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SHORT COMMUNICATION



Glycosylation of random IgG distinguishes seropositive and seronegative rheumatoid arthritis

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ABSTRACT

The N-glycosylation of human immunoglobulins, especially IgGs, plays a critical role in determining affinity of IgGs towards their effector (pro- and anti-inflammatory) receptors. However, it is still not clear whether altered glycosylation is involved in only antibody-dependent disorders like seropositive rheumatoid arthritis (RA) or also in pathologies with similar clinical manifestations, but no specific auto-antibodies like seronegative RA. The clarification of that uncertainty was the aim of the current study. Another study aim was the detection of specific glycan forms responsible for altered exposure of native glycoepitopes. We studied sera from seropositive RA ($n=15$) and seronegative RA ($n=12$) patients for exposure of glycans in native IgG molecules, followed by determination of specific glycans by capillary electrophoresis with laser-induced fluorescent detection (CE-LIF). Aged-matched groups of normal healthy donors (NHD) and samples of intravenous immunoglobulin IgG preparations (IVIG) served as controls. There was significantly stronger binding of *Lens culinaris* agglutinin (LCA) and *Aleuria aurantia* lectin (AAL) lectins towards IgG from seropositive RA compared to seronegative RA or NHD. CE-LIF analysis revealed statistically significant increases in bisecting glycans FA2BG2 ($p=.006$) and FABG2S1 ($p=.005$) seropositive RA, accompanied by decrease of bisecting monogalactosylated glycan FA2(6)G1 ($p=.074$) and non-bisecting monosialylated glycan FA2(3)G1S1 ($p=.055$). The results suggest that seropositive RA is distinct from seronegative RA in terms of IgG glycan moieties, attributable to specific immunoglobulin molecules present in seropositive disease. These glycans were determined to be bisecting GlcNAc-bearing forms FA2BG2 and FABG2S1, and their appearance increased the availability of LCA and AAL lectin-binding sites in native IgG glycoepitopes.

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Introduction

Human immunoglobulin IgG molecules possess one constant glycosylation site at Asp 297 bearing a branched N-glycan on each of two CH₂ domains of the Fc-fragment [1]. Two glycans on each chain face each other inside the polypeptide pocket [2], while bearing charged sialic acids on the end and repelling each other, they determine the shape of the Fc portion of the immunoglobulin molecule [3]. Besides, glycan chains of the IgG molecule are highly flexible [4], making the glycan chain an important factor in determining the IgG shape. The shape of the effector Fc region of IgG molecule is critical for binding with pro-inflammatory and anti-inflammatory FcγR receptors. Since different conformations of the IgG molecule (determined by the glycan) have different affinities for FcγR receptors [3], the same antibody targeted against a specific antigen can have opposite effects in the immune system depending on

whether pro- or anti-inflammatory receptors are activated. Establishment of this fact gave rise to a number of thorough studies aimed to determine how glycans of IgGs are related to disease; current state-of-the-art knowledge is summarized in several excellent reviews [5–8]. Briefly summarizing, sialylated and galactosylated IgG glycans are usually related to anti-inflammatory activity via type II FcR, while agalactosylated forms are associated with pro-inflammatory activity realized via type I FcR receptors. The appearance of the agalactosylated forms precedes the beginning of active disease in some autoimmune conditions and declines upon resolution of inflammation. In addition, antibodies (Ab) with bisecting GlcNAc attached to the N-glycan have been shown to possess increased antibody-dependent cellular cytotoxicity (ADCC) [9,10]. The absence of a core fucose can increase ADCC up to several dozen times [11,12]. As a result of investigations in the area of IgG glycans, we devised our

strategy to create safer monoclonal antibodies [13,14], as well as develop novel drug candidates with enhanced anti-inflammatory activity that would replace or substitute for intravenous immunoglobulin IgG preparations (IVIg) [15] or develop personalized medicine [16].

Despite strong progress in the field, we still do not have a clear understanding of how the complex moiety of immunoglobulins is formed and how specific glycosylation of each form is achieved [5]. Moreover, not all IgG subclasses have equal contribution, with some Ab subclasses like IgG2b and IgG2c having much greater affinity to specific receptors than others [17]. Most of the analysis has been done by denaturing techniques, involving the step of protein unfolding and inevitable destruction of the specific glycoepitope conformation that is responsible for the recognition and binding events. The destruction of the conformation can be avoided by applying a lectin-ELISA detection technique that we proposed to evaluate native glycan conformations of captured IgG molecules [18]. While focusing on autoimmune disorders we have demonstrated that Fc glycosylation of complexed native IgG molecules is associated with disease activity of systemic lupus erythematosus [19], determines the pathogenicity of Anti- β 2GP1-IgG autoAb [20] and is observed in early rheumatoid arthritis (RA) and changes during disease therapy [21]. Furthermore, exposure of sialyl-specific glycoepitopes on anti-histone IgG autoantibodies in SLE determines their capabilities to participate in the clearance of late apoptotic cells [22].

This was aimed to evaluate the impact of IgG glycosylation on RA pathogenesis and determine those glycoepitopes and related glycans that are involved in the pathology. To realize the first goal we studied two cohorts of RA patients: seronegative ($n=12$) and seropositive ($n=15$). To realize the second goal we subsequently analyzed samples from RA patients to detect the glycan composition of IgG using the non-denaturation lectin-ELISA assay followed by capillary electrophoresis with laser induced fluorescent detection (CE-LIF) [23].

Materials and methods

Blood serum samples

All analyses of human material were performed in accordance with the institutional guidelines and with the approval of the ethical committees of the participating institutions. Ethical approval for the study was received from Ethics Committees of UHO 25-1:11861-3/2012, FAU-UKER No. 52_14B, DH LNMU No. 3/2010-03-22, No. 1/2014-01-20.

This study involved samples from seronegative (12) and seropositive (15) RA patients that were collected at three research centers: Faculty of Medicine University Josip Juraj Strossmayer of Osijek (Croatia), University Hospital Erlangen (Germany) and Danylo Halytsky Lviv National Medical University (Ukraine). Age- and sex-matched NHD (11 persons) served as controls. Samples were stored at 4°C during analysis, but otherwise kept at -20°C. Discrimination of seropositive vs. seronegative RA was based on the generally accepted criteria of rheumatoid factor (RF),

Table 1. Main clinical parameters of the study participants.

	Seronegative RA	Seropositive RA	NHD
N	12	15	11
Sex (male/female)	1/11	3/12	1/10
Age (years), mean \pm SEM	58 \pm 16	53 \pm 14	50 \pm 8
Disease duration (years)	9 \pm 6	10 \pm 7	NA
CRP (mg/l), median \pm IQR	5.55 \pm 9.2	23.1 \pm 28.5	4 \pm 2
RF (IU/ml), median \pm IQR	0 \pm 12	273.1 \pm 263	8 \pm 10.6
CCP (IU/ml), median \pm IQR	5.4 \pm 4.7	340 \pm 390.1	NA

RA: rheumatoid arthritis; NHD: normal healthy donors; CRP: C-reactive protein; RF: rheumatoid factor; CCP: cyclic citrullinated peptide; CCP: anti-cyclic citrullinated peptide Ab; SEM: standard error mean.

cyclic citrullinated peptide (CCP), anti-cyclic citrullinated peptide Ab (anti-CCP Ab), and C-reactive protein (CRP) levels. The main clinical parameters of the study participants are summarized in Table 1. There were no significant differences between age and disease duration within studied groups.

To compare IgG glycoforms in the CE-LIF analysis, commercially available IVIg (intravenous immunoglobulin IgG Gamunex, Grifols, USA) was used as controls. Since by definition, IVIg is obtained by pooling a minimum of 5000 sera from NHD, we considered its value to be the best “mean” reference of studied populations.

Lectin-ELISA for IgG glycoepitopes exposure

The previously developed lectin-ELISA assay [18,20] for sensing glycoconformations of total serum IgG was used in this study implicating lectin *Aleuria aurantia* lectin (AAL; specific to core α 1,6-fucose residue of branched N-glycans) and LCA (*Lens culinaris* agglutinin; specific to fucosylated tri-mannose N-glycan core). Briefly, plates were coated with 50 μ l of a 2 μ g/ml solution of AffiniPure F(ab')₂ Fragment Goat Anti-human IgG (H+L) in 100 mM carbonate-bicarbonate buffer (pH: 9.6). The remaining unadsorbed material was washed away three times with TBS-T/0.1 M CaCl₂, MgCl₂ buffer. The plates were blocked with 3% of gelatin treated with periodic acid in TBS-T/0.1 M CaCl₂, MgCl₂ buffer for 2 h at 37°C. All serum samples were diluted 1:1000 in washing buffer and incubated at 37°C for 2 h, after that the plates were washed again. Five and 10 ng/ml of LCA, AAL- and SNA-biotinylated lectins were diluted in washing buffer and incubated for 1 h at 37°C. Streptavidin-labeled horseradish peroxidase diluted in washing buffer (1:10,000) was added to the plates and incubated at room temperature for 1 h. After the corresponding washings, the assay was developed with 3,3',5,5'-tetramethylbenzidine (TMB) containing H₂O₂ as substrate. The absorbance was read at 450 nm.

CE-LIF analysis for IgG glycan detection

a. Protein A affinity: the protein A microwell plate was washed with 300 μ l of phosphate buffer solution (PBS, pH 7.0). Two hundred micro liter of serum samples diluted in 200 μ l of PBS was applied into the protein A microwell plate. The plate was filtered, the liquid from chambers of the eluate plate was removed and the

applying procedure was repeated three times. The captured proteins were eluted with 100 μ l of 10% of acetic acid and this eluted liquid was put into 10 kDa nanosep spinfilters (VWR, Radnor, PA). The spinfilters were centrifuged at 11,000 \times g/5 min, then 100 μ l of MilliQ water (Advantage A10, MilliporeSigma, Burlington, MA, USA) was added and the spinfilters were centrifuged again. The spinfilters were put into new vials, 45 μ l of MilliQ water and 5 μ l of glycoprotein denaturation buffer were added and the vials were incubated for 10 min at 50 $^{\circ}$ C.

- b. PNGase F digestion: samples were centrifuged at 13,000 \times g/10 min. After that, 47 μ l of 20 mM NaHCO₃ buffer (pH: 7.0) and 3 μ l of N-glycanase enzyme (30 mU) were added and the samples were incubated overnight at 37 $^{\circ}$ C.
- c. APTS labeling: 100 μ l of MilliQ water was added to the samples and then the samples were centrifuged at 13,000 \times g/10 min. The filters were removed and put into new vials and centrifuged under vacuum conditions for drying. After the vacuum-centrifugation, 6 μ l of 20 mM APTS (8-amminopyrene-1,3,6-trisulfonic acid, tri-sodium salt) in 15% of acetic acid and 2 μ l of 1 M sodium-cyanoborohydride (in THF) were added to the vials and the vials were stored overnight at 37 $^{\circ}$ C.
- d. Cleaning: the labeled samples were purified using Prozyme CU cartridges (Prozyme, Hayward, CA). One hundred and fifty micro liter of clean-up buffer was added to the samples and the vials were centrifuged at 700 \times g/3 min; 150 μ l of clean-up buffer was added again and the procedure was repeated; 150 μ l of 96% of acetonitrile (Sigma-Aldrich, St. Louis, MO) was added to the CU cartridges and centrifugation was repeated. Then, the vials under the CU cartridges were changed and 50 μ l of MilliQ water was added and centrifugation was repeated. The liquid in the vials was now ready for analysis in CE-LIF.
- e. Analysis: capillary electrophoresis separation of APTS labeled N-glycans was performed on a Beckman PA800 Plus Pharmaceutical Analysis System under the control of 32 Karat software, version 9.0 (Beckman Coulter, Brea, CA) using a N-CHO neutral coated capillary. The total capillary length was 60 cm with a 50 cm effective

separation length from the injection end to the detector. The instrument was equipped with a 3-mW 488-nm solid-state laser and a 520-nm cutoff filter for laser induced fluorescence detection. For all experiments, a commercially available carbohydrate separation buffer was used (SCIEX, Brea, CA) at a separation temperature of 25 $^{\circ}$ C, using an applied potential of -30 kV (500 V/cm) with reversed polarity. The samples were injected with pressure at 1 psi for 5 s.

Statistics

To evaluate statistical significance of parametric values (e.g. glycoform content), the Student's *t*-test was used. For comparisons between two groups, the Mann-Whitney *U*-test for numerical variables was employed. All analyses were performed using Excel 2016 (Microsoft, Microsoft Corp., Redmond, WA, USA) and Prism 7.0 (GraphPad, GraphPad Software, La Jolla, CA, USA) software. A *p* value of $\leq .05$ was considered statistically significant. Three levels of significance are depicted in the figures by asterisks: **p* < .05; ***p* < .01; and ****p* < .001. Individuals with missing data were not included in the respective analyses.

Results

We employed AAL and LCA lectins that bind to the core α 1,6-fucose residue and the fucosylated tri-mannose N-glycan core, respectively, to assess the glycosyl residues exposed by complexed native IgG [24]. The lectin-ELISA assay revealed that fucosylated tri-mannose N-glycan core, detected by the LCA lectin, was differently exposed in the cohorts of NHD, seronegative and seropositive RA patients. The seropositive RA patients had significantly higher lectin binding than either seronegative RA patients or NHD (Figure 1(A)). Also, exposure of the α 1,6-fucose residue of core N-glycan was significantly stronger in the seropositive RA cohort compared to NHD, as detected by the AAL lectin (Figure 1(B)). Thus, the seropositive RA patients were characterized by altered accessibility of the N-glycan core available for lectin binding in the lectin-ELISA assay. These data

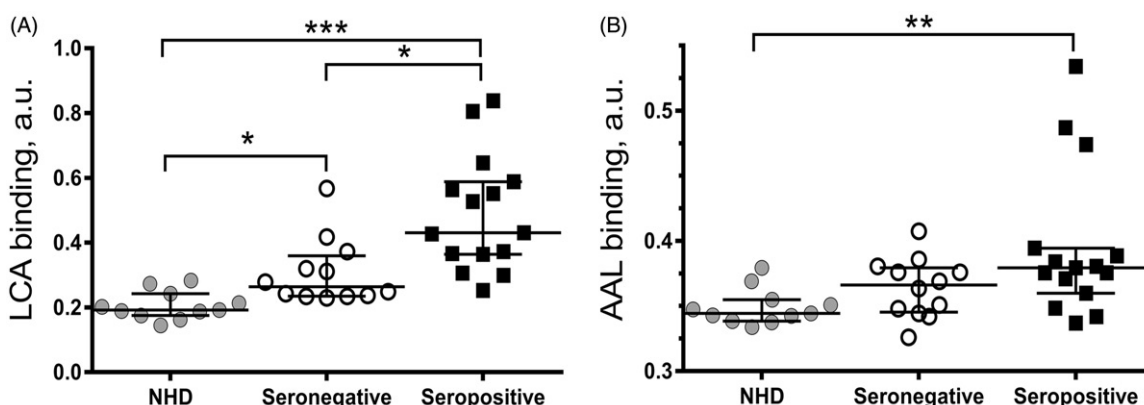


Figure 1. Binding of LCA (A) and AAL (B) lectins to captured native (non-denatured) IgG in total sera from NHD, seronegative, and seropositive rheumatoid arthritis (RA) patients. LCA: *Lens culinaris* agglutinin; AAL: *Aleuria aurantia* lectin; NHD: normal healthy donors. Significance: **p* < .05; ***p* < .01; ****p* < .001.

are in good accordance with glycan exposure reported by us for other RA cohort during long-time follow-up study [21].

Differential accessibility of core fucose residues can be the result of (a) differential configuration of glycan antennae (different glycoepitopes); (b) altered (amount of) glycans with IgG (due to changes in IgG content within specific groups (IgG1, IgG2) or subgroups (IgG2b, IgG2c) during disease developments; (c) other alterations in glycan structure of IgG molecules, such as presence of oligomannose or bisecting N-glycans; and (d) altered glycan accessibility in the formed immune complexes. With the aim to determine the composition of glycans within total serum IgG, the later was isolated by protein-A affinity chromatography, their glycans were cleaved enzymatically with PNGase F enzyme and then they were subjected to CE-LIF analysis, as described above. IVIG commercial preparations, being a pool of more

than 5000 NHD sera, were used as controls for comparison. A typical CE-LIF chromatogram with indication of glycans present on the captured IgG molecules is shown in Figure 2. The relative content of each form in seropositive and seronegative RA patients and reference IVIG values are shown in the accompanying table (Figure 2).

CE-LIF analysis of the portion of each glycan in each population (indicated in %) effectively shows re-distribution of glycans in the analyzed sera samples (since the sum of all forms is taken as 100%). Analysis of relative glycan content demonstrated significant changes of FA2BG2S1, FA2(6)G1, and FA2BG2 between seropositive and seronegative RA patients. In addition, the glycan content of A2BG2S2 and FA2(3)G1S1 was also changed between groups despite being not significant. The content of these glycans was further analyzed in groups (by applying Mann-Whitney *U*-test).

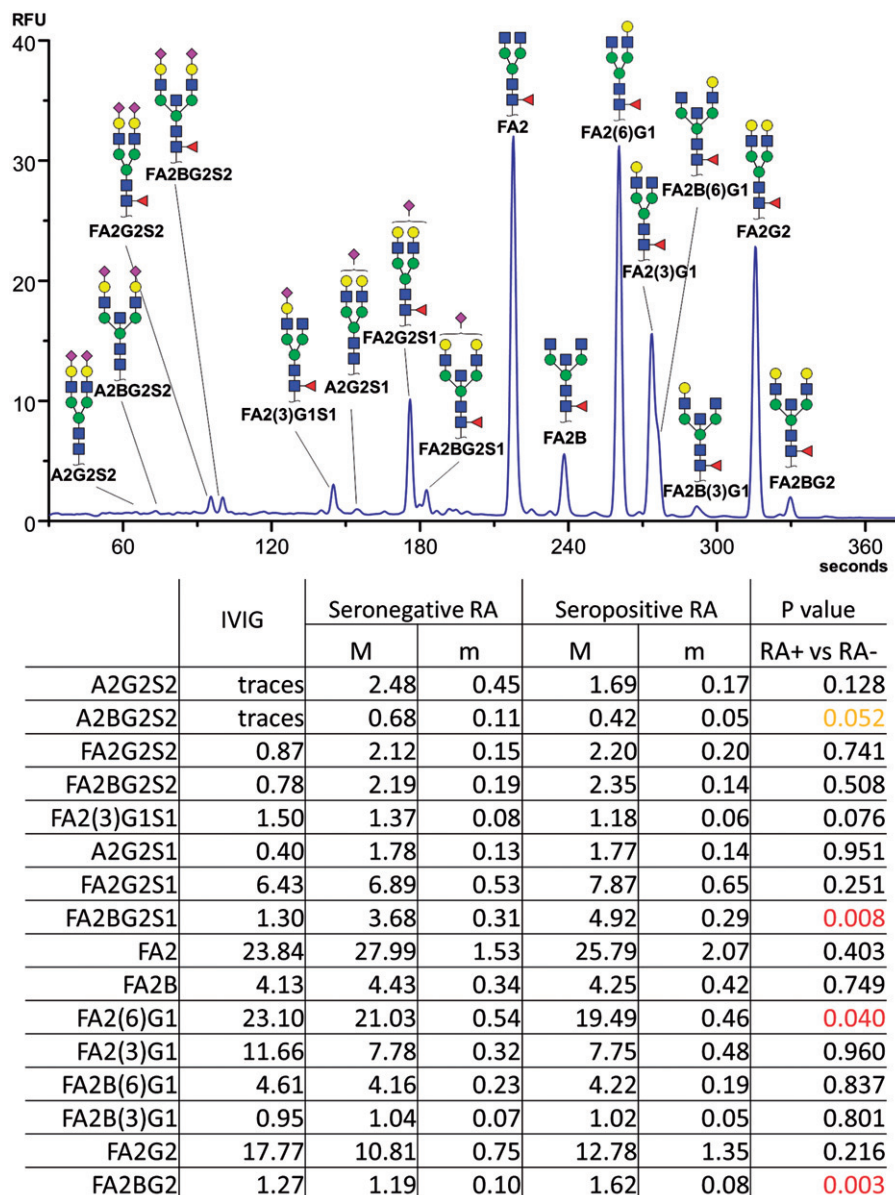


Figure 2. CE-LIF analysis of IgG glycans in patients with rheumatoid arthritis (RA). Top: typical chromatogram of glycans isolated from sera of an RA patient indicating individual glycans. Bottom: M \pm m data of each glycoform in the seronegative and seropositive RA cohorts, calculated as the relative content of each glycoform in the serum sample (in percentages). The sum of all glycoforms may not add up to 100%, as some minor forms are omitted from the analysis. Significant *p* values are highlighted in red. IVIG: intravenous immunoglobulin IgG preparations; M: mean; m: standard error mean; RA+: seropositive RA; RA-: seronegative RA.

Figure 2 also demonstrates that the content of many glycans in RA is far from the values of IVIG (representing the reference control).

Both FA2BG2 and FABG2S1 glycans, being present in significantly higher proportions in the seropositive RA cohort, possess a bisecting GlcNAc residue in the glycan core (Figure 3(A,B)). At the same time, content of the non-bisecting monogalactosylated glycan FA2(6)G1 and non-bisecting monosialylated (and monogalactosylated) glycan FA2(3)G1S1 was lower in the seropositive group and higher in the seronegative group of RA patients (Figure 3(C,D)).

Since the relative content of each glycan in the total pool is dependent on the content of other forms, we plotted them pairwise to discriminate cohorts of seropositive and seronegative RA patients based on specific glycan content. For this reason, we have selected the following pairs with the most prevalent discriminative possibilities: FA2(3)G1S1 vs. FA2BG2 (Figure 4(A)); FA2BG2S1 vs. FA2BG2 (Figure 4(B)); FA2(6)G1 vs. FA2BG2 (Figure 4(C)); and FA2(3)G1S1 vs. FA2BG2S1 (Figure 4(D)). As seen in Figure 4, the usage of two parameters representing corresponding glycans effectively separates the populations of seronegative and seropositive RA patients.

Discussion

By analyzing sera from seropositive and seronegative RA patients, we found that the type of RA (seropositive or seronegative) is tightly connected with changes of the specific glycan in the IgG pool. We observed distinct exposure of glycoepitopes related to core fucose of N-glycans that were detected by LCA and AAL lectins in native non-denatured

IgG conformations. The increased reactivity of IgG N-glycans with LCA and AAL lectins was recently demonstrated during “timely interventions in rheumatoid arthritis” study for 30 RA patients, both at the beginning of the study and 36-month later during the course of treatment [21]. Since, lectin binding by lectin is in fact due to “accessibility” of the glycan, and immune complexes bound to antibodies can influence the process, as described in study [19], we then utilized a subsequent analysis of individual glycans by CE-LIF revealed prominent redistribution of branched-core N-glycans with seropositive and seronegative RA groups. Obtained data indicate that seropositive RA is accompanied by the appearance of IgG glycans bearing bisecting GlcNAc residue at the glycan core. The presence of bisecting GlcNAc coincided with higher binding of IgG by fucosylated trimannose N-glycan core specific to LCA lectin. At the same time, utilization of specific glycans allowed us to effectively discriminate seropositive and seronegative RA populations.

We hereby combined two approaches, allowing us to evaluate the native glycoepitope configuration by lectin-ELISA followed by determination of specific glycan using the denaturation CE-LIF approach. The first approach is important since it allows evaluation of native glycoepitopes, which are also accessed by recognition molecules involved in the immune response (e.g. FcγR) and determine the configuration of the Ab molecule. The second approach allows the molecular identification of specific glycan moieties within the IgG pool.

Individual glycans were changed in both groups compared to IVIG, used as reference control. However, as seen in Figure 2, the values of the seronegative group were much closer (often within error value) to IVIG values than those of the seropositive group.

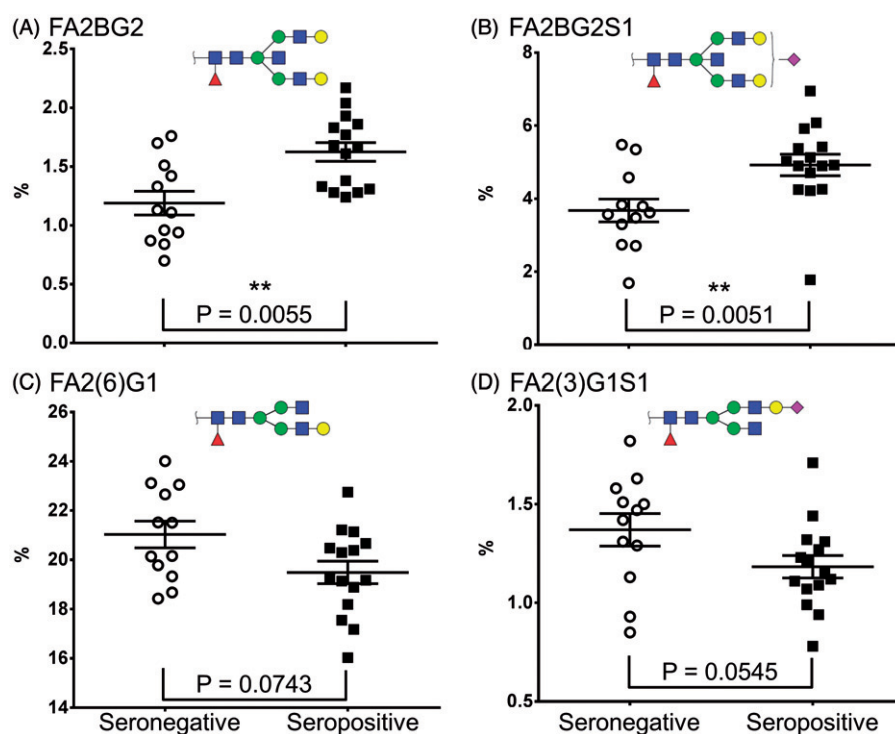


Figure 3. Distribution of four specific glycoforms with altered content between seronegative and seropositive rheumatoid arthritis (RA) serum samples, calculated by CE-LIF analysis.

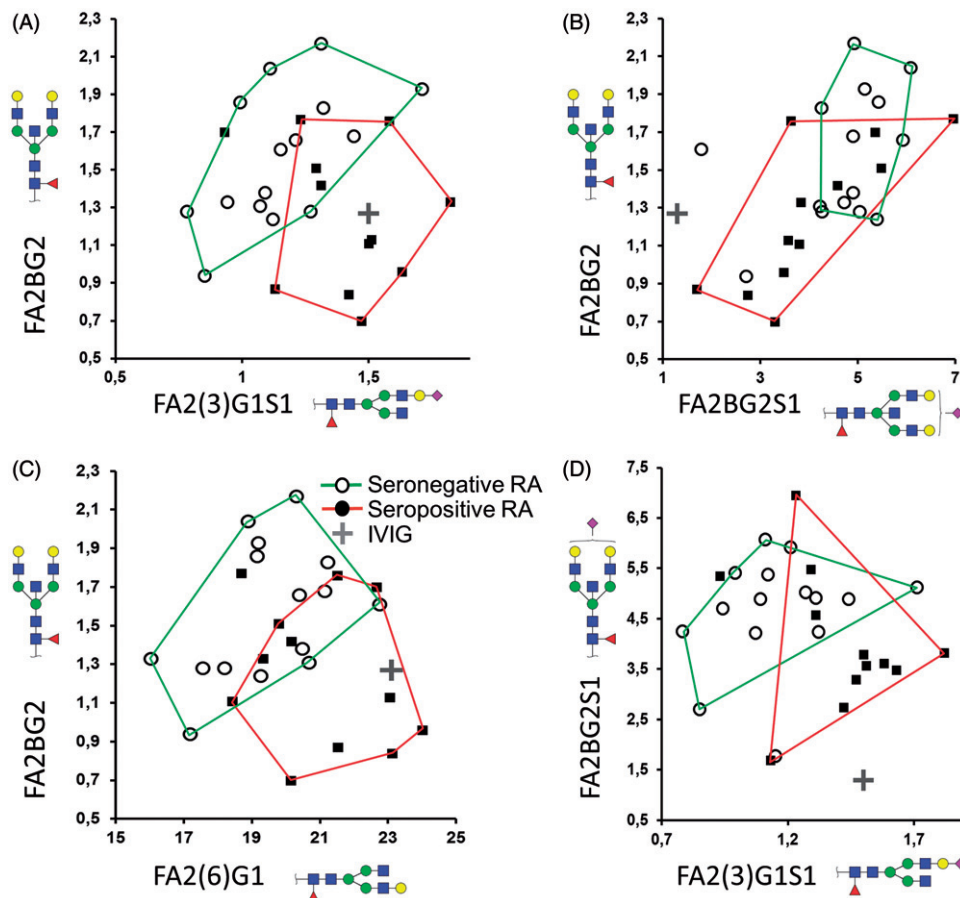


Figure 4. Discrimination of seropositive and seronegative rheumatoid arthritis (RA) patient cohorts based on the relative content of glycans, detected by CE-LIF analysis. Reference value of specific glycan for normal healthy donors (NHD) is represented by intravenous immunoglobulin IgG preparations (IVIG) mean data (grey cross).

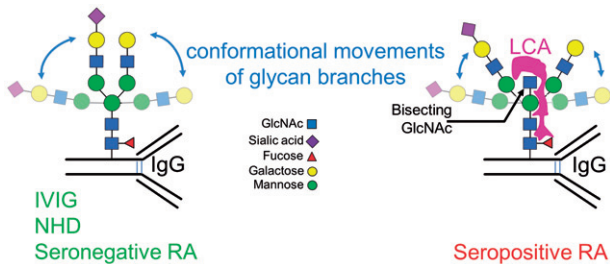


Figure 5. The proposed role of the bisecting GlcNAc residue in facilitating LCA lectin binding. LCA: *Lens culinaris* agglutinin; IVIG: intravenous immunoglobulin IgG preparations; NHD: normal healthy donors; RA: rheumatoid arthritis.

We observed that the appearance of bisecting GlcNAc residue in the core of IgG from seropositive RA samples is accompanied by binding with LCA and AAL lectins. Taking into account the data of Meier and Duus [4] demonstrating the extent of glycan movement in the native molecule, we propose that addition of bisecting GlcNAc limits the convergence of glycan branches and makes them accessible to targeting lectin molecules (Figure 5).

The exposure of fucose and of branching GlcNAc is greatly influencing the ADCC of Ab preparation, and thus a main target during the design of efficient monoclonal Ab [25]. It was demonstrated that it is the absence of fucose, not the presence of bisecting N-acetylglucosamine *per se*, that results in enhanced ADCC [9]. Bisecting N-glycans are

formed due to alterations in *de novo* glycan synthesis, particularly due to the switch from complex to hybrid N-glycan biosynthesis (then enzyme GlcNAcT-III transfers N-acetylglucosamine to the β -linked mannose in the core to generate the bisecting N-acetylglucosamine). It was shown that mutation of a single gene, encoding alpha-mannosidase II, which regulates the hybrid to complex branching pattern of extracellular N-glycans, results in a systemic autoimmune disease similar to human systemic lupus erythematosus [26]. Thus, seropositive RA was characterized by altered glycopattern, attributable to autoimmune and inflammatory conditions, while this was not true in seronegative RA. Seropositive group also was characterized by higher level of systemic inflammation, if judged by C-reactive protein level, confirming previous data that increased AAL exposure on IgG can result from inflammatory processes [24], currently not well understood. Recent data have reported the increase of N-glycosylation sites on variable regions of anti-citrullinated protein antibody (ACPA) due to somatic hypermutations as a common feature of autoimmune diseases [27]. On ACPA antibodies, these Fab glycans carry glycoforms with bisecting GlcNAc residues [28] and thus increase in specific autoimmune-attributable antibody bearing altered glycosylation can be another explanation of the observed phenomena.

Thus, we conclude that seropositive RA is distinct from seronegative RA due to altered glycan content, attributable

to pathogenic seropositive RA-specific IgG. These glycans were determined and demonstrated to be the bisecting GlcNAc-bearing glycoforms FA2BG2 and FABG2S1.

Ethical approval

Ethical approval was received from Ethics Committees of DH LNMU No.3/2010-03-22, No.1/2014-01-20, UHO 25-1:11861-3/2012, FM UJJSO (2158/61-07-13-31), FAU-UKER No. 52_14B.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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