Bitter Sweet Symphony: the impact of sugars on autoimmunity

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ACPA IgG variable domain glycosylation increases before the onset of rheumatoid arthritis and stabilizes thereafter; a cross-sectional study encompassing 1500 samples

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Abstract

The autoimmune response in Rheumatoid Arthritis (RA) is marked by anti-citrullinated protein antibodies (ACPAs). A notable feature of ACPA IgG is the abundant expression of N-linked glycans in the variable domain. However, the presence of ACPA variable domain glycosylation (VDG) across disease stages, and its' response to therapy is poorly described. To understand its dynamics, we investigated the abundance of ACPA IgG VDG in 1498 samples from individuals in different clinical disease stages.

Using liquid chromatography, we analyzed ACPA IgG VDG profiles of 7 different cohorts from Japan, Canada, The Netherlands and Sweden. We assessed 106 healthy, 228 pre-symptomatic, 277 arthralgia, 307 patients with new-onset/ early RA and 117 RA patients 4, 8 and 12 months after disease-onset and prespecified treatment regimens. Additionally, we measured VDG of 234 samples from patients whose RA did and patients whose RA did not enter long-term drug-free remission (DFR) during up to 16 years follow-up.

Our data show that ACPA IgG VDG significantly increased ($p < 0.0001$) towards disease-onset and associates with ACPA levels and epitope spreading pre-diagnosis. A slight increase in VDG was observed in established RA and a moderate influence of treatment ($p = 0.007$). Individuals in whom DFR was later achieved, ACPA IgG VDG was already reduced at the time of RA-onset.

Thus, the abundance of ACPA IgG VDG increases towards RA-onset and correlates with maturation of the ACPA response. While, ACPA IgG VDG levels are fairly stable in established disease, a lower degree of VDG at RA-onset correlates with DFR. Although the underlying biologic mechanisms remain elusive, our data support the concept that VDG relates to an expansion of the ACPA response pre-disease and contributes to disease development.

Introduction

Rheumatoid Arthritis (RA) is a prevalent, slowly evolving autoimmune disease with arthralgia as an important pre-disease manifestation. The most specific autoimmune response for RA is characterized by the presence of anti-citrullinated protein antibodies (ACPAs), which can be found several years before the onset of clinical symptoms. ACPA-positive patients have a more severe disease course and are less likely to achieve drug-free remission (DFR) as compared to seronegative patients[1]. ACPA responses are known to be dynamic during the transition towards RA, as an increase in ACPA levels combined with a broader epitope-recognition profile is associated with the development of clinical symptoms[2]. Autoantibody levels are, however, not associated with long-term treatment response and do not predict DFR[3]. Glycomic analysis revealed that ACPA IgG are abundantly glycosylated in their antigen-binding fragments expressing complex-type variable domain glycans (VDGs) that are mainly disialylated and bisected[4]. A variable domain glycosylation (VDG) on more than 90% of the autoantibodies, is an outstanding characteristic of ACPA IgG and distinguishes the molecules from conventional IgG which display, next to the conserved presence of glycans in the fragment crystallizable (Fc) region, a considerably lower VDG of ~12 to 14%[4,5]. Glycosylation sites required for the attachment of VDGs are introduced by somatic hypermutation (SHM)[6]. Although the role and dynamics of ACPA IgG Fc glycans has been studied extensively[7-10], little is known about the expression levels or potential biologic implications of the VDG on ACPAs. As carbohydrates might encode important biologic information and possibly affect cellular functions, it is important to understand VDG dynamics over time in relation to the disease course of RA. Previously, we showed that ACPA IgG VDG can occur several years before RA-onset. In a Canadian population, ACPA IgG VDG was predictive for disease development[11,12]. However, how ACPA IgG VDG changes between different clinical disease stages from healthy, symptom-free individuals to individuals with arthralgia to patients at RA-onset and with established RA has not been elucidated. Additionally, it is unclear whether VDG levels are associated with treatment outcomes, predict DFR and disease flares, or can be modified by treatment. To understand the momentum of VDGs and thereby their possible contribution to the autoreactive B-cell responses in RA, we cross-sectionally investigated the presence and abundance of ACPA IgG VDGs in 1498 samples from an ethnically diverse group of individuals in various disease stages. Importantly, by analyzing samples from a well-controlled treatment strategy trial, the Improved study, aiming to assess the most effective strategy in inducing remission in early RA, we investigated longitudinal VDG changes in established RA after treatment escalation or treatment tapering[13]. Finally, we longitudinally analyzed ACPA IgG VDG changes of individuals visiting the Leiden Early Arthritis Clinic (EAC) in whom sustained drug-free (> 1 year) remission (SDFR) was achieved and those who experienced late disease flares, with an extensive follow-up of up to 16 years[14].

Results

ACPA IgG variable domain glycosylation increases toward disease-onset and remains stable in established RA

To provide a comprehensive overview of the presence and abundance of ACPA IgG VDGs (Figure 1, A and B), we analyzed 1377 ACPA-positive and 121 ACPA-negative samples from individuals in different clinical disease stages (Figure 1C and Table 1). Comparable to the results of previous studies[11,12], we found that high percentages of VDGs (median of 56.2%) on ACPA IgG were already present in healthy individuals (n = 106) without symptoms (Figure 1D). Cross-sectional analysis revealed a significant increase in VDG (median of 74.7%) in individuals with clinically identified arthralgia (n = 277) compared to healthy individuals (Figure 1D). A further significant increase in VDG of 18% was observed when individuals were sampled at RA-onset (n = 181) (VDG median of 92.6% in the combined dataset) (Figure 1D and Table 1).

This was, however, not apparent in all individual sample sets, as VDG changes between the arthralgia phase and RA-onset could not be observed in the statistically underpowered longitudinal dataset from cohort 5 (arthralgia, Leiden), presumably because the clinically suspect arthralgia individuals were included shortly before the onset of arthralgia (Figure S3), and an increase in VDG could have occurred earlier. Patients presenting with arthralgia, regardless of whether they did or did not subsequently develop RA, displayed a lower VDG than patients at the time of RA-onset (Figure S1E). In samples from patients with established RA after prespecified treatment (n = 346), ACPA IgG VDG remained stable, with only a moderate increase after 12 months, to a median of 105.2% (Figure 1D). As previously shown[11], an increase in ACPA IgG VDG toward RA-onset was also observed in a Swedish population of ACPA-positive individuals that later developed RA. The extended dataset used here also exhibited a rise in VDG when analyzed per individual in a longitudinal manner (Figure S1D)[21], however no significance could be detected cross-sectionally (Figure S1C).

Overall, the results obtained indicate that the presence of VDG on ACPA IgG was lower in healthy individuals and increased toward RA development. However, in established disease, no further progression of ACPA IgG VDG was observed in this cross-sectional setting.

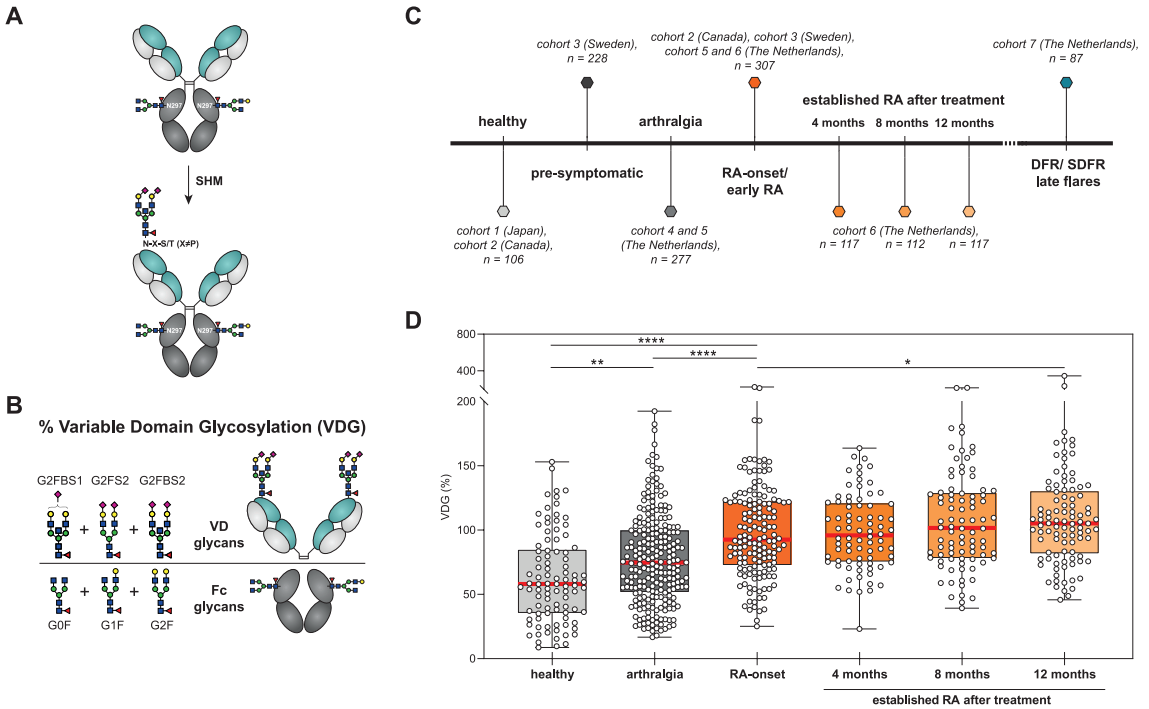

Figure 1. Percentage ACPA IgG VDG from individuals in different disease stages of RA. (**A**) Depiction of the process by which IgG carries N-glycans at each N297 residue in the Fc domain and can acquire additional N-linked glycosylation sites (N-X-S/T, X ≠ P) in the variable domain by somatic hypermutation (SHM)[6]. (**B**) Depiction of the calculation of ACPA IgG VDG, i.e., [(sum of the most abundant VDG)/(sum of the most abundant Fc glycans) × 100] or [(G2FBS1+G2FS2+G2FBS2)/(G0F+G1F+G2F) × 100]. The selected glycan traits are exclusively present on either the variable domain or the Fc domain of ACPA IgG. A-/mono-/di-galactosylated (G0/G1/G2), core fucosylated (F), bisecting GlcNAc (B), mono-/di-sialylated (S1/S2). Blue square: *N*-acetylglucosamine (GlcNAc), green circle: mannose, yellow circle: galactose, red triangle: fucose, pink diamond: *N*-acetylneuraminic acid. (**C**) "Time-line" of clinical disease stages, the corresponding analyzed cohorts and absolute numbers of analyzed samples. (**D**) Percentage ACPA IgG VDG, measured by liquid chromatography, of healthy (light gray), arthralgia (dark gray), RA-onset (orange) and established RA (light orange), 4, 8 and 12 months, individuals after prespecified treatment. Data are presented as box and whiskers including all data points. Kruskal-Wallis tests were performed for the cross-sectional datasets of *cohorts 1, 2, 4* and *5* including the Dunn's test. Generalized estimating equation (GEE) analysis was performed for the longitudinal samples of *cohort 6*. Significant differences are denoted by *(p = 0.037), **(p = 0.0032), or ****(p < 0.0001).

Table 1. Descriptive cohort data. Cohorts including samples from individuals in different disease stages from healthy symptom-free to established RA. Cohort 7 includes individuals that achieve DMARD-free remission (DFR), sustained DFR (SDFR) and individuals with late disease flares.

cohort	cohort 1, Japan (Nagasaki)	cohort 2, Canada (Manitoba)		cohort 3, Sweden (Umeå)		cohort 4, The Netherlands (Amsterdam, Reade)	cohort 5, The Netherlands (Leiden, CSA)	
disease stage	healthy, symptom-free	healthy	RA-onset	pre-symptomatic	0.5 - 1.5 years after RA-onset	arthralgia	arthralgia	RA-onset
	N = 58	N = 48	N = 25	N = 228	N = 126	N = 239	N = 38	N = 26
sex (female), n(%)	38 (66)	32 (66.7)	19 (79)	145 (64)	78 (61)	185 (77)	29 (76.3)	22 (84.6)
age (years), mean (SD)	66.7 (9.9)	37.6 (13.5)	42 (14.7)	52.2 (9.4)	59.7 (9.3)	48.3 (11.6)	48.3 (12.5)	48.1 (12.5)
arthritis/RA at follow up, n (%)	9 (15.5)	31 (64.6)	-	228 (100)	-	137 (57.3)	26 (68.4)	-
VDG-positive, n (%)	48 (83.8)	42 (33.3)	21 (84)	105 (46.1)	116 (92.1)	211 (87.9)	27 (71.1)	18 (69.2)
VDG%, median (IQR)	58.1 (35.6)	44.9 (69.3)	99.9 (46.1)	97.4 (53.5)	94.2 (50.8)	75.3 (48.9)	70.4 (28.8)	59.1 (49.1)
ACPA-positive, n (%)	58 (100)	37 (77.1)	22 (88)	168 (73.7)	125 (98.4)	239 (100)	33 (86.8)	21 (80.8)
ACPA levels (aU/ml)*, median (IQR)	35.8 (79)	54 (135.5)	200 (103.3)	126.7 (455.5)	592.9 (725.3)	358 (1351)	123 (324)	25.5 (266.8)
ACPA assay	CLEIA (STACIA MEBLux test CCP; MBL, Nagoya, Japan)[20]	CCP-2 kit (INOVA Diagnostics, (San Diego, CA, USA)[19]		Immunoscan RA anti-CCP-2 EIA from Euro-Diagnostica (Arnhem, The Netherlands)[18]		anti-CCP ELISA (Axis Shield, Dundee, UK)[17]	anti-CCP-2 ELISA (Eurodiagnostica, The Netherlands)[16]	

* ACPA levels (arbitrary units/ml) were determined with various assays/standards and are thus not directly comparable with each other.

	cohort 6, The Netherlands (Leiden, Improved)				cohort 7, The Netherlands (Leiden, EAC)					
	RA-onset	4 months after RA-onset	8 months after RA-onset	12 months after RA-onset	RA-onset, do not achieve DFR	RA-onset, achieve DFR	pre-remission	DRF	SDFR	DFR with late flares
	N = 130	N = 117	N = 112	N = 117	N = 59	N = 36	N = 52	N = 41	N = 35	N = 11
	88 (67.7)	79 (67.5)	78 (69.6)	78 (66.7)	42 (71.2)	24 (66.7)	37 (71.2)	27 (65.9)	27 (77.1)	7 (63.6)
	51.1 (12.5)	50.6 (12.8)	50.7 (12.4)	51.0 (12.4)	49.7 (14.5)	50.8 (13.1)	54.2 (14.8)	58.5 (13.6)	54.6 (15.1)	69.4 (14.8)
	-	-	-	-	-	-	-	-	-	-
	117 (90)	78 (66.7)	86 (76.8)	98 (83.8)	59 (100)	32 (89)	37 (71)	30 (73.2)	22 (62.9)	9 (81.8)
	96 (48.2)	95.9 (45.1)	101.7 (50.3)	105.2 (48.1)	83.8 (46)	61.4(35)	74.05 (30)	67.7 (41.5)	73.5 (42)	78.3 (26.9)
	130 (100)	117 (100)	112 (100)	117 (100)	59 (100)	29 (80.6)	38 (73)	33 (80)	29 (82.9)	10 (91)
	903.3 (1101.2)	449.9 (806.9)	602 (1061.8)	651.7 (962.5)	7340 (5984)	1933 (7296)	3583 (5302)	3010 (8975)	2616 (6765)	4210 (10709)
	anti-CCP-2 in house ELISA (The Netherlands)[3,15]				anti-CCP-2 in house ELISA (The Netherlands)[3,15]					

Interconnection between the increase in variable domain glycosylation and maturation of the ACPA immune response

To obtain further insights into ACPA IgG VDG, we investigated the possible association between VDG percentages and the "maturation" of the ACPA response by analyzing ACPA IgG levels and the broadness of the citrullinated epitope-recognition profile. Pearson's correlation analysis revealed a strong, highly significant correlation between VDG percentages and ACPA IgG levels among healthy individuals ($r = 0.728$ and $r = 0.672$) and among individuals with arthralgia ($r = 0.640$) (Figure 2A and S2). At RA-onset ($r = 0.214$) and in established RA after prespecified treatment ($r = 0.341$, $r = 0.362$ and $r = 0.215$), however, we observed only moderate correlations as depicted by the correlation coefficient r (Figure 2A and S2). Likewise, our data revealed that ACPA IgG with increased VDG showed a significantly broader recognition profile toward multiple citrullinated epitopes (Figure 2, B and C). Ordinal regression analyses confirmed these findings for individuals with arthralgia ($p < 0.001$) (Table S1) as well as in patients at RA-onset ($p = 0.004$) and over time after treatment ($p < 0.001$) (Table S2). Thus, ACPA IgG VDG associated with ACPA IgG levels and the breadth of the epitope-recognition profile, suggesting that these two features of the ACPA responses are interconnected.

Impact of immunosuppression on ACPA IgG variable domain glycosylation

By taking advantage of the design of the Improved study (Figure 3A), we investigated whether ACPA IgG VDG predicts early remission in RA or is associated with the intensity of immunosuppression.

First, we used the longitudinal dataset to identify ACPA IgG VDG changes over time by analyzing matched-paired individuals at RA-onset (n = 130) versus 4 (n = 117), 8 (n = 112) and 12 months (n = 117) after disease development. VDG appeared to be steadily and abundantly expressed on ACPA IgG in disease, although minor changes in expression levels were observed over time. A slight, but non-significant decrease was observed 4 months after disease-onset and initiation of methotrexate (MTX) and prednisone treatment (Figure 3, B and C, and Table S3). Previous studies have shown a similar decline of ACPA IgG levels after initiation of treatment, providing further evidence of a correlation between VDG and ACPA IgG levels[3]. After 4 months, prednisone was tapered such that patients were then treated with MTX only, if early remission (DAS < 1.6) had been achieved. If early remission was not achieved, patients were randomized to one of two treatment escalation arms (arm 1: MTX, prednisone, hydroxychloroquine, and sulfasalazine combination, arm 2: MTX and adalimumab combination) (Figure 3A)[13]. At 8 months, individuals in the early remission group either continued MTX treatment combined with prednisone (no drug-free group) or, if disease remission persisted, their medication was tapered (drug-free group). Individuals in the treatment escalation group (arm 1 and arm 2) continued MTX treatment, in combination with adalimumab. Overall, irrespective of the treatment arm, VDG increased moderately but significantly 12 months after RA-onset ($p = 0.037$) (Table S3).

When comparing the different treatment groups, marginal but statistically significant effects of immunosuppression on ACPA IgG VDG were observed, with a reduction in VDG 12 months after RA-onset (Figure 3, D and E, and S4A), though not at 4 and 8 months. This moderate but significant negative effect of immunosuppression on VDG was confirmed by a generalized estimating equation (GEE) analysis over time (8 vs. 12 months) [in the early remission, drug-free group: regression coefficient (B) 12.27 (95% confidence interval -7.32 to 31.87) vs. in the early remission, no drug-free and treatment escalation group: 6.42 (95% confidence interval -0.35 to 13.10); p = 0.007)] (Table S4) and was similar to previously reported findings with regard to ACPA IgG levels[3]. Last, we investigated whether VDG percentage at RA-onset predicts remission after 4 months and early drug-free remission within the first year. Similar to ACPA IgG levels[3], VDG percentages did not predict early (drug-free) remission (Table S5). Collectively, these results show that ACPA IgG VDG are expressed at a persistently high level in established RA and show a slight but statistically significant decrease upon immunosuppression.

Decreased VDG during active disease in patients in whom sustained DFR is later achieved

As a next step, we performed cross-sectional and longitudinal ACPA IgG VDG analyses in individuals in whom long-term DFR was achieved or who experienced DFR with late flares. We therefore made use of the unique EAC database including patients who were followed up for a period of up to 16 years after disease-onset. Using this database, we were able to identify and approach 41 individuals in whom DFR had been achieved and 35 patients in whom SDFR (> 1 year) had been achieved. The longitudinal analysis was performed for matched-paired samples from patients at RA-onset (n = 36), during active disease (pre-remission) (n = 52), during DFR (n = 41), during SDFR (> 1 year) (n = 35), and when experiencing late disease flares (n = 11). Again, the data showed that VDG are stably expressed in established RA. Intriguingly, however, patients whose RA achieved DFR during follow-up (n = 36, VDG = 61.4%) showed significantly reduced ACPA IgG VDG at the onset of disease compared to age and gender-matched patients with persistently high disease activity (DAS > 3) (n = 59, VDG = 83.8%) (Figure 4A and Table 1). In contrast, no statistically significant changes were observed when the ACPA IgG VDG percentages were determined over time in the DFR group or any of the other groups analyzed (Figure 4, B to D).

Thus, these longitudinal data, confirm that ACPA IgG express a constant amount of VDG after RA-onset. The cross-sectional data also indicate that among individuals in whom long-term DFR is achieved, fewer glycans are present in the variable domains of ACPA IgG at the time of RA-onset.

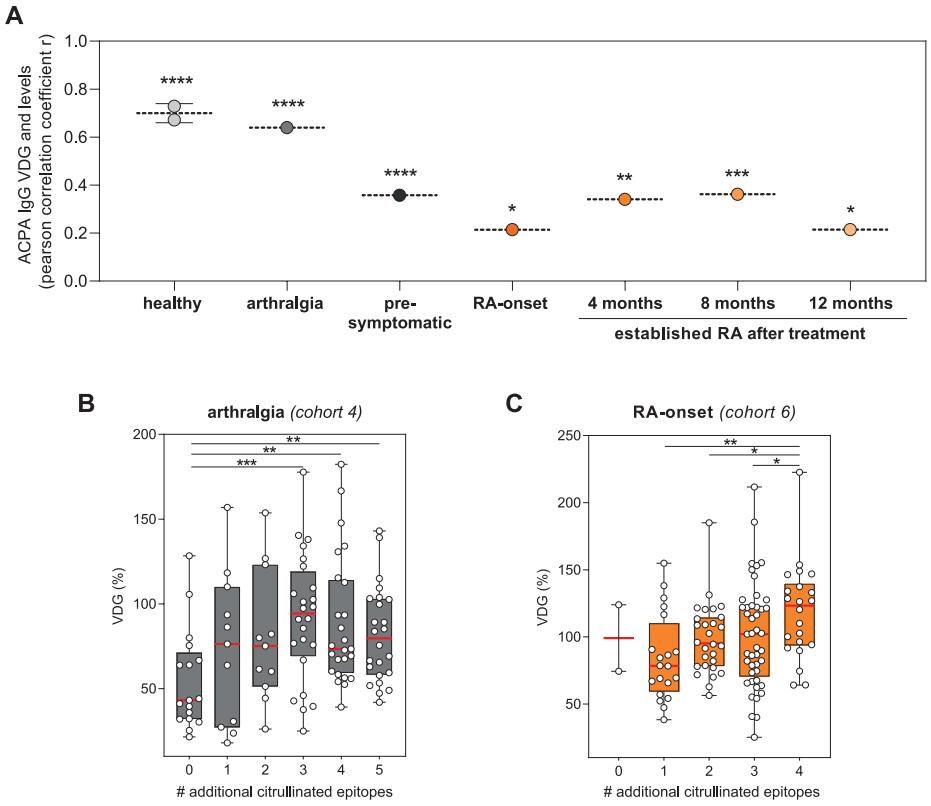

Figure 2. Correlation of ACPA IgG VDG with ACPA IgG levels and epitope spreading (maturation of the ACPA response). (**A**) Pearson's correlation coefficients r for the correlation between ACPA IgG VDG and ACPA IgG levels across different disease stages. P-values are two-tailed and significant differences are denoted by *(RA-onset) p = 0.0203, *(12 months) p = 0.0338, **p = 0.0023, ***p = 0.0006 or ****p < 0.0001. (**B**) ACPA IgG VDG percentages shown for ACPA IgG isolated using CCP2 from arthralgia individuals (*cohort 4, The Netherlands*) and tested for binding to 0 to 5 additional citrullinated antigens (citrullinated vimentin 60-75, citrullinated fibrinogen β 36-52, α 60-74 and α 36-50 and citrullinated enolase 5-21). Kruskal-Wallis tests were performed: **(0 vs. 4) p = 0.0036, **(0 vs. 5) p = 0.0096, ***p = 0.0006. (**C**) ACPA IgG VDG percentages shown for ACPA IgG isolated using CCP2 from individuals at RA-onset (*cohort 6, The Netherlands*) and tested for recognition of 0 to 4 additional citrullinated antigens (citrullinated vimentin 59-74, citrullinated fibrinogen β 36-52 and α 27-43 and citrullinated enolase 5-20). Kruskal-Wallis tests were performed: **(2 vs. 4) p = 0.0411, **(3 vs. 4) p = 0.0417, ***p = 0.0011.

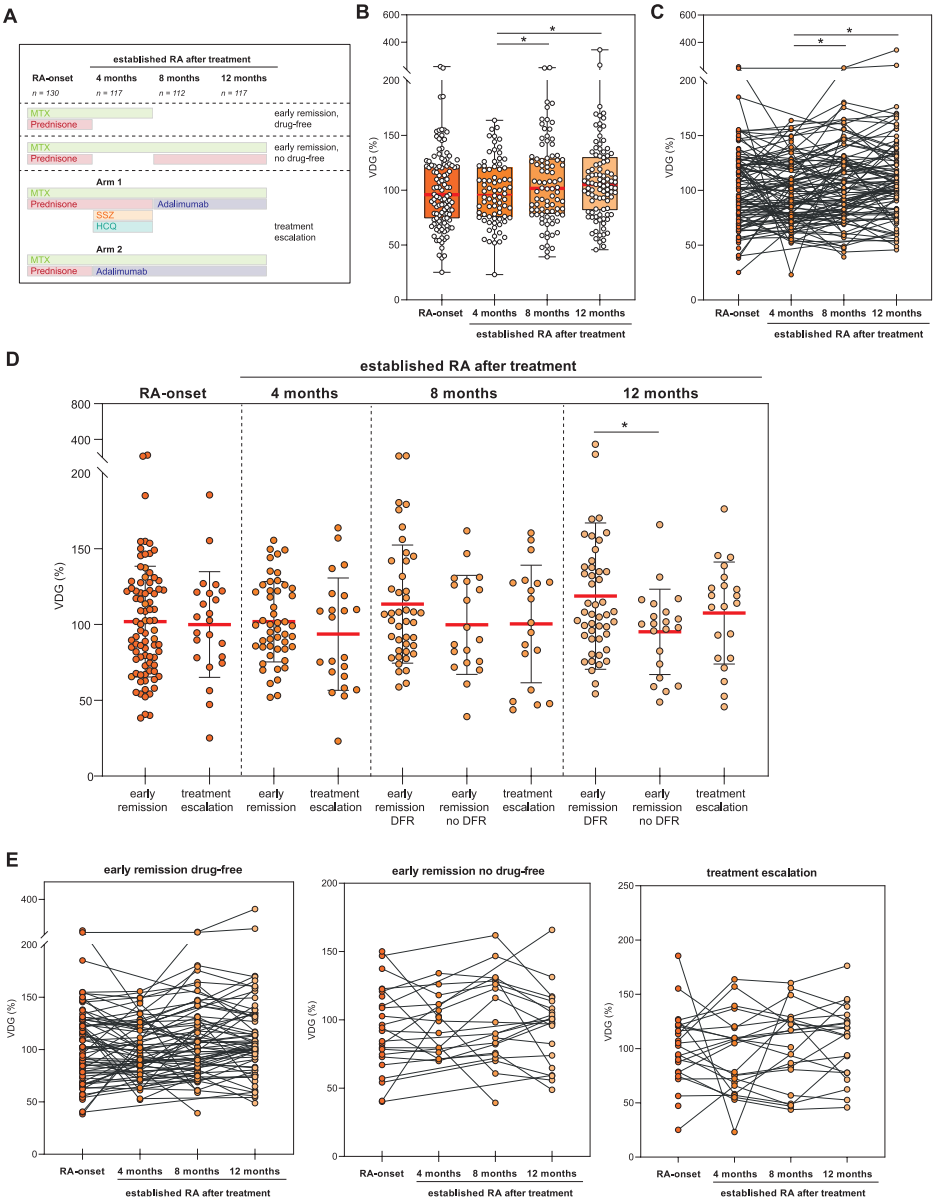

Figure 3. Longitudinal analysis of ACPA IgG VDG at RA-onset and in established RA after treatment (*cohort 6, The Netherlands*). (**A**) Treatment protocol. HCQ = hydroxychloroquine, MTX = methotrexate, SSZ = sulfasalazine (**B**) ACPA IgG VDG longitudinal data of patients at RA-onset and with established RA after prespecified treatment (*cohort 6, The Netherlands*). Data are presented as box and whiskers including all data points. Mixed-effects analysis, using Restricted Maximum Likelihood (REML), was performed including the Tukey test: *(4 vs. 8 months) p = 0.0407, *(4 vs. 12 months) p = 0.0310. (**C**) Longitudinal data from A are represented as matched pairs. (**D**) ACPA IgG VDG percentages shown by treatment group [early remission (drug-free), early remission (no drug-free) and treatment escalation]. Ordinary one-way ANOVA was performed including the Fisher's LSD test: *p = 0.015. (**E**) Longitudinal data within each treatment group, represented as matched pairs.

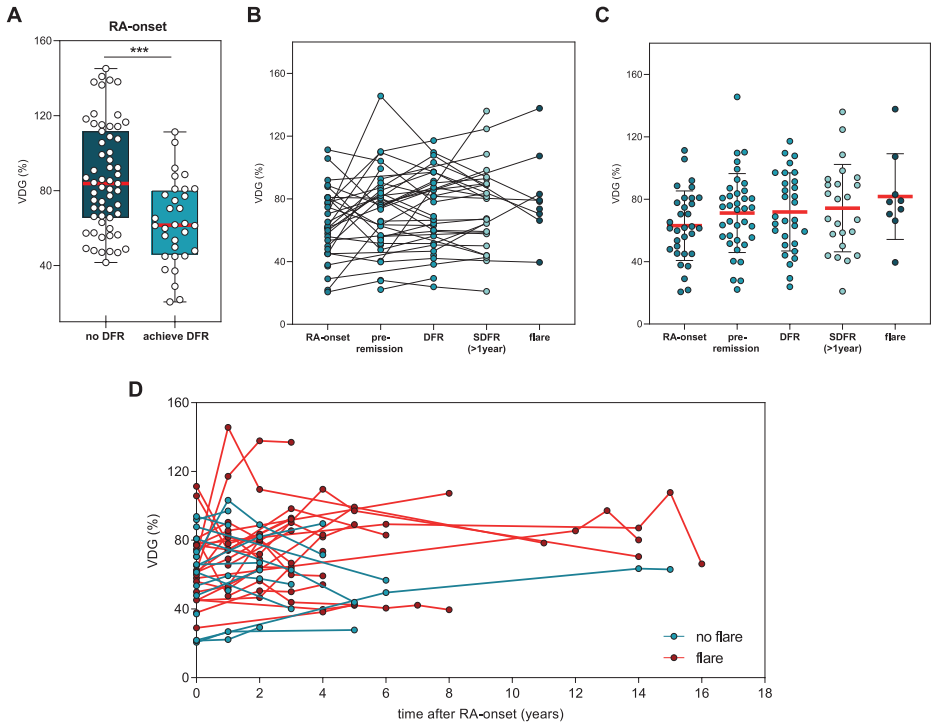

Figure 4. Cross-sectional and longitudinal analysis of ACPA IgG VDG at RA-onset and during drug-free remission (DFR) (*cohort 7, The Netherlands*). (**A**) ACPA IgG VDG percentages at RA-onset in individuals in whom DFR was not achieved and those in whom DFR was achieved. DFR was defined as the absence of clinical synovitis after discontinuation of DMARD-treatment. Mann-Whitney´s U test was performed: ****p < 0.0001. (**B**) Data on ACPA IgG VDG in matched-paired samples from patients at RA-onset, pre-remission, during DFR, during SDFR (≥ 1-year), and during late disease flares. Flare was defined as the recurrence of clinical synovitis on joint examination. (**C**) Same individuals as in B are depicted as scatter dot plots. Horizontal and vertical bars show the mean ± SD. (**D**) ACPA IgG VDG data by assessment time point in longitudinally assessed samples from patients who did (red) and those who did not experience late flares (turquoise).

Discussion

An important key characteristic of IgG autoantibodies from patients with RA is the abundant presence of bisected and disialylated glycans in the variable domain. To gain insight into the introduction and occurrence of this unusual antibody feature across different disease stages, we have captured ACPA IgG of 1500 samples from 852 individuals in different clinical disease stages. Moreover, we analyzed the effect of therapy on the degree of variable domain glycosylation (VDG) on ACPA. The large sample size increases the power of our study, and we demonstrated that ACPA IgG VDG correlates strongly with the maturation of the ACPA immune

response prior to disease-onset, while no correlation with age was observed. We found that the abundance of ACPA IgG VDG increased significantly from the time these ACPA-positive individuals were healthy and symptom-free (58.1%) toward the pre-RA phase (arthralgia) (74.7%), with a further increase toward disease-onset (92.6%). Thus, our data strongly indicate that an increase in ACPA IgG VDG already occurs in the asymptomatic phase before symptom-onset, with a further increase during progression to arthralgia and ultimately RA-diagnosis, although the latter notion requires further detailed research with longitudinal sampling.

In established RA, we noted a constant high expression of glycans on the variable domain of ACPA IgG with a slight, but significant, increase after 12 months (105.2%). These latter findings are in agreement with our previous observations, estimating > 90% VDG on ACPA IgG in RA[4], as well as the finding that > 80% of ACPA B-cell receptors in RA express N-linked glycosylation sites in the variable region[22]. Our longitudinal data from cohort 6 depict increased VDG levels in individuals in whom treatment was tapered, while patients who received more intense treatment showed reduced ACPA IgG VDG profiles over time ($p = 0.007$). This significant impact of immunosuppression was also observed for ACPA IgG levels[15], confirming the correlation between ACPA IgG levels and VDG, which was strongest in the pre-disease phase. These findings are also in accordance with the notion that VDG could have a regulatory impact on the ACPA immune response. In this respect, it is intriguing to note that the HLA-shared epitope alleles predispose to ACPA harboring VDG rather than to ACPA in general[11], thus linking ACPA VDG with the major genetic risk factor for RA. Indeed, a more in depth longitudinal analysis of the correlation between the presence of pre-disposing HLA-DR4 genes and the presence of VDG revealed a shorter "transit time" to RA in HLA-DR4-positive pre-disease individuals who still displayed relatively low levels of ACPA VDG, as compared to HLA-DR4-negative individuals[21].

Of note, we observed, in the extensive "post" RA analysis, that individuals in whom long-term DFR is achieved exhibit lower VDG profiles in active disease (61.4%) compared to patients in whom long-term DFR is not achieved (83.8%). The relevance of these findings is unknown, although it is remarkable that long-term DFR, a relatively rare event in ACPA-positive RA, was associated with lower VDG on ACPA. Importantly, reduced ACPA IgG levels are not the cause of a lower variable domain glycosylation, which was controlled by titrating ACPA IgG into healthy serum samples resulting in a maintained high degree of VDG (Figure S5B). Thus it is tempting to speculate that VDG serve as an additional "hit" determining the fate of the autoreactive B-cell response and thereby exert an impact on ACPA levels.

Together with previous data, showing that N-linked glycan sites are selectively introduced into the ACPA B-cell receptor sequences upon SHM[22] and that VDG are significantly elevated in ACPA-positive individuals who subsequently develop RA[23], our results provide evidence that a glycan attached to the variable domain fosters a breach of tolerance of autoreactive B cells.

As carbohydrates are known to affect cellular functions, ACPA-expressing B cells may gain a selection advantage when abundantly expressing glycans in their variable domains. The disialylated, and thus negatively charged glycans attached to the variable domain, which have also a large steric requirement, might modulate binding to autoantigens or affect B-cell receptor signaling of the citrullinated antigen-directed B cells. Further, it cannot be ruled out that VDG impact on effector mechanism and thereby autoantibody-mediated inflammation, similar to findings for Fc glycans. Next to these areas for further research, it would be interesting to investigate changes in specific VDG traits in more depth, as altered glycan composition could be associated with defined biologic implications, as also observed for Fc glycans. Recent studies have shown, for example, that not only Fc glycans on total IgG, but also ACPA IgG VDG show a decrease in the bisecting GlcNAc after COVID-19 infection[24,25]. Interestingly, VDG are not only a feature of ACPA IgG in RA, but have also been described in other human autoimmune responses, such as in ANCA-associated vasculitis and Sjögren's syndrome, and have been observed on anti-hinge and anti-drug antibodies[26-28].

A limitation of our study is that VDG profiles could be detected in only 70% of the samples analyzed, mainly explained by limited sample amounts or low ACPA IgG levels, as observed in the group of healthy individuals. Especially for rare disease stages, such as for the "DFR with late flares" group, only a limited number of samples were available to us. In addition, ACPA were captured using the highly sensitive and specific antigen CCP2. However, it cannot be excluded that certain ACPA molecules that recognize different citrullinated epitopes and do not interact with CCP2 were omitted from the analysis. Importantly though, we did not observe an effect of VDG on the binding affinity to CCP2 (data not shown), making a selection bias towards higher or lower glycosylated ACPA unlikely. Another limitation of our study is that conclusions are mainly based on cross-sectional data derived from samples collected at different sites. Although collection of such data from one site would be highly challenging, the analyses of samples from different sites could be hampered by site-specific effects. Importantly however, we also observed an increase in ACPA IgG VDG toward RA-onset in the longitudinal dataset of cohort 3, including paired samples obtained from pre-symptomatic individuals and RA patients over a time period of 15 years, as also previously described[21]. Furthermore, we observed concordant ACPA IgG VDG across different cross-sectional cohorts of healthy individuals (58.1% and 44.9%) or individuals with arthralgia (75.3% and 70.4%).

In summary, we have provided a comprehensive overview of the expression of VDG on ACPA IgG over various clinical disease stages in RA. Although the biologic implications of VDG attached to antibodies in general and ACPA specifically are still largely unexplored, our data show that VDG are a key characteristic of ACPA across disease stages in individuals of different ethnicities who develop RA. Our results show an increase in VDG towards disease progression and suggest,

taken together with previous data indicating a selective introduction of these N-linked glycan sites, that VDG may serve as a trigger for the maturation of the ACPA immune response. It will therefore be relevant to understand the biologic impact of VDG on the ACPA immune response and its detailed clinical implications.

Materials and Methods

Patient and public involvement – Individuals were involved in this study by donating blood when attending population surveys[18,19], medical health check-ups[14,20], were recruited to take part in the arthralgia study in the Amsterdam area of The Netherlands (*cohort 4*)[17] or the treatment strategy study "Improved" in early RA (*cohort 6*)[13].

Ethical considerations – All participants have given their written informed consent and the Regional Ethical Review Board Committees approved the studies.

Study cohorts – ACPA IgG VDG were analyzed in 1498 serum samples from individuals in different clinical disease stages including 121 ACPA-negative RA control samples. The descriptive cohort data are presented in Table 1. Additionally, 247 healthy donor and 150 ACPA-positive RA control samples, sampled at the Leiden rheumatology outpatient clinic, were assessed to verify the methodology used.

Cohort 1, healthy symptom-free (Japan, Nagasaki) – Healthy symptom-free individuals (n = 58) were included that were tested positive for the presence of ACPA and are part of the Nagasaki Island Study (NaIS) performed in Japan (a community-based prospective cohort study based on resident health check-ups)[20]. The individuals included into the study showed no joint complains at the time of the resident health check-up and were thus defined as healthy. ACPA-positive individuals were further examined and followed up for a period of up to 3 years. 9 individuals (15.5%) developed RA during follow-up.

Cohort 2, healthy and RA-onset (Canada, Manitoba) – Individuals were part of the longitudinal research project "Early Identification of Rheumatoid Arthritis in First Nations" based at the Arthritis Centre at the University of Manitoba[19]. 48 samples of healthy individuals (first degree relatives of RA patients) were included. 25 individuals were sampled prior to RA-onset, in the absence of joint-complaints, and at the time of diagnosis of clinically-apparent RA (paired samples).

Cohort 3, pre-symptomatic and after RA-onset/ early RA (Sweden, Umeå) – Individuals were recruited into the Northern Sweden Health and Disease Study (NSHDS) or the Västerbotten Intervention Project. Blood samples of the individuals were collected and stored in a

biorepository (the Northern Sweden Medical Research Biobank). RA patients were registered based on the fulfillment of the 1987 ARA classification criteria. Individuals were retrospectively identified from the cohort as having donated blood before the onset of signs of symptoms of joint disease, defined as pre-symptomatic (n = 228, median (IQR) pre-dating time: 4.7 (5.9) years) and after diagnosis of RA, defined as early RA (n = 126; 0.5 to 1.5 years)[18].

Cohort 4, arthralgia (The Netherlands, Amsterdam, Reade) – ACPA-positive individuals with arthralgia (n = 239) prospectively sampled at rheumatology outpatient clinics in the Amsterdam area of The Netherlands[17] were selected. Individuals were followed up for a period of up to 10 years and 137 (57.3%) developed arthritis during follow-up.

Cohort 5, arthralgia and RA-onset (The Netherlands, Leiden, CSA) – Individuals, at risk of RA development, were recruited for the prospective Clinically Suspect Arthralgia (CSA) cohort in the Leiden rheumatology outpatient clinic and followed longitudinally[29]. 38 individuals with arthralgia were included into this study. 26 of these individuals were sampled with arthralgia and at the time of diagnosis of clinically-apparent RA (paired samples).

Cohort 6, RA-onset and established RA after treatment (The Netherlands, Leiden, Improved) – Longitudinal samples of 130 RA patients at disease-onset as well as 4 months (n = 117), 8 months (n = 112) and 12 months (n = 117) after diagnosis and defined treatment were included. Individuals, recruited in 12 hospitals in the western area of The Netherlands, were included in the Improved (Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease) study. This multicenter, randomized control trial was aimed to achieve DFR including treatment alteration every 4 months. Initial treatment was methotrexate (MTX) and prednisone. Patients in whom early remission was achieved (defined as DAS < 1.6) tapered prednisone. If disease was still in remission at 8 months, MTX was also tapered. If DAS was ≥ 1.6 after stopping prednisone, it was restarted. Patients in whom early remission was not achieved were randomized to one of two treatment arms: MTX, prednisone, hydroxychloroquine, and sulfasalazine combination (arm 1) or MTX and adalimumab combination (arm 2)[13].

Cohort 7, RA-onset, DFR, SDFR and late disease flares (The Netherlands, Leiden, EAC) – Individuals at RA-onset in whom DMARD-free remission (DFR) was not achieved at later time-points (n = 59) and longitudinal samples (n = 175) of individuals in whom DFR or sustained DFR (SDFR) was achieved, were selected and recruited from the Leiden Early Arthritis Clinic (EAC) in the Leiden University Medical Center (The Netherlands)[14]. Samples at diagnosis of clinically apparent RA (RA-onset) (n = 36), during the pre-remission phase (n = 52), during DFR (n = 41), during SDFR (n = 35), and at the time of late disease flares (n = 11) were included with a follow-up of up to 16 years. DFR was defined as the absence of clinical synovitis (swollen-joints at physical examination) after discontinuation of DMARD-treatment (including systemic/ intra-

articular corticosteroids). In the 41 patients DMARDs were stopped after a median of 2.9 years (IQR 1.0 to 4.9 years). SDFR was defined as the absence of clinical synovitis after cessation of DMARD-treatment, that persisted for the entire follow-up of ≥ 1-year. SDFR was achieved in the 35 patients after a median of 2.8 years (IQR 2.0 to 5.2 years). SDFR was maintained for 7.1 years (IQR 4.5 to 11.2 years) after DMARD-stop, showing the sustainability of DMARD-free remission. Flare was defined as the reoccurrence of clinical synovitis on joint examination. Medical files were studied on occurrence of SDFR until September 2021.

Laboratory analyses – ACPA IgG levels in aU/ml were analyzed in serum samples using standard and commercial available anti-cyclic citrullinated peptide (CCP) assays or anti-CCP-2 in house enzyme-linked immunosorbent assays (ELISA) as previously described[3,15-20]. ACPA fine specificity for samples from cohort 6 (RA-onset and established RA, The Netherlands) was determined using anti-citrullinated vimentin 59-74, anti-citrullinated fibrinogen β 36-52 and α 27-43 and anti-citrullinated enolase 5-20 IgG in house ELISA as previously described[3]. ACPA fine specificity for samples from cohort 4 (arthralgia, The Netherlands) was determined using anti-citrullinated vimentin 60-75, anti-citrullinated fibrinogen β 36-52, α 60-74 and α 36-50 and anti-citrullinated enolase 5-21 IgG in house ELISA.

ACPA IgG capturing and VDG analysis using liquid chromatography – Capturing of ACPA IgG, total glycan release, glycan labeling and purification was performed as previously described[11]. In brief, ACPA were affinity isolated from 25 μl serum samples using NeutrAvidin Plus resin (Thermo Fisher Scientific) coupled with 0.1 μg/μl CCP2-biotin followed by IgG capturing using FcXL affinity beads (Thermo Fisher Scientific). N-linked glycans were released using 0.5 U PNGase F (Roche), subsequently labelled with 2-AA and 2-PB and HILIC SPE purified using GHP membrane filter plates (Pall Life Science). Ultra-high performance liquid chromatography (UHPLC) was performed on a Dionex Ultimate 3000 (Thermo Fisher Scientific) instrument, a FLR fluorescence detector set and an Acquity BEH Glycan column (Waters, Milford, MA). Separation and glycan peak alignment were performed as previously published[11]. HappyTools version 0.0.2 was used for calibration and peak integration[30]. The N-linked glycan abundance in each peak was expressed as the total integrated area under the curve (AUC). The cut-off was defined based on PBS control (blank) and ACPA-negative healthy control samples of individuals donating blood in the Leiden area, excluding outliers (below or above Q_1−1.5 × IQR and Q_3+1.5 × IQR). The percentage of ACPA IgG VDG was calculated based on the following formula: [(sum of the most abundant VDG)/(sum of the most abundant Fc glycans) × 100] or [(G2FBS1+G2FS2+G2FBS2)/(G0F+G1F+G2F) × 100][23]. The glycan traits were selected based on previous observations showing their exclusive presence on either the variable domain or the Fc domain of ACPA IgG molecules[4,31]. The sum of the Fc glycans, the amount of N-linked glycans attached to the conserved Asn297 in the Fc domain of IgG antibodies, remains constant.

Statistical analyses – Continuous data were analyzed using non-parametric methods (Kruskal-Wallis test for non-paired samples and Mann-Whitney´s U-test for non-paired samples) and parametric tests (Mixed-effect analysis for matched-paired samples including missing values) when appropriate. The mixed-effect analysis model using Restricted Maximum Likelihood (REML) is comparable to repeated measures ANOVA with regard to p values and multiple comparisons tests, but can handle missing values. Correlations between ACPA IgG levels (log transformed) and percentages of VDG were assessed with Pearson correlation. All p-values are two-sided and $p < 0.05$ was considered as statistically significant. Logistic and ordinal regression analyses were performed for cohort 4 (arthralgia, The Netherlands) and cohort 6 (RA-onset, The Netherlands) to investigate the association of ACPA IgG VDG/ACPA IgG levels with epitope spreading, remission and early DFR. The unstandardized coefficient (B) represents the mean change in the response given a one unit change in the predictor. The longitudinal and repeated measures data from cohort 6 (RA-onset and established RA after treatment, Leiden) were analyzed using generalized estimating equations (GEE), as specified before[3]. GEE was used to assess VDG changes over time and associations with treatment/treatment decisions. The specific covariates and dependent variables are listed in the table legends, respectively. Statistical calculations were performed using STATA (V.16.1; STATA Corp, College Station, Texas USA).

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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., R.E.M.T., T.W.J.H. Methodology: T.K., L.H., T.J.W. Software: T.K., L.H., T.J.W. Investigation: T.K., L.H., T.J.W. Visualization: T.K., T.J.W. Supervision: D.v.d.W., H.U.S., R.E.M.T. and T.W.J.H. Writing—original draft: T.K., R.E.M.T. and T.W.J.H. Writing—review and editing: L.H., T.J.W., M.T., S.Y.K., A.K., H.S.E.-G., D.v.S., S.R.-D., M.W., A.H.M.v.d.H.-v.M., C.F.A., D.v.d.W. and H.U.S.

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Supplemental Tables

Table S1. Association between the recognition of multiple citrullinated epitopes (dependent variable) and VDG or ACPA IgG levels (independent variable) in individuals with arthralgia (*cohort 4, The Netherlands*).

	B (95% CI) univariable (simple)	p value
VDG	0.03 (0.02 - 0.04)	< 0.001
log ACPA levels	0.84 (0.67 - 1.02)	< 0.001

Table S2. Association between the recognition of multiple citrullinated epitopes (dependent variable) and VDG or ACPA IgG levels (independent variable) of individuals at RA-onset and in established RA after prespecified treatment (*cohort 6, The Netherlands)*.

	B (95% CI) univariable (simple)	p value
baseline (ordinal regression analysis)		
VDG	0.142 (0.0045 - 0.0239)	0.004
ACPA levels	0.0032 (0.0025 - 0.0038)	< 0.0001
over time (GEE)		
VDG	0.0089 (0.0052 - 0.0125)	< 0.0001
ACPA levels	0.0020 (0.0017 - 0.0022)	< 0.0001

Table S3. Changes in ACPA IgG VDG after RA-onset and treatment (*cohort 6, The Netherlands*).

	VDG (%)	p value	VDG (%), adjusted[1]	p value, adjusted
continues				
per 4 months	2.85 (0.51 - 5.19)	0.017	1.29 (-1.30 - 3.89)	0.32
per month	0.71 (0.13 - 1.30)	0.017	0.32 (-0.33- 0.97)	0.048
per visit				
RA-onset	1 (ref)	-	1 (ref)	-
4 months	-3.83 (-10.79 - 3.12)	0.28	-0.06 (-8.76 - 8.87)	0.99
8 months	4.15 (-2.86 - 11.16)	0.25	1.51 (-7.50 - 10.51)	0.74
12 months	7.49 (0.47 - 14.52)	0.037	3.76 (-4.19 - 11.71)	0.35

[1] covariates: age, gender, CRP and ACPA levels

Table S4. Generalized estimating equation (GEE) analysis of VDG from individuals sampled 4 vs. 8 months and 8 vs. 12 months after RA-onset and prespecified treatment (*cohort 6, The Netherlands*). Association between VDG and prespecified treatment after 4 or 8 months respectively. The regression coefficient (B, with 95% CI) indicates changes in VDG between 4 vs. 8 months and 8 vs. 12 months for the different treatment groups.

	B (95% CI)	p value[1] univariable (simple)
VDG (%) 4 vs. 8 months		
early remission[2]	7.52 (-6.79 – 21.83)	-
treatment escalation[3]	3.27 (-6.35 – 12.90)	0.98
VDG (%) 8 vs. 12 months		
early remission, drug-free[4]	12.27 (-7.32 – 31.87)	-
early remission no drug-free and treatment escalation[5]	6.42 (-0.35 – 13.19)	0.007

[1] interaction term *treatment decision*time*

[2] methotrexate (MTX) monotherapy

[3] MTX, prednisone, hydroxychloroquine, and sulphasalazine combination (arm 1) or MTX and adalimumab combination (arm 2)

[4] MTX tapering for individuals achieving DFR

[5] continuous MTX and prednisone or adalimumab treatment

Table S5. Logistic regression analysis of individuals at RA-onset (*cohort 6, The Netherlands*). Association between VDG (dependent variable) at RA-onset and treatment response – remission at 4 months and early drug-free remission within 1 year (independent variable).

	OR (95% CI)	p value
remission at 4 months	1.003 (0.991 – 1.014)	0.65
drug free remission in the first year	1.00 (0.99 – 1.01)	0.50

Supplemental Figures

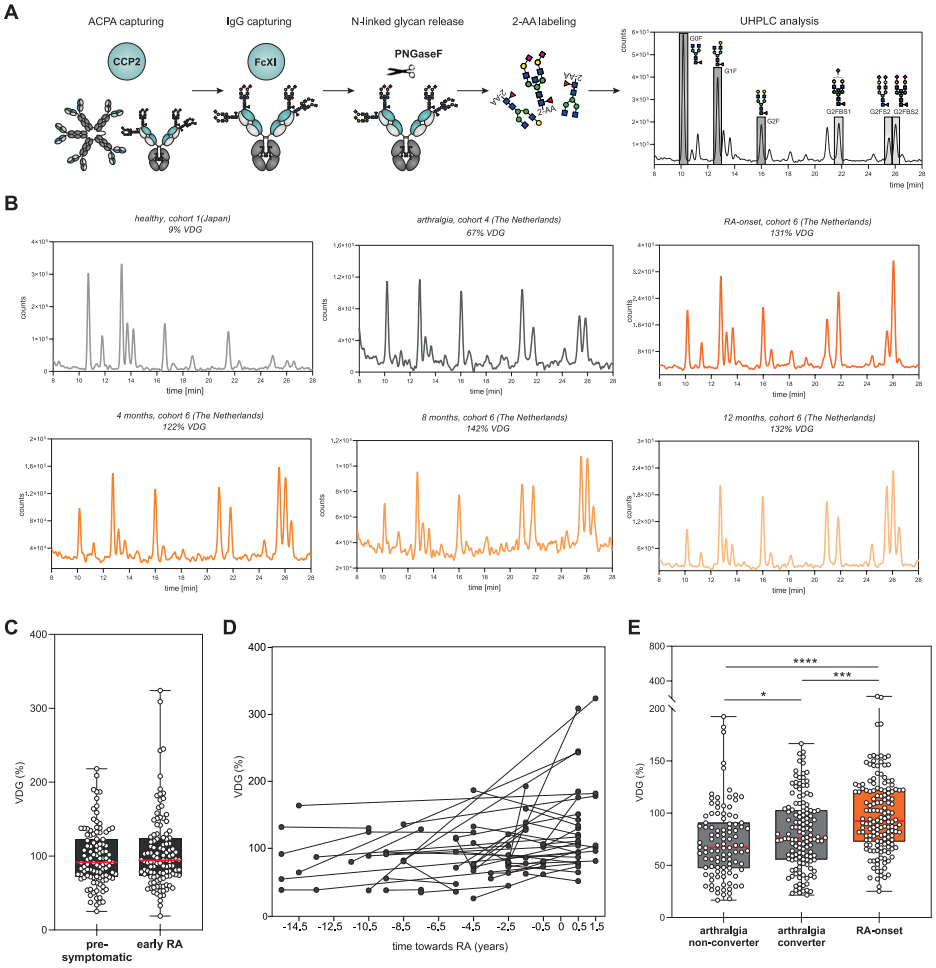

Figure S1. ACPA IgG capturing and liquid chromatography VDG analysis. (**A**) Experimental procedure: ACPA were captured using CCP2-streptavidin beads, followed by IgG capturing (FcXI beads). N-linked glycans were released using PNGase F and 2-AA labeled for liquid chromatography. G0F, G1F, G2F, G2FBS1, G2FS2 and G2FBS2 glycan peaks are highlighted. A-/mono-/di-galactosylated (G0/G1/G2), fucose attached to the core GlcNAc (F), bisecting GlcNAc (B), mono-/di-sialylated (S1/S2). Blue square: *N*-acetylglucosamine (GlcNAc), green circle: mannose, yellow circle: galactose, red triangle: fucose, pink diamond: *N*-acetylneuraminic acid. (**B**) UHPLC chromatograms of ACPA IgG VDG from a healthy individual (*cohort 1*), an individual with arthralgia (*cohort 4*) and an individual at RA-onset and 4, 8 and 12 months after disease development (*cohort 6*). (**C**) ACPA IgG VDG of individuals diagnosed with RA later in life and sampled prior to symptom-onset and after RA diagnosis (*cohort 3*). Data are presented as box and whiskers including all data points. (**D**) Matched paired samples of pre-symptomatic individuals and early RA patients (*cohort 3*). (**E**) Percentage ACPA IgG VDG of individuals with arthralgia that later convert or do not convert to RA and of patients at RA-onset. Data are presented as box and whiskers including all data points. Kruskal-Wallis tests were performed. Significant differences are denoted by *(p = 0.0406), ***(p = 0.0001), or ****(p < 0.0001).

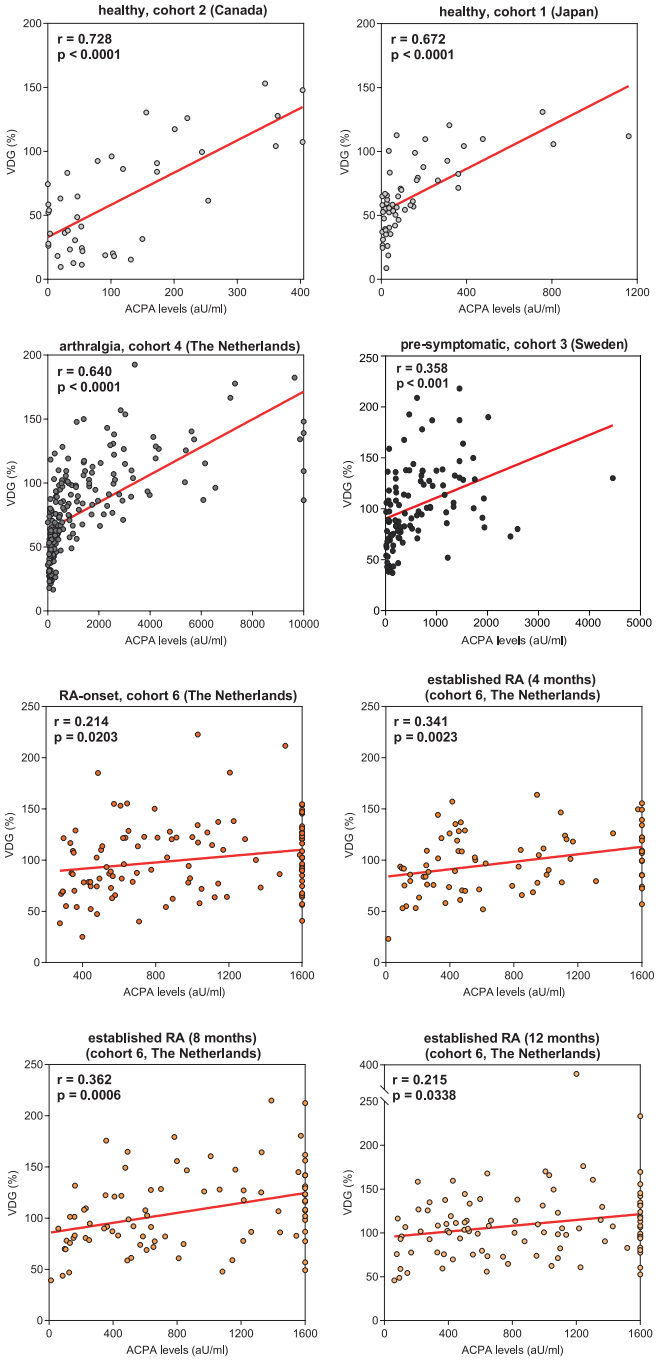

Figure S2. ACPA IgG VDG percentages correlate with ACPA IgG levels. Pearson's correlation between ACPA IgG VDG percentages and ACPA IgG levels (aU/ml). The respective p-values (two-tailed) and correlation coefficients are presented.

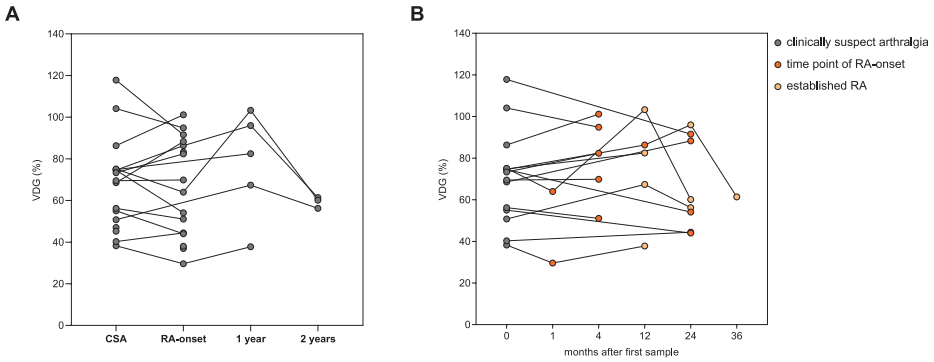

Figure S3. ACPA IgG VDG in individuals with arthralgia from cohort 5 (*The Netherlands*). (**A**) Longitudinal analysis of ACPA IgG VDG percentages from individuals with clinically suspect arthralgia (CSA), at RA-onset and 1 or 2 years after disease development. (**B**) "Time line" of VDG percentages up to 36 months after 1st visit. Time point of RA-onset is depicted in orange.

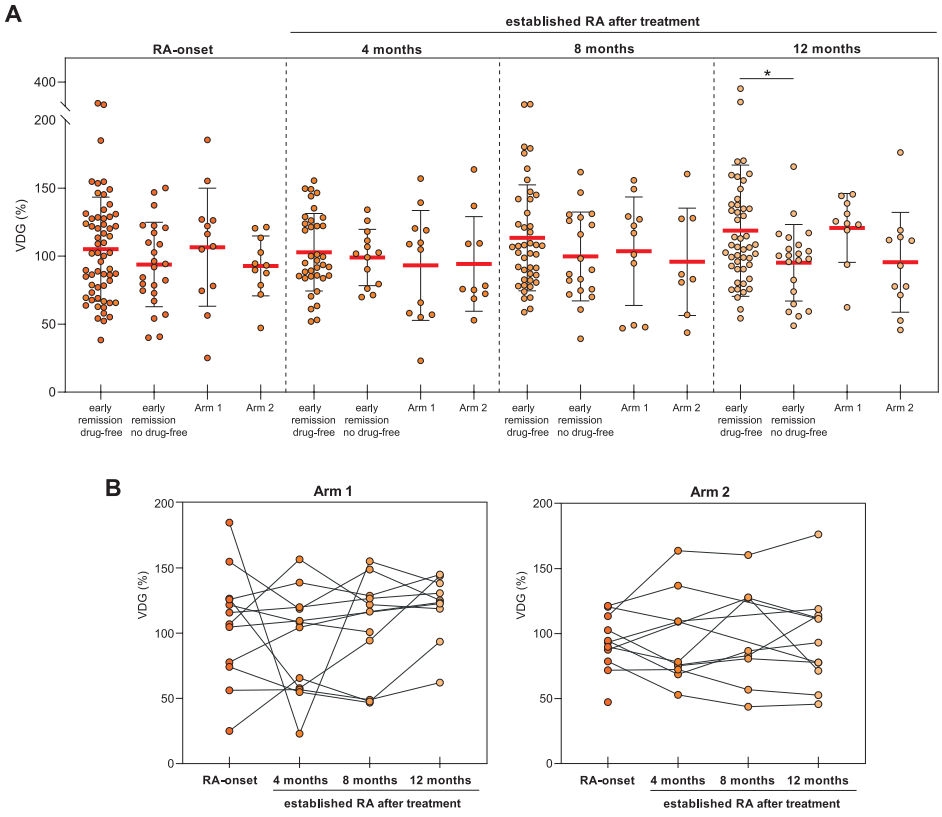

Figure S4. Longitudinal analysis of ACPA IgG VDG at RA-onset and in established RA for cohort 6 *(The Netherlands)* separated for the different treatment arms. (**A**) ACPA IgG VDG comparison of the four treatment groups: early remission, drug-free; early remission, no drug-free; arm 1 and arm 2. Treatment specifications are illustrated in figure 3A. Ordinary one-way ANOVA was performed including the Fisher's LSD test: *p = 0.015. (**B**) Longitudinal matched paired samples are shown for treatment arm 1 and 2.

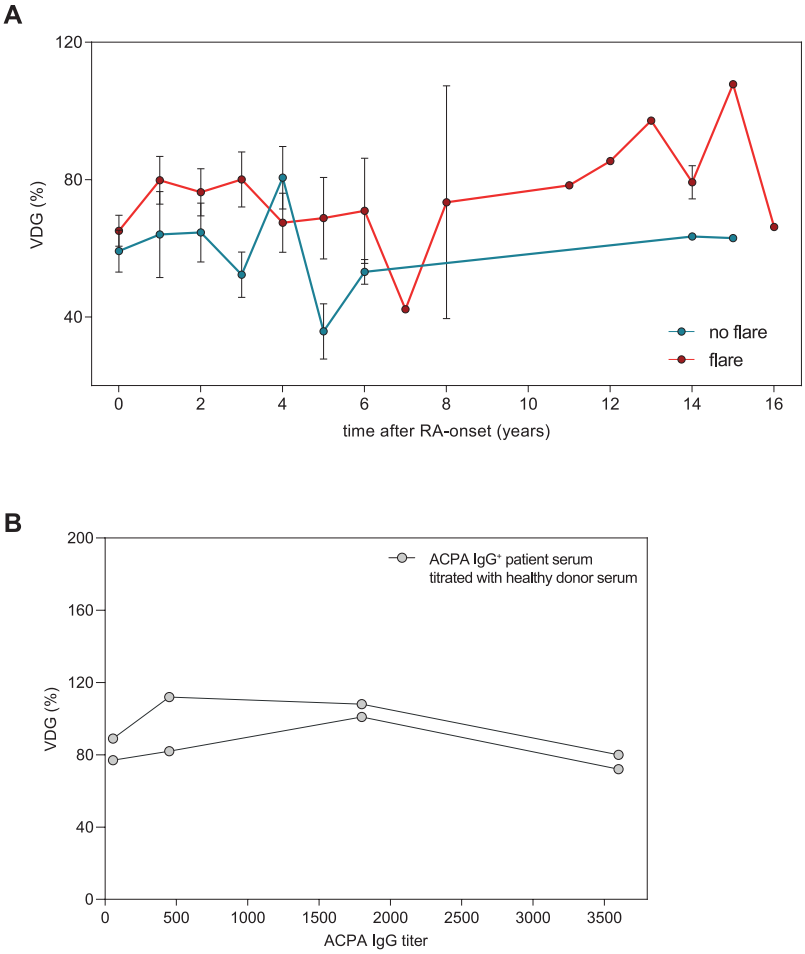

Figure S5. ACPA IgG VDG over various time-points and for different ACPA IgG titer. (**A**) "Time-line" of ACPA IgG VDG after RA-onset and towards drug-free remission (DFR) (*cohort 7, The Netherlands*). ACPA IgG VDG mean and SEM of the samples presented in figure 4D is depicted including patients who did and those who did not experience late flares. (**B**) Stable VDG of ACPA IgG positive patient serum measured with different titers. ACPA IgG titers were lowered by mixing the sera with ACPA-negative healthy donor sera. Two individual experiments are presented