Arthritis & Rheumatology
Vol. 71, No. 10, October 2019, pp 1626–1633
DOI 10.1002/art.40920

American College of Rheumatology
Empowering Rheumatology Professionals

N-Linked Glycans in the Variable Domain of IgG Anti–Citrullinated Protein Antibodies Predict the Development of Rheumatoid Arthritis

Lise Hafkenscheid,[1] Emma de Moel,[1] Irene Smolik,[2] Stacey Tanner,[2] Xiaobo Meng,[2] Bas C. Jansen,[3] Albert Bondt,[1] Manfred Wuhrer,[1] Tom W. J. Huizinga,[1] Rene E. M. Toes,[1] Hani El-Gabalawy,[2] and Hans U. Scherer[1]

Objective. Anti–citrullinated protein antibodies (ACPAs) are disease-specific biomarkers in rheumatoid arthritis (RA). More than 90% of IgG ACPAs harbor *N*-linked glycans in the antibody variable (V) domain. The corresponding *N*-glycosylation sites in ACPA V-region sequences result from somatic hypermutation, a T cell–dependent process. As ample evidence indicates that T cells drive the maturation of the ACPA response prior to arthritis onset, we undertook this study to investigate whether the presence of glycans in IgG ACPA V domains predicts the transition from predisease autoimmunity to overt RA.

Methods. We analyzed 2 independent sets of serum samples obtained from 126 ACPA-positive first-degree relatives (FDRs) of RA patients. Both sets originated from an Indigenous North American population and comprised cross-sectional and longitudinal samples of individuals who did or did not develop inflammatory arthritis. Serum IgG ACPAs were affinity-purified and subjected to ultra high-performance liquid chromatography–based glycan analysis.

Results. In both data sets, FDR-derived IgG ACPA displayed markedly lower levels of V domain glycans (<50%) compared to IgG ACPA from RA patients. Notably, FDRs who later developed RA showed extensive V-domain glycosylation before the onset of arthritis. Moreover, IgG ACPA V-domain glycosylation was strongly associated with future development of RA (hazard ratio 6.07 [95% confidence interval 1.46–25.2]; $P = 0.013$).

Conclusion. Extensive glycosylation of the IgG ACPA V domain is present in a subset of predisposed FDRs of Indigenous North American RA patients. The presence of this feature substantially increases the risk of RA development. Based on these findings, we propose that glycosylation of the IgG ACPA V domain represents a predictive marker for RA development in ACPA-positive individuals and may serve to better target prevention measures.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease. Approximately 1% of the global population is affected, but higher prevalence rates have been observed in certain defined populations, such as Indigenous North Americans (1,2). Indigenous North Americans develop RA at a younger age, experience higher disease burden, have a remarkably high prevalence of the major genetic risk factor for RA (HLA class II shared epitope [SE] alleles) (3), and develop RA that is primarily seropositive for RA-associated autoantibodies, particularly anti–citrullinated protein antibodies (ACPAs) (4).

It is now well established that ACPAs can be present for many years without evidence of clinical symptoms of RA (5).

Supported by the Canadian Institutes of Health Research (grant MOP 77700), the Netherlands Organization for Scientific Research (NWO) (projects 435000033 and 91214031), and the Innovative Medicines Initiative funded projects BeTheCure (contract 1151422) and RTCure (contract 777357). Ms Hafkenscheid's work was supported by the Dutch Arthritis Foundation (grant NR 12-2-403). Drs. Bondt and Wuhrer's work was supported by funding from the European Union's Seventh Framework Programme (contract FP7-Health-F5-2011; project HighGlycan; grant 278535). Dr. Scherer's work was supported by the NWO and the Netherlands Organization for Health Research and Development (ZonMW) (NWO-ZonMW VENI grant 91617107, ZonMW Enabling Technology Hotels grant 435002030, and an ZonMW Clinical Fellowship project 90714509) and the Dutch Arthritis Foundation (project 15-2-402).

[1]Lise Hafkenscheid, MSc, Emma de Moel, MD, Albert Bondt, PhD, Manfred Wuhrer, PhD, Tom W. J. Huizinga, MD, PhD, Rene E. M. Toes, PhD, Hans U. Scherer, MD, PhD: Leiden University Medical Center, Leiden, The Netherlands; [2]Irene Smolik, PhD, Stacey Tanner, MD, Xiaobo Meng, PhD, Hani El-Gabalawy, MD: University of Manitoba, Winnipeg, Manitoba, Canada; [3]Bas C. Jansen, PhD: Ludger Ltd., Oxfordshire, UK.

Drs. El-Gabalawy and Scherer contributed equally to this work.

Drs. Huizinga, Toes, and Scherer have submitted a provisional patent application related to research obtained in this study. No other disclosures relevant to this article were reported.

Address correspondence to Hans U. Scherer, MD, PhD, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: h.u.scherer@lumc.nl.

Submitted for publication November 29, 2018; accepted in revised form April 30, 2019.

Notably, ACPA levels, isotype usage, and the citrullinated antigen recognition profile broaden relatively close to the onset of arthritis (6). Thus, it has been postulated that the development of ACPA-positive disease is a multistep process (7–9), in which tolerance to citrullinated antigens is initially broken, followed by a putative "second hit" that leads to the expansion of the ACPA response and, ultimately, to development of clinically detectable disease. Hence, it has become of considerable interest to understand the drivers of this predisease expansion of the ACPA response and to identify markers that predict the transition from asymptomatic autoimmunity to ACPA-positive inflammatory arthritis (IA). Interestingly, recent immunogenetic evidence indicates that HLA SE alleles might contribute to the expansion of the ACPA response by facilitating the provision of T helper cell activity to ACPA-expressing B cells. This is based on the observation that HLA SE alleles are risk factors for ACPA-positive disease but do not predispose to the development of ACPA positivity in healthy individuals (10,11). Thus, it can be hypothesized that in individuals destined to develop RA, ACPA-expressing B cells receive predisease T cell "help" that initiates and drives B cell maturation, including isotype switching and somatic hypermutation (SHM) (6).

Recently, we found that almost all IgG ACPA molecules carry *N*-glycans in their B cell receptor variable (V) domains (12,13). Notably, all of the consensus *N*-glycosylation sequences (Asn-X [≠Pro]–Ser/Thr) found in these V regions were generated upon SHM and not encoded in the germline genetic repertoire (14). Moreover, we found evidence that the generation of such *N*-glycosylation sites offers an advantage to ACPA-expressing B cells that helps with their escape from selection checkpoints, as these cells acquire extensive somatic mutations despite a lack of avidity maturation. Together, these observations are consistent with the notion that T cells have a pivotal role in the selection and expansion of ACPA-expressing B cells, possibly by facilitating the introduction of *N*-glycosylation sites in IgG ACPA V domains.

Based on these considerations, we hypothesized that the detection of IgG ACPA V-domain glycosylation in ACPA-positive individuals is indicative of maturation of this autoimmune response and could potentially serve as a predictor for the development of ACPA-positive RA. To address this hypothesis, we analyzed IgG ACPA V-domain glycosylation in a longitudinal manner in unaffected ACPA-positive first-degree relatives (FDRs) of RA patients in an Indigenous North American population predisposed to the disease. We determined that there is a heterogeneity of IgG ACPA V-domain glycosylation levels in unaffected FDRs and that high levels, comparable to those seen in most RA patients, serve to substantially increase the risk of future RA development.

PATIENTS AND METHODS

Study population. The current study is nested within a longitudinal research project initiated in 2005 entitled "Early Identification of Rheumatoid Arthritis in First Nations," that was based at the Arthritis Centre at the University of Manitoba. The study and its consenting process were fully approved by the University of Manitoba Research Ethics Board and the Band Councils of the study's participating communities, the latter based on specific study agreements with the communities (15). Sera were sent in 2 sets, in a blinded manner, to Leiden University Medical Center. The first set was collected from a cross-sectional cohort consisting of 10 RA patients and 84 ACPA-positive FDRs. A second set of samples represented a longitudinal cohort of FDRs who had serial visits. As a quality control measure, there was intentional overlap, with 11 FDRs being in both sets (Table 1 and Supplementary Figure 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract). Additional information on study design and IA case definition are available in the Supplementary Methods.

Table 1. Patient characteristics*

	Age, mean ± SD years	Female sex, no. (%)	CCP-3, mean ± SD titer	Follow-up duration, mean ± SD months
Cross-sectional cohort				
RA patients (n = 10)†	50 ± 10	9 (90)	148 ± 71	–
FDRs (n = 84)	38 ± 14	55 (65)	66 ± 68	–
FDRs included based on IgG ACPA QC (n = 15)	36 ± 12	11 (73)	131 ± 124	–
Longitudinal cohort				
Transitioned FDRs (n = 13)‡	34 ± 14	9 (69)	122 ± 120	50 ± 28
Nontransitioned FDRs (n = 19)§	39 ± 15	12 (63)	49 ± 85	64 ± 24
Nontransitioned FDRs included based on IgG ACPA QC (n = 15)	40 ± 18	11 (73)	62 ± 90	45 ± 29

* For relationships between donors, quality control, and number of samples, see Supplementary Figure 1 and Supplementary Methods (http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract). CCP-3 = cyclic citrullinated peptide 3.
† Median disease duration of rheumatoid arthritis (RA) patients was 6.8 years (interquartile range 2.9–18.7). After quality control (QC) measures, samples from all 10 RA patients were included for analysis of IgG anti–citrullinated protein antibodies (ACPAs).
‡ After QC measures, samples from all 13 transitioned first-degree relatives (FDRs) were included for analysis of IgG and analysis of IgG ACPA. The mean ± SD follow-up duration for samples included for analysis of IgG ACPA was 46 ± 24 months.
§ After QC measures, samples from all 19 nontransitioned FDRs were included for analysis of IgG.

Isolation of IgG ACPA and of total IgG depleted of ACPA. IgG ACPA and IgG were captured as previously described (16). Briefly, ACPA were purified from 25 µl serum by antigen affinity chromatography using NeutrAvidin Plus UltraLink beads (Thermo Scientific) coated with cyclic citrullinated peptide 2 (CCP-2). Incubation was performed by shaking the plate for 2 hours at room temperature. The flowthrough was collected by centrifugation. Beads were washed with phosphate buffered saline, and ACPAs were eluted with formic acid and immediately neutralized (16). IgG and IgG ACPAs were subsequently isolated in a similar manner using 20 µl Protein G Sepharose (GE Healthcare Life Sciences). Two hundred microliters of ACPA elution or 15 µl of the flowthrough was loaded onto protein G beads and incubated by shaking at room temperature. Elution was performed with formic acid and centrifugation at 50*g* for 1 minute.

Glycan analysis. Glycans were isolated and analyzed as previously described (12). Briefly, IgG and IgG ACPA eluates were dried by vacuum centrifugation. Glycans were released using PNGase F (Roche) and labeled with 2-aminobenzoic acid (2-AA) and 2-picoline borane (Sigma-Aldrich). The 2-AA–labeled glycans were purified via hydrophilic interaction liquid chromatography–solid phase extraction (HILIC-SPE) using multiwell filter plates, as previously described (17,18). HILIC-SPE–purified 2-AA–labeled glycans were diluted in 100% acetonitrile and injected into a UHPLC Dionex Ultimate 3000 (ThermoFisher Scientific) equipped with an Acquity UHPLC BEH Glycan column and a fluorescence detector.

Data analysis. HappyTools was used to align, calibrate, and integrate the raw emissions of the chromatograms exported from Chromeleon, version 7.1.2.1713 (ThermoFisher Scientific) (19). The calibrations list, settings, and quality control measurements are provided in the Supplementary Methods and Supplementary Tables 1 and 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract). V-domain glycosylation was calculated using the following formula: percentage V-domain glycosylation = (V-domain glycans/Fc glycans) × 100, where the V-domain glycans were GP19 plus GP23 plus GP24, and the Fc glycans were GP4 plus GP8 plus GP14. We selected the glycans used for the calculation based on our previous observation of their respective, exclusive presence in either the Fc portion or in the V domain (12,13) (Supplementary Figure 2). IgG ACPA glycan profiles could be obtained for all 10 RA samples from the cross-sectional cohort; 15 of the 84 FDR samples showed IgG ACPA glycan profiles that met the criteria. In the longitudinal cohort, 67 of the 117 samples from the FDRs yielded sufficient signal to determine the IgG ACPA glycan profile (Table 1 and Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract).

Statistical analysis. In the cross-sectional cohort, the percentage of V-domain glycosylation between RA patients and FDRs was compared by Mann-Whitney U test. In the longitudinal cohort, levels of V-domain glycosylation over time were compared between FDRs who developed RA and those who did not, using linear mixed models with a random intercept and slope. A multivariable Cox proportional hazards regression analysis was performed, with RA diagnosis as outcome and V-domain glycosylation level as predictor. We used V-domain glycosylation at the first moment of sampling, dichotomized as above or below the group median to draw Kaplan-Meier survival curves and estimate risk of developing RA. Receiver operating characteristic curve (ROC) regression was used to calculate diagnostic properties. All analyses in the longitudinal cohort included adjustment for age and sex and were conducted using Stata SE 14.1. All reported percentage values refer to IgG ACPA V-domain glycosylation levels and not to relative changes. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were calculated.

RESULTS

Lower levels of ACPA V-domain glycosylation in ACPA-positive healthy FDRs compared to RA patients. Previously, we demonstrated that IgG ACPAs, in contrast to other antigen-specific IgG molecules (e.g., IgG against tetanus toxoid), are hyperglycosylated in the V domain in patients with established RA. These glycans are biantennary *N*-linked glycans that are fully galactosylated and sialylated (12,13). Although ACPA can be present before disease onset in individuals who will eventually develop RA, the V-domain glycosylation state of ACPA has not been studied in the predisease phase. To address this question, IgG ACPAs were isolated from the serum of ACPA-positive Indigenous North American patients with RA and their unaffected ACPA-positive FDRs. By analyzing an initial cross-sectional set of samples, we observed that FDR-derived IgG ACPA showed, on average, substantially lower levels of V-domain glycosylation than IgG ACPA from patients with established RA (Supplementary Figure 3A, http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract). Notably, the levels of V-domain glycosylation of IgG ACPA isolated from Indigenous North American RA patients were comparable to the levels we previously reported in Dutch RA patients (112% versus 93%, respectively) (12). Additionally, tetanus toxoid–specific IgG in Indigenous North American RA patients did not show the enhanced V-domain *N*-glycosylation observed in IgG ACPA, which is consistent with our previous observations in Dutch RA patients (data not shown) (13,14).

Analysis of a second set of samples derived from longitudinally followed up, unaffected, ACPA-positive FDRs essentially replicated the cross-sectional results, which demonstrated low levels of IgG ACPA V-domain glycosylation compared to those in RA patients (Supplementary Figure 3B, http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract). For subsequent group-level analyses, we combined the IgG ACPA V-domain glycosyl-

ation data from the initial cross-sectional cohort with the data from baseline samples from the FDRs included in the longitudinal cohort (Figure 1A). Thus, each individual FDR contributed only once to the analysis, but overall this approach served to increase sample size and statistical power of the analysis.

FDRs displayed heterogeneous levels of IgG ACPA V-domain glycosylation. This observation prompted us to analyze whether FDRs with high levels subsequently developed clinically detectable IA. As depicted in Figure 1B (and in Supplementary Figures 3A and B, http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract), FDRs who transitioned to a state of clinically detectable IA later in life had substantially higher levels of IgG ACPA V-domain glycosylation compared to individuals who did not transition during follow-up (89% versus 20%; $P < 0.0001$). Furthermore, a small portion of FDRs reported new arthralgia; these individuals demonstrated higher IgG ACPA V-domain glycosylation levels compared to FDRs who remained asymptomatic (Supplementary Figure 3). Therefore, the initially unaffected FDRs who subsequently developed clinically detectable IA or new-onset arthralgia displayed high levels compared to FDRs who remained in an unaffected or asymptomatic state.

Association of high IgG ACPA V-domain glycosylation levels in FDRs and development of RA. The observation that unaffected FDRs who developed IA later in life had significantly higher IgG ACPA V-domain glycosylation levels than FDRs who did not develop IA (Figure 1B) prompted us to determine the time course of glycosylation in these individuals. Intriguingly, we observed that V-domain glycosylation levels were already increased in some FDRs up to 7 years before the onset of arthritis. Other individuals who developed IA showed an increase in glycosylation ~1–2 years before disease onset (Figure 2A). When all available time points were considered, IgG ACPA V-domain glycosylation was 34% higher (95% CI 7–62%) in FDRs who developed IA compared to FDRs who did not ($P = 0.015$) (Figure 2B). Notably, FDRs with new-onset arthralgia were classified as nontransitioned. Taken together, these data indicate that ACPA-positive individuals who subsequently developed IA displayed a high degree of IgG ACPA V-domain glycosylation in the preclinical phase. The time course for development of IA, however, varied considerably between individuals.

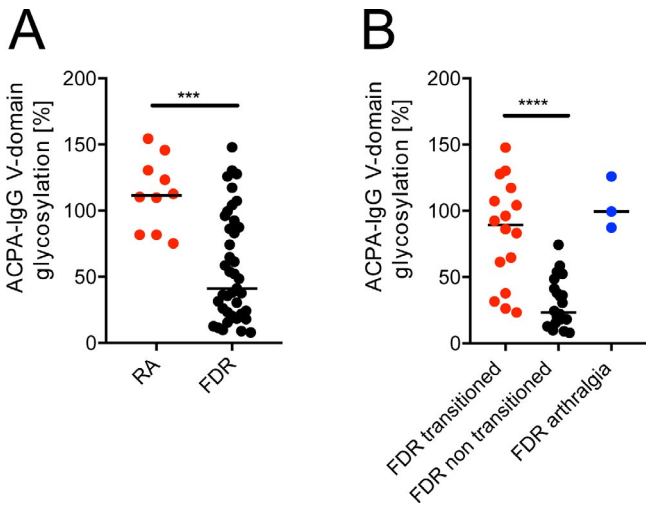


Figure 1. Anti–citrullinated protein antibody (ACPA)–positive first-degree relatives (FDRs) have lower levels of IgG ACPA variable (V)–domain glycosylation than do patients with clinically evident rheumatoid arthritis (RA). Percentages of IgG ACPA V-domain glycosylation in RA patients versus unaffected FDRs (**A**) and in FDRs who transitioned to inflammatory arthritis versus those who did not (**B**) are shown. Symbols represent individual subjects; bars show the median. *** = $P < 0.001$; **** = $P < 0.0001$. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract.

Absence of elevated V-domain glycosylation in IgG ACPA. The degree of V-domain glycosylation of IgG ACPA is remarkably high compared to other IgG molecules. To date, we have been unable to identify another autoreactive B cell response with comparably high levels of V-domain glycans (12,13). Furthermore, among the Dutch RA population, non-ACPA IgG V-domain glycosylation levels were only 17%. To confirm that non-ACPA IgG V-domain glycosylation is also low in the Indigenous North American population and to investigate a potential association with clinical outcome, we used the flowthrough of the ACPA isolation columns to further purify non-ACPA IgG from FDR-derived sera, followed by glycosylation analysis as it was performed for IgG ACPA. V-domain glycosylation of non-ACPA IgG was as low as 15–25% in these samples, with a mean of 17% for FDRs who did not develop IA and 20% for those who did (P not significant) (Figure 3). These levels are consistent with published data on IgG in healthy individuals (12,20). Moreover, the degree of V-domain glycosylation remained low over time and showed no differences in mean levels between transitioning and nontransitioning FDRs ($P = 0.33$). These data suggest that increased V-domain glycosylation is not a feature of non-ACPA IgG and that this phenomenon is inherent in the citrulline-directed immune response.

Elevated IgG ACPA V-domain glycosylation level as a predictive marker for disease development in ACPA-positive at-risk subjects. Given that FDRs who developed IA had higher levels of IgG ACPA V-domain glycosylation over time compared to those who did not, we attempted to create models of glycosylation as a predictor of IA development. To account for the variance between time points and duration of follow-up of available study subjects, 2 statistical models were established. In the first model, we used the median IgG ACPA V-domain glycosylation level in the cross-sectional analysis (58%) as a cutoff point, thus generating a binary variable. Cox regression analysis, corrected for age and sex, revealed that IgG ACPA V-domain glycosylation levels above the median were associated with development of IA (HR 6.07 [95% CI 1.46–25.2]; $P = 0.013$) (Figure 4). A similar cutoff point was

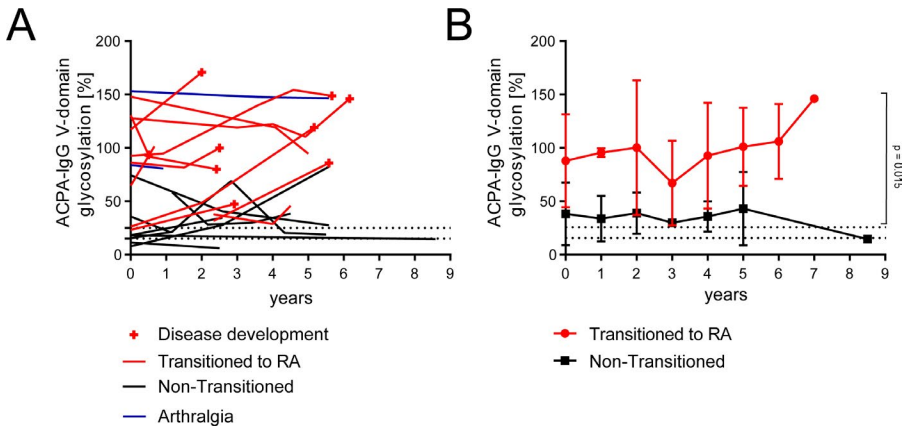


Figure 2. IgG ACPA V-domain glycosylation is elevated in FDRs who transitioned to RA during follow-up. **A**, Longitudinal data on IgG ACPA V-domain glycosylation. Time of RA diagnosis is shown with a red cross; lines without crosses indicate that the patient transitioned to RA but did not have a sample obtained at the time of diagnosis. **B**, Median IgG ACPA V-domain glycosylation levels in FDRs who transitioned to RA versus those who did not. Bars show the median and range. Dotted lines indicate the range of IgG V-domain glycosylation observed in healthy individuals. See Figure 1 for definitions.

created when ROC regression was used to determine the predictive value of high IgG ACPA V-domain glycosylation levels. At this cutoff point, IgG ACPA V-domain glycosylation had a sensitivity of 76.9% (95% CI 46.2–95.0%), a specificity of 78.6% (95% CI 49.2–95.3%), a positive predictive value (PPV) of 76.9% (95% CI 46.2–95.0%), and a negative predictive value (NPV) of 78.6% (95% CI 49.2–95.3%) for IA development.

DISCUSSION

IgG ACPA represents the most relevant prognostic and diagnostic biomarker in RA and is associated with poor prognosis and progressive joint destruction (21). A large body of evidence indicates that early intervention in RA improves clinical outcomes (22,23). Based on these observations, it is hypothesized that intervention at a stage prior to the onset of clinically detectable arthritis may result in better clinical outcomes and possibly even prevent disease (22). Currently, this preclinical phase is identified based on the presence of suggestive joint symptoms such as arthralgia, the detection of RA-associated autoantibodies, especially ACPA, or both of these factors. Imaging studies indicate that this clinical phenotype may also feature subclinical synovitis (22,24). Although arthralgia in conjunction with a broad autoantibody response is a strong predictor of imminent RA onset, a linear progression toward these features has been difficult to demonstrate (25). In particular, the existing longitudinal data suggest that a considerable proportion of individuals with ACPA do not develop RA. Thus, it has become increasingly important to delineate biomarkers that can be used to improve the risk model and that in turn provide actionable clinical information on which interventions can be based (24,26). Our current study demonstrates that the glycosylation state of the IgG ACPA V domain may serve such a purpose. We found that the presence of

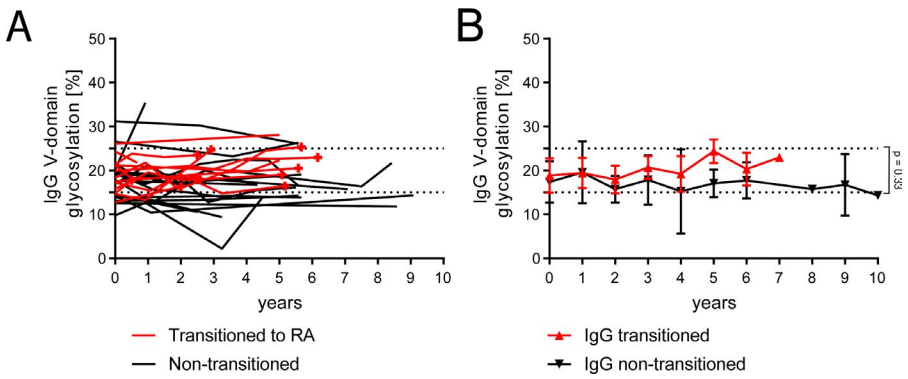


Figure 3. IgG depleted of ACPA does not display enhanced levels of V-domain glycosylation. **A**, Longitudinal data on IgG V-domain glycosylation. Time of RA diagnosis is shown with a red cross; lines without crosses indicate that the patient transitioned to RA but did not have a sample obtained at the time of diagnosis. **B**, Median IgG V-domain glycosylation levels in FDRs who transitioned to RA versus those who did not. Bars show the median and range. Dotted lines indicate the range of IgG V-domain glycosylation observed in healthy individuals. See Figure 1 for definitions.

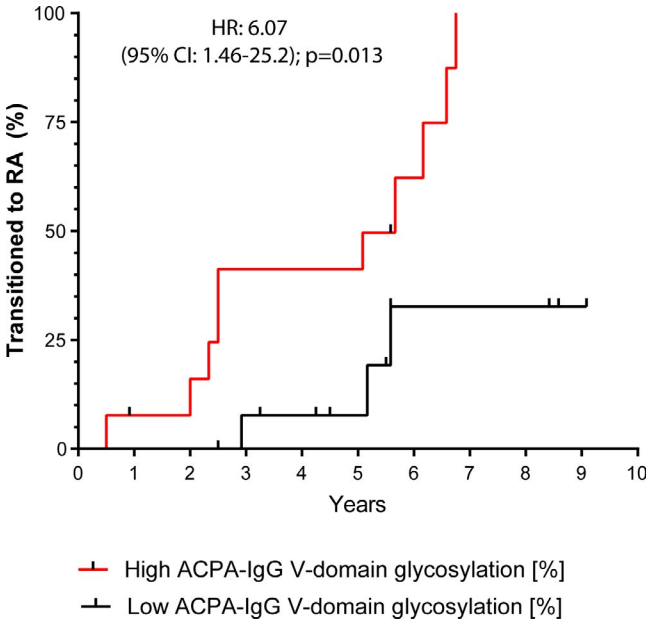


Figure 4. Development of RA in ACPA-positive FDRs, based on degree of IgG ACPA V-domain glycosylation at the first instance of sampling. IgG ACPA V-domain glycosylation levels below and above the 58.5% median are shown. HR = hazard ratio; 95% CI = 95% confidence interval (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40920/abstract.

extensive IgG ACPA V-domain glycosylation in unaffected ACPA-positive individuals is a strong predictor of progression toward disease. Indeed, a high level of IgG ACPA V-domain glycosylation had a PPV of 76.9% and an NPV of 78.6% for predicting the development of IA.

Considering these clinical implications, the present data refine our understanding of the ACPA immune response and its predisease evolution. Conceptually, the development of ACPA-positive RA has been proposed as a multistep process (7,8) in which the break in immune tolerance to citrullinated antigens develops first, followed by expansion and maturation of the autoimmune response, a so-called second hit. Recent evidence suggests that this putative second hit is driven by T helper cells that provide help to ACPA-expressing B cells. This T cell–B cell interaction, which likely mediates the increased usage of isotypes and epitope spreading observed before disease onset, may also be responsible for the introduction of *N*-glycosylation sites in the V domain of IgG ACPA and, therefore, the presence of glycans in this region. This hypothesis is further supported by the observation that the consensus sequences for *N*-glycosylation sites in IgG ACPA V domains are not encoded in their respective germline genes but introduced upon SHM (14). In fact, the presence, frequency, and distribution of these sites suggest a role of the glycans in the selection, development, and activation of the B and plasma cells that express/secrete IgG ACPA.

Notably, the ACPA-positive FDRs examined here exhibited lower levels of IgG ACPA V-domain glycosylation than their relatives with RA, but there was considerable heterogeneity in the population. As a matter of fact, a subset of FDRs had high levels of V-domain glycosylation, which in some cases was detectable years before disease onset. Individuals who exhibited fluctuation between low and normal levels over time (15–25%) rarely transitioned to disease. Given this heterogeneity, we used the available longitudinal samples to develop a kinetic understanding of how IgG ACPA V-domain glycosylation evolves in individuals who ultimately develop IA. Although the data are limited by the small number of those who transitioned to disease, it is clear that there is more than one trajectory and time course for disease development, with some individuals demonstrating a rapid increase in glycosylation and others showing high levels for an extended period of time. These observations suggest that the acquisition of *N*-glycans in the ACPA V domain is a process that requires repeated T cell–dependent B cell hypermutation events, potentially as a result of multiple hits that occur with varying kinetics. Ultimately, these may lead to the persistence, if not the pathogenicity, of the ACPA response.

Our study is limited by the relatively small number of samples with longitudinal follow-up. Also, the number of samples with low-level ACPA that did not pass quality control may have introduced bias. However, except for ACPA levels, the patients who could be included in the analysis had similar baseline characteristics compared to those who had to be excluded because of technical limitations (Table 1). Furthermore, in samples that passed our strict controls, estimates of the predictive value of IgG ACPA V-domain glycosylation were substantial and hold promise for clinical application. Nonetheless, additional studies are necessary to validate and extend these findings.

Finally, our study was conducted exclusively in an Indigenous North American population. Seropositive RA in this population was associated with HLA–DRB1 SE–encoding alleles, as it is in most other populations worldwide. The primary SE allele in the Indigenous North American population is HLA–DRB1*14:02, which is prevalent in the background population and almost unique to Indigenous North Americans. In contrast to other RA-predisposing SE alleles such as HLA–DRB1*04:01, which is seen primarily in white populations, HLA–DRB1*14:02 can accommodate both citrulline and arginine peptides with comparable affinity, but the orientation of citrulline-containing peptides is upright and directly interfaces with the T cell receptor (27,28). It is unclear whether these differences in peptide presentation to T cells impact their capacity to provide help for ACPA-expressing B cells. However, the observation that ACPA V-domain glycosylation patterns are comparable in Indigenous North American and Dutch RA patients suggests that, irrespective of how T cell autoimmunity develops, the T cell–dependent SHM of ACPA B cells is a final common mechanism in the evolution of the ACPA response. Notably, tetanus toxoid–specific IgG did not show enhanced V-domain glycosylation in the Indigenous North American RA patients, in accordance with our previous observations

in Dutch RA patients and with the absence of additional *N*-glycosylation sites in B cell receptors of tetanus toxoid–specific B cells (13,14).

In summary, we have shown that IgG ACPA V-domain glycosylation is a strong predictive biomarker for the development of ACPA-positive RA. Our findings have important implications for assessing the risk of future RA development in unaffected ACPA-positive individuals and, in turn, for stratifying these individuals for intervention studies. The results also provide mechanistic information regarding the evolution of preclinical RA autoimmunity. Future studies in this and other populations will be needed to determine the ultimate clinical utility of these findings.

ACKNOWLEDGMENTS

The authors wish to thank the Indigenous North American people from the Manitoba study communities for their participation and commitment to this longitudinal study. Furthermore, we thank Dr. Jan Wouter Drijfhout (Leiden University Medical Center) for providing the CCP-2 peptide.

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. Dr. Scherer 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. Hafkenscheid, Tanner, Huizinga, Toes, El-Gabalawy, Scherer.

Acquisition of data. Hafkenscheid, Smolik, Tanner, Meng, Wuhrer.

Analysis and interpretation of data. Hafkenscheid, de Moel, Jansen, Bondt, Wuhrer, Huizinga, Toes, El-Gabalawy, Scherer.

ADDITIONAL DISCLOSURES

Author Jansen is an employee of Ludger Ltd.

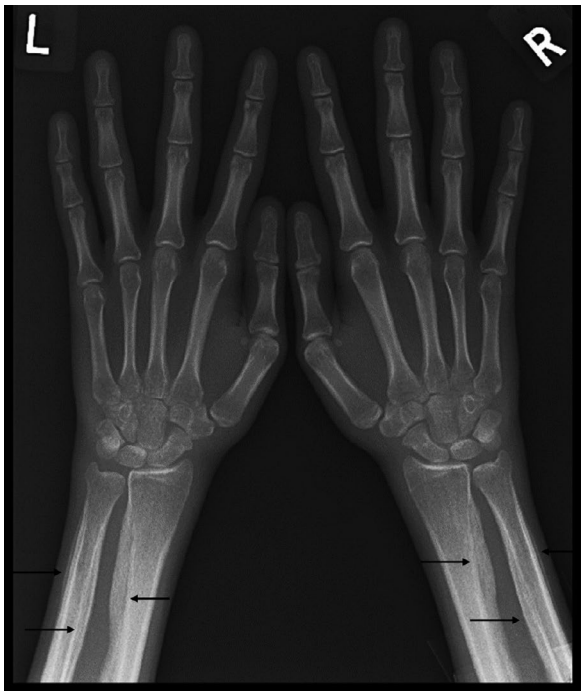
REFERENCES

1. Ferucci ED, Schumacher MC, Lanier AP, Murtaugh MA, Edwards S, Helzer LJ, et al. Arthritis prevalence and associations in American Indian and Alaska Native people. Arthritis Rheum 2008;59:1128–36.
2. Peschken CA, Esdaile JM. Rheumatic diseases in North America's indigenous peoples. Semin Arthritis Rheum 1999;28: 368–91.
3. El-Gabalawy HS, Robinson DB, Hart D, Elias B, Markland J, Peschken CA, et al. Immunogenetic risks of anti-cyclical citrullinated peptide antibodies in a North American Native population with rheumatoid arthritis and their first-degree relatives. J Rheumatol 2009;36:1130–5.
4. Peschken CA, Hitchon CA, Robinson DB, Smolik I, Barnabe CR, Prematilake S, et al. Rheumatoid arthritis in a North American Native population: longitudinal followup and comparison with a white population. J Rheumatol 2010;37:1589–95.
5. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum 2004;50:380–6.
6. Willemze A, Trouw LA, Toes RE, Huizinga TW. The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol 2012;8:144–52.
7. Kempers AC, Hafkenscheid L, Scherer HU, Toes RE. Variable domain glycosylation of ACPA-IgG: a missing link in the maturation of the ACPA response? Clin Immunol 2018;186:34–7.
8. Koning F, Thomas R, Rossjohn J, Toes RE. Coeliac disease and rheumatoid arthritis: similar mechanisms, different antigens. Nat Rev Rheumatol 2015;11:450–61.
9. Malmström V, Catrina AI, Klareskog L. The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting. Nat Rev Immunol 2017;17:60–75.
10. Wakitani S, Murata N, Toda Y, Ogawa R, Kaneshige T, Nishimura Y, et al. The relationship between HLA-DRB1 alleles and disease subsets of rheumatoid arthritis in Japanese. Br J Rheumatol 1997;36: 630–6.
11. Hensvold AH, Magnusson PK, Joshua V, Hansson M, Israelsson L, Ferreira R, et al. Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins. Ann Rheum Dis 2015;74:375–80.
12. Hafkenscheid L, Bondt A, Scherer HU, Huizinga TW, Wuhrer M, Toes RE, et al. Structural analysis of variable domain glycosylation of anti-citrullinated protein antibodies in rheumatoid arthritis reveals the presence of highly sialylated glycans. Mol Cell Proteomics 2017;16:278–87.
13. Rombouts Y, Willemze A, van Beers JJ, Shi J, Kerkman PF, van Toorn L, et al. Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. Ann Rheum Dis 2016;75:578–85.
14. Vergroesen RD, Slot LM, Hafkenscheid L, Koning MT, van der Voort EI, Grooff CA, et al. B-cell receptor sequencing of anti-citrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of N-glycosylation sites during somatic hypermutation. Ann Rheum Dis 2018;77:956–8.
15. Smolik I, Robinson DB, Bernstein CN, El-Gabalawy HS. First-degree relatives of patients with rheumatoid arthritis exhibit high prevalence of joint symptoms. J Rheumatol 2013;40:818–24.
16. Habets KL, Trouw LA, Levarht EW, Korporaal SJ, Habets PA, de Groot P, et al. Anti-citrullinated protein antibodies contribute to platelet activation in rheumatoid arthritis. Arthritis Res Ther 2015; 17:209.
17. Burnina I, Hoyt E, Lynaugh H, Li H, Gong B. A cost-effective plate-based sample preparation for antibody N-glycan analysis. J Chromatogr A 2013;1307:201–6.
18. Jansen BC, Bondt A, Reiding KR, Scherjon SA, Vidarsson G, Wuhrer M. MALDI-TOF-MS reveals differential N-linked plasma- and IgG-glycosylation profiles between mothers and their newborns. Sci Rep 2016;6:34001.
19. Jansen BC, Hafkenscheid L, Bondt A, Gardner RA, Hendel JL, Wuhrer M, et al. HappyTools: a software for high-throughput HPLC data processing and quantitation. PLoS One 2018;13:e0200280.
20. Van De Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y. The emerging importance of IgG Fab glycosylation in immunity. J Immunol 2016;196:1435–41.
21. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet 2010;376:1094–108.
22. Van Steenbergen HW, da Silva JA, Huizinga TW, van der Helm-van Mil AH. Preventing progression from arthralgia to arthritis: targeting the right patients. Nat Rev Rheumatol 2018;14:32–41.
23. Demoruelle MK, Deane KD. Treatment strategies in early rheumatoid arthritis and prevention of rheumatoid arthritis. Curr Rheumatol Rep 2012;14:472–80.

24. Boeters DM, Raza K, vander Helm-van Mil AH. Which patients presenting with arthralgia eventually develop rheumatoid arthritis? The current state of the art. RMD Open 2017;3:e000479.
25. Ten Brinck RM, van Steenbergen HW, van Delft MA, Verheul MK, Toes RE, Trouw LA, et al. The risk of individual autoantibodies, autoantibody combinations and levels for arthritis development in clinically suspect arthralgia. Rheumatology (Oxford) 2017;56:2145–53.
26. Burgers LE, Allaart CF, Huizinga TW, van der Helm-van Mil AH. Clinical trials aiming to prevent rheumatoid arthritis cannot detect prevention without adequate risk stratification: a trial of methotrexate versus placebo in undifferentiated arthritis as an example. Arthritis Rheumatol 2017;69:926–31.
27. Van Heemst J, Jansen DT, Polydorides S, Moustakas AK, Bax M, Feitsma AL, et al. Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. Nat Commun 2015;6:6681.
28. Raychaudhuri S, Sandor C, Stahl EA, Freudenberg J, Lee HS, Jia X, et al. Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. Nat Genet 2012;44:291–6.

DOI 10.1002/art.41014

Clinical Image: Hypertrophic osteoarthropathy in cystic fibrosis



The patient, a 19-year-old man with cystic fibrosis (CF), presented with pain and swelling of both wrists in association with an infective respiratory exacerbation. His CF-related complications included clubbing, multilobar bronchiectasis, chronic infection with *Pseudomonas aeruginosa*, exocrine pancreatic insufficiency, CF-related diabetes mellitus, and low body mass. Examination revealed bilateral wrist swelling and tenderness. Radiography of the hands and wrists demonstrated multi–layered periosteal reactions in the distal ulna and radius bilaterally (**arrows**), consistent with a diagnosis of hypertrophic osteoarthropathy (HOA) (1). While most commonly linked with non–small cell lung cancer, secondary HOA may occur with a range of pulmonary and extrapulmonary pathologies, including CF (2). The pathophysiologic mechanisms remain unclear. In our patient, treatment with intravenous antibiotics and oral corticosteroids was associated with significant improvement in both respiratory and joint symptoms.

1. Turner MA, Baildam E, Patel L, David TJ. Joint disorders in cystic fibrosis. J R Soc Med 1997;90 Suppl 31:13–20.
2. Yap FY, Skalski MR, Patel DB, Schein AJ, White EA, Tomasian A, et al. Hypertrophic osteoarthropathy: clinical and imaging features. Radiographics 2017;37:157–95.

Elizabeth Clarke, MBChB, MRCP
Manchester University NHS Foundation Trust
Wythenshawe Hospital
and University of Manchester
Rowland Bright-Thomas, MBChB, MRCP, MD
Manchester University NHS Foundation Trust
Wythenshawe Hospital
Manchester, UK