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https://doi.org/10.1111/cei.12987

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Original Article

Altered glycan accessibility on native immunoglobulin G complexes in early rheumatoid arthritis and its changes during therapy.

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# The present work was performed in fulfillment of the requirements for obtaining the degree “Dr. med.” for JS

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Running title: Altered glycosylation of native IgG-complexes in RA

Key words: immune complex, rheumatoid arthritis, lectin-ELISA, Aleura aurantia, Sambucus nigra, Jacalin.
Summary
The goal of this study was to investigate the glycosylation profile of native IgG in patients with rheumatoid arthritis (RA). To accomplish this, lectin binding assays, precluding accessibility of the glycan on native IgG by biotinylated lectins were developed. Lectins capturing fucosyl residues (AAL), fucosylated tri-mannose N-glycan core sites (LCA), terminal sialic acid residues (SNA) and O-glycosidically-linked galactose/N-acetylgalactosamine (GalNac-L) were used. Patients with recent-onset RA at baseline and after 3-year follow-up were investigated. We found that native IgG in RA patients is significantly more often complexed with IgM, C1q, C3c and CRP altering the structure of native IgG. The accessibility to fucose residues to respective lectins was significantly higher in patients with RA. Accessibility to fucose residues on IgG positively correlated with the levels of antibodies to cyclic citrullinated peptides (anti-CCP). We also observed a significantly higher accessibility to sialic acid residues and galactose/GalNAc glycotopes in native IgG of patients with RA at baseline. While sialic acid accessibility increased during treatment, the accessibility to galactose/GalNAc decreased. Hence, successful treatment of RA was associated with an increase in the SNA/GalNAc-L ratio. Interestingly, SNA/GalNAc-L ratio particularly increased after glucocorticoid treatment. In summary, this study shows the exposure of glycans in native IgG of patients with early RA revealing particular glycosylation patterns in RA patients and changes in respective patterns after the initiation of treatment of RA.
Introduction

The impact of the glycosylation status of immunoglobulins and IgG containing immune complexes (IC) on disease activity in rheumatoid arthritis (RA) has attracted increased attention in recent years. IC deposition in synovial membranes or blood vessels of skin and kidney among others, and the accompanying inflammation and tissue damage, are mechanistically associated with various autoimmune diseases like RA and systemic lupus erythematosus (SLE). The formation of IC containing autoreactive antibodies is discussed to play a detrimental role in the tissue-damaging processes of RA. Deposition of IC in endothelia is thought to foster atherosclerosis, and thus offers an explanation for the increased risk of patients with RA to develop cardiovascular pathology.

Moreover, there is evidence that IgM rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (anti-CCP) are independent risk factors for ischaemic heart disease and increased mortality rates. The presence of autoreactive immunoglobulins such as RF and anti-CCP antibodies is associated with a more severe course of RA. Most circulating IC are initially captured by the complement receptor 1 on the surfaces of erythrocytes. The erythrocytes transport the IC to liver or spleen where they are and phagocytosed by macrophages and thus cleared from the blood stream. Pathogenicity of autoantibody is essentially influenced by their glycosylation pattern. As depicted in supplementary figure 1 several different modifications of the glycan structure are associated with the native IgG-complexes: (1) the canonical glycans attached to the CH2-domaines (2) the glycans attached to some VH and CH1 domains (3) putative sites of hyper-glycosylation (4) increased accessibility for lectin binding of the canonical glycans attached to the CH2-domaines and (5) glycans attached to non-IgG molecules of the IgG-complex.

The aim of this study was to characterize the glycan exposure of IgG-complexes in patients with recent-onset RA. We observed significant differences in the glycan exposure in RA patients compared to healthy controls. Several of the lectins displayed an altered, mainly increased binding to the IgG-complexes.

Material and Methods

Patients and control subjects

We studied 30 patients (20 women, 10 men, mean age 57.4 years) with recent-onset rheumatoid arthritis (RA) enrolled in a prospective observational cohort designated TIRA-1 (TIRA = a Swedish acronym for “timely interventions in rheumatoid arthritis”). Symptom duration was at least 6 weeks, but less than 12 months. At baseline (BL), all patients fulfilled the American College of Rheumatology...
(ACR) criteria for RA\textsuperscript{20}. Five of 30 patients (17\%) had radiographic changes at BL. The patients were treated with disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids as considered appropriate by the physician\textsuperscript{11}. A 28-joint disease activity score (DAS28) was calculated for all patients\textsuperscript{21} and functional ability was assessed by the Health Assessment Questionnaire (HAQ)\textsuperscript{22}. Serum samples were available from BL and the 3-year follow-up (FU). Serum samples from 30 age-matched healthy blood donors (15 women, 15 men, mean age 53.0 years, p>0.05 compared to patients) from the same geographic area served as controls. Oral and written informed consent was obtained from all subjects, and the study protocol was approved by the local ethics committee in Linköping, Sweden.

**Serological analyses**

Rheumatoid factors of IgM and IgA class were analysed by EIA (Autozyme RF IgM and IgA, respectively, Cambridge Life Sciences, Cambridge, United Kingdom). Anti-CCP of IgG and IgA class were analysed as previously described\textsuperscript{11,23}.

**Analysis of IgG-complexes**

We developed an IgG capture ELISA to detect the non IgG proteins IgA, IgM, C3c, C1q and C-reactive protein (CRP) in IgG-complexes. F(ab')\textsuperscript{2}-fragment of goat anti-human IgG (Jackson Laboratories Immunoresearch) was diluted in coating buffer (0.1 M Na\textsubscript{2}CO\textsubscript{3}/NaHCO\textsubscript{3}, pH 9.6) to 2 µg/ml and applied onto ninety-six well MaxiSorp\textsuperscript{TM} microtiter plates (Nunc, F96) at 4°C overnight. After each incubation three washing steps with phosphate buffered saline (PBS) containing 0.05% Tween-20 (Roth, PBS-tween) followed. We used 3 % bovine serum albumin (BSA, Sigma-Aldrich) in PBS-tween at 37°C for two hours as blocking step. Subsequently the plates were incubated with sera from patients with RA or from NHD (1:1000 in PBS-tween) at 37°C for 2 h. Saturating binding of IgG-complexes to the plates was achieved (not shown). For the detection of IgG and for non-IgG components of IgG-complexes we employed horseradish-peroxidase (HRP)-labeled antibodies against human IgG (Southern Biotech), human IgA (Southern Biotech), human IgM (Southern Biotech), human C1q (Abcam), human C3c (Abcam) or human CRP (Abcam). The detection antibodies were diluted in PBS-tween with final concentrations as recommended by the manufacturer and incubated at 37°C for 1 h. The color reaction was started by the addition of substrate solution (0.1M Na\textsubscript{2}HPO\textsubscript{4}, 0.05M citrate acid monohydrate, 0.02% H\textsubscript{2}O\textsubscript{2} and 100µg/ml Tetra-methyl benzidine, pH=5, Merck). After 10 min the reaction was stopped with 25% sulphuric acid (Merck). We determined
the optical density values with the ELISA-reader (Tecan infinite F200 Pro) employing a 450 nm/620 nm filter/reference pair.

Detection of lectin binding sites exposed by the IgG-complexes

To detect specific glycan structures exposed by IgG-complexes we conducted an IgG capture lectin-ELISA immobilizing nIgG-complexes and detecting glycan accessibility using the lectins AAL (Aleuria aurantia lectin), LCA (Lens culinaris agglutinin), and SNA (Sambucus nigra agglutinin) specific for fucosyl residues, fucosylated tri-mannose N-glycan cores, and sialic acid, respectively 24-26. By using a N-acetylgalactosamine-lectin (GalNAc-L) we detected O-glycosidically linked (β 1-3) galactose and N-acetylgalactosamine 27. The coating of the plates with anti-human IgG F(ab’)2-fragment was performed as described above. For blocking a special blocking buffer (3% deglycosylated gelatin, 0.1% CaCl2, 0.1% MgCl2, 0.05% tween-20) was applied onto the plates at 37°C for 2. Deglycosylation of gelatin was achieved with 1% periodic acid (Merck) treatment for 24 h and dialysis against Tris buffered saline containing 0.1% CaCl2, 0.1% MgCl2 (TBS-Ca-Mg). After every incubation step, plates were washed three times with TBS-Ca-Mg and 0.05% tween-20. Sera from patients with RA or NHD were diluted 1:1000 in TBS-Ca-Mg-tween and incubated at 37°C for 2 h. A saturated binding of IgG-complexes to the ELISA plates was confirmed with anti-human-IgG detection antibody. To characterize the glycan exposure of the IgG-complexes we employed biotin-labeled lectins AAL (100 ng/ml), LCA (100 ng/ml), GalNac-L (1.1 ng/ml) and SNA (100 ng/ml), respectively. The plates were incubated at room temperature for 1 h and then washed three times. In order to detect lectin binding, HRP-streptavidin (Jackson Laboratories Immunoresearch) was used as recommended by the manufacturer. After incubation for 1 h at room temperature color reaction was started and optical density was determined as described above. The performance of IgG-complexes capture ELISA and IgG-complexes capture lectin-ELISA has previously been described in detail 19.

All ELISA were performed under native conditions in order to get relevant information about the in vivo accessibility of ligand binding sites exposed under non-denaturing conditions.

Statistical Analysis

Two-sample t-test assuming unequal variance was used for analysis of variations in IgG-complex reactivity with detection antibodies and lectins. SPSS statistical software was employed for statistical analysis of dependency of treatment and sex and for correlation analysis. Correlation between reactivity with detection antibodies or lectins and clinical and laboratory data were tested with the Spearman method. Multiple sample correction by Bonferroni was applied to t-tests and Spearman test.
The fourfold table analysis of SNA/GalNac-L reactivity ratio was performed with chi-square test and Yates correction. Differences among treatment subgroups were evaluated with the Kruskall-Wallis test with multiple sample correction by Dunns.

Results

*IgG-complexes from patients with RA in complexed with IgM-RF, CRP and the complement proteins C1q and C3c*

We first employed capture ELISA for IgG for detecting molecules attached to IgG in the serum of 30 patients with recent-onset RA at baseline (BL) and after 36 months (follow-up, FU). The reactivity of IgG-complexes with LCA, SNA, GalNAC-, C3c, C1q and CRP showed no dependency on sex and age (data for age not shown) of the patients with RA or the NHD, as shown in table 1. During the 3-years follow-up, IgG-complex reactivity with the SNA and GalNAc-L had significantly increased (male: p = 0.008; female: p = 0.003), and decreased (male: p = 0.004; female: p = 0.014), respectively. These differences were independent of gender at each time point. Interestingly, only male patients presented a significant increase of reactivity with AAL after follow-up (p = 0.03). Expectedly, when detecting IgG-associated IgM (equal to RF), we found significantly higher levels of IgG-bound IgM in RA patients compared to healthy controls (BL: p = 0.0007; FU: p = 0.0004; combined: p < 0.0001) (Figure 1A). Not surprisingly, a strong and significant correlation of IgG-bound IgM with standard IgM-RF analysis was found (BL: p = 0.002; FU: p < 0.0002). Also, there was a correlation of IgG-bound IgM with anti-CCP IgG autoantibody titres (BL: p = 0.049; FU: p < 0.04) (Tables 2a and b). In addition however, IgM was not the only protein bound to IgG in patients with RA. When detection for CRP was employed, RA patients showed increased reactivity for CRP bound to IgG (baseline: p < 0.0001; FU: p < 0.0001; combined: p < 0.0001) compared to controls. Furthermore, analysis of complement factor components showed that C1q is bound to IgG- from patients to a significantly higher extent than in normal controls (baseline: p = 0.03; FU: p = 0.03; combined: p = 0.002) (Figure 1B). Furthermore, also binding of the complement factor component C3c to IgG was higher in RA patients (BL: p = 0.04; FU: p > 0.05; combined: p = 0.02).

*Fucosyl residues and fucosylated tri-mannose N-glycan core sites on IgG-complexes from RA patients*

Lectin binding of fucosyl residues by AAL and of fucosylated tri-mannose N-glycan by LCA was higher in IgGs from RA patients than from healthy controls (AAL: BL: p = 0.02; FU: p = 0.01; combined: p = 0.002; LCA: BL: p = 0.03; FU: p = 0.01; combined: p = 0.001) (Figure 2). In male RA patients fucose reactivity measured by AAL binding significantly increased between baseline and follow-up (p = 0.03).
When correlating AAL and LCA reactivity to other clinical and laboratory parameters in RA patients we found a strong correlation between LCA and anti-CCP-IgG (p=0.001) and –IgA antibodies (p= 0.012) as well as IgM-RF (p=0.043) and IgA-RF (p=0.002) at baseline (Table 2a). Similar correlations were observed at follow-up (Table 2b). These results suggest that anti-CCP antibodies and RF are responsible for the higher exposure of fucosylated tri-mannose N-glycan core sites on IgG-complexes of patients with RA.

The SNA/GalNAc-L ratio changes during treatment of RA

We next analysed binding of IgG-complexes to SNA identifying sialic acid residues and GalNAc-L identifying galactose residues. As shown in figure 3, patients with RA show a significantly higher exposure of sialic acid (BL: p < 0.0001; FU: p < 0.0001; combined: p < 0.0001) and GalNAc-L binding sites (BL: p < 0.0001; FU: p < 0.0001; combined: p < 0.0001) on their IgG-complexes at baseline and follow-up compared to healthy individuals. Interestingly, reactivity with the SNA lectin significantly increased during treatment of RA in both genders (BL vs. FU: p = 0.001). Contrarily, the reactivity to GalNAc-L decreased significantly during therapy (BL vs. FU: p = 0.0003) in both genders.

To better demonstrate the changes in the exposure of sialic acid and O-glycosidically linked (β 1-3) galactose and N-acetylgalactosamine on IgG-complexes over time, we calculated the SNA/GalNAc-L ratio (figure 4ab). We found that this ratio clearly distinguishes patients before and after treatment (BL vs. FU: p < 0.0001). The SNA/GalNAc-L ratio remained either constant or increased in 26 patients with RA during therapy, whereas only 4 patients show a slight decrease (BL vs. FU: p<0.0001) (figure 4b).

Analyzing associations between specific anti-rheumatic treatment and the SNA/GalNAc-L ratio, we found a significant increase of the SNA/GalNAc-L ratio in patients receiving treatment with glucocorticoids (Figure 5; p < 0.001).

For control purposes we tested whether the GalNAc-L binding is related to IgG-IgA complexes. We observed neither a correlation of IgG-bound IgA molecules with the reactivity with GalNAc-L nor significant differences in the amount of IgA molecules contained in IgG-complexes (table 1). Therefore, the reactivity of IgG-complex with GalNAc-L can rather be explained by the changes of O-glycosidically linked (β 1-3) galactose and N-acetylgalactosamine exposure on IgG-complex-constituents other than IgA.

Discussion

Herein, we characterized native IgG-complexes from RA patients and determined whether it is associated with other immunoglobulins such IgM or other proteins such as complement factor
components C1q and C3c and the pentraxin CRP. Furthermore we characterized the lectin binding of native IgG to test for accessible fucosyl-, galactosyl- and sialic acid- residues. We analysed these structural features of IgG in RA patients at the start of their disease and after three years of treatment. Recently, we showed that patients with SLE exhibit nIgG-complexes with increased AAL and LCA reactivity and that AAL accessibility is associated with disease activity 19.

Complex formation of native IgG with IgM was expected in RA patients featuring IgM-RF. Indeed, results on IgM complexed to IgG were closely related to the data obtained from standard IgM-RF testing and also related to anti-CCP-IgG results, which together with RF are strongly associated with a severe disease course in RA 29. In addition, however, we also found complexes of IgG with C1q and C3c. This observation may reflect the constant activation of the complement system in RA. Clearance of ICs is mediated via C3 fragments and their binding to the erythrocyte-borne C3b/C4b receptor CR1. The circulating erythrocyte-bound ICs are subsequently cleared in the liver or spleen thus preventing IC deposition and tissue damage 30. Therefore, in patients with RA circulating IgG-complexes might expose enough C3b for their efficient clearance from the blood stream, which is not the case in patients with SLE 19. Despite the role of classical complement activation in RA remaining unclear, the presence of complement cleavage products bound to circulating ICs (CIC) suggests a certain level of complement activation in RA.

In contrast to SLE 19, IgG also forms complexes with CRP in RA patients. These complexes are based on antibodies against CRP, which have been described in lupus nephritis 31. In RA, CRP itself appears to associate with IgG. CRP is chronically elevated in RA and has shown to contribute to bone destruction 32. Nielen et al. reported that patients with RA have higher levels of serum CRP in the two years before diagnosis and that patients carrying anti-CCP autoantibodies show slightly higher concentrations of serum CRP than patients without these autoantibodies 29.

Glycosylation of IgG may essentially influence its function and pathogenicity 33. Several studies demonstrated that variations in the galactosylation and sialylation status of IgG influence disease activity in RA 1-3. However, most of these studies analysed the presence of glycans on denatured and proteolytically cleaved fragments of IgG. In this study, we characterized the carbohydrate pattern of native IgG resembling the in vivo accessibility of glycans on IgG molecules. We recently showed that patients with SLE exhibit IgG-complexes with increased AAL and LCA reactivity and that AAL accessibility is associated with disease activity 19. Herein, we show that also RA patients exhibit a stronger AAL and LCA reactivity of IgG than healthy individuals. This observation suggests an
increased exposure of fucosyl residues and the fucosylated tri-mannose N-glycan core in RA. In patients with RA, we did not find an association of increased AAL and LCA reactivity and disease activity. It is well established that in patients with RA, IgG heavy chains exhibit increased fucosylation and that fucose determines the binding to FcγRIIIA (CD16) and hence antibody dependent cellular cytotoxicity (ADCC). However, these findings were achieved using denaturing methods such as mass spectrometry or electrophoretic separation and thus do not necessarily represent the in vivo exposure of certain glycans by ICs.

We also detected a significant increase of AAL reactivity with after three years follow-up for the male RA patients. This is particularly interesting in light of previous findings that the FcγRIIIA-158VV genotype, which associated with higher IgG-IC binding affinity, is also associated with a more severe disease course in male, but not female RA patients. FcγRIIIA, expressed on natural killer cells, monocytes, macrophages and neutrophils, is known to primarily bind IgG-containing ICs. Furthermore, Copy Number Variation (CNV) of FCGR3B, the gene encoding FcγRIIIB receptor, solely expressed on neutrophils, has been associated with glomerulonephritis in SLE and with the development of systemic autoimmunity. In this context, the continuously increased accessibility of fucose residues may enhance the activation of FcγRIIIA and FcγRIIIB receptors on effector cells like neutrophils, monocytes and macrophages and thus foster concomitant tissue damage.

Besides increased AAL and LCA reactivity, IgG-complexes from patients with RA exhibited a significantly elevated exposure of both sialic acid (SNA) and GalNAc (GalNAc-L) residues compared to healthy individuals. Interestingly, the exposure of sialylated sites on IgG-complexes increased significantly during follow-up. The association of increased SNA and GalNAc-L reactivity and a clinical amelioration of the disease during the study period support the protective and anti-inflammatory role of immunoglobulin sialylation in autoimmunity. A protective effect of increased sialylation of autoreactive and total IgG has been described in anti-phospholipid syndrome and RA, respectively. A recent study revealed that sialic acid residues on IgG facilitate its binding to the C-type lectin receptor SIGN-R1 on regulatory macrophages in a RA mouse model. This resulted in an upregulation of the inhibitory FcγRIIB on effector macrophages thus dampening the pro-inflammatory actions of IgG. Furthermore, it has been shown that the production of antigen-specific IgG rich in sialic acid is driven by the induction of tolerance to the specific antigen and that this sialylated IgG prevents pro-inflammatory T- and B-cell responses. We want to stress that we did not solely determine autoantibody sialylation in our study, but measured sialic acid exposed by native IgG and IgG-
complexes. The erythrocyte-bound IgG-complexes are not to be found in sera and therefore are not analysed in our study. Nevertheless, we suggest that an altered sialylation of IgG-complexes may modify its interaction with erythrocytes and consequently alter their clearance.

Along with the sialylation changes we observed a significant increase in the exposure of O-glycosidically linked (β1-3) galactose and N-acetylgalactosamine in the serum of patients with RA. The three major components of GalNAc-L-binding O-glycosidically linked oligosaccharides are N-acetylgalactosamine, N-acetylglucosamine and galactose. The sialylation of galactose (β1-3) N-acetylgalactosamine (GalNAc) does not influence the binding of GalNAc-L, whereas a lack of galactose reduces but not abrogates the binding of GalNAc-L. This O-glycosidically linked oligosaccharide is bound to serine or threonine residues in a particular location of the Fc hinge region of the immunoglobulin to a beta-turn close to Proline residues. Moreover, GalNAc-L binds a manifold of O-glycosidically linked GalNAc oligosaccharides terminating in variations of mainly N-acetylgalactosamin (GlcNAc) and galactose, occasionally harbouring terminal fucose residues or sialic acids (Neu5Ac). GalNAc-L also presents high binding affinity to human secretory IgA. Recent studies showed that patients with RA display increased levels of N-acetylgalactosamine and N-acetylgalactosamine on circulating acute phase proteins and that this glycan configuration is associated with disease activity and the development of cardiovascular diseases in RA. However, immunoglobulin contribution to this specific glycoprotein pool is very low and it remains elusive whether these glycans are N- or O-glycosidically linked to acute phase proteins bound to IgG-complexes.

Previous studies reported on a positive correlation of disease activity and the presence of agalactosylated IgG N-glycans. The latter are always associated with a lack of terminal sialic acids. Whether this is cause or consequence of the ongoing inflammation in RA remains to be clarified. Information about O-glycan variations in RA and other autoimmune diseases is scarce. Wada et al. reported that not only N-glycans but also O-glycans on IgG displaying a significant reduction of their GalNAc content in RA patients. Altered O-glycosylation of IgA molecules is suspected to be an antigen for endogenous IgG in IgA nephropathy leading to IC formation. In contrast to our results, these data were generated applying denaturizing conditions and, therefore, were not reflecting the in vivo accessibility of glycans for surface lectins of effector cells. In our assays, the increased reactivity of IgG-complexes with GalNAc-L and its decline during therapy represents the actual alterations of the exposure of GalNAc, GlcNAc and galactose on IgG O-glycans. It has been demonstrated that Ig O-
glycosylation protects the glycoprotein from proteolytic cleavage. According to this, patients with RA might exhibit Ig molecules less vulnerable to proteolytic cleavage.

We also discovered a significant increase of the SNA/GalNAc-L ratio for patients who received glucocorticoid therapy between inclusion and follow up (p <0.0001). Also previous studies have shown increased protein sialylation in patients treated with hydrocortisone. We suggest that the alteration of the glycan of the IgG-complexes in patients with RA might be related to glucocorticoid therapy. Decline of GalNAc-L reactivity by glucocorticoid therapy may increase of the susceptibility of the IgG complex to be cleared and may therefore contribute to successful abrogation of inflammation in RA. Hence, some of the sustained anti-inflammatory properties of glucocorticoids may be due to glycan alterations. i.e. increase in IgG sialylation and decrease in GalNAc-L binding glycans.

In conclusion, we report that patients with RA exhibit a particular glycan composition of IgG-complexes including the associated non-IgG molecules. We argue that the increased exposure of fucosylated tri-mannose N-glycan core sites in the IgG of RA patients is related to higher autoantibody titers and disease severity. We also demonstrate that native IgG of RA patients shows substantial changes in the exposure of sialic acid and O-linked galactosylic residues and that these changes fluctuate over time following pharmacotherapy. Specifically, the SNA/GalNAc-L ratio appears to be an indicator for the efficacy of glucocorticoid therapy. Taken together, our study provides important information about the composition and accessibility of specific carbohydrate residues on native IgG sowing that glycans can be recognized by soluble lectins modulating thereby the complement cascade and myeloid effector cells. This ex vivo analysis of native IgG complements the molecular deciphering of the glycosylation patterns of denatured IgG heavy chain fragments.

Acknowledgements

This work was partially funded by the Volkswagen-Stiftung, by the Thyssen-Stiftung, by the German Research Foundation (DFG, CRC1181-C03, KFO257), by the EU H2020-MSCE-RISE-2015 project Nr. 690836 PANG and by the County Council of Östergötland, the Swedish Society for Medical Research, the Swedish Rheumatism Association, the Swedish Society of Medicine, the Professor Nanna Svartz foundation, and the King Gustaf V 80-year foundation.

Author contributions

JS, MHCB, IM, AS, CS and SW planed and performed ELISA experiments, conducted data analysis and wrote the manuscript. AK, AS and CS collected serum samples and clinical data, performed routine laboratory analysis and wrote the manuscript. CJ, RB, GS, MH, LEM supervised the project,
planned experiments, performed data analysis and wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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**Figure legends**

**Figure 1.** Native IgG-complexes contain additional serum proteins detected by ELISAs. a) Circulating native IgG-complexes from patients with RA show significantly higher amounts of bound autoreactive IgM and CRP as compared to NHD. This was independent of the time of assessment. b) Binding of complement products C1q and C3c to circulating IgG-ICs is enhanced in patients with RA at inclusion when compared to NHD. Only the binding of C1q remained significant along the study period. Shown are OD values from capture ELISA and the significances are presented after Bonferroni-correction.

**Figure 2.** Native IgG-complexes expose fucosyl residues and fucosylated tri-mannose N-glycan cores detected by lectin-ELISAs. The lectins AAL and LCA show significantly higher binding to native IgG-complexes from RA than to those from NHD, independent of therapy. Shown are OD values from capture ELISA and the significances are presented after Bonferroni-correction.

**Figure 3.** Native IgG-complexes expose sialic acids and O-glycosidically linked (β 1-3) galactose and N-acetylgalactosamine (GalNAc-L) detected by lectin-ELISAs. Circulating native IgG-complexes from patients with RA exhibit significantly increased binding of SNA compared to NHD. This was even increased 36 months after start of therapy. The reactivity of native IgG-complexes with GalNAc-L compared with NHD was significantly higher for the patients with RA at inclusion and it decreased significantly after 3 years of therapy. Shown are OD values from capture ELISA and the significances are presented after Bonferroni-correction.

**Figure 4.** The SNA/GalNAc-L binding ratio of the native IgG-complexes changes in dependency of the therapy. a) The SNA/GalNAc-L reactivity ratio of native IgG-complexes discriminates patients with RA before and after therapy. A contingency table analysis was performed using chi-square test and Yates correction. b) The SNA/GalNAc-L reactivity ratio increases or remains constant in course of treatment in 26/30 cases. Only 4/30 patients present a slight decrease in SNA/GalNAc-L binding ratio after the inclusion visit (p < 0.0001). Shown are SNA/GalNAc-L ratio values and p-values presented after posttest correction.

**Figure 5.** The SNA/GalNAc-L binding ratio reflects glucocorticoid therapy. The study cohort was divided into four groups according to the period of time receiving glucocorticoid therapy. The first group did not receive glucocorticoids during the whole observation period (steroid 00), patients initiating glucocorticoid therapy after enrollment (steroid 01), patients who were treated with glucocorticoids at the time of inclusion but were withdrawn later (steroid 10) and patients receiving
glucocorticoids during the whole study period (steroid 11). A significant increase of the SNA/GalNAc-L binding ratio was to be observed only for the patients that were treated with glucocorticoids over the entire period.

**Supplementary figure 1. Composition of the native IgG-complexes and their putative glycosylation sites.** This scheme shows (1) the canonical glycans attached to the IgG-CH2-domain (2) the glycans attached to some VH and CH1 domains (3) putative sites of hyper-glycosylation (4) increased accessibility for lectin binding of the canonical glycans attached to the CH2-domaines 19 and (5) glycans can also be exposed by IgG associated molecules exemplified by IgA, IgM, C1q, C3d, and CRP.

**Supplementary figure 2. The reactivities of native IgG-complexes to IgA and GalNAc-L do not correlate.** Non parametric (Spearman) correlation analysis between the binding of IgA antibodies and the binding of GalNAc-L to IgG-ICs is shown. Note, this makes it unlikely that the reactivity of the native IgG-complexes with GalNAc-L is due to its known reactivity with IgA. Correlation coefficients are presented for each time point and p-values are shown after posttest correction.
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<td>70.6 ± 17.8</td>
<td>70.3 ± 18.1</td>
<td>0.967</td>
<td>0.237</td>
<td>0.264</td>
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<td>FU Mean ± SD</td>
<td>70.6 ± 17.8</td>
<td>70.3 ± 18.1</td>
<td>0.967</td>
<td>FU Mean ± SD</td>
<td>70.6 ± 17.8</td>
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<td>0.767</td>
<td>0.709</td>
<td>0.448</td>
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<td>0.767</td>
<td>FU Mean ± SD</td>
<td>39.4 ± 15.7</td>
<td>37.9 ± 14.8</td>
<td>0.767</td>
<td>0.709</td>
<td>0.448</td>
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<td>23.5 ± 10.9</td>
<td>17.1 ± 3.6</td>
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<td>20.7 ± 5.2</td>
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<td>BL Mean ± SD</td>
<td>12.0 ± 7.8</td>
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<tr>
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<td>BL Mean ± SD</td>
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<td>8.6 ± 3.3</td>
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<td>0.855</td>
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<td>FU Mean ± SD</td>
<td>13.3 ± 8.5</td>
<td>10.2 ± 6.0</td>
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<td>FU Mean ± SD</td>
<td>33.6 ± 12.4</td>
<td>32.6 ± 10.9</td>
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<td>FU Mean ± SD</td>
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<td>FU Mean ± SD</td>
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<td>88.4 ± 20.9</td>
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<td>GalNAc-L</td>
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</table>

Abbreviations: BL: Baseline; FU: Follow up; SD: Standard Deviation; p: p-value
Table 2a. Correlation coefficients (rho) and statistical significance (p) of reactivity of complexed IgG with routine laboratory and clinical parameters for baseline samples.

<table>
<thead>
<tr>
<th></th>
<th>α-CCP-IgA</th>
<th>α-CCP-IgG</th>
<th>IgM-RF</th>
<th>IgA-RF</th>
<th>ESR</th>
<th>CRP</th>
<th>DAS 28</th>
<th>HAQ</th>
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<tbody>
<tr>
<td>IgA</td>
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Legend: upper value: Spearman correlation coefficient; lower value: p-value; IgA-CCP: IgA-antibodies to CCP; IgG-CCP: IgG-antibodies to CCP; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: Serum C-reactive protein; DAS 28: Disease Activity Score 28 for Rheumatoid Arthritis; HAQ: Health assessment Questionnaire; p-values shown after Bonferroni correction (*17).
Table 2b. Correlation coefficients (rho) and statistical significance (p) of reactivity of complexed IgG with routine laboratory and clinical parameters for follow-up samples.

<table>
<thead>
<tr>
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<th>α-CCP-IgA</th>
<th>α-CCP-IgG</th>
<th>IgM-RF</th>
<th>IgA-RF</th>
<th>ESR</th>
<th>CRP</th>
<th>DAS 28</th>
<th>HAQ</th>
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</thead>
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<tr>
<td>IgA</td>
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<td>0.134</td>
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Legend: upper value: Spearman correlation coefficient; lower value: p-value; IgA-CCP: IgA-antibodies to CCP; IgG-CCP: IgG-antibodies to CCP; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: Serum C-reactive protein; DAS 28: Disease Activity Score 28 for Rheumatoid Arthritis; HAQ: Health assessment Questionnaire; p-values shown after Bonferroni correction (*17).
Reactivity with Abs to C1q

Reactivity with Abs to C3c

OD*100

p = 0.03

p = 0.03

p = 0.002

p = 0.02

p = 0.04
**Table:**

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<th></th>
<th>BL</th>
<th>FU</th>
<th>Total</th>
</tr>
</thead>
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<td>Low SNA/N-ac-L</td>
<td>5</td>
<td>24</td>
<td>29</td>
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<tr>
<td>High SNA/N-ac-L</td>
<td>25</td>
<td>6</td>
<td>31</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>30</td>
<td>30</td>
<td>60</td>
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</table>

**Graph a:**
- Scatter plot showing reactivity with GalNAc-L against reactivity with SNA.
- Black circles represent Baseline, grey circles represent Follow up (month 36).

**Graph b:**
- Scatter plot showing SNA/GalNAc-L OD*100.
- Trend line with p < 0.0001.
SNA/GalNAc binding ratio

Steroid 0/0

Steroid 0/1

Steroid 1/0

Steroid 1/1

Baseline

Follow up (month 36)

p < 0.0001
Increased glycosylation

Increased exposure of glycosyl residues

Glycosylated IgA
Glycosylated IgM
CRP
C3c
Glycan
Reactivity with GalNAc-L

Reactivity with Ab to IgA

Baseline Follow Up

\[ \begin{array}{c|cc}
 & \text{Baseline} & \text{Follow Up} \\
\hline
\text{rho} & 0.476 & 0.188 \\
\text{p} & 0.063 & 0.99 \\
\end{array} \]