Bitter Sweet Symphony: the impact of sugars on autoimmunity

Kissel, T.

On the presence of HLA-SE alleles and ACPA IgG variable domain glycosylation in the phase preceding the development of rheumatoid arthritis

Theresa Kissel, Karin A.J. van Schie, Lise Hafkenscheid, Anders Lundquist, Heidi Kokkonen, Manfred Wuhrer, Tom W.J. Huizinga, Hans U. Scherer, René E.M. Toes, Solbritt Rantapää-Dahlqvist

Ann Rheum Dis. 2019 Dec;78(12):1616-1620.

Abstract

Anti-citrullinated protein antibodies (ACPAs) in Rheumatoid Arthritis (RA) patients display a unique feature defined by the abundant presence of N-linked glycans within the variable domains (V-domains). Recently, we showed that N-glycosylation sites, which are required for the incorporation of V-domain glycans, are introduced following somatic hypermutation. However, it is currently unclear when V-domain glycosylation occurs. Further, it is unknown which factors might trigger the generation of V-domain glycans and whether such glycans are relevant for the transition towards RA. Here, we determined the presence of ACPA IgG V-domain glycans in paired samples of pre-symptomatic individuals and RA patients.

ACPA IgG V-domain glycosylation was analyzed using UHPLC in paired samples of pre-symptomatic individuals [median (IQR) pre-dating time: 5.8 (5.9) years; n = 201; 139 ACPA-positive and 62 ACPA-negative] and RA patients (n = 99; 94 ACPA-positive and 5 ACPA-negative).

V-domain glycans on ACPA IgG were already present up to 15 years before disease in pre-symptomatic individuals and their abundance increased closer to symptom-onset. Noteworthy, HLA-SE alleles associated with the presence of V-domain glycans on ACPA IgG.

Our observations indicate that somatic hypermutation of ACPAs, which results in the incorporation of N-linked glycosylation sites and consequently V-domain glycans, occurs already years before symptom-onset in individuals that will develop RA later in life. Moreover, our findings provide first evidence that HLA-SE alleles associate with ACPA IgG V-domain glycosylation in the pre-disease phase and thereby further refine the connection between HLA-SE and the development of ACPA-positive RA.

Introduction

Rheumatoid Arthritis (RA) is hallmarked by the presence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs)[1-3]. Several genetic risk factors such as the human leukocyte antigen class II shared epitope (HLA-SE) alleles are associated with ACPA-positive RA. Noteworthy, an association with HLA-SE can only be found in ACPA-positive disease and is mostly lost in ACPA-positive healthy individuals, indicating that HLA-SE-restricted T helper cell activity is likely involved in the development of ACPA-positive disease and not the initial induction of autoimmunity. Presumably, these T cells provide help to ACPA-expressing B-cells that have been activated in an earlier phase[4,5]. ACPA IgG are glycoproteins that harbor, like all IgG, N-linked glycans in the Fc region located at Asn297[6]. Remarkably, approximately 90% of ACPA IgG molecules in sera from RA patients are also abundantly glycosylated within their variable domain (V-domain)[7]. Structural composition analysis revealed that these V-domain glycans are mostly biantennary complex-type glycans carrying sialic acids[7,8]. To undergo N-linked glycosylation, a consensus sequence in the protein backbone is required (N-X-S/T, where X ≠ P)[9]. Previously, we have shown that N-linked glycosylation sites in ACPA IgG V-domains are introduced during somatic hypermutation[10]. Furthermore, in a cross-sectional study of Indigenous North American individuals, we observed that ACPA IgG V-domain glycosylation is largely absent in ACPA-positive subjects that did not transition to RA, while N-linked glycans were found on ACPA-positive healthy subjects that later developed RA[11].

On the basis of these and other observations, we hypothesize that V-domain glycosylation conveys a selective advantage to ACPA-expressing B cells, which potentially plays a pivotal role in disease development[10,12]. To investigate and understand the presence and acquisition of ACPA V-domain glycans in the phase preceding arthritis in more depth, we now aimed to analyze the presence of V-domain glycans on ACPA IgG in paired samples of pre-symptomatic individuals and RA patients.

Results

ACPA IgG V-domain glycan profiles were detected in ACPA-positive pre-symptomatic individuals and RA patients

We analyzed individuals who were sampled before symptom-onset, after diagnosis of RA and randomly selected ACPA-positive and negative control samples from the same population. Chromatographic glycosylation peaks (Figure 1A) could be observed for captured ACPA IgG of 94 out of 201 pre-symptomatic individuals (89 ACPA-positive), 80 out of 99 RA patients (78 ACPA-positive) and 2 out of 43 control samples (1 ACPA-positive and 1 with an anti-CCP2 antibody level < 25 AU/ml). The results obtained strengthen the reliability of the method used,

as from the samples displaying glycan profiles only 8 (4.5%) were derived from ACPA-negative individuals (anti-CCP2 antibody level < 25 AU/ml) compared to 168 glycan profiles (> 95%) derived from samples obtained from ACPA-positive individuals (Table S2 and Figure S1). Of note, in 68 ACPA-positive samples (40.5%) no glycan profiles, including Fc glycans (positive control), could be detected, indicating a limitation of the assay sensitivity.

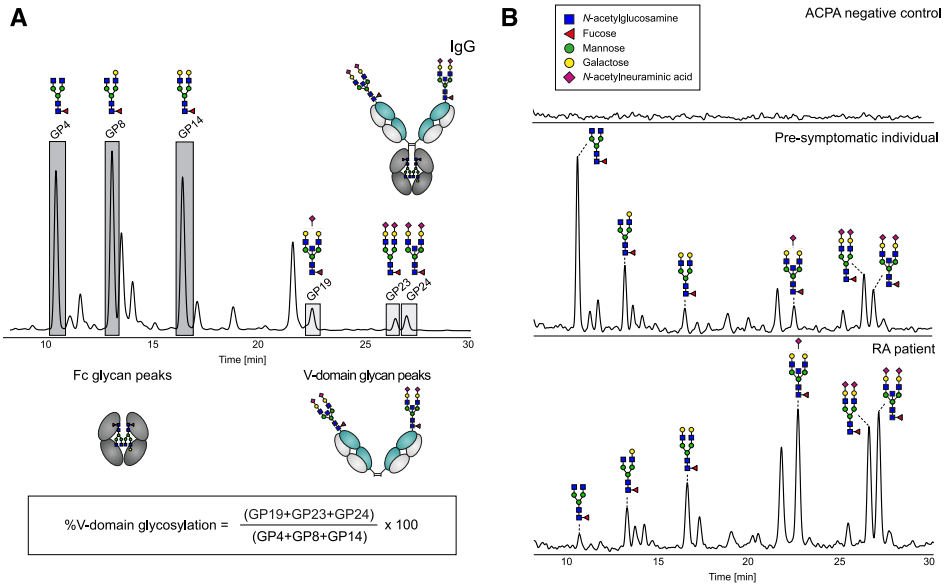

Figure 1. Representative UHPLC spectra of released N-glycans. (**A**) UHPLC chromatogram of healthy control IgG after IgG capturing and schematic representation of Fc- and V-domain derived glycosylation peaks. The 6 chromatographic peaks (alignment based on[13]) used for the calculation of %V-domain glycosylation are highlighted and the formula is visualized. (**B**) UHPLC spectra of released N-glycans after ACPA IgG capturing from an ACPA IgG negative individual (no detectable glycan peaks), an ACPA IgG positive pre-symptomatic individual (V-domain glycosylation of 55%) and an ACPA IgG positive RA patient (V-domain glycosylation of 324%). Assigned are the GP4, GP8, GP14, GP19, GP23 and GP24 chromatographic peaks of the IgG glycome based on literature[7,8]. Blue square: *N*-acetylglucosamine, green circle: mannose, yellow circle: galactose, red triangle: fucose, pink diamond: α2,6-linked *N*-acetylneuraminic acid.

ACPA IgG V-domain glycosylation rises towards symptom-onset and is already present years before

To address the question when V-domain glycosylation first appears, matched paired individuals were sampled before symptom-onset (between -15 and -0.5 years) as well as after diagnosis of RA (between +0.5 and +3 years) and analyzed for ACPA IgG V-domain glycosylation (Figure 1A)[7]. The data obtained revealed that V-domain glycosylation was already present in pre-symptomatic individuals (Figure 2A). Interestingly, ACPA IgG V-domain glycosylation increased

over time ($p < 0.001$) reaching a mean of 111.4% at symptom-onset, showing that, on average, more than one N-glycan is present within the V-domain of these ACPA IgG. Likewise, also analyses of the 29 matched pairs, with detectable V-domain glycan peaks, showed an increase of ACPA IgG V-domain glycosylation towards disease-onset ($p = 0.043$, paired t-test) (Figure 2B). Furthermore, we observed that rising V-domain glycosylation in pre-symptomatic individuals correlated moderately with anti-CCP2 antibody levels ($r_s = 0.504$, $p < 0.001$) (Figure 2C). This correlation could not be detected anymore after disease development (Figure 2D).

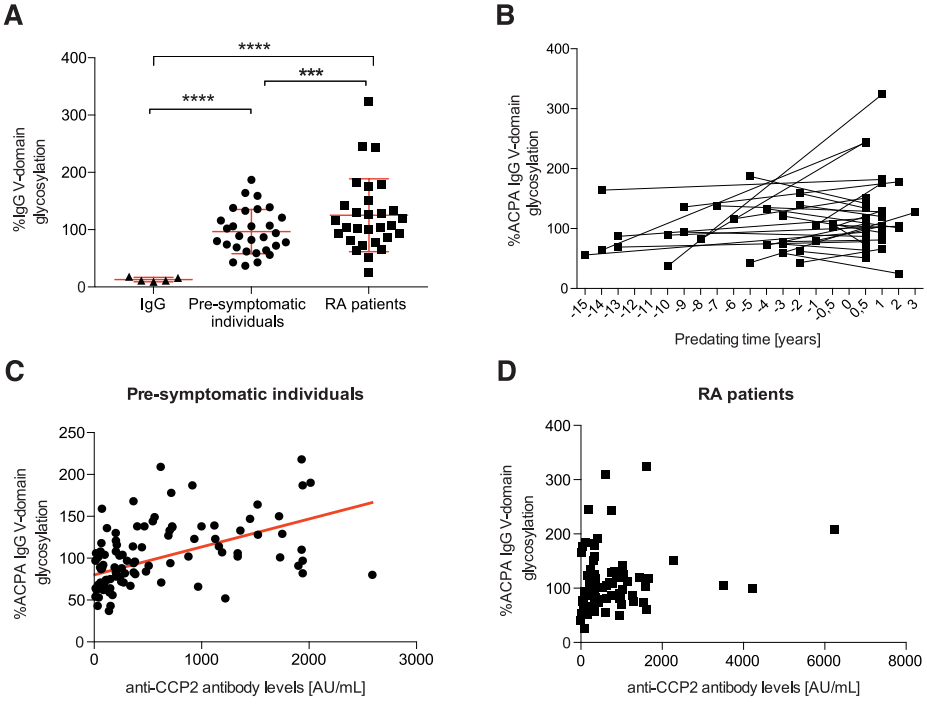

Figure 2. ACPA IgG variable domain glycosylation levels of pre-symptomatic individuals and RA patients. (**A**) Percentage V-domain glycosylation of healthy control IgG samples, captured ACPA IgG from pre-symptomatic individuals and RA patients. (**B**) Percentage ACPA IgG V-domain glycosylation followed over pre-dating time (-15 years before until +3 years after symptom-onset) for 29 matched paired pre-symptomatic and RA patient samples that showed detectable V-domain glycan profiles. 0 indicates onset of RA. ACPA IgG V-domain glycosylation increases towards disease-onset ($p = 0.043$, paired t-test). (**C**) Scatter plot of percentage ACPA IgG V-domain glycosylation and anti-CCP2 antibody levels in pre-symptomatic individuals ($r_s = 0.504$, $p < 0.001$) (**D**) Scatter plot of percentage ACPA IgG V-domain glycosylation and anti-CCP2 antibody levels in RA patients ($r_s = 0.169$, $p = 0.133$). Significant differences are indicated by *** ($p < 0.001$) and **** ($p < 0.0001$). The cut-off used for ACPA IgG V-domain glycan peaks is defined as the average of the AUC sum intensity of all blank sample peaks plus x* standard deviation (x = value defined as such that all blank and ACPA-negative healthy donor control samples fall below the cut-off).

Table 1. Logistic regression analysis of samples from pre-symptomatic individuals with detectable vs. non detectable ACPA IgG glycan profiles.

Variable	Simple		
	OR	95%CI	p-value
Anti-CCP2 ab +/-	20.29	7.63, 53.94	< 0.001
Anti-CCP2 ab levels	1.005	1.003, 1.007	< 0.001
RF +/-	7.36	3.91, 13.88	< 0.001
Sex m/f	0.78	0.44, 1.38	0.393
Ever smoking y/n	0.72	0.394, 1.317	0.286
HLA-SE +/-	1.97	1.022, 3.806	0.043
Pre-dating time, years	1.077	0.999997, 1.16	0.050008

ACPA IgG V-domain glycosylation associates with HLA-SE

To investigate possible associations of ACPA IgG V-domain glycosylation pre-disease and cohort characteristics, we performed a logistic regression analysis using detectable ACPA IgG V-domain glycan vs. non-detectable glycan profiles as an outcome. The statistical analysis showed no association between V-domain glycosylation and "sex" or "ever smoking". Interestingly, an association between ACPA IgG V-domain glycosylation and HLA-SE was observed (OR = 1.97, p = 0.043). This association remained significant after adjusting for anti-CCP2 antibody status, and pre-dating time (OR: 2.46, p = 0.023) as well as after adjusting for RF and pre-dating time (OR: 2.54, p = 0.015) (Table 1). However, this association was non-significant after correcting for anti-CCP2 antibody levels although a clear trend remained (OR = 2.06, p = 0.086). In contrast, no association was found when a reciprocal analysis was performed addressing the question whether HLA-SE associates with anti-CCP2 antibody positivity pre-disease (OR = 1.01, 95%CI = 0.51 to 1.98).

Discussion

In this study we have captured ACPA IgG from pre-symptomatic individuals and RA patients and analyzed their glycan profiles using UHPLC. The observed glycan profiles were derived from samples of ACPA-positive individuals in more than 95% of the cases. These results indicate the high reliability of the implemented methodology (Figure S1). Glycan profile detection in 8 out of 107 ACPA-negative samples may be explained by the presence of ACPA levels slightly below the ELISA cut-off. In fact, 3 out of these 8 subjects in whom ACPA V-domain glycans were detected, while displaying ACPA-negativity based on the ELISA cut-off, had been tested positive for reactivity towards other citrullinated antigens, which could explain capturing by the CCP2-coated beads. A clear limitation of the present study is that not all ACPA-positive samples could be analyzed for the presence of V-domain glycans as not all glycan-profiles, including Fc glycans, could be detected. This is likely due to technical constraints such as low ACPA levels or limited sample amounts.

Multiple			Multiple			Multiple		
OR	95%CI	p-value	OR	95%CI	p-value	OR	95%CI	p-value
32.13	10.54, 97.91	< 0.001	-			-		
-			1.005	1.003, 1.008	< 0.001	-		
-			-			7.59	3.85, 14.96	< 0.001
-			-			-		
-			-			-		
2.46	1.13, 5.33	0.023	2.06	0.90, 4.70	0.086	2.54	1.20, 5.36	0.015
0.94	0.85, 1.04	0.206	0.98	0.89, 1.08	0.747	1.01	0.93, 1.10	0.804

At present ACPA IgG is used as one of the most relevant biomarkers in RA. However, ACPA detection in subjects at risk does not always correlate with the progression to RA[14]. Our data show that N-linked V-domain glycans are a specific feature of ACPA IgG, which can be present already years before the onset of RA. These results are in line with a recent study indicating that the presence of V-domain glycans could potentially be used as a biomarker to identify ACPA-positive individuals at risk to develop RA[11]. Together our studies show that V-domain glycosylation occurs in almost all ACPA-positive individuals who will develop RA despite different ethnic and environmental backgrounds. Moreover, the current study shows that V-domain glycans appeared already up to 15 years before diagnosis (Figure 2B). Furthermore, our data reveal that V-domain glycosylation increases towards disease-onset, conceivably due to the generation of de-novo N-glycosylation sites or the expansion of N-glycosylation site-bearing clones, and that this increase associates with higher ACPA levels (Figure 2C). These results are in line with the notion that ACPA-expressing B cells gain a selective advantage through the generation of V-domain glycans.

Additionally, we did observe an association between ACPA IgG V-domain glycosylation pre-disease and HLA-SE alleles. This association remains after including anti-CCP2 status into the model. Likewise, a clear, although non-significant trend remains after correcting for anti-CCP2 antibody levels.

Unfortunately, we could not study a possible correlation between V-domain glycosylation and epitope spreading of the ACPA response as we did not have sufficient data on the citrullinated-epitope recognition profile of the samples available. Nonetheless, an association between HLA-SE status and ACPA IgG V-domain glycosylation is intriguing as it suggests that HLA-SE predispose to the formation of N-linked glycosylation sites in ACPA pre-disease and not to ACPA-positivity itself. This assumption is in line with findings indicating that the association between HLA-SE and ACPA-positivity is mostly lost in healthy individuals[4,5]. However, although

appealing, additional replication is warranted as the present study could have introduced bias due to e.g. limited sample size or assay-sensitivity. Nonetheless, we consider it highly relevant to perform such studies as it could provide novel insights into the role of HLA-SE-restricted T cells on the development of ACPA-positive RA. HLA-SE T cells might facilitate the introduction of N-linked glycosylation sites on ACPA-expressing B cells allowing their expansion as conceivably explaining the rise in ACPA levels pre-disease.

In summary, our data disclose that V-domain glycosylation precedes the development of ACPA-positive RA and may serve as aid to improve current algorithms predicting RA development thereby allowing early treatment of high risk individuals. Noteworthy, our findings suggest that the action of HLA-SE could be explained by the contribution to facilitate the introduction of N-linked glycosylation sites into ACPA IgG pre-disease.

Materials and Methods

Patient and public involvement – Patients were involved in this study by donating blood at the Medical Biobank of Northern Sweden, when attending population surveys.

Ethical considerations – All participants have given their written informed consent when included into the surveys and the Regional Ethical Review Board Committee at Umeå University approved the study.

Study cohort – Individuals, diagnosed with RA later in life, were sampled prior to symptom-onset [median (IQR) pre-dating time: 5.8 (5.9) years; n = 201; 139 ACPA-positive and 62 ACPA-negative] and after diagnosis of RA (n = 99, 94 ACPA-positive and 5 ACPA-negative as specificity control). Further, randomly selected control samples (n = 43, 3 ACPA-positive and 40 ACPA-negative) were included. The RA patients fulfilled the 1987 ARA classification criteria[15]. Descriptive cohort information is presented in supplementary Table 1.

Laboratory analyses – Anti-citrullinated protein antibodies (ACPAs) were analyzed in blood samples of pre-symptomatic individuals and RA patients using anti-cyclic citrullinated peptide 2 (CCP2) enzyme linked immunoassays from Euro-Diagnostica (Malmö, Sweden) with a positivity cut-off set at 25 AU/mL according to the manufacturer´s instructions.

ACPA IgG isolation – ACPA were isolated from plasma samples using NeutrAvidin Plus resin (Thermo Fisher Scientific) coupled with 0.1 μg/μl CCP2-biotin as previously described[16]. In brief, 50 μl of CCP2-coupled beads were transferred onto each well of a 96-well polypropylene filter plate (Orochem). Prior to sample application, bead-coated filter plates were washed with 150 μl

PBS by centrifugation (500×g for 2 min). Serum samples (25 µl) were diluted in 175 µl PBS, added to the bead-coated filter plates and incubated for 2 hours at 600 rpm on a plate shaker. Sample flowthrough was collected by centrifugation (500×g for 2 min). Washing steps with 150 µl PBS followed by 150 µl 25 mM ammonium bicarbonate (pH 7 to 7.5) and two times 150 µl PBS were performed. CCP2 specific antibodies were eluted with 100 µl 0.1 M formic acid (pH 2.5). Eluted samples were neutralized with 2 M TRIS until pH reached 7 to 7.5. ACPA isolation was followed by IgG isolation using FcXL affinity beads (Thermo Fisher Scientific) as previously described[16]. In brief, 100 µl bead slurry was transferred onto each well of a 96-well polypropylene filter plate (Orochem). 500 µl PBS was added and the plates centrifuged at 50×g for 2 min. The bead-coated plates were washed three times with 200 µl PBS. Captured ACPA from the serum samples was added to the bead-coated plates and incubated for 1 hour at 900 rpm on a plate shaker. The flowthrough was collected by centrifugation (50×g for 1 min). The plate was washed three times with 200 µl PBS. 100 µl 100 mM formic acid (pH 2.5) was added in two steps, incubated for 5 min at 900 rpm and the eluted ACPA IgG collected by centrifugation (1 min at 500 rpm). The ACPA IgG samples were evaporated using a speedvac for 3 hours at 45 °C.

N-linked glycan release, labeling and UHPLC analysis – N-linked glycans from captured ACPA IgG samples were released using PNGase F as previously described[7]. Released N-glycans were subsequently labeled with 2-aminobenzoic acid (2-AA, Sigma Aldrich) and 2-picoline borane (2-PB, Sigma Aldrich) and purified via hydrophilic interaction liquid chromatography-solid phase extraction (HILIC SPE) using GHP membrane filter plates (Pall Life Science) as previously described[17]. 2-AA labelled and HILIC SPE purified N-linked glycans were diluted in ACN to obtain a final concentration of 75% ACN and analyzed using a Dionex Ultimate 3000 (Thermo Fisher Scientific) instrument, a FLR fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm and a Acquity UHPLC BEH Glycan column (1.7 µm 2.1 mm × 100 mm; Waters, Milford, MA). Separation was performed at 60 °C with a flow rate of 0.6 ml/min. 100% ACN (solvent A) and 100 mM ammonium formate pH 4.4 (solvent B) were used for gradient generation. Column equilibration was performed for 0.5 min using 85% of solvent A. Sample loading occurred in 75% of solvent A followed by a column wash with 85% solvent A for 10 min. The separation was achieved using a start gradient of 75% solvent A and 25% solvent B, which decreased linearly to 63% solvent A within 30 min. For re-equilibration the column was flushed for 4 min with 40% solvent A and 10 min 85% solvent A. The generated chromatograms were analyzed and exported as excel files using Chromeleon version 7.1.2.1713 (Thermo Fisher Scientific). All chromatograms were aligned in the same manner and separated into 24 peaks. Glycan composition in each chromatographic peak was defined as previously described[18].

Data analysis and processing – Calibration was performed using HappyTools version 0.0.2[18] and the elution time of five calibrant peaks (GP4, GP8, GP14, GP23, GP24). Integration and baseline correction were performed using the elution time of all chromatographic peaks and

the following settings: General settings: start t = 8 min, end t = 30 min; Peak detection settings: minimum intensity: 0.05, sigma value: 2.0; calibration settings: minimum peaks: 4, minimum S/N: 1; Quantitation settings: datapoints: 100, baseline order: 1, background window: 1.0, MT slice points: 5. The amount of N-glycans in each peak was expressed as the total integrated area under the curve (AUC). A cut-off was defined using the average of the AUC sum intensity of all blank samples plus x* standard deviation (x = value defined such that all blank and healthy donor control samples fall below the cut-off). The percentage ACPA IgG V-domain glycosylation was calculated based on the three most abundant Fc-glycan peaks (positive control for IgG capturing) and the three most abundant V-domain glycan peaks using the following formula: [(GP19+GP23+GP24) / (GP4+GP8+GP14)] × 100[11,18].

Statistical analyses – Statistical calculations were performed using SPSS for Windows version 25.0 (IBM Corp., NY, USA). Continuous data were analyzed using non-parametric methods (Mann-Whitney´s U-test and Wilcoxon signed-rank test) and parametric tests when appropriate. Correlation analyses were performed using Spearman rank-order correlation (r_s). All p-values are two-sided and $p < 0.05$ was considered as statistically significant. Simple and multiple logistic regression analyses were performed for different variables associated with ACPA IgG V-domain glycosylation.

Acknowledgments

The authors would like to thank the Department of Biobank Research at Umeå University, Västerbotten Intervention Programme, the Northern Sweden MONICA study and the County Council of Västerbotten for providing data and samples. We would like to thank Dr. Jan Wouter Drijfhout (LUMC, Leiden, The Netherlands) for providing the CCP2 peptide.

Funding

This work has been financially supported by ReumaNederland 17-1-402 (to R.E.M.T.), the IMI-funded project RTCure 777357 (to T.W.J.H.), ZonMw TOP 91214031 (to R.E.M.T.), the Swedish Research Council VR 2017-00650 (to S.R-D.) as well as the King Gustaf V's 80-Year Fund, the King Gustaf V's and Queen Victoria's Fund and the Swedish Rheumatism Association (to S.R-D.).

Conflict of interest

H.U.S., T.W.J.H. and R.E.M.T. are mentioned inventors on a patent on ACPA IgG V-domain glycosylation.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Conceptualization: T.K., K.A.J.v.S., L.H., R.E.M.T. and S.R.-D. Methodology: T.K., K.A.J.v.S., L.H., A.L., H.K. Software: T.K., A.L., H.K. Investigation: T.K., K.A.J.v.S., L.H. Visualization: T.K. Supervision: H.U.S., R.E.M.T. and S.R.-D. Writing—original draft: T.K., R.E.M.T. and S.R.-D. Writing—review and editing: K.A.J.v.S., L.H., A.L., H.K., M.W., T.W.J.H. and H.U.S.

References

1 Schellekens, G. A., de Jong, B. A., van den Hoogen, F. H., van de Putte, L. B. and van Venrooij, W. J., Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest* 1998. 101: 273-281.

2 Aletaha, D., Neogi, T., Silman, A. J., Funovits, J., Felson, D. T., Bingham, C. O., 3rd, et al., 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum* 2010. 62: 2569-2581.

3 Aho, K., Heliovaara, M., Maatela, J., Tuomi, T. and Palosuo, T., Rheumatoid factors antedating clinical rheumatoid arthritis. *J Rheumatol* 1991. 18: 1282-1284.

4 Hensvold, A. H., Magnusson, P. K., Joshua, V., Hansson, M., Israelsson, L., Ferreira, R., et al., Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins. *Ann Rheum Dis* 2015. 74: 375-380.

5 Terao, C., Ohmura, K., Ikari, K., Kawaguchi, T., Takahashi, M., Setoh, K., et al., Effects of smoking and shared epitope on the production of anti-citrullinated peptide antibody in a Japanese adult population. *Arthritis Care Res (Hoboken)* 2014. 66: 1818-1827.

6 Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M. and Dwek, R. A., The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol* 2007. 25: 21-50.

7 Hafkenscheid, L., Bondt, A., Scherer, H. U., Huizinga, T. W., Wuhrer, M., Toes, R. E., et al., Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans. *Mol Cell Proteomics* 2017. 16: 278-287.

8 Rombouts, Y., Willemze, A., van Beers, J. J., Shi, J., Kerkman, P. F., van Toorn, L., et al., Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. *Ann Rheum Dis* 2016. 75: 578-585.

9 Shakin-Eshleman, S. H., Spitalnik, S. L. and Kasturi, L., The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *J Biol Chem* 1996. 271: 6363-6366.

10 Vergroesen, R. D., Slot, L. M., Hafkenscheid, L., Koning, M. T., van der Voort, E. I. H., Grooff, C. A., et al., B-cell receptor sequencing of anti-citrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of N-glycosylation sites during somatic hypermutation. *Ann Rheum Dis* 2018. 77: 956-958.

11 Hafkenscheid, L., de Moel, E., Smolik, I., Tanner, S., Meng, X., Jansen, B. C., et al., N-Linked Glycans in the Variable Domain of IgG Anti-Citrullinated Protein Antibodies Predict the Development of Rheumatoid Arthritis. *Arthritis Rheumatol* 2019. 71: 1626-1633.

12 Scherer, H. U., Huizinga, T. W. J., Kronke, G., Schett, G. and Toes, R. E. M., The B cell response to citrullinated antigens in the development of rheumatoid arthritis. *Nat Rev Rheumatol* 2018. 14: 157-169.

13 Pucic, M., Knezevic, A., Vidic, J., Adamczyk, B., Novokmet, M., Polasek, O., et al., High throughput isolation and glycosylation analysis of IgG-variability and heritability of the IgG glycome in three isolated human populations. *Mol Cell Proteomics* 2011. 10: M111 010090.

14 van Beers, J. J., Willemze, A., Jansen, J. J., Engbers, G. H., Salden, M., Raats, J., et al., ACPA fine-specificity profiles in early rheumatoid arthritis patients do not correlate with clinical features at baseline or with disease progression. *Arthritis Res Ther* 2013. 15: R140.

15 Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., Fries, J. F., Cooper, N. S., et al., The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988. 31: 315-324.

16 Habets, K. L., Trouw, L. A., Levarht, E. W., Korporaal, S. J., Habets, P. A., de Groot, P., et al., Anti-citrullinated protein antibodies contribute to platelet activation in rheumatoid arthritis. *Arthritis Res Ther* 2015. 17: 209.

17 Jansen, B. C., Bondt, A., Reiding, K. R., Scherjon, S. A., Vidarsson, G. and Wuhrer, M., MALDI-TOF-MS reveals differential N-linked plasma- and IgG-glycosylation profiles between mothers and their newborns. *Sci Rep* 2016. 6: 34001.

18 Jansen, B. C., Hafkenscheid, L., Bondt, A., Gardner, R. A., Hendel, J. L., Wuhrer, M., et al., HappyTools: A software for high-throughput HPLC data processing and quantitation. *PLoS One* 2018. 13: e0200280.

Supplemental Tables

Table S1. Descriptive information of the sample cohort.

Variables	Controls N = 43	Pre-symptomatic individuals N = 201	RA patients N = 99
Females, n (%)	32 (74.4)	126 (62.7)	67 (67.7)
Age at sampling, mean±SD, years	51.3 ± 9.6	51.4 ± 9.4	59.0 ± 9.5
Anti-CCP2 abs, n (%)	3 (6.98)	139 (69.2)	94 (95.0)
RF, n (%)	3 (6.98)	107 (53.2)	82 (82.8)
Ever smoker, n (%)	14 (32.6)	140 (69.7)	72 (72.7)
HLA-SE, n (%)	-	141 (70.2)	76 (76.8)
Pre-dating time, median (IQR), years	-	5.8 (5.9)	-

Table S2. Detectable ACPA IgG V-domain glycosylation profiles within the sample cohort.

	n	anti-CCP2 antibody level > 25AU/ml		Detectable glycan profiles	Non detectable glycan profiles
Controls	43	pos	3	1	2
		neg	40	1	39
Pre-symptomatic individuals	201	pos	139	89	50
		neg	62	5	57
RA patients	99	pos	94	78	16
		neg	5	2	3

Supplemental Figure

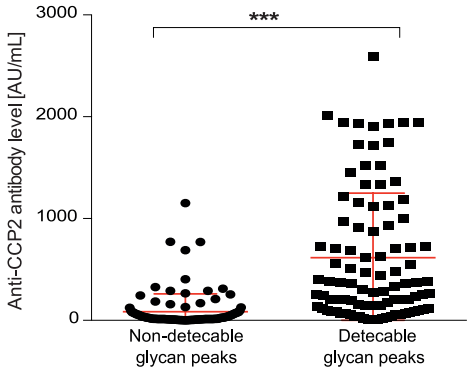

Figure S1. Detectability of ACPA IgG V-domain glycosylation correlates with anti-CCP2 antibody positivity. Anti-CCP2 antibody levels stratified for detectable vs. non-detectable ACPA IgG V-domain glycan peaks within all samples tested.