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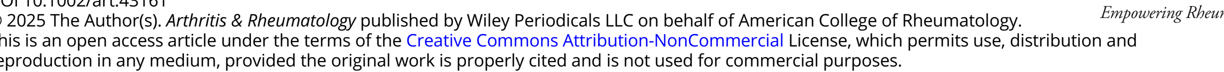
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Recognition of Glycine Versus Nonglycine Citrulline Motifs Dictating the HLA Class II Association of Anticitrullinated Protein Antibodies: Insights From Autoantibody Profiling of 6,900 Scandinavian Patients With Rheumatoid Arthritis

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Objective. Rheumatoid arthritis (RA)–associated anticitrullinated protein antibodies (ACPAs) target various antigens by binding short citrulline amino acid motifs, resulting in heterogeneous ACPA profiles among patients. Here we analyzed ACPA patterns by recognized citrulline motifs in relation to the RA risk factors HLA-DRB1 shared epitope (SE) alleles and history of smoking.

Methods. Rheumatoid factor (RF) and anticyclic citrullinated peptide (CCP2) isotypes, 15 anti-Cit- and four anti-Carb/Acet-peptide-IgG, were centrally measured in 6,907 patients from five Scandinavian RA cohorts using fluoroenzyme immunoassay and a custom-made multiplex solid-phase microarray. HLA-DRB1 SE alleles were imputed from single-nucleotide polymorphism genotyping data.

Results. Single-citrulline peptides derived from four multicitrulline peptides (Cit Fib$\alpha_{36\text{-}50}$, Cit Fib$\beta_{60\text{-}74}$, Cit TNC5, and Cit Vim$_{60\text{-}75}$) showed differential binding patterns, supporting recognition of citrulline motifs rather than long peptides. Four citrulline peptides (Cit Fib$\beta_{36\text{-}52}$, Cit Fib$\beta_{60\text{-}74\text{-}Cit3}$, Cit Fil$_{307\text{-}324}$, and Cit Vim$_{60\text{-}75\text{-}Cit1}$) captured 97% of IgG anti-CCP2+ patients. Patient subsets based on ACPA, anti-Carb/Acet, and RF displayed differences in ACPA composition and disease activity but not comorbidities. Different ACPAs overlapped, but when dichotomizing patients based on high reactivity to peptide citrulline motifs, only ACPA to nonglycine citrulline motif associated with HLA SE alleles. In IgG anti-CCP2+ patients, 90% of those with only high nonglycine ACPA were HLA SE allele carriers compared with 67% in the group with glycine motif–only ACPA (odds ratio 4.5). Smoking status associated with IgA and glycine motif ACPA.

Conclusion. Although citrulline-glycine motifs are prevalent ACPA targets, our data reveal that HLA SE alleles are primarily associated with ACPA to nonglycine citrulline motifs, providing insight in ACPA T cell dependance. Yet, the etiologic significance of ACPA targeting different protein structures remains unknown.

INTRODUCTION

More than two-thirds of all patients diagnosed with rheumatoid arthritis (RA) have "seropositive" disease as defined by the presence of RA autoantibodies, primarily rheumatoid factor (RF) reactivity (anti-Fcγ) and/or anticitrullinated protein autoantibodies (ACPAs).[1] Notably, seropositive RA has been linked to worse disease progression with more erosive disease compared

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with "seronegative" disease.[2] Important risk factors associated with seropositive RA include exposure to cigarette smoking and certain HLA-DRB1 variants denoted shared epitope (SE) alleles.[3] Interestingly, although the HLA-DR association places the adaptive immune response centrally in RA pathogenesis, and there is a strong correlation between SE and ACPA positivity, there are also indications that SE alleles are a stronger predictor of clinical arthritis than the presence of ACPA alone.[4]

ACPAs are highly specific for RA and commonly measured with cyclic citrullinated peptide (CCP2) assays using commercial synthetic peptides designed for high diagnostic accuracy. However, serum ACPAs have been found to recognize many different citrullinated proteins and peptides.[5] Proteins with disordered structures and regions enriched for glycine, serine, and threonine have been suggested to be particularly targeted.[6] Multiplex antigen panels have been developed to capture these ACPA fine specificity variations between patients with RA.[7–10] The clinical relevance of interpatient heterogeneity of ACPA peptide targets is, however, still controversial. Despite previous efforts, it has been difficult to demonstrate any clear association with disease progression or treatment outcome. Furthermore, adding to the complexity, studies using patient single cell–derived monoclonal antibodies show that individual ACPA IgG molecules can bind a multitude of different cit-peptides and that the peptide interaction is determined by only a couple of additional residues besides the citrulline (ie, cit-motifs).[11] This means that serological fine specificity patterns are not necessarily reflecting parallel ACPA clones but also include multireactive clones that create a direct dependence between different ACPA measurements.

Patients with RA, especially those positive for ACPA, can also have other IgG antimodified protein autoantibodies (AMPA) targeting additional posttranslational modifications of proteins, primarily carbamylation and acetylation.[7,12–14] AMPAs correlate with citrulline reactivity in antibody serology; certain monoclonal CCP2+ ACPAs have also been found to directly cross-react with carbamylated and acetylated peptides.[11] Moreover, although RF may not be as specific for RA as ACPA, RF is postulated to play important roles in amplifying inflammatory response. For instance, there is an additive effect of ACPA and RF positivity on the risk of bone erosions.[15] Furthermore, in patients with early RA, the presence of RF is associated with systemic inflammation in an ACPA-dependent manner.[16] In clinical practice, seropositivity is often determined based on only CCP2 IgG and RF IgM measurements, yet other isotypes or autoantibody reactivities can capture additional seropositive patients who display similar clinical risk factor associations as those defined based on CCP2 IgG and/or RF IgM.[17] Moreover, IgA ACPA and RF have been proposed to reflect a link with mucosal triggering, but the role in pathogenesis is not well understood.

To further delineate the (co-)occurrence of RF, ACPAs, other AMPAs, and their association with RA risk factors (here: smoking and HLA-DRB1 SE alleles), we investigated RA autoantibody profiles in >6,900 patients with RA from five different Scandinavian RA cohorts using new optimized assays. To do so, we first validated the array's capacity to capture distinct ACPA cit-peptide binding based on citrulline motifs and assessed the overall frequency and dependance of different ACPA and AMPA fine specificities in the patients with RA. We hypothesized that ACPA fine specificities targeting glycine-containing citrulline motif in proteins may have different immunologic origin and clinical relevance than nonglycine ACPA. To explore this hypothesis further, we investigated two key topics: (1) do different ACPA and autoantibody compositions associate with different clinical subsets of patients with RA? (2) Do ACPA fine specificities targeting certain citrulline amino acid motifs (with and without glycine) associate differently with the main RA risk factors of HLA-DRB1 SE alleles and smoking?

MATERIALS AND METHODS

Study populations with RA. Our study population was made up of patients with RA from five Scandinavian cohorts[18] (summarized in Supplementary Figure 1 and Supplementary Table 1): The Swedish Rheumatology Quality registry biobank (SRQb; N = 3,895),[19] the Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen (ARCTIC; N = 212),[20] the Danish Nationwide Clinical Rheumatology Registry (DANBIO) and corresponding samples within the Danish Rheumatologic Biobank (DRB; N = 2,324),[21] the Norwegian Antirheumatic Drug Register[22] (NOR-DMARD, N = 302), and the Ultrasound in Rheumatoid Arthritis patients starting Biologic Treatment (ULRABIT; N = 174) cohort.[23] The study was approved by the regional ethics review boards and followed the Declaration of Helsinki, and all patients gave informed consent. Inclusion in ARCTIC, NOR-DMARD, and ULRABIT required fulfillment of the American College of Rheumatology (ACR)/EULAR classification criteria for RA.[1] Because SRQb and DANBIO/DRB are clinical routine care RA registers, fulfillment of ACR/EULAR classifications is not a formal criterion for inclusion (a clinical RA diagnosis assigned by the treating rheumatologist is sufficient) but is fulfilled in most cases.

Drs Mathsson Alm and Westerlind are co-first authors and contributed equally to this work. Drs Askling and Grönwall are co-last authors and contributed equally to this work.

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Blood samples and other information. One stored plasma sample per patient (n = 6,907) was sent for centralized antibody analysis. Depending on the source cohort, this sample was taken at different points during the RA disease course permitting the stratification into early versus established RA. In an initial screening, we split the statistical analyses into (1) patients with early RA (n = 597) consisting of disease-modifying antirheumatic drug (DMARD)–naive patients with RA from the ARCTIC cohort (n = 212; baseline time point, average <7 months disease duration) and SRQb (n = 385; <90 days from time of diagnosis at the time of sampling) and (2) a cross-sectional validation dataset with blood samples taken at any later timepoint during the RA disease, consisting of 3,510 patients from the Swedish SRQb (excluding the early RA samples) and 2,800 patients from the Danish and Norwegian RA cohorts.

Smoking status was available for 3,212 patients from the SRQb cohort and 2,316 patients from the DANBIO/DRB cohort; individuals were categorized as "ever smokers" (including current and former smokers) or "never smokers" based on self-report. Clinical measurements close to the time of blood sampling (±14 days) were retrieved from the SRQ register and were in this time window available for 2,185 of the patients from the SRQb cohort. Through linkage of SRQb to the Swedish National Patient Register and the Prescribed Drug Register, we also retrieved data on comorbid conditions during the 5 years preceding donation of blood for the study. Details of the comorbidity definitions can be found elsewhere.[24]

For SRQb, *HLA-DRB1 SE alleles* were imputed using SNP2HLA[25] from genetic data for 2,652 patients, genotyped on the Illumina global screening array (deCODE Genetics, Reykjavik, Iceland).[19] Patients were determined to be SE allele positive if carrying *HLA-DRB1*01* (except **0103*), **04*, **10*, or **14:02*. For 2,735 patients from the Danish and Norwegian cohorts (DANBIO/DRB, ARCTIC, NOR-DMARD, and ULRABIT) *HLA-DRB1* SE alleles were imputed by SNP2HLA[25] from genetic data for 28 single-nucleotide polymorphisms (SNPs) from the HLA locus from a customized genotyping effort by AmpliSeq on Ion-Torrent 5S XL covering 375 SNPs in collaboration with Thermo Fisher Scientific.

Monoclonal antibodies. CCP2+ human monoclonal antibodies were generated by cloning Igs from single cells from patients with RA as previously described.[26–29] They originated from different patients, tissues, and B cell compartments (Supplementary Table 2). All clones were expressed as recombinant human IgG1 in Hek293 Expi cells (Thermo Fisher Scientific).

Autoantibody measurements. IgM, IgG, IgA RF and IgG, and IgA anti-CCP2 analyses were performed by fully automated commercial fluoroenzyme immunoassay for in vitro diagnostics (EliA, Phadia AB, Uppsala, Sweden). ACPA fine specificities were screened using a new optimized version of the previously reported custom-made multiplex solid-phase microarray platform (Thermo Fisher Scientific, ImmunoDiagnostics). The array contained four original peptides with multiple citrulline sites and 15 single-citrulline peptides derived from human fibrinogen (alpha and beta chain), vimentin, tenascin C, and filaggrin; two acetylated histone 4 peptides with naturally occurring sites; and two synthetic filaggrin (cfc1) peptides in which the arginine/citrulline site in the original peptide was replaced by either homocitrulline or acetyl-lysine as well as arginine control peptides. On the array, biotinylated peptides are immobilized by binding to streptavidin (see Supplementary Table 2 for peptide design). For autoantibody serology screenings, fluorescent intensity was normalized to arbitrary units. For each array batch, the cut-off for positivity for each antigen was determined by the 98th percentile for plasma from 235 to 570 healthy controls (Thermo Fisher Scientific). High signal was defined as three times the cut-off for positivity (Supplementary Table 3). In total, 712 healthy controls were analyzed on the array.

Statistical methods. Spearman analyses were used for investigation of direct correlations of continuous values. Differences in autoantibody levels between groups were evaluated with Mann-Whitney or Kruskal-Wallis analysis with Dunn correction for multiple comparisons. Differences in frequencies of positive tests were assessed with Fisher exact tests. Logistic regression was used for further multivariate analysis. Statistical analysis was performed using JMP 16 (SAS Institute) and Prism 9 (Graphpad). *P* values <0.05 were considered statistically significant. Unsupervised hierarchical clustering was performed using the Ward method.

RESULTS

ACPA binding is determined by recognition of single-citrulline motifs. We developed an optimized antigen array including a panel of single-citrulline peptides for improved capture of different ACPA specificities based on cit-motifs. The array was first validated using a panel of 33 previously published patient-derived CCP2+ monoclonal ACPAs (Supplementary Table 2). Most of the monoclonals bound to several cit-peptides on the array. Certain clones also bound to carbamylated (Carb) and acetylated (Acet) peptides (Supplementary Figure 2). Except for two clones, the few CCP2+ clones that were negative on the array were found to bind other cit-peptides by enzyme-linked immunosorbent assay (Supplementary Figure 2). Importantly, when studying the four peptide families of an original multi-cit peptide and corresponding single-cit derivates (TNC5, $Vim_{60\text{-}75}$, $Fib\alpha_{36\text{-}50}$, and $Fib\beta_{60\text{-}74}$), the binding preferences for the clones could be determined (Supplementary Figure 2B–E). In most cases, clones that bound to the original peptides recognized only one of the single-cit peptides or only peptides with similar cit-motifs.

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Next, we investigated the plasma IgG binding patterns in cross-sectional patients with RA (n = 6,907; cohort description in Supplementary Figure 1 and Supplementary Table 1). Binding was specific for citrulline peptides and low/no binding was detected to the arginine (Arg) version of the peptides (Supplementary Table 4). When analyzing patients positive for any citrulline peptides in the families TNC5 (n = 3,114; 45%), Vim_{60-75} (n = 4,591; 66%), $Fib\alpha_{36-50}$ (n = 2,645; 38%), or $Fib\beta_{60-74}$ (N = 4,474; 65%), we observed patient-specific binding patterns (Figure 1A–D). For example, when detecting binding to the original TNC5 peptide in a patient, a preferential strong binding could be seen for one of the four cit-sites but typically not for all. Interestingly, for the peptide families Vim_{60-75} and $Fib\beta_{60-74}$ we could detect a clear dominance of one cit-site, Cit $Vim_{60-75-cit1}$ and Cit $Fib\beta_{60-74-cit3}$. On the other hand, for the TNC5 and $Fib\alpha_{36-50}$ peptide families binding was seen to all sites but differently in different patients.

Several single-cit peptide reactivities displayed significant direct correlation with each other, but this correlation was typically stronger outside of the peptide families. Surprisingly, the strongest correlation was seen by the two peptides with unrelated sequences: Cit $Fib\alpha_{36-50-Cit1}$ and Cit $Fib\beta_{60-74-Cit2}$ (Supplementary Figure 3). Notably, in several cases one of the single-cit peptides captured significantly more patients than the original multi-cit peptide (ie, Cit $Fib\alpha_{36-50-Cit1}$ vs Cit $Fib\alpha_{36-50}$; Cit $Fib\beta_{60-74-cit3}$ vs Cit $Fib\beta_{60-74}$; and Cit $Vim_{60-75-cit1}$ vs Cit Vim_{60-75}) (Figure 1 and Supplementary Figure 2B–D).

Frequencies and overlap between different autoantibody classes and isotypes. Four single cit-peptides stood out with a high frequency of positivity: Cit $Fib\beta_{36-52}$ (51%), Cit $Fib\beta_{60-74-Cit3}$ (59%), Cit $Fil_{307-324}$ (54%), and Cit $Vim_{60-75-Cit1}$ (63%) (Table 1; Figure 2A). When we combined these four peptides that we denote "group A" peptides, they captured 97% of IgG CCP2+ patients and 22% of IgG CCP2−. The other 11 peptides ("group B" peptides) varied in frequency of positivity. Moreover, the ACPA patterns were relatively consistent between RA cohorts included in the study, as seen by dichotomizing patients into early DMARD-naive patients with RA (n = 597) and two cross-sectional replication cohorts as (1) the cross-sectional part of the Swedish cohort from the SRQb (n = 3,510) and (2) the cross-sectional part from the Norwegian and Danish cohorts (n = 2,800) (Table 1).

Notably, we also observed that 56% of IgG anti-CCP2+ patients (2,646 of 4,723) had antibody reactivity to carbamylated or acetylated peptide epitopes (Table 1). There was a positive correlation between Acet/Carb positivity and the number of detectable ACPA fine specificities (Supplementary Figure 4). Using the $Fil_{307-324}$ peptide and replacing the arginine/citrulline residue with acetyl-lysine or homocitrulline further illustrated the overlap between reactivities, with a large proportion of Cit $Fil_{307-324}$–positive patients also being positive for Carb $Fil_{307-324}$ or triple positive for the Cit, Carb, and Acet versions (Supplementary Figure 5). Reactivity to acetylated histone 4, Acet $His4_{1-18\ K5}$, and Acet $His4_{1-18\ K16}$ was less frequent but substantially overlapped with Carb/Acet filaggrin positivity (Supplementary Figure 6). Although cit-reactivity alone was common, only a few patients was uniquely positive for Carb or Acet without Cit-positivity.

When evaluating the total seropositivity, 76% of the patients with RA were seropositive based on CCP2 IgG and/or RF IgM. Including IgA CCP2 and RF isotypes further increased the size of the seropositive group to 83% (n = 5,759). Furthermore, a few additional seropositive individuals were captured when including IgG to Acet/Carb, resulting in the final proportion of 84% (n = 5,805) of patients positive for at least one of the investigated markers, which reduced the seronegative group to 16% of all included patients (Figure 3A; Supplementary Table 6). Of CCP2 IgG negative patients, 11% were positive for at least one of the ACPA fine specificities. The cit-reactivity levels in these patients were not necessarily low or just above cut-off. However, it should be emphasized that including multiple peptide tests significantly reduced the specificity as seen by high combined positivity in the analyzed healthy controls, despite high specificity for each individual test (n = 712; 22% cit-peptide positivity; Supplementary Table 4). Hence, a more appropriate approach in testing may be to use the high cut-off, which maintained specificity (4.5% positivity in controls) but increased the seropositivity to 79% in combination with CCP2 and/or RF (n = 5,460; Supplementary Tables 4 and 6).

In general, there was a large overlap between different ACPA fine specificities. A total of 91% of all patients with RA who were positive for any ACPA fine specificity were positive for two or more peptides. Yet, among patients positive for only one peptide (n = 493), the same four group A peptides dominated: Cit $Fib\beta_{36-52}$, Cit $Fib\beta_{60-74-Cit3}$, Cit $Fil_{307-324}$, and Cit $Vim_{60-75-Cit1}$ (9%, 13%, 14%, and 15% positives, respectively; Supplementary Figure 7). Notably, many individuals displayed high signals (three times the cut-off) to both group A and group B peptides (Figure 3B–C; Supplementary Table 5). Only a smaller subset (5% of all patients with RA) had high reactivity restricted to the less frequent (group B) peptides (Figure 3C).

Many cit-peptides that are commonly used as antigens for detecting ACPA fine specificities display glycine (gly) residues next to the citrulline, either in the +1 (cit-gly) or −1 (gly-cit) position. Of the 15 single-cit peptides, 4 peptides contained cit-gly or gly-cit motifs and 11 did not (Table 1). Importantly, of the dominating (group A) peptides, two contained gly motifs (Cit $Fib\beta_{36-52}$ and Cit $Fil_{307-324}$) and two did not (Cit $Fib\beta_{60-74-Cit3}$ and Cit $Vim_{60-75-Cit1}$). There was still a large overlap between these reactivities among patients: 49% had high signal for both gly-motif peptides and non-gly motif peptides. Nevertheless, 5% had high reactivity only to gly-motif peptides, and 15% of all patients had high reactivity to only peptides with non-gly motifs (Figure 3D). Interestingly, the patient group with only gly-peptide ACPAs had significantly

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Figure 1. Serological anticitrullinated protein antibody patterns reveal the preferential binding of autoantibodies to citrulline motifs. A total of 6,907 patients with rheumatoid arthritis were screened for binding to citrulline-containing peptides. The figure visualizes binding to four peptide families with the original peptide with multiple citrulline and the equivalent single-citrulline peptides. Patients with any positive signal within the families are shown: (A) Cit TNC5 in 3,115 positive patients, (B) Cit Fib$\beta_{60\text{-}74}$ in 4,474 positive patients, (C) Cit Fib$\alpha_{36\text{-}50}$ in 2,645 positive patients, and (D) Cit Vim$_{60\text{-}75}$ in 4,591 positive patients. Heatmaps display positive signals in arbitrary units with the cut-off for positivity in the assays subtracted.

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Table 1. Autoantibody frequencies in different RA cohorts*

Autoantibody assay	Peptide sequence	Single-cit	Group A/B	Gly motif	Early RA[a] (n = 597), n (%)	Cross-sectional 1[b] (n = 3,510), n (%)	*P* value[c]	Cross-sectional 2[d] (n = 2,800), n (%)	*P* value[c]	All IgG anti-CCP2− (n = 2,183), n (%)	All IgG anti-CCP2+ (n = 4,724), n (%)
Cit-peptides											
Cit Fibα$_{36-50}$	GP(**cit**)VVF(**cit**)HQSACKDSK	N	N/A	N/A	90 (15)	515 (15)	NS (0.23)	474 (17)	NS (0.30)	126 (6)	953 (20)
Cit Fibα$_{36-50\text{-Cit1}}$	GP(**cit**)VVERHQSACKNSL	Y	B	N	143 (24)	931 (27)	NS (0.19)	795 (28)	**0.03**	202 (9)	1,667 (35)
Cit Fibα$_{36-50\text{-Cit2}}$	GPRVVE(**cit**)HQSACKNSL	Y	B	N	83 (14)	494 (14)	NS (0.95)	486 (17)	**0.04**	115 (5)	948 (20)
Cit Fibβ$_{36-52}$	NEEGFFSA(**cit**)**G**HRPLDKK	Y	**A**	Y	316 (53)	1,804 (51)	NS (0.51)	1,419 (51)	NS (0.32)	168 (8)	3,371 (71)
Cit Fibβ$_{60-74}$	(**cit**)PAPPPISGGGY(**cit**)A(**cit**)	N	N/A	N/A	278 (47)	1,618 (46)	NS (0.86)	1,313 (47)	NS (0.89)	159 (7)	3,050 (64)
Cit Fibβ$_{60-74\text{-Cit1}}$	(**cit**)PAPPPISGGGYRAR	Y	B	N	26 (4)	187 (5)	NS (0.37)	119 (4)	NS (0.91)	90 (4)	242 (5)
Cit Fibβ$_{60-74\text{-Cit2}}$	RPAPPPISGGGY(**cit**)AR	Y	B	N	175 (29)	1,069 (30)	NS (0.60)	899 (32)	NS (0.19)	159 (7)	1,984 (42)
Cit Fibβ$_{60-74\text{-Cit3}}$	RPAPPPISGGGYRA(**cit**)	Y	**A**	N	318 (53)	2,001 (57)	NS (0.09)	1,734 (62)	**0.0001**	185 (8)	3,868 (82)
Cit Fil$_{307-324}$	HQCHQEST(**cit**)**G**RSRGRCGRSGS	Y	**A**	Y	312 (52)	1,904 (54)	NS (0.37)	1,533 (55)	NS (0.28)	162 (7)	3,587 (76)
Cit TNC1	VFLRRKN**G**(**cit**)ENFYQNW	Y	B	Y	137 (23)	723 (21)	NS (0.19)	800 (29)	**0.006**	93 (4)	1,567 (33)
Cit TNC5	EHSIQFAEMKL(**cit**)PSNF(**cit**)NLEG (**cit**)(**cit**)KR	N	N/A	N/A	208 (35)	1,044 (30)	**0.01**	1,147 (41)	**0.006**	163 (8)	2,236 (47)
Cit TNC5$_{\text{Cit1}}$	EHSIQFAEMKL(**cit**) PSNFRNLEGRRKR	Y	B	N	47 (8)	380 (11)	**0.03**	320 (12)	**0.01**	95 (4)	652 (14)
Cit TNC5$_{\text{Cit2}}$	EHSIQFAEMKLRPSNF(**cit**) NLEGRRKR	Y	B	N	112 (19)	628 (18)	NS (0.61)	575 (21)	NS (0.34)	75 (3)	1,240 (26)
Cit TNC5$_{\text{Cit3}}$	EHSIQFAEMKLRPSNFRNLE**G**(**cit**) RKR	Y	B	Y	171 (29)	771 (22)	**0.0005**	797 (28)	NS (0.92)	92 (4)	1,647 (35)
Cit TNC5$_{\text{Cit4}}$	EHSIQFAEMKLRPSNFRNLEGR(**cit**) KR	Y	B	N	102 (17)	456 (13)	**0.008**	401 (14)	NS (0.09)	59 (3)	900 (19)
Cit Vim$_{60-75}$	VYAT(**cit**)SSAV(**cit**)L(**cit**)SSVP	N	N/A	N/A	297 (50)	1,670 (48)	NS (0.33)	1,598 (57)	**0.001**	124 (6)	3,441 (73)
Cit Vim$_{60-75\text{-Cit1}}$	VYAT(**cit**)SSAVRLRSSVP	Y	**A**	N	342 (57)	2,186 (62)	**0.03**	1,792 (64)	**0.002**	246 (11)	4,074 (86)
Cit Vim$_{60-75\text{-Cit2}}$	VYATRSSAV(**cit**)LRSSVP	Y	B	N	73 (12)	450 (13)	NS (0.74)	500 (18)	**0.0007**	91 (4)	932 (20)
Cit Vim$_{60-75\text{-Cit3}}$	VYATRSSAVRL(**cit**)SSVP	Y	B	N	140 (23)	786 (22)	NS (0.56)	846 (30)	**0.0009**	77 (4)	1,695 (36)
Acet/Carb-peptides											
Carb Fil$_{307-324}$	HQCHQEST(**carb**)GRSRGRCGRSGS	N/A	N/A	N/A	192 (32)	1,045 (30)	NS (0.25)	1,084 (39)	**0.003**	148 (7)	2,173 (46)
Acet Fil$_{307-324}$	HQCHQEST(**acet**)GRSRGRCGRSGS	N/A	N/A	N/A	168 (28)	891 (25)	NS (0.16)	903 (32)	NS (0.05)	83 (4)	1,879 (40)
Acet His4$_{1-18\ \text{K5}}$	SGRG(**acet**) GGKGLGKGGAKRHRKVL	N/A	N/A	N/A	45 (7.5)	276 (7.9)	NS (0.87)	295 (11)	**0.03**	84 (4)	532 (11)
Acet His4$_{1-18\ \text{K16}}$	SGRGKGGKGLGKGGA(**acet**) RHRKVL	N/A	N/A	N/A	55 (9.2)	334 (9.5)	NS (0.88)	344 (12)	**0.04**	92 (4)	641 (14)
CCP2											
IgG anti-CCP2	–				385 (64)	2,437 (69)	**0.02**	1,902 (68)	NS (0.11)	N/A	N/A
IgA anti-CCP2	–				174 (29)	1,065 (30)	NS (0.59)	873 (31)	NS (0.35)	24 (1)	2,088 (44)
RF											
IgM RF	–				371 (62)	2,211 (63)	NS (0.71)	1,851 (66)	NS (0.07)	497 (23)	3,936 (83)
IgA RF	–				237 (39)	1,140 (32)	**0.0007**	936 (33)	**0.004**	208 (9.5)	2,105 (44)
IgG RF	–				147 (25)	499 (14)	**<0.0001**	483 (17)	**<0.0001**	78 (3.6)	1,051 (22)

* *P* values <0.05 are significant and are marked with bold font. CCP2, cyclic citrullinated peptide; N, no; N/A, not available; NS, not significant; RA, rheumatoid arthritis; RF, rheumatoid factor; Y, yes.

[a] Early disease-modifying antirheumatic drug–naive patients with RA from the Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen cohort and Swedish Rheumatology Quality registry biobank with <90 days after diagnosis.

[b] Swedish Rheumatology Quality registry biobank (excluding patients included in the "early RA" dataset).

[c] Fisher's exact tests comparing frequencies with the disease-modifying antirheumatic drug–naive early RA cohort IgG reactivity with Cit/Carb/Acet-peptides were measured with antigen microarray. CCP2 and RF were measured by fluorescent enzyme immunoassay.

[d] Danish and Norwegian patients (excluding Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen patients included in the "early RA" dataset).

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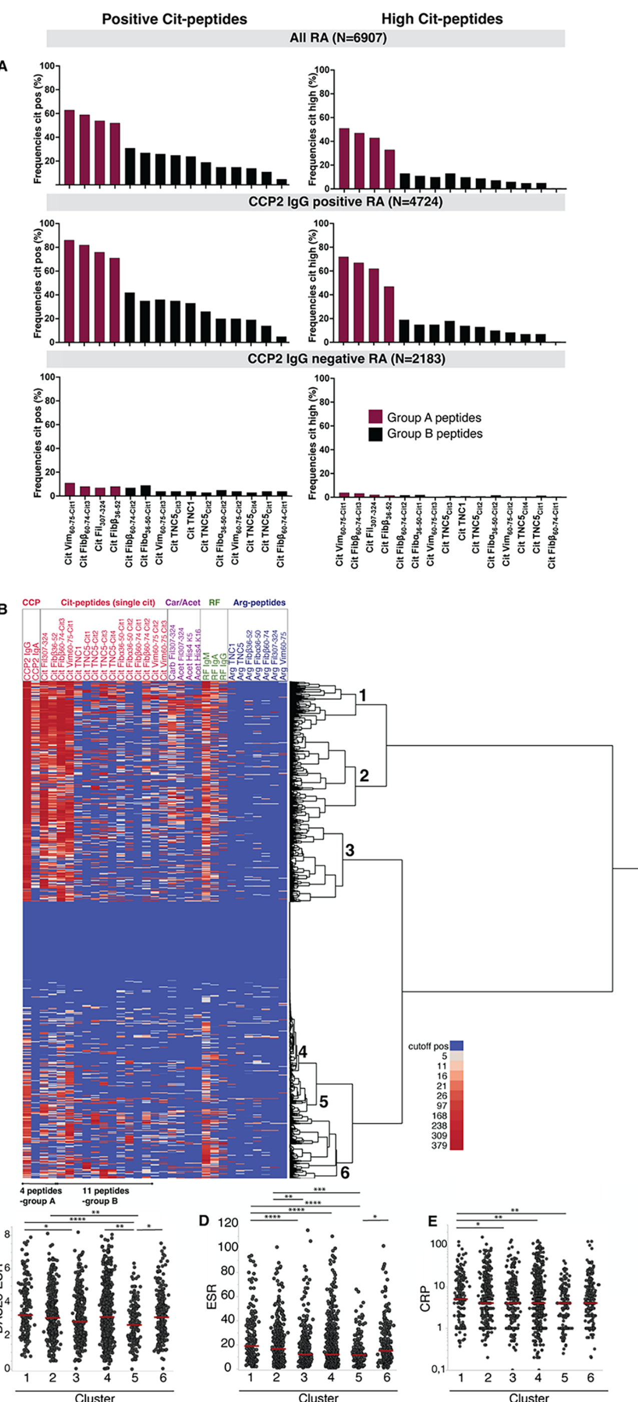

Figure 2. Autoantibody profiles in RA. (A) Frequencies of anticitrullinated protein antibody binding to different cit-peptides. Frequencies of positive cit-peptide signals (left) and high signals (right) in all patients with RA (top), IgG anti-CCP2–positive patients (middle), or IgG anti-CCP2–negative patients (low). "Group A peptides" with the highest frequency of positivity are highlighted in red. The cut-off for positivity was based on the 98th percentile of healthy controls. The cut-off for high signal was set to three times the positive cut-off. (B) Unsupervised hierarchical clustering illustrates patient subsets based on autoantibody reactivity patterns. Clustering by Ward method is shown for positive antibody signals in arbitrary units with the cut-off for positivity in the assays subtracted. The data include RF (IgM, IgA, and IgG), CCP2 (IgG and IgA), four peptides with carbamylated or acetylated sites (Carb/Acet), 15 peptides with single-citrulline sites, and seven arginine control peptides. Six distinct patient clusters could be identified as indicated. (C–E) Disease activity by DAS28, ESR (mm/h) and CRP levels (mg/L) by patient autoantibody profile cluster, including patients from the Swedish Rheumatology Quality registry biobank with available clinical data ±14 days from sampling. Statistical analysis with Kruskal-Wallis test with Dunn correction of multiple comparison. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. CCP2, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28-ESR, Disease Activity Score 28 with erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; RF, rheumatoid factor.

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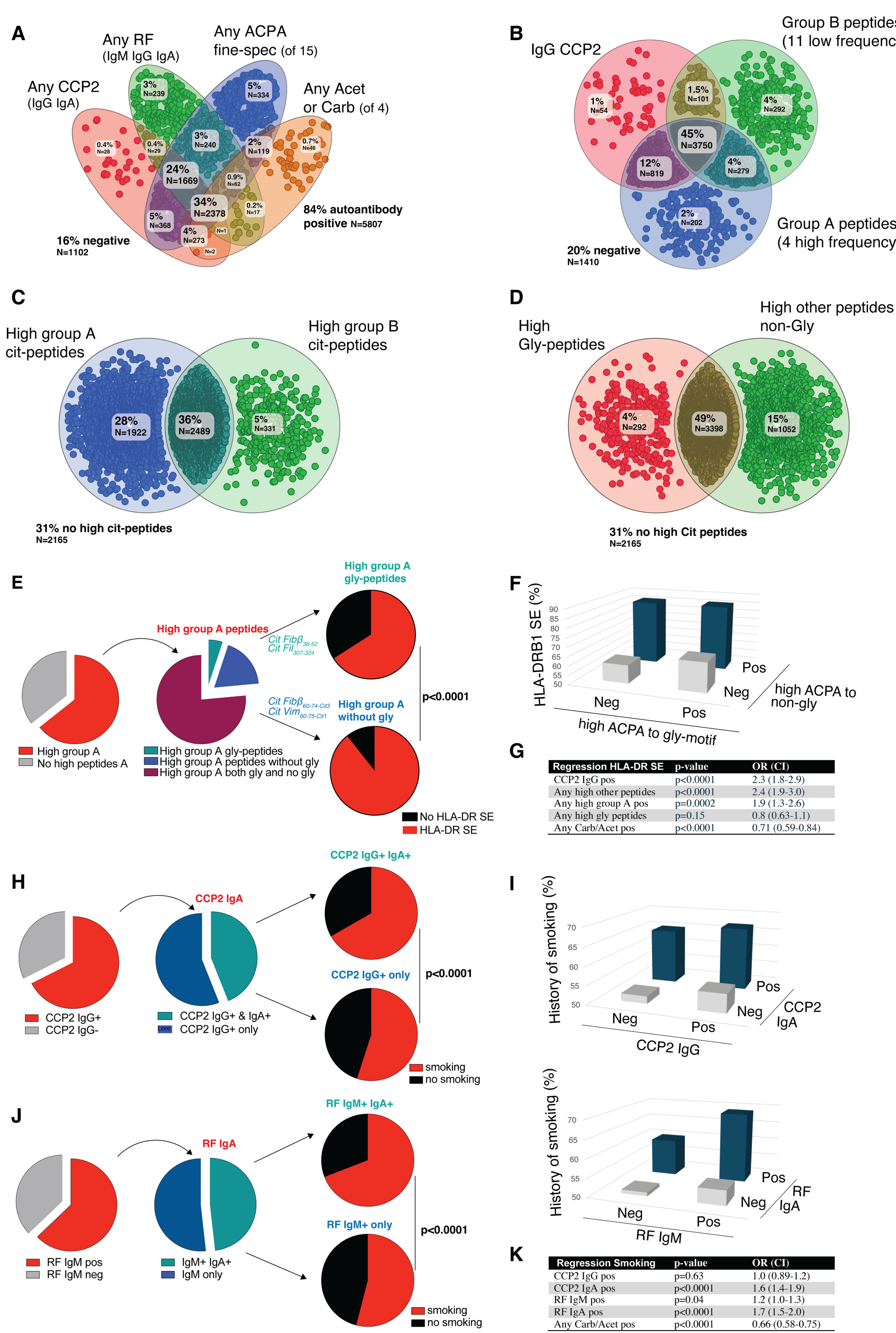

Regression HLA-DR SE	p-value	OR (CI)
CCP2 IgG pos	p<0.0001	2.3 (1.8-2.9)
Any high other peptides	p<0.0001	2.4 (1.9-3.0)
Any high group A pos	p=0.0002	1.9 (1.3-2.6)
Any high gly peptides	p=0.15	0.8 (0.63-1.1)
Any Carb/Acet pos	p<0.0001	0.71 (0.59-0.84)

Regression Smoking	p-value	OR (CI)
CCP2 IgG pos	p=0.63	1.0 (0.89-1.2)
CCP2 IgA pos	p<0.0001	1.6 (1.4-1.9)
RF IgM pos	p=0.04	1.2 (1.0-1.3)
RF IgA pos	p<0.0001	1.7 (1.5-2.0)
Any Carb/Acet pos	p<0.0001	0.66 (0.58-0.75)

Figure 3. Autoantibody subset and association with rheumatoid arthritis risk factors. Data is shown for 6,907 patients with rheumatoid arthritis. (A) Overlap between anti-CCP2 (IgG and IgA), RF (IgM, IgA and IgG), 15 ACPA fine specificities using single-citrulline peptides, and four antihomocitrulline (Carb) and anti–acetyl-lysine (Acet) reactivities. (B) Overlap between IgG anti-CCP2, four high-frequency ACPAs (group A peptides), and 11 low-frequency ACPAs (group B). (C) Overlap between group A and group B ACPAs. (D) ACPA reactivity to peptides with glycine motifs (cit-gly and gly-cit) compared with 11 peptides without glycine. (E) Analysis of HLA-DRB1 SE allele frequency based on high group A reactivity and reactivity to only gly-motif peptides (Cit Fib$\beta_{36\text{-}52}$ and Cit Fil$_{307\text{-}324}$) versus other nonglycine peptides (Cit Fib$\beta_{6F0\text{-}74\text{-}Cit3}$ and Cit Vim$_{60\text{-}75\text{-}Cit1}$). (F) Total frequency of HLA-DRB1 SE in patients based on high cit-motif reactivity (n = 5,387 with HLA data). (H and J) History of smoking in CCP2 IgG+ IgA+ versus CCP2 IgG+ only and RF IgM+ IgA+ versus RF IgM+ only, respectively. (I) Smoking in patients based on CCP2 IgG/IgA positivity or RF IgM/IgA positivity (n = 3,112 with smoking data). (G and K) Logistic regression analysis for (G) HLA-DRB1 SE or (K) smoking with parameters as categorical values. ACPA, anticitrullinated protein antibody; CCP2, cyclic citrullinated peptide; CI, confidence interval; OR, odds ratio (yes/no); RF, rheumatoid factor; SE, shared epitope.

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higher CCP2 levels (mean ± SD: 187 ± 204; median 98 vs 102 ± 135; median 46 AU/mL) (Supplementary Figure 8), but the average peptide signals on the array was slightly lower for gly-motif peptides (Supplementary Figure 9).

Patient clusters based on autoantibody profiles show differences in ACPA composition and disease activity. Unsupervised clustering with the Ward method including the 15 single-citrulline peptides, all isotypes of CCP2 and RF, and the homocitrulline and acetyl-lysine peptides illustrates the complex autoantibody profiles in the patients (Figure 2B; Table 2). Six main clusters of patients were identified, of which two captured patients with very high CCP2 IgG levels (clusters 1–2), one with moderate-to-high antibody levels (cluster 3), two with lower levels (clusters 5–6), and one cluster captured the seronegative group (cluster 4). The clusters also differed in CCP2 IgA positivity, Carb/Acet positivity, RF reactivity, and the proportion of different peptide subsets as well as HLA-DRB1 and smoking (Table 2; Supplementary Table 7). The cohort origin did not seem to affect the clustering (Supplementary Table 7). Notably, the patient subsets defined by the six clusters displayed significant differences in disease activity and inflammation by Disease Activity Score 28 (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Tender Joint Count (TJC), and Swollen Joint Count (SJC) but not in the Patient Global Assessment (PGA) or in reported pain. Of the individual components, ESR and TJC28 remained statistically significant across the clusters when adjusting for sex, age, and early DMARD-naive disease (Table 2). The subsets with moderate antibody levels had the lowest disease activity (cluster 5, average DAS28 = 2.8), and patients displayed higher RA disease activity in the high antibody cluster (cluster 1, average DAS28 = 3.6) as well as the seronegative cluster (cluster 4, average DAS28 = 3.3) (Figure 2C–E). No associations between comorbidities and antibody profiles were observed (Supplementary Table 8).

Importantly, the composition of ACPA fine specificities based on recognized cit-motifs was significantly different between the antibody-positive clusters. Regarding the ACPAs targeting different cit-motifs, we noted that even if clusters 1 and 2 had high multireactivity, the signals to the positive glycine-containing peptides were higher in cluster 2. The moderate antibody clusters 5 and 6 had a higher proportion of non-glycine cit-motif ACPA compared with others, but there were also differences in signal intensity for positive peptides between these two clusters so that glycine cit-motif signals were higher in cluster 5. In regression analysis, we found that having high reactivity to glycine cit-peptides or high reactivity to non-glycine cit-peptides independently correlated with patient clusters also when adjusting for CCP2 IgG levels, RF IgM levels, number of ACPA fine specificities, sex, and age. When instead dichotomizing the patients into four subgroups based only on ACPA fine specificity patterns of peptides with and without glycine cit-motifs (Figure 3), we found that the double positive group (gly and non-gly ACPA) displayed the highest disease activity by DAS28 followed by the gly-motif only group (Supplementary Figure 10 and Supplementary Table 9).

HLA-DRB1 SE alleles are more strongly associated with non-glycine cit-peptide ACPA specificities. The frequency of presence of SE allele positivity among patients with RA was 77% (Table 3). As expected, we observed a strong association between SE alleles and CCP2 IgG positivity, with 85% of CCP2 IgG+ patients carrying SE alleles (odds ratio [OR] = 3.9 95% confidence interval [CI] 3.4–4.5). There was a dose-dependent relationship in which patients with two copies of SE alleles had higher CCP2 IgG levels and a higher number of ACPA fine specificities (Supplementary Figure 11). RF and Carb/Acet reactivities were also associated with a higher frequency of SE alleles but had a lower OR (RF IgM OR = 2.2 95% CI 1.9–2.5; anti-Carb/Acet OR = 1.6 95% CI 1.4–1.8). However, if the patients were first stratified for CCP2 IgG positivity, so that CCP2 IgG+ patients with and without additional Carb or Acet reactivity were compared, there was no statistically significant association of Carb/Acet with SE alleles (Table 3), as most patients were concordant for SE and CCP2 IgG. Yet, patients that were CCP2 IgG negative but had high cit-peptide reactivity (n = 223) had a modest but significant increase in HLA SE allele positivity (69% vs 57%; P = 0.002; OR = 1.6 95% CI 1.2–2.2), which was diminished when excluding RF IgM positives (OR = 1.1 95% CI 0.75–1.7).

Importantly, there were striking differences in patient subsets based on ACPA fine specificities to glycine motif peptides or non-glycine ACPA in terms of association with SE alleles with the OR for non-gly motif ACPA positivity being twice as high as for ACPAs binding gly-motif peptides. To avoid bias by low-frequency ACPA fine specificities, we next compared patients with high group A peptide reactivity but with either only high non-gly motif ACPAs or only high gly-motif ACPA reactivity, and we found a significant difference in SE allele frequency in both the SRQb dataset (90% vs 62%; OR = 4.6, 95% CI 2.6–7.9) and the independent replication dataset from Danish and Norwegian patients (89% vs 67%; OR = 4.1, 95% CI 2.3–7.4). There was no difference in RF positivity between these groups. When the cohorts were combined, dichotomized for CCP2 IgG positivity, and all peptides (A and B) included in the analysis, the frequency of SE was 90% in CCP2 IgG+ patients with high non-gly motif ACPA versus 67% in CCP2 IgG+ patients with only high gly-motif ACPA (OR = 4.5, 95% CI 1.9–11.0; P = 0.0005). Regression analysis confirmed that high ACPA reactivity to gly-motif peptides did not associate with SE alleles in contrast with high ACPA to non-gly motif peptides (Figure 3).

Glycine motif ACPA are associated with a history of smoking. We also investigated ACPA fine specificity in relation to smoking. Patient-reported smoking status was available for 5,530 patients, showing a 58% overall history of cigarette

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Table 2. Autoantibody distribution in different patient clusters*

	Cluster 1 (n = 692)	Cluster 2 (n = 1,265)	Cluster 3 (n = 1,100)	Cluster 4 (n = 2,361)	Cluster 5 (n = 580)	Cluster 6 (n = 905)
Autoantibodies						
CCP2 IgG levels, mean ± SD	560 ± 104	575 ± 59	245 ± 118	6 ± 12	67 ± 45	82 ± 78
CCP2 IgG, n (%)	692 (100)	1,265 (100)	1,090 (99)	272 (16)	520 (90)	782 (86)
CCP2 IgA levels, mean ± SD	43 ± 28	34 ± 27	12 ± 16	2.2 ± 3.8	5.2 ± 9.2	6.7 ± 11
CCP2 IgA, n (%)	597 (86)	978 (77)	332 (30)	35 (1.5)	53 (9)	116 (13)
RF IgM levels, mean ± SD	164 ± 191	77 ± 100	48 ± 69	5.7 ± 11	27 ± 38	93 ± 107
RF IgM, n (%)	658 (95)	1,124 (89)	892 (81)	559 (24)	404 (70)	795 (88)
RF IgG levels, mean ± SD	65 ± 102	35 ± 42	24 ± 34	7.9 ± 15	16 ± 21	40 ± 70
RF IgG, n (%)	275 (40)	335 (26)	157 (14)	68 (2.9)	55 (9.5)	239 (26)
RF IgA levels, mean ± SD	41 ± 28	30 ± 26	24 ± 24	7.9 ± 13	18 ± 20	31 ± 27
RF IgA, n (%)	452 (65)	641 (51)	405 (37)	191 (8.1)	163 (28)	459 (51)
Carb/Acet, n (%)	623 (90)	935 (74)	573 (52)	280 (12)	184 (32)	303 (33)
High group A only, n (%)	165 (25)	572 (45)	550 (50)	161 (7)	325 (56)	148 (16)
High group B only, n (%)	1 (0.1)	14 (1)	14 (1)	135 (6)	46 (8)	121 (13)
High both A and B peptides, n (%)	527 (76)	674 (53)	509 (46)	71 (3)	178 (31)	529 (58)
High gly-peptides only, n (%)	1 (0.1)	43 (3)	80 (7)	62 (3)	79 (13)	26 (3)
High non–gly-peptides only, n (%)	3 (0.4)	56 (4)	186 (17)	247 (10)	213 (37)	347 (38)
High both gly and non-gly, n (%)	688 (99)	1,161 (92)	807 (73)	58 (3)	257/580 (44)	425 (47)
Ratio gly signal/non-gly, mean ± SD[a]	1.1 ± 0.43	2.7 ± 0.32	2.4 ± 1.7	2.9 ± 0.60	5.2 ± 0.53	1.1 ± 0.44
Cit Fibβ$_{36-52}$, n (%)	607 (88)	932 (74)	478 (43)	76 (1.7)	76 (13)	128 (14)
Cit Fil$_{307-324}$, n (%)	663 (96)	1,098 (87)	697 (63)	51 (2.1)	217 (37)	265 (29)
Cit Fibβ$_{60-74-Cit3}$, n (%)	665 (96)	1,097 (87)	830 (75)	95 (4)	245 (42)	317 (35)
Cit Vim$_{60-75-Cit1}$, n (%)	669 (97)	1,024 (81)	798 (73)	102 (4.3)	328 (57)	585 (65)
Number cit-peptide+ (of 15), mean ± SD	8.7 ± 2.6	6.6 ± 2.4	5.7 ± 2.4	1.0 ± 1.8	4.2 ± 2.2	5.3 ± 3.0
Summary autoantibodies						
	High CCP2 IgG	High CCP2 IgG	Moderate-high CCP2 IgG	Low antibody/ seronegative	Low CCP2 IgG	Moderate-low CCP2 IgG
	High CCP2 IgA	High CCP2 IgA	Moderate-low CCP2 IgA	–	Low CCP2 IgA	Low CCP2 IgA
	High cit-pep	Moderate-high cit-pep	Moderate cit-pep	–	Moderate cit-pep	Moderate cit-pep
	A and B; gly and non-gly	A>B; gly and non-gly	A>B; gly and non-gly		A>B; gly < non-gly	A and B; gly < non-gly
	High RF	High RF	Moderate RF	–	Moderate-low RF	High RF
	High Carb/Acet	Moderate Carb/Acet	Moderate Carb/Acet	–	Moderate-low Acet/Carb	Moderate-low Acet/Carb
Clinical features[b]						
DAS28-ESR, mean ± SD [n]	3.59 ± 1.6 [171]	3.26 ± 1.4 [371]	3.10 ± 1.5 [311]	3.30 ± 1.6 [660]	2.75 ± 1.3 [158]	3.2 ± 1.4 [227]
PGA, mean ± SD [n]	37 ± 27 [188]	34 ± 27 [410]	32 ± 26 [350]	36 ± 27 [735]	34 ±2 4 [34]	33 ± 26 [251]
SJC28, mean ± SD [n]	3.0 ± 4.0 [183]	2.4 ± 3.2 [406]	2.1 ± 3.6 [344]	2.6 ± 4.3[730]	2.2 ± 3.6 [170]	2.2 ± 3.6 [253]
TJC28, mean ± SD [n]	3.4 ± 5.0 [182]	2.6 ± 4.2 [406]	2.9 ± 4.3 [344]	3.5 ± 5.0 [730]	1.7 ± 2.8 [170]	2.6 ± 3.7 [253]
ESR, mean ± SD [n]	25 ± 21 [181]	21 ± 17 [388]	17 ± 17 [334]	18 ± 18 [697]	15 ± 13 [164]	20 ± 17 [238]
CRP, mean ± SD [n]	11 ± 16 [186]	9.7 ± 16 [409]	8.0 ± 14 [352]	9.1 ± 17 [740]	5.5 ± 6.6 [174]	8.4 ± 15 [250]
Pain, mean ± SD [n]	37 ± 27 [186]	34 ± 26 [407]	34 ± 28 [350]	37 ± 28 [730]	32 ± 23 [174]	34 ± 27 [251]

* CCP2, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28-ESR, Disease Activity Score 28 with erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; PGA, Physician Global Assessment; RF, rheumatoid factor; SJC28, Swollen Joint Count 28; TJC28, Tender Joint Count 28.
[a] Ratio of the average array signal for positive gly-motif peptides/average signal for positive non–gly-peptides. Only positive signals above cut-off were included in the calculation.
[b] Clinical parameter from the time of blood sampling ±14 days available in the Swedish Rheumatology Quality registry biobank (SRQb).

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Table 3. Association of autoantibody subsets with smoking and HLA-DRB1 SE alleles*

	Smoking			HLA-DRB1 SE					
Subgroup	Smoking, n (%)	*P* value[a]	OR [95% CI]	Swedish SE+ (77%, 2,055/2,652),[b] n (%)	*P* value[a]	OR [95% CI]	Norwegian / Danish SE+ (77%, 2,096/2,735),[c] n (%)	*P* value[a]	OR [95% CI]
CCP2 IgG pos	2,247/3,737 (60)	**<0.0001**	1.4 [1.2-1.5]	1,569/1,832 (85)	**<0.0001**	3.6 [3.0-4.4]	1,588/1,870 (85)	**<0.0001**	3.9 [3.3-4.8]
CCP2 IgG neg	939/1,793 (52)	–	–	509/820 (62)	–	–	508/865 (59)	–	–
Any CCP2	2,260/3,757 (60)	**<0.0001**	1.4 [1.2-1.5]	1,572/1,838 (85)	**<0.0001**	3.6 [2.9-4.4]	1,596/1,881 (85)	**<0.0001**	4.0 [3.3-4.8]
CCP2 neg	926/1,773 (52)	–	–	506/814 (62)	–	–	500/854 (59)	–	–
Any RF	2,225/3,632 (61)	**<0.0001**	1.5 [1.4-1.7]	1,458/1,762 (83)	**<0.0001**	2.1 [1.7-2.5]	1,539/1,894 (81)	**<0.0001**	2.2 [1.8-2.7]
RF neg	961/1,898 (51)	–	–	620/890 (70)	–	–	557/841 (66)	–	–
CCP2 IgG+ IgA	1,096/1,643 (67)	**<0.0001**	1.6 [1.4-1.9]	704/829 (85)	NS (0.74)	–	726/853 (85)	NS (0.85)	–
CCP2 IgG+ only	1,151/2,094 (55)	–	–	858/1,003 (86)	–	–	862/1,017 (85)	–	–
RF IgM+ IgA	1,159/1,677 (69)	**<0.0001**	1.9 [1.6-2.2]	679/832 (81)	NS (0.37)		715/906 (79)	**0.03**	0.69 [0.54-0.88]
RF IgM only	972/1,800 (54)	–	–	697/836 (84)	–	–	783/928 (84)	–	–
Any Carb/Acet	1,326/2,326 (57)	NS (0.44)	–	836/1,026 (82)	**<0.0001**	1.5 [1.2-1.8]	1,029/1,258 (82)	**<0.0001**	1.7 [1.4-2.1]
No Carb/Acet	1,860/3,204 (58)	–	–	1,219/1,626 (75)	–	–	1,067/1,476 (72)	–	–
CCP2 IgG, any Carb/Acet	1,224/2,115 (57)	**0.001**	0.8 [0.7-0.9]	781/930 (84)	NS (0.13)	–	973/1,160 (84)	NS (0.11)	–
CCP2 IgG, no Carb/Acet	1,023/1,622 (63)	–	–	781/902 (87)	–	–	615/710 (87)	–	–
Peptide A high	2,102/3,494 (60)	**<0.0001**	1.3 [1.2-1.5]	1,429/1,653 (86)	**<0.0001**	3.8 [3.1-4.6]	1,543/1,812 (85)	**<0.0001**	3.8 [3.1-4.6]
No peptide A high	1,084/2,036 (53)	–	–	626/999 (63)	–	–	553/923 (60)	–	–
Gly-peptide high	1,776/2,898 (61)	**<0.0001**	1.4 [1.2-1.5]	1,149/1,353 (85)	**<0.0001**	2.4 [2.0-2.9]	1,294/1,536 (84)	**<0.0001**	2.6 [2.2-3.2]
No gly-peptide high	1,410/2,632 (54)	–	–	906/1,299 (70)	–	–	802/1,199 (67)	–	–
Non–gly-peptide high	2,116/3,541 (60)	**<0.0001**	1.3 [1.1-1.4]	1,462/1,687 (87)	**<0.0001**	4.1 [3.4-4.9]	1,552/1,823 (85)	**<0.0001**	3.9 [3.2-4.7]
No non-gly high	1,070/1,989 (54)	–	–	593/965 (62)	–	–	544/912 (62)	–	–
Group A high, non-gly only	371/667 (56)	**0.03**	1.5 [1-2.1]	300/335 (90)	**<0.0001**	4.6 [2.6-7.9]	272/305 (89)	**<0.0001**	4.1 [2.3-7.4]
Group A high, Gly only	119/184 (65)	–	–	62/95 (65)	–	–	54/81 (67)	–	–

* Smoking status was available in the Swedish Rheumatology Quality registry biobank (SRQb) and Danish Nationwide Clinical Rheumatology Registry/Danish Rheumatologic Biobank (DANBIO/DRB). *P* values <0.05 are significant and are marked with bold font. CCP2, cyclic citrullinated peptide; CI, confidence interval; NS, not significant; OR, odds ratio; RF, rheumatoid factor; SE, shared epitope.
[a] *P* value and OR are from Fisher exact tests.
[b] SEs by Illumina Global Screening Array (deCODE Genetics).
[c] For the Danish Nationwide Clinical Rheumatology Registry/Danish Rheumatologic Biobank (DANBIO/DRB), Aiming for Remission in Rheumatoid Arthritis: A Randomised Trial Examining the Benefit of Ultrasound in a Clinical Tight Control regimen (ARCTIC), Norwegian Antirheumatic Drug Register (NOR-DMARD), and Ultrasound in Rheumatoid Arthritis patients starting Biologic Treatment (ULRABIT), SE by Thermo Fisher Scientific customized genotyping.

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smoking including both previous and current smokers. Although there was a significant association of CCP2 IgG positivity as well as group A cit-peptide positivity with smoking, it was rather modest. Intriguingly, patients with only high gly-motif ACPAs (including both A and B peptides) had a slightly higher frequency of a smoking than patients with only high non-gly motif ACPAs (65% vs 56%; OR = 1.4, 95% CI 1–2.0; *P* = 0.02).

We also used IgA positivity as a comparison in the analysis to ascertainthe robustness of the smoking data. Indeed, we could validate a significant association between a history of cigarette smoking and IgA positivity, in line with previous reports.[7,30] This was particularly striking when dichotomizing patients based on CCP2 IgA+ IgG+ double positivity compared with CCP2 IgG alone in which 67% versus 55% of the patients had a history of smoking, respectively (Table 3). A similar association was found for IgA RF. Here, we dichotomized RF IgM+ IgA+ double positivity and compared it with RF IgM+, for which 69% versus 54% had a history of smoking. Logistic regression analysis showed an independent association for positivity of CCP2 IgA (OR = 1.6, 95% CI 1.4–1.9) and RF IgA (OR = 1.7 95% CI 1.5–2.0) to smoking status (Figure 3).

DISCUSSION

In this comprehensive serologic effort, we provide a granular profiling of autoantibodies in RA and demonstrate that ACPAs have preferential binding to small citrulline-containing epitopes, which is reflected in differential binding to single-citrulline peptides originating from the same peptide backbone. Our harmonized dataset from 6,900 patients enabled us to investigate the association of different ACPA subsets with RA risk factors. Strikingly, we found significant differences in HLA-DR SE allele association with fine specificities targeting nonglycine compared with glycine peptide epitopes. We could confirm an association of IgA autoantibody positivity with a history of cigarette smoking.[7,30,31] The observed associations may provide insights into how exposure to different classes of citrullinated antigen drives specific ACPA reactivity and the T cell dependance of such responses.

A complete lack of HLA association would indicate T cell–independent responses, and the low association for glycine-containing peptides, could implicate differences in T cell dependence in epitopes spreading between different ACPA responses. In line with our data is a report of difference in HLA SE allele association for ACPAs targeting a peptide derived from vimentin (no glycine peptide) compared with a peptide from fibrinogen (citrulline-glycine motif peptide),[32] but at the time this was not attributed to glycine motifs. Glycine, a small nonpolar amino acid, is often found in flexible or disordered protein regions. Cit-gly motifs are common in identified ACPA antigens,[33] including in the filaggrin cfc1 peptide. Furthermore, large unbiased peptide array screenings have confirmed a high prevalence of glycine adjacent to the citrulline in ACPA targets.[6,28] Interestingly, arg-gly motifs have also been suggested to be preferential substrates for protein arginine deiminase 2 (PAD2) and PAD4 enzymes.[34,35] However, it remains to be elucidated if cit-gly citrullination is more dominating during physiologic conditions compared with during RA-associated hypercitrullination. Because T cell and B cell epitopes generally do not overlap, it is difficult to directly extrapolate the implications for antigen processing, presentation, and T cell tolerance. Moreover, further genetic association analysis is needed to determine if the gly-motif ACPAs may have an association with other major histocompatibility complex class II HLA alleles outside of the DRB1 or SE family. Similarly, we cannot currently deduce the biologic or temporal relation between the gly and non-gly cit-reactivities in the B cell response.

As expected based on previous studies, the inclusion of multiple antibody tests, including both RF and CCP2 isotype detection and different modified peptides, increased the sero-positive group and identified autoantibody positive patients within the classical seronegative subgroup (CCP2 IgG−, and RF IgM−).[7,17,36] Yet, for clinical applications, multipeptide tests needs to be carefully redesigned and further evaluated to reduce the false discovery rate and increase the specificity for the combination of assays. Here we use the peptide array primarily to investigate ACPA patterns in patients with RA. Overall, the investigated ACPA fine specificities were relatively stable between cohorts despite potential differences in the inclusion criteria. Our novel panel of cit-peptides showed improved discrimination between different ACPA fine specificities. Moreover, different patient subsets could be identified in unsupervised clustering, which were also characterized by RF, CCP2 IgA, and Carb/Acet autoantibody levels. However, the clinical importance of these different autoantibody profiles remains to be investigated.

Owing to the multireactive nature of ACPAs, all investigated cit-peptides should be considered pseudoantigen capturing a certain ACPA reactivity pattern. They may not represent epitopes that are exposed in the citrullinated proteins, or the affinity of the ACPA may be higher to other cit-peptides/proteins expressing a similar motif that could be the primary targets. With that being said, the high frequency of binding to the peptide Cit Fib$\beta_{60\text{-}74\text{-}Cit3}$ with a C-terminal citrulline is particularly interesting given the suggested link between RA and the periodontal pathogen *Porphyromonas gingivalis*.[37] *P. gingivalis* expresses a PAD enzyme that preferentially citrullinate C-terminal arginine.[38]

Our data further support that ACPA specificity is determined by only a few amino acids adjacent to the citrulline. However, in several cases we could observe that one of the single-cit peptides had a higher frequency of positivity than did the original peptide with multiple citrullines. This could indicate that an arginine-to-citrulline conversion, resulting in a change from a positive to a neutral charge, outside of the dominating epitope, are reducing binding. This observation may suggest a more conformational component at play. The biochemical design of synthetic peptides (eg, biotin position and linker length in biotinylated peptides) have

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also been shown to impact binding.[39] Nevertheless, available co-crystal structures of ACPA with cit-peptides show a hapten-like binding to the citrulline and more limited direct interactions with other side chains,[40,41] but certain amino acids or structures are hypothesized to sterically repulse binding.

Interestingly, the panel of 33 patient-derived monoclonal antibodies that were used for evaluations represented the serological profiles well. We observed high binding to the same preferential cit-peptides and similar binding patterns as in the patient antibody serology. Considering the multireactivity of ACPAs, it could be that patients with RA have a rather limited number of circulating ACPA clones that explains their ACPA fine specificity profiles. Indeed, a recent mass spectrometry study shows that the IgG1 ACPA response is unique to each patient and dominated by a small number of clones.[42]

Evidence links the risk of RA with smoking and other lung exposures, which implicates a central role for mucosal immunity.[3] We found that autoantibodies of the IgA isotype, both CCP2 IgA and RF IgA, were particularly strongly associated with a history of smoking, as previously reported.[7,31] However, serum IgA's direct link to mucosal responses is complex. Serum Igs are mostly expressed by long-lived cells in the bone marrow, but IgA-expressing cells can originate from prior mucosal responses.[43] IgA ACPA positivity is infrequent in IgG negative individuals and show a concordance with high IgG ACPA levels, IgG positivity for multiple cit-peptides, and IgG anti-Carb/Acet reactivity. Because human B cells class-switch from IgG to IgA, it can be hypothesized that smoking drives a strong chronic response resulting in epitope spreading and IgG to IgA class-switching.

Anti-Carb and anti-Acet autoantibodies were found in a subset of ACPA+ patients with RA and did not show any further association with SE or smoking when adjusting for CCP2 positivity. Nevertheless, this subset of patients is interesting because autoreactivity to multiple modifications (Cit, Carb, or Acet) have previously been shown to be associated with more aggressive disease and higher risk of relapse after treatment.[12,44,45] In this study, we used modified filaggrin peptides to detect Carb and Acet binding in combination with acetylated histone 4. Although the histone 4 peptides represent natural lysine sites, which we have previous found to be acetylated and targets for multireactive ACPAs,[14] the filaggrin peptides are more artificial with the arginine/citrulline residue replaced with acetyl-lysine or homocitrulline. Hence, these peptides were similarly constructed as previously reported modified vimentin (mod-Vim58-69; Orgentec Diagnostika),[7,12] but because the peptide motif is different they may capture a slightly different subset than mod-Vim. Indeed, CCP2+ ACPA mAbs were found to be restricted in their cross-reactivity to these peptides depending on the peptide backbone (Supplementary Figure 2).

The use of multiple autoantibody tests distinguishes different ACPA profiles. We propose that different ACPA fine specificity combinations may be important for disease pathogenesis. Indeed, patients with different autoantibody patterns either based on unsupervised clustering or ACPA fine specificity subtypes displayed statistically significant differences in disease activity components, in particular ESR and TJC. Patients with multipeptide reactivities had higher disease activity than patients with fewer autoantibodies, but also a subset of seronegative patients had high activity. In model systems, ACPAs have been suggested to be directly involved in arthritis pathogenesis by interactions with different synovial cells (eg, neutrophils and osteoclasts) and by inducing pain, tenosynovitis, and bone loss.[14,29,46–48] Intriguingly, certain human ACPA clones have also been observed to have anti-inflammatory and antiatherogenic properties in experimental murine models.[49–51] Hence, different subsets of ACPAs are hypothesized have different functionality depending on their antigen-recognition.

In summary, we demonstrate that redefining ACPA fine specificity based on their recognition motif, rather than individual peptides, reveal distinct HLA-DR associations that provide essential indications about the B cell and T cells citrulline responses. Moreover, mapping the autoantibody response accelerates our understanding about seropositive disease and patient subsets.

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AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Grönwall confirms that all authors have provided the final approval of the version to be published, and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

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