High-throughput IgG Fc N-glycosylation profiling by mass spectrometry of glycopeptides

Maja Pučić Baković[1], Maurice H.J. Selman[2], Marcus Hoffmann[2,3], Igor Rudan[4,], Harry Campbell[4], André M. Deelder[2], Gordan Lauc[1,5], Manfred Wuhrer[2¶]

[1] Genos Ltd., Laboratory of Glycobiology, Planinska 1, 10000 Zagreb, Croatia

[2] Leiden University Medical Center, Biomolecular Mass Spectrometry Unit, Leiden, The Netherlands

[3] Max Planck Institute for Dynamics of Complex Technical Systems, Sandtorstrasse 1, 39106 Magdeburg, Germany

[4] Centre for Population Health Sciences, The University of Edinburgh Medical School, Edinburgh, UK

[5] University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića 1, 10000 Zagreb, Croatia

To whom correspondence should be addressed at:

Manfred Wuhrer, Leiden University Medical Center, Biomolecular Mass Spectrometry Unit, Department of Parasitology, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Fax: +31-71-526-6907; E-mail: m.wuhrer@lumc.nl

Running title: IgG Fc glycosylation changes with age and sex

ABSTRACT

Age- and sex-dependence of subclass specific immunoglobulin G (IgG) Fc *N*-glycosylation was evaluated for 1709 individuals from two isolated human populations. IgGs were obtained from plasma by affinity purification using 96-well protein G monolithic plates and digested with trypsin. Fc *N*-glycopeptides were purified and analyzed by negative-mode MALDI-TOF-MS with 4-chloro-α-cyanocinnamic acid (Cl-CCA) matrix. Age-associated glycosylation changes were more pronounced in younger individuals (<57 years) than in older individuals (>57 years) and in females than in males. Galactosylation and sialylation decreased with increasing age and showed significant sex dependence. Interestingly, the most prominent drop in the levels of galactosylated and sialylated glycoforms in females was observed around the age of 45 to 60 years when females usually enter the menopause. The incidence of bisecting *N*-acetylglucosamine increased in younger individuals and reached a plateau at older age. Furthermore, we compared the results to the total IgG *N*-glycosylation of the same populations recently analyzed by hydrophilic interaction liquid chromatography (HILIC). Significant differences were observed in the levels of galactosylation, bisecting *N*-acetylglucosamine and particularly sialylation which were shown to be higher in HILIC analysis. Age- and sex-association of glycosylation features was to a large extent comparable between MALDI-TOF-MS and HILIC IgG glycosylation profiling.

Keywords: Immunoglobulin G, glycopeptides, *N*-glycosylation, glycomics, glycome, mass spectrometry, hydrophilic interaction liquid chromatography

INTRODUCTION

Immunoglobulin G (IgG) is the most abundant glycoprotein in human serum and a major effector molecule of the humoral immune response. Human IgG occurs in four subclasses (IgG1-4), and each molecule consists of two heavy and two light chains. The two light chains together with the parts of the heavy chains (V_H and C_H1 domains) form two Fab (fragment antigen-binding) moieties which are linked by a flexible hinge region to one Fc (fragment crystallizable) moiety formed by the remainders of the two heavy chains (C_H2 and C_H3 domains).[1] Each heavy chain in the Fc region carries a single covalently attached biantennary *N*-glycan at the highly conserved asparagine 297.[2] Fc glycans are essential structural components of the IgG molecule and minor changes in glycan composition can significantly alter the conformation of the Fc region changing the interaction with receptor proteins and thus modulating the effector functions of IgG.[3, 4] The lack of core fucose enhances the IgG1 binding to activating Fc receptor FcγRIIIa leading to increased antibody dependent cellular cytotoxicity (ADCC) and destruction of target cells.[2, 5-7] . Moreover, the presence of sialic acid on the Fc *N*-glycans confers anti-inflammatory properties to IgG.[8] In a mouse arthritis model the anti-inflammatory effect of sialylated IgG was shown to be mediated through interaction with SIGN-R1, a lectin receptor on mouse splenic macrophages, the human orthologue of this receptor being DC-SIGN.[9, 10] Very recently Karsten *et al.*[11] reported anti-inflammatory properties to be mediated by Fc galactosylation via the formation of immune complexes. High *N*-glycan galactosylation of IgG1 in immune complexes was shown to promote the association between the inhibitory IgG receptor FcγRIIB and C-type lectin-like receptor dectin-1, resulting in a blockage of pro-inflammatory effector functions.[11] Following numerous reports on the importance of IgG Fc *N*-glycosylation for effector functions,

engineering the Fc region of therapeutic antibodies has become a major challenge and goal for the biopharmaceutical industry.[3]

The majority of IgG *N*-glycans are attached to heavy chains of the Fc region, but 15-20% of polyclonal human IgG molecules also contain glycans within the Fab regions which were found to be more highly bisected, galactosylated and sialylated than Fc glycans.[3, 12-15]

Microheterogeneity of human IgG glycans is known to be dependent on various physiological (age, sex, pregnancy) and pathological parameters (tumors, infections, autoimmune diseases, etc.).[1] A number of earlier studies have reported that specific patterns of IgG glycosylation change with age and depend on gender. Parekh *et al.* were the first to describe decreasing levels of galactosylation with aging.[16] Subsequently, it has been shown that age-dependent galactosylation levels are sex-specific and that levels of bisected glycoforms change with age as well.[17-19] Moreover, in a recent large-scale study of IgG glycans decreased levels of agalactosylated glycoforms with bisecting *N*-acetylglucosamine (GlcNAc) were found to be an early marker of familial longevity.[19]

In this study, we performed large-scale IgG1 and IgG2&3 Fc *N*-glycosylation profiling by negative-mode MALDI-TOF-MS with 4-chloro-α-cyanocinnamic acid (Cl-CCA) matrix in 1709 individuals and investigated age- and sex-specificity of the glycosylation features: sialylation, galactosylation, fucosylation, and the occurrence of bisecting GlcNAc. We compare the results to the total IgG glycosylation recently analyzed by hydrophilic interaction liquid chromatography (HILIC) of enzymatically released and fluorescently labeled glycans.[20]

EXPERIMENTAL SECTION

Study population. The study was based on plasma samples from respondents who were residents of the Croatian Adriatic islands, Vis and Korčula, and who were recruited within a larger genetic epidemiology program previously described.[21, 22] In total, 1709 individuals were included with 795 subjects from Vis (46.5%) and 914 (53.5%) from Korčula. The age range was 18-98 years (median age 57, interquartile range 21 years). There were 61.7% females and 38.3% males. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the appropriate Ethics Board of the University of Zagreb Medical School. An informed consent was signed by all participants prior to participation.

IgG purification. Immunoglobulin G was isolated from plasma by affinity chromatography using 96-well protein G monolithic plates as previously reported.[20] Briefly, 50 µl of plasma was diluted 10x with PBS, applied to the protein G plate and instantly washed. IgGs were eluted using 1000 µl of 100 mM formic acid and immediately neutralized to pH 7.0 with 1 M ammonium bicarbonate.

Trypsin digestion of human polyclonal IgG. Aliquots (1/20; 50 µl) of the protein G eluates were brought to 96-well polypropylene V-bottom microtitration plates, and a standard IgG sample was added in six-fold to each plate to allow evaluation of inter-batch variation. TPCK trypsin (Sigma-Aldrich, St. Louis, MO) was first dissolved in ice-cold 20 mM acetic acid (Merck, Darmstadt, Germany) to a final concentration of 0.4 µg/µl after which it was further diluted to 0.02 µg/µl with ice-cold ultra-pure water. For overnight digestion at 37°C, 20 µl of diluted trypsin was added to each IgG sample.

Reverse-phase solid phase extraction (RP-SPE) of glycopeptides. Glycopeptides were purified and desalted by reverse phase (RP) solid phase extraction (SPE) using Chromabond C_{18}ec beads (Marcherey-Nagel, Düren, Germany) as described previously.[23] Briefly, C-18 beads were activated with 80% ACN containing 0.1% trifluoroacetic acid (TFA; Fluka) and conditioned with 0.1% TFA. Tryptic IgG digests were diluted with 0.1% TFA, loaded onto C-18 beads and washed with 0.1% TFA. IgG glycopeptides were eluted with low concentration of ACN (18%) containing 0.1% TFA to minimize coelution of interfering peptides. Eluates were dried by vacuum centrifugation and stored at -20°C until mass spectrometric analysis. The heating and acid steps of the sample preparation method were evaluated and found not to lead to any noticeable degradation of the Fc glycan moieties with regard to sialylation and fucosylation.

MALDI-TOF-MS of IgG glycopeptides. Large-scale IgG glycosylation analysis was performed using MALDI-TOF-MS. Samples were dissolved in 20 µl of water, and 3 µl aliquots were spotted onto MTP 384 polished steel target plates (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Subsequently 1 µL of 5 mg/mL 4-chloro-α-cyanocinnamic acid (Cl-CCA; 95% purity; Bionet Research, Camelford, Cornwall, UK) in 50 % acetonitrile was applied on top of each sample and allowed to dry.[24, 25] Glycopeptides were analyzed on an UltrafleX II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) which was operated in the negative-ion reflectron mode. Ions between *m/z* 1000 and 3800 were recorded. To allow homogeneous spot sampling a random walk laser movement with 50 laser shots per raster spot was applied and each IgG glycopeptide sum mass spectrum was generated by accumulation of 2000 laser shots. Mass spectra were internally calibrated using a list of known glycopeptides. Data processing and evaluation were performed with FlexAnalysis Software (Bruker Daltonics) and Microsoft Excel, respectively. The data were

baseline subtracted and the intensities of a defined set of 27 glycopeptides (16 glycoforms for IgG1 and 11 for IgG2&3) were automatically defined for each spectrum (Table 1). IgG2 and IgG3 have identical peptide moieties (E_{293}EQFNSTFR_{301}) of their tryptic Fc glycopeptides and are, therefore, not distinguished by the profiling method.[23]

Relative intensities of IgG Fc glycopeptides were obtained by integrating and summing four isotopic peaks followed by normalization to the total subclass specific glycopeptide intensities. An exception was made for the IgG1 G1S1 (monogalactosylated, monosialylated biantennary glycan without core fucose, see Table 1) glycoform for which only the first isotopic peak could be reliably determined as the 2nd, 3rd and 4th isotopic peaks overlap with peaks of the IgG4 G2F glycoform and thus cannot be used to calculate the sum intensity. Instead, the signal intensity for the sum of four isotopic peaks of the IgG1 G1S1 glycoform was determined by multiplying the signal of the 1st isotopic peak of the IgG1 G1S1 glycoform with a correction factor (4.348, which is the average ratio of the sum of all four isotopic peaks versus the 1st isotopic peak determined for the related G2S1 glycoform of 126 standard samples).The level of galactosylation was calculated from the relative intensities of various Fc N-glycoforms (Table 1) according to the formula: (G1+G1F+G1FN+G1N+G1S1+G1FS1)*0.5+G2+G2F+G2FN+G2N+G2S1+G2FS1 for the IgG1 subclass and (G1F+G1FN+G1S1+G1FS1)*0.5+G2F+G2FN+G2FS1 for the IgG2&3 subclasses. The prevalence of bisecting GlcNAc was determined by summing the relative intensities of all bisected glycoforms (G0N, G1N, G2N, G0FN, G1FN, and G2FN for IgG1 and G0N, G0FN, G1FN, and G2FN for IgG2&3). The level of sialylation was defined by summation of all sialylated glycoforms (G1S1, G1FS1, G2S1, and G2FS1 for IgG1 and G1S1, G1FS1, and G2FS1 for the IgG2&3 subclasses). The incidence of IgG1 fucosylation was evaluated by summing all fucosylated IgG1 Fc *N*-glycoforms (G0F+G0FN+G1F+G1FN+G1FS1+G2F+G2FN+G2FS1). The

incidence of IgG2&3 fucosylation was not evaluated, as a large portion of the afucosylated IgG2&3 glycoforms could not be determined due to mass spectrometric overlap with isomeric IgG4 glycoforms (Table 1).[23]

HILIC of 2-AB labeled *N*-glycans with fluorescence detection. Total IgG *N*-glycans were enzymatically released, fluorescently labeled with 2-aminobenzamide and analyzed by hydrophilic interaction liquid chromatography (HILIC) on a Waters Acquity UPLC instrument (Waters, Milford, MA).[20] 2-AB labeled *N*-glycans were separated on a Waters BEH Glycan chromatography column, 100 x 2.1 mm i.d., 1.7 µm BEH particles, with 100 mM ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B. A linear gradient of 75-62% of acetonitrile at flow rate of 0.4 ml/min in 20 min was used to separate *N*-glycans into 24 peaks.

Comparison of MALDI-TOF-MS and chromatography analysis. In order to compare MALDI-TOF-MS IgG Fc *N*-glycopeptide profiles with the HILIC IgG *N*-glycan profiles we recalculated glycosylation features of the MS measurement to be in accordance with published HILIC calculations. Hence, galactosylation and bisecting GlcNAc levels were determined only from neutral glycoforms. From MALDI-TOF-MS analysis, 12 out of 16 detected IgG1 glycoforms and 8 out of 11 detected IgG2&3 glycoforms were included in this comparison, and a normalization was performed (total signal intensity per subclass was set to 100%). As for HILIC glycan analysis, 12 neutral glycoforms from 14 chromatographic peaks (G1F and G1FN glycoforms were separated into two peaks each) were considered, and normalization was performed (total signal of the 14 chromatographic peaks = 100%). The level of galactosylation in neutral glycoforms was calculated as follows: (G1+G1F+G1FN+G1N)*0.5+G2+G2F+G2FN+G2N for the IgG1 subclass, (G1F+G1FN)*0.5+G2F+G2FN for the IgG2&3 subclasses and (G1+G1F+G1FN)*

0.5+G2+G2N+G2F+G2FN for the total IgG. The incidence of bisecting GlcNAc in neutral glycoforms was determined by summing the relative intensities of all neutral bisected glycoforms: (G0N+G1N+G2N+G0FN+G1FN+G2FN) for the IgG1, (G0N+G0FN+G1FN+G2FN) for the IgG2&3 and (G0FN+G1FN+G2N+G2FN) for the total IgG. For comparison of sialylation levels between MALDI-TOF-MS and HILIC analysis, we used a degree of sialylation of fucosylated glycoforms without bisecting GlcNAc (FGS/(F+FG+FGS)) calculated from the total detected glycoforms: (G1FS1+G2FS1)/(G0F+G1F+G2F+G1FS1+G2FS1)*100 for IgG1 and IgG2&3 and (G1FS1+G2FS1+G2FS2)/(G0F+G1F+G2F+G1FS1+G2FS1+G2FS2)*100 for total IgG (HILIC data).

Additionally, we have compared MALDI-TOF-MS profiles of 2-AA-labeled *N*-glycan with HILIC profiles of 2-AB-labeled *N*-glycans for two standard samples, i.e., IgG and IgG Fc (Athens Research & Technology, Athens, GA). Sample preparation and analysis of 2-AB glycans was as described above. Details on sample preparation and analysis of 2-AA glycans can be found with Supplementary Figure 5. Comparison of *N*-glycopeptide patterns with HILIC profiles was performed for IgG1 and IgG Fc standard samples as described with Supplementary Figure 6.

Statistical analysis. IgG glycosylation variables were tested for normality using the Kolmogorov-Smirnov test, and nonparametric statistical tests were further used. Correlations were determined with Spearman's rank test and gender differences were tested with the Mann-Whitney test. Statistical analysis was performed with SPSS 13 (SPSS Inc, Chicago, IL).

RESULTS

MALDI-TOF-MS glycopeptide profiling. IgG was purified from plasma samples of 1709 individuals (61.7% female and 38.3% male) ranging in age between 18 and 98 years. All four human IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were obtained by high-throughput affinity purification using 96 well protein G monolithic plates. After tryptic digestion IgG Fc *N*-glycopeptides were enriched and desalted by RP-SPE and analyzed by negative-ion reflectron mode MALDI-TOF-MS using 4-chloro-α-cyanocinnamic acid as matrix substance.[24] IgG3 Fc *N*-glycopeptides have a peptide moiety which is identical to the one of IgG2 (Table 1).[26] Therefore, these two subclasses were determined together. IgG4 Fc *N*-glycopeptides were not determined due to the low abundance of their signals and overlap with other signals (Table 1).[26] Mass spectra were processed automatically which resulted in the determination of 16 IgG1 Fc glycoforms and 11 IgG2&3 Fc glycoforms (Table 1 and Figure 1). Glycoforms were assigned on the basis of their composition and literature data on human plasma IgG glycosylation.[13, 20, 27] Relative intensities of glycoforms were obtained by integration and summation of four isotopic peaks followed by normalization to the total subclass specific glycopeptide intensities.

To determine inter- and intra-batch variation of the analytical method, a standard IgG sample was added in six replicates to each sample plate. The intra-batch and inter-batch variation were determined for the five major glycoforms of the standard IgG samples, and the relative standard deviation (RSD) was found to be ≤ 6% for each sample plate (intra-batch) and below 10% over the entire 20 plates (inter-batch) for both IgG1 and IgG2&3. No batch correction was performed.

Association of IgG glycosylation with age and sex. We evaluated the correlation of IgG glycosylation features and glycoforms with age (Table 2). The obtained data showed a pronounced decrease of IgG1 and IgG2&3 Fc galactosylation with age. The relative abundances of agalactosylated IgG glycoforms (represented by G0F glycoform) increased at higher age (Supplementary Figure 1A and D). The opposite was observed for glycoforms with one or two galactoses (represented by G1F and G2F, respectively) which abundances decreased with age (Supplementary Figure 1B, C, E and F). Previously, it has been shown that IgG glycosylation changes with age are sex-specific and that those associations are more evident and stronger in individuals of up to 50-60 years of age.[18, 19, 28] Therefore, to reveal more details we divided our whole population into two age groups at median age (57 years of age). Younger individuals, both female and male, showed stronger negative correlation between age and galactosylation in all tested IgG subclasses. Stronger correlation coefficients in the younger group were also observed in all of the rest of glycosylation features, independent of the sex and subclass (Table 2). Statistically significant differences ($p \leq 0.001$) between the two age groups were revealed for all the glycosylation features that showed significant age dependence (Table 3).

When galactosylation was evaluated for the entire age range no sex difference was revealed (data not shown). However, by analyzing the data stratified for ages below and above the median age statistically significant differences in IgG galactosylation between females and males emerge. Younger females showed a stronger correlation between galactosylation and age than males for both IgG1 and IgG2&3 (Table 2). The level of galactosylation reached similar values for both sexes around median age after which sex differences in galactosylation changes with age were less obvious (Figure 2A and D). Interestingly, while the younger group of females (age ≤57) appeared to have higher galactosylation than males

(median of 43.0% versus 39.0%, respectively), at older age this relationship seemed to be reversed with males showing higher level of galactosylation than females (median of 33.9% versus 31.3%, respectively; Table 3).

Association of IgG sialylation with age showed a trend similar to that of galactosylation. The level of sialylated glycoforms significantly decreased with increasing age for both IgG1 and IgG2&3 (Table 2). This negative correlation was more pronounced for younger individuals than for older ones. Correlation of age with sialylation was stronger in females than in males. Moreover, just like in the case of galactosylation, IgG sialylation for younger individuals was higher in females than in males (Figure 2C and F). Around the age of 60 sialylation reached similar levels for both sexes after which males exhibited higher level of sialylation.

The incidence of bisecting GlcNAc increased with age (Table 2; Figure 2B and E). However, significant positive correlations were observed only for the younger individuals, while in the older age group the abundance of bisecting GlcNAc-containing glycoforms seemed to reach a plateau (Figure 2B and E). Within both age groups, no sex differences were found except for the IgG2&3 subclasses which showed higher level of bisecting GlcNAc in older females compared to males of similar age.

In addition, age- and sex-dependent variations in IgG1 core fucosylation were evaluated. A weak but nevertheless significant negative age effect on the level of IgG1 core fucosylation was observed only in younger individuals (Table 2). Within our cohort, there was no clear difference in fucosylated IgG1 glycoforms between females and males (Table 3).

Next, we analyzed correlations between different IgG glycosylation features with Spearman's rank test (Table 4). The strongest relationship was found between galactosylation and the level of sialylation for IgG1 (r = 0.68) as well as IgG2&3 (r = 0.84) (Figure 3A and C). IgG1 galactosylation showed a negative correlation (r = -0.17) with the level of core fucosylation

(Supplementary Figure 2A). An even stronger negative correlation was observed between IgG1 sialylation and core fucosylation, r = -0.38 (Figure 3B). Over the whole age range the galactosylation of the analyzed IgG subclasses exhibited a weak negative (r = -0.09 for IgG1 and r = -0.11 for IgG2&3) but significant correlation with the incidence of bisecting GlcNAc (Supplementary Figure 2D, E and F). Interestingly, this effect showed a pronounced age-dependence for the IgG1 subclass: while younger individuals revealed a negative correlation (r = -0.12), at higher age galactosylation appeared to positively correlate (r = 0.13) with bisecting GlcNAc. We additionally observed a negative correlation (r = -0.30) between the level of core fucosylated IgG1 *N*-glycans and the incidence of bisecting GlcNAc (Figure 3D). In addition, a weak positive correlation (r = 0.13) was observed between the bisecting GlcNAc and sialylated IgG1 *N*-glycans (Supplementary Figure 2B). A similar positive correlation (r = 0.16) was observed for bisecting GlcNAc and sialylation of IgG2&3 subclasses but only for older individuals (Supplementary Figure 2C).

Comparison of MALDI-TOF-MS and HILIC IgG glycosylation profiles. In order to compare the subclass-linked IgG Fc glycosylation analyzed by MALDI-TOF-MS with the total IgG (Fc and Fab of all subclasses) *N*-glycosylation profiles analyzed by HILIC, we chose to use calculations of glycosylation features as performed previously with HILIC data.[20] Hence, we compared IgG1 Fc glycosylation, IgG2&3 Fc glycosylation and total IgG glycosylation by evaluating the levels of galactosylation and bisecting GlcNAc from neutral glycoforms and the degree of sialylation of fucosylated glycoforms (without bisecting GlcNAc) from the total measured glycoforms. When levels of glycosylation features calculated from MALDI-TOF-MS profiles were correlated with the features calculated from HILIC analysis, highly significant and strong positive correlation coefficients of IgG1 Fc or IgG2&3 Fc with the total IgG were obtained (Supplementary Figure 3). The strongest correlation was observed for the

galactosylation of neutral glycoforms of total IgG and subclass-specific Fc glycopeptides (r ≥0.90). The degree of sialylation of total fucosylated glycoforms as well as the levels of bisecting GlcNAc in neutral glycoforms analyzed by the two methods also revealed a positive correlation but with weaker coefficients (r ≥0.51 and r ≥0.70, respectively).

Next, MALDI-TOF-MS and HILIC data were compared with respect to age- and sex-dependence. Correlation coefficients of age and glycosylation features observed for IgG1 Fc, IgG2&3 Fc and the total IgG (Supplementary Table 1) were very similar to those described above for the total Fc glycoforms. Correlation of age with the level of galactosylation was almost the same for all three data sets (IgG1 Fc, IgG2&3 Fc and total IgG). As for the association of the degree of sialylation with age, IgG2&3 Fc and the total IgG were rather similar showing a strong negative correlation, evident even in the older group of individuals, while this correlation was much less pronounced for IgG1 Fc. Minor distinctions were noticed in the strength of the correlations of age with bisecting GlcNAc. The total IgG (Fc and Fab of all four subclasses) measured by HILIC showed stronger positive correlation of bisecting GlcNAc with age than IgG1 Fc and IgG2&3 Fc measured by MALDI-TOF-MS. Similarly to the total glycoforms, this age effect on bisecting GlcNAc was only significant for the younger individuals (≤ 57 years) as seen for both methods.

Very similar differences regarding sex and age groups were consistently observed for IgG1 Fc, IgG2&3 Fc, and the total IgG (Supplementary Table 2). Significant differences between younger and older females were observed for the levels of galactosylation, bisecting GlcNAc and the degree of sialylation. In males, only the galactosylation levels measured by both methods notably differed between the two age groups (below and above age 57). As for the bisecting GlcNAc, the difference between younger and older males was revealed only for IgG1 Fc, while IgG2&3 Fc and the total IgG showed a difference in the degree of sialylation.

Regardless of the analytical method, older individuals showed sex differences in all glycosylation features, with the exception of bisecting GlcNAc of IgG1. Similarly, younger females and males had significantly different levels of galactosylation and sialylation. Median levels of glycosylation features for the whole studied population were compared between the total IgG and subclass-specific Fc fragments (Supplementary Table 2). Levels of galactosylation and bisecting GlcNAc as well as the degree of sialylation were higher for the total IgG than for the IgG Fc glycosylation data sets (Figure 4). The difference was most prominent in the degree of sialylation of fucosylated glycoforms since the total IgG exhibited approximately 7 times higher level of sialylation than IgG1 Fc and IgG2&3 Fc. For all three data sets the level of galactosylation and the level of sialylation showed the same trend of sex-associated differences in both age groups: while younger females had higher levels of galactosylation and sialylation than males, at older age males showed higher levels of both features than females (Supplementary Figure 4). Another trait for which similar results were obtained in the different data sets is the incidence of the bisecting GlcNAc (Supplementary Table 2) which was increasing only in younger individuals and showed no difference between the sexes. At older age (>57 years) no significant change in the incidence of bisecting GlcNAc was observed anymore, but sex differences became apparent for IgG2&3 Fc and total IgG, with females showing higher level of this feature.

In the next analysis step, we looked at the relations between glycosylation features for each of the three data sets and compared these relations between IgG1 Fc, IgG2&3 Fc and total IgG (Supplementary Table 3). In all three cases a strong positive correlation between the level of galactosylation and sialylation was noticed ($r \geq 0.65$ for the whole age range). Negative association ($r \geq -0.17$) of the galactosylation and the incidence of the bisecting GlcNAc for the total IgG was observed over the whole age range and in younger individuals

following the stratification at median age. Similarly, IgG2&3 Fc showed a negative association, while in the case of IgG1 Fc the only significant correlation was a positive one in older individuals. Correlations of the degree of sialylation and the level of bisecting GlcNAc showed the opposite direction for the Fc fragments and the total IgG. IgG1 Fc and IgG2&3 Fc had a significant positive correlation ($r \geq 0.14$) between those two features for the whole age range as well as for the stratified age groups. By contrast, total IgG showed a negative correlation significant for the whole age range ($r = -0.19$) as well as for the younger group of individuals ($r = -0.17$).

In addition, to evaluate the observed difference in sialylation between Fc glycopeptides (MALDI-TOF-MS) and total IgG glycans (HILIC) we analyzed two sialylated IgG standards by both methods. HILIC glycan profiles of IgG1 and IgG Fc standards were compared with their MALDI-TOF-MS Fc glycopeptide profile obtained in reflectron and linear negative-ion mode (Supplementary Figure 6). The most abundant sialylated glycan, G2FS1, of IgG1 standard showed 2x higher relative intensity in negative linear mode in comparison to negative reflectron mode MS. Relative abundance of this glycoform as determined by HILIC of 2-AB glycans (Supplementary Table 6) was even 3x higher than in linear negative mode MS analysis of glycopeptides.

Next, we analyzed the total released glycans of IgG and IgG Fc standards by negative linear mode MALDI-TOF-MS (after 2-AA-labeling; see Supplementary Table 4 for mass list) and HILIC (after 2-AB-labeling; Supplementary Figure 5). The coefficient of variation (CV) between MS and HILIC data for IgG standard of the major sialylated species, G1FS1 and G2FS1, was found to be less than 5%. Less abundant sialylated glycans (<1%), show much higher CV between two methods (data not shown).

DISCUSSION

We performed a large-scale IgG glycopeptide profiling by MALDI-TOF-MS with Cl-CCA matrix for 1709 individuals from two isolated human populations. In accordance with literature findings, our results showed a clear tendency of decreased galactosylation and sialylation with increasing age.[16-20, 23, 28] In both age groups (above and below the median age of 57 years) we observed sex-related differences with females showing higher levels at young age, and males showing slightly higher levels at older age. The most prominent drops in the levels of galactosylation and sialylation in females were observed around the age of between 45 and 60 years, which might be linked to changes in the hormonal status due to the transition from pre- to post-menopausal stage. Altered IgG galactosylation and sialylation levels have been reported during pregnancy[29] suggesting that changes in hormone levels could in part explain observed changes in IgG glycosylation. While the regulation of IgG glycosylation is still largely not understood, a recent *in vitro* study using a primary human B cell culture has established regulatory roles for various systemic or microenvironmental factors such as cytokines and all-*trans* retinoic acid.[30]

In line with the parallel decrease of galactosylation and sialylation we observed, as expected, a strong positive and highly significant correlation between these two features for all the analyzed subclasses. It was reported that GalT-I (β-1,4-galactosyltransferase I) and ST6Gal-I (α-2,6-sialyltransferase I) form a complex which increases their enzymatic activity, suggesting that these two *N*-glycosyltransferases act cooperatively in *N*-glycan synthesis.[31] Additionally, we noticed that both galactosylation and sialylation of IgG1 tend to negatively correlate with the level of core fucosylation. IgG galactosylation and fucosylation changes going in different directions have been previously described for juvenile chronic arthritis and rheumatoid arthritis.[32, 33]

For both sexes and both IgG1 Fc *N*-glycans and IgG2&3 Fc *N*-glycans we confirmed an increasing incidence of bisecting GlcNAc with age.[17-20, 23] In accordance with Yamada *et al.* who have reported the level of bisecting GlcNAc reaching a plateau at the age of 50 years[18] we did not detect a correlation between age and bisecting GlcNAc in older individuals.

Addition of a bisecting GlcNAc by GnT-III (β-1,4-*N*-acetylglucosaminyltransferase III) has been shown to prohibit the subsequent addition of a core fucose.[34] In line with this we found for the IgG1 subclass a significant negative correlation between bisection and fucosylation. Furthermore, GnT-III has also been reported to negatively affect the addition of galactose by GalT (β-1,4-galactosyltransferase).[35] In our study, we observed a weak negative correlation between galactosylation and bisection for both IgG1 Fc and IgG2&3 Fc over the whole age range. Interestingly, upon stratification to younger and older groups of individuals, correlation coefficients of almost the same strength but different direction were revealed. While the younger group followed the direction of the whole age range showing a negative correlation of IgG1 galactosylation and bisection, at older age this correlation appeared to be positive, indicating the occurrence of pronounced changes in the regulation of IgG Fc glycosylation with increasing age.

IgG glycosylation of our cohort was analyzed by hydrophilic interaction chromatography and by MALDI-TOF mass spectrometry. In the first approach, a mixture of released Fab and Fc *N*-glycans of all four IgG subclasses was analyzed in detail.[20] In the second approach, presented in this paper, we have thoroughly analyzed IgG Fc glycosylation in a subclass-specific manner. Very strong correlation between galactosylation levels determined by HILIC and those determined by MS of glycopeptides unambiguously demonstrates the high quality of both data sets. One should keep in mind that the very limited scattering of the data is only in

part caused by measurement inaccuracies, and may for a large part be caused by the inclusion of Fab glycans in the HILIC but not in the MS analyses.

By comparing glycosylation features measured by both methods, we observed many parallels, next to some striking differences: HILIC analysis of total IgG revealed higher levels of galactosylation and bisection in neutral glycoforms, and sialylation which was on average seven-fold higher for HILIC than for IgG1 Fc and IgG2&3 Fc. Increased levels of these glycosylation features observed in the HILIC analysis may largely be linked to the inclusion of Fab glycans, as Fab glycans are known to show an increased incidence of fully galactosylated, sialylated and bisected structures compared to the Fc.[12, 14, 15, 36] However, the analysis of sialylation levels by mass spectrometry is complicated by two phenomena: first, the charge introduced by the sialic acid will influence ionization. It may be assumed that negative-mode ionization of the sialylated species was more efficient than the ionization of glycopeptides with non-sialylated, neutral glycan chains. Second, sialic acids are known to be labile and will, therefore, be lost to some extent upon MALDI-ionization via in-source decay or metastable decay.[37] These phenomena may explain the lower relative signal heights of sialylated species observed in negative linear mode MALDI-TOF-MS as compared to their detection using HILIC with fluorescence analysis.

HILIC glycosylation profiling of the total IgG revealed a significant negative correlation of bisecting GlcNAc and sialylation while in subclass-specific Fc glycosylation profiling this association was significantly positive for both IgG1 and IgG2&3 regardless of the age. The presence of bisecting GlcNAc is known to negatively affects the addition of a galactose[35] and since galactosylated glycoforms represent a substrate for sialylatransferase, the negative effect of bisecting GlcNAc on galactosylation could also inhibit sialylation by decreasing the level of galactosylated substrate.

The HILIC and Fc glycopeptide MALDI-TOF-MS IgG glycosylation profiling methods differ in various respects. First, sample preparation is different. After the common IgG affinity purification step, HILIC of fluorescently labeled glycans requires enzymatic glycan release, labeling and sample clean-up, while the glycopeptides profiling with MALDI-TOF-MS stays more closely to a proteomics workflow with trypsin treatment and SPE. Alternatively, trypsin treatment may be directly followed by reverse phase LC-MS analysis of glycopeptides, thereby minimizing sample preparation steps.[38] Second, HILIC and MALDI-TOF-MS differ in sample throughput. While HILIC has been tremendously speeded up by UPLC technology allowing the analysis of a couple of samples per hour, the speed of MALDI-TOF-MS in mass spectrometric profiling is much faster. Third, the sample purity requirements of the two analysis methods are very different. HILIC of released glycans is quite sensitive to contaminants such as other glycoproteins, as the released glycans from glycoprotein contaminants will interfere with the IgG glycosylation profile. Hence, high sample purity is pivotal. In contrast, MALDI-TOF-MS of glycopeptides allows distinguishing between IgG Fc glycopeptides and glycopeptides of other glycoproteins on the basis of the mostly different masses of the peptide moieties, and the presence of low quantity amounts of contaminating glycoproteins will therefore, in most cases, not interfere with IgG Fc glycosylation profiling. Fourth, the assignment of glycans to the specific Fc glycosylation sites of IgG subclasses as achieved by the MALDI-TOF-MS method is pivotal for deducing the functional implications of the observed glycosylation features. IgG Fc glycans have very distinct functions as they modulate the interaction with Fc receptors[2, 5-7, 11] and other cell-surface receptors of immune cells.[9, 10] Moreover, most of the modulating effects of IgG Fc glycans have been reported for IgG1[9, 11, 39] and may not apply to IgG2, stressing the relevance of subclass- and site-specific IgG glycosylation profiling as achieved by mass spectrometry of glycopeptides.

Financial Support

This work was supported by the EC FP7 programme (HighGlycan project) under grant agreement 278535 and the Croatian Ministry of Science, Education and Sport grant #309-0061194-2023. Maja Pučić Baković gratefully acknowledges financial support of the Federation of European Biochemical Societies for her study visit to the LUMC where mass spectrometric analysis was performed.

REFERENCES

1. Arnold, J. N.; Wormald, M. R.; Sim, R. B.; Rudd, P. M.; Dwek, R. A., The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* **2007,** 25, 21-50.
2. Huhn, C.; Selman, M. H.; Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M., IgG glycosylation analysis. *Proteomics* **2009,** 9, (4), 882-913.
3. Jefferis, R., Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* **2005,** 21, (1), 11-6.
4. Mimura, Y.; Sondermann, P.; Ghirlando, R.; Lund, J.; Young, S. P.; Goodall, M.; Jefferis, R., Role of oligosaccharide residues of IgG1-Fc in Fc gamma RIIb binding. *J Biol Chem* **2001,** 276, (49), 45539-47.
5. Iida, S.; Misaka, H.; Inoue, M.; Shibata, M.; Nakano, R.; Yamane-Ohnuki, N.; Wakitani, M.; Yano, K.; Shitara, K.; Satoh, M., Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcgammaRIIIa. *Clin Cancer Res* **2006,** 12, (9), 2879-87.
6. Shields, R. L.; Lai, J.; Keck, R.; O'Connell, L. Y.; Hong, K.; Meng, Y. G.; Weikert, S. H.; Presta, L. G., Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* **2002,** 277, (30), 26733-40.
7. Ferrara, C.; Grau, S.; Jager, C.; Sondermann, P.; Brunker, P.; Waldhauer, I.; Hennig, M.; Ruf, A.; Rufer, A. C.; Stihle, M.; Umana, P.; Benz, J., Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A* **2011,** 108, (31), 12669-74.
8. Kaneko, Y.; Nimmerjahn, F.; Ravetch, J. V., Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* **2006,** 313, (5787), 670-3.
9. Anthony, R. M.; Wermeling, F.; Karlsson, M. C.; Ravetch, J. V., Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci U S A* **2008,** 105, (50), 19571-8.
10. Anthony, R. M.; Kobayashi, T.; Wermeling, F.; Ravetch, J. V., Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature* **2011,** 475, (7354), 110-3.
11. Karsten, C. M.; Pandey, M. K.; Figge, J.; Kilchenstein, R.; Taylor, P. R.; Rosas, M.; McDonald, J. U.; Orr, S. J.; Berger, M.; Petzold, D.; Blanchard, V.; Winkler, A.; Hess, C.; Reid, D. M.; Majoul, I. V.; Strait, R. T.; Harris, N. L.; Kohl, G.; Wex, E.; Ludwig, R.; Zillikens, D.; Nimmerjahn, F.; Finkelman, F. D.; Brown, G. D.; Ehlers, M.; Kohl, J., Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcgammaRIIB and dectin-1. *Nat Med* **2012,** 18, (9), 1401-6.
12. Holland, M.; Yagi, H.; Takahashi, N.; Kato, K.; Savage, C. O.; Goodall, D. M.; Jefferis, R., Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochim Biophys Acta* **2006,** 1760, (4), 669-77.

13. Stadlmann, J.; Pabst, M.; Kolarich, D.; Kunert, R.; Altmann, F., Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics* **2008,** 8, (14), 2858-71.
14. Wormald, M. R.; Rudd, P. M.; Harvey, D. J.; Chang, S. C.; Scragg, I. G.; Dwek, R. A., Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. *Biochemistry* **1997,** 36, (6), 1370-80.
15. Youings, A.; Chang, S. C.; Dwek, R. A.; Scragg, I. G., Site-specific glycosylation of human immunoglobulin G is altered in four rheumatoid arthritis patients. *Biochem J* **1996,** 314 (Pt 2), 621-30.
16. Parekh, R.; Roitt, I.; Isenberg, D.; Dwek, R.; Rademacher, T., Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. *J Exp Med* **1988,** 167, (5), 1731-6.
17. Shikata, K.; Yasuda, T.; Takeuchi, F.; Konishi, T.; Nakata, M.; Mizuochi, T., Structural changes in the oligosaccharide moiety of human IgG with aging. *Glycoconj J* **1998,** 15, (7), 683-9.
18. Yamada, E.; Tsukamoto, Y.; Sasaki, R.; Yagyu, K.; Takahashi, N., Structural changes of immunoglobulin G oligosaccharides with age in healthy human serum. *Glycoconj J* **1997,** 14, (3), 401-5.
19. Ruhaak, L. R.; Uh, H. W.; Beekman, M.; Koeleman, C. A.; Hokke, C. H.; Westendorp, R. G.; Wuhrer, M.; Houwing-Duistermaat, J. J.; Slagboom, P. E.; Deelder, A. M., Decreased levels of bisecting GlcNAc glycoforms of IgG are associated with human longevity. *PLoS One* **2010,** 5, (9), e12566.
20. Pučić, M.; Knežević, A.; Vidič, J.; Adamczyk, B.; Novokmet, M.; Polašek, O.; Gornik, O.; Šupraha-Goreta, S.; Wormald, M. R.; Redžić, I.; Campbell, H.; Wright, A.; Hastie, N. D.; Wilson, J. F.; Rudan, I.; Wuhrer, M.; Rudd, P. M.; Josić, D.; Lauc, G., High Throughput Isolation and Glycosylation Analysis of IgG-Variability and Heritability of the IgG Glycome in Three Isolated Human Populations. *Mol Cell Proteomics* **2011,** 10, (10), M111 010090.
21. Rudan, I.; Campbell, H.; Rudan, P., Genetic epidemiological studies of eastern Adriatic Island isolates, Croatia: objective and strategies. *Coll. Antropol.* **1999,** 23, (2), 531-46.
22. Rudan, I.; Biloglav, Z.; Vorko-Jovic, A.; Kujundzic-Tiljak, M.; Stevanovic, R.; Ropac, D.; Puntaric, D.; Cucevic, B.; Salzer, B.; Campbell, H., Effects of inbreeding, endogamy, genetic admixture, and outbreeding on human health: a (1001 Dalmatians) study. *Croat. Med. J.* **2006,** 47, (4), 601-10.
23. Selman, M. H.; McDonnell, L. A.; Palmblad, M.; Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M., Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem* **2010,** 82, (3), 1073-81.
24. Selman, M. H.; Hoffmann, M.; Zauner, G.; McDonnell, L. A.; Balog, C. I.; Rapp, E.; Deelder, A. M.; Wuhrer, M., MALDI-TOF-MS analysis of sialylated glycans and glycopeptides using 4-chloro-alpha-cyanocinnamic acid matrix. *Proteomics* **2012,** 12, (9), 1337-48.
25. Jaskolla, T. W.; Lehmann, W. D.; Karas, M., 4-Chloro-alpha-cyanocinnamic acid is an advanced, rationally designed MALDI matrix. *Proc Natl Acad Sci U S A* **2008,** 105, (34), 12200-5.
26. Selman, M. H.; Niks, E. H.; Titulaer, M. J.; Verschuuren, J. J.; Wuhrer, M.; Deelder, A. M., IgG fc N-glycosylation changes in Lambert-Eaton myasthenic syndrome and myasthenia gravis. *J Proteome Res* **2011,** 10, (1), 143-52.
27. Parekh, R. B.; Dwek, R. A.; Sutton, B. J.; Fernandes, D. L.; Leung, A.; Stanworth, D.; Rademacher, T. W.; Mizuochi, T.; Taniguchi, T.; Matsuta, K.; et al., Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* **1985,** 316, (6027), 452-7.
28. Vanhooren, V.; Desmyter, L.; Liu, X. E.; Cardelli, M.; Franceschi, C.; Federico, A.; Libert, C.; Laroy, W.; Dewaele, S.; Contreras, R.; Chen, C., N-glycomic changes in serum proteins during human aging. *Rejuvenation Res* **2007,** 10, (4), 521-531a.
29. van de Geijn, F. E.; Wuhrer, M.; Selman, M. H.; Willemsen, S. P.; de Man, Y. A.; Deelder, A. M.; Hazes, J. M.; Dolhain, R. J., Immunoglobulin G galactosylation and sialylation are associated with pregnancy-induced improvement of rheumatoid arthritis and the postpartum flare: results from a large prospective cohort study. *Arthritis Res Ther* **2009,** 11, (6), R193.

30. Wang, J.; Balog, C. I.; Stavenhagen, K.; Koeleman, C. A.; Scherer, H. U.; Selman, M. H.; Deelder, A. M.; Huizinga, T. W.; Toes, R. E.; Wuhrer, M., Fc-glycosylation of IgG1 is modulated by B-cell stimuli. *Mol Cell Proteomics* **2011,** 10, (5), M110 004655.
31. Hassinen, A.; Pujol, F. M.; Kokkonen, N.; Pieters, C.; Kihlstrom, M.; Korhonen, K.; Kellokumpu, S., Functional organization of Golgi N- and O-glycosylation pathways involves pH-dependent complex formation that is impaired in cancer cells. *J Biol Chem* **2011,** 286, (44), 38329-40.
32. Flogel, M.; Lauc, G.; Gornik, I.; Macek, B., Fucosylation and galactosylation of IgG heavy chains differ between acute and remission phases of juvenile chronic arthritis. *Clin Chem Lab Med* **1998,** 36, (2), 99-102.
33. Gornik, I.; Maravic, G.; Dumic, J.; Flogel, M.; Lauc, G., Fucosylation of IgG heavy chains is increased in rheumatoid arthritis. *Clin Biochem* **1999,** 32, (8), 605-8.
34. Ferrara, C.; Brunker, P.; Suter, T.; Moser, S.; Puntener, U.; Umana, P., Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1, 4-N-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. *Biotechnol Bioeng* **2006,** 93, (5), 851-61.
35. Fukuta, K.; Abe, R.; Yokomatsu, T.; Omae, F.; Asanagi, M.; Makino, T., Control of bisecting GlcNAc addition to N-linked sugar chains. *J Biol Chem* **2000,** 275, (31), 23456-61.
36. Mimura, Y.; Ashton, P. R.; Takahashi, N.; Harvey, D. J.; Jefferis, R., Contrasting glycosylation profiles between Fab and Fc of a human IgG protein studied by electrospray ionization mass spectrometry. *J Immunol Methods* **2007,** 326, (1-2), 116-26.
37. Harvey, D. J.; Hunter, A. P.; Bateman, R. H.; Brown, J.; Critchley, G., Relationship between in-source and post-source fragment ions in the matrix-assisted laser desorption (ionization) mass spectra of carbohydrates recorded with reflectron time-of-flight mass spectrometers. *International Journal of Mass Spectrometry* **1999,** 188, (1-2), 131-146.
38. Selman, M. H.; Derks, R. J.; Bondt, A.; Palmblad, M.; Schoenmaker, B.; Koeleman, C. A.; van de Geijn, F. E.; Dolhain, R. J.; Deelder, A. M.; Wuhrer, M., Fc specific IgG glycosylation profiling by robust nano-reverse phase HPLC-MS using a sheath-flow ESI sprayer interface. *J Proteomics* **2011**.
39. Davies, J.; Jiang, L.; Pan, L. Z.; LaBarre, M. J.; Anderson, D.; Reff, M., Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. *Biotechnol Bioeng* **2001,** 74, (4), 288-94.

Figure legends

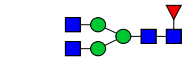
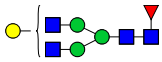
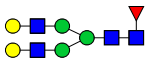
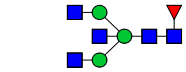
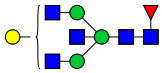
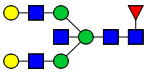
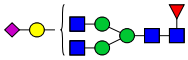
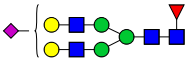
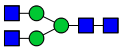
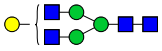
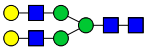
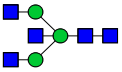
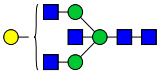
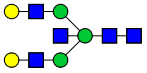
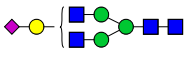
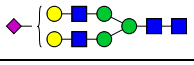
Figure 1. MALDI-TOF-MS spectra of tryptic IgG Fc *N*-glycopeptides. Dashed arrows represent IgG2&3 glycopeptides, while continued arrows represent IgG1 glycopeptides. Glycan species are given in terms of number of galactoses (G0, G1, G2), fucose (F), bisecting *N*-acetylglucosamine (N) and *N*-acetylneuraminic acid (S). Structural schemes are given in terms of pep (peptide moiety), blue square (*N*-acetylglucosamine), red triangle (fucose), green circle (mannose), yellow circle (galactose), and purple diamond (*N*-acetylneuraminic acid).

Figure 2. Age dependence of IgG1 and IgG2&3 glycosylation features. Females are plotted in red with a fitted line in dark red, while males are plotted in blue with a fitted line in dark blue. Both lines were fitted using the loess (locally weighted scatterplot smoothing) method.

Figure 3. Correlations between IgG Fc glycosylation features stratified for sex. Females are plotted in red with a fitted line in dark red, while males are plotted in blue with a fitted line in dark blue. R^2 - coefficient of determination.

Figure 4. Boxplot representations of the levels of glycosylation features analyzed by MALDI-TOF-MS (IgG1 Fc and IgG2&3 Fc) and by HILIC (total IgG). Gal_n – level of galactosylation in neutral glycoforms; BisGlcNAc_n – incidence of bisecting GlcNAc in neutral glycoforms; FGS/(F+FG+FGS) – degree of sialylation of fucosylated glycoforms without bisecting GlcNAc.
The bottom of the box represents the lower quartile while the top represents the upper quartile (25^{th} and 75^{th} percentile, respectively). The middle line represents the median. The whiskers extend to the minimum and to the maximum values.

Table 1. Theoretical masses of tryptic glycopeptides of human IgG subclasses 1, 2 and 3. Glycan compositions are given in terms of hexose (H), *N*-acetylhexoamine (N), deoxyhexose (fucose; F), and N-acetylneuraminic acid (sialic acid; S). Structural schemes are given as in Figure 1.

Glycan species	Glycan structure	IgG1 E_{293}EQYNSTYR_{301}[a] [M-H]$^-$	IgG2&3 E_{293}EQFNSTFR_{301}[a] [M-H]$^-$
no glycan	-	1189.5120	1157.5222
G0F		2632.0460[b]	2600.0561
G1F		2794.0988[b]	2762.1089
G2F		2956.1516	2924.1617
G0FN		2835.1253[b]	2803.1354
G1FN		2997.1781[b]	2965.1882
G2FN		3159.2309	3127.2410
G1FS1		3085.1942[b]	3053.2043
G2FS1		3247.2470	3215.2571
G0		2485.9880	2453.9981
G1		2648.0408	n.d.[c]
G2		2810.0936	n.d.[c]
G0N		2689.0673	2657.0774
G1N		2851.1201	n.d.[c]
G2N		3013.1729	n.d.[c]
G1S1		2939.1362	2907.1463
G2S1		3101.1890	n.d.[c]

[a] Tryptic IgG glycopeptide sequence.

[b] Isomeric glycopeptide species of IgG1 and IgG4.

[c] Not determined due to the occurrence of isomeric glycopeptide species of IgG2&3 and IgG4.

Table 2. Correlation coefficients of IgG glycosylation features and age stratified for sex.

Positive correlation coefficients (*r*) for age indicate increased levels with increasing age, while negative correlation coefficients indicate decreased levels with increasing age. Correlations found to be significant after Bonferroni correction for gender and glycosylation features ($P \leq 0.006$ for IgG1 and $P \leq 0.008$ for IgG2&3) are in bold. Gal - level of galactosylation, Bis GlcNAc - level of bisecting *N*-acetylglucosamine, Core F - level of core fucosylation, and Sial - level of sialylation.

IgG subclass	Glycosylation feature	All ages		Ages ≤ 57		Ages > 57	
		Female	Male	Female	Male	Female	Male
		r (P)	*r (P)*	*r (P)*	*r (P)*	*r (P)*	*r (P)*
IgG1 Fc	Gal	**-0.66** (<0.001)	**-0.44** (<0.001)	**-0.54** (<0.001)	**-0.39** (<0.001)	**-0.21** (<0.001)	**-0.23** (<0.001)
	Bis GlcNAc	**0.27** (<0.001)	**0.19** (<0.001)	**0.28** (<0.001)	**0.21** (<0.001)	0.04 (0.440)	0.07 (0.262)
	Core F	-0.01 (0.880)	-0.12 (0.009)	**-0.14** (0.003)	**-0.19** (0.003)	0.01 (0.767)	-0.03 (0.580)
	Sial	**-0.43** (<0.001)	**-0.17** (<0.001)	**-0.39** (<0.001)	-0.13 (0.044)	-0.01 (0.910)	-0.06 (0.372)
IgG2&3 Fc	Gal	**-0.69** (<0.001)	**-0.46** (<0.001)	**-0.55** (<0.001)	**-0.34** (<0.001)	**-0.27** (<0.001)	**-0.27** (<0.001)
	Bis GlcNAc	**0.17** (<0.001)	**0.14** (<0.001)	**0.18** (<0.001)	**0.16** (0.005)	-0.05 (0.267)	0.04 (0.451)
	Sial	**-0.60** (<0.001)	**-0.32** (<0.001)	**-0.48** (<0.001)	**-0.27** (<0.001)	**-0.22** (<0.001)	-0.15 (0.009)

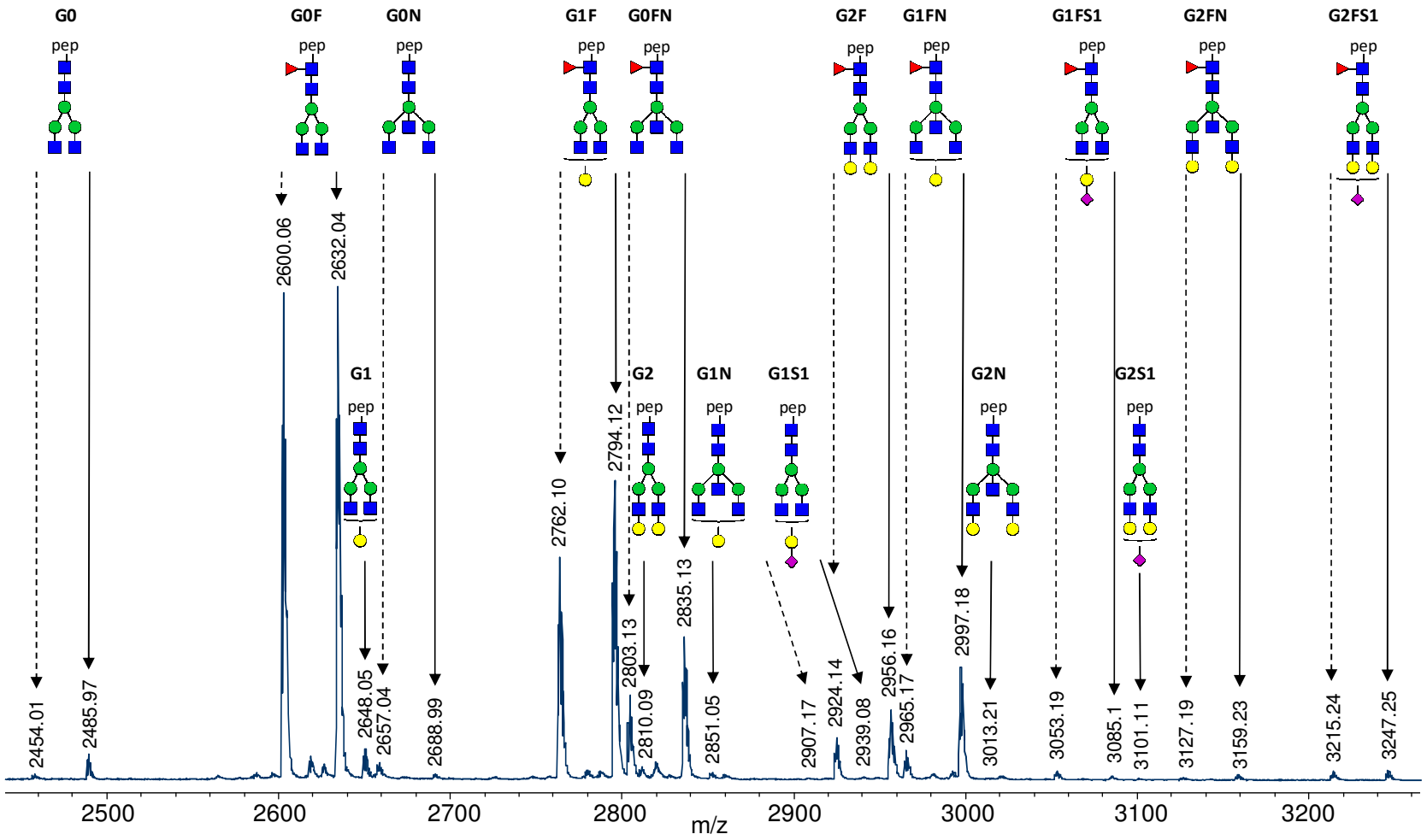
Table 3. Descriptives of glycosylation features in females and males with statistical significance (*P*) of sex differences and differences between age groups. Sex differences found to be significant after Bonferroni correction for age and glycosylation features (*P* ≤0.006 for IgG1 and *P* ≤0.008 for IgG2&3), and differences between age groups found to be significant after Bonferroni correction for sex and glycosylation features (*P* ≤0.006 for igG1 and *P* ≤0.008 for IgG2&3) are in bold. IQR - interquartile range. Abbreviations of glycosylation features as in Table 2.

IgG subclass	Glycosylation feature	Ages ≤ 57			Ages > 57			Differences between age groups (*P*)	
		Female	Male	Sex differences (*P*)	Female	Male	Sex differences (*P*)		
		Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)		Female	Male
IgG1 Fc	Gal	43.0 (12.8)	39.0 (8.9)	**<0.001**	31.3 (8.9)	33.9 (9.6)	**<0.001**	**<0.001**	**<0.001**
	Bis GlcNAc	11.8 (4.4)	11.9 (4.2)	0.785	13.6 (4.8)	12.9 (4.3)	0.008	**<0.001**	**0.001**
	Core F	92.0 (5.4)	92.6 (5.4)	0.122	92.4 (4.2)	91.9 (5.2)	0.147	0.159	0.117
	Sial	4.4 (2.2)	3.8 (2.1)	**<0.001**	3.0 (1.8)	3.2 (1.9)	**0.004**	**<0.001**	**0.001**
IgG2&3 Fc	Gal	33.0 (13.9)	29.7 (9.3)	**<0.001**	21.2 (7.6)	24.1 (7.9)	**<0.001**	**<0.001**	**<0.001**
	Bis GlcNAc	8.3 (3.3)	8.2 (3.1)	0.051	9.3 (3.6)	8.5 (3.1)	**<0.001**	**<0.001**	0.010
	Sial	3.8 (2.6)	3.4 (1.9)	**0.002**	2.0 (1.3)	2.6 (1.5)	**<0.001**	**<0.001**	**<0.001**

Table 4. Correlation coefficients (*r*) of glycosylation features.

Correlations found to be significant after Bonferroni correction ($P \leq 0.004$ for IgG1 and $P \leq 0.008$ for IgG2&3) are in bold. Abbreviations of glycosylation features as in Table 2.

IgG subclass	Glycosylation feature	All ages			Ages ≤ 57			Ages > 57		
		Bis GlcNAc *(r)*	Core F *(r)*	Sial *(r)*	Bis GlcNAc *(r)*	Core F *(r)*	Sial *(r)*	Bis GlcNAc *(r)*	Core F *(r)*	Sial *(r)*
IgG1 Fc	Gal	**-0.09** (<0.001)	**-0.17** (<0.001)	**0.68** (<0.001)	**-0.12** (<0.001)	**-0.15** (<0.001)	**0.67** (<0.001)	**0.13** (<0.001)	**-0.24** (<0.001)	**0.60** (<0.001)
	Bis GlcNAc		**-0.30** (<0.001)	**0.13** (<0.001)		**-0.31** (<0.001)	**0.13** (0.001)		**-0.31** (<0.001)	**0.28** (<0.001)
	Core F			**-0.38** (<0.001)			**-0.35** (<0.001)			**-0.44** (<0.001)
IgG2&3 Fc	Gal	**-0.11** (<0.001)		**0.84** (<0.001)	-0.08 (0.026)		**0.83** (<0.001)	-0.01 (0.078)		**0.75** (<0.001)
	Bis GlcNAc			0.04 (0.081)			0.07 (0.049)			**0.16** (<0.001)



A graphic – a designated figure for Abstract

Fig. 1

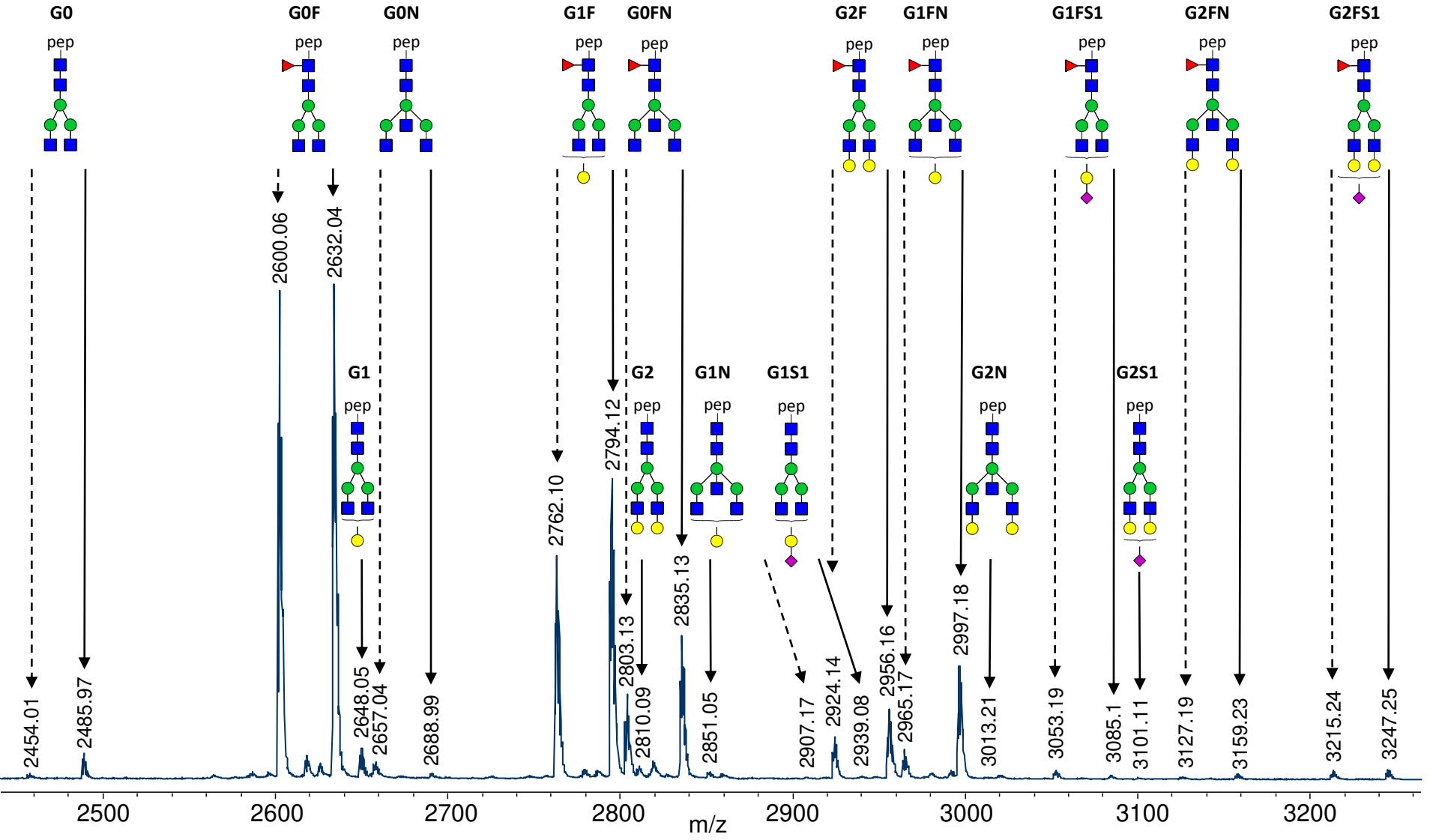

G0
pep
G0F
pep
G0N
pep
G1F
pep
G0FN
pep
G2F
pep
G1FN
pep
G1FS1
pep
G2FN
pep
G2FS1
pep
G1
pep
G2
pep
G1N
pep
G1S1
pep
G2N
pep
G2S1
pep
2454.01
2485.97
2600.06
2632.04
2648.05
2657.04
2688.99
2762.10
2794.12
2803.13
2810.09
2835.13
2851.05
2907.17
2924.14
2939.08
2956.16
2965.17
2997.18
3013.21
3053.19
3085.1
3101.11
3127.19
3159.23
3215.24
3247.25
2500
2600
2700
2800
2900
3000
3100
3200
m/z

Fig. 2

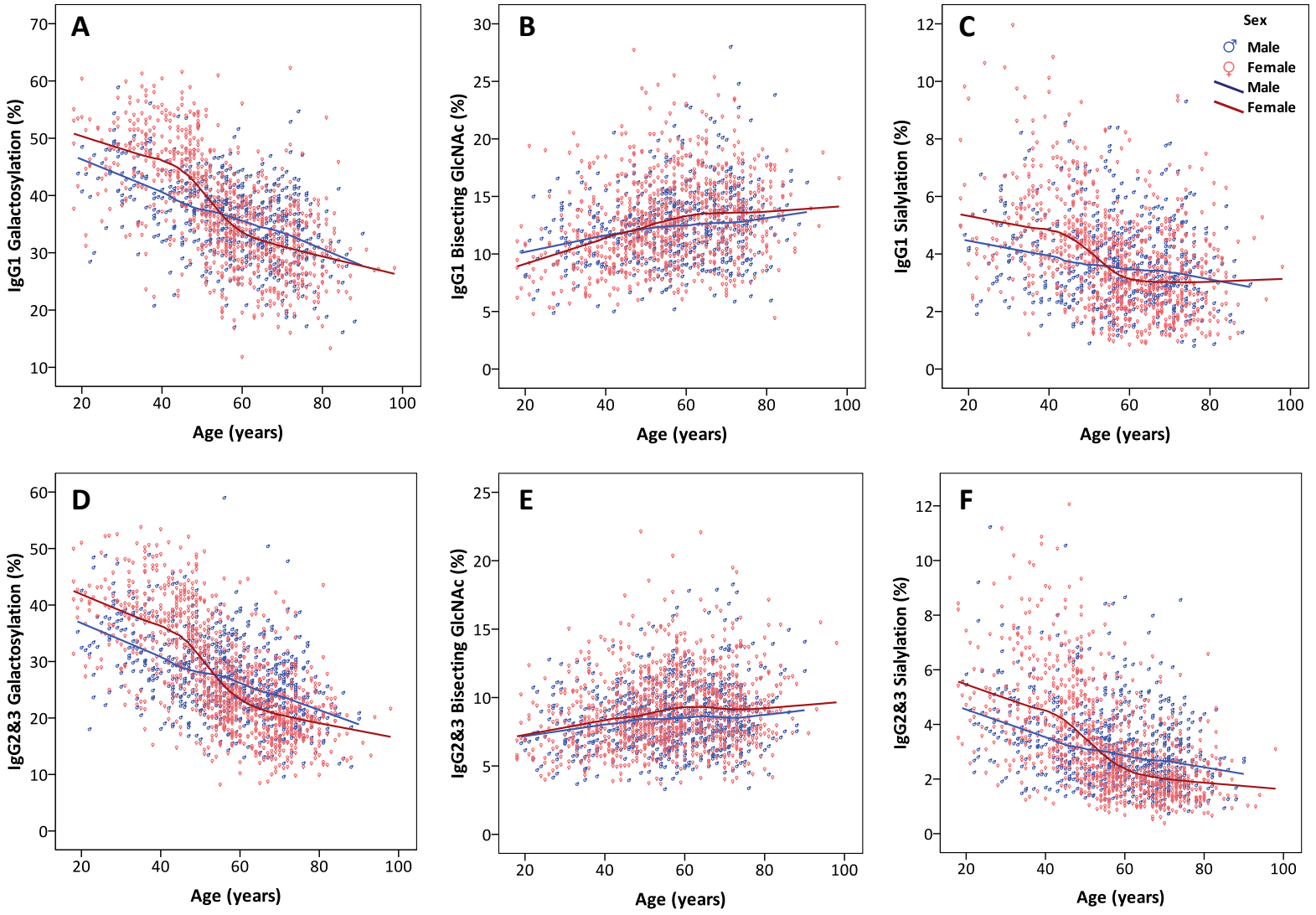

A
IgG1 Galactosylation (%)
Age (years)
B
IgG1 Bisecting GlcNAc (%)
Age (years)
C
Sex
Male
Female
Male
Female
IgG1 Sialylation (%)
Age (years)
D
IgG2&3 Galactosylation (%)
Age (years)
E
IgG2&3 Bisecting GlcNAc (%)
Age (years)
F
IgG2&3 Sialylation (%)
Age (years)

Fig. 3

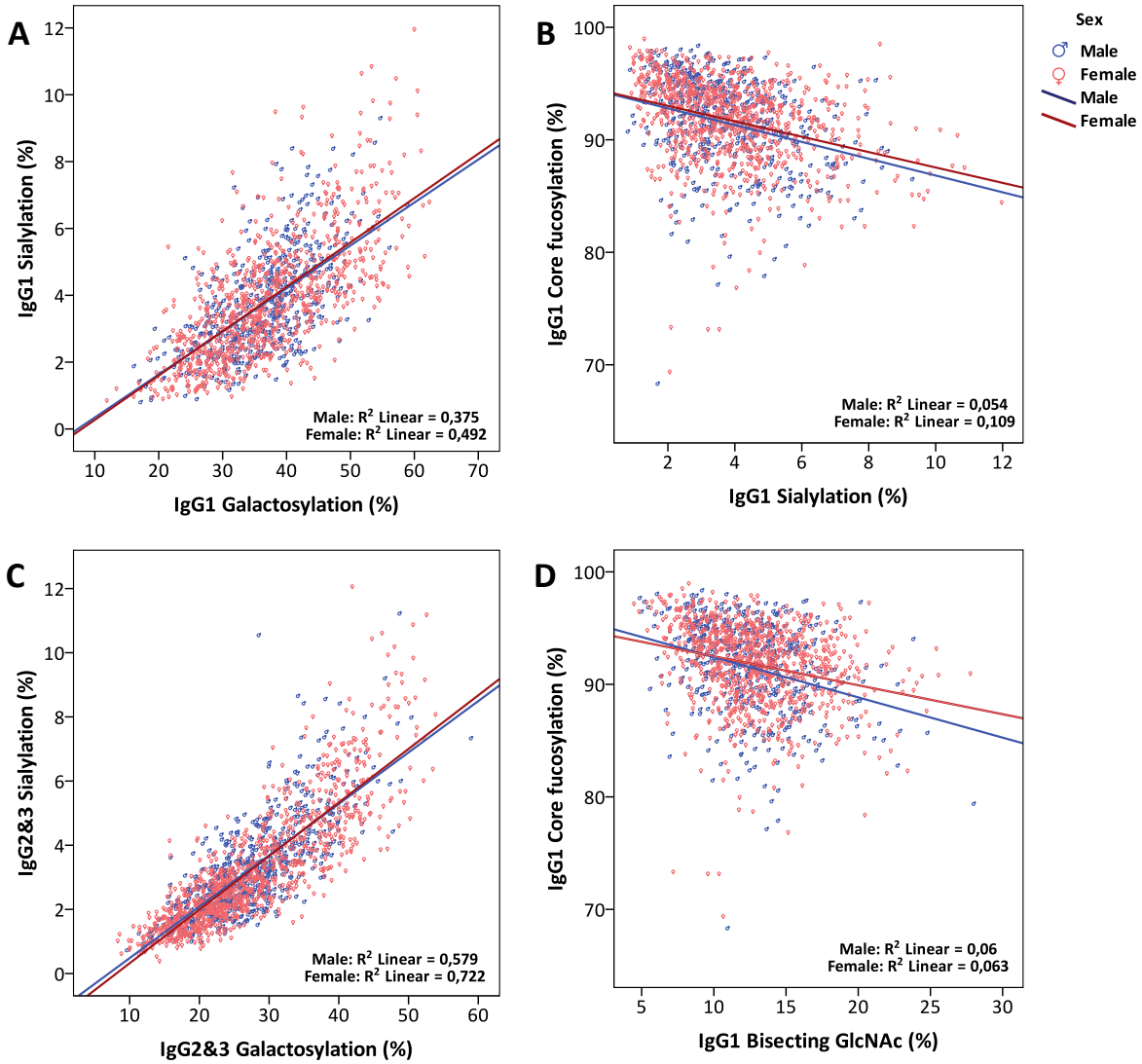
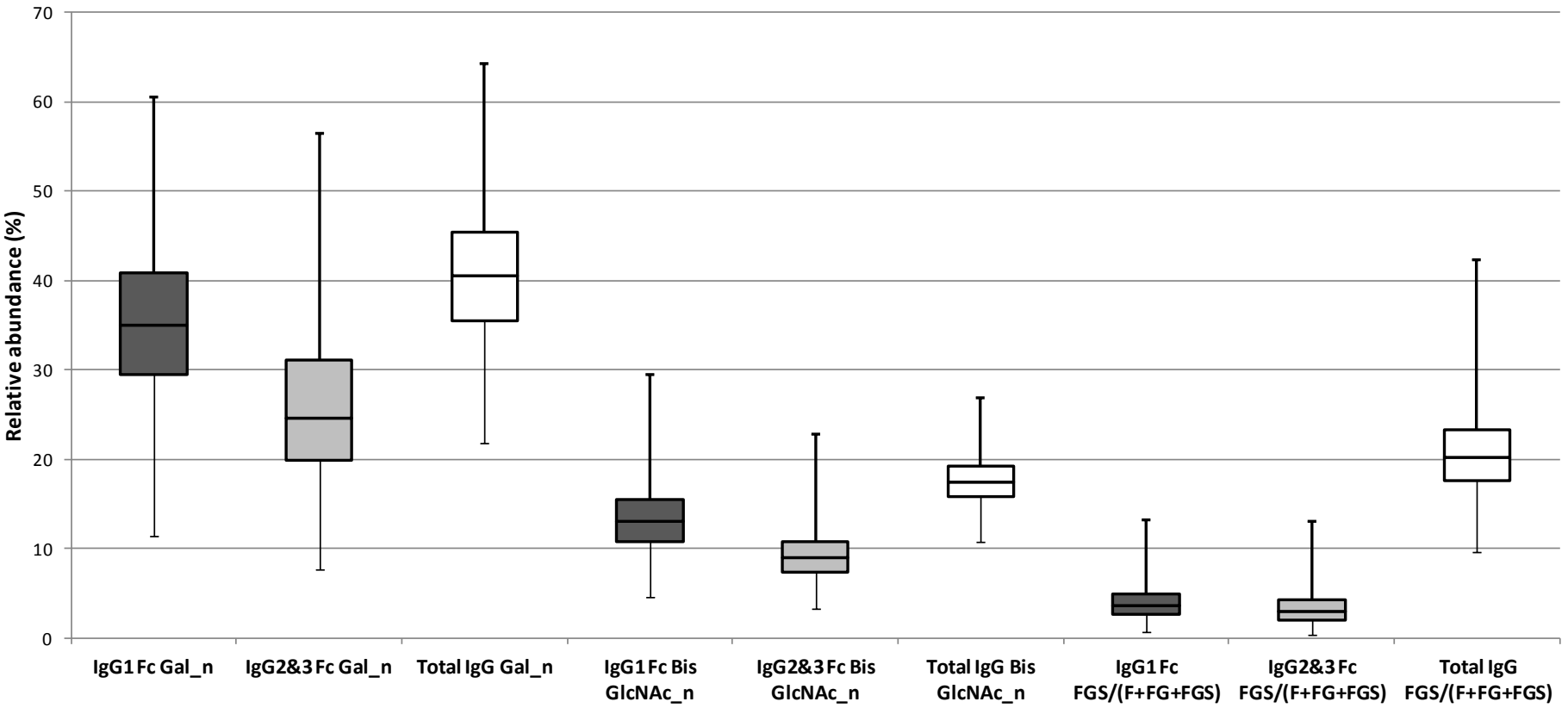
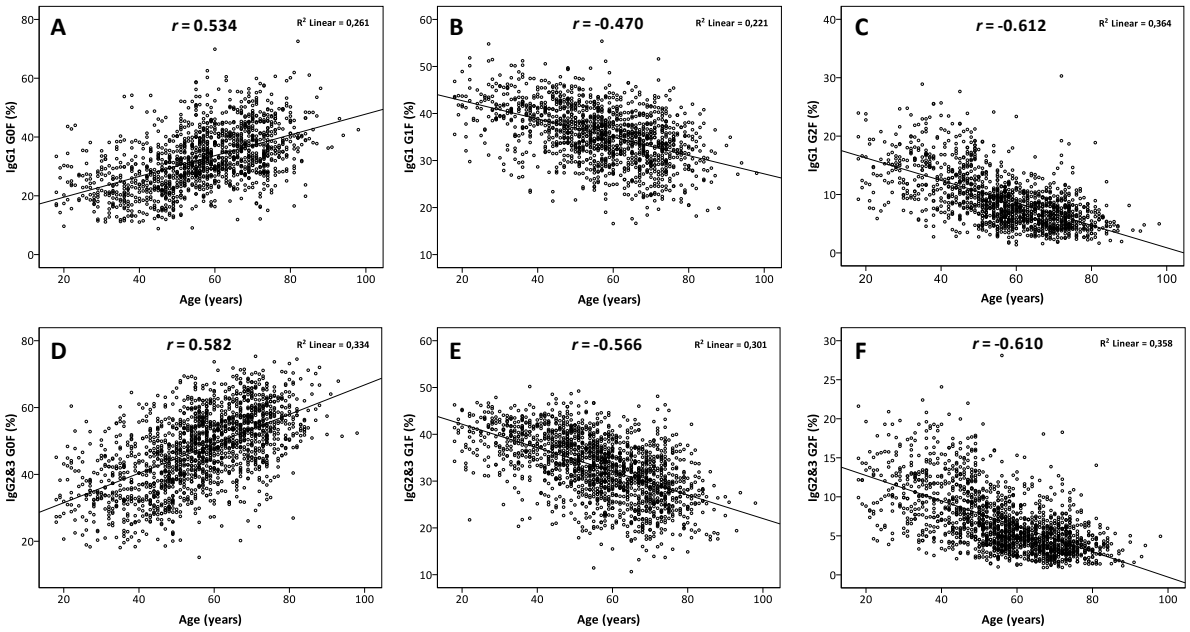

A
IgG1 Sialylation (%)
IgG1 Galactosylation (%)
Male: R² Linear = 0,375
Female: R² Linear = 0,492
B
IgG1 Core fucosylation (%)
IgG1 Sialylation (%)
Male: R² Linear = 0,054
Female: R² Linear = 0,109
Sex
Male
Female
Male
Female
C
IgG2&3 Sialylation (%)
IgG2&3 Galactosylation (%)
Male: R² Linear = 0,579
Female: R² Linear = 0,722
D
IgG1 Core fucosylation (%)
IgG1 Bisecting GlcNAc (%)
Male: R² Linear = 0,06
Female: R² Linear = 0,063

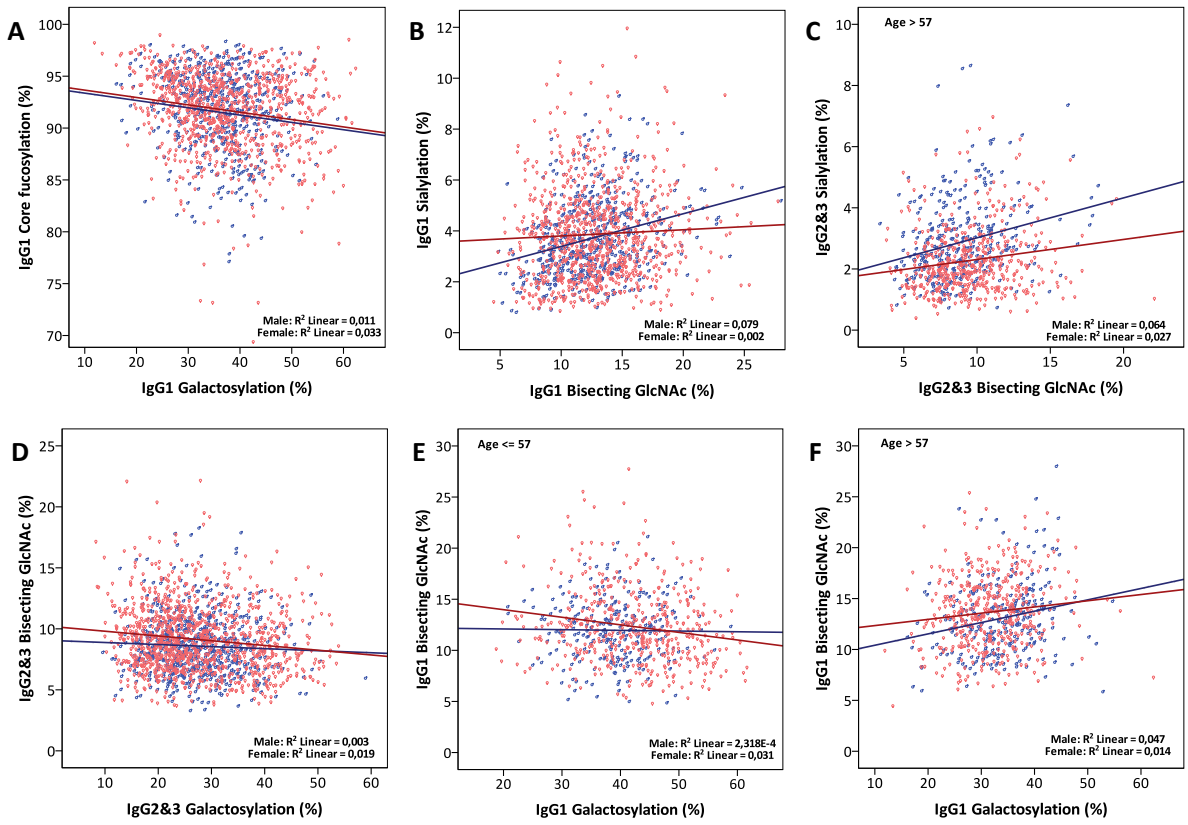
Fig. 4


70
60
50
40
30
20
10
0
Relative abundance (%)
IgG1 Fc Gal_n
IgG2&3 Fc Gal_n
Total IgG Gal_n
IgG1 Fc Bis GlcNAc_n
IgG2&3 Fc Bis GlcNAc_n
Total IgG Bis GlcNAc_n
IgG1 Fc FGS/(F+FG+FGS)
IgG2&3 Fc FGS/(F+FG+FGS)
Total IgG FGS/(F+FG+FGS)

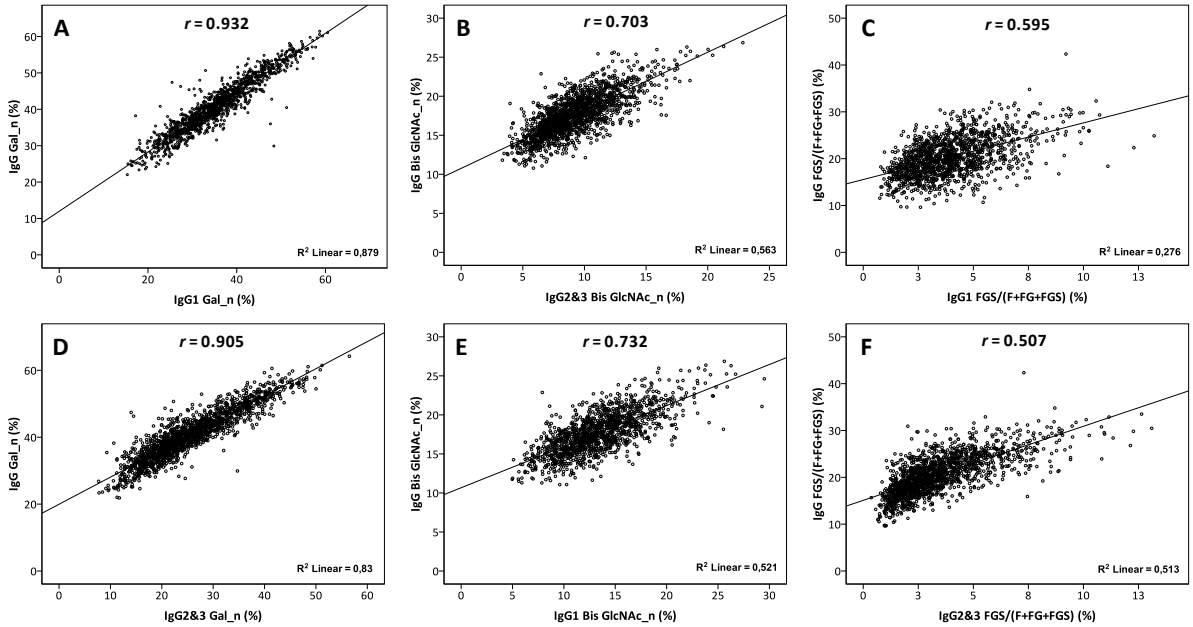
Supplementary Figure 1



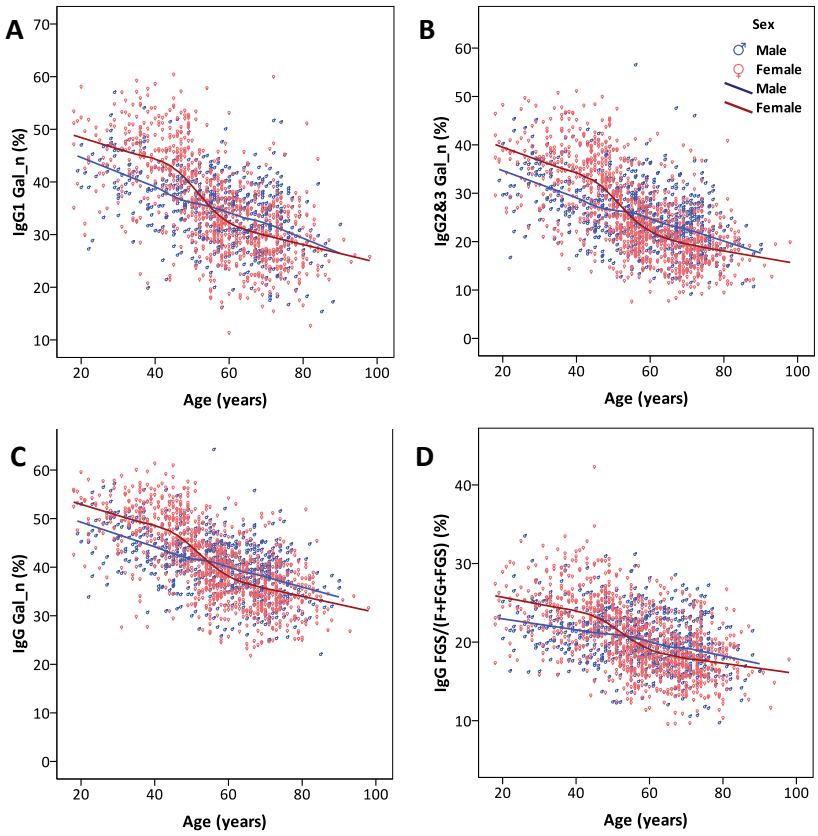
Supplementary Figure 1. Age dependence of IgG1 and IgG2 &3 glycoforms with correlation coefficients (*r*) and coefficients of determination (R^2).



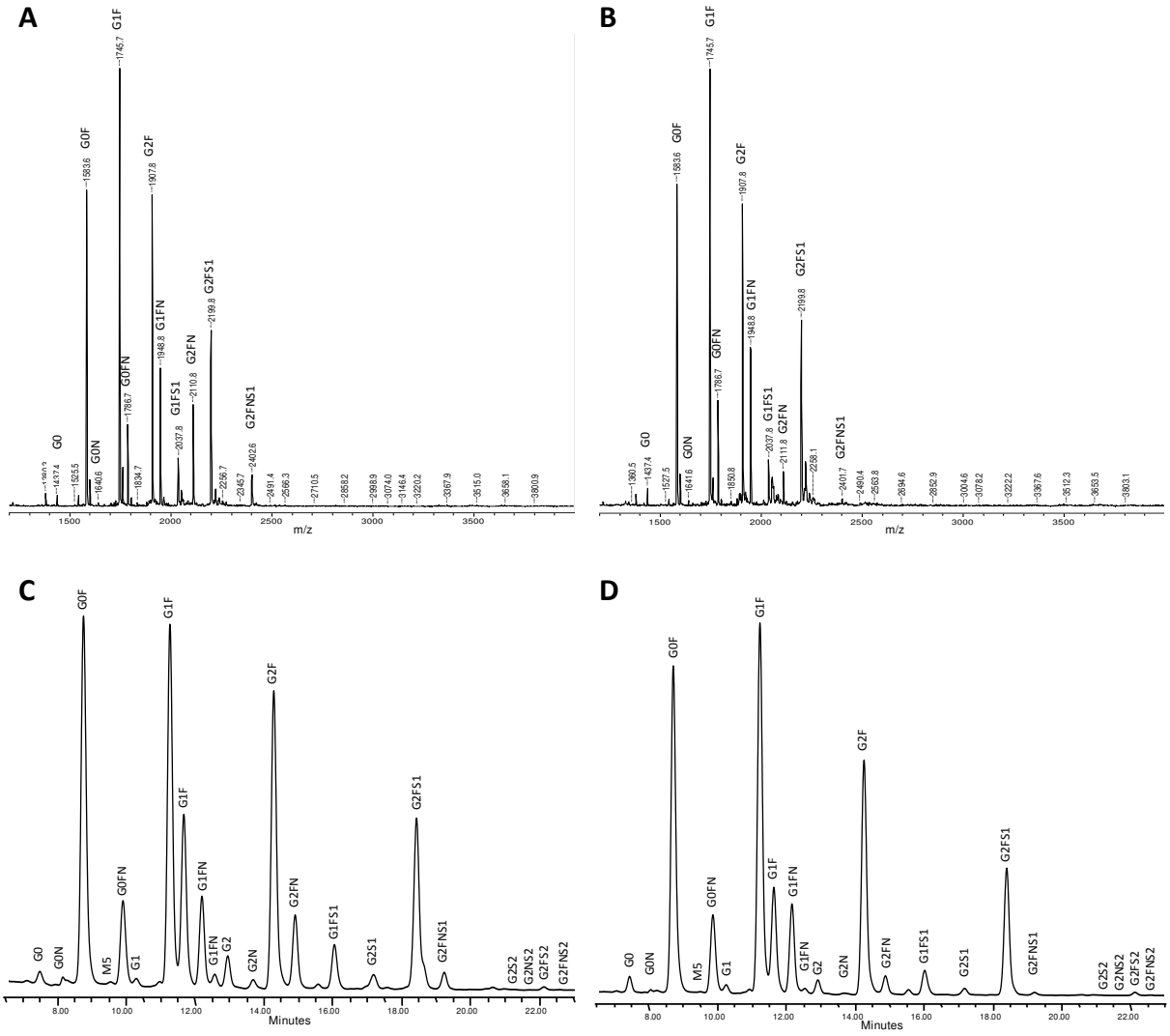
Supplementary Figure 2. Statistically significant correlations between IgG Fc glycosylation features stratified for sex. Females are plotted in red with a fitted line in dark red, while males are plotted in blue with a fitted line in dark blue. R^2 - coefficient of determination.



Supplementary Figure 3. Correlations (*r* - correlation coefficients) between glycosylation features from MALDI-TOF-MS and HILIC profiling. R^2 - coefficient of determination.



Supplementary Figure 4. Age dependence of MS (IgG1 Fc, IgG2&3 Fc) and HILIC (total IgG) galactosylation levels in neutral glycoforms and percentage of sialylation of fucosylated structures. Females are plotted in red with a fitted line in dark red, while males are plotted in blue with a fitted line in dark blue. Both lines were fitted using the loess (locally weighted scatterplot smoothing) method.



Supplementary Figure 5. Linear-negative ion mode MALDI-TOF-MS spectra of 2-AA-labeled *N*-glycan species of IgG standard (A) and IgG Fc standard (B) and HILIC chromatogram of 2-AB *N*-glycan species of IgG standard (C) and IgG Fc standard (D). Glycan species are given as in Table 1.

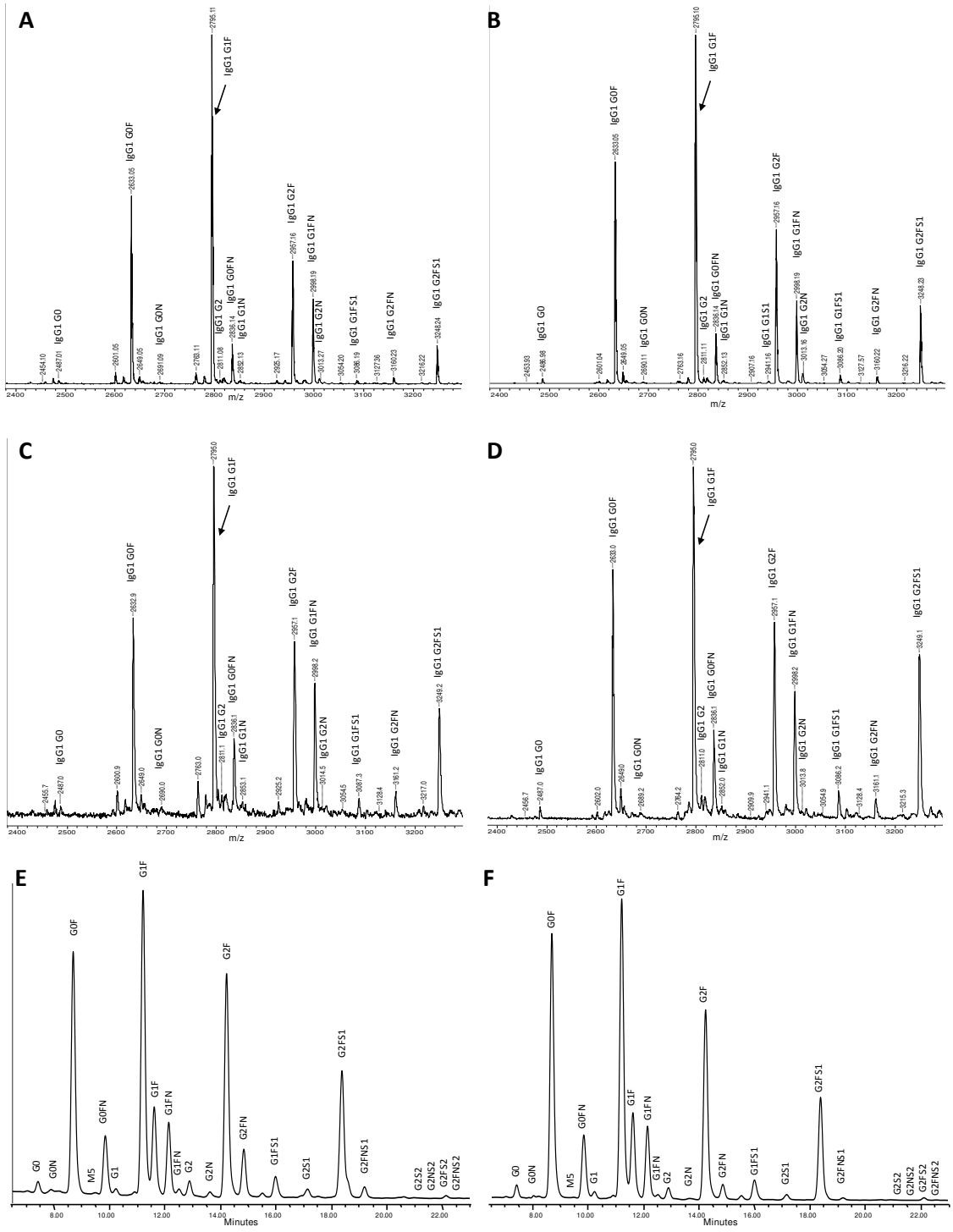
2-AB-labeled *N*-glycans were analyzed by HILIC as described in Experimental section. 2-AA-labeled glycans were prepared and analyzed as follows:

***N*-glycan release and 2-AA labeling of IgG standards.** To 100 µg of dried human IgG and IgG Fc standards 30 µl of 1.33% SDS was added followed by a 10 min incubation at 60°C to allow protein denaturation. Subsequently, 20 µl of a *N*-glycan release mixture was added containing 2% NP-40, 2.5x PBS and 0.5 mU PNGase-F (Roche, Manheim, Germany) followed by an overnight incubation at 37°C. To the released *N*-glycans, 25 µl labeling mixture (0.35M 2-AA in DMSO with 15% glacial acetic acid) and 25 µl reducing agent (1M 2-picoline borane in DMSO) was applied and incubated for 2h at 65°C.

Cotton HILIC microSPE of released *N*-glycans of IgG standards. Cotton HILIC microSPE was performed as described previously (Selman, M.H., *et al.*, Cotton HILIC SPE microtips for microscale

purification and enrichment of glycans and glycopeptides, *Anal. Chem*. 2011, 83(7):2492-9), with minor modifications. Briefly, 5 µl of the 2-AA labeled *N*-glycans from the human IgG and IgG Fc standards was diluted with ACN to a final ACN concentration of 84%. *N*-glycans were adsorbed to the cotton HILIC microSPE by aspirating and dispensing the sample 20x. The *N*-glycans were washed 3x with 84% ACN containing 1% TFA, and eluted directly onto an AnchorChip MALDI target (Bruker Daltonics, Bremen, Germany) with 2 µl ultrapure water. Spotted samples were allowed to dry, overlaid with 1 µL of 5 mg/mL 2,5-dihidroxybenzoic acid (DHB; Bruker-Daltonics) in 50 % acetonitrile and allowed to dry in a controlled manner underneath a pierced cap containing 5 holes of ca. 5 mm.

MALDI-TOF-MS. 2-AA labeled *N*-glycans of the IgG and IgG Fc standards were analyzed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) which was operated in the negative-ion linear mode). Ions between *m/z* 1000 and 4500 were recorded. To allow homogeneous spot sampling a random walk laser movement with 50 laser shots per raster spot was applied and each sum mass spectrum was generated by accumulation of 4000 laser shots. Mass spectra were internally calibrated using a list of known 2-AA labeled *N*-glycans. Data processing and evaluation were performed with FlexAnalysis Software (Bruker Daltonics) and Microsoft Excel, respectively. The data were baseline subtracted and the intensities of a defined set of 2-AA labeled N-glycans (47 plasma N-glycans; Supplementary Table 4) were automatically determined for each spectrum.



Supplementary Figure 6. Reflectron-negative (A, B) and linear-negative (C, D) ion mode MALDI-TOF-MS spectra of tryptic glycopeptides, and HILIC chromatograms of 2-AB-labeled *N*-glycan species (E, F). IgG1 standard (A, C, E) and IgG Fc standard (B, D, F) were used for the analyses. Glycan species are given as in Table 1.

2-AB-labeled *N*-glycans were analyzed by HILIC as described in the Experimental section. *N*-glycopeptides were prepared and analyzed as follows:

Trypsin digestion of IgG standards. Twenty µg of a human IgG1 and IgG Fc standard (Athens Research & Technology, Athens, GA) was digested overnight at 37°C with 200 ng sequencing grade trypsin (Promega, Madison, WI).

Reverse-phase solid phase extraction (RP-SPE) of glycopeptides. *N*-glycopeptides were purified and desalted as described in the Experimental section.

MALDI-TOF-MS. *N*-glycopeptides of the IgG1 and IgG Fc standards were spotted onto MTP 384 polished steel target plate (Bruker Daltonics), overlaid with 1 µL of 5 mg/mL 4-chloro-α-cyanocinnamic acid (Cl-CCA; 95% purity; Bionet Research) in 50 % acetonitrile and allowed to dry at room temperature. N-glyopeptides were analyzed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) which was operated in the negative-ion reflectron and leniear mode. Ions between *m/z* 1000 and 4500 were recorded. To allow homogeneous spot sampling a random walk laser movement with 50 laser shots per raster spot was applied and each sum mass spectrum was generated by accumulation of 2000 laser shots. Mass spectra were internally calibrated using a list of known glycopeptides. Data processing and evaluation were performed with FlexAnalysis Software (Bruker Daltonics) and Microsoft Excel, respectively. The data were baseline subtracted and the intensities of a defined set of glycopeptides (16 IgG1 glycoforms) were automatically defined for each spectrum.

Supplementary Table 1. Correlation coefficients of IgG glycosylation features and age stratified for sex for the IgG1 Fc, IgG2&3 Fc and the total IgG.

IgG subclass	Glycosylation feature	All ages		Ages ≤ 57		Ages > 57	
		Female	Male	Female	Male	Female	Male
		r (P)	*r (P)*	*r (P)*	*r (P)*	*r (P)*	*r (P)*
IgG1 Fc	Gal_n	**-0.66** (<0.001)	**-0.45** (<0.001)	**-0.53** (<0.001)	**-0.40** (<0.001)	**-0.21** (<0.001)	**-0.24** (<0.001)
	Bis GlcNAc_n	**0.24** (<0.001)	**0.18** (<0.001)	**0.25** (<0.001)	**0.20** (0.002)	0.04 (0.450)	0.06 (0.306)
	FGS/(F+FG+FGS)	**-0.42** (<0.001)	**-0.15** (0.001)	**-0.37** (<0.001)	-0.11 (0.088)	-0.01 (0.957)	-0.07 (0.252)
IgG2&3 Fc	Gal_n	**-0.69** (<0.001)	**-0.46** (<0.001)	**-0.55** (<0.001)	**-0.34** (<0.001)	**-0.27** (<0.001)	**-0.28** (<0.001)
	Bis GlcNAc_n	**0.13** (<0.001)	**0.12** (0.002)	**0.14** (0.001)	0.14 (0.010)	-0.06 (0.208)	0.03 (0.549)
	FGS/(F+FG+FGS)	**-0.59** (<0.001)	**-0.31** (<0.001)	**-0.47** (<0.001)	**-0.26** (<0.001)	**-0.22** (<0.001)	**-0.15** (0.008)
IgG	Gal_n	**-0.68** (<0.001)	**-0.49** (<0.001)	**-0.56** (<0.001)	**-0.37** (<0.001)	**-0.28** (<0.001)	**-0.26** (<0.001)
	Bis GlcNAc_n	**0.30** (<0.001)	**0.23** (<0.001)	**0.34** (<0.001)	**0.32** (<0.001)	0.03 (0.498)	0.07 (0.201)
	FGS/(F+FG+FGS)	**-0.57** (<0.001)	**-0.34** (<0.001)	**-0.45** (<0.001)	**-0.16** (0.005)	**-0.20** (<0.001)	**-0.16** (0.004)

Positive correlation coefficients (*r*) for age indicate increased levels with increasing age, while negative correlation coefficients indicate decreased levels with increasing age. Correlations found to be significant after Bonferroni correction for gender and glycosylation features ($P \leq 0.008$) are in bold. Gal_n - level of galactosylation in neutral glycoforms, Bis GlcNAc_n - level of bisecting *N*-acetylglucosamine in neutral glycoforms and FGS/(F+FG+FGS) - degree of sialylation of fucosylated glycoforms without bisecting *N*-acetylglucosamine.

Supplementary Table 2. Descriptives of glycosylation features in females and males with statistical significance (*P*) of sex differences and differences between age groups for the IgG1 Fc, IgG2&3 Fc and the total IgG.

IgG subclass	Glycosylation feature	Ages ≤ 57			Ages > 57			Differences between age groups (*P*)	
		Female	Male	Sex differences (*P*)	Female	Male	Sex differences (*P*)		
		Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)		Female	Male
IgG1 Fc	Gal_n	41.4 (12.2)	37.5 (8.4)	**<0.001**	30.0 (8.6)	32.5 (9.3)	**<0.001**	**<0.001**	**<0.001**
	Bis GlcNAc_n	12.4 (4.7)	12.5 (4.3)	0.633	14.2 (4.9)	13.2 (4.6)	0.013	**<0.001**	**0.002**
	FGS/(F+FG+FGS)	4.4 (2.4)	3.8 (2.2)	**<0.001**	3.0 (1.9)	3.3 (2)	**0.003**	**<0.001**	0.159
IgG2&3 Fc	Gal_n	31.0 (13)	28.2 (8.7)	**<0.001**	20.0 (7.3)	22.6 (7.6)	**<0.001**	**<0.001**	**<0.001**
	Bis GlcNAc_n	8.7 (3.5)	8.5 (3.1)	0.028	9.5 (3.7)	8.7 (3.2)	**0.001**	**<0.001**	0.105
	FGS/(F+FG+FGS)	3.9 (2.8)	3.5 (2.0)	**0.002**	2.2 (1.4)	2.7 (1.6)	**<0.001**	**<0.001**	**0.004**
IgG	Gal_n	45.9 (10.9)	43.2 (7.4)	**<0.001**	35.9 (7.7)	38.4 (6.8)	**<0.001**	**<0.001**	**<0.001**
	Bis GlcNAc_n	17.0 (3.4)	16.8 (3.7)	0.139	18.5 (3.5)	17.6 (2.9)	**<0.001**	**<0.001**	0.008
	FGS/(F+FG+FGS)	22.7 (5.9)	21.4 (4.6)	**<0.001**	18.0 (4.5)	19.0 (4.9)	**<0.001**	**<0.001**	**<0.001**

P-values of sex differences ($P \leq 0.006$) and differences between age groups ($P \leq 0.006$) found to be significant after Bonferroni correction for multiple comparisons are in bold. IQR – interquartile range. Gal_n - level of galactosylation in neutral glycoforms, Bis GlcNAc_n - level of bisecting *N*-acetylglucosamine in neutral glycoforms and FGS/(F+FG+FGS) - degree of sialylation of fucosylated glycoforms without bisecting *N*-acetylglucosamine.

Supplementary Table 3. Correlation coefficients of glycosylation features for the IgG1 Fc, IgG2&3 Fc and the total IgG.

IgG subclass	Glycosylation feature	All ages		Ages ≤ 57		Ages > 57	
		Bis GlcNAc_n	FGS/(F+FG+FGS)	Bis GlcNAc_n	FGS/(F+FG+FGS)	Bis GlcNAc_n	FGS/(F+FG+FGS)
		r (P)	*r (P)*	*r (P)*	*r (P)*	*r (P)*	*r (P)*
IgG1 Fc	Gal_n	-0.63 (0.018)	**0.65** (<0.001)	-0.09 (0.013)	**0.63** (<0.001)	**0.15** (<0.001)	**0.57** (<0.001)
	Bis GlcNAc_n		**0.26** (<0.001)		**0.26** (<0.001)		**0.40** (<0.001)
IgG2&3 Fc	Gal_n	**-0.07** (0.003)	**0.80** (<0.001)	-0.04 (0.244)	**0.78** (<0.001)	0.02 (0.965)	**0.70** (<0.001)
	Bis GlcNAc_n		**0.14** (<0.001)		**0.17** (<0.001)		**0.24** (<0.001)
IgG	Gal_n	**-0.22** (<0.001)	**0.82** (<0.001)	**-0.21** (<0.001)	**0.79** (<0.001)	-0.04 (<0.278)	**0.75** (<0.001)
	Bis GlcNAc_n		**-0.19** (<0.001)		**-0.17** (<0.001)		-0.05 (<0.166)

Correlations found to be significant after Bonferroni correction ($P \leq 0.008$) are in bold. Gal_n - level of galactosylation in neutral glycoforms, Bis GlcNAc_n - level of bisecting *N*-acetylglucosamine in neutral glycoforms and FGS/(F+FG+FGS) - degree of sialylation of fucosylated glycoforms without bisecting *N*-acetylglucosamine.

Supplementary Table 4. Calculated monoisotopic *m/z* values of 2-AA labeled glycans.

Glycan species	Glycan composition	$[M-H]^-$
M5	H5N2	1354.4789
FA1	H3N3F1	1379.5106
G0	H3N4	1436.5320
M6	H6N2	1516.5317
M2A1G1S1	H3N3S1	1524.5481
G0F	H3N4F1	1582.5899
G1	H4N4	1598.5848
G0N	H3N5	1639.6114
M7	H7N2	1678.5848
M3A1G1S1	H4N3S1	1686.6009
M5A1G1	H6N3	1719.6111
G1F	H4N4F1	1744.6428
G2	H5N4	1760.6377
G0FN	H3N5F1	1785.6693
G1N	H4N5	1801.6642
FM3A1G1S1	H4N3H1S1	1832.6588
M8	H6N2	1840.6374
M4A1G1S1	H5N3S1	1848.6537
G1S1	H4N4S1	1889.6803
G2F	H5N4F1	1906.6956
G1FN	H4N5F1	1947.7221
G2N	H5N5	1963.7170
M9	H9N2	2002.6902
M5A1G1S1	H6N3S1	2010.7065
G1FS1	H4N4F1S1	2035.7382
G2S1	H5N4S1	2051.7331
G2FN	H5N5F1	2109.7750
G2FS1	H5N4F1S1	2197.7910
G2NS1	H5N5S1	2254.8125
G3F	H6N5F1	2271.8278
G2S2	H5N4S2	2342.8285
G2FNS	H5N5F1S1	2400.8704
G3NS1	H6N6S1	2416.8653
G2FS2	H5N4F1S2	2488.8864
G2NS2	H5N5S2	2545.9079
G3FS1	H6N5F1S1	2562.9232
G2FNS2	H5N5F1S2	2691.9658
G3S2	H6N5S2	2707.9607
G3FS2	H6N5F1S2	2854.0186
G3S3	H6N5S3	2999.0561
G4S2	H7N6S2	3073.0929
G3FS3	H6N5F1S3	3145.1140
G4FS2	H7N6F1S2	3219.1508
G4S3	H7N6S3	3364.1883
G4FS3	H7N6F1S3	3510.2462
G4S4	H7N6S4	3655.2837
G4FS4	H7N6F1S4	3801.3417

Supplementary Table 5. Comparison of relative abundance (%) of *N*-glycan species of IgG and IgG Fc standards measured by MALDI-TOF-MS of 2-AA labeled *N*-glycans and HILIC of 2AB labeled *N*-glycans.

Glycan species	IgG		Fc	
	MALDI-TOF-MS of 2-AA *N*-glycans	**HILIC of 2-AB *N*-glycans**	**MALDI-TOF-MS of 2-AA *N*-glycans**	**HILIC of 2-AB *N*-glycans**
G0F	18.09	20.29	18.87	21.52
G1F	25.06	28.62	26.38	32.60
G2F	17.82	16.14	17.80	16.53
G0FN	4.65	4.84	5.65	5.52
G1FN	7.88	5.62	8.41	6.55
G2FN	5.79	4.10	1.87	1.45
G1FS1	2.74	2.86	2.60	2.29
G2FS1	10.06	10.63	11.60	9.19
G2FNS1	1.78	0.99	0.35	0.22
G2FNS2	0.01	0.06	0.05	0.05
G2FS2	0.04	0.19	0.13	0.23
G2NS2	0.01	0.02	0.06	0.01
G0	0.68	0.71	0.87	0.97
G1	1.50	0.46	1.79	0.60
G2	2.21	1.79	1.42	1.07
G0N	0.20	0.60	0.21	0.32
G2N	0.50	0.49	0.18	0.14
G2S1	0.91	1.16	1.58	0.54
G2S2	0.07	0.12	0.11	0.07
M5	0.03	0.29	0.04	0.12

Glycan species are given as in Table 1.

Supplementary Table 6. Comparison of relative abundance (%) of *N*-glycan species of IgG1 and IgG Fc standards measured by MALDI-TOF-MS of *N*-glycopeptides and HILIC of 2-AB labeled *N*-glycans.

Glycan species	IgG1			Fc		
	RN ion mode MALDI-TOF-MS of glycopeptides	LN ion mode MALDI-TOF-MS of glycopeptides	HILIC of 2-AB *N*-glycans	RN ion mode MALDI-TOF-MS of glycopeptides*	LN ion mode MALDI-TOF-MS of glycopeptides*	HILIC of 2-AB *N*-glycans
G0F	22.27	18.28	18.49	22.68	19.65	21.67
G1F	41.49	32.92	30.45	35.67	27.88	32.83
G2F	14.55	16.00	17.71	15.73	15.40	16.65
G0FN	4.62	6.58	4.75	5.10	6.70	5.56
G1FN	10.01	11.98	6.25	8.43	9.84	6.60
G2FN	0.65	1.51	3.94	0.67	1.21	1.46
G1FS1	0.29	0.82	2.23	0.85	1.84	2.31
G2FS1	4.44	9.50	11.47	7.99	12.82	9.26
G0	0.31	0.06	0.80	0.48	0.55	0.98
G1	0.76	1.18	0.46	1.17	2.02	0.61
G2	0.37	1.12	1.31	0.62	1.48	1.08
G0N	0.09	0.04	0.66	0.13	0.10	0.32
G2N	0.13	0.00	0.43	0.33	0.11	0.14
G2S1	0.03	0.00	1.05	0.16	0.39	0.54

Glycan species are given as in Table 1.

* only IgG1 glycopeptides measured