Elevated Fab glycosylation of anti-hinge antibodies

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Objective: Rheumatoid arthritis (RA) is characterized by systemic inflammation and the presence of anti-citrullinated protein antibodies (ACPAs), which contain remarkably high levels of Fab glycosylation. Anti-hinge antibodies (AHAs) recognize immunoglobulin G (IgG) hinge neoepitopes exposed following cleavage by inflammation-associated proteases, and are also frequently observed in RA, and at higher levels compared to healthy controls (HCs). Here, we investigated AHA specificity and levels of Fab glycosylation as potential immunological markers for RA.

Method: AHA serum levels, specificity, and Fab glycosylation were determined for the IgG1 hinge cleaved by matrix metalloproteinase-3, cathepsin G, pepsin, or IdeS, using enzyme-linked immunosorbent assay and lectin affinity chromatography, in patients with early active RA (n = 69) and HCs (n = 97).

Results: AHA reactivity was detected for all hinge neoepitopes in both RA patients and HCs. Reactivity against CatG-IgG₁-F(ab')₂ and pepsin-IgG₂-F(ab')₂ was more prevalent in RA. Moreover, all AHA responses showed increased Fab glycosylation levels in both RA patients and HCs.

Conclusions: AHA responses are characterized by elevated levels of Fab glycosylation and highly specific neoepitope recognition, not just in RA patients but also in HCs. These results suggest that extensive Fab glycosylation may develop in response to an inflammatory proteolytic microenvironment, but is not restricted to RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder marked by joint erosions. This state of chronic inflammation generates a plethora of neoepitopes derived from inflammation-associated post-translational protein modifications that can be targeted by an assembly of autoantibodies known as the anti-modified protein antibodies (AMPAs). Of these, the anti-citrullinated protein antibodies (ACPAs) have been characterized extensively and provide an indication of the disease course, disease activity, and treatment strategy (1). ACPAs show cross-reactivity between different citrullinated proteins and peptides and a striking increase in Fab glycosylation (> 90% vs 15–25% on total immunoglobulin G (IgG)) (2, 3). During inflammation and infection, endogenous [e.g. matrix metalloproteinases (MMPs) and cathepsins] or exogenous proteases [e.g. IgG-degrading enzyme of Streptococcus pyogenes (IdeS), produced by infectious microbes] are abundantly expressed and able to cleave IgG at protease-specific locations within the hinge region into an F(ab')₂ and an Fc fragment (4, 5). Another type of AMPA, the anti-hinge antibodies (AHAs), specifically recognizes neoepitopes exposed in the hinge of proteolytically cleaved IgG molecules, which are absent in intact IgG (5, 6). AHAs are found more frequently in RA patients than in healthy controls (HCs) (7). In particular, a predominant, subclass-specific reactivity for the pepsin/MMP-7-cleaved IgG₄ hinge was observed in a subset of RA patients, which was essentially absent in HCs (8, 9). Increased levels of several MMPs and cathepsins have been demonstrated in the synovial fluid of RA patients. However, AHA responses against IgG cleaved by RA-associated proteases MMP-3 or cathepsin G (CatG) have not been studied in RA. Furthermore, whether AHAs, like ACPAs, also have aberrant Fab glycosylation levels is unclear.

Here, we study AHA responses that may play a role in RA pathology and investigate their potential as immunological markers for RA. Therefore, the levels and specificity of AHAs directed against inflammation (MMP-3, CatG, pepsin) and infection (IdeS)-related IgG hinge neoepitopes and their levels of IgG Fab glycosylation were studied in patients with early active RA.
Method

Patients

In this study, we included 69 baseline serum samples from individuals diagnosed with active early rheumatoid arthritis (RA) who were included in the ‘COBRA-light’ trial (10). These patients had not been treated with disease-modifying anti-rheumatic drugs during baseline sample collection. RA was diagnosed according to the 1987 criteria of the American Rheumatism Association (11). To validate the increased reactivity for CatG-IgG1-F(\(ab\)\(^{-}\))\(_{2}\) in RA (Figure 1C) an additional RA cohort was measured, composed of 100 baseline serum samples from consecutive RA patients with established disease, with a median disease duration of 8 years (interquartile range 3–16 years) and a mean 28-joint Disease Activity Score (DAS28) of 5.2 (12). These patients have not been treated with biologics but, in most cases, have been treated with conventional anti-rheumatic drugs, such as methotrexate. Serum samples from 97 randomly selected healthy individuals were included in all measurements except for Figure 1(C). Here, another 100 HCs were measured to validate CatG-IgG1-F(\(ab\)\(^{-}\))\(_{2}\) reactivity.

Ethical approval

RA patients gave written informed consent for the use of serum samples and clinical data. No informed consent was obtained for the samples from the HCs, because materials used for this study were leftovers from samples taken for routine diagnostic purposes. HC materials were used anonymously without any connection to clinical or person-specific data.

Antibodies

The anti-biotin IgG1/IgG3/IgG3/IgG4 were generated in house as described previously (13). In brief, synthetic variable heavy (\(V_{H}\)) and variable light (\(V_{L}\)) DNA constructs were separately cloned into pcDNA3.1 expression

![Figure 1](https://example.com/figure1.png)

Figure 1. Presence and levels of anti-hinge antibody (AHA) reactivity in rheumatoid arthritis (RA) patients and healthy controls (HCs). (A) Schematic representation of two alternative methods for F(\(ab\)\(^{-}\))\(_{2}\) generation. Amino acid sequences of immunoglobulin G\(_{1}\) (IgG\(_{1}\)) and IgG\(_{4}\) lower hinge regions with assigned cleavage sites for matrix metalloproteinase-3 (MMP-3) (1), cathepsin G (CatG) (2), pepsin/MMP-7 (3), and IdeS (4). (B) Scatterplots showing the levels of AHA reactivity against six different F(\(ab\)\(^{-}\))\(_{2}\) targets generated by four different proteases: MMP-3, CatG, pepsin-, and IdeS-IgG\(_{1}\)-F(\(ab\)\(^{-}\))\(_{2}\)s, and pepsin- and IdeS-IgG\(_{4}\)-F(\(ab\)\(^{-}\))\(_{2}\)s determined using the AHA enzyme-linked immunosorbent assay in early RA patients (n = 69) and HCs (n = 97). (C) Scatterplot showing CatG-IgG1-F(ab\(^{-}\))\(_{2}\) reactivity in additional validation cohorts of established RA patients (n = 100) and HCs (n = 100). Dashed lines represent cut-off levels at 4 AU/mL for positivity. Differences in AHA levels between RA patients and HCs were analysed with a Mann–Whitney U-test. The tables show the frequency of AHA positivity (% pos.) in early RA and HCs for the six different F(\(ab\)\(^{-}\))\(_{2}\) targets and for the CatG-IgG\(_{1}\) validation cohorts at a cut-off of 4 AU/mL.

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vectors containing the corresponding constant domain gene segments, and cotransfected in HEK293F cells. After a 5 day incubation at 37°C in humidified 8% CO₂, antibodies were purified from the culture supernatant using protein G Sepharose (ThermoFisher, Waltham, MA, USA). For inhibition experiments, we used the therapeutic antibodies adalimumab (IgG1, Humira; AbbVie, Lake Bluff, IL, USA) and natalizumab (IgG4, Tysabri; Biogen Idec International, Cambridge, MA, USA).

Generation of proteolytically cleaved F(ab’)_2 fragments

IgG₁ and IgG₄ F(ab’)₂ fragments yielded by proteolysis with either pepsin (isolated from porcine gastric mucosa; Sigma-Aldrich, St. Louis, MO, USA) or IdeS (Streptococcus pyogenes; FabRICATOR; Genovis, Lund, SE) were generated as described previously (1). Non-digested IgG was removed with a HiTrap protein G or A column. Purified F(ab’)₂ fragments were dialysed against phosphate-buffered saline (PBS).

Generation of recombinant F(ab’)₂ fragments

Besides proteolytically cleaved F(ab’)₂ fragments, recombinant F(ab’)₂ fragments were also produced. Synthetic V₄₄ and V₄₁ DNA constructs were separately cloned into pcDNA3.1 expression vectors containing the corresponding constant CH₁ and κ (light-chain) domain gene segment, and cotransfected in HEK293F cells. After a 5 day incubation at 37°C in humidified 8% CO₂, F(ab’)₂ fragments were purified from the culture supernatant using a-κ Sepharose (CaptureSelect; ThermoFisher). Fab by-products were extracted from the purified F(ab’)₂ fragments by size-exclusion chromatography (Figure S1A). The size and purity of all proteolytically cleaved and recombinantly expressed F(ab’)₂s were evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S1B) by loading 5 µg F(ab’)₂s on precast 4–12% Bis-Tris gels (NuPAGE; ThermoFisher) and visualized with Coomassie Blue. When the AHA reactivities for proteolytically cleaved IgG₁-F(ab’)₂ s versus recombinantly expressed IgG₁-F(ab’)₂s of the same protease were compared in a group of 86 HCs, they were highly similar (Figure S2).

Synthetic peptides

Synthetic peptide analogues ( pep) of the IgG₁4 lower hinge were designed from the C-terminal amino acids exposed by the protease (MMP-3, CatG, pepsin, or IdeS) up to 14 amino acids upstream. Synthetic peptides were ordered and produced at GenScript, Piscataway, NJ, USA (Table S1), and dissolved in ultrapure H₂O and pH adjusted to pH 7–8 using 4 M NaOH. In inhibition experiments, peptides were preincubated with the serum samples for 1 h before testing in the AHA ELISA, or peptides were used as the coating antigen in the AHA peptide ELISA.

AHA ELISA

Levels of serum AHAs in RA patients and HCs were measured as described previously (9). To reduce assay background reactivity, PBS/0.02% Tween-20 was replaced by high-performance ELISA (HPE) buffer (Sanquin Reagents, Amsterdam, the Netherlands) in the F(ab’)₂, serum, and IgG–horseradish peroxidase (HRP) incubation steps. In brief, 96-well plates were coated with biotinylated human serum albumin (HSA-biotin), washed, and pepsinized with either proteolytically cleaved or recombinantly expressed anti-biotin F(ab’)₂ fragments. After washing, plates were incubated with serum samples (diluted 1:400) and then washed, and AHAs were detected using mouse anti-human IgG-HRP (MH16-1; Sanquin) and developed using tetramethylbenzidine (TMB) substrate. The reaction was stopped by the addition of 0.2 M H₂SO₄ and absorbance was measured at 450 and 540 nm. AHA concentrations were calculated relative to a calibration curve of a reference serum showing high reactivity for pepsin-IgG₁-F(ab’)₂ [defined to contain 200 AU/mL (8)] present on every plate. This calibration curve was used to determine the AHA concentration of all studied AHA reactivities as we observed that all reactivities diluted equally. As the calculated values are non-proportional they were expressed in arbitrary units per millilitre (AU/mL). For most subjects tested, a serum dilution of 1:400 gave AHA levels within the dynamic range of the assays. When necessary, samples were tested at higher dilutions to reach the dynamic range. The cut-off for AHA positivity was determined by inhibition experiments using a selection of sera, including both positive and negative for a specific AHA reactivity, as described before (9). Cut-offs were set at the level where all positive sera were negative after inhibition. AHA ELISAs for the different AHAs had similar background reactivities, as expected, since F(ab’)₂ targets are highly similar and only differ by a few amino acids. This resulted in a consistent cut-off value across all F(ab’)₂ targets of 4 AU/mL. Individuals who showed high reactivity to the HSA-biotin coat were excluded from the data set (HC one sample; RA four samples). The reproducibility of the AHA ELISA is shown in Figure S3 as an example for anti-CatG-IgG₁-F(ab’)₂ reactivity in RA patients (r = 0.87****).

AHA peptide ELISA

To improve the specificity of the AHA ELISA, we exchanged the anti-pepti pF(ab’)₂ fragments for biotinylated synthetic peptides (GenScript) (Table S1). Streptavidin-coated ELISA plates (ThermoFisher) were prewashed and pepsinized with 0.1 µg/mL biotinylated synthetic peptide in PBS for 1 h and washed. Subsequent
sample incubation and detection were performed in the identical way as described above for the AHA ELISA. For the CatG-IgG1 and pepsin-IgG4 peptide ELISAs shown in Figure 2(D, E), samples were diluted 1:400 in HPE buffer enriched with 25 µg/mL pepsin-cleaved adalimumab (IgG1) to prevent detection of cross-reactive pepsin-IgG1-AHAs. Detection and quantification were performed as described above for the AHA ELISA.

Detection of Fab glycosylation on AHAs

We analysed the AHA Fab glycosylation levels for individuals who showed a moderate to high AHA level. Fab glycosylated AHAs were purified from serum as described previously (14), using *Sambucus nigra* agglutinin (SNA) affinity chromatography, which makes use of the fact that the SNA lectin enriches for Fab glycans, but not for Fc glycans, through

Figure 2. Specificity of anti-hinge antibody (AHA) responses in rheumatoid arthritis (RA) patients and healthy controls (HCs). (A) Specificity determined for each reactivity (indicated above each panel) in five early RA patients (upper graph) and five HC (bottom graph) with established positivity for the tested F(ab')2 target. Serum is preincubated with excess amounts of synthetic peptides (pep; Table S1) or F(ab')2’s (generated by cleaving therapeutic antibodies [adalimumab, immunoglobulin G1 (IgG1); natalizumab, IgG4] with pepsin or IdeS) and tested in the AHA enzyme-linked immunosorbent assay. Obtained optical density (OD) values were normalized to the situation without inhibitor. Bars indicate the means of five samples. Inhibitors are generated from the IgG1 sequence unless stated otherwise in the legend. (B) Inhibition of AHA reactivity against CatG-IgG1-F(ab')2 using pepsin-cleaved adalimumab (IgG1) in early RA patients (n = 69) and HCs (n = 97). (C) As in (B) but for pepsin-IgG1-F(ab')2 reactivity. (D) AHA reactivity for CatG-IgG1-F(ab')2 versus CatG-IgG1-pep in early RA patients (n = 69) and HCs (n = 97). (E) As in (D) but for anti-pepsin-IgG4 reactivity. Similarity for F(ab')2 versus peptide reactivity was determined using Spearman’s rank correlation coefficient (r); ****p < 0.0001.

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their terminal 2,6-linked sialic acid residues. SNA affinity chromatography provides a good estimate for the level of Fab glycosylation as more than 90% of Fab glycans carry terminal sialic acid residues (14–18). In brief, RA patient and HC serum samples were applied over an SNA column. The bound fraction of sialic acid containing proteins was eluted with 0.5 M lactose in 0.2 M acetic acid and dialysed against PBS. In the AHA ELISA set-up, the percentages of Fab glycosylated AHAs were calculated by dividing the amount of AHAs in the SNA-enriched fraction by the amount of AHAs in the combined SNA-depleted and SNA-enriched fractions. AHA concentrations in both SNA-depleted and SNA-enriched fractions were determined using the calibration curve as described above for the AHA ELISA. As reference measurements, the levels of IgG Fab glycosylation for total IgG (pan-IgG) and anti-tetanus toxoid (TT), a typical vaccine antigen which is often detectable in the general population, were also determined for all samples. To confirm the presence of Fab glycans, size-exclusion chromatography was performed on sera that showed normal, moderate, and high SNA binding. Sera were fractionated using a high-performance liquid chromatography column (Agilent 1260 Infinity II) equipped with a Superdex 200 (GE Healthcare, Chicago, IL, USA), and analysed by ELISA to detect total IgG and AHAs using in-house protocols, as described above and previously (14).

Statistical analysis

Differences between groups were analysed with a Mann–Whitney U-test or a Kruskal–Wallis test, and (non-parametric) correlations were analysed with a Spearman rank correlation test. A p value < 0.05 was considered significant. The statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

Results

Anti-hinge antibodies that target cathepsin G-IgG₁-F(ab’)₂s are more frequent and have higher levels in RA

We evaluated the level of AHA reactivity for a panel of IgG hinge neoeptopes in baseline serum samples from 69 treatment-naïve consecutive RA patients with active disease (< 2 years) (10) and 97 HCs. To obtain a homogeneous fraction of F(ab’)_2s with defined C termini, F(ab’)_2s were recombinantly expressed with truncated hinge regions, representing IgG₁ or IgG₄ proteolytically cleaved by MMP-3, CatG, pepsin, or IdeS (Figure 1A; Figure S1A, B) (5). AHA reactivity for the RA-associated proteases MMP-3- and CatG-IgG₁-F(ab’)₂s could be detected in both RA patients and HCs (Figure 1B). AHA reactivity was also observed for pepsin- and IdeS-IgG₁₄-F(ab’)₂s, in line with previous results (9). Reactivity against CatG-IgG₁-F(ab’)₂s was most frequent for both, but more prevalent in RA patients than in HCs (57% vs 44%, fold difference 1.3), and with significantly higher levels (p = 0.03). Pepsin-IgG₄-F(ab’)₂ reactivity was also more prevalent in RA, as observed previously. The increased AHA level and frequency for CatG-IgG₁-F(ab’)₂s in RA were validated in an additional cohort of established RA patients (11) and HCs (Figure 1C) (p = 0.0003; fold difference 2.4).

Anti-hinge antibodies are predominantly 'protease restricted'

Next, we evaluated the specificity of AHAs targeting IgG hinge neoeptopes. Pairwise comparisons of all AHA reactivities revealed that single positivity was found more frequently than double positivity (Figure S4). Inhibition experiments were performed to evaluate more extensively the specificity of AHA responses in RA patients and HCs. With few exceptions, little to no inhibition of AHA binding was observed using either excess amounts of F(ab’)_2s or single-chain hinge peptides with a non-identical C-terminus (Figure 2A; Figure S5, Table S1). By contrast, identity inhibitor(s) invariably showed strong inhibition. In some cases, cross-reactivity was observed between AHAs recognizing MMP-3- and CatG-IgG₁-F(ab’)₂s, as well as between pepsin- and CatG-IgG₁-F(ab’)₂s. Also, some cross-reactivity was observed for the AHA response against pepsin-IgG₁-F(ab’)₂s versus pepsin-IgG₄-F(ab’)₂s in HCs.

As both CatG-IgG₁ and pepsin-IgG₄ reactivities are more prevalent in RA, we examined whether a more RA-specific subset of these reactivities may be detected by performing the AHA ELISAs for both targets while including a pepsin-IgG₄-F(ab’)₂ inhibitor in a larger panel of subjects. For CatG-IgG₁-F(ab’)₂ reactivity (Figure 2B), we found little evidence for cross-reactivity in either HCs or RA patients. On the other hand, for pepsin-IgG₄-F(ab’)₂s, we observed cross-reactivity in most HCs (five out of nine) versus fewer in RA patients (five out of 24) (Figure 2C). We also compared neoeptope reactivity to single-chain peptide versus double-chain F(ab’)₂. We observed a moderate correlation for AHA binding to the peptide versus the F(ab’)_2 in RA (CatG-IgG₁: r = 0.67, p < 0.0001; pepsin-IgG₄: r = 0.61, p < 0.0001) (Figure 2D, E), while for HCs, the correlation was weaker (CatG-IgG₁: r = 0.49, p < 0.0001) or absent (pepsin-IgG₄: r = 0.17, p = 0.086), indicating a qualitative difference in pepsin-IgG₄ AHAs between HCs and RA patients, in addition to a quantitative difference.

Elevated levels of Fab glycosylation on anti-hinge antibodies

To estimate IgG Fab glycosylation levels on AHAs, we fractionated sera from RA patients and HCs using SNA affinity chromatography, making use of the fact that SNA enriches for Fab glycans, but not for Fc glycans.
through the terminal 2,6-linked sialic acid residues found in > 90% of Fab glycans (14–18), and determined the AHA IgG SNA-bound fraction by ELISA. A significant increase in IgG sialylation was observed across all AHA responses compared to reference total IgG (pan-IgG, RA 14%, HCs 11%) and anti-TT (RA 16%, HC 14%), with high interpatient variability for most IgG hinge neoepitopes (Figure 3). No significant difference was observed when AHA responses were compared between RA patients and HCs, and AHA levels and IgG sialylation levels did not correlate (Figure S6). The presence of Fab glycans on AHAs was confirmed by size-exclusion chromatography and subsequent ELISA (Figure S7). Thus, elevated IgG Fab glycosylation is a hallmark of AHAs in both RA patients and HCs, and was found to be increased for both infection (IdoS)- and inflammation (MMP-3, CatG, and pepsin)-associated AHA responses.

**Discussion**

In this study, we explored the molecular characteristics of AHAs from RA patients compared with HCs. We found that both RA patients and HCs showed reactivity against all evaluated IgG-F(ab’)_2S with rather restricted specificity and limited cross-reactivity with F(ab’)_2S generated by different proteases. Reactivity against CatG-IgG1-F(ab’)_2S and pepsin-IgG4-F(ab’)_2S was found more frequently and with higher levels in RA patients. Strikingly, all AHA responses were characterized by elevated Fab glycosylation, not only in RA patients but also in HCs. The higher frequency of CatG-IgG1-F(ab’)_2 reactivity in RA patients may be explained by increased levels and activity of cathepsin G in the synovial fluid of these patients, resulting in high levels of immunogenic CatG-cleaved IgG (19). However, this was not observed for MMP-3, the levels and activity of which are also increased in RA patients. Differential AHA levels may be caused by a difference in immunogenicity towards the exposed hinge neoepitope or the context in which these autoantibodies develop (e.g., severity of inflammation). To study the relationship between AHA levels and disease activity (DAS28), associations with other autoantibodies or inflammation (C-reactive protein levels), and to differentiate patient subgroups, a larger group of subjects will be necessary.

Besides higher frequencies and levels for both CatG-IgG1-F(ab’)_2S and pepsin-IgG4-F(ab’)_2S in RA, AHAs in RA patients also recognized these neoepitopes with higher specificity as they showed less cross-reactivity with pepsin-IgG1-F(ab’)_2S and were more efficient in recognizing the single-chain peptide, compared to AHAs in HCs. This suggests that AHAs in HCs do not solely recognize the neoepitope and that the double-chain conformation of the antibody C-terminus is crucial for epitope recognition. Thus, the AHAs produced by RA patients and HCs differ both quantitatively and qualitatively. Importantly, specific recognition of pepsin-IgG4-F(ab’)_2S is rare in HCs, which implies its potential as an immunological marker for RA.

All AHA responses show elevated Fab glycosylation levels, albeit with high interpatient variability and being less elevated on average (55%) than observed for ACPAs, which are prevalent autoantibodies in RA with high levels of Fab glycans (> 90%) (3). For another prevalent autoantibody in RA, rheumatoid factor (RF), Fab glycosylation levels have not been determined. However, analysis of RF variable region sequences (20) revealed that most non-isotype switched

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**Figure 3.** Levels of Fab glycosylation on anti-hinge antibodies in rheumatoid arthritis (RA) patients and healthy controls (HCs). Graphs show the percentage of immunoglobulin G (IgG) Fab glycosylated antibodies for total IgG (pan-IgG), anti-tetanus toxoid (TT), and AHAs directed against the six different IgG1=F(ab’)_2 targets for a total of 24 early RA patients and 27 HCs who tested seropositive for the respective F(ab’)_2 target. *Sambucus nigra* agglutinin (SNA; lectin) affinity chromatography of serum results in a sialic acid-positive fraction (enriched for Fab glycosylated IgG) and a sialic acid-negative fraction (almost devoid of Fab glycosylated IgG) (14). Specific and total IgG was measured in both fractions by AHA enzyme-linked immunosorbent assay (ELISA) (see Method), TT antigen binding test, and IgG ELISA [as described previously (14)]. For each reactivity, more than five subjects were included, and bars indicate medians. A Kruskal–Wallis test was performed for pan-IgG or TT versus all AHA reactivities.
sequences displayed few N-glycosylation sites and, although in a limited number of sequences, also the switched sequences did not show aberrant levels of N-glycosylation sites (unpublished data). Importantly, elevation of AHA Fab glycosylation levels was not found exclusively in RA patients but occurred to a similar degree in HCs. This suggests that selection for Fab glycosylated AHAs can occur in (chronic) inflammatory states independent of it being induced by infection or autoimmunity. As antibody responses can persist over time, elevated AHA Fab glycosylation can be a sign of active but also of previous inflammation, which may explain the observed elevation in HCs. The reason for certain (auto)antibody responses acquiring (elevated) Fab glycosylation remains incompletely understood. A proposed mechanism for the selection of Fab glycan-expressing B cells involves the engagement of Fab glycosylated B-cell receptors with glycan binding molecules, e.g. CD22 on the B-cell surface, which may modify the activation threshold of these B cells, leading to a selection advantage (21).

For most studied parameters, we did not observe large differences between RA patients and HCs. Presumably, AHAs develop in response to IgG cleaved by proteases, which may be of bacterial or endogenous origin. In particular, (chronic) inflammation may induce a proteolytic microenvironment and one may speculate that elevated Fab glycosylation is a characteristic thereof, rather than an autoantibody-specific feature.

**Conclusion**

In this study, we have expanded the molecular characterization of the AHA repertoires in RA patients and HCs. Both cathepsin G-cleaved IgG1 and pepsin-cleaved IgG4 AHA responses were found more frequently and with higher levels in RA and, since pepsin-cleaved IgG4 recognition was also more specific in RA, this response has the potential to be an immunological marker for disease. Furthermore, elevated IgG Fab glycosylation is a hallmark of AHAs in both RA patients and HCs. Future research should provide more insights into the contribution of the serological AHA status, combined with the presence of other AMPAs and disease activity, to provide the best information regarding diagnosis, prognosis, and therapeutic efficacy in RA.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Supplementary material**

Supplemental data for this article can be accessed online at https://doi.org/10.1080/03009742.2021.1986959.

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**References**

1. Bugatti S, Manzo A, Montecucco C, Caporali R. The clinical value of autoantibodies in rheumatoid arthritis. Front Med 2018;5:339.
2. Lloyd KA, Steen J, Amara K, Ticecombe PJ, Israelsson L, Lundström SL, et al. Variable domain N-linked glycosylation and negative surface charge are key features of monoclonal ACA: implications for B-cell selection. Eur J Immunol 2018;48:1030–45.
3. Rombouts Y, Willemze A, Van Beers JJ, Shi J, Kerkman PF, van Toorn L, et al. Extensive glycosylation of ACA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. Ann Rheum Dis 2016;75:578–85.
4. Ribbens C, Martin y Porras M, Franchimont N, MJ K, JM J, Dams P, et al. Increased matrix metalloproteinase-3 serum levels in rheumatic diseases: relationship with synovitis and steroid treatment. Ann Rheum Dis 2002;61:161–6.
5. Ryan MH, Petrone D, Nemeth JF, Barnathan E, Björk L, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008;45:1837–46.
6. Brezski RJ, Knight DM, Jordan RE. The origins, specificity, and potential biological relevance of human Anti-IgG hinge autoantibodies. Sci World J 2011;11:1153–67.
7. Birdsell HH, Lidsky MD, Rossen RD. Anti-Fab’ antibodies in rheumatoid arthritis. Measurements of the relative quantities incorporated in soluble immune complexes in sera and supernatants from cultured peripheral blood lymphocytes. Arthritis Rheum 1983;26:1481–92.
8. Van De Stadt LA, De Vrieze H, Derksen NIL, Brouwer M, Wouters D, van Schaardenburg D, et al. Antibodies to IgG4 hinge can be found in rheumatoid arthritis patients during all stages of disease and may exacerbate chronic antibody-mediated inflammation. Arthritis Rheumatol 2014;66:1133–40.
9. Falkenburg WJJ, van Schaardenburg D, Ooijevaar-de Heer P, Tsang-A-Sjoe MW, Bultink IE, Voskuyl AE, et al. Anti-hinge antibodies recognize IgG subclass- and protease-restricted neoepitopes. J Immunol 2017;198:82–93.
10. Den UD, Ter WM, Boers M, Kerstens P, Voskuyl A, Nurmohamed M, et al. A non-inferiority trial of an attenuated combination strategy (‘COBRA-light’) compared to the original COBRA strategy: clinical results after 26 weeks. Ann Rheum Dis 2014;73:1071–8.
11. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. Classification criteria: the 1987 American Rheumatism Association revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
12. Jamnitski A, Knieckaert CL, Nurmohamed MT, Hart MH, Dijkmans BA, Aarden L, et al. Patients non-responding to etanercept obtain lower etanercept concentrations compared with responding patients. Ann Rheum Dis 2012;71:88–91.
13. Kohen F, Bagci H, Barnard G, Bayer EA, Bayer G, Schindler DG, et al. Preparation and properties of anti-biotin antibodies. Methods Enzymol 1997;279:451–63.
14. van de Bovenkamp FS, Derksen NIL, Ooijevaar-de Heer P, van Schie KA, Kruithof S, Berkowska MA, et al. Adaptive antibody diversification through N-linked glycosylation of the immunoglobulin variable region. Proc Natl Acad Sci U S A 2018;115:1901–6.
15. Stadlmann J, Weber A, Pabst M, Anderle H, Kunert RJ, Ehrlich H, et al. A close look at human IgG sialylation and subclass distribution after lectin fractionation. Proteomics 2009;9:4143–53.
16. Guhr T, Bloem J, Derksen NIL, Wuhrer M, Koenderman AHL, Aalberse RC, et al. Enrichment of sialylated IgG by lectin fractionation does not enhance the efficacy of immunoglobulin G in a murine model of immune thrombocytopenia. PLoS One 2011;6:e21246.
17. Käsermann F, Boerema DJ, Rüegsegger M, Hofmann A, Wymann S, Zuercher AW, et al. Analysis and functional consequences of increased Fab-sialylation of intravenous immunoglobulin (IVIG) after lectin fractionation. PLoS One 2012;7:e37243.
18. Bondt A, Rombouts Y, Selman MHJ, Hensbergen PJ, Reiding KR, Hazes JM, et al. Immunoglobulin G (IgG) Fab glycosylation analysis using a new mass spectrometric high-throughput profiling method reveals pregnancy-associated changes. Mol Cell Proteomics 2014;13:3029–39.
19. Gao S, Zhu H, Zuo X, Luo H. Cathepsin G and its role in inflammation and autoimmune diseases. Arch Rheumatol 2018;33:498–504.
20. Falkenburg WJJ, von Richthofen HJ, Rispens T. On the origin of rheumatoid factors: insights from analyses of variable region sequences. Semin Arthritis Rheum 2019;48:603–10.
21. Vletter EM, Koning MT, Scherer HU, Veelken H, Toes REM. A comparison of immunoglobulin variable region N-linked glycosylation in healthy donors, autoimmune disease and lymphoma. Front Immunol 2020;11:241.