UNIVERSITY OF BIRMINGHAM
University of Birmingham Research at Birmingham

# The impact of autoantibodies against citrullinated, carbamylated, and acetylated peptides on radiographic progression in patients with new-onset rheumatoid arthritis

Nijjar, Jagtar S ; Morton, Fraser R ; Bang, Holger ; Buckley, Christopher; van der Heijde, Désirée ; Gilmour, Ashley ; Paterson, Caron ; McInnes, Iain B; Porter, Duncan; Raza, Karim; Scottish Early Rheumatoid Arthritis Inception Cohort Investigators

*DOI:*
10.1016/S2665-9913(20)30381-7

*License:*
Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

*Document Version*
Peer reviewed version

*Citation for published version (Harvard):*
Nijjar, JS, Morton, FR, Bang, H, Buckley, C, van der Heijde, D, Gilmour, A, Paterson, C, McInnes, IB, Porter, D, Raza, K & Scottish Early Rheumatoid Arthritis Inception Cohort Investigators 2021, 'The impact of autoantibodies against citrullinated, carbamylated, and acetylated peptides on radiographic progression in patients with new-onset rheumatoid arthritis: an observational cohort study', *The Lancet Rheumatology*, vol. 3, no. 4, pp. e284-e293. https://doi.org/10.1016/S2665-9913(20)30381-7

Link to publication on Research at Birmingham portal

**General rights**
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

**Take down policy**
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 21. Jan. 2025

# The impact of the presence of autoantibodies against citrullinated, carbamylated and acetylated peptides on radiographic progression in patients with new onset rheumatoid arthritis: an observational study.

Jagtar S. Nijjar MBChB PhD[1#], Fraser R. Morton MRes[2#], Scottish Early Rheumatoid Arthritis Inception Cohort Investigators, Holger Bang PhD[3], Christopher D. Buckley FRCP DPhil[4¶], Désirée van der Heijde MD[5¶], Ashley Gilmour MSc[2], Caron Paterson BSc[2], Iain B. McInnes FRCP PhD[2¶], Duncan Porter MD[6*¶], Karim Raza BMBCh PhD[4,7*¶]

# joint first authors
* joint senior authors
¶ these authors are full professors

1. Department of Medicine, University of Cambridge 2. Research into Inflammatory Arthritis Centre Versus Arthritis, Institute of Infection, Immunity and Inflammation, University of Glasgow. 3. Orgentec Diagnostika, Mainz, Germany. 4. Research into Inflammatory Arthritis Centre Versus Arthritis and MRC Versus Arthritis Centre for Musculoskeletal Ageing Research, Institute of Inflammation and Ageing, University of Birmingham. 5. Leiden University Medical Centre, The Netherlands. 6. NHS Greater Glasgow and Clyde, Glasgow, United Kingdom. 7. Sandwell and West Birmingham NHS Trust, Birmingham.

Corresponding author: Professor Karim Raza, Professor of Rheumatology supported by Versus Arthritis, Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK.

## Abstract

### Background

A range of anti-modified peptide autoantibodies (AMPA) are associated with rheumatoid arthritis (RA). We assessed the relationship between AMPA profiles and radiographic progression in new onset RA.

### Methods

AMPAs (targeting citrullinated, carbamylated and acetylated peptides) were measured by ELISA in 362 patients with new onset RA from the Scottish Early Rheumatoid Arthritis Inception Cohort and Biobank (SERA). Radiographic progression in hands and feet was determined using the Sharp-van der Heijde (SvH) method. 233 patients with RA had AMPA status and radiographic progression scores after quality control. Differences in radiographic progression between groups were determined using least square means changes with baseline value of radiographic variable, rheumatoid factor, gender, age of onset of disease, symptom duration and baseline DAS28-CRP included as covariates.

### Findings

Four main autoantibody groupings by class of modification were identified in RA patients (n=362) with reactivities against modified peptides as follows: citrullinated only (n=73, 20%), citrullinated and acetylated (n=45, 12%), citrullinated, carbamylated and acetylated (n=151, 42%) and AMPA negative (n=74, 20%). In RA patients with both antibody and radiographic data, those with antibodies against all three post-translational modifications (least square mean 1·7, n=97) had greater radiographic progression over 12 months compared to those with anti-citrullinated peptide antibodies (ACPA) only (least square mean 0·5, n=48) (least square mean difference of total SvH score of 1·2, 95% CI 0·05-2.40). There was no difference in progression comparing ACPA only with seronegative patients (least square mean 0.7, n=47) (least square mean difference -0.2, 95% CI -1·09-0·67).

### Interpretation

RA patients with all three classes of AMPA have more rapid radiographic progression over 12 months than those with ACPA alone. Patients with ACPA alone had similar radiographic progression over 12 months to AMPA negative patients.

## Funding

EU FP7 HEALTH programme, Scottish Translational Medicine Research Collaboration, Scottish Chief Scientific Office.

## Keywords

Rheumatoid arthritis, acetylated, carbamylated, citrullinated, ACPA, radiographic progression.

## Research in context

### Evidence before this study

- RA patients with ACPA and anti-carbamylated antibodies have more rapid radiographic progression than those who are antibody negative

### Added value of this study

- In patients with newly presenting RA, there are four major patterns of AMPA
- Increased radiographic progression in patients with ACPA is confined to those with multiple AMPA reactivities
- Those with ACPA alone have similar radiographic progression to those who are seronegative for all AMPAs

### Implications of all the available evidence

- Current data suggest that optimal prediction of future rates of radiological progression will require the assessment of autoantibodies against multiple post-translationally modified proteins/peptides. Future observational and interventional studies in which radiological progression is measured as an outcome variable should consider incorporating a measurement of anti-acetylated and anti-carbamylated peptide antibodies as well as ACPA to allow an appropriate description of the cohort or of the different study arms.

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis that causes pain, disability and reduced life expectancy, to the detriment of the patient and wider society (1). Autoantibodies including rheumatoid factors and anti-citrullinated peptides antibodies (ACPA) inform both diagnosis and prognosis in RA (2-5).

Recently, it has become clear that immune dysfunction in RA leads to the generation of antibodies to proteins that have undergone other post-translational modifications (PTM) including carbamylation (6,7) and acetylation (8,9). Multiple anti-modified protein antibodies (AMPA) can occur in individual patients, though the roles of their combinations in diagnosis and prognosis remain uncertain.

Citrullination of self-proteins occurs in the lung, periodontal tissue and joint by the enzyme peptidyl arginine deiminase (PAD), which converts arginine residues to citrulline. Smoking in combination with certain HLA-DR haplotypes has been implicated in the development of ACPA and RA pathogenesis (10). ACPA often pre-dates the onset of clinical disease, and the presence of autoantibodies can identify a pre-RA state (11). Furthermore, RA patients with ACPA are more likely to have erosive progression and joint damage compared to seronegative patients (4,12).

Homocitrullination (or carbamylation) occurs when cyanate ions react with lysine residues to form homocitrulline in a non-enzymatic reaction. It is unclear if there is a physiological role for carbamylation, but urea is a source of cyanate ions and therefore carbamylation can occur more often in uraemic states. Antibodies to carbamylated proteins have been associated with a poorer prognosis in RA, an increased risk of relapse and increased radiographic progression (6).

Acetylation is a more complex PTM that can occur irreversibly at the N-terminus of proteins but can also occur when the activated acetyl group from acetyl-coA is consumed at lysine residues. This modification may be modified by

inflammatory and metabolic states, and is potentially reversible. Lysine acetylation is widely studied at the genome-wide level with the interplay of histone acetyltransferases and histone deacetylases being crucial in the epigenetic control of gene expression, although other roles of amino acid acetylation are less well defined.

Recent work has shown that mice can develop a wide cross-reactive AMPA response when immunised with a peptide expressing only one post-translational modification (13). Immunisation with acetylated peptides generated autoantibodies against citrullinated, carbamylated and acetylated peptides suggesting that a broad AMPA response could be generated by breaking tolerance to acetylated peptides and not only citrullinated peptides as originally hypothesised.

It is possible that different AMPAs have distinct and complementary roles in the aetiopathogenesis of RA. Thus far, limited data are available regarding the significance of the presence of AMPA against citrullinated, carbamylated and acetylated modifications, acting in isolation or in combination. To our knowledge this is the first time that the effect of broader AMPA combinations on erosive progression in RA have been reported, in this case by using the prospectively acquired, population wide Scottish Early Rheumatoid Arthritis Inception Cohort and Biobank (SERA) (14).

## Methods

### SERA cohort

The Scottish Early Rheumatoid Arthritis Inception Cohort and Biobank (SERA) is a national inception cohort of >1100 patients with newly diagnosed RA or undifferentiated arthritis, recruited from 16 centres across Scotland. Patients with a new clinical diagnosis of RA or undifferentiated arthritis, who had at least one swollen joint were invited to participate. Patients were excluded if their joint swelling could have been explained by an alternative diagnosis (e.g. psoriatic arthritis) or if they were carriers of blood borne viruses. Detailed demographic, clinical, laboratory

and radiographic data and biological material have been collected (14). Treatment was at the discretion of the treating rheumatologist and was recorded in a web-based portal by research nurses at six monthly intervals. The study was approved by the West of Scotland Research Ethics Committee 4 (reference 10/S0704/20) and all participants gave written informed consent.

### Measurement of AMPA

AMPAs were measured in 362 baseline plasma samples from patients with RA in the SERA cohort who were started on methotrexate within six weeks of their baseline visit, and enrolled in a sub-study to predict their response to methotrexate. Modified peptides were coated on microtiter plates as previously described (7) and the actual experimental performance of the assay was conducted according to the standard protocol for the Orgentec immunometric enzyme immunoassay.

Briefly, 0·5 µg/ml of biotinylated vimentin peptide either citrullinated (GRVYAT-Citrulline-SSAVR), carbamylated (GRVYAT-HCit-SSAVR) or acetylated (GRVYAT-Acetylated lysine-SSAVR and GRVYAT-Acetylated ornithine-SSAVR) were coated on streptavidin pre-coated cavities of a standard microtiter plate (Thermo Scientific, Denmark) and blocked with PBS supplemented with 1% bovine serum albumin. Patient samples were diluted 1:100 in PBS/0·05% Tween 20/1% albumin and incubated for 30 min. Unbound antibodies were washed out with PBS/0.05% Tween. Bound autoantibodies were detected with horseradish peroxidase conjugated anti-human IgG (Dianova, Hamburg, Germany) and visualized with TMB as substrate for the peroxidase. Optical density was measured with a standard microtiter plate reader (Tecan, Germany).

Standard curves were established by using patient serum from the outpatient Department of Rheumatology, Dresden. The reference range was defined in healthy volunteers (n=300) as mean antibody reactivity plus three standard deviations (SD). Detection of rheumatoid factor IgM, anti-modified citrullinated vimentin (MCV) IgG and anti-CCP IgG antibodies were measured using commercially available ELISA kits (Orgentec Diagnostika, Germany) and a cut off of 20 IU was used to define antibody positivity according to the manufacturer's instructions. For the AMPA

assays, the optimal ELISA cut-off values were determined by comparing the mean of antibody reactivity plus 3 standard deviations (SD) for both positive and negative samples, using sera samples from 200 RA patients (fulfilling 1987 ACR classification criteria for RA; from the Specialized Practice Rheumatology and Clinical Immunology, Dresden, Germany) and from 121 outpatients with other rheumatic disease from the same department. 300 healthy subjects were considered as controls. Based on these results, for the carbamylated, acetylated lysine and acetylated ornithine assays, a cut off of 25 IU was used to define antibody positivity.

### Radiographic scoring

Out of the 362 patients with RA who had AMPA profiles determined, 233 had paired radiographs of hands and feet that were available and of sufficient quality for scoring at baseline and one year (Supplemental Figure 1). The images were scored by one assessor, blinded to the order of the radiographs, using the Sharp-van der Heijde (SvH) method (Imaging Rheumatology International). Prior to scoring, the inter-reader (between the reader who scored radiographs for this study and a second reader) intraclass correlation coefficient for status scores had been determined as 0·97 and the smallest detectable change 1·75. Patients were not analysed if paired radiographs were not available (n=17), surgery had been performed on one or more joints (n=3) or because the radiographs were deemed to be of insufficient quality for scoring (n=109).

### Statistical analysis

The SvH erosion, joint space narrowing and total progression scores followed a negative binomial distribution. To allow for the high number of cases where there was no change in score, a zero-inflated negative binomial model was fitted to the data using the pscl R package (15) for each of the progression scores with the baseline value of the measured variable, rheumatoid factor, gender, age of onset of disease, symptom duration and baseline DAS28-CRP included as covariates. Any patients with a negative change in progression were given a score of zero. Least Square Means changes in total SvH progression, erosion score, and joint space narrowing were determined according to antibody profile using the R lsmeans package (16). Comparisons were performed between triple positive (Cit, Carb and Acet), single

positive (Cit only) and AMPA negative patients to investigate whether the presence of antibodies against citrullinated peptides alone was associated with a worse radiographic prognosis or whether these antibodies were only associated with a worse prognosis if other AMPA were also present. Confidence intervals were adjusted using Tukey's method. Rapid radiographic progression was defined as a change in SvH score of ≥5 units. Parametric and non-parametric tests were employed to determine differences in clinical characteristics and the use of each test is described in each table.

### Patient and Public Involvement

The use of biological and clinical data from the SERA Biobank and SERA dataset for the purposes of this study was reviewed by the SERA Access Committee. This Committee has four patient members, who were involved in the development and execution of the SERA Access Policy and who reviewed the proposed study, the data and samples required and the analysis plan.

### Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The following had access to the totality of the raw data: JSN, FRM, IBM, DP. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

## Results

Patients were recruited to the SERA study between March 2011, and April 2015. The 362 patients selected for this study all had RA, had all commenced methotrexate therapy within 42 days of baseline assessment and had attended the month 12 assessment appointment. 244 (67·4%) were female, their mean age at recruitment was 58·2 years (SD 13·4) and the mean duration of symptoms was 14·4 months (SD 31·1).

Six autoantibody groupings were identified: single positivity for CCP (n=27), double positivity for CCP and citrullinated vimentin (n=45), triple positivity which includes acetylated ornithine (n=35), quadruple positivity including carbamylation (n=41), those with all five autoantibodies (n=104) and negative patients (n=74) (Figure 1A). 36 patients out of a total of 362 with RA had other antibody profiles.

These patterns were simplified by combining patients who were positive for antibodies against CCP and/or citrullinated vimentin into a ACPA positive group, and those who were positive for antibodies against acetylated ornithine and/or acetylated lysine into an anti-Acetylated Peptide Antibody positive group. This generated four major groupings in the whole cohort: single ACPA positivity (n=73; from herein referred to as single positive), double positivity with ACPA/ Anti-Acetylated Peptide Antibody (n=45; from herein referred to as double positive), triple positivity with ACPA/ Anti-Acetylated Peptide Antibody / Anti-Carb Antibody (n=151; from herein referred to as triple positive) and AMPA negative patients (n=74) (Figure 1B).

Out of 362 patients with a diagnosis of RA, 284 were positive for antibodies to citrullinated peptides (78%) and 78 were negative (22%) in keeping with other RA cohorts. In this cohort, only 15 patients had antibodies to citrullinated and carbamylated peptides alone (4%).

ACPA concentrations are higher in patients with more AMPA classes (Figure 2). Median ACPA concentration is higher in triple positive (n=151) compared to single positive patients (n=73) (1000 IU vs 96 IU, fdr adjusted p value $=2 \times 10^{-16}$).

To evaluate whether autoantibody status is associated with variation in risk of radiographic progression, we combined the data from the AMPA assays with radiographic progression data that had been derived from baseline and 12 month hand and foot radiographs. Out of the 362 patients with RA who had their autoantibodies measured, 233 had corresponding hand and foot radiographs that were of sufficient quality to score for radiographic progression between baseline and 12 months (Supplemental figure 1). This subset of patients had similar baseline characteristics to the 362 patients with RA (Supplemental table 1) and similar differences in ACPA

concentrations between the different AMPA groups (Supplemental figure 2). Baseline characteristics of the radiographic cohort are outlined in Table 1.

RA patients who were triple positive (n=97) had higher radiographic progression over 12 months (least square mean 1·7, 95% CI 0·90–2·61) than those who were single positive (n=48) (least square mean 0·5, 95% CI 0·05–1·01) (difference of total SvH score of 1·2, 95% CI 0·05-2.40) (Figure 3B and Table 2). There was no statistical difference in radiographic progression between RA patients who were single positive (n=48) and those who were AMPA negative (n=47) (least square mean 0·7, 95% CI 0.14-1.35) (Table 2). There were no differences between males and females (Supplemental Table 2).

Fewer patients in the single positive group showed radiographic progression, defined as an increase in the total SvH score of >=1, compared to the triple positive group (9 (18·8%) of 48 vs 35 (36·1%) of 97, p=0·03, Chi-Sq). The difference in number of patients with rapid radiographic progression (increase in SvH >=5) over 12 months between single positive and the triple positive groups (1 (2·1%) of 48 vs 11 (11·3%) of 97, p=0·06, Chi-Sq) failed to reach statistical significance.

Higher ACPA concentration has been associated with increased radiographic progression in patients with RA. We compared the ACPA concentration in patients with radiographic progression compared to those who did not, within each AMPA group, and did not find any differences (Supplemental figure 3).

There was no difference in age at RA onset between the patients in the single positive group and in the triple positive group (56·6 years vs 57·8 years). Mean symptom duration prior to recruitment was 12·0 months for the single positive group and 13·3 months for the triple positive group. Both age of onset and symptom duration were included as covariates in the model.

Baseline disease activity was comparable between patients in the single positive group and in the triple positive group when measured by DAS28-ESR (4·8 vs 5·2) or DAS28-CRP (4·5 vs 4·7). The change in disease activity score over 12 months and proportion of patients achieving EULAR response criteria at 12 months were not

different, although the number of complete cases (n=141 for delta DAS28-CRP and n=173 for delta DAS28-ESR and EULAR response) due to missing data was less than the full radiographic cohort (n=233).

Baseline HAQ, HAD and EQ5D scores were also similar between groups. There was no difference between patients who had ever smoked and those who had never smoked (Table 1) or in baseline laboratory test values in the patients according to AMPA status (Supplemental Table 3).

IgM rheumatoid factor was measured to determine whether the relationship between radiological progression and autoantibody profiles were limited only to patients seropositive for rheumatoid factor. Similar proportions of patients were single positive and double positive in IgM RF positive and IgM RF negative groups (Supplemental table 4). More patients in the IgM RF positive group were triple positive and conversely, in the IgM RF negative group, more patients were negative for all AMPA. In the patients who were IgM RF positive, those who were triple positive had higher radiographic progression over 12 months than those who were single positive (difference in total SvH score of 1.0 p=0·01) (Supplemental figure 4 and Supplemental table 5). In patients who were IgM RF negative there was no statistically significant difference in SvH score between triple positive patients versus those who were single positive (Supplemental table 5). We also assessed radiographic progression in patients in the negative, single positive and triple positive groups and compared progression between RF positive and RF negative patients in each of these groups. For each of the radiographic measures (total SvH change, erosion score change, and joint space narrowing score change) there was no significant difference between RF positive and RF negative patients in any of the analysed autoantibody groups (Supplemental table 6).

DMARD use is shown in table 3. Thirty two out of 48 patients in the single positive group were on csDMARD monotherapy at one year compared to 47 out of 97 patients in the triple positive group (67% vs 49% Chi-Sq p=0·04). Furthermore, only two patients out of 48 in the single positive group were on therapy with three DMARDs (methotrexate, sulfasalazine and hydroxychloroquine) compared to 14 out of 97 in the

triple positive group although this result was not statistically significant. Thus, more rapid radiological progression in the triple positive group compared with the patients with ACPA only was not a consequence of the former group receiving less intense therapy.

## Discussion

To our knowledge this is the first study to investigate the relationship between multiple autoantibodies to citrullinated, carbamylated and acetylated peptides and radiographic progression in RA. In the SERA cohort, we found four major AMPA groups: negative for all antibodies measured, single positivity to citrullinated peptides, double positivity to citrullinated and acetylated peptides and triple positivity to citrullinated, acetylated and carbamylated peptides. Our key result shows that there is greater radiographic progression in RA patients with antibodies to all three classes of post-translational modification compared with those with ACPA only. Furthermore, radiographic progression in the ACPA only group is similar to RA patients who are seronegative for all AMPAs, suggesting that the effect, on radiographic progression, of being ACPA positive is restricted to those patients who also have other AMPAs.

Different rates of change in radiological variables (total SvH score or erosions score or joint space narrowing score) between the triple positivity and single positivity groups were not explained by baseline values of the radiological variable of interest, rheumatoid factor, age of onset, gender, symptom duration prior to recruitment or baseline DAS28-CRP. We did show that the ACPA concentration was higher in patients who were triple positive compared to those who were single positive. However, within each autoantibody grouping, we did not see a difference in ACPA concentration between those patients with radiographic progression and those without.

Comparator studies of radiographic progression in ACPA positive patients have shown increased radiographic progression in seropositive patients. At face value our results are at odds with this, however 185 (79%) of 233 patients in our radiographic

cohort are ACPA positive, when this antibody is considered on its own. When antibodies to carbamylated and acetylated vimentin are measured, we found that the radiographic progression burden was greater in the patients who had all three classes of autoantibody than in those with ACPA only.

Shi et al (6) explored the influence of anti-carbamylated antibodies on radiographic progression and found that these antibodies associated with more severe radiographic progression in the total and ACPA negative group. We did not find this effect in the ACPA negative group; this may be due to our assay specifically evaluating autoantibodies to only one carbamylated peptide, derived from vimentin, rather than a modified heterogeneous mixture in fetal calf serum. Furthermore, the ACPA and anti-Carb antibodies only group, in our cohort, was small compared to the others (n=11) and therefore we could not draw any conclusions about radiographic progression.

In contrast with other results, we find that only a very small proportion of ACPA negative RA patients had anti-Carb antibodies (1% in our subgroup of the SERA cohort vs 16% in the Leiden EAC cohort (6)). Inter-cohort differences could be due to cohort related factors but may be because we are measuring autoantibodies that are specific for a post-translational modification of a vimentin derived peptide. Our results are in keeping with a recent report from the RETRO study (8) where the largest subgroup of patients had autoantibodies to all three classes of post translational modification.

Cross reactivity of autoantibodies has been addressed by Juarez et al with inhibition studies and this is crucial given the structural similarity of the epitopes from the various post-translational modifications (9). As in this study, they demonstrated that the anti-acetylated antibodies typically occurred concurrently with anti-citrullinated peptide antibodies and did not help with further sub classification of the ACPA negative group. Recent work by Kampstra et al has confirmed that both ACPA and anti-carbamylated antibodies react to citrullinated and carbamylated antigens (13). Their work also showed that both antibody classes can recognise acetylated antigens

which is surprising given the difference in structure between acetyl-lysine and citrulline or homocitrulline.

Further work is required to assess whether subgroups of patients, defined on the basis of different combinations of AMPA, behave differently with regard to other outcome measures. Work from the RETRO study (8) has demonstrated that relapse rate is higher in those patients with multiple autoantibody specificities.

An important limitation of this work is that it only considers autoantibodies to modified vimentin derived peptides and not other proteins such as fibronectin and α-enolase. We would suggest that modifications to other peptides should be assessed in the future with a view to developing a clinical diagnostic such as an AMPA3 or AMPA4 assay with the number designating the number of anti-modified protein/peptide antibodies measured. Furthermore, the findings of this study are restricted to patients treated with conventional synthetic (cs) DMARDs (only two patients were on biologic (b) DMARD therapy at the 12 month time point). Future work should address whether these findings are replicated in other cohorts of patients treated with csDMARD and also whether they are replicated in cohorts in which bDMARDs are introduced at an earlier stage of disease. Finally, the autoantibody data presented herein are from samples collected at baseline in RA patients presenting with clinically apparent synovitis. Future work should address the evolution of the serological profile over time, both in the arthralgia phase before the development of joint swelling and also following the introduction of DMARD therapy. This could provide important insights for clinicians, for instance clarifying whether there is utility in assessing these antibodies at earlier (as has already been assessed for anti-carbamylated protein antibodies and ACPA (17)) and later stages of the disease in order to predict future rates of radiographic progression.

Our study adds to the literature by showing that patients with antibodies to citrullinated, carbamylated and acetylated peptides have a higher rate of radiographic progression than those with antibodies to citrullinated peptides alone. Interestingly, there is a group of patients with antibodies to citrullinated peptides only who progress at a rate comparable to patients negative for ACPA and for other AMPAs. We

believe that, based on these findings, further prospective work is required to understand the effects of multiple autoantibody specificities in RA and in particular whether such baseline autoantibody status should be used to stratify patients in therapeutic trials in RA.

## Tables

**Table 1. Demographic, clinical and radiographic progression data for each autoantibody group in patients with RA**. Single positive=ACPA positive only. Double positive=ACPA and anti-acetylated peptide antibody positive. Triple positive= ACPA, anti-acetylated peptide antibody and anti-carbamylated peptide antibody positive. Mann-Whitney-Wilcoxon U test was calculated for each row and p values between Cit only and Cit, Carb and Acet group are shown in the right hand column. § denotes Chi-Squared test for proportions. Significant values of P <0.05 are denoted in bold.

| | **Negative** | **Single positive** | **Double positive** | **Triple positive** | **P value Triple vs Single positive** |
|---|---|---|---|---|---|
| | n=47 | n=48 | n=29 | n=97 | |
| **Demographic characteristics** | | | | | |
| Female, no. (%) | 34 (72·3) | 36 (75·0) | 21 (72·4) | 57 (58·8) | 0·06§ |
| Age, mean ± SD years | 63·7 ± 12·4 | 56·6 ± 13·7 | 54·4 ± 15·0 | 57·8 ± 12·6 | 0·61 |
| Never Smoked, no. (%) | 25 (53·2) | 15 (31·2) | 12 (41·4) | 30 (30·9) | 0·97§ |
| Alcohol intake (units per week), mean ± SD | 5·1 (7·5) | 5·1 (11·3) | 4·9 (7·2) | 7·1 (15·7) | 0·29 |
| **Baseline characteristics** | | | | | |
| Disease duration, mean (range) months | 14·1 (1-280) | 12·0 (2-68) | 15·3 (1-148) | 13·3 (1-149) | 0·61 |
| Swollen joints (0–28), mean ± SD | 7·5 ± 5·6 | 6·7 ± 5·8 | 5·2 ± 3·6 | 7·0 ± 4·8 | 0·33 |
| Tender joints (0–28), mean ± SD | 12·1 ± 6·8 | 9·6 ± 7·3 | 8·4 ± 7·3 | 10·2 ± 7·3 | 0·66 |
| ESR, mean ± SD | 33·2 ± | 27·5 ± | 37·2 ± | 31·8 ± | 0·37 |

| | | | | | |
|---|---|---|---|---|---|
| mm/hour (complete data) | 26·3 (39) | 20·9 (40) | 27·8 (27) | 22·6 (84) | |
| CRP, mean ± SD mg/liter (complete data) | 24·7 ± 28·6 (41) | 14·8 ± 12·0 (43) | 29·6 ± 36·7 (25) | 24·6 ± 38·1 (83) | 0·84 |
| HAQ DI score (0–3), mean ± SD (complete data) | 1·3 ± 0·8 (47) | 1·1 ± 0·8 (48) | 1·2 ± 0·8 (29) | 1·1 ± 0·8 (96) | 0·86 |
| HAD score (0-21), mean ± SD (complete data) | 12·5 ± 7·4 (46) | 13·1 ± 7·8 (47) | 11·8 ± 8·7 (29) | 11·7 ± 7·3 (96) | 0·36 |
| EQ5D index value, mean ± SD (complete data) | 0·45 ± 0·30 (47) | 0·46 ± 0·36 (48) | 0·52 ± 0·36 (29) | 0·52 ± 0·31 (96) | 0·42 |
| DAS VAS (0-100mm) mean ± SD | 51·1 ± 26·0 | 46·3 ± 24·7 | 48·9 ± 24·5 | 49·9 ± 27·1 | 0·42 |
| IgM RF high positive (>3x ULN), no. (%) | 3 (6·4) | 21 (43·8) | 13 (44·8) | 43 (44·3) | |
| IgM RF low positive, no. (%) | 7 (14·9) | 5 (10·4) | 4 (13·8) | 25 (25·8) | |
| IgM RF negative, no. (%) | 37 (78·7) | 22 (45·8) | 12 (41·4) | 29 (29·9) | 0·05[§] |
| CCP high positive (>3x ULN), no. (%) | 0 (0) | 34 (70·8) | 27 (93·1) | 95 (97·9) | |
| CCP low positive, no. (%) | 0 (0) | 14 (29·2) | 2 (6·9) | 2 (2·1) | **<0·01**[§] |
| CCP negative, no. (%) | 47 (100) | 0 (0) | 0 (0) | 0 (0) | NA |
| DAS28-ESR (0–9.4), mean ± SD (complete data) | 5·4 ± 1·2 (39) | 4·8 ± 1·4 (40) | 4·9 ± 1·5 (27) | 5·2 ± 1·4 (84) | 0·16 |
| DAS28-CRP (0– | 5·0 ± 1·5 | 4·5 ± 1·2 | 4·5 ± 1·8 | 4·7 ± 1·7 | 0·23 |

| | | | | | |
|---|---|---|---|---|---|
| 9.4), mean ± SD (complete data) | (41) | (43) | (25) | (83) | |
| **Change in disease activity over one year** | | | | | |
| DAS28-ESR delta mean ± SD (complete data) | -1·8 ± 1·5 (35) | -1·7 ± 1·3 (37) | -1·3 ± 1·9 (23) | -1·5 ± 1·5 (78) | 0·43 |
| DAS28-CRP delta mean ± SD (complete data) | -2·5 ± 1·6 (28) | -2·4 ± 1·3 (31) | -2·0 ± 2·2 (16) | -2·0 ± 2·0 (66) | 0·34 |
| **EULAR response over one year using DAS28-ESR** | | | | | |
| Complete Data | 35 | 37 | 23 | 78 | |
| Good (percentage within group) | 13 (37·1) | 16 (43·2) | 8 (34·8) | 29 (37·2) | |
| Moderate (percentage within group) | 14 (40) | 12 (32·4) | 7 (30·4) | 22 (28·2) | |
| No Response (percentage within group) | 8 (22·9) | 9 (24·3) | 8 (34·8) | 27 (34·6) | 0·54[§] |
| **Baseline radiographic scores** | | | | | |
| Erosion score (0–280), mean ± SD | 3·9 ± 6·6 | 2·3 ± 4·1 | 2·1 ± 3·8 | 4·0 ± 8·5 | 0·55 |
| JSN score (0–168), mean ± SD | 4·7 ± 5·0 | 3·25 ± 6·2 | 2·5 ± 4·7 | 3·9 ± 7·9 | 0·66 |
| Total SvH score (0–448) mean ± SD | 8·6 ± 10·7 | 5·6 ± 9·3 | 4·6 ± 7·4 | 7·9 ±15·5 | 0·86 |
| **Radiographic progression** | | | | | |
| Change in Total SvH score over 12 months LSmean ± SE | 0·8 ± 0·4 | 0·5 ± 0·3 | 1·1 ± 0·5 | 1·7 ± 0·5 | **0·03** |

| | | | | | |
|---|---|---|---|---|---|
| Change in erosion score over 12 months LSmean ± SE | 0·3 ± 0·2 | 0·2 ± 0·1 | 0·5 ± 0·2 | 1·0 ± 3·0 | **0·01** |
| Change in Joint Space Narrowing score over 12 months LSmean ± SE | 0·5 ± 0·2 | 0·5 ± 0·3 | 0·1 ± 0·1 | 1·1 ± 0·4 | 0·22 |
| Patients with radiographic progression (total SvH score increase ≥ 1) no. (%) | 12 (25·5) | 9 (18·8) | 7 (24·1) | 35 (36·1) | **0·03**[§] |
| Patients with Rapid Radiographic Progression (RRP) (total SvH score ≥ 5) no. (%) | 2 (4·3) | 1 (2·1) | 2 (6·9) | 11 (11·3) | 0·06[§] |

**Table 2. Radiographic progression.** Least squared mean differences in radiographic progression scores for triple positive patients (ACPA, anti-acetylated peptide antibody and anti-carbamylated peptide antibody positive), single positive patients (ACPA positive only) and AMPA negative patients.

| Comparison | Least square mean difference | Asymptotic lower 95% CI | Asymptotic upper 95% CI |
|---|---|---|---|
| **Difference in total SvH change between baseline and 12 months** | | | |
| Triple positive vs single positive | 1·2 | 0·05 | 2·40 |
| Triple positive vs Negative | 0·9 | -0·23 | 2·26 |
| Single positive vs Negative | -0·3 | -1·09 | 0·67 |
| **Difference in Erosion Score change between baseline and 12 months** | | | |
| Triple positive vs single positive | 0·8 | 0·07 | 1·62 |
| Triple positive vs Negative | 0·7 | -0·06 | 1·59 |
| Single positive vs Negative | -0·1 | -0·56 | 0·40 |
| **Difference in Joint Space Narrowing Score change between baseline and 12 months** | | | |
| Triple positive vs single positive | 0·6 | -0·26 | 1·47 |
| Triple positive vs Negative | 0·6 | -0·26 | 1·32 |
| Single positive vs Negative | 0·0 | -0·69 | 0·53 |

**Table 3. Medication usage by RA patients.** Single positive=ACPA positive only. Double positive=ACPA and anti-acetylated peptide antibody positive. Triple positive=ACPA, anti-acetylated peptide antibody and anti-carbamylated peptide antibody positive. DMARD usage is at the one year time point. Steroid usage is over the course of one year. Chi-Sq test used to determine differences in proportions between Triple positive and Single positive patients. Results in bold denote P <0.05.

| | **Negative** | **Single positive** | **Double positive** | **Triple positive** | **P value Triple positive vs Single positive** |
|---|---|---|---|---|---|
| | n=47 | n=48 | n=29 | n=97 | |
| Methotrexate monotherapy, % of total group, (number of patients) | 25·5 (12) | 52·1 (25) | 27·6 (8) | 32·0 (31) | **0·02** |
| Sulfasalazine monotherapy, % of total group, (number of patients) | 6·4 (3) | 8·3 (4) | 6·9 (2) | 12·4 (12) | 0·46 |
| Hydroxychloroquine monotherapy, % of total group, (number of patients) | 8·5 (4) | 6·2 (3) | 0·0 (0) | 4·1 (4) | 0·58 |
| Any monotherapy, % of total group, (number of patients) | 40·4 (19) | 66·7 (32) | 34·5 (10) | 48·5 (47) | **0·04** |
| Two DMARDs, % of total group, (number of | 23·4 (11) | 29·2 (14) | 27·6 (8) | 27·8 (27) | 0·86 |

| | | | | | |
|---|---|---|---|---|---|
| patients) | | | | | |
| Three or more DMARDs, % of total group, (number of patients) | 8·5 (4) | 4·2 (2) | 17·2 (5) | 14·4 (14) | 0·07 |
| Biologics, % of total group, (number of patients) | 0·0 (0) | 0·0 (0) | 0·0 (0) | 2·1 (2) | NA |
| Total number of patients on DMARDs or biologics at 12 months, % of total group (number of patients) | 72·3 (34) | 100 (48) | 79·3 (23) | 91·7 (89) | NA |
| Oral prednisolone use at 12 months, % of total group, (number of patients) | 21·3 (10) | 25·0 (12) | 34·5 (10) | 34·0 (33) | 0·27 |
| Patients who had had any steroid injection IM or IA, % of total group, (number of patients) | 48·9 (23) | 62·5 (30) | 58·6 (17) | 56·7 (55) | 0·51 |

## Figure legends

**Figure 1. Autoantibody patterns. A:** 362 RA patients had autoantibodies to post translationally modified peptides measured. CCP denotes autoantibodies against the synthetic CCP peptide. Cit.Vim, Carb, Lys(ac) and Orn(ac) denote autoantibodies to post translational modification of a vimentin peptide where Cit.Vim=citrullinated vimentin, Carb=carbamylated vimentin, Lys(ac)=acetylated (at a lysine residue) vimentin, Orn(Ac)=acetylated (at an ornithine residue) vimentin. Six main groups emerge where the numbers within each group are more than 5% of the total sample; relevant Venn segments are coloured, with the majority of patients being positive for all five assays. **B:** Assay results were summarised into post-translational modification classes; patients with CCP or Cit.Vim positivity were combined into the Cit group and those with Orn(ac) or Lys(ac) positivity were combined into the Acet group. Venn segments are coloured when more than 5% of the population lies within a segment and this results in four groupings: single positivity to citrullinated peptides, double positivity to citrullinated and acetylated peptides, triple positivity (anti-cit/acet/carb) and negative patients.

**Figure 2. Anti-CCP antibody concentrations in RA patients.** In 362 RA patients, median anti-CCP concentration is higher in samples from triple positive patients with antibodies against citrullinated (Cit), carbamylated (Carb) and acetylated (Acet) vimentin (n=151) compared to single positive patients with antibodies against citrullinated (Cit) vimentin only (n=73) (1000 IU vs 96 IU, fdr adjusted p= $2{\cdot}0 \times 10^{-16}$). There are also significant differences in ACPA concentration between the Cit, Carb and Acet group and the Cit and Acet group (1000 IU vs 449 IU, fdr adjusted p value = 0·0003) and the Cit and Acet and Cit only group (449 IU vs 96 IU, fdr adjusted p value = 0·0002).

**Figure 3. Radiographic progression according to AMPA status. A:** Four AMPA reactivity groups are confirmed in RA patients in whom radiographic data were available. Groups were coloured and analysed if they made up more than 5% of the total population (n=233). **B:** Change in total SvH score. Triple positive RA patients with autoantibodies to citrullinated (Cit), carbamylated (Carb) and acetylated (Acet)

peptides have higher radiographic progression over one year compared to those who are single positive i.e. have autoantibodies to citrullinated peptides only (least squared mean difference 1.2, p=0·03). There is no statistical difference between single positive patients and AMPA negative patients (p=0·48). **C:** Change in SvH erosion score. Triple positive RA patients have a higher change in erosion score over one year compared to single positive patients (least squared mean difference 0·8, p=0·01). There is no statistically significant difference in erosion score change between single positive patients and AMPA negative patients (p=0·51). **D:** Change in SvH joint space narrowing score. No statistical difference was found between triple positive patients and single positive patients (p=0·22) or single positive patients and AMPA negative patients (p=0·95).

## Competing interests

**Jagtar S Nijjar** reports personal fees from Janssen Pharmaceutical and UCB outside the submitted work. **Holger Bang** reports grants and other from FP7 HEALTH programme during the conduct of the study; HB is an employee of Orgentec Diagnostika GmbH, which manufactures and sells in vitro diagnostics. Christopher D Buckley reports grants from Roche, other from Pfizer, other from GSK outside the submitted work. **Désirée van der Heijde** reports personal fees from AbbVie, Amgen, Astellas, AstraZeneca, BMS, Boehringer Ingelheim, Celgene, Cyxone, Daichii, Eisai, Elly-Lilly, Galapagos, Gilead, GSK, Janssen, Merck, Novartis, Pfizer, Regeneron, Roche, Sanofi, Takeda, UCB Pharma outside the submitted work; DVDH is Director Imaging Rheumatology BV. **Iain B McInnes** reports personal fees from AbbVie, grants from AstraZeneca, grants and personal fees from Celgene, grants and personal fees from Compugen, personal fees from Galvani, personal fees from Lilly, grants from Novartis, personal fees from Pfizer, grants from Roche, grants and personal fees from UCB outside the submitted work. **Duncan Porter** reports grants from Pfizer and grants from Chief Scientist's Office, Scotland, during the conduct of the study. **Karim Raza** reports grants and personal fees from Abbvie, grants and personal fees from Pfizer, and personal fees from Sanofi, Lilly, Bristol-Myers Squibb, UCB, Janssen, Roche Chugai outside the submitted work. **Other authors** have no disclosures.

## Contributorship

**Jagtar S Nijjar** and **Fraser R Morton** analysed the data and drafted the manuscript. Scottish Early Rheumatoid Arthritis Inception Cohort Investigators recruited participants. **Holger Bang** coordinated the antibody assays, interpreted the data and contributed to the development of the manuscript. **Christopher D Buckley** contributed to the development of the study and the manuscript. **Désirée van der Heijde** coordinated the radiographic scoring, interpreted the data and contributed to the development of the manuscript. **Ashley Gilmour** and **Caron Paterson** coordinated the collection of data from SERA participants and to the development of the manuscript. **Iain B McInnes** and **Duncan Porter** are lead investigators for the SERA cohort; they contributed to the development of the study, data interpretation

and the development of the manuscript. **Karim Raza** conceived and coordinated this study, interpreted the data and contributed to the development of the manuscript.

## Acknowledgements

Scottish Early Rheumatoid Arthritis Inception Cohort Investigators are: Cosimo di Bari, Aberdeen Royal Infirmary, Aberdeen; Margaret Duncan, Ayr Hospital; Susan Fraser, Southern General Hospital, Glasgow; Mohini Gray, Western General Hospital, Edinburgh; Lisa Hutton, Inverclyde Royal Hospital; John Harvie, Raigmore Hospital, Inverness; Vinod Kumar, Ninewells Hospital, Dundee; Mike McMahon, Dumfries & Galloway Royal Infirmary; Robin Munro, Wishaw General Hospital; John Larkin, Victoria Infirmary Glasgow; Neil McKay, Western General Hospital, Edinburgh; John McLaren, Whyteman's Brae Hospital, Fife; Stuart Ralston, Western General Hospital, Edinburgh; David M Reid, Aberdeen Royal Infirmary; Duncan Porter, Gartnavel General Hospital, Glasgow; Ruth Richmond, Borders General Hospital, Melrose; Gillian Roberts, Vale of Leven Hospital; Sarah Saunders, Glasgow Royal Infirmary; Hilary Wilson, Stobhill Hospital, Glasgow.

We are grateful to Louise Bennett for her assistance with extracting samples from the SERA biobank and to Peter Nightingale for his statistical insights.

## Funding information

The research leading to these results was funded within the FP7 HEALTH programme under the grant agreement FP7-HEALTH-F2-2012-305549 (EuroTEAM). This work was supported by awards (INF-GU-168) from 1) the Translational Medicine Research Collaboration – a consortium made up of the Universities of Aberdeen, Dundee, Edinburgh and Glasgow. Karim Raza and Christopher D Buckley are funded by the Birmingham NIHR BRC. This work has been supported by the Research into Inflammatory Arthritis Centre Versus Arthritis and the MRC / Versus Arthritis Centre for Musculoskeletal Ageing Research. The SERA cohort was supported by awards (INF-GU-168) from 1) the Translational Medicine Research Collaboration – a consortium made up of the Universities of Aberdeen, Dundee,

Edinburgh and Glasgow, the four associated NHS Health Boards (Grampian, Tayside, Lothian and Greater Glasgow & Clyde), and Pfizer and 2) from the Chief Scientific Office (Ref ETM-40).

## Data sharing statement

Data are available upon reasonable request; access to data would be subject to approval by the Scottish Early RA inception cohort (SERA) Data Access Committee.

## References

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011 Dec 8;365(23):2205–19.

2. van Venrooij WJ, van Beers JJBC, Pruijn GJM. Anti-CCP antibodies: the past, the present and the future. Nat Rev Rheumatol. 2011 Jun 7;7(7):391–8.

3. Bang H, Egerer K, Gauliard A, Lüthke K, Rudolph PE, Fredenhagen G, et al. Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis. Arthritis Rheum. Wiley Subscription Services, Inc., A Wiley Company; 2007 Aug;56(8):2503–11.

4. Mathsson L, Mullazehi M, Wick MC, Sjöberg O, van Vollenhoven R, Klareskog L, et al. Antibodies against citrullinated vimentin in rheumatoid arthritis: higher sensitivity and extended prognostic value concerning future radiographic progression as compared with antibodies against cyclic citrullinated peptides. Arthritis Rheum. Wiley Subscription Services, Inc., A Wiley Company; 2008 Jan;58(1):36–45.

5. Raza K, Breese M, Nightingale P, Kumar K, Potter T, Carruthers DM, et al. Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis. The Journal of Rheumatology. Europe PMC Funders; 2005 Feb;32(2):231–8.

6. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GMC, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci USA. 2011 Oct 18;108(42):17372–7.

7. Martínez G, Gómez JA, Bang H, Martínez-Gamboa L, Roggenbuck D, Burmester G-R, et al. Carbamylated vimentin represents a relevant autoantigen in Latin American (Cuban) rheumatoid arthritis patients. Rheumatol Int. Springer Berlin Heidelberg; 2016 Jun;36(6):781–91.

8. Figueiredo CP, Bang H, Cobra JF, Englbrecht M, Hueber AJ, Haschka J, et al. Antimodified protein antibody response pattern influences the risk for

disease relapse in patients with rheumatoid arthritis tapering disease modifying antirheumatic drugs. Ann Rheum Dis. 2017 Feb;76(2):399–407.

9. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. BMJ Publishing Group Ltd and European League Against Rheumatism; 2016 Jun;75(6):1099–107.

10. Makrygiannakis D, Hermansson M, Ulfgren A-K, Nicholas AP, Zendman AJW, Eklund A, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Ann Rheum Dis. 2008 Oct;67(10):1488–92.

11. Tracy A, Buckley CD, Raza K. Pre-symptomatic autoimmunity in rheumatoid arthritis: when does the disease start? Semin Immunopathol. 2017 Jun;39(4):423–35.

12. Syversen SW, Goll GL, van der Heijde D, Landewé R, Lie BA, Odegård S, et al. Prediction of radiographic progression in rheumatoid arthritis and the role of antibodies against mutated citrullinated vimentin: results from a 10-year prospective study. Ann Rheum Dis. BMJ Publishing Group Ltd; 2010 Feb;69(2):345–51.

13. Kampstra ASB, Dekkers JS, Volkov M, Dorjée AL, Hafkenscheid L, Kempers AC, et al. Different classes of anti-modified protein antibodies are induced on exposure to antigens expressing only one type of modification. Ann Rheum Dis. 2019 Jul;78(7):908–16.

14. Dale J, Paterson C, Tierney A, Ralston SH, Reid DM, Basu N, et al. The Scottish Early Rheumatoid Arthritis (SERA) Study: an inception cohort and biobank. BMC Musculoskelet Disord. BioMed Central; 2016 Nov 9;17(1):461.

15. Jackman S. pscl:Classes and Methods for R Developed in the Political Science Computational Laboratory. R package version 1.5.2 [Internet]. United States Studies Centre, University of Sydney; 2017. Available from: https://github.com/atahk/pscl/

16. Lenth RV. Least-Squares Means: The R Package lsmeans. J Stat Soft. 2016;69(1):1–33.

17. Brink M, Verheul MK, Rönnelid J, Berglin E, Holmdahl R, Toes REM, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. BioMed Central; 2015 Feb 7;17(1):25–8.