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**Title:** Anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis : linking genetic predisposition to clinical outcome

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# Chapter 6

## Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid

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## ABSTRACT

**Objective** Anti-citrullinated protein antibodies (ACPA) exhibit unique specificity for RA. Whether and how ACPA contribute to disease pathogenesis, however, is incompletely understood. The Fc part of human IgG carries two N-linked glycan moieties which are crucial for the structural stability of the antibody and modulate its binding affinity to Fc $\gamma$  receptors and its ability to activate complement. We have purified ACPA from serum and synovial fluid and analyzed Fc glycosylation profiles in a specific manner.

**Methods** ACPA were isolated by affinity purification using cyclic citrullinated peptides as antigen. IgG<sub>1</sub> Fc glycosylation was analyzed by mass spectrometry. ACPA glycan profiles were compared to glycan profiles of total serum IgG<sub>1</sub> obtained from 85 well-characterised patients. Glycan profiles of paired synovial fluid and serum samples were available from 11 additional patients.

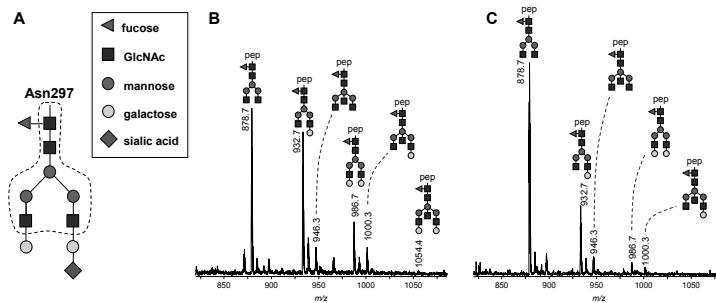
**Results** Compared to the pool of serum IgG<sub>1</sub>, ACPA IgG<sub>1</sub> lack terminal sialic acid residues. In synovial fluid, ACPA are highly agalactosylated and lack sialic acid residues, a feature that was not detected for total IgG<sub>1</sub> in synovial fluid. Moreover, differential ACPA glycan profiles were detected in RF-positive versus -negative patients.

**Conclusion** ACPA IgG<sub>1</sub> exhibit a specific Fc-linked glycan profile which is distinct from total serum IgG<sub>1</sub>. Moreover, Fc glycosylation of ACPA differs markedly between synovial fluid and serum. As Fc glycosylation directly affects the recruitment of Fc-mediated effector mechanisms, these data could further our understanding of the contribution of ACPA to disease pathogenesis.

## INTRODUCTION

Antibodies relevant to tissue pathology in autoimmune diseases are identified based on antigen binding specificity of the variable region. Only very few autoantibodies, however, mediate pathology by direct interaction with the antigen. In most other cases, the constant region (Fc part) determines antibody-mediated effector functions such as complement activation, antibody-dependent cellular cytotoxicity (ADCC) and engagement of activating or inhibitory Fc receptors. These Fc-mediated effects are influenced by the Fc part's amino acid sequence (i.e. antibody isotype and sub-class) and by Fc-linked carbohydrate structures. The latter are located in the C $\gamma$ 2 domain of the heavy chain in close vicinity to amino acids that interact with Fc receptors and the complement system. Accordingly, Fc-linked carbohydrate structures have recently received increasing attention, as modification of Fc-linked glycan residues of therapeutic antibodies has been shown to strongly influence the antibodies' therapeutic potential<sup>1-6</sup>.

The Fc part of human immunoglobulin G (IgG) carries two N-linked, highly heterogeneous glycan moieties interposed between the heavy chains. These carbohydrate structures are crucial for the stability of the antibody and influence its biological activity<sup>7-9</sup>. Attached to amino acid Asn297 of each heavy chain is a conserved, biantennary glycan backbone of N-acetylglucosamine (GlcNAc) and mannose residues, which is modified by core fucose, additional N-acetylglucosamine, galactose and terminating sialic acid residues to yield a large variety of glycoforms (Figure 1). The most prevalent glycoforms are characterized by



**Figure 1A:** Schematic depiction of a monosialylated glycan chain linked to Asn297 of the IgG heavy chain and legend for symbols used in this manuscript.

The dotted line indicates the conserved sequence of glycan residues. An additional sialic acid residue can be attached to the second galactose residue, and an additional N-acetylglucosamine residue ('bisecting GlcNAc') to the central mannose (not depicted).

**Figure 1B, 1C:** Typical mass-spectrometry profiles of IgG<sub>1</sub> tryptic glycopeptides obtained after isolation of ACPA from serum (B) and synovial fluid (C). Sum mass spectra of the elution range of the IgG<sub>1</sub> glycopeptides with neutral glycans are shown<sup>34</sup>. pep = peptide moiety; m/z = mass/charge.

presence or absence of galactoses, so called G0 (no galactose), G1 (one galactose) and G2 (two galactoses) glycoforms, which make up around 20-35%, 35% and 16% of all IgG Fc-linked glycoforms in healthy subjects, respectively <sup>7</sup>. Lack of galactose residues results in a concomitant lack of terminal sialic acid residues and increases the affinity for activating Fcγ receptors (FcγR) <sup>10, 11</sup>. In contrast, presence of the fully processed glycan structure terminating in sialic acid residues has anti-inflammatory effects <sup>1, 3</sup>. Presence of an additional, bisecting GlcNAc residue and absence of core fucose residues was found to enhance ADCC through high-avidity interaction with FcγRIIIa <sup>5, 12-14</sup>. Hydrolysis of the entire glycan backbone by glycosidase treatment or mutational deletion of the entire Asn297 N-linked glycosylation site leads to a change of the Fc part's quaternary structure. This yields a non-immunogenic antibody unable to interact with FcγR and, consequently, to elicit significant cytokine release <sup>2, 6, 8</sup>. Of interest, immune complexes containing such aglycosylated IgG fail to be eliminated rapidly from the circulation <sup>15</sup>.

In line with these structural observations, both human and murine studies indicate a strong impact of Fc-linked glycan variants on the pathogenic potential of IgG antibodies in inflammatory disorders such as rheumatoid arthritis. Early studies have demonstrated a predominance of IgG-G0 glycoforms in sera of RA patients that correlates with disease activity and reverses to normal levels in patients who undergo (spontaneous) remission <sup>16-19</sup>. Removal of galactose residues from murine IgG by β-galactosidase treatment resulted in increased arthritogenicity of transferred IgG in a murine arthritis serum transfer model <sup>20</sup>. Likewise, pregnancy-induced remission in the pristane-induced arthritis model was paralleled by a decrease of IgG-G0 glycoforms, while the postpartum period was accompanied by a respective increase in agalactosylated glycoforms <sup>21</sup>. While these studies have narrowed the focus on the presence or absence of galactose residues, more recent studies indicate that not a lack of galactose residues itself, but rather the concomitant absence of terminal sialic acid residues may be responsible for the enhanced inflammatory activity exerted by G0 glycoforms <sup>1, 3</sup>. Indeed, a receptor specifically recognizing IgG Fc-linked sialic acid residues has newly been described on a population of murine regulatory splenic macrophages, a cellular subset that may be involved in downregulation of inflammatory responses <sup>22</sup>. While these latter findings await confirmation in the human system, relevance of sialic acid residues for modulating immune responses is demonstrated by the finding that intravenous immunoglobulin preparations (IVIg) completely lose their immunosuppressive capacity upon removal of sialic acid residues by neuraminidase treatment <sup>1, 3</sup>. Together, these findings underline the importance of IgG Fc-linked glycan structures for antibody-mediated inflammatory responses.

Anti-citrullinated protein antibodies (ACPA) are highly specific autoantibodies for a subgroup of rheumatoid arthritis patients that suffer from severe erosive disease. A body of evidence points to a crucial role for ACPA in disease pathogenesis, but the pathogenic potential of ACPA and the mechanism by which ACPA could cause tissue pathology are

ill-defined. ACPA are present in serum years before the onset of clinical symptoms, ACPA production has been detected in synovial membrane explants, and increased ACPA levels were found in synovial fluid as compared to serum<sup>23-25</sup>. ACPA-positive RA patients suffer from more severe disease than patients without a citrulline-specific immune response, and presence of ACPA favours the development of overt RA in patients with undifferentiated arthritis<sup>26-29</sup>. Intriguingly, however, ACPA levels do not or only moderately correlate with disease activity, and depletion of CD20<sup>+</sup> B cells improves clinical symptoms while ACPA levels are only moderately affected<sup>30, 31</sup>. The observation that ACPA can be present without signs of inflammation, both before and during disease, raises the possibility that the quality rather than the quantity of the ACPA immune response influences disease. So far, studies have focussed on ACPA fine specificity and isotype usage, but data on ACPA Fc-linked glycan residues have not been obtained. In light of the strong influence of Fc-linked glycans on the inflammatory potential of antibodies, we have recently developed a technique that allows analysis of Fc-linked glycans in an antigen specific manner<sup>32</sup>. We have now used this technique to analyze Fc glycosylation of IgG<sub>1</sub>-ACPA in serum and synovial fluid of early arthritis patients in order to further characterize the immune response to citrullinated antigens.

## PATIENTS AND METHODS

### Serum and synovial fluid samples

Serum samples of 85 clinically well-defined, ACPA-positive arthritis patients participating in the Leiden Early Arthritis Clinic (EAC) were collected following informed consent of study participants and study-approval by the local institutional review board. The Leiden EAC is a population-based inception cohort that includes patients with self-reported symptom duration of  $\leq 2$  years<sup>33</sup>.

Paired serum and synovial fluid samples were collected from 11 additional ACPA-positive rheumatoid arthritis patients. 7 samples originated from the outpatient clinic of the Department of Rheumatology and Clinical Immunology at Charité Hospital in Berlin (Germany), 4 samples were collected at the respective outpatient clinic of the Department of Rheumatology of Leiden University Medical Center (The Netherlands).

ACPA positivity was determined based on reactivity of sera against a second generation cyclic citrullinated peptide (CCP2) in a commercially available ELISA system (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). Serum and synovial fluid samples were aliquoted and stored at -80°C until further use.

### Isolation of ACPA and total IgG from serum and synovial fluid samples

ACPA were isolated from total human serum and synovial fluid as previously described<sup>32</sup>. In brief, sera and synovial fluid samples were incubated in CCP2-coated ELISA plates (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) for 1 hour at 37°C. Supernatants were discarded and plates were washed thoroughly. Bound antibodies were eluted from the plates by adding 100mM formic acid (pro analysi for mass spectroscopy; Merck Darmstadt, Germany) for 15 minutes at room temperature. Eluates were collected in 96-well V-bottom plates, dried in a vacuum centrifuge and subjected to tryptic digest by adding 200ng trypsin (sequencing grade; Promega, Leiden, The Netherlands) in 40µl ammonium bicarbonate to each well followed by incubation at 37°C overnight. Digested samples were stored at -20°C until further use. Purity of eluted ACPA was verified by subjecting ACPA-negative control sera to the same isolation procedure. Non-trypsinized eluates of ACPA-negative samples were tested for the presence of human IgG by ELISA. None of the ACPA-negative eluates contained detectable amounts of IgG, indicating that only citrulline-specific IgG-molecules were eluted from the CCP2-plates (data not shown; for methodological details see<sup>32</sup>).

Purification of total IgG was achieved by incubating serum or synovial fluid samples with Protein A-sepharose beads (GE Healthcare, Eindhoven, The Netherlands) in 96-well filter plates (Multiscreen Solvintert, 0.45 µm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA) on a shaker for 1 hour. Beads were thoroughly washed and bound IgG-molecules (IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>4</sub>) were eluted into a 96-well V-bottom plate using 100mM formic acid. Samples were dried by vacuum centrifugation, digested with trypsin and stored at -20°C until further use<sup>32, 34</sup>.

### Fc glycosylation analysis

Analysis of Fc-linked N-glycans was performed as previously described<sup>32, 34</sup>. In brief, trypsinized samples obtained from the ACPA and total serum IgG isolation procedures were applied to an RP column (C18 PepMap 100 Å, 3 µm, 75 µm x 150 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate3000 nanoLC (Dionex/LC Packings). The LC system was coupled via an online nanospray source to an Esquire HCTultra ESI-IT-mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electron transfer dissociation module (PTM Discovery System™) and was operated in the positive ion mode. Ions from mass/charge (*m/z*) 600 to *m/z* 1800 were registered. The high-performance liquid chromatography method resulted in resolution of the glycopeptides based on the peptide moiety, with IgG<sub>1</sub> glycopeptides eluting first, followed by IgG<sub>4</sub> and IgG<sub>2</sub> glycopeptides. Glycopeptides with neutral glycan moieties tended to elute earlier than glycopeptides with antenna sialylation. Average mass spectra were generated over a 1-minute elution range for both the neutral and the acidic glycopeptides of each IgG subclass.

As the most frequent isotype in ACPA-positive RA patients is IgG<sub>1</sub>, only IgG<sub>1</sub> Fc-linked N-glycans were analyzed in this study. Furthermore, it is important to note that also the Fab-fragment of IgG-molecules can carry N-glycans<sup>35</sup>. Most previous studies have analyzed N-glycans released enzymatically from total human serum IgG, which results in a mixture of both Fc-linked and Fab-linked N-glycans. However, Fab-linked glycoforms can differ considerably from Fc-linked N-glycans, and vary between IgG subclasses<sup>36</sup>. In our approach, the specific mass of the Fc peptide portion obtained after tryptic cleavage of the IgG molecule allows to exclusively analyze Fc-linked N-glycans, whilst Fab-linked glycopeptides exhibit a heterogeneous group of signals that do not interfere with Fc glycosylation analysis at the glycopeptide level. Due to the exclusive analysis of Fc-linked N-glycans, the total number of glycoforms detected was lower than that of previous reports, and some glycoforms were not detected at all.

### Statistical analysis

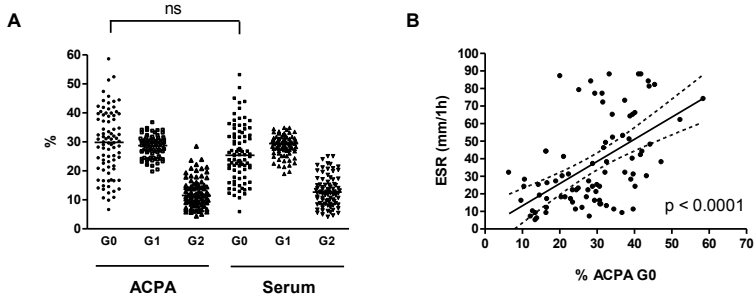
In order to account for measurement variability, the relative peak intensity of the glycoform containing one galactose residue (G1) identified by mass spectrometry was deliberately set at 1, and peak intensities of all other glycoforms were normalized accordingly. Frequencies of individual glycoforms are reported as percent of the total number of detectable glycoforms based on this normalization procedure. Differences in frequencies of glycoforms obtained from different samples were evaluated for statistical significance using the non-parametric Kruskal-Wallis test (in case of unpaired samples) followed by Dunn's post hoc test to adjust for errors introduced by multiple testing. Dunn's test generalizes the Bonferroni adjustment procedure for multiple testing to maintain the probability of a type-I error at  $\leq 5\%$ . For glycoforms of paired serum/synovial fluid samples, groups were compared using the non-parametric Mann-Whitney rank-sum test. This is indicated where appropriate. P-values  $<0.05$  were considered statistically significant. Data were analyzed using SPSS version 16.0.2 and GraphPad Prism Software 4.0.

## RESULTS

### Fc-linked galactosylation of ACPA IgG<sub>1</sub> isolated from human serum

Specific Fc glycosylation profiles of ACPA IgG<sub>1</sub> and respective profiles of total serum IgG<sub>1</sub> were obtained from 85 ACPA-positive Dutch early arthritis patients (mean age  $52.6 \pm 14.4$  years; 69.4% female). Based on previous observations reporting a predominance of agalactosylated glycoforms in patients with rheumatoid arthritis, we first analyzed frequencies of IgG<sub>1</sub> Fc-linked galactose residues (Figure 2). A median of 25.4% (IQR 19.8-32.3) of serum IgG<sub>1</sub> molecules were found to lack galactose residues, consistent with previous reports (Figure 2A)<sup>17</sup>. ACPA IgG<sub>1</sub> contained slightly higher frequencies of G0-glycoforms





**Figure 2: Fc galactosylation of ACPA IgG<sub>1</sub> and serum IgG<sub>1</sub>.**

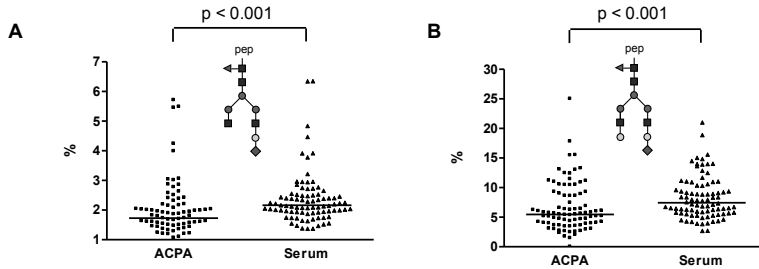
**Figure 2A:** Frequencies of G0 (no galactose), G1 (1 galactose) and G2 (2 galactoses) glycoforms for ACPA IgG<sub>1</sub> and serum IgG<sub>1</sub> as the percentage of all detected glycoforms. Horizontal lines indicate the medians.

**Figure 2B:** Correlation between the frequency of ACPA-G0 glycoforms and erythrocyte sedimentation rate (ESR). Dashed lines represent the 95% confidence interval of the linear regression (solid line). ns = not significant.

as compared to total serum IgG<sub>1</sub> (29.8% (IQR 20.9-38.8)), a nominal difference that did not remain significant after correction for multiple testing. Almost two thirds (62.4%) of patients carried ACPA IgG<sub>1</sub> that exhibited higher G0-frequencies than their respective serum IgG<sub>1</sub>. Patients of whom these ACPA were isolated did not differ significantly in age (data not shown), but did have slightly higher ESR than those with ACPA G0-frequencies below those of serum IgG<sub>1</sub> (median ESR 32.0 mm/1h (IQR 21.5 - 65.0) vs. 24.5 (IQR 15.5 - 40),  $p = 0.06$ ). This is in line with a linear correlation between the frequency of ACPA G0-glycoforms and ESR (Spearman  $\rho = 0.54$ ;  $p < 0.0001$ ; Figure 2B). A similar correlation, although weaker, was found for G0-glycoforms of serum IgG<sub>1</sub> (Spearman  $\rho = 0.38$ ;  $p < 0.0003$ ) and has been reported previously<sup>18</sup>. Importantly, the frequency of ACPA G0-glycoforms did not correlate with ACPA-titres ( $p = 0.28$ ; data not shown). Furthermore, no significant difference was noted between ACPA IgG<sub>1</sub> and total serum IgG<sub>1</sub> with respect to the presence of G1- or G2-glycoforms. Taken together, we observed a trend towards more agalactosylated glycoforms on ACPA IgG<sub>1</sub> than on serum IgG<sub>1</sub> that did not reach statistical significance at the available sample size.

### ACPA-specific Fc-linked sialylation and fucosylation patterns

Given the reported impact of sialic acid and core fucose residues on the biological activity of antibodies, we next determined the frequency of IgG<sub>1</sub> Fc-linked terminal sialic acid and core fucose residues (Figures 3 & 4). Absence of sialic acid residues increases the affinity for activating Fc $\gamma$ R, while presence of sialic acid accounts for anti-inflammatory effects, as has been shown for IVIG<sup>3,11</sup>. The frequency of sialic acid residues attached to ACPA IgG<sub>1</sub> was found to be significantly reduced as compared to serum IgG<sub>1</sub> for both G1- (Figure 3A; ACPA median 1.73% (IQR 1.36-2.16), serum median 2.16% (IQR 1.94-2.53);  $p < 0.001$ ) and G2-glycoforms (Figure 3B; ACPA median 5.47% (IQR 3.94-8.96), serum median

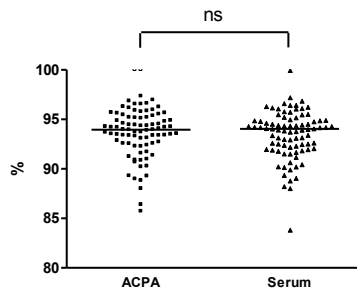


**Figure 3: ACPA-specific Fc-linked sialylation patterns.** Frequencies of monosialylated G1- (A) and G2-glycoforms (B) for both ACPA IgG<sub>1</sub> and serum IgG<sub>1</sub>. No Fc-linked disialylated glycoforms were detected. Horizontal lines indicate the medians. pep = peptide moiety (see Figure 1 for other definitions).

7.43% (5.82-10.44);  $p < 0.001$ ). As terminal sialic acid residues require galactose residues for linkage, sialic acid can only be detected on G1- or G2-, but not on G0-glycoforms. We did not detect glycoforms carrying two sialic acid residues in our samples.

Fucosylation strongly influences ADCC by modulating the Fc part's binding avidity to Fc $\gamma$ R11a. In the absence of core fucose residues ADCC is significantly increased<sup>13, 14, 37</sup>. The vast majority of IgG<sub>1</sub>-antibodies analyzed here contained core fucose residues with no significant overall difference between ACPA IgG<sub>1</sub> and total serum IgG<sub>1</sub> (Figure 4; ACPA median 93.9% (IQR 92.5-95.3); serum median 94.0% (IQR 92.1-94.9),  $p = 0.66$ ).

Overall, these data indicate that the glycosylation profile of ACPA IgG<sub>1</sub> differs considerably from total serum IgG<sub>1</sub> in the degree of sialylation.

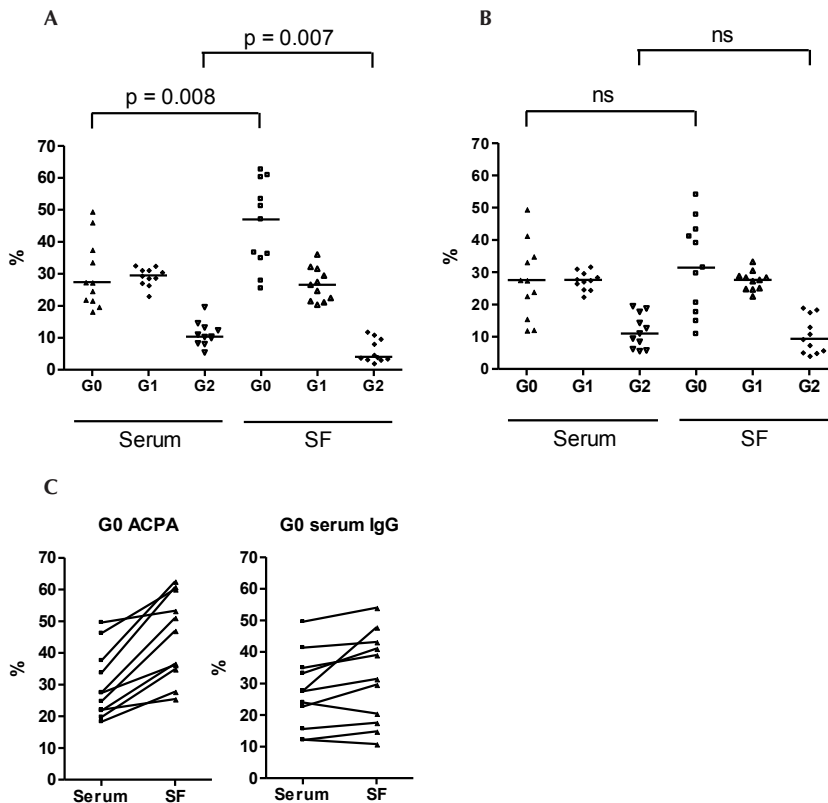


**Figure 4: ACPA-specific Fc-linked fucosylation patterns.** Overall frequency of fucosylated ACPA IgG<sub>1</sub> glycoforms (G0+G1+G2) versus fucosylated serum IgG<sub>1</sub> glycoforms (G0+G1+G2). Horizontal lines indicate the medians.

#### Fc-linked glycosylation of ACPA isolated from synovial fluid

RA is primarily characterized by synovial inflammation and progressive joint destruction. ACPA are thought to be involved in this process. However, the origin of ACPA circulating in serum is still unknown, and it remains to be determined to what extent serum ACPA contribute to synovial inflammation in the joint. In fact, ACPA titres in synovial fluid (SF) exceed those in serum, and accumulating evidence suggests that ACPA can also be gener-

ated locally by plasma cells within the synovial membrane<sup>24</sup>. So far, however, qualitative differences between serum ACPA and ACPA in SF have not been determined. In order to investigate whether Fc glycosylation differs between ACPA in SF and ACPA circulating in serum, we isolated ACPA from serum and synovial fluid of 11 paired samples. ACPA IgG<sub>1</sub> glycosylation profiles were compared to profiles of total IgG<sub>1</sub> isolated from the same compartments. In contrast to ACPA in serum, ACPA isolated from SF were found to be highly agalactosylated (Figure 5A; serum-ACPA: G0 median 27.4% (IQR 21.7-37.6), SF-ACPA: G0 median 47.0% (IQR 34.9-60.1), Mann-Whitney  $p = 0.008$ ). This finding was specific for ACPA IgG<sub>1</sub>, as no such predominance of G0-glycoforms was observed for the pool of total synovial fluid IgG<sub>1</sub> (Figure 5B; serum IgG<sub>1</sub>: G0 median 27.5% (IQR 15.6-35.0); SF-IgG<sub>1</sub>: G0 median 31.4% (IQR 17.6-43.2), Mann-Whitney  $p = 0.5$ ). Increased frequencies of ACPA G0-glycoforms in synovial fluid as compared to serum were observed in



**Figure 5:** ACPA-specific Fc glycosylation in serum versus synovial fluid.

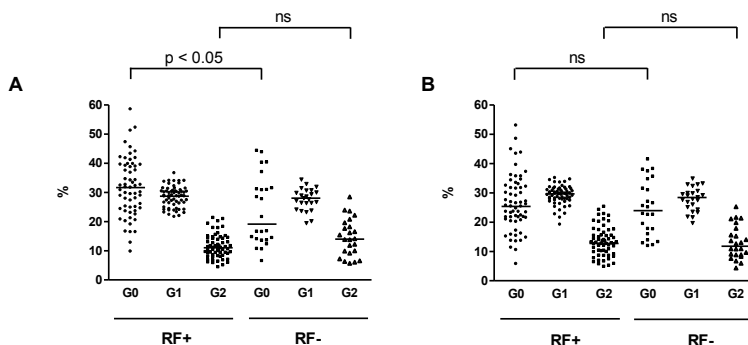
**Figure 5A and 5B:** G0-, G1- and G2-glycoforms of ACPA IgG<sub>1</sub> (A) and total serum IgG<sub>1</sub> (B) isolated from 11 paired samples of serum and synovial fluid of. Horizontal lines indicate the medians.

**Figure 5C:** ACPA IgG<sub>1</sub> and total serum IgG<sub>1</sub> G0-frequencies in serum and synovial fluid from each individual patient. Significance levels were determined by comparing individual groups using the non-parametric Mann-Whitney rank sum test.

all 11 patients analyzed (Figure 5C). In contrast, G0-frequencies of total synovial fluid IgG<sub>1</sub> were higher than in serum for some patients, but lower in others. Due to the lack of galactose residues, SF-ACPA also exhibited a significantly lower degree of sialylation (data not shown). All glycoforms examined in SF exhibited a high degree of fucosylation (up to 100%) with no significant difference between ACPA and total IgG<sub>1</sub> (data not shown).

In summary, ACPA IgG<sub>1</sub> in synovial fluid differ from ACPA IgG<sub>1</sub> in serum in terms of Fc glycosylation. Synovial fluid ACPA are highly agalactosylated and lack terminal sialic acid residues. This differential glycan profile appears to be a specific feature of ACPA, as no significant difference in Fc glycosylation was observed for total IgG<sub>1</sub> isolated from the same compartments.

Differential ACPA IgG<sub>1</sub> Fc glycosylation profile in RF-positive and -negative patients  
 Previous studies indicate that the specificity and positive predictive value of ACPA for RA are increased in the presence of rheumatoid factors (RF)<sup>25</sup>. Individuals with both ACPA and IgM-RF are significantly more likely to develop RA than those who test positive for ACPA only, suggesting that ACPA and RF directly or indirectly interact<sup>38, 39</sup>. Intriguingly, RF binding sites include the C $\gamma$ 2 domain of the Fc part of human IgG which is in close proximity to amino acid Asn297 to which the carbohydrate chains are attached. Therefore, it has been suggested that Fc glycosylation could influence RF-binding to IgG-Fc, and indeed fractions of RF exhibiting high affinity for agalactosyl IgG have been identified in RA patients<sup>40, 41</sup>. Based on these observations, we were interested in investigating whether ACPA Fc glycosylation differs between IgM-RF-positive and -negative patients (Figure 6). We observed a significant predominance of ACPA IgG<sub>1</sub> G0-glycoforms in RF-positive (n = 61; G0 median 31.6% (IQR 24.4-39.3)) as compared to RF-negative patients (n = 24; G0 median 19.1% (IQR 13.7-31.3), Figure 6A). This RF-dependent difference was only observed for ACPA, but not for total serum IgG<sub>1</sub> (Figure 6B). In order to exclude that the



**Figure 6: Differential ACPA Fc glycosylation in rheumatoid factor (RF)-positive and RF-negative patients.** Galactosylation profiles for ACPA IgG<sub>1</sub> (A) and total serum IgG<sub>1</sub> (B) molecules in IgM-RF-positive and -negative patients. Horizontal lines indicate the medians.

observed difference could be due to the co-elution of agalactosylated RF bound to ACPA during the ACPA isolation procedure, we also isolated ACPA from RF-negative/ACPA-positive sera after mixing them with RF-positive/ACPA-negative sera. Fc glycan profiles of ACPA isolated from these mixing experiments did not differ from the profiles obtained without additional RF (data not shown), making it unlikely that our results are confounded by RF bound to ACPA.

We also noted a significant lack of the sialylated ACPA-linked G2-glycoform in RF-positive patients (RF-positive: median 5.2% (IQR 3.6-6.5), RF-negative: median 8.6% (4.4-11.1),  $p = 0.038$ ), while the sialylated G1-glycoform was equally frequent in both subgroups (data not shown). No difference within these subgroups was observed for core fucosylation. Of note, RF-positive and -negative subgroups did not differ significantly in age or the degree of ESR-/C-reactive protein elevation (data not shown).

## DISCUSSION

N-glycans are crucial determinants of IgG Fc-mediated antibody effector functions. Modification of the Fc-linked carbohydrate backbone by addition of galactose, sialic acid, fucose and N-acetylglucosamine residues has a differential impact on Fc-mediated immune responses both in-vivo and in-vitro<sup>10-14, 20</sup>. Serum IgG molecules of RA patients have long been known to lack Fc-linked galactose residues when compared to serum IgG molecules of age-matched healthy controls, but whether antibodies of defined antigenic specificity differ in their degree of Fc glycosylation remained undetermined in these patients. Given recent insights in RA pathogenesis based on the identification of anti-citrullinated protein antibodies, we sought to determine whether these RA-specific autoantibodies exhibit specific Fc glycosylation profiles that would help to elucidate their role in disease pathogenesis.

Comparing Fc-linked glycosylation profiles of ACPA IgG<sub>1</sub> to the pool of total serum IgG<sub>1</sub> in early arthritis patients, we found a significant lack of sialic acid residues on ACPA IgG<sub>1</sub> molecules. Furthermore, we observed a non-significant trend towards a lower degree of ACPA-galactosylation which could result in an additional reduction in sialic acid residues. This finding points to an increased inflammatory potential of ACPA IgG<sub>1</sub> and is in line with the recent observation that sialic acid residues rather than galactose residues determine the anti-inflammatory activity of human IgG<sup>3</sup>. Sialylated IgG molecules have reduced affinity to activating FcγR and, at least in the mouse, have been described to bind to a specific receptor on regulatory macrophages in the spleen<sup>22</sup>. Likewise, a complete lack of Fc-linked sialic acid residues favours the pathogenic potential of IgG as demonstrated by the loss of anti-inflammatory activity of IVIG upon treatment with neuraminidase<sup>3</sup>. Thus, ACPA IgG<sub>1</sub> circulating in human serum exhibit, based on functional data from murine

studies, a more pro-inflammatory Fc glycosylation profile than the pool of total serum IgG<sub>1</sub>.

With regard to core-fucosylation, we found that ACPA IgG<sub>1</sub> G0-glycoforms were highly fucosylated, an observation previously reported for total serum IgG G0-glycoforms of RA patients when compared to healthy controls<sup>42,43</sup>. Based on in-vitro studies this high core fucose content is associated with a low potential to induce ADCC<sup>13</sup>, but the functional relevance for RA pathogenesis is currently unclear and warrants further study.

Extending our ACPA-specific glycan analysis to synovial fluid we observed both a reduction in ACPA IgG<sub>1</sub>-linked galactose residues as well as a concomitant absence of sialic acid residues. This lack of galactose and sialic acid residues was specific for ACPA, as no such difference in Fc glycosylation was observed for the pool of total IgG<sub>1</sub>. Although this finding is suggestive of an increased inflammatory potential of SF-ACPA as compared to serum ACPA, we cannot conclude that this ACPA Fc glycosylation profile is cause rather than consequence of inflammation. Despite this limitation, our observations demonstrate for the first time that ACPA in serum and in synovial fluid are not only quantitatively but also qualitatively different. Fc-linked glycan residues are unlikely to be enzymatically modified post-secretion in SF, as such modifications would be independent of antigen specificity and also affect the pool of total IgG in SF. Therefore, differential ACPA Fc glycosylation in SF as compared to serum suggests that SF-ACPA originate predominantly from specific B cell subsets in synovial tissue. Such ACPA-specific B cells could be under the influence of local cytokines that regulate glycan processing in plasma cells susceptible to the respective cytokine signal. Local ACPA-production has been postulated previously and is in line with the histological presence of germinal centers in synovial tissue of RA patients<sup>23,24,44</sup>. RA B and T cells are known to exhibit reduced enzymatic activity of  $\beta$ 1, 4-galactosyltransferase, the enzyme responsible for adding galactose residues to terminal GlcNAc residues in N-glycans<sup>45,46</sup>. Whether even more complex differences in N-glycan processing exist among B cell subsets in different compartments in RA is unknown. In the context of RA and other autoimmune disorders such as SLE, it would be of particular interest to investigate whether newly generated and long-lived plasma cells differentially process Fc-linked N-glycans.

We noted differential ACPA Fc glycosylation profiles in IgM-RF-positive versus -negative patients. Serum-ACPA of RF-positive patients lacked galactose residues when compared to ACPA isolated from RF-negative patients. This differential Fc glycosylation profile was only observed for ACPA but not for total serum IgG, the latter being in line with previous results<sup>47</sup>. Our finding is intriguing, as it fuels the hypothesis of an interaction between ACPA and RF. ACPA associate with RA independently of RF, but the risk to develop RA increases markedly (>30 fold) if RF are additionally present. In contrast, IgM-RF alone do not seem to associate with RA in the absence of ACPA<sup>39</sup>. RF binding sites have been mapped to the C $\gamma$ 2 and C $\gamma$ 3 domains of human IgG. Not all RF recognize the same antigenic

determinant, but at least one binding site involves Asn297 which carries the Fc-linked N-glycans <sup>41</sup>. Thus, it has been postulated that Fc glycosylation can influence RF-binding affinity, and indeed RF with high affinity for agalactosylated IgG were repeatedly identified in RA patients <sup>40, 41</sup>. Moreover, IgG aggregates in synovial fluid of RA patients were found to contain high amounts of agalactosylated IgG, suggesting that a lack of galactoses facilitates association of IgG molecules <sup>48</sup>. It is conceivable that lack of galactose residues leads to a conformational change in IgG-Fc structure <sup>9</sup> which could favour high affinity RF binding and even reveal novel epitopes that promote the generation of high affinity RF. So far, binding activity of IgG-RF was found to increase with decreasing RF Fc galactosylation (and -sialylation), compatible with the idea that Fc-glycosylation influences “antigenicity” of the Fc-tail <sup>49</sup>. Whether RF directly or even preferentially also bind to ACPA that lack galactose residues cannot be concluded from our data, but our data suggest that the IgG recognized by RF exhibits a similar glycosylation profile to the one detected on ACPA. Thus, it is intriguing to speculate that ACPA G0-glycoforms help in the generation of RF with high affinity for agalactosylated IgG, thereby facilitating immune complex formation and complement activation.

Finally, as others before, we observed a strong correlation between the frequency of serum IgG-G0 glycoforms and ESR as well as age (data not shown). This association was even more pronounced for ACPA-G0 glycoforms (Figure 2). Of note, we accounted for these correlations in our subgroup analyses, rendering it unlikely that our results are influenced by these confounding factors.

In summary, we describe a detailed analysis of Fc-linked glycosylation profiles specific for ACPA. Fc glycosylation profiles differ between antibodies of different antigenic specificities and between antibodies isolated from different compartments. These data enhance our understanding of the citrulline-specific immune response and may open up novel strategies for therapeutic interventions.

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