

## **The extensive glycosylation of the ACPA variable domain observed for ACPA-IgG is absent from ACPA-IgM**

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### **Note:**

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Recently, we described the presence of highly sialylated *N*-linked glycans in the antigen-binding fragment (Fab) of almost all anti-citrullinated protein antibody (ACPA) IgG molecules [1, 2]. These glycans could not be found on several other autoantibody systems analysed. Given the low affinity of ACPA [3], this observation raises the intriguing possibility that citrullinated antigen-specific B cells could be selected based on the presence of glycans in the variable domain, rather than on affinity for their cognate antigen. *N*-glycosylation requires the presence of specific consensus sequences in the amino acid backbone of proteins [4]. However, only few human germline Ig variable region genes encode for such sequences [5]. So far, we could identify several *N*-glycosylation sites in ACPA-IgG Fab-domains using mass spectrometry, but none of these were encoded in the germline sequence [1]. This suggests that the extensive presence of *N*-glycans in ACPA-IgG Fab-domains results from somatic mutations. Moreover, it indicates that the ACPA response matures under the influence of T-cell help, presumably in germinal centres, and makes it conceivable that the introduction of *N*-glycosylation sites might be a crucial step by which tolerance checkpoints are breached. In this respect, it is relevant to note that ACPA are frequently present before the onset of clinical symptoms, and that recent epidemiological data indicate that the HLA-region is not a genetic risk factor for the development of ACPA, but associates strongly with ACPA-positive RA [6, 7]. Thus, the HLA-region, and thereby T-cells, contribute primarily to the maturation of the ACPA response rather than to its presence. If correct, additional glycosylation of the variable domain should be absent from ACPA-IgM. Here, we tested this hypothesis to gain insight in the biological processes underlying the extensive Fab-glycosylation of ACPA-IgG. ACPA-positive sera from patients with established RA were fractionated by size using gel-filtration chromatography and tested for the presence of ACPA isotypes by ELISA [1]. Furthermore, ACPA were affinity-purified from synovial fluid and plasma followed by the heavy chain detection of ACPA isotypes by western blot [8]. Finally, the presence or absence of sialylated Fab-glycans on ACPA was tested by *Sambucus nigra* lectin (SNA) chromatography [9]. ACPA-IgG and -IgG1 consistently showed an increased molecular weight compared to non-citrulline specific IgG, consistent with the presence of Fab-glycans

(figure 1A, B [1]). In contrast, ACPA-IgM were identical in size compared to their non-citrulline specific counterparts (figure 1B). ACPA-IgG Fab-glycans are highly sialylated [2] and therefore likely to interact with SNA. Consequently, ACPA-IgG could be strongly enriched upon SNA-purification, while ACPA-IgM remained in the SNA-negative fraction (figure 1C) indicating the absence of such glycan-species from ACPA-IgM. We conclude that the absence of a molecular “size-shift” for ACPA-IgM suggests that this ACPA isotype lacks additional glycosylation in the variable region. Unlike ACPA-IgG, ACPA-IgM seems to have an overall lower degree of sialylation independent of Fab glycosylation. These results are compatible with the notion that the acquisition of Fab-glycans in the variable domain of ACPA-IgG reflects a T-cell dependent process in the development of citrullinated antigen-specific B cells in RA. In future studies, it will be crucial to understand the potential selective survival signals these glycans confer on ACPA-expressing B cells.

**Figure 1. Molecular size analysis of ACPA-IgG and -IgM.** (A) Presence of ACPA-IgG (black dots) and non-citrulline specific IgG (white dots) in gel-filtration chromatography fractions (lower fraction number indicates larger size; representative example of 7 donors). (B) Western blot of ACPA and non-citrulline specific control Ig isolated from patients (Pt) with established RA and stained for IgM, IgG and IgG1 (n=7). (C) Ratio of ACPA-IgM and -IgG (in AU) per non-citrulline specific IgM and IgG (in  $\mu\text{g}$ ) in SNA-positive (SNA+) and -negative (SNA-) fractions (obtained using sera from n=8 additional donors; non-parametric Wilcoxon test for matched-pairs, \*\*  $p < 0.01$ ).

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## **Contributorship**

AK contributed to the design of the study, the acquisition, interpretation and analysis of data, and wrote the manuscript. LH participated in the analysis and interpretation of data. AD and EM participated in the acquisition, analysis and interpretation of data. FvdB and TR provided SNA samples and participated in the interpretation of data. MvO provided synovial fluid samples. LT and TH contributed to the conception and design of the study, and the interpretation of data. RT and HUS contributed to the conception and design of the study, interpretation of data, and wrote and revised the manuscript. All authors read, revised and approved the final manuscript.

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## **Ethical approval**

Sample acquisition for the study was approved by the LUMC ethical review board. Patients gave written informed consent for participation.

## **Data sharing statement**

Data presented as “data not shown” or additional data for which only representative examples are provided in this manuscript are available from the authors upon request.



Figure 1.

