Arthritis & Rheumatology
Vol. 74, No. 7, July 2022, pp 1147–1158
DOI 10.1002/art.42098

American College of Rheumatology
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IgG Anti–Citrullinated Protein Antibody Variable Domain Glycosylation Increases Before the Onset of Rheumatoid Arthritis and Stabilizes Thereafter: A Cross-Sectional Study Encompassing ~1,500 Samples

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Objective. The autoimmune response in rheumatoid arthritis (RA) is marked by the presence of anti–citrullinated protein antibodies (ACPAs). A notable feature of IgG ACPA 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, are poorly described. To understand its dynamics, we investigated the abundance of IgG ACPA VDG in 1,498 samples from individuals in different clinical stages.

Methods. Using liquid chromatography, we analyzed IgG ACPA VDG profiles in 7 different cohorts from Japan, Canada, The Netherlands, and Sweden. We assessed 106 healthy individuals, 228 individuals with presymptomatic RA, 277 individuals with arthralgia, 307 patients with new-onset/early RA, and 117 RA patients after prespecified treatment regimens. Additionally, we measured VDG in 234 samples from patients with RA who did or did not achieve long-term drug-free remission (DFR) during up to 16 years follow-up.

Results. IgG ACPA VDG significantly increased ($P < 0.0001$) toward disease onset and was associated with ACPA levels and epitope spreading prior to diagnosis. A slight increase in VDG was observed in patients with established RA, with a moderate influence of treatment ($P = 0.007$). In patients in whom DFR was later achieved, IgG ACPA VDG was already reduced at the time of RA onset.

Conclusion. The abundance of IgG ACPA VDG increases toward RA onset and correlates with maturation of the ACPA response. While IgG ACPA 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 in the pre-disease phase and contributes to disease development.

Supported by ReumaNederland (grants 17-1-402 and 08-1-34), the IMI funded project RTCure (777357), ZonMw TOP (grant 91214031), Target to B! (LSHM18055-5GF), the Swedish Research Council (VR 2017-00650), King Gustaf V's 80-Year Fund, King Gustaf V's and Queen Victoria's Fund, the Swedish Rheumatism Association, and the Canadian Institutes of Health Research MOP grant (77700). Dr. Scherer is recipient of an NWO-ZonMW clinical fellowship (90714509), an NWO-ZonMW VENI grant (91617107), an NWO-ZonMW VIDI grant (09150172010067), and a ZonMW Enabling Technology Hotels grant (435002030) and has received support from the Dutch Arthritis Foundation (15-2-402 and 18-1-205). Dr. Toes is recipient of a European Research Council (ERC) advanced grant (AdG2019-884796).

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Author disclosures are available at https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fart.42098&file=art42098-sup-0001-Disclosureform.pdf.

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Submitted for publication August 26, 2021; accepted in revised form February 15, 2022.

INTRODUCTION

Rheumatoid arthritis (RA) is a prevalent, slowly evolving autoimmune disease in which arthralgia is an important pre-disease manifestation. The autoimmune response that is the most specific for RA is characterized by the presence of anti–citrullinated protein antibodies (ACPAs), which can be present 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 toward 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 has revealed that IgG ACPAs are abundantly glycosylated in their antigen-binding fragments, expressing complex-type variable domain glycans that are mainly disialylated and bisected (4). Variable domain glycosylation (VDG) on >90% of the autoantibodies is a notable characteristic of IgG ACPA and distinguishes the molecules from conventional IgG antibodies, which display, next to the conserved presence of glycans in the Fc region, a considerably lower VDG of ~12–14% (4,5). Glycosylation sites required for the attachment of variable domain glycans are introduced by somatic hypermutation (6).

Although the role and dynamics of IgG ACPA Fc glycans have been studied extensively (7–10), little is known about the expression levels or potential biologic implications of variable domain glycans on ACPA. 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 IgG ACPA VDG can occur several years before RA onset. In a Canadian population, IgG ACPA VDG was predictive of disease development (11,12). However, how IgG ACPA VDG changes between clinical disease states 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 characteristics and action of variable domain glycans and thereby their possible contribution to autoreactive B cell responses in RA, we cross-sectionally investigated the presence and abundance of IgG ACPA VDG in 1,498 samples from an ethnically diverse group of individuals in various stages of disease (Table 1). By analyzing samples from a well-controlled treatment strategy trial (the Improved [Induction Therapy with Methotrexate and Prednisone in Rheumatoid or Very Early Arthritic Disease] study) that aimed to assess the most effective strategy for inducing remission in early RA (13), we investigated longitudinal changes in VDG in established RA after treatment escalation or treatment tapering. Finally, we longitudinally analyzed IgG ACPA VDG changes in patients from the Leiden Early Arthritis Clinic (EAC) in whom sustained (>1 year) DFR (SDFR) was achieved and those who experienced late disease flares, with an extensive follow-up of up to 16 years (14).

PATIENTS AND METHODS

Study cohorts. IgG ACPA VDG was analyzed in 1,498 serum samples from individuals in different clinical disease stages including 121 ACPA-negative RA control samples. Descriptive data on the cohorts are presented in Table 1. Additionally, 247 healthy donor and 150 ACPA-positive RA control samples, obtained at the Leiden University Medical Center outpatient rheumatology clinic, were assessed to verify the methodology used. Details on patient and public involvement and ethical considerations in the recruitment of individuals and of the study cohorts are available in Supplementary Methods, on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42098.

Cohort 1, healthy, symptom free (Nagasaki, Japan). Cohort 1 consisted of healthy symptom-free individuals (n = 58) enrolled in the Nagasaki Island Study (a community-based prospective cohort study based on resident health examinations) (15) who tested positive for ACPA. The individuals included in the study cohort had no joint symptoms at the time of the most recent resident health examination. These individuals were followed up for a period of up to 3 years. Nine of them (15.5%) developed RA during follow-up.

Cohort 2, healthy and RA onset (Manitoba, Canada). Members of cohort 2 were part of the longitudinal research project Early Identification of Rheumatoid Arthritis in First Nations, based at the Arthritis Centre, the University of Manitoba (16). Forty-eight samples from healthy individuals (first-degree relatives of patients with RA) were included, as were paired samples obtained from 25 individuals prior to RA onset and in the absence of joint- symptoms, and at the time of diagnosis of clinically apparent RA.

Cohort 3, presymptomatic and after RA onset/early RA (Umeå, Sweden). Cohort 3 comprised 354 samples from the Northern Sweden Health and Disease Study or the Västerbotten Intervention Project. Blood samples were collected and stored in a biorepository (the Northern Sweden Medical Research Biobank). Individuals were considered to have RA if they fulfilled the 1987 American College of Rheumatology classification criteria (17). Two hundred twenty-eight individuals from the cohort were retrospectively identified as having donated blood before the onset of signs or symptoms of joint disease (defined as presymptomatic), with a median time from blood sampling to onset of signs/symptoms of 4.7 years (interquartile range [IQR] 5.9 years). For 126 individuals (defined as having early RA), blood was obtained 0.5–1.5 years after RA diagnosis (18).

Table 1. Characteristics of the study cohorts*

Cohort (location), diagnosis	Female	Age, mean ± SD years	Arthritis/RA at follow-up	VDG positive	VDG, median (IQR) %	ACPA positive	ACPA level, median (IQR) AU/ml†	ACPA assay
Cohort 1 (Nagasaki, Japan), healthy, symptom free (n = 58)	38 (66)	67 ± 9.9	9 (15.5)	48 (83.8)	58.1 (35.6)	58 (100)	35.8	CLEIA (STACIA MEBLux CCP test; MBL) (15)
Cohort 2 (Manitoba, Canada)								
Healthy (n = 48)	32 (66.7)	37.6 ± 13.5	31 (64.6)	42 (33.3)	44.9 (69.3)	37 (77.1)	54 (135.5)	CCP-2 kit (Inova Diagnostics) (16)
RA onset (n = 25)	19 (76)	42 ± 14.7	–	21	99.9 (46.1)	22	200 (103.3)	CCP-2 kit (Inova Diagnostics) (16)
Cohort 3 (Umeå, Sweden)								
Presymptomatic (n = 228)	145 (64)	52.2 ± 9.4	228 (100)	105 (46.1)	97.4 (53.5)	168 (73.7)	126.7 (455.5)	Immunoscan RA anti–CCP-2 EIA (Euro Diagnostica) (18)
0.5–1.5 years after RA onset (n = 126)	78 (61)	59.7 ± 9.3	–	116 (92.1)	94.2 (50.8)	125 (98.4)	592.9 (725.3)	Immunoscan RA anti–CCP-2 EIA (Euro Diagnostica) (18)
Cohort 4 (Amsterdam, Reade, The Netherlands), arthralgia (n = 239)	185 (77)	48.3 ± 11.6	137 (57.3)	211 (87.9)	75.3 (49)	239 (100)	358 (1,351)	Anti-CCP ELISA (Axis-Shield) (19)
Cohort 5 (Leiden, The Netherlands; CSA Study)								
Arthralgia (n = 38)	29 (76.3)	48.3 ± 12.5	26 (68.4)	27 (71.1)	70.4 (28.8)	33 (86.8)	123 (324)	Anti–CCP-2 ELISA (Euro Diagnostica) (23)
RA onset (n = 26)	22 (84.6)	48.1 ± 12.5	–	18 (69.2)	59.1 (49.1)	21 (80.8)	25.5 (266.8)	Anti–CCP-2 ELISA (Euro Diagnostica) (23)
Cohort 6 (Leiden, The Netherlands; Improved study)								
RA onset (n = 130)	88 (67.7)	51.1 ± 12.5	–	117 (90)	96 (48.2)	130 (100)	903.3 (1,101.2)	In-house anti–CCP-2 ELISA (3,22)
4 months after RA onset (n = 117)	79 (67.5)	50.6 ± 12.8	–	78 (66.7)	95.9 (45.1)	117 (100)	449.9 (806.9)	In-house anti–CCP-2 ELISA (3,22)
8 months after RA onset (n = 112)	78 (69.6)	50.7 ± 12.4	–	86 (76.8)	101.7 (50.3)	112 (100)	602 (1,061.8)	In-house anti–CCP-2 ELISA (3,22)
12 months after RA onset (n = 117)	78 (66.7)	51.0 ± 12.4	–	98 (83.8)	105.2 (48.1)	117 (100)	651.7 (962.5)	In-house anti–CCP-2 ELISA (3,22)
Cohort 7 (Leiden, The Netherlands; EAC)								
RA onset, DFR not achieved (n = 59)	42 (71.2)	49.7 ± 14.5	–	59 (100)	83.8 (46)	59 (100)	7,340 (5,984)	In-house anti–CCP-2 ELISA (3,22)
RA onset, DFR achieved (n = 36)	24 (66.7)	50.8 ± 13.1	–	32 (89)	61.4 (35)	29 (80.6)	1,933 (7,296)	In-house anti–CCP-2 ELISA (3,22)
Pre-remission (n = 52)	37 (71.2)	54.2 ± 14.8	–	37 (71)	74.05 (30)	38 (73)	3,583 (5,302)	In-house anti–CCP-2 ELISA (3,22)
DFR (n = 41)	27 (65.9)	58.5 ± 13.6	–	30 (73.2)	67.7 (41.5)	33 (80)	3,010 (8,975)	In-house anti–CCP-2 ELISA (3,22)
SDFR (n = 35)	27 (77.1)	54.6 ± 15.1	–	22. (62.9)	73.5 (42)	29 (82.9)	2,626 (6,765)	In-house anti–CCP-2 ELISA (3,22)
DFR with late flares (n = 11)	7 (63.6)	69.4 ± 14.8	–	9 (81.8)	78.3 (26.9)	10 (91)	4,210 (10,709)	In-house anti–CCP-2 ELISA (3,22)

* Except where indicated otherwise, values are the number (%). RA = rheumatoid arthritis; VDG = variable domain glycosylation; IQR = interquartile range; CCP = cyclic citrullinated peptide; EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; CSA = Clinically Suspect Arthralgia; EAC = Early Arthritis Cohort; DFR = drug-free remission; SDFR = sustained DFR.

†Anti–citrullinated protein antibody (ACPA) levels were determined with various assays/standards and thus are not directly comparable with one another.

Cohort 4, arthralgia (Amsterdam, Reade, The Netherlands). Patients in cohort 4 were ACPA-positive individuals with arthralgia (n = 239) who were seen at rheumatology outpatient clinics in the Amsterdam area (19). These individuals were followed up for a period of up to 10 years, during which 137 (57.3%) developed arthritis.

Cohort 5, arthralgia and RA onset (Leiden, The Netherlands). Individuals at risk of RA development were recruited for the prospective Clinically Suspect Arthralgia (CSA) cohort at the Leiden University Medical Center outpatient rheumatology clinic and followed up longitudinally (20). Thirty-eight patients with arthralgia from the CSA were included as cohort 5 in the present study. Paired samples from 26 of these patients were studied, i.e., a sample obtained at the time of arthralgia pre-RA diagnosis and a sample obtained at the time of diagnosis of clinically apparent RA.

Cohort 6, RA onset and established RA after treatment (Leiden, The Netherlands). Cohort 6 consisted of patients with RA who were recruited at 12 hospitals in the western area of The Netherlands and were included in the Improved study. The aim of this multicenter randomized controlled trial was to assess the achievement of DFR with treatment alteration every 4 months. Initial treatment was methotrexate (MTX) and prednisone. Prednisone was tapered in patients whose RA was in early remission (defined as a Disease Activity Score [DAS] of <1.6) (21) at 4 months. If disease was still in remission at 8 months, MTX was also tapered. If the DAS was ≥1.6 after prednisone was stopped, it was restarted. Patients in whom early remission was not achieved were randomized to 1 of 2 treatment arms: MTX, prednisone, hydroxychloroquine, and sulfasalazine combination (arm 1) or MTX and adalimumab combination (arm 2) (13). Serum samples obtained at the time of RA onset (n = 130) and at 4 months (n = 117), 8 months (n = 112), and 12 months (n = 117) after diagnosis and prespecified treatment were assessed in the present study.

Cohort 7, RA onset, DFR, SDFR, and late disease flares (Leiden, The Netherlands). Members of cohort 7 were patients from the Leiden EAC (14). Samples obtained at the time of RA onset from individuals in whom DFR was not later achieved (n = 59) and individuals in whom DFR was later achieved (n = 36) were assessed. Patients in whom DFR was later achieved were followed up longitudinally for up to 16 years; samples obtained at RA onset (n = 36), during the pre-remission phase (n = 52), DFR (n = 41), SDFR (n = 35), and at the time of late disease flares (n = 11) were included. DFR was defined as the absence of clinical synovitis (swollen joints at physical examination) after discontinuation of disease-modifying antirheumatic drug (DMARD) treatment (including systemic/intraarticular glucocorticoids). In the 41 patients in whom DFR was achieved, DMARD treatment was stopped after a median of 2.9 years (IQR 1.0–4.9 years). SDFR was defined as the absence of clinical synovitis after cessation of DMARD treatment, that persisted for ≥1 year. SDFR was achieved in the 35 patients after a median of 2.8 years of DMARD treatment (IQR 2.0–5.2 years) and was maintained for a median of 7.1 years (IQR 4.5–11.2 years) after DMARD cessation, demonstrating the sustainability of DMARD-free remission. Flare was defined as the recurrence of clinical synovitis on joint examination. Among patients in whom SDFR was achieved, data in the medical records were reviewed through September 2021.

Laboratory analyses. IgG ACPA levels in serum samples were analyzed using standard and commercially available anti–cyclic citrullinated peptide (anti-CCP) assays or in-house anti-CCP2 enzyme-linked immunosorbent assays (ELISAs) as previously described (3,15–17,19,22,23). ACPA fine specificity of samples from cohort 6 was determined using in-house anti–citrullinated vimentin 59–74, anti–citrullinated fibrinogen β36–52 and α27–43, and anti–citrullinated enolase 5–20 ELISAs as previously described (3). ACPA fine specificity of samples from cohort 4 was determined using in-house IgG anti–citrullinated vimentin 60–75, anti–citrullinated fibrinogen β36–52, α60–74, and α36–50, and anti–citrullinated enolase 5–21 ELISAs.

IgG ACPA capture and VDG analysis using liquid chromatography. Capture of IgG ACPA, total glycan release, and glycan labeling and purification were performed as previously described (11). Briefly, ACPAs were affinity isolated from 25-μl serum samples using NeutrAvidin Plus resin (Thermo Scientific) coupled with 0.1 μg/μl CCP-2–biotin followed by IgG capture using FcXL affinity beads (Thermo Scientific). *N*-linked glycans were released using 0.5 units of PNGaseF (Roche), subsequently labeled with 2-anthranilic acid and 2-picoline borane, and hydrophobic interaction liquid chromatography–solid-phase extraction purified using GHP membrane filter plates (Pall Life Sciences). Ultra high-performance liquid chromatography was performed on a Dionex Ultimate 3000 instrument (ThermoFisher Scientific), an FLR fluorescence detector set, and an Acquity BEH Glycan column (Waters). Separation and glycan peak alignment were performed as previously described (11). HappyTools, version 0.0.2, was used for calibration and peak integration (24). The *N*-linked glycan abundance in each peak was expressed as the total integrated area under the curve. The cutoff was defined based on phosphate buffered saline control (blank) and blood samples from ACPA-negative healthy control subjects in the Leiden area, excluding outliers (below 1.5× the 25th percentile or above 1.5× the 75th percentile). The percentage IgG ACPA VDG was calculated based on the formula (sum of the most abundant variable domain glycans/sum of the most abundant Fc glycans) × 100, or ([G2FBS1 + G2FS2 + G2FBS2]/[G0F + G1F + G2F]) × 100, where G0/G1/G2 represents A-/mono-/di-galactosylated, F represents core fucosylated, B represents bisecting *N*-acetylglucosamine (GlcNAc), and S1/S2 represents mono-/disialylated (12). The glycan traits were selected based on previous observations showing their exclusive presence on either the variable domain or the Fc domain of IgG ACPA molecules (12,25). 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 analysis. Continuous data were analyzed using nonparametric methods (Kruskal-Wallis test for unpaired groups and Mann-Whitney U test for 2 unpaired groups) and parametric tests (mixed-effects analysis for matched paired samples accounting for missing values) when appropriate. The mixed-effects analysis model using restricted maximum likelihood is comparable to repeated-measures analysis of variance with regard to *P* values and multiple comparisons tests, but can accommodate missing values. Correlations between IgG ACPA levels (log transformed) and percentages of VDG were assessed with Pearson's correlation coefficient. *P* values (all 2-sided) less than 0.05 were considered significant. Logistic and ordinal regression analyses were performed in cohort 4 and cohort 6 to investigate the association of IgG ACPA VDG/IgG ACPA levels with epitope spreading, remission, and early DFR. The unstandardized coefficient (B) represents the mean change in the response given a 1-unit change in the predictor. The longitudinal and repeated-measures data from cohort 6 were analyzed by generalized estimating equation (GEE) analysis, as previously described (3). GEE analysis was used to assess VDG changes over time and associations with treatment. The specific covariates and dependent variables are listed in the supplementary tables (https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Statistical calculations were performed using Stata, version 16.1.

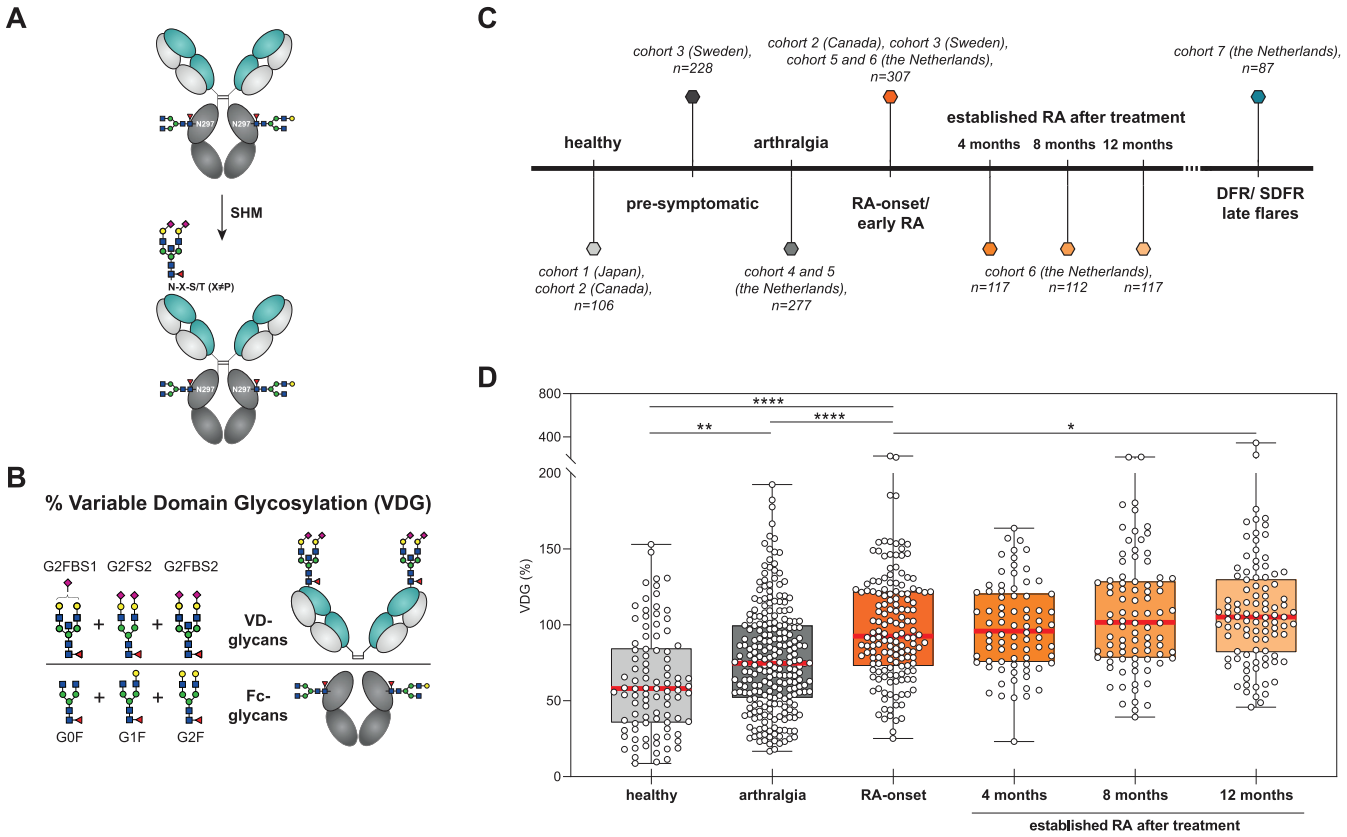

Figure 1. Percentage variable domain glycosylation in IgG anti–citrullinated protein antibodies (ACPAs) from healthy individuals, patients with arthralgia, and patients with rheumatoid arthritis (RA) in different disease stages**. A,** Depiction of the process by which IgG carries *N*-glycans at each 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 IgG ACPA VDG, i.e., (sum of the most abundant variable domain glycans/sum of the most abundant Fc glycans) × 100, or ([G2FBS1 + G2FS2 + G2FBS2]/[G0F + G1F + G2F]) × 100, where G0/G1/G2 represents A-/mono-/di-galactosylated, F represents core fucosylated, B represents bisecting *N*-acetylglucosamine (GlcNAc), and S1/S2 represents mono-/di-sialylated. The selected glycan traits are exclusively present on either the variable domain or the Fc domain of IgG ACPA. GlcNAc is shown as blue squares, mannose as green circles, galactose as yellow circles, fucose as red triangles, and *N*-acetylneuraminic acid as pink diamonds. **C,** "Timeline" of clinical disease stages, the corresponding analyzed cohorts, and numbers of samples analyzed. **D,** Percentage IgG ACPA VDG, measured by liquid chromatography, in healthy individuals, patients with arthralgia, patients at the time of RA onset, and patients with established RA 4 months, 8 months, and 12 months after institution of prespecified treatment. Data are presented as box and whisker plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers represent the minimum to maximum values. Circles represent individual samples. The cross-sectional data sets from cohorts 1, 2, 4, and 5 were analyzed by Kruskal-Wallis test with Dunn's post hoc test. The longitudinally obtained data from cohort 6 were analyzed by generalized estimating equation. * = $P = 0.037$; ** = $P = 0.0032$; **** = $P < 0.0001$. DFR = drug-free remission; SDRF = sustained drug-free remission.

RESULTS

IgG ACPA variable domain glycosylation increases toward disease onset and remains stable in established RA. To provide a comprehensive overview of the presence and abundance of IgG ACPA VDG (Figures 1A and B), we analyzed 1,377 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 variable domain glycans were already present in high percentages (median 56.2%) on IgG ACPA from healthy individuals without symptoms (n = 106) (Figure 1D). Cross-sectional analysis revealed a significant increase in VDG (median 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 in samples obtained at the time of RA onset (n = 181) (median VDG 92.6% in the combined data sets) (Figure 1D and Table 1). This was, however, not apparent in all individual sample sets, as changes in VDG between the arthralgia phase and RA onset could not be observed in the statistically underpowered longitudinal data set from cohort 5, presumably because the individuals with clinically suspected arthralgia were tested only shortly before the onset of arthralgia (Supplementary Figure 1, on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42098), and an increase in VDG could have occurred

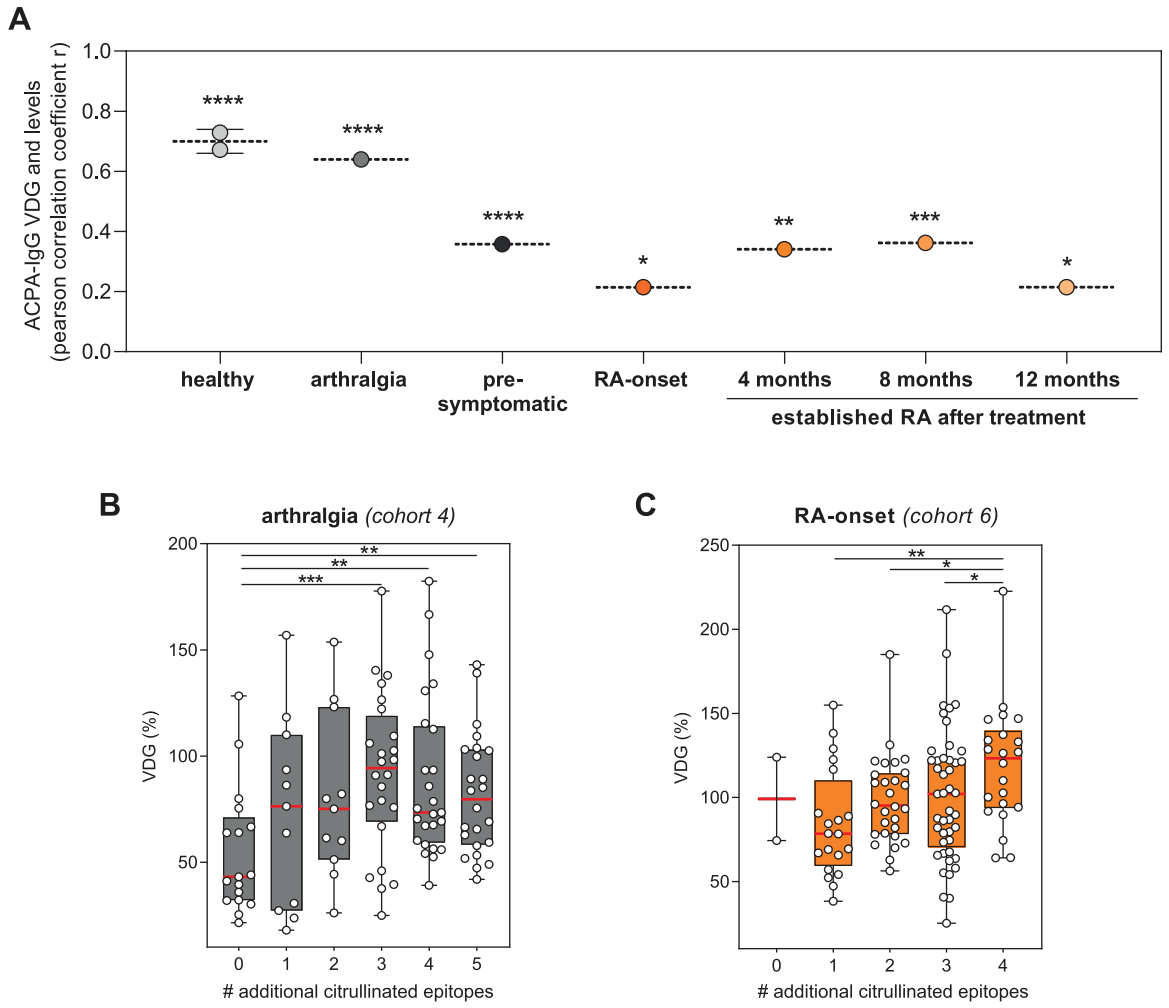

Figure 2. Correlation of IgG ACPA VDG with IgG ACPA levels and epitope spreading (maturation of the ACPA response). **A,** Pearson's correlation coefficients for the correlation between IgG ACPA VDG and IgG ACPA levels across different disease stages. * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$; **** = $P < 0.0001$. For healthy individuals, values from 2 different cohorts are shown. **B,** VDG percentage on IgG ACPA from individuals with arthralgia, isolated using cyclic citrullinated peptide 2 and tested for binding to 5 additional citrullinated antigens (citrullinated vimentin 60–75, citrullinated fibrinogen β36–52, α60–74, and α36–50, and citrullinated enolase 5–21). ** = $P < 0.01$; *** = $P = 0.0006$. **C,** VDG percentage in IgG ACPA from patients at the time of RA onset, isolated using cyclic citrullinated peptide 2 and tested for recognition of 4 additional citrullinated antigens (citrullinated vimentin 59–74, citrullinated fibrinogen β36–52 and α27–43, and citrullinated enolase 5–20). * = $P < 0.05$; ** = $P = 0.001$. In **B** and **C**, data are presented as box and whisker plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers represent the minimum to maximum values. Circles represent individual samples. See Figure 1 for definitions.

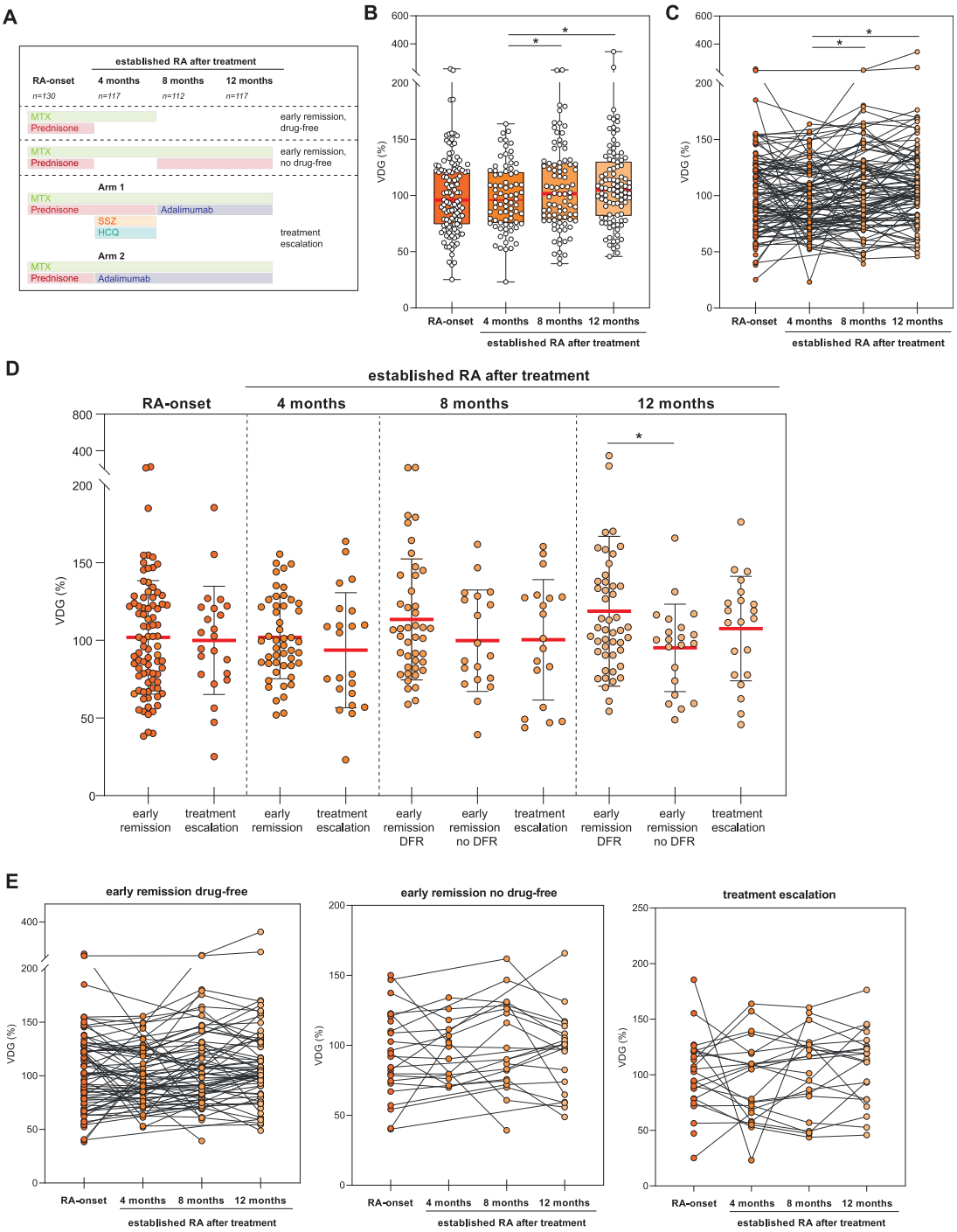

Figure 3. Longitudinal analysis of IgG ACPA VDG at the time of RA onset and in established RA after treatment (cohort 6). **A,** Treatment protocol. **B,** Longitudinal data on VDG percentage on IgG ACPA by time since RA onset. Data are presented as box and whisker plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers represent the minimum to maximum values. Circles represent individual samples. Data were analyzed by mixed-effects analysis using restricted maximum likelihood and Tukey's test. **C,** Longitudinal data by time since RA onset, represented as matched pairs. **D,** VDG percentage in IgG ACPA by treatment group (early remission [drug-free], early remission [no drug-free], and treatment escalation) at each assessed time point since RA onset. Data were analyzed by one-way analysis of variance with Fisher's least significant difference test. Horizontal and vertical bars show the mean ± SD. Circles represent individual samples. **E,** Longitudinal data by time since RA onset within each treatment group, represented as matched pairs. * = $P < 0.05$. MTX = methotrexate; SSZ = sulfasalazine; HCQ = hydroxychloroquine (see Figure 1 for other definitions).

earlier. Patients presenting with arthralgia, regardless of whether they did or did not subsequently develop RA, displayed lower VDG than patients tested at the time of RA onset (Supplementary Figure 2E, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). In samples from patients with established RA after prespecified treatment (n = 346), IgG ACPA 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 IgG ACPA VDG toward the time of RA onset was also observed in a Swedish population of ACPA-positive individuals who later developed RA. The extended data set used here also exhibited a rise in VDG when analyzed per individual in a longitudinal manner (26) (Supplementary Figure 2D); however, there was no significant difference on cross-sectional analysis (Supplementary Figure 2C).

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

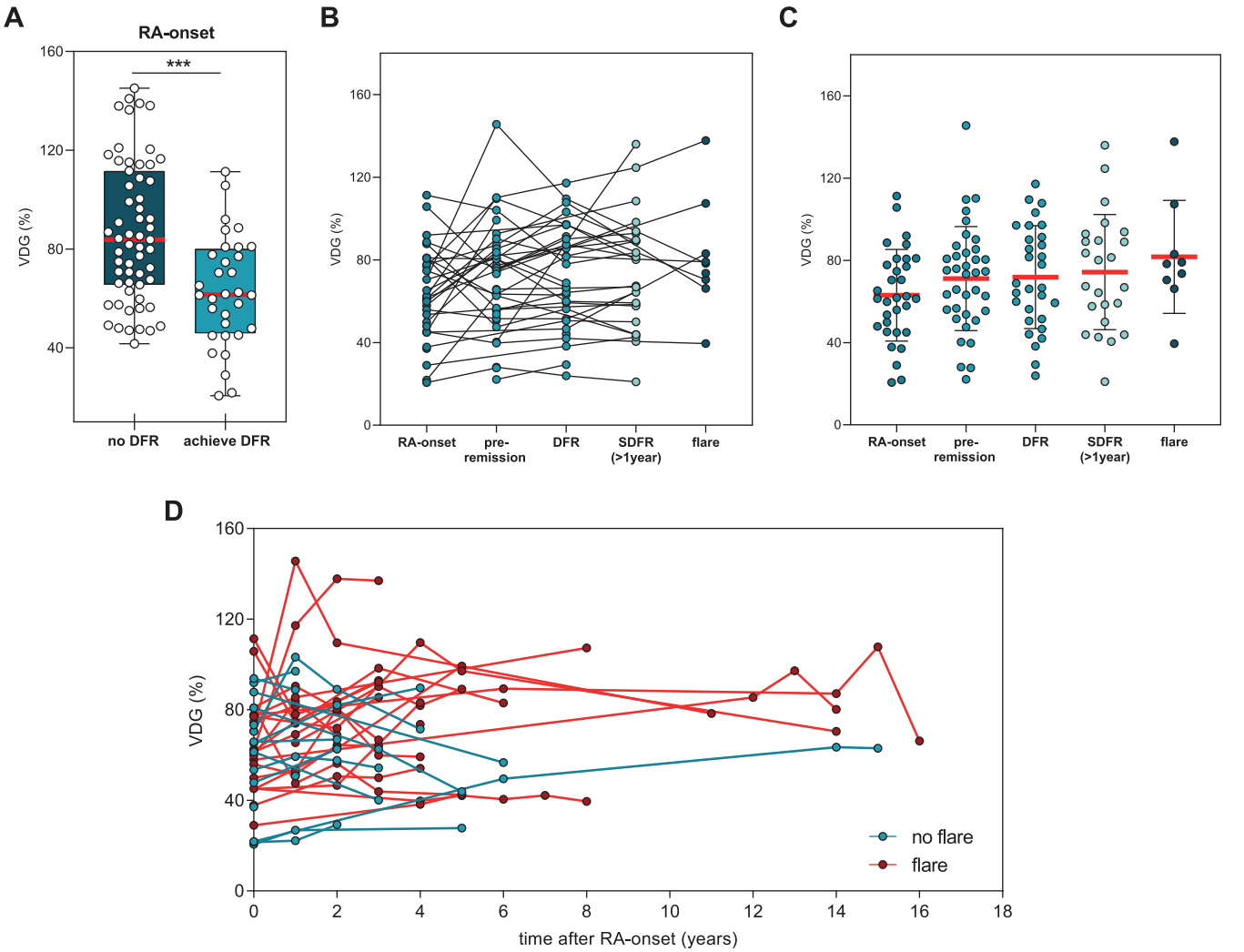

Figure 4. Cross-sectional and longitudinal analysis of IgG ACPA VDG at the time of RA onset and during DFR (cohort 7). **A,** VDG percentage on IgG ACPA at the time of RA onset in individuals in whom DFR was not achieved and those in whom DFR was achieved. DFR was defined as the absence of clinical synovitis after discontinuation of disease-modifying antirheumatic drug treatment. Data are presented as box and whisker plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the whiskers represent the minimum to maximum values. *** = $P < 0.0001$ by Mann-Whitney U test. **B,** Data on VDG percentage on IgG ACPA in matched paired samples from patients at the time of RA onset, pre-remission, during DFR, during SDFR, and during late disease flares. Flare was defined as the recurrence of clinical synovitis on joint examination. **C,** IgG ACPA VDG data by group (RA onset, pre-remission, DFR, SDFR, flare). Horizontal and vertical bars show the mean ± SD. Circles represent individual samples. **D,** IgG ACPA VDG data by assessment time point in longitudinally assessed samples from patients who did and those who did not experience late flares. Circles represent individual samples. See Figure 1 for definitions.

Interconnection between the increase in variable domain glycosylation and maturation of the ACPA immune response. To obtain further insights into IgG ACPA VDG, we investigated the possible association between VDG percentages and the "maturation" of the ACPA response by analyzing IgG ACPA levels and the broadness of the citrullinated epitope recognition profile. Pearson's correlation analysis revealed a strong, highly significant correlation between VDG percentages and IgG ACPA levels among healthy individuals (r = 0.672 and r = 0.728 in cohorts 1 and 2, respectively) and among individuals with arthralgia (r = 0.640) (Figure 2A and Supplementary Figure 3, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). At RA onset and in established RA after prespecified treatment, however, we observed only moderate correlations (r = 0.214, 0.341, 0.362, and 0.215 at RA onset and after 4, 8, and 12 months of treatment, respectively) (Figure 2A and Supplementary Figure 2E, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Likewise, our data revealed that IgG ACPA with increased VDG showed a significantly broader recognition profile toward multiple citrullinated epitopes (Figures 2B and C). Ordinal regression analyses confirmed these findings in individuals with arthralgia ($P < 0.001$) (Supplementary Table 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42098) as well as in patients at the time of RA onset ($P = 0.004$) and over time after treatment ($P < 0.001$) (Supplementary Table 2, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Thus, IgG ACPA VDG is associated with IgG ACPA levels and the breadth of the epitope recognition profile, suggesting that these two features of the ACPA response are interconnected.

Impact of immunosuppression on IgG ACPA variable domain glycosylation. We took advantage of the design of the Improved study (Figure 3A) to investigate whether IgG ACPA VDG predicts early remission in RA or is associated with the intensity of immunosuppression. First, we used the longitudinal data set to identify changes in IgG ACPA VDG over time by analyzing paired samples from patients at RA onset (n = 130) versus at 4 months (n = 117), 8 months (n = 112), and 12 months (n = 117) after disease development. Variable domain glycans appeared to be steadily and abundantly expressed on IgG ACPA after the onset of RA, although minor changes in expression levels were observed over time.

A slight but nonsignificant decrease was observed 4 months after disease onset and initiation of treatment with MTX and prednisone (Figures 3B and C and Supplementary Table 3, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Previous studies have shown a similar decline of IgG ACPA levels after initiation of treatment (3), providing further evidence of a correlation between VDG and IgG ACPA levels. 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 1 of 2 treatment escalation arms, i.e., combination treatment with MTX, prednisone, hydroxychloroquine, and sulfasalazine or combination treatment with MTX and adalimumab (13) (Figure 3A). 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 (arms 1 and 2) continued MTX treatment, in combination with adalimumab. Overall, irrespective of the treatment arm, VDG had increased moderately but significantly at 12 months after RA onset ($P = 0.037$) (Supplementary Table 3).

When the different treatment groups were compared, marginal but statistically significant effects of immunosuppression on IgG ACPA VDG were observed, with a reduction in VDG 12 months after RA onset (Figures 3D and E and Supplementary Figure 4A, https://onlinelibrary.wiley.com/doi/10.1002/art.42098), though not at 4 months or 8 months. This moderate but significant negative effect of immunosuppression on VDG was confirmed by GEE analysis of changes over time (8 months versus 12 months) (regression coefficient [B] 12.27 [95% confidence interval –7.32, 31.87] in the early remission, drug-free group versus 6.42 [95% confidence interval –0.35, 13.10] in the early remission, no drug-free and treatment escalation group; $P = 0.007$) (Supplementary Table 4, https://onlinelibrary.wiley.com/doi/10.1002/art.42098) and was similar to previously reported findings with regard to IgG ACPA levels (3). Last, we investigated whether VDG percentage at RA onset predicts remission after 4 months and drug-free remission within the first year. Similar to IgG ACPA levels (3), VDG percentages did not predict early drug-free remission (Supplementary Table 5, https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Collectively, these results show that IgG ACPA variable domain glycans 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 analyses of IgG ACPA VDG in individuals in whom long-term DFR was achieved or who experienced DFR with late flares. We 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 41 individuals in whom DFR had been achieved and 35 patients in whom SDFR (>1 year) had been achieved. Longitudinal samples obtained from the same patient at RA onset (n = 36), during active disease (pre-remission) (n = 52), during DFR (n = 41), during SDFR (n = 35), and when experiencing late disease flares (n = 11) were assessed. Again, the data showed that variable domain glycans are stably expressed in established RA. Intriguingly, however, patients in whom DFR was achieved during follow-up (n = 36) showed significantly reduced IgG ACPA VDG at the onset of disease compared to age- and sex-matched patients with persistently high disease activity (DAS >3) (n = 59) (median VDG at disease onset 61.4%

versus 83.8%) (Figure 4A and Table 1). In contrast, no statistically significant changes were observed when the IgG ACPA VDG percentages were determined over time in the DFR group or any of the other groups analyzed (Figures 4B–D). Thus, these longitudinal data confirm that IgG ACPAs express a constant amount of variable domain glycans after RA onset. The cross-sectional data also indicate that among individuals in whom long-term DFR is achieved, fewer glycans are present on IgG ACPA variable domains at the time of RA onset.

DISCUSSION

A key important 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 assessed IgG ACPAs in ~1,500 samples from 852 individuals in different clinical disease stages. Moreover, we analyzed the effect of therapy on the degree of VDG on ACPAs. The large sample size increased the power of our study, and we demonstrated that IgG ACPA 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 IgG ACPA 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 around the time of disease onset (92.6%). Thus, our data strongly indicate that an increase in IgG ACPA VDG occurs in the asymptomatic phase, 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 constant high expression of glycans on the variable domain of IgG ACPA, with a slight, but significant, increase after 12 months (105.2%). This is consistent with our previous observations, estimating >90% VDG on IgG ACPA 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 (27). Our longitudinal data from cohort 6 depict increased VDG levels in individuals in whom treatment was tapered, while patients who received more intensive treatment showed reduced IgG ACPA VDG profiles over time ($P = 0.007$). This significant impact of immunosuppression was also observed for IgG ACPA levels (22), confirming the correlation between IgG ACPA levels and VDG, which was strongest in the pre-disease phase. These findings are also in accordance with the notion that variable domain glycans 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 predisposing 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 variable domain glycans, as compared to HLA–DR4–negative individuals with similar ACPA variable domain glycan levels (26).

Of note, in the longitudinal analysis we observed that individuals in whom long-term DFR is achieved exhibit lower VDG profiles at disease onset (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 ACPAs.

Importantly, reduced IgG ACPA levels are not the cause of lower VDG, which was controlled by titrating IgG ACPA into healthy serum samples to enable maintenance of a high degree of VDG (Supplementary Figure 5B, on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42098). Thus it is tempting to speculate that variable domain glycans 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 ACPA B cell receptor sequences upon somatic hypermutation (27) and that variable domain glycan levels are significantly elevated in ACPA-positive individuals who subsequently develop RA (12), 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 also have a large steric requirement, might modulate binding to autoantigens or affect B cell receptor signaling of citrullinated antigen–directed B cells. Further, we cannot rule out a possible role of variable domain glycans in effector mechanism, and thereby, autoantibody-mediated inflammation, similar to findings for Fc glycans. In addition to these areas for further research, it would be interesting to investigate changes in specific variable domain glycan 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 variable domain glycans on IgG ACPA, show a decrease in the bisecting GlcNAc after COVID-19 (28,29). Interestingly, variable domain glycans are not only a feature of IgG ACPA in RA, but have also been described in other human autoimmune responses, such as in antineutrophil cytoplasmic antibody–associated vasculitis and Sjögren's syndrome, and have been observed on anti-hinge and antidrug antibodies (30–32).

A limitation of our study is that VDG profiles could be detected in only 70% of the samples analyzed, mainly due to a limited amount of serum available for the IgG ACPA capturing

and subsequent glycan analysis or to low IgG ACPA 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, ACPAs were captured using the highly sensitive and specific antigen CCP-2. However, it cannot be excluded that certain ACPA molecules that recognize different citrullinated epitopes and do not interact with CCP-2 were omitted from the analysis. Importantly though, we did not observe an effect of VDGs on binding affinity to CCP-2 (data not shown), making selection bias toward higher or lower glycosylated ACPAs unlikely. Another limitation of the 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 IgG ACPA VDG toward the time of RA onset in the longitudinal data set from cohort 3, including paired samples obtained from individuals when they were presymptomatic and after RA onset, over a period of 15 years, as also previously described (26). Furthermore, our findings of IgG ACPA variable domain glycan levels were concordant 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 variable domain glycans on IgG ACPA over various clinical disease stages in RA. Although the biologic implications of variable domain glycans attached to antibodies in general and to ACPAs specifically are still largely unexplored, our data show that they are a key characteristic of ACPAs across disease stages in individuals of different ethnicities who develop RA. Our results demonstrate an increase in VDG toward the time of disease onset and, taken together with previous data indicating a selective introduction of these *N*-linked glycan sites, suggest that variable domain glycans may serve as a trigger for the maturation of the ACPA immune response. It will therefore be useful to understand the biologic impact of variable domain glycans on the ACPA immune response and its detailed clinical implications.

ACKNOWLEDGMENTS

The authors would like to thank personnel of the Department of Biobank Research (Umeå University), the Västerbotten Intervention Programme (Västerbotten, Sweden), the Northern Sweden MONICA study, and the County Council of Västerbotten for providing data and samples. We are grateful to Dr. Jan Wouter Drijfhout (Leiden University Medical Center) for providing the CCP-2 peptide, Carolien Koeleman (Leiden University Medical Center) for expert assistance with liquid chromatography, and Ellis Niemantsverdriet and Marloes Verstappen (Leiden University Medical Center) for assistance with sample and data collection.

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. Ms. Kissel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kissel, Hafkenscheid, Tamai, Kawashiri, Kawakami, El-Gabalawy, van Schaardenburg, Rantapää-Dahlqvist, Wuhrer, van der Helm-van Mil, Allaart, van der Woude, Scherer, Toes, Huizinga.

Acquisition of data. Kissel, Hafkenscheid.

Analysis and interpretation of data. Kissel, Wesemael, Wuhrer, van der Helm-van Mil, van der Woude, Scherer, Toes, Huizinga.

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