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(54) Title: FAB-LINKED GLYCANS AS BIOMARKER FOR THE TRANSITION FROM A PRE-DISEASE "AT-RISK-PHASE" TO RHEUMATOID ARTHRITIS; AAV OR SJÖGREN SYNDROME

(57) Abstract: The invention provides means and methods for determining whether an individual that does not have rheumatoid arthritis, AAV or Sjogren syndrome at the moment of sampling is at risk of developing said disease, the method comprises determining whether an antibody containing sample of said individual comprises an autoantibody associated with said disease that comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method further comprising determining the risk of the individual for developing said disease. The disease is preferably rheumatoid arthritis.

Title: Fab-linked glycans as biomarker for the transition from a pre-disease "at-risk-phase" to Rheumatoid Arthritis; AAV or Sjögren syndrome.

The invention is related to the field of autoimmune disease, in particular anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV); Sjögren syndrome and arthritis, in particular to the field of rheumatoid arthritis. It also relates to methods for monitoring individuals for the development of said autoimmune disease or the treatment of autoimmune disease, preferably (rheumatoid) arthritis.

IgGs are glycoproteins that contain a conserved glycosylation site located at Asn297 present in the Fc-portion. From a structural point of view, these Fc-glycans serve as an internal scaffold and are crucial for maintaining the conformation of the Fc tail of the IgG molecule. Fc-glycosylation can modulate the interaction with Fcγ-receptors (FcγR) and can be involved in other effector functions, since specific glycoforms can activate complement pathways (C1q and MBL mediated) and/or modulate FcγR-binding. For instance, core-fucose residues can influence IgG binding to FcγRIIIa and lack of core-fucose is responsible for enhanced antibody dependent cellular cytotoxicity. Likewise, low content of sialic acid and galactose residues in Fc-glycans confers important pro-inflammatory properties to IgG, as it favors the binding of IgG to activating FcγRs.

In addition to Fc-linked N-glycans, ~15-25% of IgG molecules in human serum contain N-linked glycans present in the Fab-region. Fab-glycans can also modulate cellular function and have been implicated in the emergence of lymphoma's such as follicular lymphoma, diffuse large B-cell lymphoma and Burkitt's lymphoma B-cells, presumably through the provision of aberrant Fab-glycosylated B-cell receptor cross-linking via the glycan to lectins.

Antibodies that can bind post-translational modifications (AMPA) such as citrullinated protein antigens (ACPA), homo-citrullinated protein antigens (anti-CarP) and acetylated lysine protein antigens (AAPA) have recently been found in patients with rheumatoid arthritis (RA). Such antibodies have been implicated in disease pathogenesis. Malondialdehyde-acetaldehyde adduct (MAA-adduct) formation is another post-translational modification that is increased in RA. The modification results in antibody responses that are associated with ACPAs (Thiele et al 2015: Arthritis Rheumatol Vol 67(3): 645-655: doi 10.1002/art.38969).Various post-translational modifications that are implicated in the development of autoimmune diseases such as RA (reviewed in Trouw et al (2017; Nature reviews Rheumatology doi: 10.1058/nrrheum.2017.15). Recently, the inventors of the present invention made the intriguing observation that ACPA isolated from RA patients are extensively Fab-glycosylated. ACPA are highly specific for RA and their presence associates with disease severity and predicts the development of RA in subjects at risk (Scott 2010; Willemze, A., et al. "New biomarkers in rheumatoid

arthritis." Neth J Med 70.9 (2012): 392-9). Although it is unknown whether the Fab-glycans on IgG molecules can mediate specific functions in normal immune responses, evidence has been obtained supporting the notion that their presence can influence epitope recognition as well as half-life of antibodies in vivo (Goletz 2012; Co 1993; Leibiger 1999)

To undergo N-linked glycosylation, proteins need to have an N-linked glycosylation consensus sequence (typically N-X-S/T, where X≠P; herein N = Asparagine; S = Serine; T = Threonine; P = Proline and X is any amino acid but not Proline. S/T in the formula means an S or a T at that position; sometimes there is a C (cysteine) at the position of S/T). Importantly, the inventors previously showed that N-linked glycosylation consensus sites in ACPA-IgG were not germline-encoded but introduced during somatic hypermutation (Rombouts 2015).

In the present invention, the inventors identified the structure of N-linked glycans in the Fab-domain of autoantibodies associated with rheumatoid arthritis, Sjögren syndrome and AAV such as AMPA antibodies such as ACPA, anti-CarP, anti-MAA-adduct antibodies and AAPA antibodies in rheumatoid arthritis. The inventors also observed that ACPA-IgG molecules of ACPA-positive individuals that have not yet shown clinical signs of arthritis, i.e. “individuals at-risk”, exhibit a lower degree of Fab glycosylation as compared to ACPA-IgG in patients with established RA. Thus, the appearance of ACPA Fab glycans and/or the degree of ACPA Fab-glycosylation marks the transition from the pre-clinical phase to the onset of clinically overt arthritis. As such, the detection of ACPA Fab glycans by appropriate bioassays can be used to identify this transition and to guide treatment strategies for the prevention or delay of disease onset. The same is true for autoantibodies associated with Sjögren syndrome and AAV. Autoantibodies of autoantibody-positive individuals that have not yet shown clinical signs of Sjögren syndrome and/or AAV, i.e. “individuals at-risk” of developing said disease, exhibit a lower degree of Fab glycosylation as compared to autoantibodies in patients with established Sjögren syndrome and/or AAV.

SUMMARY OF THE INVENTION

In one embodiment is provided a method of determining whether an individual that does not have rheumatoid arthritis, Sjögren syndrome or anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) at the moment of sampling is at risk of developing said disease, the method comprising determining whether an antibody containing sample of said individual comprises an autoantibody associated with said disease and determining whether the antibody comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method further comprising determining the risk of the individual for developing said disease on the basis of said determinations.

Also provided is a method of analyzing an antibody containing sample of an individual, the method comprising determining whether said sample comprises an

autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV that comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method characterized in that the sample is a sample of an individual that does not have rheumatoid arthritis symptoms, Sjögren syndrome symptoms or AAV symptoms at the moment of sampling.

Further provided is a method of preventing or delaying the development of a rheumatoid arthritis symptom, a Sjögren syndrome symptom or an AAV symptom in an individual, the method comprising

- determining whether a sample of said individual contains an autoantibody associated with said disease;
- and determining whether the antibody has an N-linked glycosylated Fab-portion;
- wherein the sample is an antibody containing sample of the individual and the individual did not have rheumatoid arthritis, Sjögren syndrome and AAV at the time of sampling; and
- treating the individual with a medicament for said disease prior to or at the onset of the individual presenting with said disease.

Also provided is a method of monitoring an individual at risk of developing rheumatoid arthritis, Sjögren syndrome or AAV, the method comprising monitoring the presence and/or the onset of an autoantibody associated with said disease in periodic antibody containing samples of an individual, the method characterized in that the method further comprises determining whether a detected autoantibody associated with said disease comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody.

Also provided is a rheumatoid arthritis, Sjögren syndrome or AAV medicament for use in a method of treatment of an individual at risk of developing one or more of said diseases wherein the individual is determined to be at risk by the detection of an autoantibody associated with said disease which antibody comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody in an antibody containing sample of said individual. In an embodiment the individual does not have said disease at the moment of administering the medicament.

Also provided is a method of determining whether an antibody containing sample comprises an anti-modified protein antibody (AMPA), preferably a citrulline, a homo-citrulline and/or an acetylated lysine binding AMPA, the method comprising

- contacting antibodies of the sample with a peptide or protein that comprises a modified protein epitope, preferably comprising a citrulline, homo-citrulline, or acetylated lysine;

- contacting antibodies of the sample with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody; and
- determining whether an antibody with a N-linked glycan on a Fab-portion of the antibody has bound to the modified protein epitope in the peptide or protein, wherein the modified protein epitope preferably comprises citrulline, a homo-citrulline or an acetylated lysine.

Further provided is a kit of parts useful in the detection of an AMPA, preferably an ACPA, an anti-CarP and/or an AAPA antibody in a sample, the kit comprising a peptide or protein that comprises a peptide or protein with a post-translationally modified epitope, preferably a citrullinated, a homo-citrullinated and/or an acetylated lysine epitope and a molecule that can bind an N-linked glycan on a Fab-portion of an antibody.

Further provided is a kit of parts useful in the detection of an autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV such as an AMPA in a sample, the kit comprising a peptide or protein that can bind said autoantibody comprises such as a peptide or protein comprising a post-translational modification and a molecule that can bind an N-linked glycan on a Fab-portion of an antibody.

In one embodiment is also provided a method of analyzing an antibody containing sample of an individual, the method comprising determining whether said sample comprises an AMPA, preferably an ACPA antibody, an anti-CarP antibody and/or an AAPA antibody; and which antibody comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody, the method characterized in that the sample is a sample of an individual that does not have rheumatoid arthritis signs or symptoms at the moment of sampling.

Also provided is a method of determining whether an individual that does not have rheumatoid arthritis at the moment of sampling is at risk of developing rheumatoid arthritis, the method comprising determining whether an antibody containing sample of said individual comprises an anti-modified protein antibody (AMPA), preferably an ACPA antibody, an or anti-CarP antibody and/or an AAPA antibody; and determining whether the antibody comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody, the method further comprising determining the risk of the individual for developing said arthritis on the basis of said determinations.

The individual that does not have rheumatoid arthritis at the moment of sampling is preferably an AMPA, preferably an ACPA; an anti-CarP and/or an AAPA -positive individual, preferably with arthralgia (joint complaints/pain) without signs of clinically and/or radiographically detectable joint inflammation or arthritis or an individual that is asymptomatic and where ACPA, anti-CarP and/or

AAPA serology is detected as an accidental finding or as part of a screening test. The individual preferably does not have chronic arthritis symptoms.

Further provided is a method of treating an individual for a rheumatoid arthritis symptom or the development thereof, the method comprising

- determining whether a sample contains an AMPA, preferably an ACPA, an anti-CarP and/or an AAPA antibody that has an N-linked glycosylated Fab-portion;
- wherein the sample is an antibody containing sample of the individual and the individual did not have rheumatoid arthritis at the time of sampling; and
- treating the individual, preferably with a rheumatoid arthritis medicament or other targeted intervention prior to or at the onset of the individual presenting with a rheumatoid arthritis symptom. The treatment prevents or at least delays the onset of chronic arthritis, and/or the onset of a rheumatoid arthritis symptom. The treatment may also reduce the severity of the chronic arthritis, and/or rheumatoid arthritis symptom.

Also provided is a method of treating an individual for a rheumatoid arthritis symptom or the development thereof, the method comprising

- determining whether a sample contains an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody;
- and determining whether the antibody has an N-linked glycosylated Fab-portion;
- wherein the sample is an antibody containing sample of the individual and the individual did not have rheumatoid arthritis at the time of sampling; and
- treating the individual with a rheumatoid arthritis medicament prior to or at the onset of the individual presenting with a rheumatoid arthritis symptom.

Also provided is a method of monitoring an individual at risk of developing arthritis, the method comprising monitoring the presence and/or the onset of an AMPA, preferably an ACPA, an anti-CarP and/or an AAPA antibody in periodic antibody containing samples of said individual, the method characterized in that the method further comprises determining whether a detected AMPA, preferably ACPA, anti-CarP and/or AAPA antibody comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody.

Further provided is a medicament, preferably an arthritis medicament, for use in a method of treatment of an individual comprising determining the presence of an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody, that comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody in an antibody containing sample of the individual, and treating the individual when an AMPA that comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody has been detected.

Further provided is a method of determining whether an individual comprises an autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV with an N-linked glycan on a Fab-portion of the antibody, the method comprising

- contacting a B-cell containing sample of said individual with a peptide or protein that can bind said autoantibody;
- separating B-cells bound to said peptide or protein from unbound B-cells; and
- sequencing nucleic acid encoding the variable region of a heavy chain or a part thereof and/or the variable region of a light chain variable region or a part thereof of an antibody or B-cell receptor of said bound B-cells; and
- determining whether the nucleic acid sequence codes for an N-linked glycosylation consensus amino acid sequence.

Further provided is a method of determining whether an individual comprises an anti-modified protein antibody (AMPA) with an N-linked glycan on a Fab-portion of the antibody, the method comprising

- contacting a B-cell containing sample of said individual with a peptide or protein that comprises a modified protein epitope;
- separating B-cells bound to said peptide or protein from unbound B-cells; and
- sequencing nucleic acid encoding the variable region of a heavy chain or a part thereof and/or the variable region of a light chain variable region or a part thereof of an antibody or B-cell receptor of said bound B-cells; and
- determining whether the determined nucleic acid sequence codes for an N-linked glycosylation consensus amino acid sequence.

The part of the variable region can be any part, such as but not limited to a framework region, such as FR1, FR2, FR3, or FR4. The part can also be a complementarity determining region (CDR) such as CDR1, CDR2 or CDR3. The part of the variable region of the heavy chain is preferably a CDR of said variable region, preferably the CDR1, preferably CDR1 and CDR2, preferably all of the CDRs. The part of the variable region of the light chain is preferably a CDR of said variable region, preferably the CDR1, preferably CDR1 and CDR2, preferably all of the CDRs.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Typical profile of glycans found on total IgG (top) and ACPA IgG (bottom). IgG or ACPA were isolated from the same patients. Glycans were released and analysed by HPLC. ACPA F(ab)-fragments contain bi-antennary N-linked glycans that are highly sialylated (purple diamonds:= sialic acid).

Figure 2: (A) Serum samples from ACPA+ RA patients and their healthy ACPA+ first degree relatives (Healthy relatives) were analysed for the presence of F(ab) glycans on ACPA. The frequency of F(ab)-glycans on ACPA is remarkably lower on ACPA derived from healthy donors as compared to ACPA from RA patients. Figure (B) Predictive value of the percentage of the ACPA-IgG Fab glycosylation before RA diagnosis: 26 ACPA-positive healthy first degree relatives of RA patients were followed over time. Every circle represents the median of the ACPA Fab glycosylation of one individual that is followed until disease onset of rheumatoid arthritis (RA; black circles) or is followed over a similar time span without RA development (HC; healthy control; white circle). Of these 26 followed individuals, 46% developed RA over time. The percentage of Fab glycosylation of general IgG is 15-25%. IgG-Fab glycosylation >55% is considered " abnormal". Healthy individuals that developed RA at the end of the study period demonstrated higher percentages of Fab-glycosylation before RA diagnosis compared to the individuals that did not develop RA.

Figure 3: Approach A (method 2) SNA, a lectin that specifically binds to alpha2,6-linked sialic acids found on Fab-glycan structures binds to ACPA purified from serum of RA patients. (A) Method to first capture ACPA with CCP2-coated beads from serum of ACPA-positive and ACPA-negative RA patients (left). Next, the ACPA-positive elution was added to an ELISA plate coated with anti-human-IgG. In the next step, biotinylated-SNA was added to the bound ACPA-IgG to analyze levels of Fab-glycosylation of ACPA (right). As control for the amount of ACPA that was present in each well, a total IgG ELISA was performed (data not shown). (B) The ratio glycosylated ACPA-Fab/total ACPA-IgG (for methods, see Figure 3A) demonstrated a high number of Fab-linked glycans on ACPA from ACPA-positive patients, whereas the CCP-coated elution from ACPA-negative patients didn't demonstrate SNA binding. (C) To prove that SNA in our ELISA as depicted in figure 3A (right) specifically binds to Fab-glycosylated antibodies, we used commercially available IVIg (Sanquin). We incubated IVIg with SNA-coated beads and we added the flow through (FT1) or the elutions 1 (E1; eluted with PBS and lactose) or elution 2 (E2; eluted with lactose in acetic acid which is supposed to contain Fab-glycosylated antibodies) on an ELISA plate coated with anti-human IgG. Levels of Fab glycosylation on IgG were determined by SNA binding to the different fractions (ELISA method depicted in Figure 3A, right). Indeed SNA binding was highest to the fraction that contained Fab-glycosylated antibodies. An additional HPLC analysis with the fractions confirmed our finding.

Figure 4: A) Approach B (method 3): first capturing sialylated antibodies and next detection of ACPA enrichment. Methodology to assess ACPA-IgG Fab glycosylation using protein G and, subsequently SNA agarose beads. The right panel demonstrates enrichment of Fab-glycosylated ACPA in a set of serum samples of ACPA-positive RA patients.

(B) ACPA and anti-CarP antibodies are captured by SNA. IgG was isolated from serum of ACPA- and anti-CarP antibody positive RA patients by prot G beads. By SNA agarose beads, the SNA-binding IgG antibodies were isolated and resulted in an SNA-binding (eluate) and non-SNA-binding (flow through) fraction (left panel of figure 4A. The SNA+ fraction (eluate left panel of figure 4A) or the SNA-negative fraction (flow through left panel of figure 4A) were added to an ELISA plate either coated with CCP2 (left; to detect ACPA) or carbamylated FCS (right; to detect anti-CarP antibodies) and IgG binding was analysed. Indeed the SNA+ fraction contained ACPA and anti-CarP auto-antibodies, suggesting that Fab-glycocsylation is elevated on ACPA and anti-CarP antibodies compared to general IgG.

Figure 5: Size shift of ACPA and anti-carbamylated protein antibodies with respect to normal antibodies, i.e. not directed towards (homo-)citrullinated proteins (in this case anti-tetanus toxoid).

Figure 6: Amino acid sequence of Fibrinogen alpha.

Figure 7: Amino acid sequence of Fibrinogen beta.

Figure 8: Amino acid sequence of Fibrinogen gamma.

Figure 9: Scheme of the purification and analysis of the glycosylation of ACPA-IgG and IgG. 1). ACPA antibodies were purified by affinity chromatography on CCP2 (citrullinated cyclic peptide (CCP Cit) or the arginine control (CCP-Arg) followed by Protein G and Protein A capture to obtain ACPA $IgG_{1,2,4}$ as well as non-citrulline specific $IgG_{1,2,4}$ (depleted of ACPA). 2) (ACPA)-IgG F(ab')2 fragments were generated by digesting purified antibodies with Ides. The resulting Fc part was purified using anti-Fc antibodies, whereas the F(ab')2 fragments were isolated using anti-CH1 domain antibodies. 3) The *N*-glycans of antibodies and fragments were labelled with 2-aminobenzoic acid (2AA) and analyzed by UHPLC and MALDI-TOF-MS whereas the glycopeptides were analyzed by LC-MS.

Figure 10: The glycosylation of heavy chain (HC) and light chain (LC) derived from ACPA-IgG and IgG isolated from RA patients. A) SDS-PAGE of ACPA-IgG and IgG under reducing condition. Compared to NCS-IgG exhibiting one HC and one LC, ACPA-IgG showed multiple HC (HC1 to HC3) and LC bands (LC to LC2) due to *N*-linked glycosylation.(13) B) UHPLC chromatograms of the *N* glycans extracted from the different electrophoretic bands.

Figure 11: ACPA-IgG is differentially glycosylated in the Fc compared to the Fab glycosylation. MALDI-TOF spectra of the A) Fc and B) F(ab')2 fragments of ACPA-IgG purified from a representative donor.

Figure 12: The Fab-linked glycosylation patterns differ between ACPA-IgG and non-citrulline specific IgG isolated from RA patients. A) UHPLC chromatograms of ACPA-IgG and IgG F(ab')2 glycans of a representative RA patient. B) Differences in glycan-derived traits of ACPA-IgG and IgG Fab glycosylation represented in the relative abundance of galactosylation, sialylation, fucosylation.

Figure 13: ACPA-IgG are highly Fab-glycosylated compared to non-citrulline specific IgG. A) MALDI-TOF-MS spectra of ACPA-IgG and IgG B) Comparison of ACPA-IgG and IgG Fab glycosylation levels derived from UHPLC and LC-MS data. C) Comparison of the Fab glycosylation of ACPA-IgG or non-citrulline specific IgG from synovial fluid (n=3) and plasma (n=6).

Figure 14: Amino acid sequences of VH or VL regions comprising an exposed N-linked glycosylation consensus site(s) which are highlighted and underlined.

Figure 15: Elisa with various antigens and antibody preparations.

Figure 16: human albumin is sensitive to post-translational modifications such as citrullination and carbamylation. Figure depicts the sequence of the human albumin protein accession code Q56G89 of the Uniprot database.

Figure 17: human alpha-1-antitrypsin is sensitive to post-translational modifications such as carbamylation. Figure depicts the sequence of human alpha-1-antitrypsin accession code AAB59375.1 in the NCBI database.

Figure 18: Size shift as a result of N-glycosylation of an autoantibody Fab. The autoantibodies are PR3-ANCA antibodies which are correlated AAV. Depicted are HPLC size fractionation fractions of total IgG and PR3-ANCA antibodies of one patient (Panel A) and another patient (Panel B). The autoantibodies are involved in bodies (ANCA) associated with vasculitis (AAV).

Figure 19: Localization of N-glycosylation sites in ACPA BCR sequences compared to sites in BCR sequences obtained from healthy individuals. (A) IgG heavy chain (B) Ig kappa light chain (C) Ig lambda light chain.

Figure 20: CD22 as alternative for SNA to detect ACPA Fab glycans. F(ab')2 fragments of ACPA-IgG and IgG isolated from RA patients were loaded in equal amounts on gel (left figure). Next, an SDS-PAGE was performed where ACPA-IgG and IgG F(ab')2 fragments were loaded and thereafter blotted on a membrane. After this the membrane was incubated with CD22-Fc and then stained

with labelled anti-human-Fc (right figure). Only reactivity was shown for ACPA-IgG and the reactivity was gone when sialic acid was removed with sialidase.

Figure 21: Correlation ACPA-Fab glycosylation and RA development. Analysis the ACPA-IgG Fab-glycosylation in ACPA+ indigenous North American population first degree relatives (FDR) that developed RA (FDR RA) overtime and in ACPA+ FDR that did not developed RA (FDR HC) thus far. A) The FDR RA individuals have a higher ACPA-IgG Fab glycosylation whereas the FDR HC keeps normal levels of ACPA-IgG Fab glycosylation. B) The ACPA-IgG Fab glycosylation is already high before the disease onset. C) Whereas the ACPA-IgG Fab glycosylation of FDR HC stays low over time.

Figure 22: High-end UHPLC and mass spectrometry analyses of purified ACPA-IgG.

Figure 23: IVIG was fractionated using the setup detailed in figure 4, followed by UPLC analysis (upper graph) of total IgG molecules and of IgG Fab fragments (lower graph). Data show that SNA-purification enriched for IgG molecules containing highly sialylated Fab fragments (top lines in the graphs), whereas these were absent in the SNA-flow through fraction (bottom lines in the graphs). Of note, IVIG contains ~15% of Fab-glycosylated IgG molecules (middle lines in the graphs).

Figure 24: Identification of triantennary glycans on ACPA-IgG using UHPLC, LC-MS and MALDI-TOF-MS-MS. A) UHPLC chromatogram of the released and 2AA labelled glycans of ACPA-IgG where a glycan peak was eluting after the already reported GP24 glycan peak. By collecting the GPx, LC-MS was performed to investigate the masses present in this peak fraction. It was determined that the peak was corresponding to a glycan with a mass of N5H6F1S2. B) conformation of the structure with MALDI-TOF-MS-MS. The mass 2812.792 is corresponding with the glycan mass of N5H6F1S2 and by fragmentation a tri-antennary glycan with two a2.6 linked sialic acids was confirmed.

DETAILED DESCRIPTION OF THE INVENTION

The individual is preferably a human individual.

An autoimmune disease is a condition arising from an abnormal immune response to a normal body part. Autoimmune diseases can affect almost any part of the body, including the heart, brain, nerves, muscles, skin, eyes, joints, lungs, kidneys, glands, the digestive tract, and blood vessels. There are at least 80 types of autoimmune diseases. Nearly any body part can be involved. Common symptoms include low grade fever and feeling tired. Often symptoms come and go.

Some autoimmune diseases such as systemic lupus erythematosus run in families, and certain cases may be triggered by infections or other environmental factors. Some common diseases that are generally considered autoimmune include celiac disease, diabetes mellitus type 1, Graves' disease, inflammatory bowel disease, multiple sclerosis, psoriasis, rheumatoid arthritis, and systemic lupus erythematosus.

Treatment depends on the type and severity of the condition. Nonsteroidal anti-inflammatory drugs (NSAIDs) and immunosuppressants are often used. Intravenous immunoglobulin may also occasionally be used. While treatment usually improves symptoms they typically do not cure the disease.

In one embodiment is provided a method of determining whether an individual that does not have a particular autoimmune disease at the moment of sampling is at risk of developing said particular autoimmune disease, the method comprising determining whether an antibody containing sample of said individual comprises an autoantibody that is associated with said particular autoimmune disease and determining whether the autoantibody comprises an N-linked glycosylation at one or more positions in a Fab-portion of the autoantibody, the method further comprising determining the risk of the individual for developing said particular autoimmune disease on the basis of said determinations.

The risk of the individual for developing said particular autoimmune disease is high when the autoantibody that is associated with said particular autoimmune disease is detected and said antibody comprises an N-linked glycosylation at one or more positions in a Fab-portion of the autoantibody. The risk is higher when a higher fraction of the autoantibody comprises an N-linked glycosylation at one or more positions in a Fab-portion.

In one embodiment a method of analyzing an antibody containing sample of an individual is provided wherein the method comprising determining whether said sample comprises an autoantibody that is associated with a particular autoimmune disease and which autoantibody comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method characterized in that the sample is a sample of an individual that does not have symptoms of said particular autoimmune disease at the moment of sampling.

Also provided is a method of treating an individual for a particular autoimmune disease symptom or the development thereof, the method comprising

- determining whether a sample contains an autoantibody that is associated with said particular autoimmune disease; and
- determining whether the antibody has an N-linked glycosylated Fab-portion;

- wherein the sample is an antibody containing sample of the individual and the individual did not have said autoimmune disease at the time of sampling; and
- treating the individual with a medicament for the treatment of said particular autoimmune disease prior to or at the onset of the individual presenting with a symptom for said particular autoimmune disease.

Further provided is a method of monitoring an individual at risk of developing a particular autoimmune disease, the method comprising monitoring the presence and/or the onset of an autoantibody associated with said particular autoimmune disease in periodic antibody containing samples of said individual, the method characterized in that the method further comprises determining whether the autoantibody comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody.

Also provided is a medicament for use in a method of treatment of an individual comprising determining the presence of an autoantibody associated with a particular autoimmune disease and that comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody in an antibody containing sample of the individual, and treating the individual when an autoantibody associated with said particular autoimmune disease that comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody has been detected.

In one embodiment the autoimmune disease is one or more of Sjögren syndrome; anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV); and Arthritis. The arthritis is preferably rheumatoid arthritis. Sjögren syndrome is associated with a number of other medical conditions, many of which are autoimmune or rheumatic disorders, such as celiac disease, SLE (lupus), autoimmune thyroiditis, multiple sclerosis and spondyloarthropathy. Sjögren syndrome is also associated with non-Hodgkin lymphoma. Where herein reference is made to Sjögren syndrome in the context of the present invention the reference is to primary Sjögren syndrome only. The typical autoantibodies with specificity for Sjögren's syndrome are SS-A and SS-B. The reference to primary Sjögren syndrome is with the exclusion of secondary Sjögren syndrome which is associated with various auto-immune diseases.

An autoantibody is an antibody that is directed against one or more of the individual's own proteins (is directed towards a self-antigen). Autoantibodies can be directed towards a number of different self-antigens. An autoantibody is said to be associated with an autoimmune disease if the frequency with which autoantibodies with the indicated specificity are detected in individuals having said autoimmune disease is significantly higher than in the normal/healthy population. In Sjögren syndrome the autoantibody is typically an SS-A or SS-B antibody (also referred to as anti-Ro/La). Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of autoimmune diseases characterized by the abnormal infiltration of neutrophils, accumulation of

unscavenged leucocytoclasis in perivascular tissues and fibrinoid necrosis of the vessel walls. Patients with AAV frequently exhibit rapidly progressive renal failure caused by crescentic glomerulonephritis. Myeloperoxidase (MPO) and proteinase 3 (PR3) have been shown to be two major ANCA antigens. Autoantibodies in AAV are typically directed towards one or both of said proteins. The lysosomal membrane protein-2 (LAMP-2) autoantibody represents an additional ANCA subtype. In RA the autoantibody is typically an AMPA or Rheumatoid Factor.

Sjögren syndrome is a long-term autoimmune disease in which the moisture-producing glands of the body are affected. This results primarily in the development of a dry mouth and dry eyes. Other symptoms can include dry skin, a chronic cough, vaginal dryness, numbness in the arms and legs, fatigue, muscle and joint pains, and thyroid dysfunction. Those affected are at an increased risk (5%) of lymphoma. Sjögren syndrome diagnosis is made by combining clinical symptoms, results of measurements that test glandular function, biopsy of moisture-producing glands and blood tests looking for specific antibodies. On biopsy there are typically lymphocytes within the glands.

Vasculitis is a group of disorders that destroy blood vessels by inflammation. Both arteries and veins are affected. Lymphangitis is sometimes considered a type of vasculitis. Vasculitis is primarily caused by leukocyte migration and resultant damage. ANCA-associated vasculitis is a vasculitis subtype associated with autoantibodies against antigens derived from neutrophil granulocytes. Such anti-neutrophil cytoplasmic antibodies are also referred to as ANCA.

Arthritis is among the more common forms of autoimmune disease. There are over 100 different forms of arthritis. The most common form is osteoarthritis (degenerative joint disease). Osteoarthritis has a variety of causes, albeit that there are also not readily identifiable causes. The latter are often collectively referred to as age related osteoarthritis. Other arthritis forms are for example rheumatoid arthritis, psoriatic arthritis, and related autoimmune diseases.

A major complaint of individuals who have arthritis is joint pain. Pain is often a constant and may be localized to the joint affected. The pain from arthritis is often the result of the damage that is induced to the joint or the result of the inflammation that occurs in and around the joint. Other complaints are pain as a result of muscle strains caused by forceful movements against stiff, painful joints and fatigue. Rheumatoid arthritis is a debilitating and progressive disease if left untreated. Symptoms of disease and treatments for RA are detailed in Scott et al 2010 The lancet 376, Pages 1094-1108, which is incorporated by reference herein. The present invention refers to this publication in particular for the description of symptoms of RA and RA medicaments. Although the review is extensive the described symptoms and medicaments should non but read as limitative. A summary non-limitative list of symptoms is given herein below. Rheumatoid arthritis affects joints. Arthritis of joints involves inflammation of the synovial

membrane. Joints become swollen, tender and warm, and stiffness limits their movement. Most commonly involved are the small joints of the hands, feet, but larger joints like the shoulder and knee and the cervical spine can also be involved. RA typically manifests with signs of inflammation, with the affected joints being swollen, warm, painful and stiff, particularly early in the morning on waking or following prolonged inactivity. Increased stiffness early in the morning is often a prominent feature of the disease and typically lasts for more than an hour. As the pathology progresses the inflammatory activity leads to tendon tethering, erosion and destruction of the joint surface. This impairs the range of movement and leads to deformity. The rheumatoid nodule, which is sometimes in the skin, is the most common non joint feature. They occur in a large minority of the patients. It is a type of inflammatory reaction known to pathologists as a "necrotizing granuloma".

Anti-citrullinated protein antibodies (ACPA) are autoantibodies (antibodies to an individual's own proteins). The antibodies are directed against peptides and/or proteins that are citrullinated. They are present in the majority of patients with rheumatoid arthritis. Clinically, cyclic citrullinated peptides (CCP) are frequently used to detect these antibodies in patient serum or plasma.

Citrullination or deimination is the conversion of the amino acid arginine in a protein into the amino acid citrulline. Enzymes called peptidylarginine deiminases (PADs) replace the primary ketimine group (=NH) by a ketone group (=O). Citrullination performs a function in normal individuals. However, the immune system can attack citrullinated proteins, which happens specifically in rheumatoid arthritis.

Citrulline is not one of the 20 standard amino acids encoded by DNA in the genetic code. Instead, it is the result of a post-translational modification. Citrullination is distinct from the formation of the free amino acid citrulline as part of the urea cycle or as a by-product of enzymes of the nitric oxide synthase family.

Arginine is positively charged at a neutral pH, whereas citrulline is uncharged. In the reaction from arginine to citrulline, one of the terminal nitrogen atoms of the arginine side chain is replaced by an oxygen. The change in charge increases the hydrophobicity of the protein, leading to changes in protein folding. Therefore, citrullination can change the structure and function of proteins. Fibrin and fibrinogen may be favored sites for arginine deimination within rheumatoid joints.

Tests for the presence of ACPA-IgG are about as sensitive as IgM rheumatoid factor for the diagnosis of RA. Such ACPA are detectable before the onset of clinical disease. ACPA tests are presently routinely incorporated in the diagnostic scheme for RA. However, considering that such antibodies can be present for years prior to development of disease they are not on their own conclusive.

Homocitrulline is one methylene group longer than citrulline, but similar in structure. The metabolite is generated from a lysine residue. It is believed that most carbamylation during inflammation takes place when the enzyme MPO is released from neutrophils. Autoantibodies against homocitrullinated peptides and proteins (anti-CarP) are associated with RA. Anti-CarP antibodies can be detected also in pre-symptomatic individuals long before symptoms develop (Shi et al, Ann Rheum Dis. 2014 Apr;73(4):780-3.).

Citrullination and carbamylation are examples of post-translational modifications of proteins to which auto-antibodies can be produced by individuals. Lysine acetylation is another post-translational modification to which individuals can produce auto-antibodies (Juarez, et al., 2015. Annals of the rheumatic diseases: annrheumdis-2014). Acetylation occurs as a post-translational modification of a protein, for example, histones, p53, and tubulins. Among these proteins, chromatin proteins and metabolic enzymes are highly represented. Acetylation is sometimes also referred to as a co-translation modification. In the present invention it is referred to as a post-translational modification. Proteins can be acetylated on lysine residues. Lysine acetylation is thought to have a regulatory function in at least some types of proteins. Lysine acetylation modifies the end of the side chain of lysine. Where the side chain of lysine ends in -NH2, an acetylated lysine ends in NH=O-CH3.

Other types of post-translational modifications include but are not limited to phosphorylation, methylation, ubiquitination, glycosylation and/or sumoylation. In the present invention the post-translational modification that is detected by auto-antibodies is typically not a glycosylation. Preferred post-translational modifications are citrullination, homo-citrullination and lysine acetylation. A peptide or protein with a post-translational modification is also referred to as a modified peptide or protein, or a peptide or protein comprising a modified epitope. An antibody that specifically binds an epitope that comprises a post-translational modification is referred to as anti-modified protein antibody (AMPA). The AMPA binds the peptide or protein only when it comprises the post-translational modification. Such a modified epitope is also referred to as a modified protein epitope. An AMPA is an antibody that can bind a post-translationally modified epitope in a peptide or protein. The AMPA is typically not an antibody that binds a glycosylated epitope. The AMPA is preferably an antibody that binds an epitope comprising a citrulline, a homocitrulline and/or an acetylated lysine. In recent years it has become apparent that auto-immunity in RA targets citrullinated proteins and extends to other protein modifications such as protein homo-citrullination, also known as carbamylation, and acetylation (Shi et al Proc. Natl Acad Sci 2011: 108:17372-17377; Juarez et la 2016 Ann. Rheum. Dis 75:1099-1107). In another aspect the AMPA is an antibody that binds an MAA-adduct on a protein. Preferred epitopes that the AMPA can bind are epitopes comprising a citrulline, a homo-citrulline, an acetylated lysine residue and/or an

malondialdehyde-acetaldehyde adduct. Preferred epitopes that the AMPA can bind are epitopes comprising a citrulline, a homo-citrulline and/or an acetylated lysine residue. The post-translationally modified epitope in a peptide or protein can be a normal peptide or protein that is generated and subsequently modified. The post-translationally modified epitope can also be introduced directly into the peptide or protein during artificial synthesis of the peptide or if desired the protein, using an artificial amino-acid comprising the desired side chain.

In the present invention it was found that detection of an AMPA, with N-linked glycosylation at one or more positions in the Fab-portion of the antibody correlated well with the onset of rheumatoid arthritis. The AMPA is preferably an antibody that can bind a citrullinated, or a homo-citrullinated and/or an acetylated lysine epitope in a peptide or protein. The antibody is preferably an ACPA, an anti-CarP and/or an AAPA antibody. It is known that the presence of AAPA, ACPA or anti-CarP antibodies is indicative for a risk of developing the disease. However, such antibodies and in particular AAPA, ACPA and anti-CarP antibodies can generally be present for years prior to development of RA. On the other hand, N-linked glycosylation at one or more positions in the Fab-portion of such antibodies more accurately predicts the onset of disease and the development of symptoms. The moment that an AMPA comprising N-linked glycosylation at one or more positions in the Fab-portion appears in serum is indicative for the appearance of symptoms and development of the disease. The same phenomenon was detected in individuals at risk of developing Sjögren syndrome or AAV. Knowledge of the imminence of the appearance of symptoms is advantageous as early treatment of Sjögren syndrome, AAV or RA symptoms with, for instance, disease-modifying antirheumatic drugs (DMARDs) in the case of RA has been shown to be beneficial to the patients. Also, pre-symptomatic treatment is likely to be beneficial to the individual in the long term. RA symptoms can at least be delayed, and/or the severity of the symptoms can be ameliorated when compared to those of patients that were not treated or received treatment after the onset of RA symptoms. It is also possible that pre-disease treatment prior to the appearance of Fab-glycosylated antibodies that can bind a post-translationally modified epitope in a peptide or protein such as an AAPA, ACPA and/or anti-CarP antibodies could prevent the development of RA.

N-linked glycosylation is a post-translational modification that can occur at certain amino-acid motifs in a protein. The first ACPA and anti-CarP antibodies to appear in the blood typically do not have glycans in the Fab-portion of the antibody and lack a suitable consensus sequence. N-glycans attach to an asparagine which must be located in a specific consensus sequence in the primary structure (Asn-X-Ser; Asn-X-Thr or in rare instances Asn-X-Cys; X may not be proline). The Asn must be located on the surface of the antibody and the Asn must be found in the luminal side of the endoplasmic reticulum for N-linked glycosylation to be initiated.

Motifs that meet these criteria often only appear upon maturation of the antibody by means of somatic hypermutation.

The present invention provides a method for determining whether an individual comprises an antibody that comprises an N-linked glycosylation in a Fab-portion of the antibody the method comprising amplifying nucleic acid molecules that code for an antibody VH and/or VL or portion thereof in a sample comprising B-cells of said individual and determining whether an amplified nucleic acid molecule codes for an amino acid sequence that is an N-linked glycosylation consensus site. The three dimensional structure of Fab-portions of an antibody is well known. It is also known which part of the amino acids in a Fab-portion of an antibody are exposed and therefore accessible (exposed to the outside of the molecule) to post-translational N-linked modification. In a preferred embodiment of a method as described, it is determined whether an amplified nucleic acid molecule comprises a sequence that codes for an accessible consensus site for N-linked glycosylation. In a preferred embodiment the B-cells are B-cells that comprise a B-cell receptor (BCR) that can bind a post-translationally modified epitope in a peptide or protein. The presence of such a BCR indicates that the individual comprises an AMPA. Such B-cells can be purified from a B-cell population on the basis of the modified protein binding capability of the B-cell. For instance by means of beads that have a modified protein epitope on their surface. The inventors have found that accessible consensus sites for N-linked glycosylation are not randomly distributed over the VH or VL region. Such consensus sites can be clustered a framework region, such as FR1, FR2, FR3, or FR4, both in the heavy chain and the light chain. The part can also be a complementarity determining region (CDR) such as CDR1, CDR2 or CDR3. In some embodiments consensus sites are clustered around the CDR regions, most often in or around the CDR1 region or the CDR3 region of the VH or VL, typically in or around the CDR1 region. It is therefore preferred that the sequence of at least the VH CDR1 is determined, preferably the VH CDR1 and the VL CDR1; preferably at least further including determining the VH CDR3 is determined, preferably the VH CDR3 and the VL CDR3; Preferably at least the VH sequence is determined, preferably both the VH and the VL sequence is determined. In one embodiment the invention provides a method for determining whether an individual comprises an AMPA that comprises an N-linked glycosylation in a Fab-portion of the antibody; the method comprising collecting B-cells with B-cell receptors that comprise an AMPA from said individual; amplifying nucleic acid molecules that code for the VH and/or VL or portion thereof of said AMPA and determining whether an amplified nucleic acid molecule codes for an amino acid sequence that is an N-linked glycosylation consensus sequence. Said VH and/or VL portion is preferably a CDR coding sequence, preferably a CDR1 and/or CDR3 coding sequence. For examples of the sequencing of B-cell receptors of anti-citrullinated protein antibody IgG-expressing B-cells reference is made to Vergoesen et al (2017) Ann Rheum Dis. Doi: 10.1136/annrheumdis-2017-212052.

Detection of an AMPA, preferably an AAPA, ACPA or anti-CarP antibody with N-linked glycosylation at one or more positions in the Fab-portion of the antibody is typically predictive for the individual developing rheumatoid arthritis shortly after the collection of the sample. Detection of the "immunological conversion", i.e. the appearance of AMPA comprising an N-linked glycan in a Fab-portion of the antibody is preferably done as early as possible, preferably long-enough to be able to initiate effective (and ideally preventive) treatment. Detection of an AMPA with an N-linked glycan in a Fab-portion of the antibody is typically predictive for the individual developing rheumatoid arthritis within a limited time frame from collection of the sample from the individual.

In one aspect, the invention provides a new method of determining N-linked glycosylation at a Fab-portion of an AMPA. The method comprises contacting antibodies of a sample with a protein or peptide that comprises an epitope comprising a post-translational modification; contacting antibodies of the sample with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody; and determining whether an antibody with a N-linked glycan on a Fab-portion of the antibody has bound to the epitope comprising the post-translational modification in the protein/peptide.

An epitope comprising a post-translational modification (herein referred to with the term "modified protein epitope") is preferably a citrullinated epitope; a homo-citrullinated epitope and/or an acetylated lysine epitope. Antibodies to these epitopes are referred to as ACPA, anti-CarP and AAPA, respectively. In some embodiments the post-translational modification is an MAA or AA –adduct. Malondialdehyde (MDA) and its breakdown product acetaldehyde (AA) are highly reactive aldehydes, and together have been demonstrated to modify proteins to produce an MDA-AA protein adduct, termed malondialdehyde-acetaldehyde (MAA-adduct). MAA-adducts are highly immunogenic.

In nature, the modification can be introduced in the peptide or protein after synthesis of the peptide or protein, or during synthesis. In the latter case the modification is introduced in the part of the protein that has already been synthesized by the ribosome. In the laboratory it is possible to introduce the modification also by incorporating a modified version of the amino acid in the nascent amino acid chain. In the laboratory it is preferred that the peptide or protein is synthesized in the presence of an artificial amino acid that comprises the modification, which is then incorporated into the nascent peptide or protein chain.

The sample is typically a blood sample, preferably a serum or plasma sample. Such samples are typically antibody containing samples. Other antibody containing samples are for instance synovial fluid and sputum. For sequencing purposes it is preferred that the sample contains cells that produce the antibody. In such embodiments it is preferred that the sample is a sample that comprises B-cells. B-

cell receptor positive B-cells contain antibodies that are excreted and/or that are present as part of the B-cell receptor on the cell surface of the B-cell. The B-cell receptor or BCR is a transmembrane receptor protein located on the outer surface of B cells. The receptor's binding moiety is composed of a membrane-bound antibody that, like all antibodies, has a unique and randomly determined antigen-binding site. Antibody containing samples that have BCR positive cells can be used, for instance, to sequence the variable region of the expressed BCR or expressed antibody, for instance, to determine whether the variable domain comprises a consensus sequence for N-linked glycosylation. B-cell containing sample is for instance synovial fluid. IgG antibodies are found in all body fluids. They are the isotype most commonly used for the determination of AAPA, ACPA and anti-CarP antibodies and form a suitable source to determine glycosylation of a Fab-portion. The sample can be used directly in a method for detecting as described herein, or antibodies can be purified from the sample and are then used in the method. The sample is preferably a sample of an individual that does not have RA at the moment of sampling. Preferably the individual does not express an RA-classifying symptom, in particular arthritis, at the moment of sampling.

Various methods are available to determine whether a sample comprises an ACPA or an anti-CarP antibody. Most methods use a protein or peptide that comprises a citrullinated or homo-citrullinated epitope. The peptide is typically a peptide of between 6-50 amino acids. Preferably said peptide is a peptide of between 12 and 30 amino acids, more preferably of between 18 and 22 amino acids, most preferably of about 21 amino acids. The mentioned ranges include the number mentioned i.e. a range of between 12 and 30 amino acids includes peptides of 12 and 30 amino acids, respectively. The peptide may or may not be a cyclic peptide depending on the sensitivity and/or specificity of the comparable linear peptide. Circular peptides can be generated in any molecular composition as to generate the cyclic nature. A protein typically comprises 30 or more amino acids. Typically 50 or more amino acids. Examples of proteins that can be used in a method of the invention are depicted in the figures, i.e. fibrinogen alpha (Figure 6), fibrinogen beta (Figure 7) or fibrinogen gamma (Figure 8), human albumin (Figure 16) and human alpha-1-antitrypsin (Figure 17). Particularly preferred peptides are the CCP1 and CCP2 peptides, preferably CCP2. A kit of the invention preferably comprises a CCP1 and/or CCP2 peptide.

In a preferred embodiment, a peptide or protein for use in a method of the invention is a (part of) a human protein that is known to be subject to post-translational modification such as citrullination, homo-citrullination and/or acetylation in patients with RA. In a preferred embodiment the peptide is a peptide derived from human fibrinogen. The peptide preferably comprises a contiguous amino acid of between 12 and 30 amino acids, more preferably of between 18 and 22 amino acids, most preferably of about 21 amino acids present in the amino acid sequence of any one of fibrinogen alpha (Figure 6), fibrinogen beta (Figure 7) or fibrinogen gamma (Figure 8). In another preferred embodiment the peptide is a peptide derived from human albumin (Figure 16) or human alpha-1-antitrypsin

(Figure 17). The peptide or protein comprises an epitope with a post-translational modification. The epitope is preferably an epitope with wherein at least one lysine (anti-CarP or AAPA) or arginine (ACPA) present in an unmodified protein, is now a citrulline, an acetylated lysine or a homo-citrulline. The acetylated lysine, citrulline or homo-citrulline can be introduced by the appropriate acetylation or (homo)-citrullination of a peptide. More suitably the peptide is synthesized with the acetylated lysine, citrulline or homo-citrulline at the correct position.

The peptide, protein or the molecule that can bind an N-linked glycan on a Fab-portion of an antibody is typically coupled to a surface. The surface is typically a solid surface. The solid surface can be flat surface as typically present in a plate. It can also be a three-dimensional structure such as a bead. The solid surface may also be gel-matrix. A solid surface to which antibodies can be bound facilitates easy separation of specific material and non-specific material. Any method may be used to couple peptides and/or proteins in carbamylated, citrullinated or native form to the surface. Non-limiting examples are direct coating or biotin-streptavidin coating. Other methods to couple peptides or proteins to a surface are available to the person skilled in the art.

Various molecules are available that can bind an N-linked glycan on a Fab-portion of an antibody. Examples of natural proteins that can bind glycans can be found at the Functional Glycomics homepage(http:/ /www.functionalglycomics.org/ glycomics/molecule/jsp/gbpMolecule-home.jsp). SIGLECs (Sialic acid-binding immunoglobulin-type lectins) are a family of cell surface proteins that bind sialic acid. They are found primarily on the surface of immune cells and are a subset of the I-type lectins. There are 14 different mammalian Siglecs, providing an array of different functions. The family was previously numbered SIGLEC1, SIGLEC2, ... SIGLEC14. Presently many SIGLECs have been renamed. CD22 or cluster of differentiation-22, is also known as SIGLEC2. It is found on the surface of mature B cells and to a lesser extent on some immature B cells. Generally speaking, CD22 is a regulatory molecule that prevents the overactivation of the immune system and the development of autoimmune diseases. Of interest in the present invention is the fact that CD22 is a sugar binding transmembrane protein, which specifically binds sialic acid with an immunoglobulin (Ig) domain located at its N-terminus. Figure 20 shows the specific N-glycan modified Fab binding characteristics of a CD22-Fc molecule wherein at least the sialic acid binding domain is physically linked to an Fc tail. A sialic acid binding part of a SIGLEC typically contains the extracellular part of the respective SIGLEC. Fc hybrids can, for instance be made easily. A preferred SIGLEC is CD22. Antibodies are another source of molecules that can bind N-linked glycans. Lectins are preferred molecules. Most lectins can easily be produced and many are indeed commercially available. The antibody or lectin is preferably a sialic acid binding antibody or a sialic acid binding lectin, preferably a sialic acid binding lectin. A description of various members of the sialic acid family of monosaccharides, structural diversity, and linkage to the

underlying glycan chain is given in Varki A, Cummings RD, Esko JD, et al., editors. (2009) Essentials of Glycobiology chapter 14. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press. The chapter also describes various sialic acid binding lectins and their specificity. In the present invention it has been found that Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA) are particularly suited to distinguish Fc portion N-linked glycans from Fab-portion N-linked glycan. The sialic acid binding lectin that can bind an N-linked glycan on a Fab-portion of an antibody therefore preferably comprises SNA or MAA, preferably SNA (Stadlmann et al., 2010. Journal of Clinical Immunology. 30(S1):15-9). For the sake of clarity maackia amurensis agglutinin is abbreviated with MAA whereas MAA-adduct stands for the malondialdehyde-acetaldehyde adduct on proteins.

In a preferred embodiment the step of contacting antibodies of the sample with a peptide or protein that comprises an epitope with a post-translational modification such as an acetylated lysine epitope or a citrullinated or homo-citrullinated epitope is performed with a peptide or protein that is coupled to a surface. The peptide/protein bound fraction is preferably washed to remove unbound material. The peptide/protein bound fraction containing the preferably AAPA, ACPA and/or anti-CarP antibodies, if any, is then collected and contacted with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody. Subsequently, it can be determined if the sample contained ACPA and/or anti-CarP antibodies with N-linked glycans on a Fab-portion thereof. This can be done in various ways. Preferably this is done by contacting the molecule containing sample with a molecule that binds human antibodies, preferably human IgG. Unbound molecule is subsequently washed and bound molecule, if any, can be detected with a label. Preferably the molecule comprises the label. If label is detected it is determined that an antibody with a N-linked glycan on a Fab-portion of the antibody has bound to the citrullinated epitope (ACPA) or a homo-citrullinated epitope (anti-CarP) in the peptide, and that thus the sample contained ACPA and/or anti-CarP with N-linked glycan on a Fab-portion thereof.

In another preferred embodiment the method of determining whether an antibody containing sample comprises an AMPA, preferably an antibody that can bind an acetylated lysine epitope, a citrullinated or homo-citrullinated epitope in a peptide, comprises

contacting antibodies of the sample with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody and collecting bound antibodies, and

contacting collected antibodies, if any, with a peptide or protein that comprises a post-translationally modified epitope, preferably an acetylated lysine epitope, a citrullinated or homo-citrullinated epitope. In a preferred embodiment antibodies of the sample are first separated from other material in the sample and collected by contacting the sample with a molecule that binds human antibodies. In this way other molecules that may comprise N-linked glycans are not entered into

the method. Subsequently it can be determined if the sample contained AMPA, preferably AAPA, ACPA and/or anti-CarP antibodies with N-linked glycan on a Fab-portion thereof. This can be done in various ways. Preferably this is done by a method that comprises an Elisa specific for AMPA, preferably specific for AAPA, ACPA and/or anti-CarP. Antibodies of the sample are preferably contacted with a peptide/protein that comprises an acetylated lysine, a citrullinated or homo-citrullinated epitope. The peptide/protein is preferably coupled to a surface. Bound antibodies are subsequently detected by means of a molecule that can bind human antibodies, preferably IgG. The molecule preferably comprises a label.

In a preferred embodiment antibodies of the sample are first separated from other material in the sample and collected by contacting the sample with a molecule that binds human antibodies. In this way other molecules that may comprise N-linked glycans are not entered into the procedure.

The invention further comprises a method for determining whether an antibody sample of an individual comprises an AMPA, preferably an AAPA, an ACPA and/or anti-CarP antibody that has N-linked glycan on a Fab-portion, the method characterized in that N-linked glycan on a Fab-portion is detected using a molecule that can bind N-linked glycans on a Fab-portion of an antibody, preferably a sialic acid binding molecule, preferably SNA or MAA.

The step of detecting an N-linked glycan on a Fab-portion of the antibody can advantageously done by contacting the antibody or a Fab-portion thereof with a molecule that specifically binds sialic acid as indicated herein. This facilitates high throughput testing of antibody containing samples. One can also perform mass spectrometry on glycan preparations obtained from purified antibody preparations. As indicated in various figures of the present application mass spectrometry of such preparations yields spectra that disclose the structure of the glycans obtained from the antibody. The sialic acid containing glycans can easily be discriminated in such spectra. The autoantibody or antigen binding fragment thereof can be purified from other antibodies in a preparation for instance by allowing binding to specific antigen coated on beads followed by one or more washes to remove unbound antibody. N-linked glycans can be collected from such beads or eluted antibody (fragments) by enzymatic cleavage as demonstrated in the examples. The collected glycans can subsequently be identified by means of mass spectrometry. Where mass spectrometry used to be a time consuming endeavor it is now rapidly being optimized and streamlined so that becomes useful for medium to high throughput applications. Examples of suitable mass spec systems are the systems marketed by Waters, for instance under the tradename Glycoworks RapiFluor-MS N-Glycan kit.

The molecule that can bind a human antibody can be an antibody, for instance a goat anti human IgG antibody. Other molecules are protein A and protein G. Protein A is a 42 kDa surface protein originally found in the cell wall of the bacteria Staphylococcus aureus. Protein G is an immunoglobulin-binding

protein expressed in group C and G Streptococcal bacteria much like Protein A but with differing binding specificities.

The invention further comprises a kit of parts useful in the detection of an preferably a citrulline, a homo-citrulline and/or an acetylated lysine binding AMPA, preferably an AAPA, ACPA and/or antiCarP antibody in a sample. The kit preferably comprises a peptide that comprises an epitope with a post-translational modification, preferably an acetylated lysine epitope, a citrullinated or homo-citrullinated epitope and a molecule that can bind an N-linked glycan on a Fab-portion of an antibody. The peptide or the molecule that can bind an N-linked glycan is preferably linked to a surface. The kit preferably further comprises a molecule that can bind a human antibody. The molecule that can bind an N-linked glycan on a Fab-portion of an antibody is preferably a sialic acid binding lectin, preferably SNA or MAA, preferably SNA. The molecule preferably comprises a label.

The antibody sample is preferably a sample of an individual. Preferably of an individual that does not have RA, Sjögren syndrome or AAV. In a preferred embodiment the sample is a sample from an individual of which an earlier sample tested positive for the presence of an autoantibody associated with said disease such as an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody and in which the antibody did not comprise an N-linked glycan on a Fab-portion of the antibody. An autoantibody such as an AMPA is considered to be devoid of (or negative for) N-linked glycan on a Fab-portion of the antibody when 10% or less of the autoantibody comprises an N-linked glycan on a Fab-portion thereof. Thus in one aspect 10% or less of the autoantibody, preferably an AMPA, in the earlier sample comprises an N-linked glycan on a Fab-portion thereof. The invention further provides a method of monitoring an individual at risk of developing arthritis, Sjögren syndrome or AAV, the method comprising monitoring the presence and/or the onset of an autoantibody associated with said disease such as an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody in periodic antibody containing samples of said individual, the method characterized in that the method further comprises determining whether detected antibodies comprise an N-linked glycosylation at one or more positions in a Fab-portion of the antibody. Methods of the invention are particularly suited in screening a population of individuals for the conversion from a pre-disease "at-risk phase" into disease phase, such as RA. To this end the sample that is tested in a method of the invention is preferably a sample from an individual that has been tested previously for RA. The antibodies are preferably AAPA, ACPA and/or anti-CarP antibodies.

Upon detection of AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody, with an N-linked glycan on a Fab-portion of an antibody, the individual can be treated for arthritis, preferably rheumatoid arthritis, preferably with an arthritis medicament, preferably a rheumatoid arthritis medicament. As mentioned herein above, early treatment is beneficial to the patients. Also pre-

symptomatic treatment is beneficial to individuals at risk of developing RA. Treatments are expensive and typically not completely without side effects, or the potential thereof. It is preferred to start pre-symptomatic treatment as soon as possible, i.e. when symptoms of RA are expected but at least when symptoms are imminent.

A person can always attract a disease. A person is said to be at risk using a method as described herein if that person has an increased risk over the normal population. For instance, a person that does not have RA but that does have an AMPA has an increased risk of developing RA. When such an individual is tested with a method as described herein and found to have an AMPA with an N-linked glycan on a Fab-portion thereof, that person has an increased risk of developing RA when compared to the population of AMPA positive RA negative individuals as a whole. A person has an AMPA with an N-linked glycan on a Fab-portion thereof if more than 10% of the AMPA antibodies in an antibody containing sample of said individual has an N-linked glycan on a Fab-portion thereof, preferably more than 20%, preferably more than 30%, preferably more than 50%, preferably more than 55% of the AMPA antibodies in an antibody containing sample of said individual has an N-linked glycan on a Fab-portion thereof. The same holds for an individual at risk of developing Sjogren syndrome or AAV. For instance, a person that does not have Sjogren syndrome or AAV but that does have an autoantibody associated with said disease has an increased risk of developing Sjogren syndrome or AAV. When such an individual is tested with a method as described herein and found to have the autoantibody with an N-linked glycan on a Fab-portion thereof, that person has an increased risk of developing Sjogren syndrome or AAV when compared to the population of autoantibody positive Sjogren syndrome or AAV negative individuals as a whole. A person has an autoantibody with an N-linked glycan on a Fab-portion thereof if more than 10% of the autoantibodies in an antibody containing sample of said individual has an N-linked glycan on a Fab-portion thereof, preferably more than 20%, preferably more than 30%, preferably more than 50%, preferably more than 55% of the autoantibodies in an antibody containing sample of said individual has an N-linked glycan on a Fab-portion thereof.

The invention thus further provides an arthritis medicament for use in a method of treatment of an individual comprising determining the presence or absence of an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody that comprises N-linked glycosylation at one or more positions in a Fab-portion of the antibody in an antibody containing sample of the individual, and treating the individual when said antibody, preferably AAPA, ACPA or anti-CarP antibody comprising N-linked glycosylation in a Fab-portion has been detected.

Also provided is a method of treating an individual for a rheumatoid arthritis symptom or the risk of development thereof, the method comprising

- determining whether a sample contains an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody that comprises N-linked glycosylation at one or more positions in a Fab-portion thereof;
- wherein the sample is an antibody containing sample of the individual and the individual did not have rheumatoid arthritis at the time of sampling; and
- treating the individual with a rheumatoid arthritis medicament prior to or at the onset of the individual presenting with a rheumatoid arthritis symptom.

RA is a disease for which many different medicaments are available. A preferred medicament is a Disease Modifying Anti-Rheumatic Drug (DMARD). DMARDs is category of otherwise unrelated drugs defined by their use in rheumatoid arthritis to slow down disease progression. The term is often classified as synthetic DMARDs (sDMARDs, conventional or targeted) or biological DMARDs and used in contrast to non-steroidal anti-inflammatory drugs (which refers to agents that treat the inflammation but not the underlying cause) and steroids (which blunt the immune response but are insufficient to slow down the progression of the disease). In one embodiment the rheumatoid arthritis medicament as referred to in the present invention is a non-steroidal anti-inflammatory drug or a steroid. In a preferred embodiment the rheumatoid arthritis medicament is a sDMARD. Methotrexate is a preferred sDMARD. Other preferred DMARDs are abatacept; adalimumab; tocilizumab; azathioprine; chloroquine and hydroxychloroquine;; etanercept golimumab; infliximab; leflunomide; methotrexate;; rituximab and sulfasalazine. In addition, small molecule inhibitors such as tofacitinib represent a novel class of kinase inhibitors that are available in some countries. In a preferred embodiment the DMARD is a monoclonal antibody that can bind tumor necrosis factor alpha (TNF-α). The rheumatoid arthritis medicament may also be a combination of two or more medicaments wherein one or more of the combination is a DMARD.

The tested individual is preferably provided with the rheumatoid arthritis medicament prior to or at the onset of the individual presenting with a rheumatoid arthritis symptom.

A medicament for the treatment of AAV or Primary Sjögren syndrome is preferably one or more of hydroxychloroquine methotrexate, azathioprine, leflunomide, a glucocorticoid, rituximab, cyclophosphamide or mycophenolate.

The antigen-binding (Fab) portion comprises a region on an antibody that binds to antigens, the so-called variable domain. It preferably further comprises a constant domain that is associated with the variable domain in an antibody (Together often referred to as a Fab-fragment). The Fab-portion is composed of a part of the heavy chain and a part of the light chain of the antibody. Fc and Fab fragments can be generated in the laboratory. The enzyme papain can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment. The enzyme pepsin cleaves below the hinge region, so a F(ab')2 fragment and a pFc' fragment is formed. Recently another enzyme for generation of F(ab')2 has been

commercially available. The enzyme IdeS (Immunoglobulin degrading enzyme from Streptococcus pyogenes, trade name FabRICATOR) cleaves IgG in a sequence specific manner at neutral pH.

Many of the methods can be performed with a Fab-portion or a Fab-fragment in part or all of the method, instead of a complete antibody. Such methods are therefore also provided in the present invention. This is typically clear to the skilled person.

Antibodies are purified when they are separated from other antibodies in the sample. The purified antibodies are typically separated from other antibodies on the basis of one or more characteristics. As a result purified antibodies share the one or more characteristics used for the separation. The purified antibodies do not have to contain only one type of antibody. In the case of AMPA antibodies it is perfectly possible that the purified antibodies all bind CCP2 (for instance) but nonetheless have different variable domains. Similarly, antibodies that are purified on the basis of binding to a molecule that binds an N-linked glycan on a fab-portion of the antibody can have different fab-portion linked glycans, as long as all thus purified antibodies bind to the molecule. A method is a method of purifying an antibody if it separates antibodies of a sample into fractions wherein at least one fraction has a percentage of purified antibody relative to all antibody in the fraction that is higher than the percentage in the sample prior to purification. In other words the purified antibodies do not have to be essentially pure. Typically, however, it is preferred that a purified sample comprises at least 70%, more preferably at least 80%, preferably at least 90% and more preferably at least 95% of the purified type relative to all antibodies in the sample.

Determining the risk of an individual developing RA in a given time period is done by determining that the individual has an AMPA, preferably an AAPA, ACPA and/or anti-CarP antibody that comprises N-linked glycosylation at one or more positions in a Fab-portion thereof. The identification of such antibodies indicates the increased risk of the patient to develop RA in the indicated time period. The level at which such antibodies are detected is a measure for the actual time until development of RA symptoms. A high level indicates that the onset of disease is expected. Levels are preferably determined relative to the total amount of antibodies in the sample. Preferably they are determined relative to total AAPA, ACPA and/or anti-CarP in the sample.

The invention also provides a method of purifying antibodies for the measurement of antibodies comprising N-linked glycan on a Fab-portion thereof comprising

providing an antibody sample;

contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;

removing unbound antibody and eluting bound antibody from said solid surface;
incubating eluted antibody with a solid surface comprising a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
removing unbound molecules; and
determining whether an antibody has bound to said solid surface,
wherein a bound antibody indicates the presence of an N-linked glycan on a Fab-portion of an antibody in said sample. It is not necessary to purify complete antibodies. Antibody in the sample can be fragmented into Fab-portion and Fc fragments, for instance, and subsequently these fab-portion fragments are purified.

The invention also provides a method of purifying antibodies for the measurement of antibodies that have an N-linked glycan on a Fab-portion comprising
providing an antibody sample;
contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
removing unbound antibody;
incubating said solid surface with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
removing unbound molecules; and
determining whether a molecule has bound to said solid surface,
wherein a bound molecule indicates the presence of an N-linked glycan on a Fab-portion of an antibody in said sample.

Further provided is a method of purifying antibodies for the measurement of antibodies that have an N-linked glycan on a Fab-portion comprising
providing an antibody sample;
contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
removing unbound antibody;
incubating bound antibody with an enzyme that separates glycan from said antibody;
collecting glycans; and
determining whether the collected glycans comprise a Fab-portion specific glycan;
the method characterized in that the sample is an antibody sample of an individual at risk of developing RA, Sjögren syndrome or AAV.

Also provided is a method of purifying antibodies with N-linked glycosylation on a Fab-portion of the antibody, the method comprising
providing an antibody sample;
incubating said sample with a solid surface that comprises a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
washing said solid surface and collecting bound antibody;

incubating collected antibody with a peptide or protein that comprises a modified protein epitope; removing unbound antibody; and
determining whether said peptide or protein had bound antibody.

In certain embodiments the sample comprises purified antibodies. In such cases the antibodies were typically separated from other proteins of the sample by means of a binding agent that binds antibodies. Suitable agents are protein A or protein G.

Also provided is a method comprising
providing an antibody sample;
contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
removing unbound antibody;
incubating said solid surface with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
removing unbound molecules; and
determining whether a molecule has bound to said solid surface.

The sample is preferably an antibody sample of an individual at risk of developing RA, Sjögren syndrome or AAV. Preferably wherein the sample is an antibody sample of an individual of which an earlier antibody sample was tested positive for an autoantibody. Preferably, 10% or less of the autoantibody of said earlier antibody sample comprises an N-linked glycan on a Fab-portion thereof. Said autoantibody is preferably an AMPA. In certain embodiments the antibodies are cleaved to produce Fab and Fc fragments.

Antibody can be eluted from a solid surface by various means. Typically this is achieved by changing the pH and/or the salt concentration of the surrounding fluid. The antibody is generally absorbed, bound to, an absorbent on a solid phase or solid surface. Elution is the process of removing analytes from the adsorbent by running a solvent, called an "eluent", past the adsorbent/antibody complex. As the solvent molecules "elute", or travel down through the column, they can either pass by the adsorbent/analyte complex or they can displace the analyte by binding to the adsorbent in its place. After the solvent molecules displace the analyte, the analyte can be carried out of the column for analysis.

Unbound antibody is typically removed by washing the solid phase with a buffer. Suitable buffers are buffers used to load to the antibody on the solid surface. Phosphate buffered saline is a suitable buffer.

Periodic antibody containing samples of an individual are samples that are taken at different time point in the life the individual. The interval between the time points can vary. The time between respective samples can be a month, two

months, 6 months, a year, or even more. The time periods between samples can be longer when the autoantibody is negative for an N-linked glycan on a Fab-portion thereof. Time periods between sample of a series of periodic samples can vary and can be very short (days) to very long more than a couple of years also of periodic samples of one individual.

The moment of sampling is the day on which a sample of an individual has been collected. The taking of a sample can be part of the claim but is typically not part of the claim. The sample provided in the methods referred to in the claims is typically collected by an authorized person and subsequently handed over to provide it for a method as described herein.

For the purpose of clarity and a concise description features are described herein as part of the same or separate embodiments, however, it will be appreciated that the scope of the invention may include embodiments having combinations of all or some of the features described.

The invention further provides a method for determining whether an antibody comprises an N-linked glycan, the method comprising purifying AMPA from an antibody containing sample and detecting whether said AMPA comprises an H5N4S2; H5N5F1S1, H5N4F1S2; H5N5S2; H5N5F1S2 and/or H6N5F1S2 glycan of table 1. In a preferred embodiment the method further comprises determining whether the glycan is present on a FAB-portion of the antibody when one or more of said glycans have been detected.

name	name in figures of patent	stucture	UHPLC GP number
H5N2			GP5
H3N3F1			GP1
H3N4			GP2
H6N2			
H3N4F1	G0F		GP4
H4N4	G1		GP7
H7N2			
H3N5	G0B		GP3
H4N3S1			GP16
H4N4F1	G1F		GP8/9
H5N4	G2		GP12
H3N5F1	G0FB		GP6
H4N5	G1B		
H4N3F1S1[a]			
H8N2			
H4N4S1[a]			
H5N4F1	G2F		GP14
H4N5F1	G1FB		GP10/11
H5N5	G2B		GP13
H9N2			
H4N4F1S1[a]	G1FS1		GP16
H5N4S1[a]	G2S1		GP17
H4N5S1[a]	G1S1B		
H5N5F1	G2FB		GP15
H5N4F1S1[a]	G2FS1		GP18
H4N5F1S1[a]	G2FS1B		
H5N5S1[a]	G2S1B		
H5N4S2[a]	G2S2		GP21
H5N5F1S1[a]	G2FS1B		GP19
H5N4F1S2[a]	G2FS2		GP23
H5N5S2[a]	G2S2B		GP22
H5N5F1S2[a]	G2FS2B		GP24
H5N4F1E1L1[a,b]			
H5N5F1E1L1[a,b]			
H6N5F1S2[a]			GPx

a: α2,6 sialic acid, confirmed by EE-glycans in MALDI-TOF-MS
b: α2,3 sialic acid, confirmed by EE-glycans in MALDI-TOF-MS
H: Hexoses
N: N-acetylhexosamine
F: Fucose
B: Bisecting N-acetylglucosamine
S: Sialic acid
E: ethyl esterified α2,6-linked N-acetylneuraminic acid (sialic acid)
L: lactonized α2,3-linked N-acetylneuraminic acid

Table 1: Nomenclature of glycans found on ACPA-IgG and IgG. G2S2, G2FS1B, G2FS2, G2S2B, G2FS2B are considered as Fab-glycans because these glycan structures are highly expressed by Fab fragments of ACPA-IgG and IgG, whereas these structures are low- or not expressed on Fc fragments.

EXAMPLES

Example 1

Material and Methods

Patient samples

For experiments to compare ACPA glycosylation with total IgG glycosylation, plasma (n=6) and synovial fluid (n=3) samples from 9 ACPA-positive RA patients were collected at the outpatient clinic of the rheumatology department at LUMC. All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA.

For the experiments to compare ACPA-Fab glycans of ACPA derived from ACPA-positive RA patients and their ACPA-positive healthy relatives serum samples were collected from 53 ACPA-positive RA patients and their unaffected ACPA-positive first degree relatives at rheumatology clinics in Canada. The prevalence of RA is considerably higher in these communities than in the general Caucasian population, and ACPA are present at increased frequency in healthy relatives of patients [1, 2]. RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA.

Purification of antibodies

ACPA-IgG and IgG of samples collected in Leiden were purified on fast protein liquid chromatography (ÄKTA, GE Healthcare) as described previously [3]. Briefly, samples were loaded on a biotinylated CCP2-arginine-HiTrap-streptavidin column (GE Healthcare) followed by a biotinylated CCP2-citrulline-HiTrap-streptavidin column connected in series. The flow through (FT) and ACPA-eluted fractions were further loaded on a HiTrap protein G and subsequently on a HiTrap protein A column (both from GE Healtcare). The purified fractions of ACPA-depleted IgG (control IgG) and of ACPA-IgG were concentrated and desalted by size exclusion chromatography.

ACPA-IgG was purified from Canadian samples using an micro-bead system. Briefly, 25 ul plasma or serum was loaded on neutravidine beads which was coupled to a biotinylated CC(cit)P peptide. The samples were incubated for 2 hours and ACPA was eluted with formic acid and neutralized to a Ph of 7,5. The ACPA-eluted fractions were further loaded on Prot G beads to end up with ACPA-IgG.

Structural analysis

The structural analysis to compare ACPA with general IgG was performed on F(ab)$_2$ or Fc fragments of the isolated ACPA-IgG and IgG. F(ab)$_2$ and Fc fragments were generated by antibody digestion with IdeS (FabRICATOR; Genovis) and purified by IgG-Fc/CH1 CaptureSelect affinity beads (Thermo Fisher). *N*-glycans from F(ab)$_2$ and Fc fragments of (ACPA)-IgG were released in solution using PNGase F. Glycans were labelled with 2-aminobenzoic acid (2-AA), purified by hydrophilic interaction chromatography solid-phase extraction (HILIC-SPE) and characterized by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) and Ultra-high performance liquid chromatography (UHPLC).

For the experiments to compare ACPA-Fab glycans of ACPA derived from ACPA-positive RA patients and their healthy relatives, structural analysis was performed on the isolated ACPA-IgG. N-glycans of the ACPA-IgG were released in solution using PNGase. In addition, the glycans were labelled with 2-aminobenzoic acid (2-AA), purified by hydrophilic interaction chromatography solid-phase extraction (HILIC-SPE) and characterized by Ultra-high performance liquid chromatography (UHPLC).

Data and statistical analysis

UHPLC data were analysed with Chromeleon 7. The software calculates the area under the curve of the chromatograms. Glycan peaks and glycosylation-derived traits were defined as previously described.[4] The percentage of galactosylation, sialylation, fucosylation and the frequency of bisecting *N*-acetylglucosamine residues of IgG were calculated. In addition the percentage Fab glycosylation is calculated with the following formula: (sum of GP19 till GP24)/(sum of GP1 till GP14)*100%. The statistical analysis was performed using GraphPad Prism 6. A non-parametric paired Wilcoxon test was applied with a significance limit at $p<0.05$.

Results

Recently, we discovered that ACPA-IgG obtained from RA-patients exhibit a 10-20 kDa higher molecular weight compared with non-autoreactive IgG. This feature also distinguished ACPA-IgG from antibodies against recall antigens or other disease-specific autoantibodies. Structural analysis showed that the presence of N-linked glycans in the (hyper)variable domains (F(ab) domains) of ACPA is responsible for this observation. Elucidation of the precise sites where the N-linked glycans are located revealed that the N-linked consensus sequence required for N-linked glycosylation of proteins was not germline encoded but had been introduced upon somatic hypermutation [5]. Structural analysis of the N-linked Fab-glycans

present on ACPA showed that the composition of Fab-linked glycans differed from Fc-linked sugars and, more importantly, that these are highly sialylated (Figure 1). Moreover, based on quantification, we estimate that over 90% of ACPA molecules present in serum harbor F(ab)-glycans, a percentage that is even higher on ACPA in synovial fluid.

Remarkably, our preliminary data show that the frequency of hyperglycosylated ACPA is considerably lower on ACPA derived from healthy ACPA-positive relatives (Figure 2). This is intriguing as it indicates that ACPA present in healthy individuals have not yet introduced the N-linked glycosylation sites in the ACPA variable regions required for glycosylation.

Although the methodology described above can be converted into an high-throughput assay, the current assay to detect ACPA F(ab)-hyperglycosylation is time-consuming and requires high-end mass spectrometry and expertise. Therefore, a more accessible method that can be used in day-to-day routine would be preferred. It has been demonstrated that the binding of antibodies to the lectin SNA (Sambuccus Nigra Agglutinin) is primarily mediated by F(ab) glycosylation and that two sialic residues are required for binding to SNA. SNA will only bind to the Fc part under reducing conditions (which opens up the interface between CH2 domains)[6-8]. As most ACPA F(ab)-glycans contain two sialic acid residues, it is highly likely that SNA-binding of serum antibodies from RA-patients will enrich for ACPA. Therefore, an SNA-binding-based approach to detect ACPA F(ab)-glycans represents a promising strategy to visualize the presence of these glycans without the need of high-end mass spectrometry. Therefore, we embarked on two approaches to establish a high-throughput method based on SNA-detection. These approaches aim to develop a standardized protocol allowing the detection of ACPA F(ab)-glycans in a high-throughput manner.

In the first approach (Figure 3), we will first capture ACPA using CCP-coated microbeads (left panel). After elution of ACPA from these beads, we will visualize the presence of F(ab) glycans using labelled SNA (right panel). As our preliminary data indicate that the F(ab) glycans do not directly interact with antigen and hence might be accessible for SNA, it is conceivable that elution of ACPA from the beads is not necessary and that SNA can directly bind to ACPA F(ab)-glycans. Therefore, also this possibility will be tested.

In the second approach (Figure 4, left panel), a reverse strategy will be taken by first immobilizing F(ab)-glycosylated IgG (ACPA) on SNA, followed by detection of ACPA using a CCP-ELISA. Our preliminary data indicate that this approach is feasible and can be used in an high throughput manner (Figure 4, right panel). At present this approach includes pre-isolation of IgG by protein A in order to prevent "overloading" of SNA by other molecules present in serum that carry sialic acid molecules. Our preliminary data suggest that this might not be required, a possibility that will also be investigated in this context.

Example 2

Material and methods

Patient samples

Plasma (n=6) and synovial fluid (n=3) samples from nine ACPA-positive RA patients were collected at the outpatient clinic of the rheumatology department at Leiden University Medical Center. All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA and gave written informed consent [9]. Treatment included disease-modifying anti-rheumatic drugs, biological agents and glucocorticoids.

Chemicals, solvents and enzymes used

TFA, SDS, disodium hydrogen phosphate dihydrate, HCl, Glycine, ß-mercaptoethanol, acetic acid and NaCl were purchased from Merck (Darmstadt, Germany). Fifty percent sodium hydroxide and Nonidet P-40 substitute, Hyaluronidase from bovine testes type IV, EDTA, 2-aminobenzoic acid, 2-picoline borane complex, ammonium hydroxide, DMSO and formic acid were obtained from Sigma-Aldrich (St Louis, USA). Tris was purchased from Roche (Indiana, USA) and the Laemmli buffer was obtained from Bio-Rad (California USA). Peptide:N-glycosidase F (PNGase F) was bought from Roche Diagnostics (Mannheim, Germany), 2,5-dihydroxybenzoic acid from Bruker Daltonics (Bremen, Germany) and HPLC SupraGradient ACN from Biosolve (Valkenswaard, Netherlands). MQ (Milli-Q deionized water; R > 18.2 MΩ cm-1; Millipore Q-Gard 2 system, Millipore, Amsterdam, The Netherlands) was used throughout. CaptureSelect anti-IgG Fc affinity matrix and anti-CH1 affinity matrix were bought from Life Technologies (Leiden, The Netherlands). Empty Spin Column with closed screw cap, inserted plug and large 10 um filter were provided from MoBiTec (Goettingen, Germany). The PBS was obtained from B. Braun (Meslungen Germany) and the IdeS enzyme (trade name FabRICATOR) from Genovis (Lund, Sweden). The CCP2 arginine (control) and CCP2 citrulline peptides were kindly provided by Dr. J.W. Drijfhout, Department of IHB, Leiden University Medical Center (LUMC), The Netherlands.

Purification of ACPA-IgG and ACPA-depleted IgG.

ACPA-IgG and IgG were purified on fast protein liquid chromatography (ÄKTA, GE Healthcare) as previously described [5]. Briefly, samples were loaded on a biotinylated CCP2-arginine-HiTrap126 streptavidin column (GE Healthcare) followed by a biotinylated CCP2-citrulline-HiTrap-streptavidin column connected in series. The flow through (FT) and ACPA-eluted fractions were further loaded on a HiTrap protein G and subsequently on a HiTrap protein A column (both from GE Healthcare). The purified IgG and ACPA-IgG of the isotypes 1,2 and 4 were then

concentrated and desalted by size exclusion chromatography (ZebaSpin Desalting Column, 7K MWCO, Pierce Thermo Scientific) according to the manufacturer's instructions.

Generation and purification of Fc and F(ab')2 fragments

ACPA-IgG and ACPA-depleted IgG were specifically cleaved into Fc and F(ab')2 portions by using the recombinant streptococcal IdeS enzyme. The supplier's protocol was adjusted to simplify the procedure as previously described [5]. Briefly, for each sample, 30 µg of (ACPA)-IgG antibodies were dried under centrifugal evaporator and digested by adding 200 µL digestion buffer (50 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA) containing 30U of IdeS followed by incubation at 37°C for overnight. The Fc portion was then separated from the F(ab')2 by affinity chromatography on anti-IgG Fc affinity matrix (bead slurry) loaded on a 10µM filter spin column. The Fc fragments were eluted from beads with 100 mM formic acid and neutralized with 2 M Tris. In order to capture the F(ab')2 domain, the FT fraction resulting from the Fc purification was purified on anti-IgG-CH1 affinity matrix using a similar protocol as for the anti-IgG Fc affinity matrix. Elution fractions were neutralized with 2 M TRIS and desalted by size exclusion chromatography (Zeba Spin Desalting Columns, 7 kDa MWCO, Pierce Thermo Scientific). Following purification, 6 µg of the purified Fc and F(ab')2 samples were analyzed for their purity by SDS-PAGE and quantified by bicinchoninic acid Protein Assay Reagent (Pierce Thermo Scientific). For glycan analysis, the samples were dried by vacuum centrifugation.

Structural analysis

The structural analysis was performed on of either the total molecule, F(ab)2 or Fc fragments of the isolated ACPA-IgG and IgG from nine RA patients. In addition, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed of (ACPA)-IgG. *N*-glycans form total molecule, F(ab)2 and Fc fragment of (ACPA)-IgG were released in solution using PNGase F, whereas the heavy and light chain (HC/LC) glycans were obtained following in-gel digestion with PNGase F. Labelling of glycans was performed by mixing the samples (in 25 µL) with 12.5 µL of 2-aminobenzoic acid (2-AA; 48 mg/mL) in DMSO with 15% glacial acetic acid and 12.5 µL 2-picoline borane (107 mg/mL) in DMSO. The mixture was incubated for 2 h at 65 °C, cooled down to room temperature and diluted to 85% ACN prior to purification. The 2-AA labelled glycans were purified by HILIC SPE using cotton tips as described previously with some modifications [10]. Briefly, for each sample, 500 µg of cotton were packed into a 200 µL pipette tip and conditioned by pipetting three times 150 µL MQ, followed by 150 µL 85% ACN 0.1% TFA and two times 150 µL 85% ACN. The sample (in 85% ACN) was loaded by pipetting 25 times into the reaction mixture. The tips were washed three times with, three times with 150 µL 85% ACN 0.1% TFA and two times 150 µL

85% ACN. The 2-AA labelled glycans were finally eluted from the cotton with 30 µL MQ and identified by MALDI-TOF-MS and UHPLC. For MALDI-TOF-MS analysis, 2 µL of glycan sample purified by cotton HILIC SPE were mixed on spot with 1 µL of 2,5-dihydroxybenzoic acid matrix (20 mg/mL in 50% ACN, 50% water) on a Bruker AnchorChip plate (800 µm anchor; Bruker Daltonics, Bremen, Germany) and allowed to dry at ambient temperature. Measurement was performed in linear negative mode on an UltrafleXtreme MALDI-TOF-MS (Bruker Daltonics) using FlexControl 3.4 software (Bruker Daltonics). A peptide calibration standard (Bruker Daltonics) was used for external calibration. For each spectrum, a mass window of *m/z* 1000 to 4000 was used and a minimum of 5000 laser shots were accumulated. Regarding UHPLC analysis, 5 µL of purified 2-AA labelled *N*-glycan solution were separated and analyzed by HILIC-UHPLC on a Dionex Ultimate 3000 (Thermo Fisher Scientific) equipped with a 1.7 µm 2.1x100 mm Acquity UHPLC BEH Glycan column (Waters) and with a fluorescent detector. Separation was performed at 60°C with a flow rate of 0.6 mL/min. Two solutions were used for gradient generation, ACN as solution A, and 100 mM ammonium formate pH 4.4 (prepared as formic acid buffered to pH 4.4 by ammonium hydroxide) as solution B. The column was equilibrated by 85% solution A for 0.5 min. The samples were then loaded in 75% A, and excess of fluorescent reagent was eluted from the column by washing with 85% A 47 for 10 min. The separation gradient started at 75% A and decreased linearly to 63% A in 30 min. The column was then flushed at a flow rate of 0.4 mL/min with 40% A for 4 min followed by 10 min of 85% A for re-equilibration. For fluorescent detection, 330 nm was used for excitation and the emission recorded at 420 nm. The resulting chromatograms were analyzed using Chromeleon version 7.1.2.1713 (Thermo Fisher Scientific). Finally, to analyze the Fc-linked glycosylation of (ACPA)-IgG at the glycopeptide level, antibodies were digested with trypsin and analyzed by LC-MS as described [11].

Data and statistical analysis

UHPLC data was analyzed with Chromeleon 7; the program calculates the area under the curve of the UHPLC chromatograms. Glycan peaks and glycosylation-derived traits were defined as previously described [4]. The percentage of galactosylation (non-galactosylated G0, monogalactosylated G1 and digalactosylated G2), sialylation (non-sialylated N, mono-sialylated S1 and Disialylated S2), fucosylation (F) and the frequency of bisecting N-acetylglucosamine (GlcNAc, B) residues of IgG were calculated as followed : G0=GP1+GP2+GP4+GP5+GP6, G1=GP7+GP8+GP9+GP10+GP11+GP16, G2=GP12+GP13+GP14+GP15+GP17+GP18+GP19+GP21+GP22+GP23+GP24, N=GP1+GP2+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11+GP12+GP13+GP14+ GP15, S1=GP16+GP19, S2=GP21+GP24, F= GP1+GP4+GP6+GP8+GP9+GP10+GP11+GP14+GP15+GP16+GP18+GP19+GP23+ GP24 and B=GP6+GP10+GP11+GP13+GP15+GP19+GP22+GP24. Analysis of the glycan traits of the LC-MS were previously described [11]. For the processing of

LC-MS data the total intensity of the first three isotopes of every observed analyte charge state was extracted within a window of ±0.06 Da around the theoretical mass and ±20 s around the manually extracted average retention time as described earlier [12]. Glycan identification by MALDI-TOF-MS were defined as previously described [13]. The statistical analysis was performed using GraphPad Prism 6. A non-parametric paired Wilcoxon test was applied with a significance limit at $p<0.05$.

Results

Quantification of the N-glycans expressed by IgG and ACPA-IgG.

We have demonstrated that ACPA-IgG produced by RA patients are extensively *N* glycosylated in the variable region as compared to other IgG (auto)antibodies [5]. Here, we performed a comprehensive quantitative and qualitative analysis of the glycosylation of ACPA-IgG and its fragments and compared it to that of non-citrulline specific IgG (i.e. depleted of ACPA hereafter named control IgG). To this end, (ACPA)-IgG were purified by affinity chromatography and their glycans were analyzed by UHPLC, MALDI-TOF-MS and/or LC-MS according to the scheme presented in Figure 9. Following purification, the purity of (ACPA)-IgG was assessed by SDS-PAGE under reducing conditions (Figure 10A). As expected, control IgG was characterized by two electrophoretic bands corresponding to the heavy and light chains (HC and LC), whereas ACPA-IgG showed several HC and LC bands with higher molecular weights as described previously(13).Released *N*-glycans from both the HC of IgG and the HC1 of ACPA-IgG displayed a typical Fc-linked glycan profile in UHPLC (21, 25), while no *N*-glycans were detected in the LC of IgG and the LC1 of ACPA-IgG (Figure 10B). In contrast, *N*-glycans released from LC2 of ACPA-IgG showed a different profile, indicating the presence of diantennary glycoforms that were highly sialylated (Figure 10B). Likewise, the glycosylation profiles derived from HC2 and HC3 of ACPA-IgG showed the presence of a mixture of Fc-glycans but also of additional glycans usually not present in the Fc-domain (Figure 10B).

Fc-linked and Fab-linked glycans of IgG (auto)antibodies exhibit typical antibody glycan patterns.

To determine if the glycan pattern detected in the additional HC band of ACPA-IgG, i.e. HC2 and HC3, truly reflects the glycosylation of the IgG variable region [14]. We investigated the *N* glycosylation of (ACPA)-IgG and its fragments (Total/Fc/Fab) or glycopeptides (for Fc only) (Figure 9). We first analyzed and compared the structure of *N*-glycans released from Fc and F(ab')2 fragments of ACPA-IgG and control IgG (from the same donor). The *N* glycosylation profile derived from (ACPA)-IgG Fc fragments exhibited typical Fc-linked *N*-glycan structures that consisted of diantennary, often core fucosylated complex type

species with a variable number of antenna galactose (0 to 2) and sialic acid (0 to 1) residues (Figure 11A). Part of the Fc-linked *N*-glycans also contained a bisecting GlcNAc. Of note, a relatively high proportion of agalactosylated glycans (G0) was observed as previously described [11]. The *N* glycans released from (ACPA)-IgG F(ab')2 fragments consisted of highly galactosylated and sialylated diantennary glycoforms, that may carry bisecting GlcNAc and/or a core fucose (Figure 11B). Together, the results demonstrate that the *N*-glycan species attached to the Fc and Fab fragments of IgG (auto)antibody differ with a striking presence of highly sialylated glycan species in the glycans linked to the Fab-domain of ACPA-IgG.

The Fab-linked glycosylation pattern of ACPA-IgG differs from the pattern on "conventional" IgG.

We have shown that Fc-linked *N*-glycans of ACPA-IgG isolated from patients present a more pronounced reduction in the level of galactosylation and sialylation but an increased degree of core fucosylation than those of other IgG molecules [11, 15]. In agreement, the Fc-glycans of ACPA IgG purified in this study exhibit a lower level of sialylation (S 12% [IQR9-16%] for ACPA-IgG versus 16% [IQR13-17.5%] for control IgG) as well as a higher frequency of core fucosylation in comparison with that of control IgG (F 99.3% [IQR98.7-99.7%] for ACPA-IgG versus 91.8% [IQR90.3-99.7%]) IgG. In addition, however, our data revealed important differences between the Fab-linked *N*-glycan profile of ACPA-IgG and that of control IgG (Figure 12). Especially, ACPA-IgG Fab *N*-glycans displayed a high frequency of di-galactosylated species (G2; 73%[IQR69.5-80%] for IgG versus 84%[IQR74-87%] for ACPA-IgG) and di-sialylated 259 species (S2; 27%[IQR19-30%] for IgG versus 44%[IQR34-48.5%] for ACPA-IgG), as also exemplified by an increase in the ratio of sialic acid per galactose (SA/Gal; 36%[IQR33-37%] versus 30%[IQR27-31.5%] for ACPA-IgG and IgG). In addition, we found higher levels of core fucose and bisecting GlcNAc residues in 7 out of 9 samples. In general, stronger glycan differences were observed between the glycan structures derived from the Fab domain of ACPA-IgG and control IgG than between the glycans from the Fc portions.

ACPA-IgG exhibit a higher level of Fab glycosylation.

We quantified the amount of Fab glycosylation present on ACPA-IgG and IgG depleted from ACPA. To estimate the level of Fab glycosylation, glycans were released from ACPA-IgG and ACPA depleted IgG, characterized by MALDI-TOF-MS and their relative abundance was measured by UHPLC. Whereas the glycan profile of total IgG was dominated by Fc-linked *N*-glycans (G0F, G1F and G2F), the total glycan profile of ACPA-IgG exhibited a large quantity of Fab-linked *N*-glycan (G2FBS1, G2FS2 and G2FBS2) (Figure 13A). Importantly, the identification of a number of these glycoforms specific for either the Fc- or the F(ab')2-fragment, and the quantification of these glycoforms released from the entire antibody molecule,

enabled us to determine the overall frequency of Fab glycans on either ACPA-IgG or ACPA-depleted IgG (Figure 13B). All ACPA-IgG samples (n=9) exhibited an increased frequency of Fab glycosylation compared to control IgG. The median Fab-glycosylation level of IgG depleted of ACPA was estimated at 17% [IQR12%-26%], with large differences between donors. In contrast, the median Fab glycosylation of ACPA-IgG reached 93% [IQR77-123%]. Together, these data indicate that the median Fab glycosylation of ACPA-IgG is 5 times higher than that of control IgG.

(ACPA)-IgG derived from plasma and synovial fluid display different Fab glycosylation profiles.

We demonstrated that ACPA-IgG derived from the synovial fluid display a more proinflammatory Fc glycosylation profile than ACPA-IgG purified from serum [16]. Given this observation, we hypothesized that differences may also occur in the Fab-linked glycan structures and/or Fab-glycosylation levels of ACPA-IgG and control IgG. As compared to the plasma ACPA-IgG (n=6) Fab-linked glycans, the composition of SF derived ACPA-IgG (n=3) Fab glycans exhibited a trend towards lower levels of galactosylation, sialylation and bisecting GlcNAc. A similar trend was observed for the glycan profile of SF control IgG compared to plasma IgG. We next quantified the level of Fab-glycosylation of plasma (ACPA)-IgG and their counterparts from the SF. As shown in Figure 13C, a significantly higher level of Fab glycosylation was found in ACPA IgG from the SF as compared to plasma ACPA-IgG (138% vs. 80%). Of note, such a difference was not observed for control IgG (20% vs. 20%). Together, these observations indicate in quantitative terms that the level of Fab-glycosylation is even more pronounced on ACPA-IgG form SF as compared to ACPA-IgG from blood.

Example 3

Material and methods

Determination of N-linked glycosylation in the Fab-portion of IgG for ACPA or antiCarP IgG.

Biotinylated CCP2 or arginine control peptides are conjugated to different fluorochrome labelled streptavidin tetramers. By fluorescence activated cell sorting (FACS) tetramer-positive B cells are sorted. With two different methods B cell receptor (BCR) sequences are determined. The general method for isolating ACPA-specific B-cells is described in Kerkman et al., June 2, 2015; Ann. Rheum. Dis 0:1-7; doi: 10.1136/annrheumdis-2014-207182.

For the first method tetramer positive single B cells are cultured in vitro for 10-12 days in IMDM medium with a cytokine cocktail on a layer of CD40L expressing L-cells. Supernatants of these cultures are analysed for antibodies with

CCP2-reactivity by ELISA. Besides, the CCP2 positive supernatants are screened for the absence of reactivity against the control peptide. Consequently, of the CCP2-reactive, control peptide negative cultures mRNA is isolated with TRIzol. cDNA is synthesised and an Anchoring Reverse Transcription of Immunoglobulin Sequences and Amplification by Nested (ARTISAN) PCR is performed to eventually determine the BCR sequence with Sanger sequencing.

For the second method ten to thirty tetramer positive B cells are directly sorted in lysis buffer to obtain mRNA, followed by cDNA synthesis and preamplification according to the SMARTSeq protocol. As with the first protocol immunoglobulin products were obtained by the ARTISAN PCR. In contrast with the first method products were sequenced on the PACBIO platform for next generation sequencing.

Results

82% (23/28) IgG, 0% (0/3) IgM and 0% (0/1) IgA ACPA antibodies sequenced with the single cell sorting method, had a N-glycosylation site in the Fab-portion. With the multi cell sorting and next generation sequencing method 94% (17/18) IgG, 40% (2/5) IgM and 100% (9/9) IgA ACPA antibodies had a N-glycosylation site in the Fab-portion. In comparison, sequence analysis of the BCR repertoire of total B cells obtained from healthy donors indicates that only around 9% of the antibodies contain a N-glycosylation site. From this data it is clear that the percentage of ACPA's with a N-glycosylation site in the Fab region is significantly higher than the percentage in other sequences from healthy individuals.

The sequence data is supported by earlier obtained data of increase of molecular weight by hyperglycosylation of ACPA-IgG in comparison to total or anti-tetanus IgG [5].

Example 4

ACPA can recognize and bind to acetylated-peptides (lysine and ornitine)

Material and methods

It was determined whether monoclonal and polyclonal ACPA antibodies can bind to a mutated vimentin peptide with different PTM. Monoclonal ACPA E4 IgG1 [17] provided by Dr Rispens (Sanquin) was analyzed for reactivity towards PTM-modified vimentin peptides. Polyclonal ACPA from RA patients was previously purified by gel filtration columns, purified ACPA 2.93 and 2.77.

For detection of reactivity towards PTM-modified vimentin peptide an ELISA kit of Orgentec Diagnostica was used consisting of coated microplates containing PTM-modified vimentin peptides: HC52-Homocitrulline, P62-Arginine, P18-Citrulline, HC55-Acetylated Lysine, HC56-Lysine, Acetylated-Ornithine, and

Ornithine. Buffers were provided by the ELISA kit of Orgentec Diagnostica and consist of sample diluent buffer, conjugate anti-Human IgG-HRP + reference secondary IgG conjugate, TMB substrate and stop solution. Purified ACPA and monoclonal ACPA were diluted in Orgentec Diagnostica sample diluent buffer until the desired concentrations (30ug/ml for purified ACPA and ACPA E4 mAbs) and incubated on the ELISA plate. ACPA binding was detected by conjugate anti-Human IgG-HRP and TMB. The optical density was measured at 450nm (reference 600-690nm).

Results

Monoclonal ACPA E4 is reactive towards the mutated vimentin peptide with posttranslational modifications of citrulline, acetylated lysine and acetylated ornithine. Polyclonal ACPA from RA patients 2.93 is reactive towards mutated vimentin peptide with posttranslational modifications of citrulline and acetylated ornithine. Polyclonal ACPA 2.93 also harbors reactivity towards acetylated lysine and homocitrulline although to a lesser extent. Polyclonal ACPA from RA patient 2.77 also binds mutated vimentin peptide with posttranslational modifications of acetylated ornithine, acetylated lysine, citrulline and to a lesser extent homocitrulline (Figure 15).

Conclusion

Monoclonal ACPA E4 and polyclonal ACPA obtained from RA patients (2.93 and 2.77) are reactive towards mutated vimentin peptide with posttranslational modifications of citrulline, acetylated lysine and acetylated ornithine. Binding towards these different amino acids indicate that ACPA might be cross-reactive towards different PTMs.

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Example 5

Material and methods

Patients and healthy individuals

Peripheral blood samples were obtained from ACPA-positive patients with established RA. Patients were recruited from the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC) and gave written informed consent. Healthy donor samples were obtained from leftover material collected for allogeneic stem cell transplantation and sequenced as described before.[1]

Isolation and culture of antigen-specific B cells

ACPA-expressing B cells were isolated from peripheral blood mononuclear cells (PBMC) as previously described.[2] Tetanus-toxoid (TT)-specific B cells were isolated using directly labelled TT (Statens Serum Institute) prepared with the AnaTagTM Labeling Kit (ThermoFisher). Cells were sorted either in pools of 10 cells or as single cells as described.[3] One patient sample was processed following both methods. Presence of ACPA-IgG in culture supernatants was assessed by ELISA.[2]

mRNA isolation and cDNA processing

Cells sorted as pools were directly lysed using Triton X-100 (Sigma) followed by mRNA isolation. mRNA from single cell cultures was isolated using TRIzol (Thermo Fisher). Following either isolation procedure, cDNA was synthesized as described.[4]

ARTISAN PCR and sequencing

Ig transcripts were amplified using Anchoring Reverse Transcription of Immunoglobulin Sequences and Amplification by Nested (ARTISAN) PCR, with modifications.[4] PCR products of pooled cells were sequenced on the PacBio RSII system (Pacific Biosciences, Menlo Park, CA, USA). PCR products obtained from single cell cultures were sequenced with Sanger sequencing.[5] Sequence data were analyzed with Geneious R9.1.5[6] and IMGT (High)V-QUEST tools[7].

Results

Localization of N-glycosylation sites in ACPA BCR sequences

BCR sequences of citrulline-specific B cells show a remarkable frequency of *N*-glycosylation sites. Their independence from the SHM rate suggests that *N*-

glycans in the variable region confer selective advantages to ACPA-expressing B cells during development and/or maturation. To obtain more insight into this possibility, we studied the distribution of sites and compared the pattern to *N*-glycosylation sites identified in healthy donor B cell receptor (BCR) repertoires. For ACPA-IgG sequences we observed a predominance of sites in the CDR1 region, and a relative absence in CDR3 regions of ACPA-IgG. These results suggest that the *N*-glycosylation site distribution pattern of ACPA-IgG is skewed away from the CDR3 region and indicate a certain preference for glycans in the CDR1 region.[8] More specifically, by assessing the V genes of the IgG heavy chain, kappa light chain and lambda light chain in detail, we can see enrichment of sites on specific positions in the BCR sequence and lack of sites on other positions. Considering the V-gene of the IgG heavy chain and kappa light chain we see a similar pattern, enrichment of sites on positions 29 and 77 and a lower abundance of sites on several positions in the CDR3 region. In the lambda light chain there seems to be a lack of sites in positions 37, 51, 56, which are highly present in BCR sequences obtained from healthy individuals. (Figure 19)

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Example 6

Methods for the detection of ACPA-Fab glycans

Method 1:

We used high-end UHPLC and mass spectrometry analyses of purified ACPA-IgG (Figure 22).

ACPA-IgG was isolated with the CCP2 microbeads assay as it described before[1]. Briefly we coupled the CCP2-biotinylated peptide to neutravidin beads by incubating the beads with peptide for 1 hour at room temperature (RT) while shaking 850rpm. After the incubation the beads were washed with PBS to remove uncouples peptide. Then 25ul of the beads slurry (25% beads per ml) was placed in an orochem filter plate. Thereafter the 25-75ul serum/plasma was loaded and PBS was added to an end volume of 200ul per well and incubated for 2 hours at RT while shaking at 600rpm. After collecting the Flow through by spinning the plate at 500g 1min the beads were again washed with PBS for 3 times by spinning the plate at 500g 1min. ACPA was eluted by 2x100ul 100mM Formic acid (FA) and neutralize with 2M Tris to a pH of 7. The ACPA elution was further purified by using a similar technique as described above, but instead of CCP2 beads 20ul of 50% slurry prot G beads was used. The ACPA elution were incubated 1 hour at RT at 900rpm and again eluted in 100ul 100mM formic acid. The elution's, now containing ACPA-IgG, were dried using a Speedvac. Glycans were released by resolbilizing the dried ACPA-IgG in 10ul 2%SDS and 5ul PBS and denatured for 30min at 60°C. Then 10ul PNGaseF solution (1:1 1%NP-40/5xPBS containing 0.5U PNGaseF) was added and incubated overnight at 37°C. The next day 12.5ul 2-PB buffer and 12.5ul 2AA-label was added and incubated 2 hours at 60°C to label the released glycans[2,3]. The 2-AA labelled glycans were purified by HILIC SPE using cotton tips as described previously with some modifications[4]. Briefly, for each sample, 500 ug of cotton were packed into a 200 ul pipette tip and conditioned by pipetting three times 150 ul MQ, followed by 150 ul 85% ACN 0.1% TFA and two times 150 ul 85% ACN. The sample (in 85% ACN) was loaded by pipetting 25 times into the reaction mixture. The tips were washed three times with, three times with 150 ul 85% ACN 0.1% TFA and two times 150 ul 85% ACN. The 2-AA labelled glycans were finally eluted from the cotton with 30 ul MQ and identified by MALDI-TOF-MS and/or UHPLC.

The protocol described in Method 1 is time-consuming and requires high-end UHPLC analysis and expertise. Therefore, a more accessible method for use in day-

to-day routine is preferable. Method 2 and 3 are preferable, however optimization experiments are required. The lectin SNA (Sambuccus Nigra Agglutinin) binds antibodies, primarily if these carry two sialic acid residues in the Fab domain[5,6]. SNA binds the antibody Fc tail only under reducing conditions (which opens up the interface between CH2 domains)[7,8]. ACPA F(ab)-glycans contain a high degree of di-sialylated glycans which are virtually absent from the (ACPA-)IgG Fc tail. Therefore, SNA-binding to serum antibodies from RA-patients to detect ACPA F(ab)-glycans represents a promising strategy to visualize the presence of glycosylated antibodies. Method 2, as well as method 3 describe two approaches to establish a high-throughput method based on SNA-detection.

Method 2:

For method 2 (figure 3), ACPA is captured using CCP2 coated microbeads (left panel) as described above. To calculate the presence of F(ab) glycans on ACPA IgG molecules two different ELISA's are used. The first ELISA to visualize sialylated ACPA by a SNA-based lectin is depicted in figure 3, right panel. For the second ELISA, to calculate the amount of ACPA IgG, a kit from Bethyl is used (Human IgG ELISA Kit, E88-104). For both ELISA's, we first coat plates with 10ug/ml peroic acid treated goat-anti-human IgG Fc capture antibody. 200mM peroic acid was incubated for 30 min at 4°C to destroy the sialic acid present on the goat-anti-human antibody for 1 hour at RT. The plates were washed 3x with PBS 0,05% tween buffer and then blocked with 1%BSA-PBS (again treated with 20mM PA overnight at 4°C) for 1 hour at RT. After washing the plates, the eluted ACPA elution (Figure 3 left panel) were added to both plates and incubated for 1 hour at RT. After incubation and washing, one plate was incubated with 2ug/ml biotinylated SNA for 1 hour at RT and the other plate was incubated with goat-anti-human IgG HRP for 1 hour at RT. Again after the incubation the plates were washed. Subsequently, ABTS was added to the plate previously incubated with goat-anti-human IgG HRP, and the absorbance was measured at 415nm. To the plate previously incubated with SNA, Strep-HRP was added and incubated for 1 hour at RT. After the incubation the absorbance was measured by a similar approach. For the analysis of the results both plates contained a standard curve.

SNA binding per ug ACPA-IgG was calculated by dividing the SNA binding to ACPA-IgG on plate 1 by the ug ACPA-IgG captured on plate 2. The higher the binding of SNA per ug ACPA-IgG the higher the amount of ACPA Fab glycosylation. The results clearly show enhanced SNA-to-IgG ratio in the ACPA-positive samples, visualizing the high glycosylated content in Fab from ACPA.

Method 3:

For method 3, a reverse strategy is used. First, total IgG from serum or plasma is isolated by a similar approach as the micro bead assay described before. This is followed by immobilizing IgG on SNA by SNA agarose beads. Finally, ACPA-IgG is detected using a CCP ELISA on the SNA elution and flow through fractions (figure 4). Due to the high amount of di-sialylated Fab glycans present on

ACPA-IgG, an enrichment of CCP reactivity is expected in the SNA elution fraction. This approach is feasible for ACPA-IgG and can be used in a high throughput manner, as shown in figure 4 (right panel). Importantly, we confirmed that SNA agarose beads indeed capture Fab glycosylated IgG in this set-up, as UHPLC analysis of IVIG samples indicates that we could clearly enrich for Fab glycosylated IgG molecules contained in IVIG in the SNA elution fractions (figure 23). Thus, the data depicted in figure 4 also show the feasibility of this approach and visualize the presence or highly-glycosylated Fab-domains of ACPA. Of note, pre-isolation of IgG by protein G is important to prevent "overloading" of SNA beads by other molecules present in serum that carry sialylated glycans. However with adjusting the amount of serum loading on SNA beads the method can also be used in reverse.

Method 2 and 3 both show the robustness, specificity and reliability to detect F(ab) glycans. Together, these experiments show that we have establishedan assay system that quickly and reliably identifies ACPA F(ab) glycans.

Cited Art (Example 6)

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4 Selman, M. H. et al. Fc specific IgG glycosylation profiling by robust nano-reverse phase HPLC-MS using a sheath-flow ESI sprayer interface. Journal of proteomics 75, 1318-1329, doi:10.1016/j.jprot.2011.11.003 (2012).

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6 Guhr, T. 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 6, e21246, doi:10.1371/journal.pone.0021246 (2011).

7 Stadlmann, J. et al. A close look at human IgG sialylation and subclass distribution after lectin fractionation. Proteomics 9, 4143-4153, doi:10.1002/pmic.200800931 (2009).

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CLAIMS

1. A method of determining whether an individual that does not have rheumatoid arthritis, Sjögren syndrome or anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) at the moment of sampling is at risk of developing said disease, the method comprising determining whether an antibody containing sample of said individual comprises an autoantibody associated with said disease and determining whether the antibody comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method further comprising determining the risk of the individual for developing said disease on the basis of said determinations.

2. A method of analyzing an antibody containing sample of an individual, the method comprising determining whether said sample comprises an autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV that comprises an N-linked glycosylation at one or more positions in a Fab-portion of the antibody, the method characterized in that the sample is a sample of an individual that does not have rheumatoid arthritis symptoms, Sjögren syndrome symptoms or AAV symptoms at the moment of sampling.

3. A method of preventing or delaying the development of a rheumatoid arthritis symptom, a Sjögren syndrome symptom or an AAV symptom in an individual, the method comprising
- determining whether a sample of said individual contains an autoantibody associated with said disease;
- and determining whether the antibody has an N-linked glycosylated Fab-portion;
- wherein the sample is an antibody containing sample of the individual and the individual did not have rheumatoid arthritis, Sjögren syndrome and AAV at the time of sampling; and
- treating the individual with a medicament for said disease prior to or at the onset of the individual presenting with said disease.

4. A method of monitoring an individual at risk of developing rheumatoid arthritis, Sjögren syndrome or AAV, the method comprising monitoring the presence and/or the onset of an autoantibody associated with said disease in periodic antibody containing samples of an individual, the method characterized in that the method further comprises determining whether a detected autoantibody associated with said disease comprises an N-linked glycan at one or more positions in a Fab-portion of the antibody.

5. A rheumatoid arthritis, Sjögren syndrome or AAV medicament for use in a method of treatment of an individual at risk of developing one or more of said diseases wherein the individual is determined to be at risk by the detection of an autoantibody associated with said disease which antibody comprises N-linked

glycosylation at one or more positions in a Fab-portion of the antibody in an antibody containing sample of said individual.

6. The medicament for use according to claim 5, wherein the individual is treated prior to or at the onset of the individual presenting with arthritis symptoms.

7. A method of purifying antibodies for the measurement of antibodies comprising N-linked glycan on a Fab-portion thereof comprising

- providing an antibody sample;
- contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
- removing unbound antibody and eluting bound antibody from said solid surface;
- incubating eluted antibody with a solid surface comprising a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
- removing unbound molecules; and
- determining whether an antibody has bound to said solid surface,

wherein a bound antibody indicates the presence of an N-linked glycan on a Fab-portion of an antibody in said sample.

8. A method of purifying antibodies for the measurement of antibodies that have an N-linked glycan on a Fab-portion comprising

- providing an antibody sample;
- contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
- removing unbound antibody;
- incubating said solid surface with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
- removing unbound molecules; and
- determining whether a molecule has bound to said solid surface,

wherein a bound molecule indicates the presence of an N-linked glycan on a Fab-portion of an antibody in said sample.

9. A method of purifying antibodies for the measurement of antibodies that have an N-linked glycan on a Fab-portion comprising

- providing an antibody sample;
- contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
- removing unbound antibody;
- incubating bound antibody with an enzyme that separates glycan from said antibody;
- collecting glycans; and
- determining whether the collected glycans comprise a Fab-portion specific glycan;

the method characterized in that the sample is an antibody sample of an individual at risk of developing RA, Sjögren syndrome or AAV.

10. A method of purifying antibodies with N-linked glycosylation on a Fab-portion of the antibody, the method comprising
- providing an antibody sample;
- incubating said sample with a solid surface that comprises a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
- washing said solid surface and collecting bound antibody;
- incubating collected antibody with a peptide or protein that comprises a modified protein epitope; removing unbound antibody; and
- determining whether said peptide or protein had bound antibody.

11. The method of claim 10, wherein said sample comprises purified antibodies.

12. A method comprising
- providing an antibody sample;
- contacting said sample with a solid surface that comprises a peptide or protein that comprises a modified protein epitope;
- removing unbound antibody;
- incubating said solid surface with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody;
- removing unbound molecules; and
- determining whether a molecule has bound to said solid surface.

13. The method of any one of claims 1-12, characterized in that the sample is an antibody sample of an individual at risk of developing RA, Sjögren syndrome or AAV.

14. The method of any one of claims 1-13, wherein the sample is an antibody sample of an individual of which an earlier antibody sample was tested positive for an autoantibody.

15. The method of claim 14, wherein 10% or less of the autoantibody of said earlier antibody sample comprises an N-linked glycan on a Fab-portion thereof.

16. The method of claim 14 or claim 15, wherein said autoantibody is an anti-modified protein antibody (AMPA).

17. The method of any one of claims 1-16, wherein the antibodies are cleaved to produce Fab and Fc fragments.

18. A method of determining whether an antibody sample comprises an anti-modified protein antibody (AMPA), preferably a citrulline, a homo-citrulline and/or an acetylated lysine binding AMPA, the method comprising
- contacting antibodies of the sample with a peptide or protein that comprises a modified protein epitope, preferably comprising a citrulline, homo-citrulline, or acetylated lysine;
- contacting antibodies of the sample with a molecule that can bind an N-linked glycan on a Fab-portion of an antibody; and
- determining whether an antibody with a N-linked glycan on a Fab-portion of the antibody has bound to the modified protein epitope in the peptide or protein, wherein the modified protein epitope preferably comprises citrulline, a homo-citrulline or an acetylated lysine.

19. The method of claim 18, characterized in that the sample is a sample of an individual that does not have rheumatoid arthritis at the moment of sampling.

20. The method of claim 18 or claim 19, characterized in that an AMPA, preferably an citrullinated protein antigens antibody (ACPA), lysine acetylated protein antigens antibody (AAPA) and/or anti-CarP antibody had been detected in a similar earlier sample of the individual.

21. The method of claim 20, wherein the AMPA in the earlier sample tested negative for the presence of N-linked glycosylation on a Fab-portion of the antibodies.

22. The method of any one of claims 18-21, characterized in that the individual is at risk of developing arthritis, preferably rheumatoid arthritis.

23. The method of any one of claims 18-22, wherein the glycan-binding molecule comprises a lectin, preferably a sialic acid residue binding lectin, preferably a member of the SIGLEC family, preferably CD22 or the sialic acid binding part of said lectin.

24. The method of claim 23, wherein the lectin can simultaneously bind two sialic acid residues, preferably Sambuccus Nigra Agglutinin (SNA) .

25. A kit of parts useful in the detection of an autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV such as an AMPA in a sample, the kit comprising a peptide or protein that can bind said autoantibody such as a peptide or protein comprising a post-translational modification and a molecule that can bind an N-linked glycan on a Fab-portion of an antibody.

26. A kit of parts according to claim 25, wherein the peptide; protein or the molecule that can bind an N-linked glycan is linked to a solid surface.

27. A method of determining whether an individual comprises an autoantibody associated with rheumatoid arthritis, Sjögren syndrome or AAV with an N-linked glycan on a Fab-portion of the antibody, the method comprising

- contacting a B-cell containing sample of said individual with a peptide or protein that can bind said autoantibody;
- separating B-cells bound to said peptide or protein from unbound B-cells;
- sequencing nucleic acid encoding at least the CDR1 of the heavy chain variable region and/or the CDR1 of the light chain variable region of a B-cell receptor of said bound B-cells; and
- determining whether the nucleic acid sequence codes for an N-linked glycosylation consensus amino acid sequence.

28. The method of claim 27, comprising sequencing at least the CDRs of the heavy chain variable region and/or the CDRs of the light chain variable region.

Figure 1

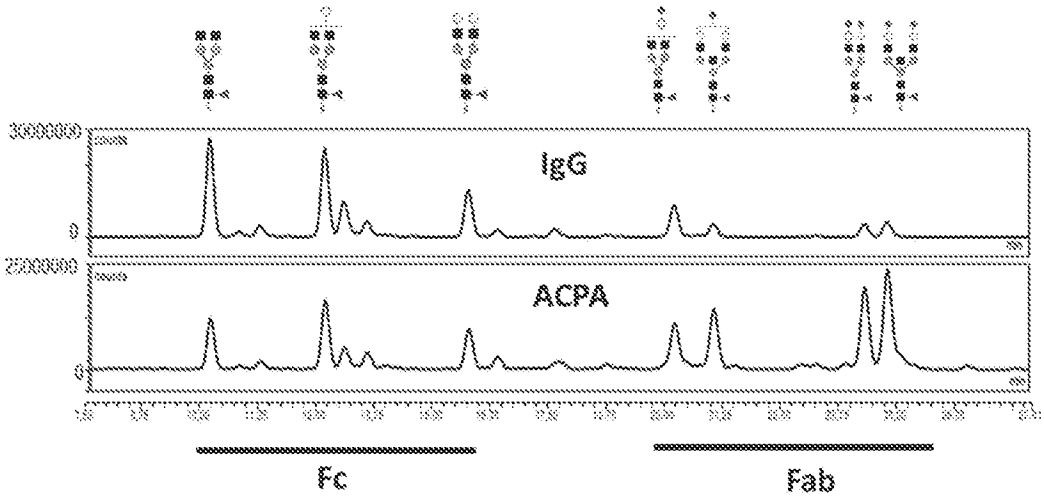


Figure 2A

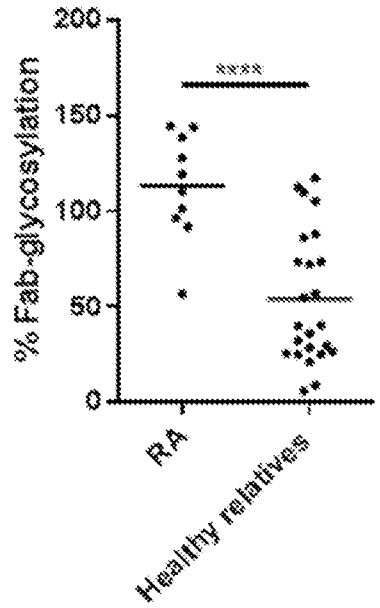


Figure 2B

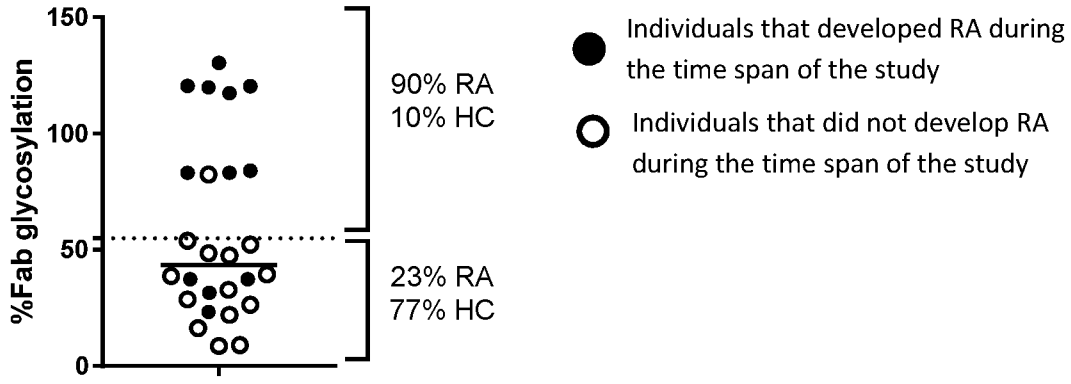
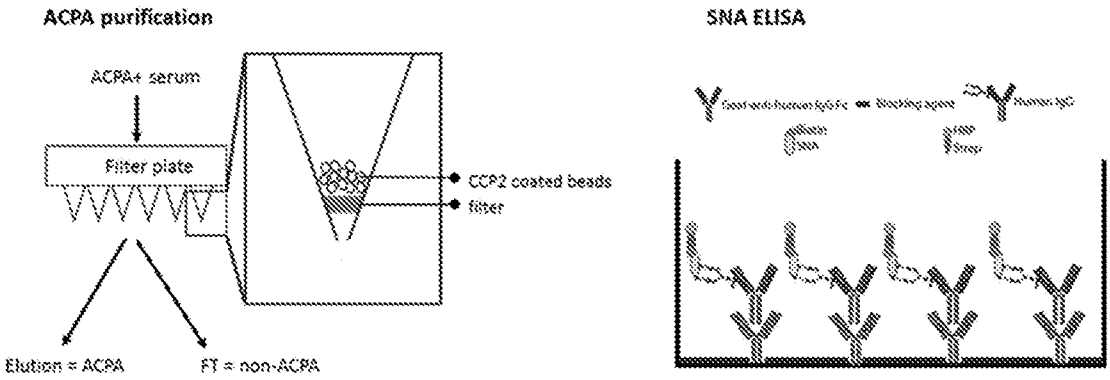
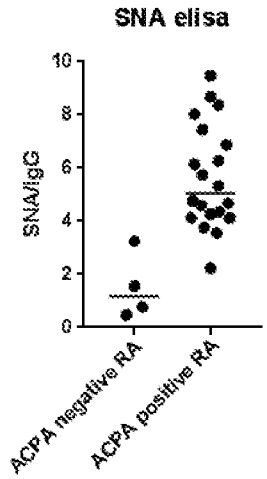


Figure 3

A


ACPA purification
ACPA+ serum
Filter plate
CCP2 coated beads
filter
Elution = ACPA
FT = non-ACPA
SNA ELISA

B


SNA elisa
SNA/IgG
0
2
4
6
8
10
ACPA negative RA
ACPA positive RA

C

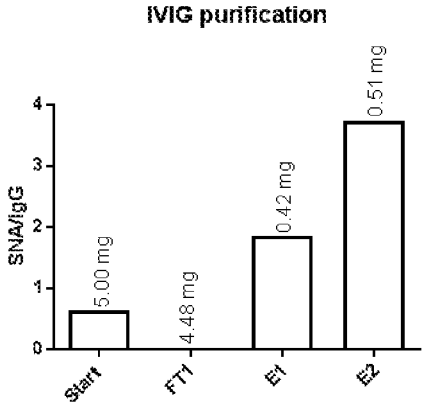
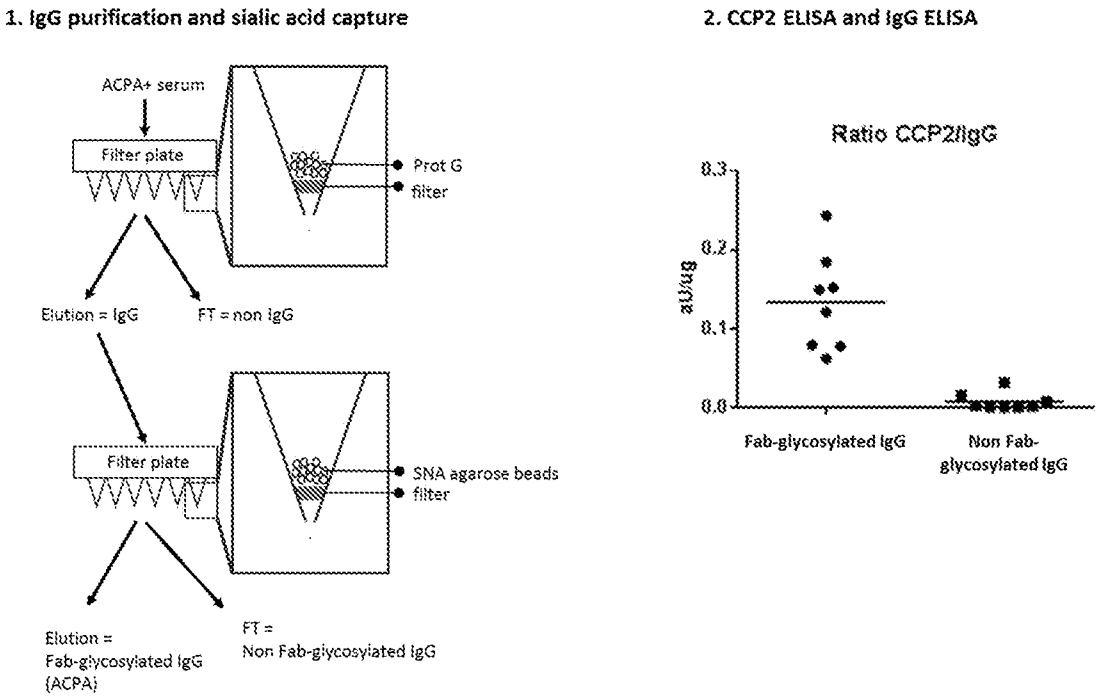

IVIG purification
SNA/IgG
0
1
2
3
4
5.00 mg
4.48 mg
0.42 mg
0.51 mg
Start
FT1
E1
E2

Figure 4

A


1. IgG purification and sialic acid capture
ACPA+ serum
Filter plate
Prot G
filter
Elution = IgG
FT = non IgG
Filter plate
SNA agarose beads
filter
Elution =
Fab-glycosylated IgG
(ACPA)
FT =
Non Fab-glycosylated IgG
2. CCP2 ELISA and IgG ELISA
Ratio CCP2/IgG
aU/ug
0.3
0.2
0.1
0.0
Fab-glycosylated IgG
Non Fab-
glycosylated IgG

B

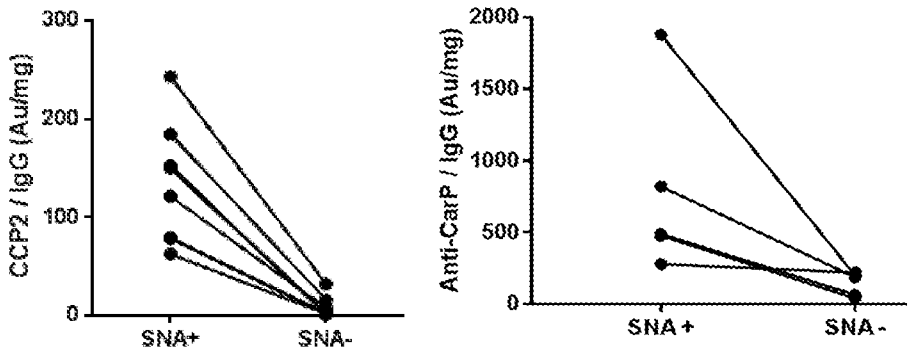
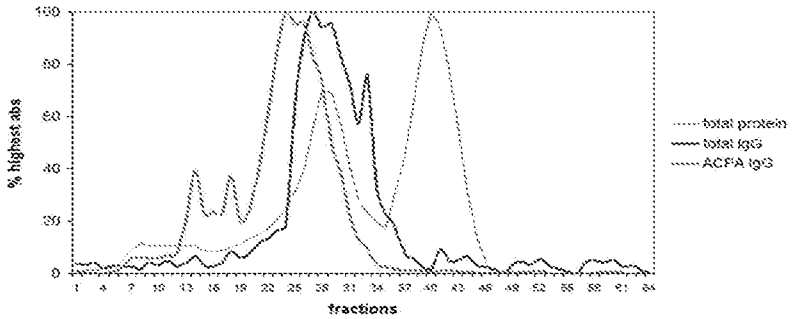
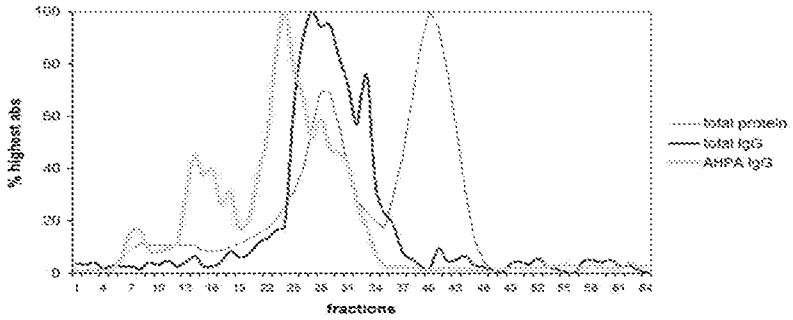

CCP2 / IgG (Au/mg)
300
200
100
0
SNA+
SNA-
Anti-CarP / IgG (Au/mg)
2000
1500
1000
500
0
SNA +
SNA -

Figure 5

Size shift ACPA IgG



Size shift anti-CarP IgG



No size shift anti-TT IgG

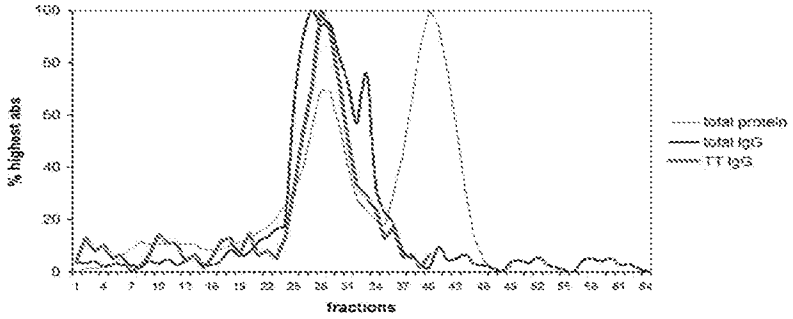


Figure 6 Amino acid sequence of Fibrinogen alpha

```
         10         20         30         40         50         60         70
MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS DWPFCSDEDW NYKCPSGCRM
         80         90        100        110        120        130        140
KGLIDEVNQD FTNRINKLKN SLFEYQKNNK DSHSLTTNIM EILRGDFSSA NNRDNTYNRV SEDLRSRIEV
        150        160        170        180        190        200        210
LKRKVIEKVQ HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC RGSCSRALAR EVDLKDYEDQ QKQLEQVIAK
        220        230        240        250        260        270        280
DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ LQKVPPEWKA LTDMPQMRME LERPGGNEIT RGGSTSYGTG
        290        300        310        320        330        340        350
SETESPRNPS SAGSWNSGSS GPGSTGNRNP GSSGTGGTAT WKPGSSGPGS TGSWNSGSSG TGSTGNQNPG
        360        370        380        390        400        410        420
SPRPGSTGTW NPGSSERGSA GHWTSESSVS GSTGQWHSES GSFRPDSPGS GNARPNNPDW GTFEEVSGNV
        430        440        450        460        470        480        490
SPGTRREYHT EKLVTSKGDK ELRTGKEKVT SGSTTTTRRS CSKTVTKTVI GPDGHKEVTK EVVTSEDGSD
        500        510        520        530        540        550        560
CPEAMDLGTL SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF VSETESRGSE SGIFTNTKES
        570        580        590        600        610        620        630
SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS YKMADEAGSE ADHEGTHSTK RGHAKSRPVR
        640        650        660        670        680        690        700
DCDDVLQTHP SGTQSGIFNI KLPGSSKIFS VYCDQETSLG GWLLIQQRMD GSLNFNRTWQ DYKRGFGSLN
        710        720        730        740        750        760        770
DEGEGEFWLG NDYLHLLTQR GSVLRVELED WAGNEAYAEY HFRVGSEAEG YALQVSSYEG TAGDALIEGS
        780        790        800        810        820        830        840
VEEGAEYTSH NNMQFSTFDR DADQWEENCA EVYGGGWWYN NCQAANLNGI YYPGGSYDPR NNSPYEIENG
        850        860
VVWVSFRGAD YSLRAVRMKI RPLVTQ
```

Figure 7 Amino acid sequence of Fibrinogen beta

```
        10         20         30         40         50         60
MKRMVSWSFH KLKTMKHLLL LLLCVFLVKS QGVNDNEEGF FSARGHRPLD KKREEAPSLR
        70         80         90        100        110        120
PAPPPISGGG YRARPAKAAA TQKKVERKAP DAGGCLHADP DLGVLCPTGC QLQEALLQQE
       130        140        150        160        170        180
RPIRNSVDEL NNNVEAVSQT SSSSFQYMYL LKDLWQKRQK QVKDNENVVN EYSSELEKHQ
       190        200        210        220        230        240
LYIDETVNSN IPTNLRVLRS ILENLRSKIQ KLESDVSAQM EYCRTPCTVS CNIPVVS
       250        260        270        280        290        300
CEEIIRKGGE TSEMYLIQPD SSVKPYRVYC DMNTENGGWT VIQNRQDGSV DFGRKWDPYK
       310        320        330        340        350        360
QGFGNVATNT DGKNYCGLPG EYWLGNDKIS QLTRMGPTEL LIEMEDWKGD KVKAHYGGFT
       370        380        390        400        410        420
VQNEANKYQI SVNKYRGTAG NALMDGASQL MGENRTMTIH NGMFFSTYDR DNDGWLTSDP
       430        440        450        460        470        480
RKQCSKEDGG GWWYNRCHAA NPNGRYYWGG QYTWDMAKHG TDDGVVWMNW KGSWYSMRKM
       490
SMKIRPFFPQ Q
```

Figure 8 Amino acid sequence of Fibrinogen gamma

```
         10         20         30         40         50         60
MSWSLHPRNL ILYFYALLFL SSTCVAYVAT RDNCCILDER FGSYCPTTCG IADFLSTYQT
         70         80         90        100        110        120
KVDKDLQSLE DILHQVENKT SEVKQLIKAI QLTYNPDESS KPNMIDAATL KSRKMLEEIM
        130        140        150        160        170        180
KYEASILTHD SSIRYLQEIY NSNNQKIVNL KEKVAQLEAQ CQEPCKDTVQ IHDITGKDCQ
        190        200        210        220        230        240
DIANKGAKQS GLYFIKPLKA NQQFLVYCEI DGSGNGWTVF QKRLDGSVDF KKNWIQYKEG
        250        260        270        280        290        300
FGHLSPTGTT EFWLGNEKIH LISTQSAIPY ALRVELEDWN GRTSTADYAM FKVGPEADKY
        310        320        330        340        350        360
RLTYAYFAGG DAGDAFDGFD FGDDPSDKFF TSHNGMQFST WDNDNDKFEG NCAEQDGSGW
        370        380        390        400        410        420
WMNKCHAGHL NGVYYQGGTY SKASTPNGYD NGIIWATWKT RWYSMKKTTM KIIPFNRLTI
        430        440        450
GEGQQHHLGG AKQVRPEHPA ETEYDSLYPE DDL
```

Figure 9

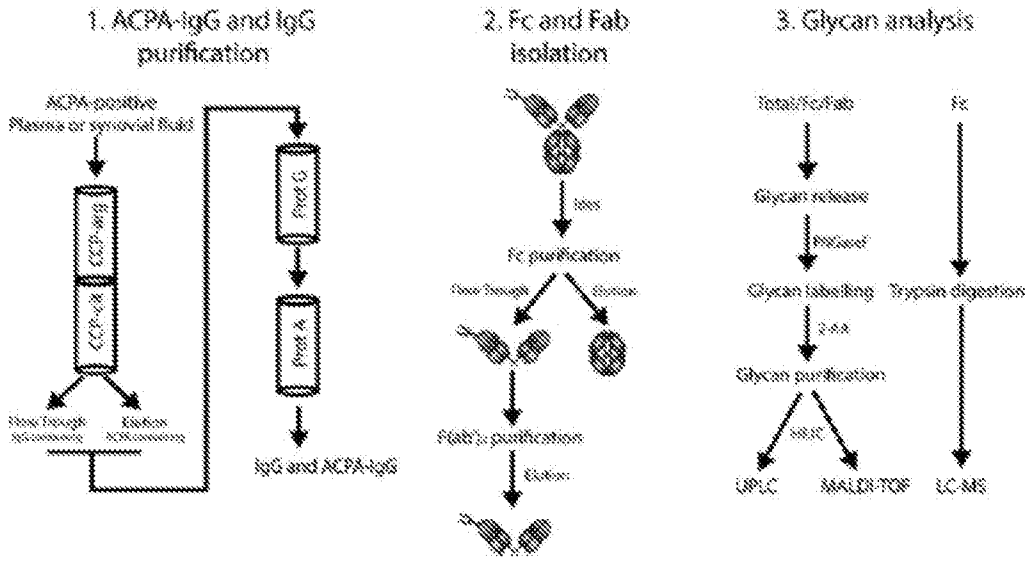

1. ACPA-IgG and IgG purification
2. Fc and Fab isolation
3. Glycan analysis
IgG and ACPA-IgG
Fc purification
Glycan release
Glycan labelling
Glycan purification
Trypsin digestion
UPLC
MALDI-TOF
LC-MS

Figure 10

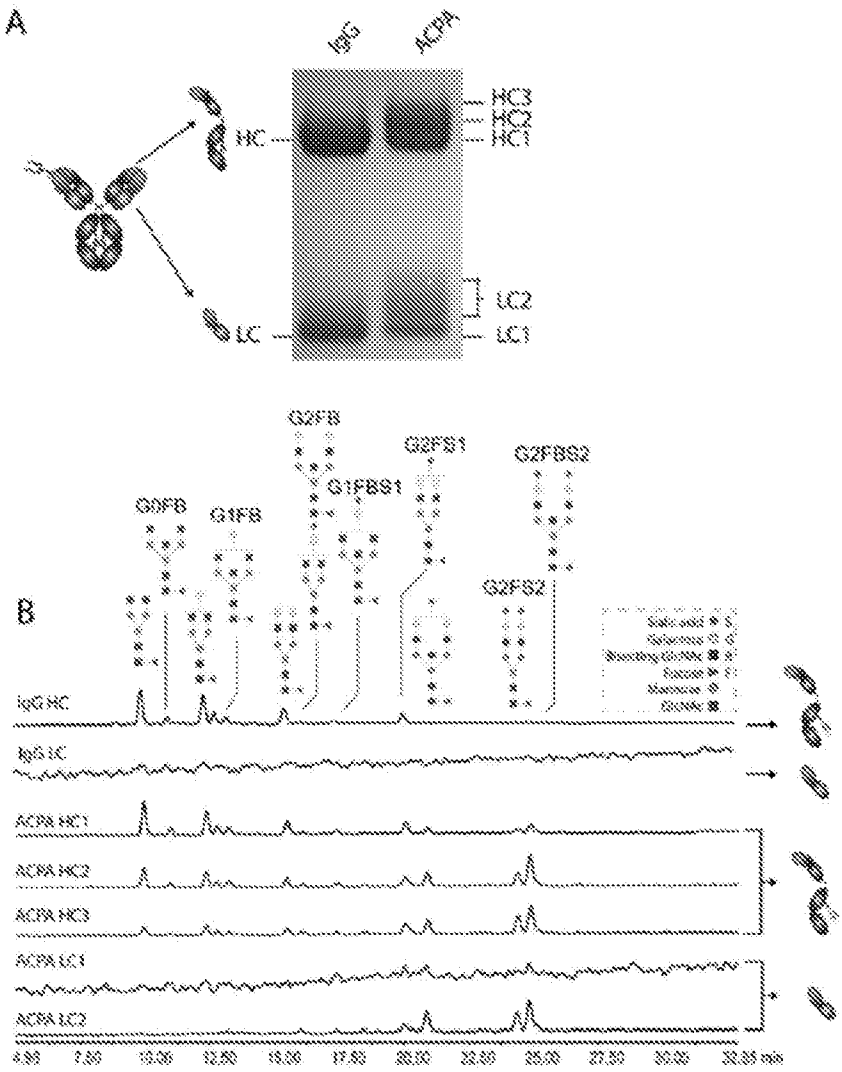

A
IgG
ACPA
HC
LC
HC3
HC2
HC1
LC2
LC1
B
G0FB
G1FB
G2FB
G1FBS1
G2FS1
G2FBS2
G2FS2
IgG HC
IgG LC
ACPA HC1
ACPA HC2
ACPA HC3
ACPA LC1
ACPA LC2

Figure 11

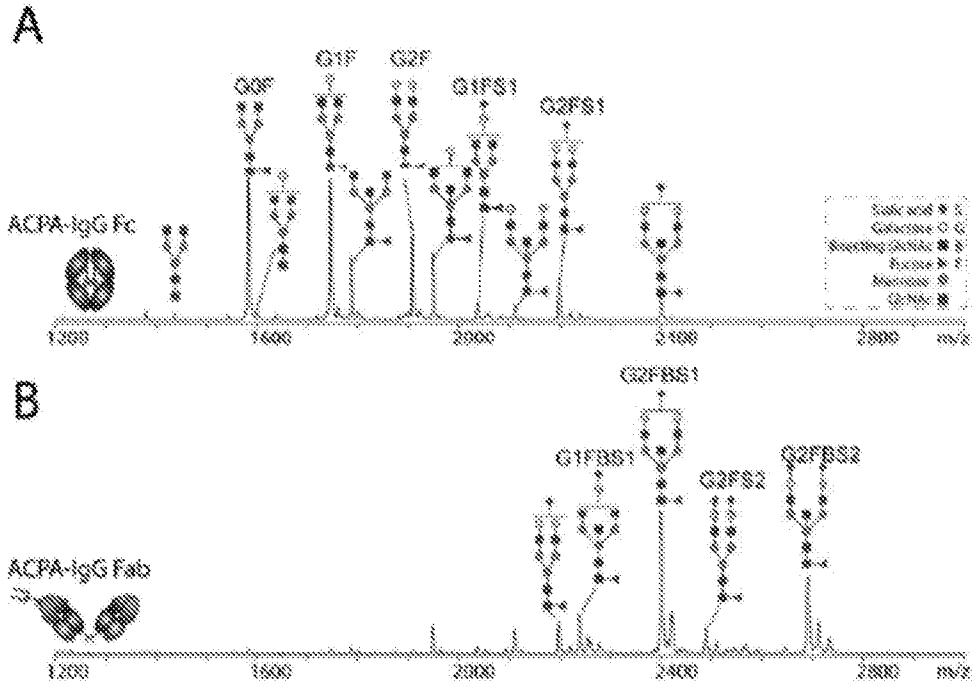

A
G0F
G1F
G2F
G1FS1
G2FS1
ACPA-IgG Fc
1200
1600
2000
2400
2800
m/z
B
G2FBS1
G1FBS1
G2FS2
G2FBS2
ACPA-IgG Fab
1200
1600
2000
2400
2800
m/z

Figure 12

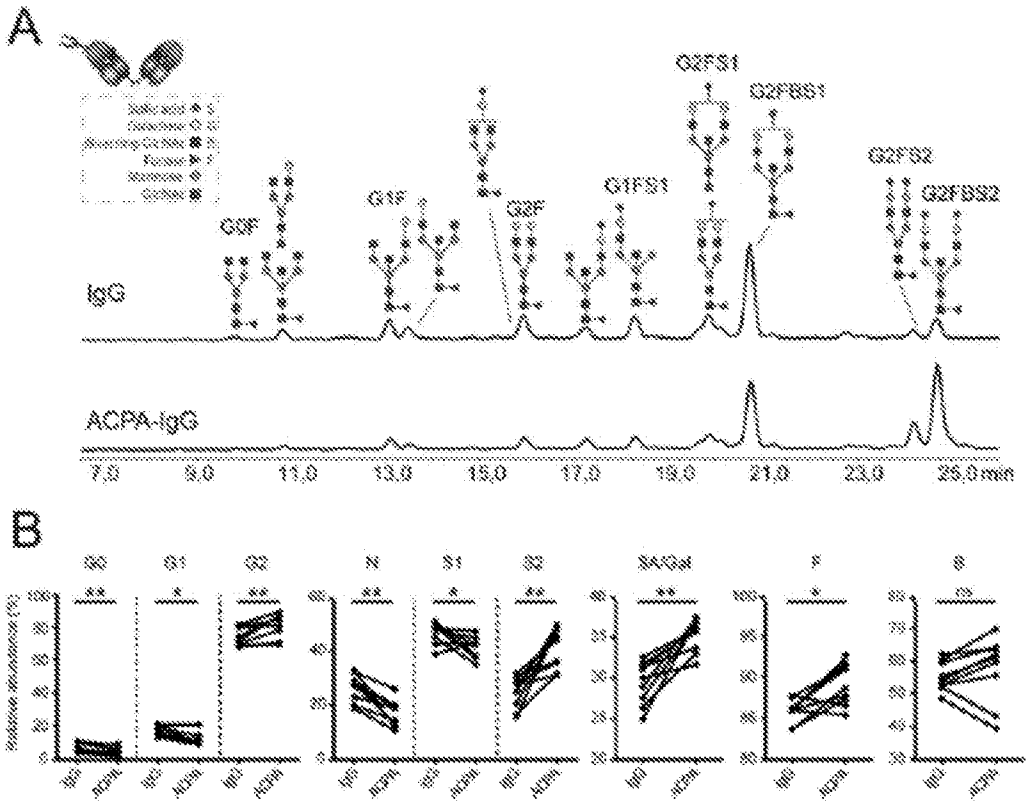

A
G0F
G1F
G2F
G1FS1
G2FS1
G2FBS1
G2FS2
G2FBS2
IgG
ACPA-IgG
7.0
9.0
11.0
13.0
15.0
17.0
19.0
21.0
23.0
25.0 min
B
G0
G1
G2
N
S1
S2
SAGal
F
B

Figure 13

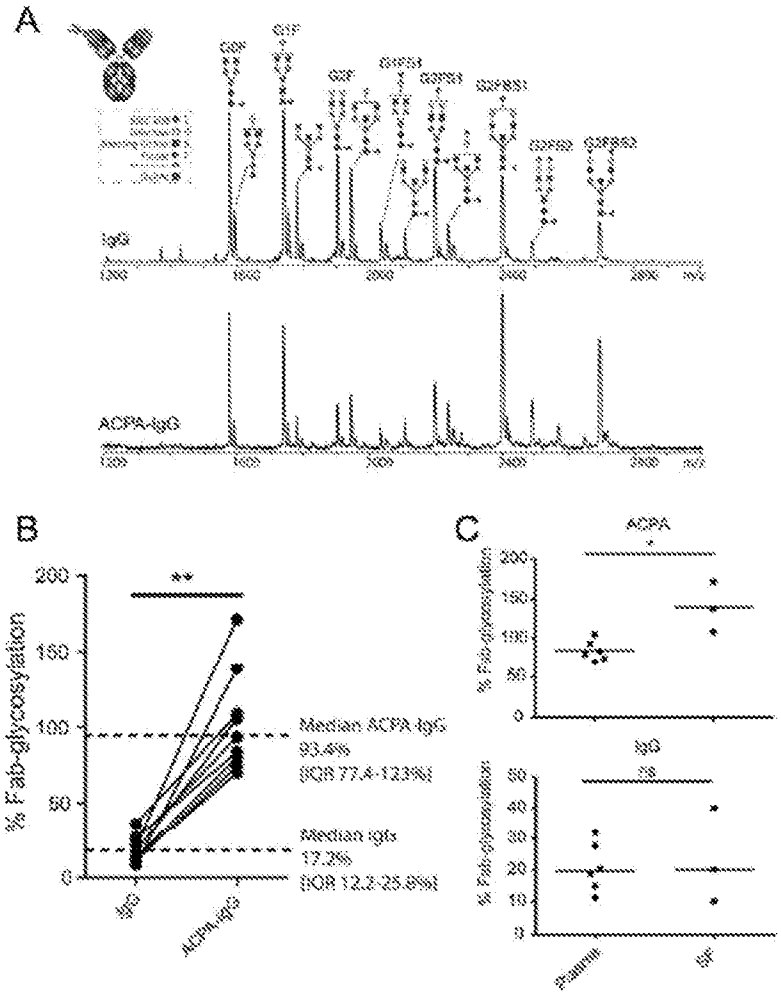

A
IgG
ACPA-IgG
B
% Fab-glycosylation
**
Median ACPA-IgG
93.4%
(IQR 77.4-123%)
Median IgG
17.2%
(IQR 12.2-25.6%)
IgG
ACPA-IgG
C
ACPA
IgG
ns
% Fab-glycosylation
Plasma
IgG

Figure 14.

RL0633-H
IGHV
QVQLEESGPGLVRPSETLSLTCSVSGVSLSEISYFWGWVRQPPGKGLEWIGTIHYSARIYY
TPSLQSRVSMSVDTSKNQFSLNVTSVTAADTAVYYCAISYDYGDFFDYWGQGILVTVSS
IGLV
IFILAQPHSVSESAGKTVTISCTRSSGSIASTYVQWYQQRPGSSPSTVVFQNDQRPSGVPD
RFSGSIDSSSNSASLTISGLKTEDEADYYCQSYDTANHVLFGGGTKLTVL

RL0676-B
IGHV
QVQLQESGPGLVKPSETLSLTCNVSGGSLKSDNFYWSWIRQRPGQGLEFIGYYVYSDITY
FNPSLKSRVNISLDTSKRQLSLQVRSVTAADTGIYYCARGIGLGDVIICEGFDVWGRGTTV
TVSS
IGLV
NFLLAQPHSVSESPGKTITLSCTRSSGNVASESVQWYQQRPGSSPTTVILQNNRRPSGVP
DRFSGSIDTSSNSASLTISGLRPEDEADYFCQSFDSSGLIFGGGTKLTVL

RL0758-E
IGHV
QVQLVESGGGVVQPGKSLRLSCVASGFTFKNFALHWVRQAPGRGLEWLAVISDDGSESH
YADSVQGRFLISRDNSTNTLVLQMNHLRSDDTAHYYCARDLSKIFPLYYGMDVWGQGTT
VIVSA
IGLV
EVVLTQSPGTLSLSPGERATLSCRASRHVSSTYLVWYQHKPGQPPRLLISGASRRATGIPD
RFNGSGSGTDFTLTIASLEPEDFAVYYCHHYGFSPCSFGQGTKLEIK

Figure 15.

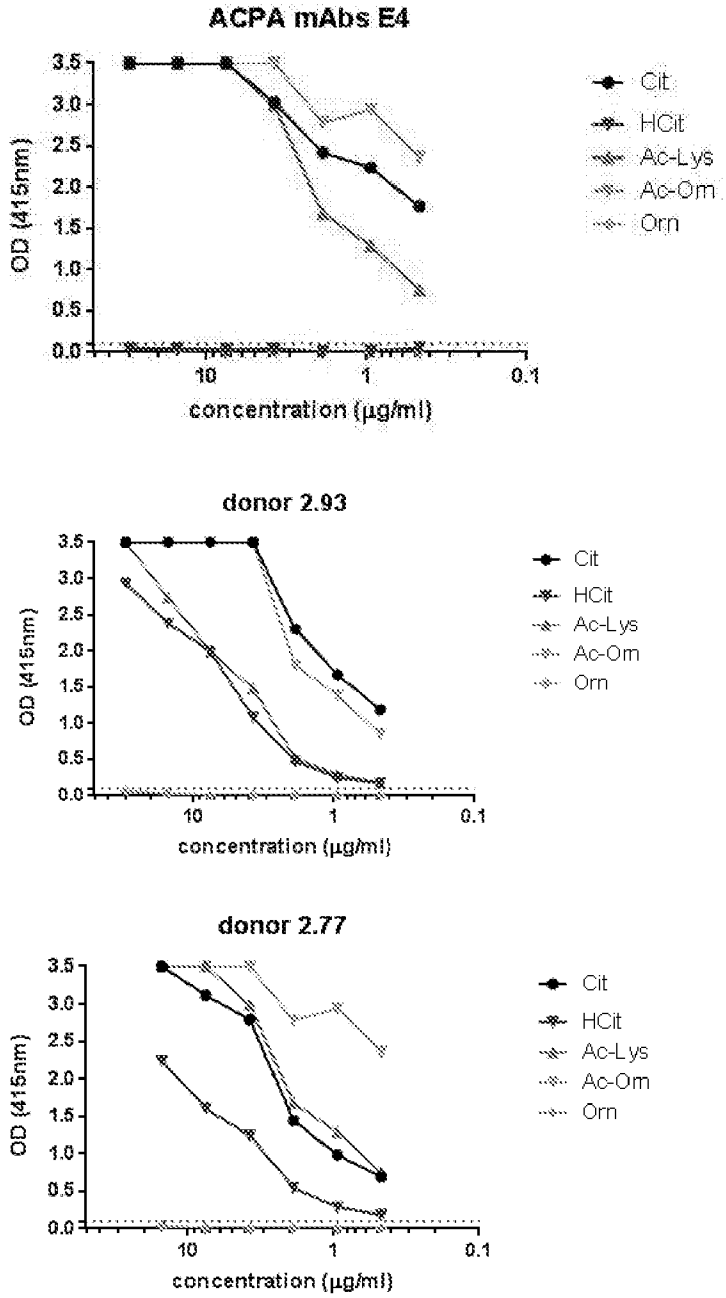

ACPA mAbs E4
OD (415nm)
concentration (µg/ml)
Cit
HCit
Ac-Lys
Ac-Orn
Orn
donor 2.93
donor 2.77

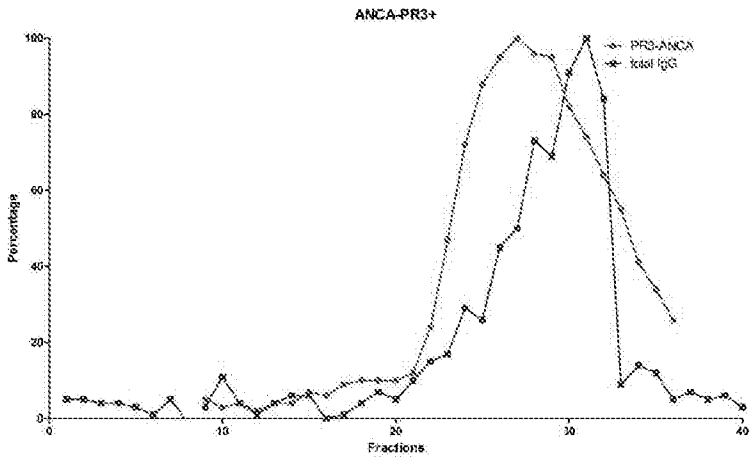
Figure 16. Human Albumin

MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVT
EFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRP
EVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEG
KASSAKQGLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADL
AKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVGSKDVCKNYAEAKDVFLGMFL
YEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYK
FQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDCLSVFLNQLCVLHEKTPVSDRV
TKCCTESLVNGRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL

Figure 17. Alpha-1-antitrypsin

```
  1 MPSSVSWGIL LLAGLCCLVP VSLAEDPQGD AAQKTDTSHH DQDHPTFNKI TPNLAEFAFS
 61 LYRQLAHQSN STNIFFSPVS IATAFAMLSL GTKADTHDEI LEGLNFNLTE IPEAQIHEGF
121 QELLRTLNQP DSQLQLTTGN GLFLSEGLKL VDKFLEDVKK LYHSEAFTVN FGDTEEAKKQ
181 INDYVEKGTQ GKIVDLVKEL DRDTVFALVN YIFFKGKWER PFEVKDTEEE DFHVDQVTTV
241 KVPMMKRLGM FNIQHCKKLS SWVLLMKYLG NATAIFFLPD EGKLQHLENE LTHDIITKFL
301 ENEDRRSASL HLPKLSITGT YDLKSVLGQL GITKVFSNGA DLSGVTEEAP LKLSKAVHKA
361 VLTIDEKGTE AAGAMFLEAI PMSIPPEVKF NKPFVFLMIE QNTKSPLFMG KVVNPTQK
```

Figure 18.
A


ANCA-PR3+
PR3-ANCA
total IgG
Percentage
Fractions
0
10
20
30
40

B

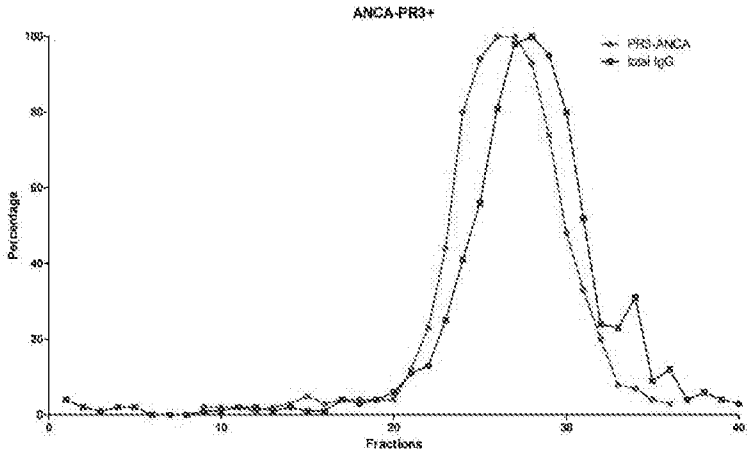
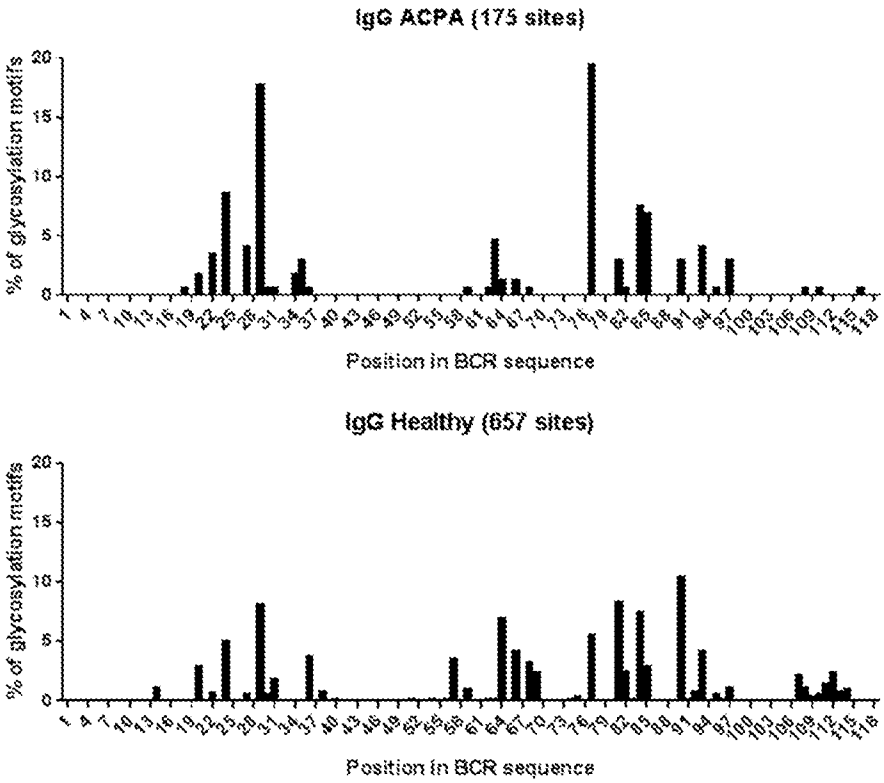
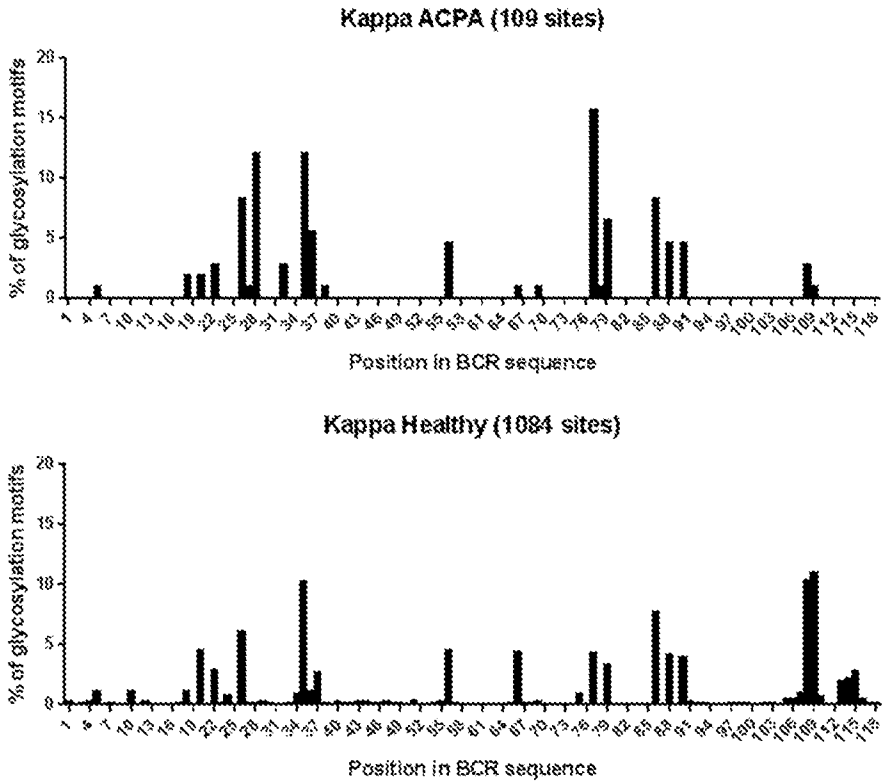

ANCA-PR3+
PR3-ANCA
total IgG
Percentage
Fractions
0
10
20
30
40

Figure 19.

A


IgG ACPA (175 sites)
% of glycosylation motifs
Position in BCR sequence
IgG Healthy (657 sites)
% of glycosylation motifs
Position in BCR sequence

B


Kappa ACPA (109 sites)
% of glycosylation motifs
Position in BCR sequence
Kappa Healthy (1084 sites)
% of glycosylation motifs
Position in BCR sequence

C

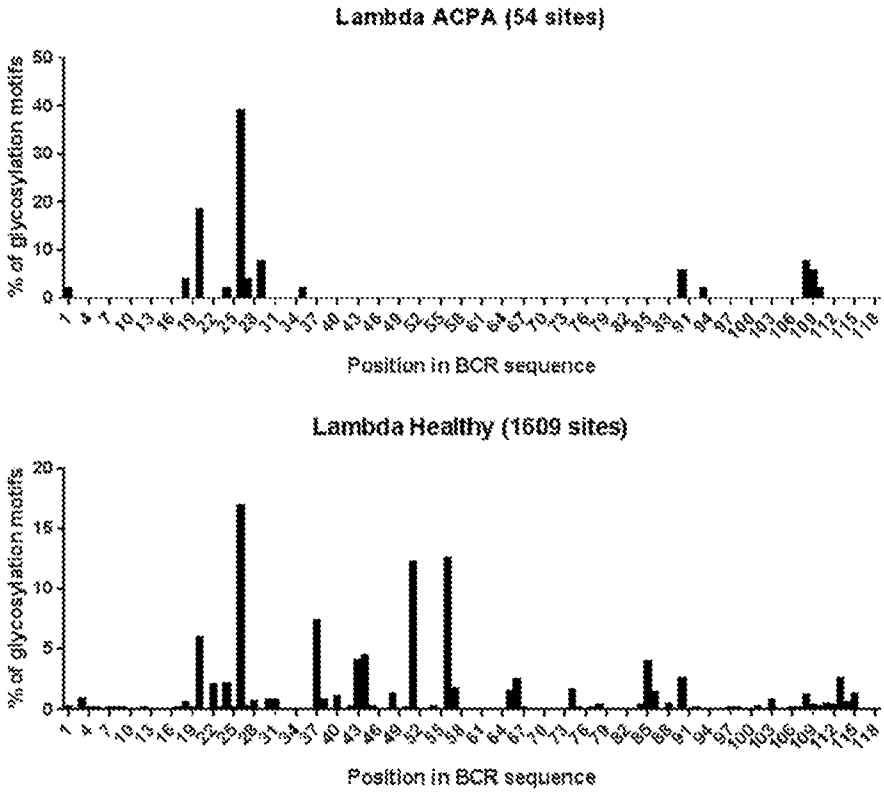

Lambda ACPA (54 sites)
% of glycosylation motifs
Position in BCR sequence
Lambda Healthy (1609 sites)
% of glycosylation motifs
Position in BCR sequence

Figure 20.

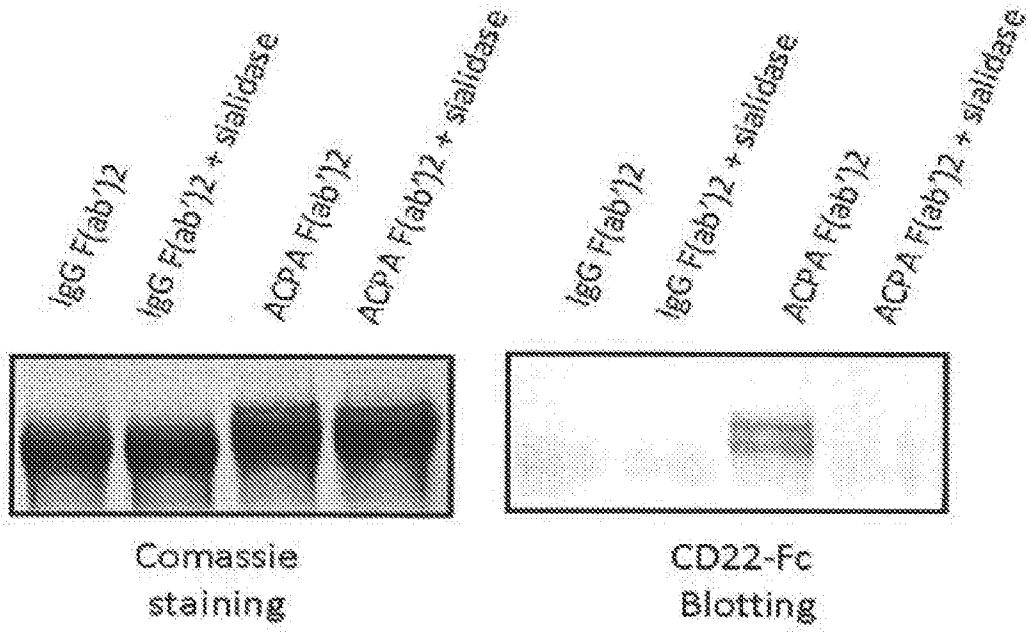

IgG F(ab')2
IgG F(ab')2 + sialidase
ACPA F(ab')2
ACPA F(ab')2 + sialidase
IgG F(ab')2
IgG F(ab')2 + sialidase
ACPA F(ab')2
ACPA F(ab')2 + sialidase
Comassie staining
CD22-Fc Blotting

Figure 21.

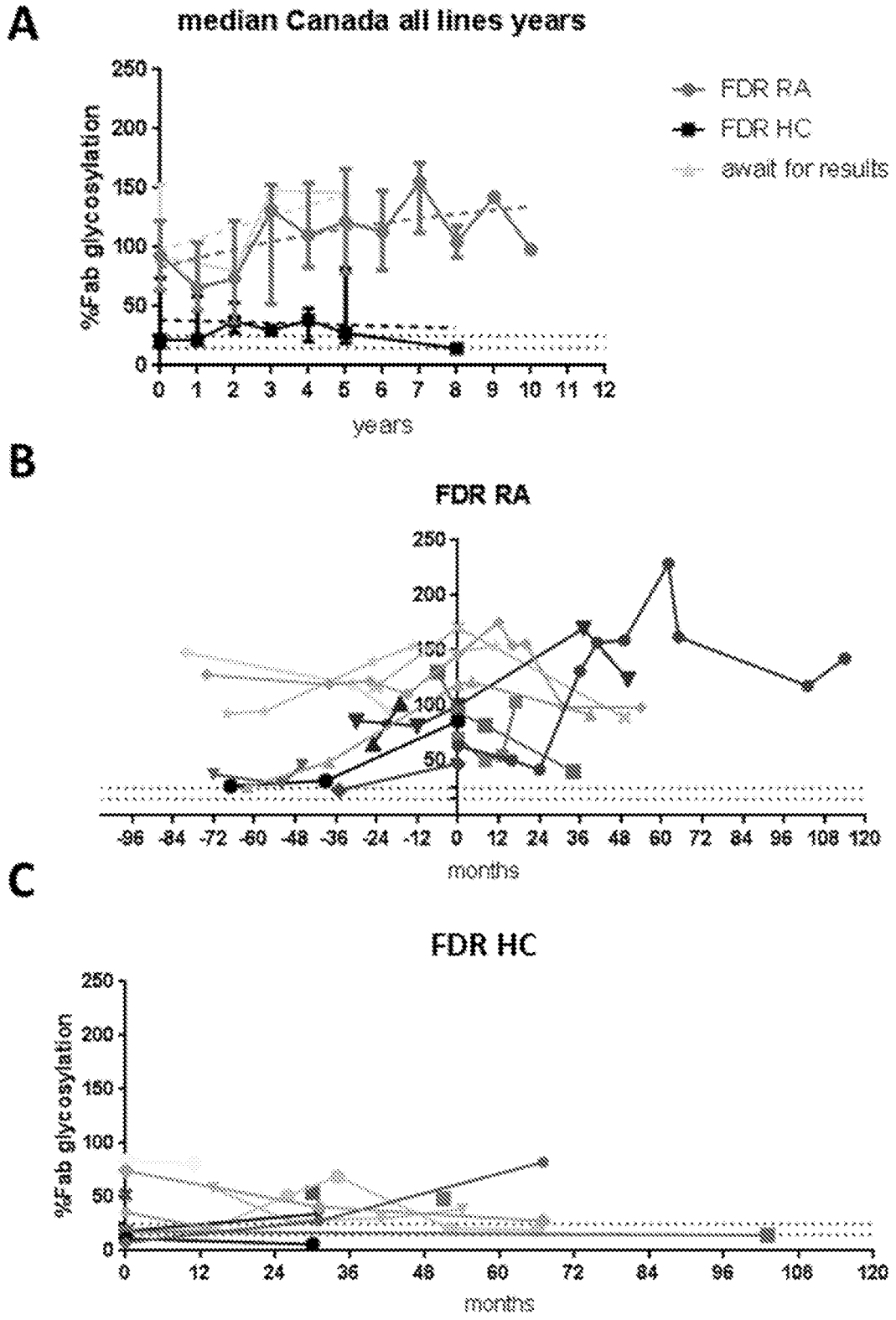

A
median Canada all lines years
%Fab glycosylation
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
years
FDR RA
FDR HC
await for results
B
FDR RA
250
200
150
100
50
-96 -84 -72 -60 -48 -36 -24 -12 0 12 24 36 48 60 72 84 96 108 120
months
C
FDR HC
%Fab glycosylation
250
200
150
100
50
0
0 12 24 36 48 60 72 84 96 108 120
months

Figure 22

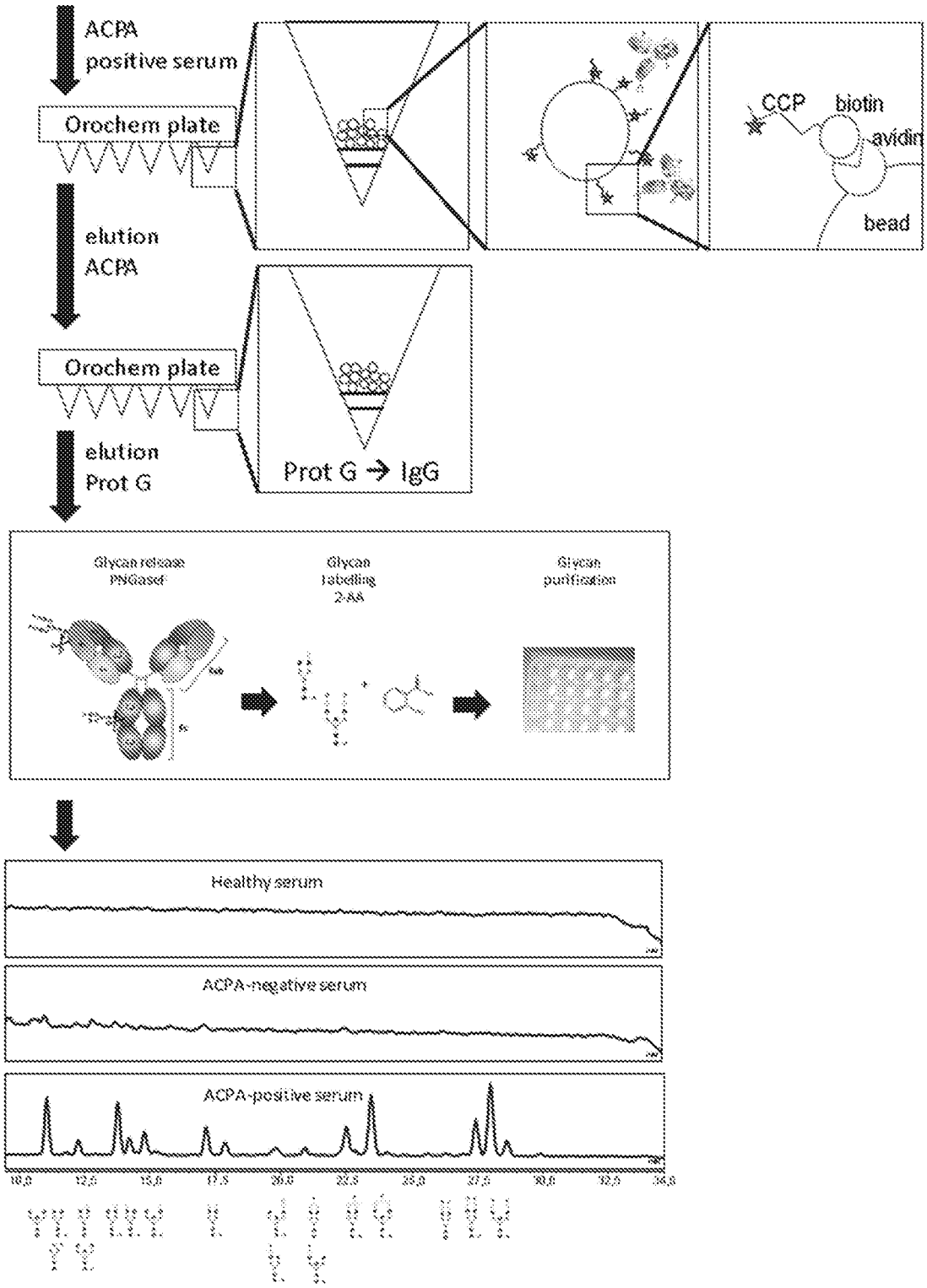

ACPA
positive serum
Orochem plate
elution
ACPA
Orochem plate
elution
Prot G
Prot G → IgG
CCP
biotin
avidin
bead
Glycan release
PNGase F
Glycan
labelling
2-AB
Glycan
purification
Healthy serum
ACPA-negative serum
ACPA-positive serum

Figure 23

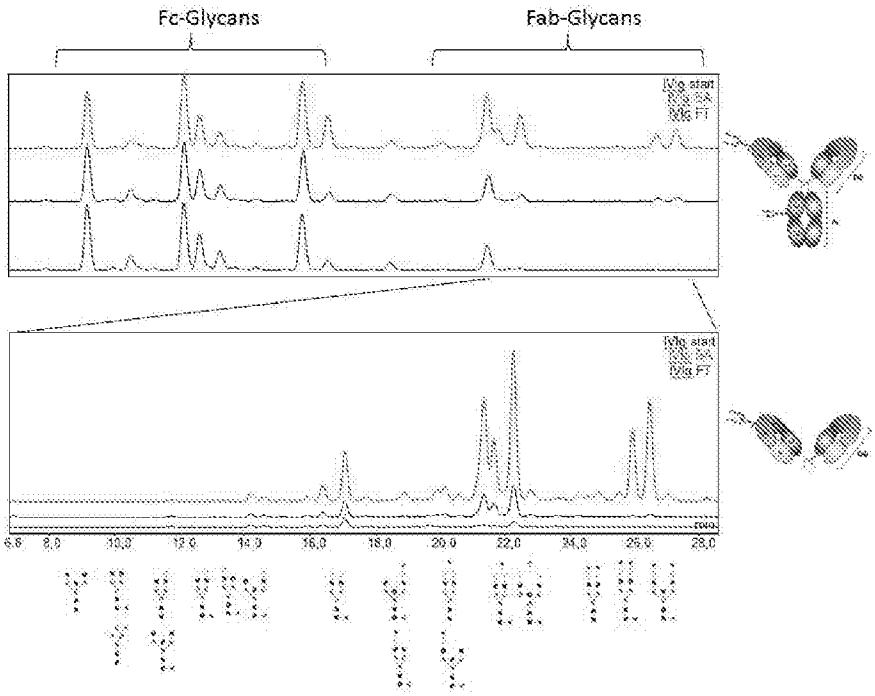
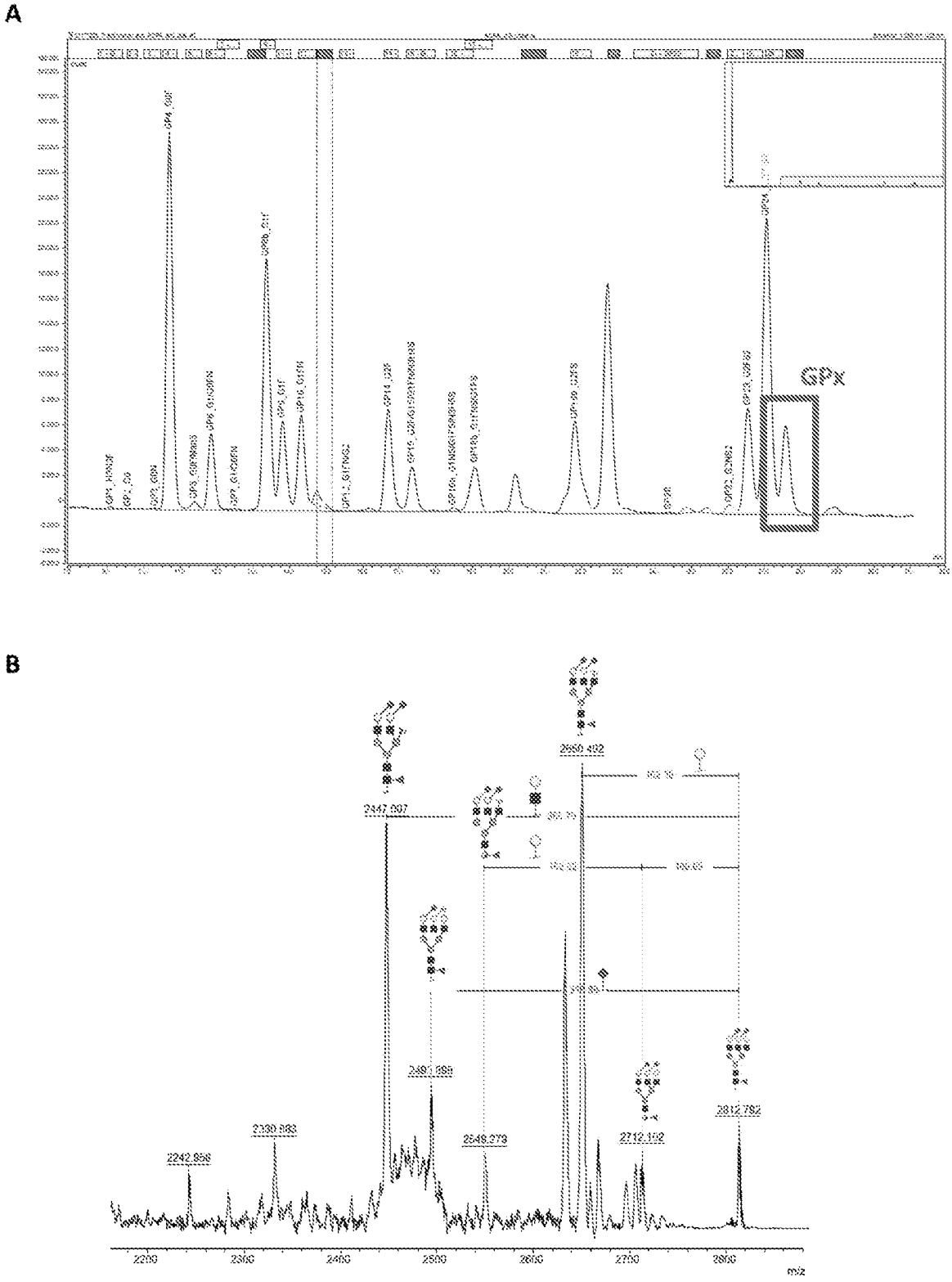


Figure 24


A
GPx
B
2650.402
2447.307
2891.099
2549.279
2712.102
2812.782
2330.883
2242.856
2200
2300
2400
2500
2600
2700
2800
m/z

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050773

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/564 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLLAND M ET AL: "Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - GENERAL SUBJECTS, ELSEVIER, AMSTERDAM, NL, vol. 1760, no. 4, 1 April 2006 (2006-04-01), pages 669-677, XP025014812, ISSN: 0304-4165, DOI: 10.1016/J.BBAGEN.2005.11.021 [retrieved on 2006-04-01] abstract ----- -/--	1-28

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2018	21/02/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Moreno de Vega, C

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. SHI ET AL: "Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 108, no. 42, 10 October 2011 (2011-10-10), pages 17372-17377, XP055339360, US ISSN: 0027-8424, DOI: 10.1073/pnas.1114465108 the whole document	1-28
A	WO 2012/105838 A1 (ACADEMISCH ZIEKENHUIS LEIDEN [NL]; TROUW LEENDERT ADRIANUS [NL]; TOES) 9 August 2012 (2012-08-09) claims 1-15	1-28
A	RADJIV GOULABCHAND ET AL: "Impact of autoantibody glycosylation in autoimmune diseases", AUTOIMMUNITY REVIEWS, vol. 13, no. 7, 1 July 2014 (2014-07-01), pages 742-750, XP55448739, NL ISSN: 1568-9972, DOI: 10.1016/j.autrev.2014.02.005 the whole document	1-28
X	SCHERER H U ET AL: "A HIGH FREQUENCY OF N-GLYCANS IN THE ACPA-IGG VARIABLE DOMAIN MODULATES REACTIVITY TO CITRULLINATED ANTIGENS", ANNALS OF THE RHEUMATIC DISEASES, BRITISH MEDICAL ASSOCIATION, GB, vol. 73, no. Suppl. 2, 1 June 2014 (2014-06-01), page 129, XP009193273, ISSN: 0003-4967, DOI: 10.1136/ANNRHEUMDIS-2014-EULAR.4717	7-13,17, 18,22, 25,26
Y	the whole document	1-28
A	FRANS G.M. KROESE ET AL: "Autoimmunity: Break-through in the diagnosis and treatment of immune-mediated inflammatory diseases", IMMUNOLOGY LETTERS., vol. 162, no. 2, 1 December 2014 (2014-12-01), pages 150-162, XP55448742, NL ISSN: 0165-2478, DOI: 10.1016/j.imlet.2014.10.013 the whole document	1-28

2 -/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROMBOUTS YOANN ET AL: "Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis", ANNALS OF THE RHEUMATIC DISEASES, BRITISH MEDICAL ASSOCIATION, GB, vol. 75, no. 3, 1 March 2016 (2016-03-01), pages 578-585, XP009193271, ISSN: 0003-4967	7-13,17, 18,22, 25,26
Y	the whole document	1-28

Y	DEKKERS JACQUELINE ET AL: "The role of anticitrullinated protein antibodies in the early stages of rheumatoid arthritis", CURRENT OPINION IN RHEUMATOLOGY, CURRENT SCIENCE, LONDON, GB, vol. 28, no. 3, 1 May 2016 (2016-05-01), pages 275-281, XP009193272, ISSN: 1040-8711 the whole document	1-28

X	LISE HAFKENSCHEID ET AL: "Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans", MOLECULAR & CELLULAR PROTEOMICS, vol. 16, no. 2, 16 November 2016 (2016-11-16), pages 278-287, XP055448713, US ISSN: 1535-9476, DOI: 10.1074/mcp.M116.062919	7-13,17, 18,22, 25,26
Y	the whole document	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2017/050773

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012105838 A1	09-08-2012	AU 2012211537 A1	15-08-2013
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		JP 6154748 B2	28-06-2017
		JP 2014505884 A	06-03-2014
		US 2014162297 A1	12-06-2014
		US 2017234869 A1	17-08-2017
		WO 2012105838 A1	09-08-2012