulatory mechanisms involved in inducing pregnancy-associated glycosylation changes as well as the immunological role and implications of these alterations.

In conclusion, the here described nano-reversed phase HPLC-MS method allows sensitive, fast and reproducible analysis of subclass-specific IgG glycosylation and is combined with automated data processing. Moreover, the sheath-flow ESI sprayer provides a sensitive and robust plug-and-play interface that minimizes contamination of the mass spectrometer. The system is currently being applied to analyze large clinical cohorts, and no decrease in signal intensity has been observed after 2000 injections.

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