Different Hierarchies of Anti–Modified Protein Autoantibody Reactivities in Rheumatoid Arthritis

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Objective. Anti–citrullinated protein antibodies (ACPAs) are a hallmark of seropositive rheumatoid arthritis (RA). Yet, the precise disease-relevant autoantigens that are targeted by ACPAs remains a matter of debate. This study utilized patient-derived monoclonal ACPAs, rather than serum autoantibody analysis, to characterize the multireactivity to different protein modifications and to reveal autoantibody subsets in patients with RA.

Methods. Twelve human monoclonal ACPAs (positive by the second-generation cyclic citrullinated peptide test) were generated from 6 RA patients, and a head-to-head comparison of their reactivities was performed. For profiling, we used a complementary DNA–based protein array (Engine GmbH) and 3 peptide-screening platforms with RA autoantigens (Thermo Fisher Scientific), citrullinated and carbamylated peptides (NimbleGen/Roche), or histone-derived peptides with different posttranslational modifications (JPT Histone Code), covering >207,000 peptides (>7,800 gene products).

Results. The fine-specificity profiles of the investigated ACPAs varied, but all of the monoclonal ACPAs displayed multireactivity to a large number of citrullinated peptides/proteins, each characterized by specific binding properties. ACPA subsets could be defined by clone-distinct consensus binding motifs (e.g., Cit–Gly, Gly–Cit, or Arg–Cit–Asp), with the most common ACPA recognition being that of a Gly in the +1 flanking position, but with additional amino acid preferences. For ACPA protein recognition, we observed a preference for citrullinated RNA-binding proteins with high Arg/Gly content. Six of the 12 ACPA clones also bound acetylated-lysine (KAc) or homocitrulline peptide motifs, displaying a similar affinity or higher apparent affinity than that for Cit peptides.

Conclusion. ACPAs and anti–modified protein autoantibodies represent overlapping facets of RA autoimmunity and bind to a wide variety of modified proteins, extending well beyond the historically recognized set of RA autoantigens. So far, KAc reactivity has been detected only in the context of anti–Carb and anti–Cit peptide autoantibody responses, postulating the existence of hierarchies of autoreactivity in RA. Future investigations of ACPA fine specificities and functionality should take into consideration the presence of consensus Cit/Carb/KAc motifs and the multireactivity of these autoantibodies in patients with RA.
seropositivity (i.e., when the patient is positive for ACPAs and/or rheumatoid factor (RF)) may also, to some extent, instruct the choice of therapy.

The origin and contribution of autoantibodies to the development of RA represent a conundrum, given the well-replicated observation that positivity for both ACPAs and RF can occur many years before the emergence of any signs of arthritis (2–4). However, these autoantibodies have been implicated in the development of both noninflammatory and inflammatory symptoms of the disease (for review, see ref. 5). Thus, there has been a mounting interest in studying ACPAs, e.g., analyzing serum from large, well-defined patient cohorts (4,6–9) and using purified patient autoantibodies (10,11) for downstream studies. As demonstrated by many different investigators over the years, ACPAs from RA patients are known to recognize a broad range of citrullinated proteins and peptides, including both ubiquitous antigens and inflammation- and joint-specific targets (12–17). Autoreactivities to other posttranslational modifications (PTMs), in addition to reactivity to citrullinated peptides, have also been reported (18–20).

In order to understand the evolution, specificity, and functionality of different autoantibodies, our studies, in addition to those of other investigators, have focused on analysis of paired heavy- and light-chain immunoglobulin sequences from single B cells from different compartments from patients in different stages of RA development, and generation of monoclonal antibodies (mAb). For several of these monoclonal ACPAs, unique functional activities relevant to the pathogenesis of RA have been demonstrated (21–24). However, the relationship between their target antigen specificities and their functional effects remains unclear. Therefore, a deeper understanding of the binding reactivities to these modified proteins could serve to define the most relevant autoantigen targets in RA.

Thus, in the current study, we used novel array platforms, which together cover a total of 7,898 antigens, to study a set of 12 human citrulline–binding recombinant monoclonal ACPAs generated from 6 RA patients. We evaluated the extensive multi-reactivities of these ACPAs, extending well beyond the current list of recognized autoantigens, and defined different subsets of ACPAs based on their consensus-recognition epitopes. We also delineated the relationship of autoreactivity to different PTMs (i.e., citrullination, carbamylation, and acetylation) and classified the PTM peptide reactivities in a hierarchy based on ACPA subsets. Multireactivity of ACPAs to acetylated peptides/proteins was found only in the context of reactivity to citrulline and homocitrulline.

**MATERIALS AND METHODS**

**Expression and quality control of monoclonal ACPAs.** The included ACPA mAb have been described previously and have been verified to have citrulline reactivity down to a concentration of at least 100 ng/ml (22,24–26). All recombinant ACPA mAb were expressed as human IgG1 in Expi 293 cells (Life Technologies), and the mAb were purified with protein G resin (GE Life Sciences). The critical steps involved in the cloning and expression of these mAb have been outlined by us in a recent report (27). In brief, quality control tests of the purified IgG included use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, size-exclusion chromatography with a fast protein liquid chromatography system for ensuring nonaggregated IgG (>90% monomeric), polyreactivity testing (binding to insulin, double-stranded DNA, and lipopolysaccharide [LPS]), Limulus amebocyte lysate endotoxin tests (<5 EU/mg IgG), and validation by enzyme-linked immunosorbent assay (ELISA) with titration of IgG (5,000–50 ng/ml) for comparison of Cit-containing peptides to Arg-containing peptides (22,24). In this study, an additional polyreactivity assay was included that utilizes the soluble membrane protein fraction from HEK 293 cells (fractionated using Proteo-Extract subcellular proteome extraction kit; Calbiochem) coated at 5 μg/ml in carbonate buffer to ELISA high-binding wells, for evaluation of ACPA mAb IgG at 5 μg/ml in phosphate buffered saline–0.1% bovine serum albumin.

**RA autoantigen array.** The ACPA mAb were evaluated in autoantigen arrays at concentrations of 25 μg/ml and 5 μg/ml, using a previously described custom-made multiplex solid-phase microarray platform (Thermo Fisher Scientific–ImmunoDiagnostics) containing citrullinated peptides that have been previously recognized to be RA autoantigens, as well as control antigens (native peptides and autoantigens targeted in other autoimmune diseases) (8,22). Purified polyclonal anti-CCP2+ ACPAs were evaluated in the array at a concentration of 10 μg/ml.

**Peptide array.** We utilized a custom-designed peptide array (Roche NimbleGen) that contained a total of 53,019 citrulline and 49,022 homocitrulline in situ–synthesized peptides and their corresponding native arginine or lysine peptides. The polyclonal anti-CCP2+ ACPA preparations were investigated at a concentration of 15 μg/ml, and the monoclonal ACPA preparations were investigated at a concentration of 1 μg/ml, as previously reported for a few clones (22). The 16-mer peptides covered a total of 1,439 extracellular matrix and RA-related proteins. In these arrays, the established cutoff value for positivity (i.e., positive binding signal) was defined as 5 times the 98th percentile of fluorescence intensity (FI) values for all peptides in control experiments with a set of non-ACPA mAb (FI >1,000 arbitrary units [AU] for citrullinated peptides, and FI >850 AU for carbamylated peptides). We utilized the Weblogo server (https://weblogo.berkeley.edu/logo.cgi) to illustrate the antibody consensus binding motif from all positive peptides (with citrulline/homocitrulline residues) that were devoid of any native peptide background binding. For analyses of the consensus binding motifs, we excluded peptides with fewer than 4 flanking C-terminal or N-terminal residues (compared to the structure of the citrullinated/carbamylated peptides), whereas in all other analysis steps, all peptides with a positive signal in the peptide arrays were
Table 1. Summary of ACPA mAb reactivities to common RA Cit peptide antigens*

| Cit peptide | ACPA clone | Peptide sequence |
|-------------|------------|-----------------|
|              | 14CFCT     |                |
|              | 37CEP      |                |
|              | 37CEP      |                |
|              | 1325:04C00 |                |
|              | 1325:01B09 |                |
|              | 1325:05C06 |                |
|              | 14CFCT     |                |
|              | 2H12       |                |
|              | 07E07      |                |
|              | BVCA1      |                |
|              | 2D09       |                |
|              | 1E04       |                |
|              | 1003:      |                |

**Group 1**

|            | ACPA clone | Peptide sequence          |
|------------|------------|---------------------------|
|            | Fiba30-50  | GP(Cit)VVE(Cit)HQSAKDS     |
|            | Fiba30-50  | APPPISGGYRA(Cit)PAKAAT     |
|            | Fiba30-50  | APPPISGGY(Cit)ARPAAAT      |

**Group 2**

|            | ACPA clone | Peptide sequence          |
|------------|------------|---------------------------|
|            | Fiba30-50  | ST(Cit)SVSSSSY(Cit)MFGG    |
|            | Fiba30-50  | VYAT(Cit)SSAV(Cit)L(Cit)SSVP |
|            | Fiba30-50  | (Cit)PAPPISSGGY(Cit)(Cit) |

**Group 3**

|            | ACPA clone | Peptide sequence          |
|------------|------------|---------------------------|
|            | Fiba30-50  | SHQEST(Cit)GRSRGRSGRSGS   |
|            | Fiba30-50  | SKQFTSSTSY(Cit)GDFTESKS   |
|            | Fiba30-50  | KIHA(Cit)EFD5(Cit)GNTVE   |
|            | Fiba30-50  | (Cit)SHAKS(Cit)PV(Cit)STHTS |
|            | Fiba30-50  | NEEFFSA(Cit)GHRPLDKK      |
|            | Fiba30-50  | HHPGAEFPS(Cit)KSSSSYKQF   |

* Results are shown for a selection of citrulline peptides analyzed on the Thermo Fisher Scientific rheumatoid arthritis (RA) autoantigen array platform. The monoclonal anti-citrullinated protein antibody (ACPA) clones are ordered by number of peptides that showed a positive signal for binding to each monoclonal antibody (mAb). The peptides are ordered into reactivity groups 1–3. Citrullinated o-enolase peptide 1 (CEP-1) and filaggrin (Fil307–324) peptides were analyzed as autocyclic, while all other peptides were analyzed as linear. Glycine (G) in position +1 or −1 as compared to citrulline is underlined in the peptide sequence. Fib = fibrinogen; Vim = vimentin.

† Represents the number of positive citrulline peptides of 18 analyzed. The total peptide number also includes 6 type II collagen citrulline peptides (data not shown).
included. Detailed information on the peptides in these arrays is provided in the Supplementary List of Antigens in Array Platforms (available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

Protein macroarray. The hEXselect macroarray platform (Engine GmbH), consisting of 20,776 Escherichia coli on-array expressed protein fragments from 6,885 genes (with 1–37 fragments per gene) from human fetal brain complementary DNA (cDNA), was used to citrullinate proteins with 15 μl/ml rabbit skeletal muscle peptidyl arginine deiminase (PAD) (Sigma-Aldrich). ACPA mAb binding was detected at a human IgG concentration of 1 μg/ml, and purified anti-CCP2 polyclonal antibody binding was detected at a concentration of 10 μg/ml. Alkaline phosphatase–conjugated anti-human IgG-Fc with Atto phosph substrate was used for detection of protein binding, with the results expressed as the spectral FI, which was scored on a scale of 0–3 utilizing a visual scoring (VS) system with a visual grid software. All scoring was performed by a single individual at the company site. Clones with in-frame protein expression (>90% homology with gene products in the UniProt or NCBI databases, based on protein BLAST alignment; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and with a VS score of 2 or 3 were considered positive hits. Detection of the N-terminal RGS-His6-Tag was used as an expression control on a separate array. Classification of positive proteins by molecular function and cellular compartment was performed using the Panther classification web service (http://www.pantherdb.org).

Histone Code peptide array. The ACPA mAb were analyzed at a concentration of 1 μg/ml on a Histone Code peptide microarray (JPT Peptide Technologies) at the company site in Berlin, Germany. The array library contains 20-mer peptides presenting histone modifications, assessed in triplicate, from all histone isoforms, including H2A (15 isoforms), H2B (15 isoforms), H3 (5 isoforms), and H4 (1 isoform). Single modifications of lysine (KAc, Kme1, Kme2, Kme3, Kbut, Kprop, Kmal, and Ksuc) and arginine (Rme1, Rme2a, Rme2s, and Cit), and phosphorylation of threonine, serine, and tyrosine were incorporated, generating 2,819 peptides. When native peptides and peptides with multiple modifications were included, the resulting total number of peptides was 3,873. The cutoff for positivity was based on the values for control mAb, and set to 3 times the 98th percentile of FI values for all peptides in the control experiments.

ELISA evaluation of mAb binding. Detailed protocols for the validation ELISA, modified-peptide competition studies, and ELISA chaotropic salt-elution experiments are provided in the Supplementary Methods (available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

RESULTS

Broad multireactivity of monoclonal ACPAs from different anatomic compartments and different patients.

We conducted a systematic head-to-head comparison of the binding profiles of 12 previously reported monoclonal ACPAs (22,24–26). The mAb, derived from single B cells from 6 RA patients, were generated from different compartments (blood versus synovial fluid) and different B cell subsets (memory B cells versus antibody-secreting cells), and all 12 mAb were, to a large extent, encoded by different V(D)J rearrangements (for details on the ACPA clones, see Supplementary Table 1, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). All 12 monoclonal ACPAs displayed high levels of somatic hypermutation (SHM), contained SHM-introduced Fab glycosylation sites, and reacted with several Cit-containing peptides when evaluated using an array with 18 Cit peptides derived from RA candidate autoantigens (8) (Table 1) (further details are provided in Supplementary Figure 1 and Supplementary Table 2, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

All of the ACPA mAb demonstrated positive reactivity in the clinical CCP2 or CCP3 assays, while they generally did not have any significant binding to Arg-containing peptides and control antigens, nor did they show any nonspecific “stickiness” or background reactivity (i.e., characteristics of polyreactivity) (see Supplementary Table 2 and Supplementary Figure 2, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). Interestingly, 1 clone, mAb 1003:10A01, bound CCP2 but not CCP3. Many of the traditionally used RA peptides from fibrinogen, flaggrin, α-enolase, and type II collagen share similar features (e.g., a Cit–Gly site), and thereby capture the reactivity of the same antibody clones. Cit-vimentin peptides, which lack these features, capture a different subset of mAb with more restricted binding patterns, and yet other mAb display multireactivity that also extends between these peptide groups (Table 1, and Supplementary Figure 1 [http://online library.wiley.com/doi/10.1002/art.41385/abstract]).

Distinct consensus motifs explaining the differences in ACPA citrulline multitargeting. To further compare the ACPA subsets, we used a large-scale custom-designed peptide array platform (NimbleGen Roche) and investigated binding to 53,019 Cit-containing peptides and corresponding Arg-containing peptides (Figures 1A–F and Table 2). We found that a majority of the 12 ACPAs indeed bound large numbers of the Cit-containing peptides (ranging from 75 to 3,275 peptides) derived from extracellular matrix proteins and known RA autoantigens, and exhibited significantly lower or undetectable binding to Arg-containing peptides. Control mAb showed negligible binding (control results are shown in Supplementary Figure 3, available on the Arthritis & Rheumatology website at
as expected, affinity-purified polyclonal anti-CCP2 IgG from RA patient plasma (10) reacted with a large number of citrulline peptides, with binding patterns similar to those displayed by some of the monoclonal ACPAs, but with lower binding signals than several of the mAb (Figure 1A and Supplementary Figure 4, available on the Arthritis & Rheumatology website at http://onlineibrary.wiley.com/doi/10.1002/art.41385/abstract).

We noted that both the number of positive peptides and the average binding intensities differed between the individual ACPA clones, and found that by using the peptides that were positive for ACPA binding, we could derive the consensus Cit-containing
| Table 2. Summary of ACPA mAb reactivities using multiple screening platforms* |
|---------------------------------|
|                      | Total no. of peptides/ proteins assessed | mAb clone 1325:01B09 syn-ASC | mAb clone 1325:04C03 syn-ASC | mAb clone 1325:05C06 syn-ASC | mAb clone 1325:07E07 syn-ASC | mAb clone 37CEPT2C04 syn-ASC | mAb clone 37CEPT1G09 syn-ASC | mAb clone 14CFCT3G09 syn-ASC | mAb clone 37CEPT1G09 syn-ASC | mAb clone 37CEPT2C04 syn-ASC | mAb clone 1003:10A01 syn-ASC | mAb clone 14CFCT2D09 syn-ASC | mAb clone 14CFCT2H12 syn-ASC | mAb clone 1325:04C03 syn-ASC | mAb clone BVCA1 p- mB | Anti-CCP pool† |
| CCP2 assay           | –                                  | 1,460                        | 1,170                        | 2,790                        | 670                        | 300                        | 2,350                        | 2,350                        | 310                        | 2,400                        | 370                        | 350                        | 790                        | –                              |                          |
| CCP3 assay           | –                                  | 370                        | 340                        | 310                        | 520                        | 320                        | 370                        | 520                        | 430                        | 520                        | 340                        | 0                        | 550                        | –                              |                          |
| RA autoantigen array†| Pos. RA Cit peptides               | 18                          | 10                          | 12                          | 10                          | 5                          | 15                          | 15                          | 16                          | 4                          | 9                          | 3                          | 2                          | 4                              | –                              |
| NimbleGen array      | Pos. Cit peptides†                 | 53,019                      | 2,828                      | 3,047                      | 2,225                      | 2,611                      | 3,275                      | 1,145                      | 4,933                      | -938                      | 1,810                      | 75                          | 75                          | 398                           | 10,548                        |
| RV2 S pair           | Pos. Arg peptides (Arg+Cit)‡       | 318                          | 12                          | 0                            | 2                          | 53                          | 142                         | 1                          | 0                          | 10                        | 2                          | 428                         | 302                         | 418                           | 348                           |
| Primary Cit motif    | –                                  | -Cit-G                      | G-Cit-                     | -Cit-G                      | Complex                    | -Cit-G                      | -Cit-G                      | -Cit-G                      | -Cit-G                      | A/V-Cit-X-R                 | -Cit-N-G                    | R-Cit-D                     | Cit-G                         | –                              |                          |
| Primary Carb motif   | –                                  | -Carb-G                     | NA                          | G-Carb-                     | <50                        | -Carb-<xxx-K                | -Carb-<50                   | -Carb-<50                   | -Carb-<50                   | L-xxx-Carb                  | Carb-D                       | Cit-G                         | –                              |                          |
| Engine macroarray    | Total pos. Cit proteins‡          | 20,776                      | 1,790                      | 402                          | NA                         | NA                         | 792                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                            | 674                           |
| Total pos. native proteins | 20,776                  | 73                        | 19                          | NA                          | NA                         | 22                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                            | –                              |
| Pos. in-frame Cit proteins | NA                      | 993                        | 144                          | NA                          | NA                         | 389                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                            | 304                           |
| Pos. in-frame native [Nat+Cit+] | NA                | 3621                     | 73                          | [3]                         | [0]                        | 2811                       | 4219                       | 16                          | 31                         | 10                          | 4                          | 41                          | 70                          | 161                           | 60                            |
| VS 1 in-frame Cit   | –                                  | 168                        | 48                          | NA                          | NA                         | 210                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                            | 150                           |
| VS 2 in-frame Cit   | –                                  | 725                        | 82                          | NA                          | NA                         | 111                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                         | NA                            | 89                            |
| JPT Histone Code array | Pos. Cit peptides#             | 132                        | 2                            | 18                          | NA                          | 4                           | 6                           | 18                          | 17                         | 0                          | NA                         | 0                          | 0                          | 8                              | –                              |
| Pos. KAc peptides# | 314                          | 12                        | 0                            | NA                          | 2                           | 53                          | 142                         | 1                          | 0                          | NA                         | 0                          | 2                          | 0                              | –                              |
| Pos. Arg/Lys peptides§ | 318                       | 0                         | 0                            | NA                          | 0                           | 0                           | 10                          | 0                          | 0                          | 0                          | 0                          | 0                          | 0                              | –                              |
| Primary histone motif | –                               | K-Ac-G                     | R-xox-Cit                  | Cit-                         | -K-Ac-R                    | -KAc                         | -Cit-<8                     | NA                         | <8                         | <8                         | Cit-<DM                         | –                              | –                              | –                              |

* Values are the number of peptides/proteins with a positive (pos.) signal for reactivity with the indicated monoclonal antibody (mAb) clones derived from antibody-secreting cells (syn-ASC), citrullinated α-enolase peptide tetramer–positive memory B cells (CEP+ mB), citrullinated filaggrin cyclic peptide tetramer–positive memory B cells (CFC+ mB), synovial memory B cells (syn-mB), and peripheral memory B cells (p-mB), as determined using a second-generation or third-generation anti–cyclic citrullinated peptide antibody assay (CCP2 and CCP3, respectively; each in RU/ml at 5 μg/ml), the Thermo Fisher Scientific rheumatoid arthritis (RA) autoantigen array, the NimbleGen peptide array, the Engine macroarray, and the JPT Histone Code array. NA = not applicable; VS = visual score; KAc = acetylated lysine.
† Comprising polyclonal anti–citrullinated protein antibodies (ACPAs) purified from plasma using a CCP2 affinity column.
‡ Single-positive citrulline (Cit) or homocitrulline (Carb) peptides and no reactivity to native Arg/Lys peptides (fluorescence intensity [FI] cutoff >1,000 arbitrary units [AU] for Cit, and FI >850 AU for Carb).
§ All positive Arg or Lys peptides (peptides double-positive in both Arg/Cit or both Lys/Carb format).
¶ Values are the theoretical fragment number, and all may not be expressed to an adequate level; approximately one-third should theoretically be in the correct frame.
# Cutoff FI based on 3 times the 98th percentile of FI values for control IgG (FI 10,000 AU) on the Cit/Carb array. The same FI level was used as the cutoff for the native peptides.

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binding motifs for each mAb. While some of the ACPA mAb with similar clonotypes from the same patients (i.e., clones 14CFCT or 37CEPT [24] [see Supplementary Table 1 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]]) had similar motifs, we did not observe any association with the B cell differential stage (antibody-secreting cells or memory B cell–derived clones). We found that several individual ACPA clones showed preferential recognition of peptides with a glycine in the +1 position. This Cit–Gly reactivity was also dominating in the polyclonal anti-CCP2 IgG preparations (results shown in Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). However, certain ACPA clones instead preferred other motifs (e.g., clone 1325:04C03 with Gly–Cit, or clone BVCA1 with Arg–Cit–Asp) (Figures 1C and D).

The 9 amino acid consensus motifs in which citrulline was centered also revealed that other residues outside of positions –1 and +1 could be critical for some mAb (e.g., clones 1003:10A01 and 62CFCT1E04). For several ACPAs, an increased frequency of serine/threonine residues could be seen in the Cit-flanking regions, but this did not dominate enough to be visualized in the consensus motif representations for individual mAb (Figure 1C).

Moreover, when amino acid frequencies in the Cit-flanking residues (+1 and –1 positions) were analyzed, it was evident that mAb sharing the overall Cit–Gly consensus motif may still have different amino acid preferences in the –1 position (e.g., Pro in ACPA clone 1325:05C06 versus Asp/Arg in ACPA clone 37CEPT1G09), and therefore these may differ in their antigen preference (Figure 1C, and Supplementary Figure 5, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). The mAb varied in their signal intensity for binding to different peptides, suggesting that there are differences in their binding affinity (see Supplementary Figures 5 and 6 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). Importantly, none of the ACPAs bound to all peptides that contained their preferential binding motif, and this was attributable to the biochemical properties of the amino acids adjacent to the motif. For example, ACPA clone 1325:01B09 did not bind to Cit–Gly peptides with a negatively charged or aromatic amino acid in position –1, while it was more likely to bind to Cit–Gly peptides with a positively charged amino acid in position –1 (see Supplementary Figure 7, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

The peptides with the strongest mAb binding (according to FI values) were derived from a wide range of extracellular proteins, including a number of targets not previously described (listed in Supplementary Table 3, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). We acknowledge that analysis of the Cit-flanking regions alone may give a limited picture of reactivity, and we cannot formally exclude the possibility that there may also be structural elements in the binding.

**Differential binding of ACPA clones on a cDNA library-based protein array.** Only a few large, unbiased screenings of ACPA binding to full-length antigens have been performed to date. To address this, we used a large-scale macroarray (Engine GmbH) based on a cDNA library from >6,800 genes expressed on a PVDF membrane. When 3 selected ACPA mAb with different binding profiles (mAb clones 1325:01B09, 1325:04C03, and 37CEPT2C04) were investigated, we found a few positive reactions on the native membrane (range 10–36 peptides) but a large number of positive reactions when the membrane was citrullinated (Figures 2A–E and Table 2). Positive reactivity was scored by the manufacturer on a scale of 0–3 using a VS scoring system, where VS0 was found to have a median FI of 1.4 × 10^7, VS1 was found to have a median FI of 2.3 × 10^7, VS2 was found to have a median FI of 4.5 × 10^7, and VS3 was found to have a median FI of 1.1 × 10^8 AU.

For our subanalysis, high-intensity mAb binding (VS2 or VS3) to a citrullinated protein fragment with no native reactivity was considered a positive hit (resulting in 811 positive hits with mAb clone 1325:01B09, 90 positive hits with mAb clone 1325:04C03, and 186 positive hits with mAb clone 37CEPT2C04) (Figure 2). Not surprisingly, we could detect an association between the mAb binding signal intensity and the protein expression level on the membrane (see Supplementary Figure 8, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

Selected cDNA clones from the array were re-expressed for ELISA validation, which confirmed a significant correlation between ACPA binding to citrullinated on-array expressed proteins and ELISA reactivity to citrullinated purified proteins (see Supplementary Figure 9, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). Notably, the cDNA library also contains clones with out-of-frame insertions, which were excluded in the analysis of positive hits. Yet, the ACPA clones also bound some of these random citrullinated peptides/short fragments (data not shown).

Purified polyclonal anti-CCP2 IgG displayed reactivity, as expected, to a range of enzymatically citrullinated protein fragments, with considerable overlap with the reactivities of monoclonal ACPAs (see Supplementary Figure 10 and Supplementary Tables 4 and 5 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). All 3 ACPA mAb showed preference for binding nuclear antigens and DNA/RNA-binding proteins (Figures 2D and E, and Supplementary Table 5 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). For mAb clones 1325:01B09 and 37CEPT2C04, RNA-binding proteins such as heterogeneous nuclear RNP s (hnRNP s) were overrepresented,
and for mAb clone 1325:04C03 there was an overrepresentation of ribosomal proteins. Notably, the proteins with the highest ACPA signal intensity had a high frequency of both Arg–Gly and Gly–Arg sequences, which may result in multiple Cit–Gly or Gly–Cit binding motifs (see Supplementary Figure 11, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). However, protein fragments highly recognized by mAb clone 1325:04C03 had significantly more binding motifs (see Supplementary Figure 11, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). However, protein fragments highly recognized by mAb clone 1325:04C03 had significantly more

**Figure 2.** Wide range of ACPA binding to citrullinated proteins on a cDNA library–based macroarray. Three monoclonal ACPA clones (1 μg/ml) were evaluated for binding to full-length proteins/protein fragments using the Engine macroarray platform with *Escherichia coli* on-array expressed proteins from cDNA clones. A, Binding of ACPA clone 1325:01B09 to the untreated (native) array or the rabbit peptidyl arginine deiminase–citrullinated array shows how the reactivity to peptides was assessed on a visual scoring (VS) system, with VS scores of 0–3. B and C, Venn diagrams show the overlap of positive binding of 3 ACPA clones (1325:01B09, 1325:04C03, and 37CEPT2C04) to the in-frame citrullinated protein fragments having a citrulline VS score of 2 or 3 and no native binding, based on cDNA clones (B) or proteins (C). D and E, ACPA-positive proteins were analyzed by cellular component (D) or molecular function (E) based on the frequency (freq) of ACPA binding to gene products represented by the cDNA clones on the array. The cDNA clones with low/no expression on the array (10th percentile of anti-His signals) were excluded before the analysis. ** = P < 0.01; *** = P < 0.001; **** = P < 0.0001 versus the frequency among all proteins represented on the array, by Fisher's exact test. Extr. = extracellular; ER = endoplasmic reticulum; DNA bind. (RNA pol-II) = DNA binding to RNA polymerase II; transf = transferase (see Figure 1 for other definitions).
Gly–Arg motifs than fragments recognized by the other mAb, which is consistent with the binding preference of this antibody (Supplementary Figure 11 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). Although the array displays some bias for protein representation (i.e., number of cDNA clones per gene), there was no significant difference in expression level between protein groups (see Supplementary Figure 12, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). Hence, histones and hnRNPs seem to be frequent ACPA citrullinated protein targets that were independently detected across arrays.

Monoclonal ACPAs are characterized by differential multireactivity to carbamylation and acetylation but not to other PTMs. In addition to ACPAs, RA can also be associated with other anti–modified protein autoantibodies (AMPAs), such as anti–carbamylated protein (anti-Carb) antibodies. We therefore investigated to what extent AMPA multireactivity occurred among the investigated monoclonal ACPAs, and whether such reactivities were related to their Cit-peptide fine specificities. First, we evaluated the Carb peptide reactivity of the 12 ACPA mAbs in the NimbleGen Roche peptide array, including 59,211 homocitrulline peptides and corresponding native lysine control peptides. This array covered the same antigens as the NimbleGen citrulline array but included a different set of peptides, which were based on lysine residues instead of arginine. Interestingly, we observed that only a subset (6 of 12 ACPA clones) showed significant binding to the peptides on the Carb-peptide array (Figures 1E and F). The polyclonal purified anti-CCP2 IgG pool also showed significant specific Carb peptide binding, although with a lower signal intensity than was observed on the citrulline peptide array (Figure 1, and Supplementary Figure 4 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). Yet, for some of the mAbs, the binding to citrulline and homocitrulline peptides was equally strong, both in terms of number of positive peptides and intensity.

For certain clones (ACPAs clones 1325:01B09 and BVCA1), the Carb peptide consensus motif corresponded well to the Cit peptide motif, and yet the amino acid variation in the Cit-flanking residues in the positive peptides may suggest some differences (see Supplementary Figure 5 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). Other mAbs showed a more promiscuous Carb-peptide binding without a defined motif (ACPAs clones 37CEPT2C04 and 37CEPT1G09) or showed binding to a significantly different Carb peptide motif compared to the Cit peptide motif (ACPAs clones 1325:05C06 and 1003:10A01).

Modified histones are interesting as RA autoantigens, and consequently, a range of histone peptides were represented on the NimbleGen array platform (results of the subanalysis are shown in Supplementary Figure 13, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). Only certain ACPAs recognized citrullinated histone peptides, and only a subset of these antibodies also bound to carbamylated histone peptides. To further explore the extent of AMPA binding to different histone modifications, we next used the Histone Code array platform (JPT Peptide Technologies), which utilized a large number of histone peptides with different modified residues. We detected significant similarities between the NimbleGen and Histone Code platforms, with the same ACPA clones showing citrullinated histone reactivity (Figures 3A–E, and Supplementary Figure 13 [http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]). Certain clones (mAb 1325:01B09, 37CEPT1G09, and 37CEPT2C04) displayed a strong preferential binding to acetylated peptides while others had a citrulline-restricted histone binding (mAb clones 1325:04C03, 14CFCT3G09, and BVCA1) (Figure 3, and Supplementary Table 7, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). For peptides that had other PTMs on the array, limited or no reactivity was detected (Figure 3, and Supplementary Figure 14, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

Differences and similarities between the ACPA clones are further illustrated in Figures 4A and B, when we compared the motifs generated by the 20 peptides with the highest binding on the different array platforms. As demonstrated earlier, some antibodies that shared the overall Cit–Gly motif displayed differences (e.g., in the −1 position) in the motif generated from the top 20 peptides with highest binding. These findings reveal how the mAb may have different affinity for different Cit–Gly peptides.

In a similar way, the mAb with a Cit–Gly motif and AMPA properties did not always share the same multireactivity profiles. Importantly, for most antigens, citrullinated protein reactivity does not correlate with carbamylated or acetylated protein reactivity, since the arginine or lysine regions may look completely different. This was evident when, for example, we compared citrullinated to carbamylated full-length fibrinogen reactivity (see Supplementary Figure 15, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract). Moreover, when we used the Orgentec synthetic peptide assay system, in which the arginine/citrulline in a vimentin peptide is replaced with acetylated lysine or homocitrulline (see Supplementary Figure 16, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract), it was shown that some of the ACPAs bound to carbamylated but not acetylated residues in the right peptide context (e.g., mAb clone BVCA1), while others bound to all modifications (e.g., mAb clone 37CEPT2C04).

Chaotropic salt elution was used to calculate an avidity index for binding to the different PTMs determining a 50% inhibitory concentration of NaSCN, and the results further showed the differences in preferential binding of the mAb and emphasized that citrulline is not necessarily mediating the strongest interaction (see Supplementary Figures 16 and 17, available on the Arthritis & Rheumatology website at
In fact, of the 3 mAb positive for binding to multiple peptides in the Orgentec assay, mAb clones 37CEPT1G09 and 37CEPT2C04 had a similar apparent affinity to different modifications. Only mAb clone BVCA1 displayed citrulline as the clear preferential PTM, with a moderately higher avidity index than for carbamylation (avidity index 1.1 for citrullination compared to 0.76 for carbamylation). Yet, it is important to acknowledge that the apparent affinity of ACPA clones for different modifications are difficult to compare accurately, due to potential differences in the motif preferences. One approach was to select Cit and Carb peptides with the highest NimbleGen array reactivity and replace the homocitrulline-lysine with acetyl-lysine (KAc) for competition experiments. These experiments confirmed that there can be significant variation in affinity for the different PTMs. Notably, mAb clone 1325:01B09 had more than 6 times higher apparent affinity for KAc peptides than for citrulline peptides (inhibitory apparent affinity $K_i$ of 130 nM for His4-KAc5 compared to $K_i$ of 840 nM for Cit-His2AFY [see Supplementary Figure 17, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]).

All of the ACPA mAb in the study carry verified Fab glycosylation sites (26). However, consistent with our previous results for the homocitrulline-lysine with acetyl-lysine (KAc) for competition experiments. These experiments confirmed that there can be significant variation in affinity for the different PTMs. Notably, mAb clone 1325:01B09 had more than 6 times higher apparent affinity for KAc peptides than for citrulline peptides (inhibitory apparent affinity $K_i$ of 130 nM for His4-KAc5 compared to $K_i$ of 840 nM for Cit-His2AFY [see Supplementary Figure 17, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract]), and nearly 3 times higher affinity for the carbamylated peptides ($K_i$ of 360 nM for His4-CarbK5).
citrullinated antigens, the presence of Fab glycosylation did not significantly affect interaction of the AMPA clones with acetylated or carbamylated peptides (see Supplementary Figure 18, available on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41385/abstract).

DISCUSSION

The complexity of the ACPA responses in RA continues to fascinate investigators, and we are only beginning to unravel their role in RA pathogenesis. A large focus of attention in the scientific
community has been the search for a specific citrullinated antigen for which immune recognition may mediate pathogenic functionality. Ever since it was deduced that autoreactivity to citrullinated filaggrin can be revealed with the anti-iperinuclear factor test (28), a large number of ACPA targets have been identified in different studies and highlighted as particularly important, including vimentin, type II collagen, fibrinogen, α-enolase, hnRNPs, and histones (12–17). Multiplex serologic screenings have further demonstrated the heterogeneity of ACPA responses in RA patients (4,6–9). However, mounting evidence also reveals that the RA citrullinome includes a wide range of proteins (29,30). Unbiased screenings have shown that RA patient serum contains anticitrulline reactivity that expands widely beyond that seen in response to classic RA citrullinated antigens (8,31,32). Several independent investigations have previously shown that individual monoclonal ACPAs can display cross-reactivity to multiple citrullinated proteins and peptides (21,22,24,33–35).

In the current study using multiple large array platforms, we deciphered in a systematic way the multireactivity of human ACPAs. The results of our investigation support and strengthen previous results demonstrating that individual patient–derived ACPA clones have distinct reactivity profiles, and that these can be explained by recognition of certain consensus peptide motifs that are present across multiple proteins (e.g., Cit–Gly, Gly–Cit, Arg–Cit–Asp). While previous serologic studies demonstrated the importance of citrulline-adjacent glycine (36), we studied the reactivity of individual B cell clones with single cell-derived mAb. Our findings highlight the presence of other binding motifs beyond Cit–Gly, and demonstrate how mAb sharing the Cit–Gly motif can have different recognition patterns, e.g., due to the amino acid preferences in the −1 position of the motif. Evidence of these recognition patterns reveals that certain ACPAs have preferential binding to specific citrullinated antigens (e.g., citrullinated filaggrin, α-enolase, vimentin, or fibrinogen), and yet the binding is in no way restricted to these proteins, nor is there anything suggesting that they would necessarily be the primary target of recognition in vivo.

Here we have shown that for certain anti-CCP2+ ACPA clones, but not all, this multireactivity also extends to PTMs of lysine residues, specifically carboxymethylation and acetylation. In addition, we were able to rule out the possibility, at least in the context of histones, that other naturally occurring protein modifications serve as targets of cross-reactive ACPAs. Multireactivity of polyclonal ACPAs to other PTMs has been previously reported (21,22,37,38), and we have recently demonstrated that ACPA multireactivity can exist on the monoclonal level (21,22).

In the extended studies presented herein, we were able to demonstrate that some ACPA clones had a binding profile largely restricted to citrulline-containing peptides, while others reacted to multiple PTMs. In this way, the clones are forming a hierarchy of reactivities, comprising either 1) multireactivity restricted to citrulline, 2) multireactivity to citrullinated and carbamylated antigens, or 3) multireactivity that covers all 3 PTMs (citrulline, homocitrulline, and acetyl-lysine) (as illustrated in Figure 4B). Thus, among the 12 mAb clones, all KAc-reactive clones also had reactivity to citrullinated and carbamylated peptides/proteins. Notably, when screening different RA-derived mAb, we only found acetyl-lysine or homocitrulline reactivity among clones that were citrulline reactive (data not shown). Although we have denoted all of the clones in this investigation as ACPAs on the basis of their citrulline reactivity, and how they were originally described, the PTM–cross-reactive clones should, in reality, be considered AMPAs. Indeed, for some of the clones, the carbamylated protein or acetylated protein binding is more prominent and of higher apparent affinity than the citrullinated protein binding.

Different AMPA classes are presently gaining an increasing interest in RA. Reactivity to modified amino acids, including carbamylated lysine and acetylated lysine, in the context of a vimentin backbone peptide have been reported to be frequent in RA and provide some prognostic value (18). In serologic screenings, anti-CarP, detected with carbamylated fetal calf serum, have been associated with more severe disease (20), thereby potentially narrowing the serologic gap between seropositive and seronegative RA (39). Yet, the observed multireactivity of the antibodies assessed herein suggests that anti-CarP responses should not be considered a separate autoreactivity, but rather a different flavor of the ACPA/AMPA profiles.

Human polyclonal anti-CCP2 Igg purified from RA patients contains antibody binding to both acetylated and carbamylated epitopes (21,37,38). Yet, we observed a lower signal intensity for binding to homocitrulline and acetyl-lysine–containing peptides than to citrulline peptides (Figure 1 and Supplementary Figure 4; see also ref. 21). IgG reactivity to Carb peptides in the RA serum is also reported to be lower than that to Cit peptides, supporting the notion that only a subset of anti-CCP IgG cross-react to other PTMs (32,40). We have previously shown that certain ACPA clones with AMPA reactivity to acetylated histones can bind neutrophil extracellular traps in a PAD-independent manner (21). The current study further emphasizes that these AMPAs bind significantly stronger to KAc peptides than to Cit peptides. Notably, since citrulline is an arginine modification and acetylation is a lysine modification, there is not necessarily any correlation between the antigens that are recognized by AMPAs through acetylation and those recognized by ACPAs through citrullination. Yet, histones contain both Arg–Gly and Lys–Gly sites, and therefore they may be recognized by autoantibodies with a KAc/Cit–Gly motif. Although the recently recognized crystal structure of human ACPAs in complex with citrullinated peptides provides some insights into the structural properties of ACPA multireactivity (41), more data are still required to fully understand how binding to different modifications can be mediated.

The ACPA recognition of consensus linear motifs, in which only a few flanking amino acids besides the modified site seems to be critical, is a unique human ACPA feature. This may further inform us about the B cell selection processes,
evolution of autoreactivity, and epitope spreading in RA. These features of human autoimmunity would not be fairly reflected in murine immunization experiments. It has been commonly recognized in the literature that a majority of B cell epitopes in protein recognition are conformational, a finding that was recently confirmed in computational analyses of the increasing number of available antibody–antigen structures (42). However, when linear epitopes are recognized, they contain on average 4–7 residues that are critical for the interaction (43). The human AMPA clones do not seem to fit into this dogma. Moreover, high SHM levels, as detected in ACPAs, reflect multiple B cell germinal center selection rounds, which, in most responses, would be expected to yield a more restricted binding, rather than the multireactivity seen for ACPAs in patients with RA. It is tempting to speculate that the evolution of AMPA reactivity is sequential, starting with citrulline recognition, but a parallel scenario is also possible, given the high KAc signals and the observation that both ACPAs and AMPAs have a similar number of mutations.

We also had the opportunity to investigate possible structural epitopes targeted by ACPAs by utilizing a large cDNA library–based protein array and in vitro citrullination. This array format covers >6,800 expressed proteins but is associated with some limitations, including the following: out-of-frame clones that had to be excluded from the analyses, a bias toward proteins more readily expressed in E. coli (i.e., more nuclear and less membrane proteins), and possibly the presence of endogenous modifications of antigens by E. coli that could explain the observed background reactivity on the native membrane. The cDNA library originates from the human fetal brain, which, on the one hand, is not disease-relevant, but on the other hand, ensures high proteome coverage due to high transcript levels. Moreover, proteins are expressed directly on the array, and although efforts are taken to ensure refolding after bacterial lysis, some proteins may be unfolded or incorrectly folded due to the bacterial expression, lysis, or citrullination process.

Nevertheless, we could verify several known targets, such as histone 2A and hnRNP A1 (21), but could also identify a previously unappreciated bias for 40S ribosomal protein S14 and RNA-binding proteins. Interestingly, a previous study using a similar macroarray format suggested that 40S ribosomal protein was a preferential PAD4 target (44). Other published screenings have demonstrated that both PAD2 and PAD4 have preferences for modification of Arg peptides with Gly in the +1 position, with PAD4 having additional restriction in peptide substrates (45). Theoretically, unstructured flexible segments with a large Arg/Lys/Gly content, including those in RNA-binding proteins, may also be more accessible for enzymatic modification. In fact, it was recently reported that serum ACPAs preferentially bind to disordered Cit peptides on a NimbleGen array (40). Taken together with the observations of high ACPA reactivity in different peptide assays, these findings postulate that ACPAs primarily bind continuous epitopes that are located in exposed protein structures (e.g., loops, or flexible regions) in citrullinated targets. Interestingly, it could be that the preferential ACPA motifs are originating from the most commonly physiologically modified peptides to which the immune system is exposed.

When working with autoantibodies that display multireactivity, it is imperative to rule out any nonspecific polyreactive interactions that may cause false positivity, which has historically been problematic (46). In the current study, we therefore followed a strict methodology, including extensive quality control of the recombinant IgG and stringent assays (details provided in ref. 27). Thus, the 12 validated ACPA mAb used in the current study displayed no signs of biologically irrelevant “stickiness” or polyreactivity. These mAb also have features seen in multiple previous studies of ACPAs, including Fab glycosylation and high SHM levels (22,24,26,33–35,47). This is in contrast to 2 RA clones (mAb 1276:01D10 and 1103:01B02) that were originally described as citrulline-reactive but were later found to be nonreactive (46), and were also found to be negative in the citrulline, homocitrulline, and acetylated-lysine assays herein.

It is intriguing to hypothesize that ACPA subsets, based on reactivity fingerprints and consensus motifs, may be associated with different pathogenic properties. Polyclonal ACPAs have been observed to modulate both osteoclast and fibroblast functionality, have proinflammatory properties, and mediate pain behavior (22,24–26,48–50). Our previous studies demonstrated that the ACPA clone 1325:04C03 (with the Gly–Cit motif) have osteoclastogenic properties and have no effect on fibroblasts (22,23,51). In contrast, clone 1325:01B09 (with the Cit–Gly motif) mediates fibroblast migration but has no effect on osteoclasts (22,23,51).

The mAb clones 37CEPT1G09 and 37CEPT2C04, with the Cit–Gly motif and widely promiscuous homocitrulline/acetylated lysine peptide binding, can augment LPS-induced arthritis and mediate pain behavior in vivo (24). In contrast, the 14CFCT3G09 clone, with its Cit–Gly motif, lacks Carb/KAc peptide reactivity and fails to enhance arthritis and pain signs. It is possible that recognition of both modified arginine and modified lysine sites may facilitate pathogenic immune complex formation. Nevertheless, not all ACPAs may be pathogenic and some may even be protective. Moreover, other subsets of RA-related autoantibodies may also trigger similar functionality as ACPAs, but may act through distinct pathways (19).

Taken together, these emerging data emphasize the unique value of using patient-derived mAb to gain a deeper understanding of the adaptive immune response in RA. The striking properties of the hierarchies of AMPA reactivities and the multireactivity profiles can teach us about the evolution of human autoimmune responses and may further elucidate the etiology behind the break-of-tolerance to modified antigens in autoimmune disease.

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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. Grönwall 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. Sahlsström, Hansson, Steen, Piccoli, Lundberg, Klæreskog, Mueller, Catrina, Skriner, Malmström, Grönwall. Acquisition of data. Sahlsström, Hansson, Steen, Amara, Titcombe, Forsström, Stäleisen, Israelsson. Analysis and interpretation of data. Sahlsström, Hansson, Steen, Amara, Titcombe, Forsström, Stäleisen, Israelsson, Piccoli, Lundberg, Klæreskog, Mueller, Catrina, Skriner, Malmström, Grönwall.

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