Glycoproteomics characterization of immunoglobulins in health and disease
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Chapter 4:

Galactosylation and sialylation levels of IgG predict relapse in patients with PR3-ANCA associated vasculitis

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Table of Contents

4.1: Summary

Objective:

The objective of our study is to investigate the Fc glycosylation profiles of both antigen-specific IgG targeted against proteinase 3 (PR3-ANCA) and total IgG as prognostic markers of relapse in patients with Granulomatosis with Polyangiitis (GPA).

Methods:

Seventy-five patients with GPA and a PR3-ANCA rise during follow-up were included, of whom 43 patients relapsed within a median period of 8 (2-16) months. The *N*-glycan at Asn297 of affinity-purified and denatured total IgG and PR3-ANCA was determined by mass spectrometry of glycopeptides in samples obtained at the time of the PR3-ANCA rise and at the time of the relapse or time-matched during remission.

Results:

Patients with total IgG1 exhibiting low galactosylation or low sialylation were highly prone to relapse after an ANCA rise (HR 3.46 [95%-CI 1.73-6.96], p<0.0001 and HR 3.22 [95%-CI 1.52-6.83], p=0.002, respectively).

In relapsing patients, total IgG1 galactosylation, sialylation and bisection significantly decreased and fucosylation significantly increased from the time of the PR3-ANCA rise to the relapse (p<0.0001, p=0.0087, p<0.0001 and p=0.0025), while the glycosylation profile remained similar in non-relapsing patients. PR3-ANCA IgG1 galactosylation, sialylation and fucosylation of PR3-ANCA IgG1 decreased in relapsing patients (p=0.0073, p=0.0049 and p=0.0205), but also in non-relapsing patients (p=0.0007, p=0.0114 and p=0.0002), while bisection increased only in non-relapsing patients (P<0.0001).

Conclusion:

While Fc glycosylation profiles have been associated with clinically manifest autoimmune diseases, in the present study we show that low galactosylation and sialyation in total IgG1 but not PR3-ANCA IgG1 predicts disease reactivation in patients with GPA who experience an ANCA rise during follow-up. We postulate that glycosylation profiles may be useful in pre-emptive therapy studies using ANCA rises as guideline.

4.2: Introduction

Granulomatosis with Polyangiitis (GPA; Wegener's), Microscopic Polyangiitis (MPA) and Eosinophilic Granulomatosis with Polyangiitis (EGPA; Churg Strauss Syndrome) are inflammatory disease entities affecting small to medium vessels. They are characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase-3 (PR3) or myeloperoxidase (MPO) and are frequently grouped together under the term ANCA-associated vasculitis (AAV) (1).

The pathogenic potential of ANCA to cause necrotizing glomerulonephritis (NCGN) is well established in mouse models (2-4). Patients with severe NCGN are almost always positive for either PR3- or MPO-ANCA (5), while ANCA are less often detected in patients with localized forms of vasculitis (6, 7). After remission induction, a rise in the ANCA titer is detected in some patients and disease reactivation may occur shortly thereafter (8). However, the relation between longitudinal ANCA measurements and disease reactivation is far from absolute since many ANCA rises are not followed by a relapse and relapses may occur without a preceding ANCA rise (9, 10). Recently, it has been shown that ANCA rises are highly predictive for disease activity in patients with severe vasculitic disease, e.g. NCGN or alveolar hemorrhage, but not in patients with limited granulomatous disease (11-14). Our current hypothesis is that the pathogenicity of an ANCA rise is modulated by the quality of the auto-antibody, for which the glycosylation profile is an important factor.

The glycosylation profile of the crystallizable fragment (Fc) of the immunoglobulin G (IgG) is characterized by a single *N*-linked glycan attached to each heavy chain at the asparagine-297 (15). The Fc *N*-glycan composition affects Fcγ receptor (FcγR) affinity (15-18) and can influence complement activation (19). The lack of a core fucose, *N*-acetylneuraminic (sialic) acids and galactose residues on the Fc *N*-glycan have been found to increase the inflammatory capacity of IgG, at least in mice (20-22). The Fc *N*-glycan is essential for the pathogenicity of the antibody, since deglycosylation of MPO-ANCA significantly attenuates ANCA-mediated neutrophil activation and reduces glomerular crescent formation in a mouse model (23).

Already in the early eighties, it was recognized that total IgG in patients with rheumatoid arthritis (RA) contained less galactose and sialic acid at the non-reducing termini compared

to healthy controls (24). A difference in the glycosylation profile of total IgG has since been demonstrated in patients with various other auto-immune diseases when compared to healthy controls, including systemic lupus erythematosus, inflammatory bowel disease, myasthenic gravis, ankylosing spondylitis, primary Sjögren's syndrome, psoriatic arthritis and multiple sclerosis (25-29). Furthermore, significant differences have been observed in the glycosylation profile of total IgG and specific auto-antibodies, such as anti-citrullinated protein antibodies (ACPA), anti-β2GP1 and anti-histone IgG (30-33). Finally, Fc glycosylation of auto-antibodies may change during disease development. For example, the glycosylation profile of ACPA changes prior to the onset of RA towards a more inflammation-associated phenotype (31, 34).

In patients with PR3-ANCA, it was previously shown with lectin assays that total IgG exhibits a lower degree of galactosylation when compared to healthy controls (35, 36). In addition, the degree of sialylation of PR3-ANCA is lower during active disease compared to inactive disease (37). Recently, it has been shown with mass spectrometric IgG Fc glycosylation analysis that total IgG Fc of patients with severe AAV exhibits lower levels of galactosylation, sialylation and bisecting N-acetylglucosamine (GlcNAc) compared to healthy controls (38). This finding was more pronounced for PR3-ANCA compared to total IgG (38). Correlations were observed between the glycosylation profile of PR3-ANCA and several cytokine concentrations, suggesting that the glycosylation of ANCA may be driven by T-cell activation in an antigen-specific manner (38). Potential differences and similarities in the glycosylation profile of total IgG and antigen-specific IgG between patients with PR3-ANCA and patients with MPO-ANCA associated vasculitis have not yet been investigated.

The objective of our study is to investigate differences in the glycosylation profile of both PR3-ANCA and total IgG and the prognostic value at the time of a rise in PR3-ANCA in patients with GPA. The primary question is whether patients with a particular glycosylation profile are more prone to relapse. Furthermore, upon an ANCA rise, changes in glycosylation profiles associated with a relapse are examined. To increase the homogeneity of our cohort, we included only GPA patients that are PR3-ANCA positive, the disease subgroup that is most prevalent in our area.

4.3: Methods

4.3.1: Inclusion criteria

All patients who visited the clinic at the Maastricht University Medical Center (MUMC) between January 1, 2000 and November 1, 2011 were evaluated. Inclusion criteria were a diagnosis of GPA according to the EMA classification system and a rise in PR3-ANCA during remission (11, 39, 40).

Clinical characteristics were recorded in all subjects according to the Dutch law on Medical Treatment Act (WGBO), the Personal Data Protection Act (Wbp) and the Code of Conduct for Health Research (Federa) (41). Ethics approval was waived by our local ethics committee.

4.3.2: Classification of patients

Renal involvement was preferably determined by a kidney biopsy showing pauci-immune necrotizing glomerulonephritis (42, 43). However, surrogate markers such as haematuria in combination with red cell casts, dysmorphic erythrocytes (>10) and/or proteinuria sufficed (40). All patients have been treated according to the European League against Rheumatism (EULAR) guidelines (43, 44).

The definitions recommended by the EULAR of 2007 were applied to define disease activity states (39). Remission was defined as absence of disease activity attributable to active disease during maintenance immunosuppressive therapy of a prednisone dosage of 7.5mg or lower. A relapse was defined as re-occurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment. Relapses were further subdivided in 'major' or 'minor' depending on whether the relapse was potentially organ- or life-threatening or not (39).

4.3.3: ANCA measurements

Patients were routinely evaluated during follow-up, generally every three months during the first two years after diagnosis and/or a relapse and 2-3 per year later on. At every visit, patients were screened for potential symptoms of a relapse (39) and blood was drawn.

Antigen-specific solid-phase ANCA tests were performed for the detection and quantification of PR3-ANCA. Initially, commercially available direct PR3-ANCA enzyme linked immunosorbent assays (ELISA) were used (Euro Diagnostica, Malmö, Sweden) (45). On October 1, 2005, this assay was replaced by a fluorescent-enzyme immune-assay (FEIA) for

PR3-ANCA (EliA, Thermo Fisher, Freiburg, Germany) (46). During the transition, ANCA measurements were performed using both methods.

4.3.4: Definition of an ANCA rise

For the detection of an ANCA rise, the ANCA titer was compared to all measurements made with the same assay in the past 6 months. We defined a rise using the slope of an increase as previously described (11), thereby taking into account the relative increase (in %) and the time between measurements (in days). A receiver operating characteristics (ROC) curve was calculated to determine the optimal cut-off value of the slope. The chosen cut-off values as determined by the ELISA and FEIA method were 2.56 and 2.25 %/day, respectively. This is equivalent to a relative increase of 78% and 68% over one month or 233% and 205% over three months.

To ensure that small elevations were above the intra-assay coefficient of variation, a rise had to constitute to a relative increase of at least 25% and an absolute increase equivalent to a doubling of the lowest value of a borderline result (at least 10 AU for the ELISA and 5 U/ml for the FEIA). Because our analysis is focused on patients in remission, only serum samples drawn at least 3 months after the previous disease activity were eligible for detection of an ANCA rise (11).

4.3.5: Serum selection

In all 75 patients, a serum sample was selected at the time of an ANCA rise ('T1') (11). In relapsing patients, either renal or non-renal, a second serum sample was selected prior to the start of the immunosuppressive induction therapy at the time of the relapse ('T2rel'). In non-relapsing patients, a second serum sample was selected after the ANCA rise during remission ('T2rem'), of which the time between the first and second sample was matched with the time between the first and second sample in the renal relapsing patients. For 4 relapsing patients no serum sample was available at the time of the relapse, and therefore only 71 T2 serum samples were analyzed.

4.3.6: Total IgG purification

For total IgG purification, the wells of two filter plates (Multiscreen filter plates with Durapore membrane, pore size 0.65 µm; Merck Millipore, Darmstadt, Germany) were filled with 15 µl of Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) in 200 µl PBS, followed by the addition of 2 µl of the serum samples. Furthermore, 10 of the

serum samples were purified in duplicate. Fifteen wells were filled with 2 µl Milli-Q-purified water to serve as negative control and 20 wells were filled with 2 µl of the serum of a healthy donor to serve as positive control. The plates were incubated on a shaker at room temperature for 1 hour. The samples were then washed on a vacuum manifold with 4x 200 µl PBS and 3x 200 µl Milli-Q-purified water, followed by the addition of 100 µl 100 mM formic acid (Fluka, Steinheim, Germany) for elution into a V-bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany), which has been shown to result in near-complete denaturation of IgG (47). Eluates were dried in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany) at 60 °C for approximately 2 hours.

4.3.7: PR3-ANCA purification

An ELISA kit (Wieslab PR3-ANCA; Euro Diagnostica) was used for purification of PR3-ANCA. Eighty µl diluent containing PBS (Wieslab kit) and 20 µl of the serum samples were added to two PR3-coated 96-well plates. Ten of the serum samples were purified in duplicate in separate wells. Fifteen wells were filled with 20 µl Milli-Q-purified water to serve as negative control and 20 wells were filled with 20 µl of the serum of a healthy donor to serve as a further negative control. The samples were incubated on a shaker at room temperature for 1 hour, followed by washing 4x with 250 µl wash buffer (Wieslab kit) and 1x with 150 µl 50 mM ammonium bicarbonate buffer (Sigma-Aldrich, St. Louis, MO). The PR3-ANCA was eluted by adding 100 µl 100 mM formic acid to the wells and collected into a V-bottom 96-well plate. The samples were then dried in a centrifugal vacuum concentrator at 60°C for approximately 2 hours.

4.3.8: PR3-ANCA purification

The dried total IgG and PR3-ANCA samples were resuspended in 20 µl 50 mM ammonium bicarbonate and incubated on a shaker at room temperature for 15 minutes. Twenty µl of 0.01 µg/µl trypsin (sequencing grade modified trypsin, Promega, Madison, WI) was then added to each well. The samples were again incubated on a shaker at room temperature for 15 minutes, followed by overnight incubation at 37°C. Samples were stored at -20°C.

4.3.9: LC-MS/MS analysis

The digested samples were analyzed by nanoLC-reversed phase (RP)-electrospray ionization (ESI) –quadrupole time-of-flight (qTOF)-MS on an Ultimate 3000 HPLC system (Dionex/Thermo Scientific, Sunnyvale, CA) coupled to a MaXis Impact (Bruker Daltonics, Bremen, Germany). The samples were concentrated on a Dionex Acclaim PepMap100 C18

trap column (particle size 5 µm, internal diameter 300 µm, length 5 mm) and separated on an Ascentis Express C18 nano column (2.7 µm HALO fused core particles, internal diameter 75 µm, length 50 mm; Supelco, Bellefonte, PA). The following linear gradient was applied, with solvent A consisting of 0.1% trifluoroacetic acid (TFA; Fluka) and solvent B of 95% acetonitrile (Biosolve, Valkenswaard, The Netherlands): t=0 min, 3% solvent B; t=2, 6%; t=4.5, 18%; t=5, 30%; t=7, 30%; t=8, 1%; t=10.9, 1%. The sample was ionized in positive ion mode with a CaptiveSprayer (Bruker Daltonics) at 1100 V. A nanoBooster (Bruker Daltonics) was used to enrich the nitrogen gas with acetonitrile to enhance the ionization efficacy. A mass spectrum was acquired every second (frequency of 1 Hz), with the ion detection window set at *m/z* 550-1800. In between every 12 measurements an external IgG standard was run. A mass spectrum of both total IgG and PR3-ANCA can be seen in Figure 4.1.

4.3.10: Data processing

Mass spectrometric identification and processing of tryptic IgG glycopeptide data was done as described previously (48), with a few differences as stated below, and was performed blindly in order to prevent bias. The LC-MS data files were examined in Compass DataAnalysis 4.2 software (Bruker Daltonics). Negative controls were inspected and found to contain no significant intensity of IgG signals.

First, the *m/z* axis of the mass spectra was calibrated internally using several known IgG glycopeptides masses. Next, the files were converted to the .mzXML data format using the MSconvert program from the ProteoWizard 3.0 suite (49). The m/z and retention times of all the tryptic IgG glycopeptides were manually determined using MZmine 2.10 (50) and can be found in Supplemental Table S4.1. The time axes were aligned based on a list of the most prominent IgG glycopeptide peaks and their retention times, using an alignment tool designed in-house (51). The intensity of the first three isotopes of every analyte was extracted using a window of ± 0.04 Thomson and ± 10 s around the manually determined retention time. This was achieved using the in-house developed 3D Total Extractor (3DTE) program (software code can be found in the supplemental data). A list of analytes is provided to 3DTE, which then uses a binary search to find the retention time and mass region around each analyte. Afterwards, the maximum intensity of each analyte is determined per mass spectrum that falls within the retention time window. The value that is reported for each analyte by 3DTE is the sum of the highest intensity values from the individual mass spectra. Lastly, the program

generates a tab-separated output file that lists all the compositions and their respective total intensities. Background correction was optimized to minimize intensity biases in the determination of glycopeptide ratios. In Excel 14.0, the background-corrected signal intensities of the three isotopic peaks in both 2+ and 3+ charge state were summed to obtain a single value for each glycopeptide. IgG1 G2 and G2S2 were excluded because of overlap with contaminant peaks. Isotopic peaks for several other IgG1 glycopeptides were also excluded due to peak overlap, and these values were replaced by an estimate based on the remaining isotopic peaks and the theoretical isotopic pattern (these isotopic peaks account for less than 3% of the total glycopeptide signal intensity). Afucosylated IgG4 glycopeptides were excluded due to peak overlap with IgG1 glycopeptides. In order to obtain a percentage value for each glycopeptide, this value was then divided by the sum of all signal intensities of glycopeptides, and this was done separately for IgG1, IgG2/3 and IgG4. In Caucasian populations the tryptic glycopeptide of IgG3 generally has the same peptide sequence as IgG2 (52). Therefore it was not possible to distinguish between IgG2 and IgG3 glycopeptides with the methods we used, and these glycopeptides will be referred to as IgG2/3. The IgG percentage data can be seen in Supplemental Table S4.2. Several glycosylation features were calculated from this data: fucosylation (% of glycans bearing a core fucose), bisection (% of glycans with a bisecting GlcNAc), galactosylation (% of antennae carrying a galactose) and sialylation (% of antennae carrying a sialic acid, see supplemental data for the exact definitions).

In order to guard the quality of the acquired data, an intensity threshold was set. If the sum of the signal intensities of G0F, G1F and G2F in triple charge state for an IgG subclass did not exceed this threshold, all data for that subclass was excluded. Additionally, data was excluded if the signal/background of the highest peak per subclass did not exceed 20. Finally, because of the chance of overlap between IgG4 glycopeptide peaks and later eluting IgG2/3 peaks, IgG2/3 data was excluded if more IgG4 was present than IgG2/3 (as determined by G0F+G1F+G2F (3+) signals). Lastly, we determined the average relative standard deviation (RSD) of eight prominent glycans (G0F, G1F, G2F, G0FN, G1FN, G2FN, G1FS and G2FS) to further examine the quality of the data.

4.3.11: Statistical analysis

Numerical variables were expressed as median (interquartile range [IQR]) and categorical variables as relative abundances (percentages). Two unpaired variables were compared with

the Mann Whitney U test. Two paired variables were compared with the Wilcoxon signed rank test. Continuous variables were correlated with the Spearman test.

Receiver operating characteristic (ROC) curves were constructed for galactosylation, sialylation, fucosylation and bisection at the time of an ANCA rise to discriminate relapsing patients from patients who remained in remission. If a trend towards significance was observed in an ROC curve, an optimal cut-off value was derived that was closest to the upper left corner (the cut-off value with the highest sum of sensitivity and specificity). Patients were subsequently categorized as high (above or equal to the cut-off) or low (below cut-off) level for the concerning glycosylation trait. Differences in the time to relapse between high and low were assessed using the log-rank test. The time to relapse was estimated using the Kaplan-Meier method. An event was defined as a relapse at the time of the start or increase of immunosuppressive treatment. Subjects were censored at the time of last follow-up. Hazard ratios (HR) with the 95% confidence interval were derived using the Cox regression analysis, adjusted for age and sex. The proportional hazards assumption was assessed by visually inspecting log-log plots.

All statistical analyses were performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA) and SPSS statistics for Windows, version 23.0 (IBM, Armonk, NY, USA). Bonferroni corrections were applied to the statistical testing, in which a p-value of <0.0125 was considered significant and <0.05 was considered as a trend.

4.4: Results

4.4.1: Patient characteristics

Seventy-five patients with Granulomatosis with Polyangiitis positive for PR3-ANCA were included in this study, of whom 51 patients had renal involvement in the past and 24 patients did not (see Table 4.1). Forty-three (57.3%) patients relapsed within a median time of 8 (2-16) months since the ANCA rise.

Table 4.1: Patient characteristics. The organ involvement refers the organ involvement during any previous periods of disease activity in the past (at the diagnosis or any previous relapse). The induction treatment refers to the treatment regimen that was used to induce remission during the most recent period of disease activity only. Abbreviations: SD, standard deviation. GC, glucocorticosteroid therapy. *This patient was included after a minor relapse which was treated with GC monotherapy, while remission was induced after diagnosis using cyclophosphamide + GC. ±The follow-up time refers to the time from the most recent disease activity to the endpoint of the study (at the time of relapse or at the last time of follow-up).

	Renal		Non-renal	
	Relapse (n=31)	Remission (n=20)	Relapse (n=12)	Remission (n=12)
Demographics				
Age (in years + SD)	58.2 (14.5)	54.7 (14.2)	58.1 (9.0)	66.2 (11.3)
Male	23 (74.2%)	13 (65%)	6 (50%)	4 (33.3%)
Organ involvement at previous disease activities				
Arthralgia	19 (61.3%)	10 (50%)	7 (58.3%)	5 (41.7%)
Cutaneous	13 (41.9%)	3 (15%)	2 (16.7%)	0 (0%)
Eyes	8 (25.8%)	5 (25%)	3 (25%)	3 (25%)
Ear, nose, throat	26 (83.4%)	17 (85%)	10 (83.3%)	11 (91.7%)
Lung	23 (74.2%)	14 (70%)	10 (83.3%)	10 (83.3%)
Cardiovascular	1 (3.2%)	2 (10%)	0 (0%)	0 (0%)
Renal	31 (100%)	20 (100%)	0 (0%)	0 (0%)
Central nervous system	1 (3.2%)	0 (0%)	0 (0%)	1 (8.3%)
Peripheral nervous system	7 (22.6%)	4 (20%)	1 (8.3%)	2 (16.7%)
Induction treatment at previous disease activity resulting in remission				
Cyclophosphamide + GC	19 (61.3%)	14 (70%)	6 (50%)	10 (83.3%)
Rituximab + GC	1 (3.2%)	3 (15%)	0 (0%)	0 (0%)
Methothrexate + GC	5 (16.1%)	0 (0%)	4 (33.3%)	1 (8.3%)
Mofetil mycophenolate + GC	3 (9.7%)	3 (15%)	0 (0%)	0 (0%)
Gusperimus + GC	2 (6.4%)	0 (0%)	1 (8.3%)	0 (0%)
GC monotherapy	1 (3.2%)*	0 (0%)	1 (8.3%)	1 (8.3%)
Immunosuppressive therapy at the time of the ANCA rise				
Immunosuppressive therapy	27 (87.1%)	16 (80%)	8 (66.7%)	8 (66.7%)
None	4 (12.9%)	4 (20%)	4 (33.3%)	4 (33.3%)
Follow-up				
Follow-up time (in months + SD) ±	12.9 (20.4)	42.0 (20.8)	13.9 (16.1)	45.6 (35.3)
Persistently ANCA positive	10 (32.3%)	6 (30%)	2 (16.7%)	3 (25%)
Relapse				
Major	18 (58.1%)	-	4 (33.3%)	-
Minor	13 (41.9%)	-	8 (66.7%)	-

Out of 156 samples total (75 at the time of ANCA rise, 71 at a second time point, and 10 duplicates), PR3-ANCA IgG1 data was excluded in 16 samples, total IgG1 data in 0 samples, total IgG2/3 data in 14 samples and total IgG4 in 20 samples. Because the signal intensity of PR3-ANCA IgG2/3 and IgG4 in the majority of samples did not surpass the threshold, we excluded this data group altogether. For 8 prominent glycoforms, the external IgG standard

run in between every 12 measurements showed an average RSD of 4.2% for IgG1, 4.0% for IgG2/3 and 7.1% for IgG4. The on-plate healthy donor serum-derived total IgG samples exhibited an average RSD of 4.0% for IgG1, 3.0% for IgG2/3 and 3.5% for IgG4. Finally, for the aforementioned 8 glycoforms, the 10 ANCA serum samples which were processed in duplicate showed an average RSD of 2.3%, 1.5% and 1.3% for total IgG1, IgG2/3 and IgG4, and 3.1% for PR3-ANCA IgG1. Two LC-MS spectra of total IgG and PR3-ANCA IgG, belonging to an AAV patient at the time of relapse, can be seen in Figure 4.1.

For better readability, the results of total IgG1 and PR3-ANCA IgG1 are presented in the manuscript, while the results of total IgG2/3 and total IgG4, which exhibit the same general trend as total IgG1, can be found in the supplemental data.
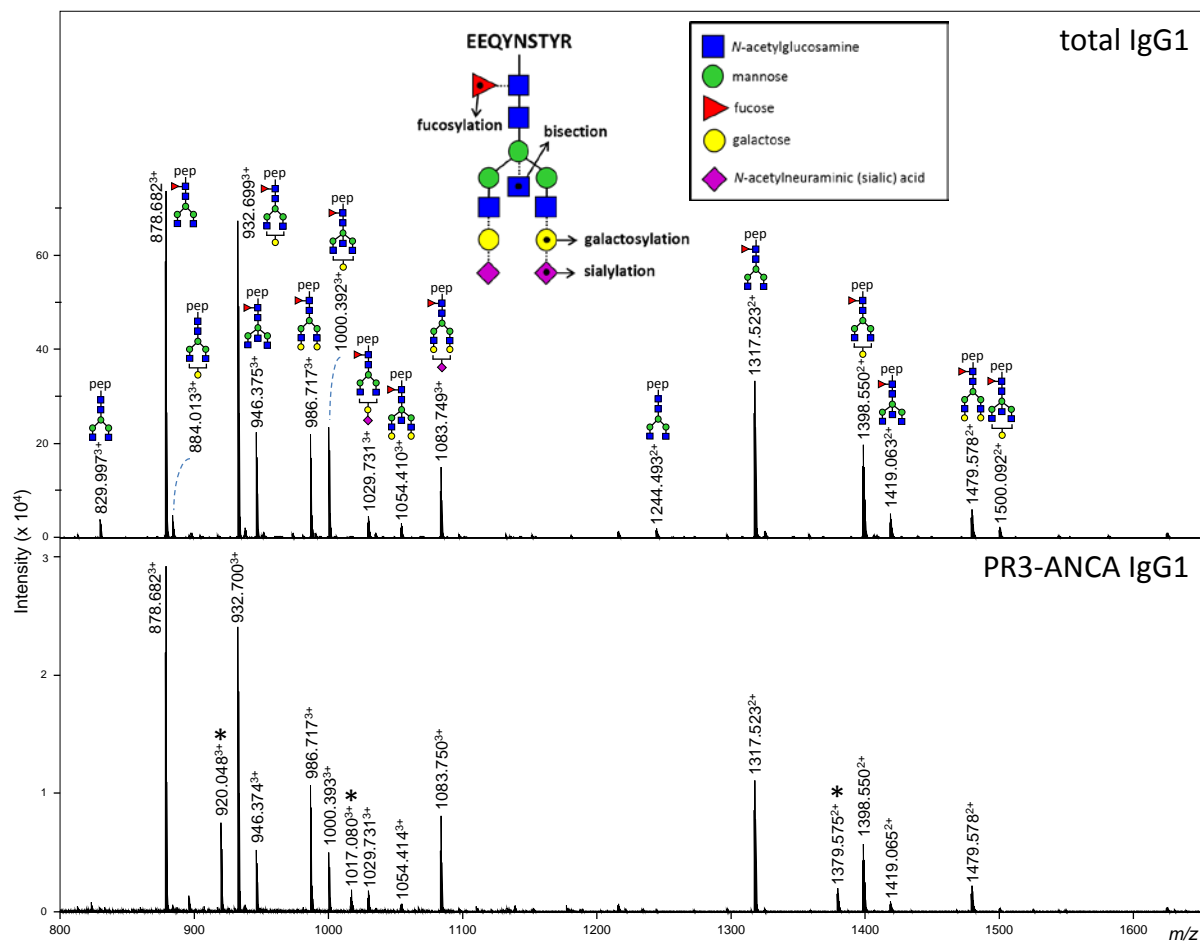

Figure 4.1: LC-MS spectra showing tryptic IgG1 glycopeptides for total IgG and PR3-ANCA belonging to an AAV patient (#9; details in Supplemental Table 4.1) at the time of relapse. The peaks denoted with an asterisk belong to a co-enriched contaminant (an apolipoprotein *O*-glycopeptide). Pep=peptide.

4.4.2: IgG1 Fc glycosylation of total IgG and PR3-ANCA at the time of the ANCA rise

At the time of the ANCA rise, we analyzed whether there were differences in the glycosylation profile of total IgG1 and PR3-ANCA IgG1. The degree of galactosylation, sialylation and fucosylation of total IgG1 was lower compared to PR3-ANCA IgG1 (46.0% [39.1-49.7%] versus 47.4% [40.4-57.5%], p=0.0175; 6.2% [5.2-7.1%] versus 6.9 [5.1-9.0%]; p=0.0289 and 93.3% [89.9-95.5%] versus 98.4% [97.3-99.0%], p<0.0001, respectively). In contrast, the level of bisection of total IgG1 was higher compared to PR3-ANCA IgG1 (19.0 [15.9-21.7%] versus 11.4% [10.1-13.9%], p<0.0001, see Supplemental Figure S4.1).

4.4.3: IgG1 Fc glycosylation as a predictor of a relapse at the time of an ANCA rise

Thereafter, we investigated whether patients with a particular IgG1 Fc glycosylation profile at the time of an ANCA rise are more prone to relapse after an ANCA rise. The ROC curve of total IgG1 galactosylation and sialylation were found to differentiate relapsing patients from non-relapsing patients (Supplemental Figure S4.2; AUC 0.6533, p=0.02385 and AUC 0.6563, p=0.02131, respectively). Total IgG1 bisection and fucosylation did not yield significant discrimination between relapsing and non-relapsing patients (p=0.9488 and p=0.9402, respectively; data not shown). The ROC curve of PR3-ANCA IgG1 was not significant for any of the glycosylation traits (data not shown). An optimal cut-off value was derived from the significant ROC curves, and patients were classified as 'low galactosylation' if the galactosylation rate of total IgG1 was lower than 46.1% and as 'low sialylation' if the sialylation rate of total IgG1 was lower than 6.9% (see Supplemental Figure S4.2).

Forty-one of 75 (54.7%) patients were classified as low galactosylation, of whom 30 (73.2%) patients relapsed within a median of 6 (1-15) months after the ANCA rise. In comparison, only 13 of 34 (38.2%) patients who were classified as high galactosylation relapsed in a median of 10 (2.5-17.5) months. Thus, patients with low galactosylation total IgG1 were highly prone to relapse after an ANCA rise (HR 3.46 [95%-CI 1.73-6.96], p<0.0001, adjusted for age and sex, see Figure 4.2A).
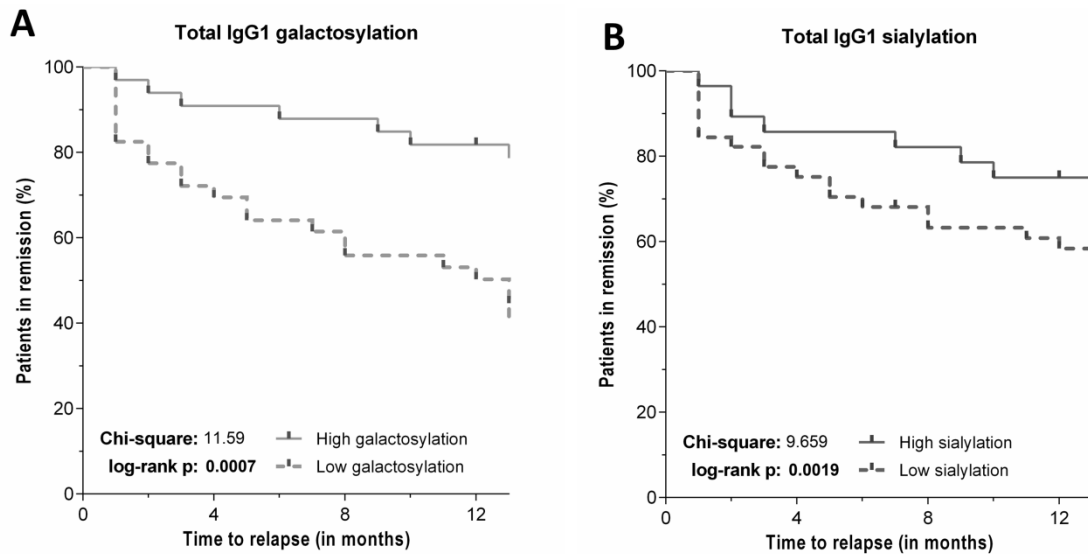
Figure 4.2: Time to relapse after an ANCA rise, (A) according to the degree of galactosylation of total IgG 1 Fc and (B) according to the degree of sialylation of total IgG 1 Fc.

Similar results were found for the degree of sialylation. Forty-seven of 75 (62.7%) patients were classified as low sialylation, of whom 34 (72.3%) patients relapsed within a median of 8 (1-17) months after the ANCA rise. In comparison, only 9 of 28 (32.1%) patients with a high sialylation relapsed in a median of 7 (2-12) months. Low sialylation patients were highly prone to relapse after an ANCA rise (HR 3.22 [95%-CI 1.52-6.83], p=0.002, adjusted for age and sex; see Figure 4.2B).

Because the degree of sialylation and galactosylation are highly correlated (terminal *N*-acetylneuraminic acid is attached to galactose), we in addition calculated the sialic acid per galactose. No association was observed between the sialic acid per galactose and the time to relapse (see Supplemental Figure S4.3 and S4.4).

4.4.4: Changes in IgG1 Fc glycosylation during follow-up

Next, we looked at potential changes of the glycosylation profile during follow-up. With regards to the total IgG1, the degree of galactosylation, sialylation and bisection significantly decreased and fucosylation significantly increased in relapsing patients (p<0.0001, p=0.0087, p<0.0001 and p=0.0025, respectively), while the glycosylation profile remained similar in patients who remained in remission (see Figure 4.3 and figure S4.5 and S4.6 in the supplemental data).
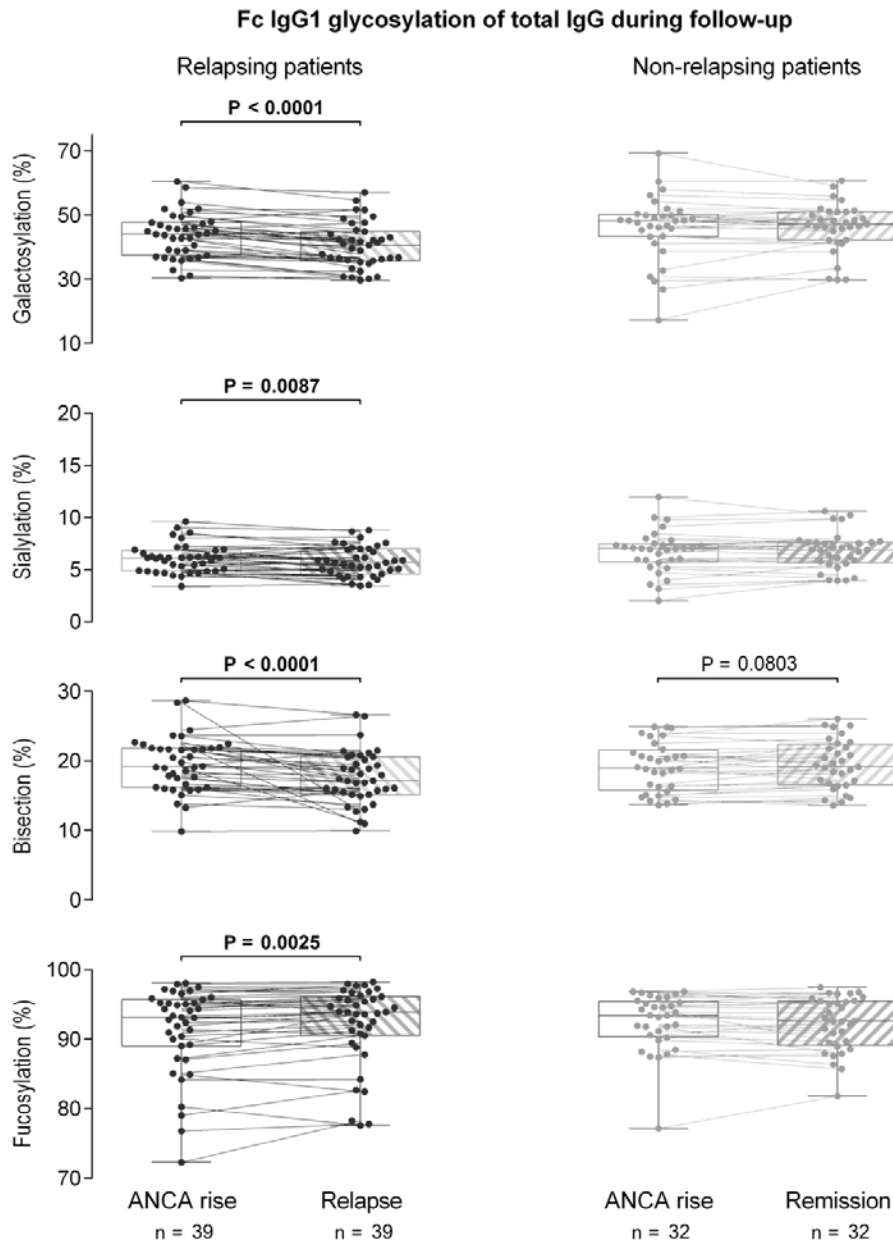
Figure 4.3: The glycosylation profile of total IgG1 Fc at the time of an ANCA rise (T1) and at the time of a relapse (T2rel) in relapsing patients (black dots) and time-matched during remission (T2rem) in patients who remain in remission (gray dots). Dots represent individual patients, lines indicate corresponding pairs. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Wilcoxon signed rank test, P-values are shown if <0.10 and in bold if <0.0125.

With regard to PR3-ANCA IgG1, in relapsing patients, a significant reduction in the degree of galactosylation and sialylation and a trend towards a reduction in fucosylation were observed from the ANCA rise to the time of the relapse (p=0.0073, p=0.0049 and p=0.0205, respectively, see Figure 4.4). Similarly, in patients who remained in remission, a significant reduction in the degree of galactosylation, sialylation and fucosylation was observed from the

ANCA rise to the time of the second sample (p=0.0007, p=0.0114 and p=0.0002, respectively). Moreover, the proportion of bisection of PR3-ANCA IgG1 significantly increased from the ANCA rise to the time-matched sample during remission (p<0.0001).
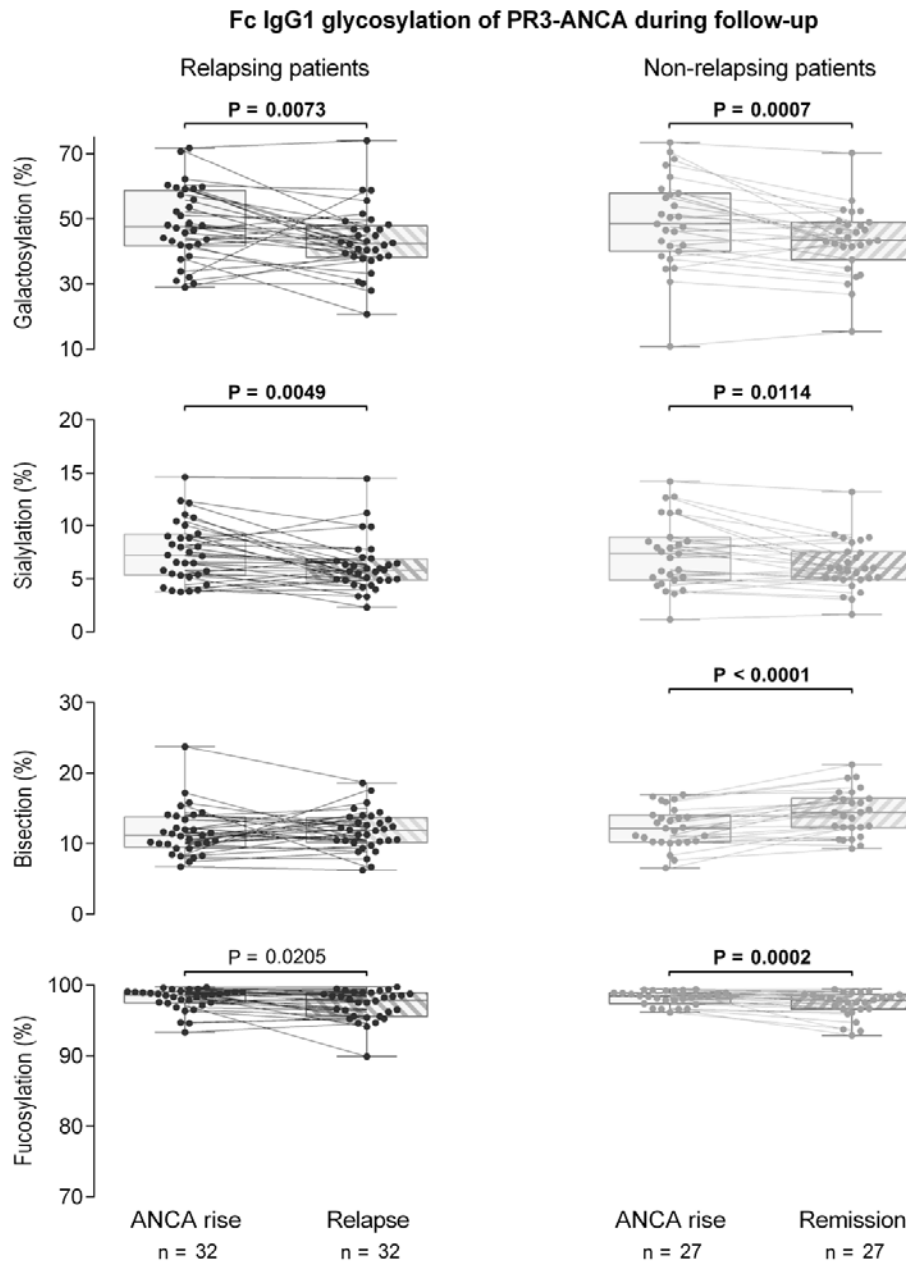
Figure 4.4: The glycosylation profile of antigen specific PR3-ANCA IgG1 Fc at the time of an ANCA rise (T1) and at the time of a relapse (T2rel) in relapsing patients (black dots) and time-matched during remission (T2rem) in patients who remain in remission (gray dots). Dots represent individual patients, lines indicate corresponding pairs. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Wilcoxon signed rank test, P-values are shown if <0.10 and in bold if <0.0125.

4.4.5: IgG1 Fc glycosylation at the time of relapse or time-matched during remission

The changes in the glycosylation profile over time lead to significant differences between patients in relapse or in remission at the second time point. With regard to total IgG1, a significantly lower degree of galactosylation and sialylation and a trend towards a lower degree of bisection was observed in relapsing patients compared to patients who remain in remission ($p=0.0015$, $p=0.0120$ and $p=0.0443$, respectively, see Figure 4.5 and Figure S4.7 in the supplemental data). Conversely, the glycosylation profile of PR3-ANCA was only significantly different with regard to bisection, which was lower in relapsing patients compared to patients who remained in remission ($p=0.0009$).
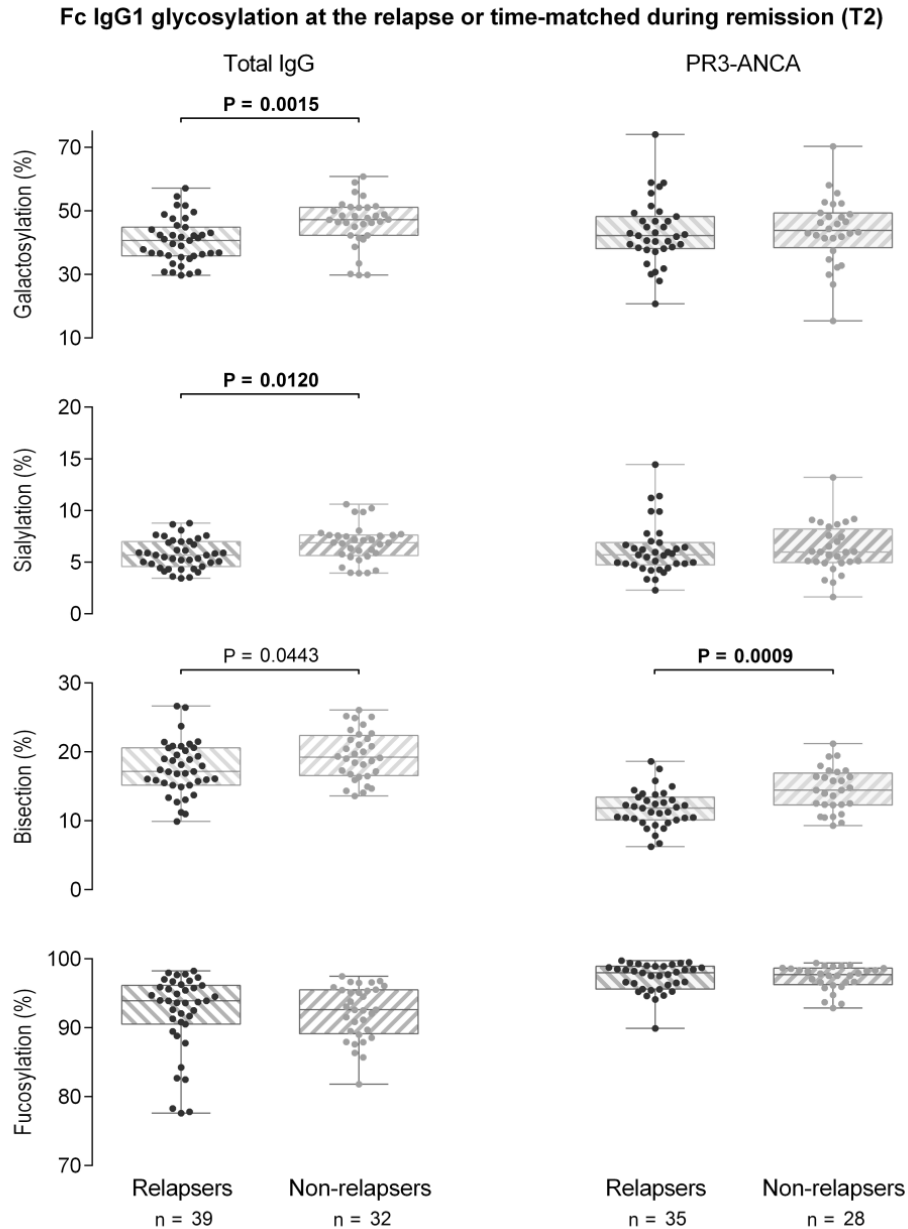
Figure 4.5: The glycosylation profile at the time of a relapse in relapsing patients and time-matched during remission in patients who remain in remission (gray dots). IgG1 Fc glycosylation of total IgG (left side, white background) and antigen specific PR3-ANCA (right side, yellow background) is shown. Dots represent individual patients. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Mann Whitney U test, P-values are shown if <0.10 and in bold if <0.0125.

4.5: Discussion

In this study we investigate with mass spectrometry the changes in Fc glycosylation over time in both total IgG and PR3-ANCA of patients with AAV. In patients who were in clinical remission, a first serum sample was taken at the time of an ANCA rise, whereas a second sample was acquired either after relapse but before therapy was started, or for patients who remained in remission, after a similar length of time. Analysis of the two longitudinal samples revealed a significant reduction in galactosylation, sialylation and bisection and an increase in fucosylation in total IgG Fc of relapsing patients, while these glycosylation traits did not change significantly in patients who remain in remission. Most importantly, the Fc glycosylation profile of total IgG at the time of an ANCA rise predicts a relapse. Namely, patients with low galactosylation or low sialylation in total IgG are more prone to relapse compared to patients with high galactosylation or sialylation in total IgG. In addition, we observed that the level of sialylation and galactosylation of PR3-ANCA is decreased over time, both in relapsing and non-relapsing patients. Therefore, it appears that PR3-ANCA gains a more inflammation-associated phenotype during follow-up in both groups of patients, independent of disease course.

While IgG with a low degree of galactosylation has repeatedly been found to be associated with pro-inflammatory autoimmune responses, the underlying mechanisms are still largely elusive (53-56). In addition, highly galactosylated IgG may confer anti-inflammatory activities through the association with the inhibiting receptor FcγRIIb and the C-type lectin-like receptor Dectin-1 in mice (21). This latter pathway has been shown to block C5a effector functions in vitro and C5a-dependent inflammatory responses in animal mouse models (21). This is highly relevant in AAV, since C5a plays a pivotal role in the pathophysiology (1, 57). Mouse models have shown that the blocking of C5a or C5a receptor (C5aR) ameliorates anti-MPO induced necrotizing glomerulonephritis (58, 59). The safety and efficacy in the treatment of non-life-threatening AAV with CCX168, a C5aR inhibitor, is currently being tested in a phase 2 study (EudraCT Number: 2011-001222-15). The addition of galactose to the glycan structure also no longer enables the interaction of MBL with IgG, and may thereby block the lectin pathway (19). The clinical relevance of the lectin pathway in AAV is questionable however, since mannose-binding lectin (MBL) deposition in the kidney was found in only a minority of patients and complement activation in AAV occurs predominantly via the alternative pathway (57).

Sialylated IgG has been reported to have anti-inflammatory properties likely mediated through interaction with the murine C-type lectin receptor SIGN-R1 (a homologue of the human DC-SIGN) and FcγRIIb (22, 60). In our study, we found that total IgG preserved an anti-inflammatory glycosylation profile in patients who remained in remission, but changed to an inflammation-associated phenotype in relapsing patients. It remains unclear whether this change merely represent a bystander acute-phase reaction or whether the loss of anti-inflammatory effector function of total IgG enabled PR3-ANCA to induce disease reactivation. Notably, low galactosylation and sialylation at the ANCA rise predicts disease reactivation, suggesting that the change of total IgG towards an inflammation-associated phenotype precedes the onset of disease reactivation. We hypothesize that these anti-inflammatory mechanisms of total IgG are involved in the suppression of active disease in our AAV patients who remain in remission.

One may wonder what causes the change of the glycosylation profile of total IgG from an anti-inflammatory towards a pro-inflammatory phenotype. One study observed an average reduction of total IgG sialylation by 40% upon antigenic challenge in a mouse model (22). Based on our findings, we speculate that a second hit that is not related to the presence of PR3-ANCA nor the glycosylation profile of PR3-ANCA is required for a relapse after an ANCA rise (the "first hit"). Several candidates for such a second hit have been postulated, such as microorganisms (61), environmental factors (62, 63), and/or other auto-antibodies (64). No functional data are currently available regarding the role of the glycosylation profile of total IgG and/or antigen-specific IgG in the capacity of PR3-ANCA to induce inflammation and this should be further studied.

A recent study reported a novel correlation between bisection of PR3-ANCA and disease state (38). In our study we find a correlation between bisection and relapse/remission. The level of bisection of total IgG decreases significantly in relapsing patients, while it stays stable in non-relapsing patients. In PR3-ANCA the level of bisection remains stable in relapsing patients, while it increases in non-relapsing patients. While the effect of a bisecting GlcNAc on IgG effector functions is minor compared to that of the other glycosylation features, it has been reported that bisection can enhance antibody-dependent cellular cytotoxicity (ADCC) through increased FcγRIIIa affinity (65, 66). Since a reduction in bisection is seen during AAV relapse, it is likely that the minor anti-inflammatory effect of decreased bisection is overshadowed by effects of other changes in glycosylation and that

ADCC may only play a minor role in the pathogenesis of AAV (67). A decrease in IgG bisection has not been reported for any other autoimmune disorders, while an increase in bisection has been observed for Lambert-Eaton Myasthenic Syndrome (LEMS) (29). A slight increase in fucosylation over time was seen in total IgG of relapsing patients. In contrast, PR3-ANCA showed a minor decrease in fucosylation for both relapsing and non-relapsing patients. While the absence of a core fucose can greatly enhance the inflammatory properties of IgG through increased FcγRIIIa affinity, the differences in fucosylation in our study cohort are likely too small to be of much influence.

Our findings regarding the glycosylation profile of total IgG compared to antigen-specific PR3 markedly differ from those of a previous study, which reported a reduction of galactosylation, sialylation and bisection of PR3-ANCA as compared to total IgG, while we observe that bisection, but not galactosylation and sialylation is reduced in PR3-ANCA. These differences may be largely caused by the pronounced differences in study design (38). First, we included patients with severe AAV as well as patients with more limited forms of AAV, while the previous report only included patients with severe AAV (38). Second, our patients were in remission at the time of sampling, while the patients of Wuhrer et al. were sampled at the time of active disease (38). Interestingly, both studies observe a lower degree of bisection in PR3-ANCA compared to total IgG.

Our study is limited by the amount of included patients, hence only the *N*-glycan analysis was included in the statistical evaluation. Future validation in other, larger study cohorts is warranted in which the predictive value of a multitude of factors, in particular the IgG *N*-glycan analysis, should be evaluated using multivariate techniques, including principle components analysis. A strong aspect of our study is that our patients are highly characterized and we only included patients with GPA positive for PR3-ANCA. Yet differences still remain in organ involvement and immunosuppressive therapy. Our findings, however, may not apply to patients with MPA or patients positive for MPO-ANCA and this should be further investigated.

Most importantly, we addressed the clinical value of the aberrant glycosylation which we observe in AAV patients. Changes in IgG glycosylation, especially galactosylation and sialylation, might be useful to screen patients for their risk of relapse. Our data indicates that analysis of total IgG would be sufficient for this purpose. Already in the first serum sample,

acquired a median time of 8 months before the time of relapse, we could identify patients that are at risk for a future disease relapse. Differences in the glycosylation profile between relapsing and non-relapsing patients becomes more pronounced as the time of relapse approaches. Longitudinal acquisition of serum samples taken every few months would reveal changes in the personal glycosylation profile of each patient that could possibly help as a guide when to start treatment in these patients. It remains to be studied, however, whether treatment based on this information will be able to minimize tissue and organ damage.

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Supplemental Information

A complete overview of the supplemental information is available online at

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5360573.
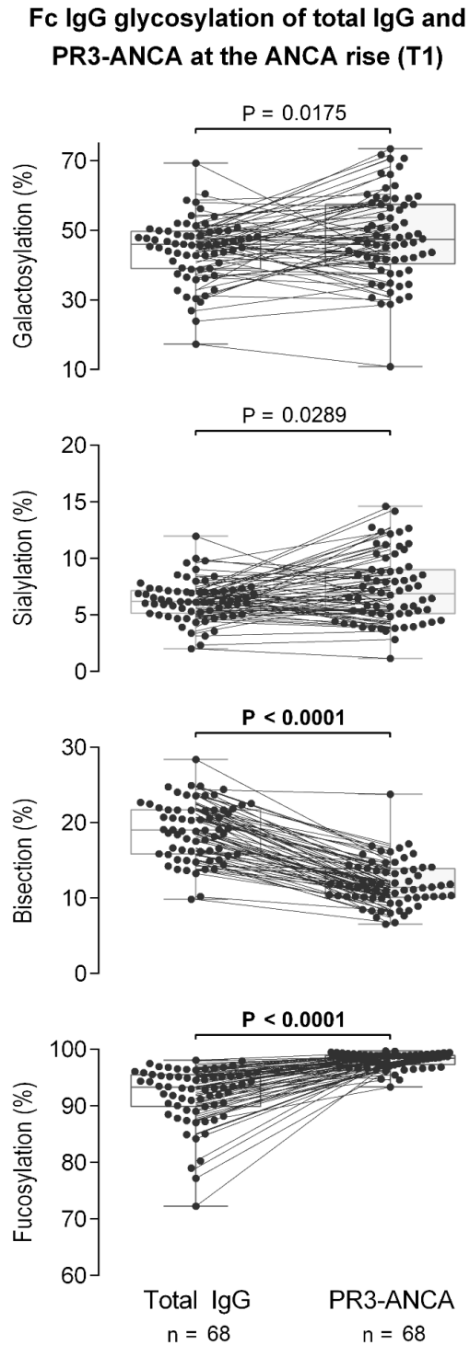
Supplemental Figure S4.2: The glycosylation profile of total IgG (white background) differs significantly compared to PR3-ANCA (yellow background) at the time of an ANCA rise (T1). IgG1 Fc glycosylation is shown. Dots represent individual patients. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Mann Whitney U test, P-values are shown if <0.10 and in bold if <0.0125.