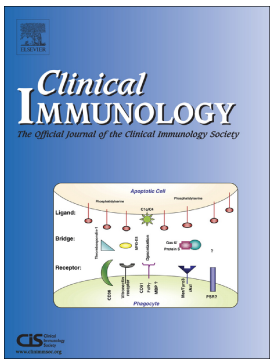

Combination of anti-citrullinated protein antibodies and rheumatoid factor is associated with increased systemic inflammatory mediators and more rapid progression from preclinical to clinical rheumatoid arthritis

Nithya Lingampalli, Jeremy Sokolove, Lauren J. Lahey, Jess D. Edison, William R. Gilliland, V. Michael Holers, Kevin D. Deane, William H. Robinson

PII: S1521-6616(18)30246-8
DOI: doi:10.1016/j.clim.2018.05.004
Reference: YCLIM 8048

To appear in: *Clinical Immunology*

Received date: 9 April 2018
Revised date: 22 May 2018
Accepted date: 22 May 2018

Please cite this article as: Nithya Lingampalli, Jeremy Sokolove, Lauren J. Lahey, Jess D. Edison, William R. Gilliland, V. Michael Holers, Kevin D. Deane, William H. Robinson , Combination of anti-citrullinated protein antibodies and rheumatoid factor is associated with increased systemic inflammatory mediators and more rapid progression from preclinical to clinical rheumatoid arthritis. Clinical Immunology(2017), doi:10.1016/j.clim.2018.05.004

Combination of Anti-Citrullinated Protein Antibodies and Rheumatoid Factor is Associated with Increased Systemic Inflammatory Mediators and More Rapid Progression from Preclinical to Clinical Rheumatoid Arthritis

Nithya Lingampalli[1,2], Jeremy Sokolove[1,2], Lauren J. Lahey[1,2], Jess D. Edison[4], William R. Gilliland[5], V. Michael Holers[3], Kevin D. Deane[3]*, William H. Robinson[1,2,*]

Email Addresses:

Nithya Lingampalli: nlingampalli@gmail.com

Jeremy Sokolove: sokolove@stanford.edu

Lauren J. Lahey: llahey@stanford.edu

Jess D. Edison: jess.d.edison.mil@mail.mil

William R. Gilliland: wgilliland@usuhs.edu

V. Michael Holers: michael.holers@ucdenver.edu

Kevin D. Deane: kevin.deane@ucdenver.edu

William H. Robinson: w.robinson@stanford.edu

Institutional Addresses:

[1]VA Palo Alto Health Care System, 3801 Miranda Ave, C4-191, Palo Alto, CA 94304

[2]Division of Immunology and Rheumatology, Stanford University, 269 Campus Drive, CCSR 4135, Stanford, CA 94305

[3]Division of Rheumatology, University of Colorado Denver School of Medicine, 1775 Aurora Court, Aurora, CO 80045

[4]Rheumatology Service, Department of Medicine, Walter Reed National Military Medical Center, 8901 Wisconsin Avenue, Bethesda, MD 20889

[5]Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814

*W.H.R. and K.D.D. contributed equally to this manuscript. Correspondence should be addressed to K.D.D. at kevin.deane@ucdenver.edu or W.H.R. at w.robinson@stanford.edu.

Abstract

The development of rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPAs) can be observed years prior to clinical diagnosis of rheumatoid arthritis (RA). Nevertheless, the interaction between these two autoantibodies and their combined effect on development of RA is unclear. We measured RF, cytokines, and ACPA subtypes in serial pre-clinical serum samples collected from 83 US veterans who all developed RA. Levels of cytokines and ACPAs were compared between the following groups: anti-cyclic citrullinated peptide (anti-CCP)-/RF- (double negative), anti-CCP+/RF-, anti-CCP-/RF+, or anti-CCP+/RF+ (double-positive). The double-positive subgroup had significantly higher levels of 20 inflammatory cytokines and 29 ACPA reactivities, and the shortest interval, 1.3 years, between the preclinical sample timepoint and diagnosis of RA. Thus, the combined presence of ACPAs and RF is associated with a more rapid progression to RA, suggesting that anti-CCP+/RF+ individuals have a more advanced preclinical disease state and that the onset of RA may be imminent.

Keywords

Rheumatoid arthritis; Rheumatoid factor (RF); anti-citrullinated protein antibodies (ACPA), cytokines

1. INTRODUCTION

Rheumatoid arthritis (RA) affects approximately 0.5% of the general population [1, 2], and is characterized by inflammation and destruction of the synovial joints [3, 4]. The window of opportunity for reducing the severity of joint damage with treatment comes early in the course of the clinically apparent disease [5]. As a result, much effort is underway to identify predictive biomarkers for the future development and diagnosis of RA prior to the onset of clinical symptoms as well as biomarkers to classify the mechanisms underlying the development of RA [6, 7].

It is now well established that elevations of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), and cytokines precede the onset of the clinical findings of inflammatory arthritis that can be classified as RA during a period that can be termed 'Preclinical RA' [8-20]. ACPAs are highly specific for RA and are detected in 60-70% of RA patients [21-24]. Anti-CCP tests capture several overlapping reactivity of ACPA targets and thus do not represent a single ACPA specificity [25]. RF is also present in nearly 70% of RA patients; however, it is less specific than ACPA for RA and its role in RA pathogenesis is not fully defined [26, 27].

Previous studies found that RF or ACPAs had the potential to serve as biomarkers predictive for RA disease severity [28, 29] as well as for the conversion of the preclinical state to clinical rheumatoid arthritis [16, 19]. Further, it has been shown that the presence of both ACPAs and IgA RF predicted the most rapid progression from the preclinical state to clinical rheumatoid arthritis [19]. Others have shown that higher concentrations of either RF or ACPAs are associated with a more aggressive disease course, characterized by greater disease activity and lower remission rates [23, 30, 31]. Indeed, both RF and ACPAs are known to contribute to the pathogenesis of RA, and we recently demonstrated that they interact to promote inflammation in experimental models of RA [32]. Still incompletely understood, however, is the potential interactions and thus relationship between the detection of these autoantibodies, individually as compared to together, and the timing of the transition from preclinical to clinically apparent and classified RA.

Defining the specific autoantibody specificities, and combinations of autoantibody specificities, as they relate to the development of clinical RA in these asymptomatic preclinical patients could yield key biomarkers for identifying individuals at high risk for the imminent development of RA [33]. Several studies have described the presence of RF and ACPAs in the blood prior to the onset of arthritis and the clinical diagnosis of RA [8-16]. Given that prior work demonstrated that ACPA and

RF are both elevated in the pre-clinical phase of RA in this pre-clinical RA cohort [8, 9], here we focus on how ACPA and RF positivity and the co-occurrence of these autoantibodies promote the transition from preclinical to clinical onset of RA.

In this manuscript, we evaluate the relationship between positivity for RF and/or ACPA with the presence of multiple cytokines in preclinical RA to test the hypothesis that positivity for both RF and ACPA in preclinical RA is associated with imminent onset of clinically apparent inflammatory arthritis and higher levels of inflammation. Specifically, we examined the presence of RF by nephelometry and anti-CCP ACPA autoantibodies detected by commercial assays, as well 34 different ACPA autoantigens and 48 cytokines and chemokines detected by multiplex arrays, in serial serum samples collected from a cohort of patients during the preclinical phase of RA. We sought to elucidate the temporal relationships between autoantibody status, systemic inflammation, and progression to clinical RA. An additional goal was to determine which autoantibodies may be most useful in predicting the likelihood and timing of onset of clinical RA as a precedent for future studies to further evaluate these connections. We demonstrate that the co-occurrence of ACPAs and RF in preclinical RA is associated with an increase in systemic inflammation, as well as a shorter transition period from preclinical to clinical RA.

2. METHODS

2.1. Patient samples and clinical measures

The study protocol was approved by the Institutional Review Boards at the Walter Reed Army Medical Center (WRAMC), Stanford University, and the University of Colorado. The protocol now resides at WRNMMC and remains an active protocol. Informed consent was not possible due to the retrospective analysis of this serum repository cohort, and the requirement for informed consent was waived by the ethics committees at WRAMC, the University of Colorado and Stanford University. All investigators conformed to the principles expressed in the 1975 Declaration of Helsinki [34].

As previously described [8, 9], samples studied included serial serum samples obtained from the Department of Defense Serum Repository (DoDSR), which stores serum samples obtained from the United States Armed Forces personnel during enlistment and deployment on average every year.

Samples are stored in a central repository at -30°C. The subjects studied in this analysis were members of the United States Armed Forces who were assigned to the Walter Reed National Military Medical Center (WRNMMC) Rheumatology Clinic in 1989-2003, and were diagnosed with RA during the longitudinal sample collection. At clinical diagnosis, all subjects satisfied the 1987 American College of Rheumatology classification of RA or were considered to have RA by a board-certified rheumatologist [35].

The samples studied were serial serum samples collected at time points up to 14 years before and at the time of clinical diagnosis of RA. A total of 288 banked serum samples from a representative cohort of 83 patients were analyzed using a bead-based multiplex cytokine assays and a bead-based rheumatoid arthritis autoantigen array, which includes a CCP antigen bead, as previously described [8, 9, 36]. Clinical data, such as age, gender, race, and presence of joint erosions, were available for each of the subjects, as were the results of RF tests by nephelometry performed at the Rheumatology Clinical Research Laboratory (positivity defined as ≥15 IU/ml) and anti-CCP2 tests to measure IgG antibodies to CCP (CCP2 assay with positivity defined as ≥5 units/ml) [8]. Investigators at Stanford University were blinded to the samples' disease classification at the time of antibody and cytokine profiling. Only after testing was complete was the coding key provided to link the serum samples to the corresponding data on the subject.

2.2. Multiplex cytokine analysis

As previously described [9, 37-39], multiplex analysis of 48 cytokines and chemokines was performed on the serum samples using the Bio-Plex™ bead array (Bio-Rad) run on a Luminex 200 system (Luminex Corporation) according to the manufacturer's instructions, with the exception that the proprietary Bio-Rad assay dilution buffer was modified to contain reagents that reduce the effect of heterophilic antibodies (RF) in multiplex immunoassays [40]. Data processing was performed with the Bio-Plex Manager 5.0 software (Bio-Rad), and serum concentrations (pictograms per milliliter) were interpolated from standard curves for each respective cytokine or chemokine. This protocol and data generated were MAIME compliant and were deposited in the Gene Expression Omnibus Repository (accession number GSE32021; http:/www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32021).

2.3. Multiplex autoantigen arrays

As previously described[38], serum levels of ACPA antibodies targeting 44 putative RA-associated autoantigens were measured by using a custom bead-based immunoassay on a Bio-Plex platform

[36, 39]. Of the 44 antigens contained in the bead-based array, 34 are citrullinated and 10 are native (Figure 6). Serum was diluted to a 1:30 ratio in a proprietary sample dilution buffer provided by Bio-Rad, mixed with the antigens conjugated to spectrally distinct fluorescent microspheres (Bio-Rad), and then incubated with an anti-human phycoerythrin (Cy3)–labeled antibody (Jackson ImmunoResearch). The resulting fluorescence intensities were analyzed on a Luminex 200 platform (Luminex Corporation).

The number of ACPA reactivities detected in each of the samples by multiplex antigen array was determined by calculating the z-score of the reactivity (fluorescence intensity) of the sample to each antigen. Positivity was defined as a z-score of 1.5 standard deviations above the mean reactivity to a given antigen in the entire sample set.

2.4. Statistical analysis

For all analyses other than that presented in Figure 4, samples were categorized by autoantibody status (using the CCP2 results for CCP): anti-CCP−/RF− (double-negative; *n*=68), anti-CCP+/RF− (*n*=28), anti-CCP−/RF+ (*n*=37), and anti-CCP+/RF+ (double-positive; *n*=98). For Figure 4, all samples, including those that remaining seronegative throughout collection time, were utilized: anti-CCP−/RF− (double-negative; *n*=125), anti-CCP+/RF− (*n*=28), anti-CCP−/RF+ (*n*=37), and anti-CCP+/RF+ (double-positive; *n*=98). Statistical analysis of the differences in patient characteristics between the different groups was performing using a Kruskal-Wallis test (Table 1). The measured cytokine data were normally distributed, and levels of each cytokine and chemokine were compared between groups using unpaired t-test with Welch's correction (Figure 2). *P* values less than 0.05 were considered significant. The statistical analyses were made using PRISM (GraphPad Software).

Additional comparisons were performed on multiplex cytokines as well as multiplex ACPAs of samples using Significance Analysis of Microarrays (SAM) version 3.08 (Figures 1 and 3) [41]. Output was sorted on the basis of false discovery rates (FDRs) in order to determine which antigens had the greatest differences in autoantibody reactivity between groups of samples. The use of FDRs obviates the need to adjust for multiple comparisons. Cluster 3.0 [42] was used to subject SAM results to unsupervised hierarchical clustering according to similarities in ACPA specificities, cytokines, or chemokines detected, and Java TreeView 1.1.3 [43] was used to visualize the results as a heatmap.

In some patients, certain markers were detected at an early time point but not at a later time point (this was observed in 10% or fewer of samples). For descriptive analyses relating to autoantibody status and relative days before clinical RA diagnosis, if a patient tested positive for a given antibody marker (e.g. anti-CCP or RF) at one time point but negative for that same marker at all subsequent time points, then that patient was designated negative for that marker. Conversely, if a patient initially tested positive for a given marker (e.g. anti-CCP or RF), subsequently negative, but at an even later time point tested positive once more, then that patient was considered positive for that marker throughout the time course studied. This trend was observed in less than 3% of patients that were subsequently defined negative, and in less than 2% of patients described as positive.

3. Results

3.1. ACPAs and RF in the military cohort

The characteristics of the 83-patient cohort at the time of clinical diagnosis are shown in Table 1, with patients grouped based on their CCP (based on the CCP2 test results) and RF (based on RF test results by nephelometry) autoantibody status. The studied cohort was 59% males, and had a mean age of clinical diagnosis of 40 years. Furthermore, there was no significant difference in gender or age at diagnosis between the four autoantibody status groups as tested using a Kruskal-Wallis test. At the time of initial sample collection, 19% of the patient samples were classified as anti-CCP+/RF+ (double-positive), 7% were anti-CCP+/RF−, 7% were anti-CCP−/RF+, and 67% were anti-CCP−/RF− (double-negative). The time of sample collection relative to age at clinical RA diagnosis, race, and erosion presence did not differ significantly between the groups as tested using a Kruskal-Wallis test.

A total of 288 samples were used from the 83 patients from the cohort. Of these samples, 34% were classified as double-positive, 10% were anti-CCP+/RF−, 13% were anti-CCP−/RF+, and 43% were double-negative.

Within the overall cohort, 19 patients, 22.9% of the total number, remained seronegative (CCP-/RF-) throughout the time of sample collection and observation. Given that these individuals were never positive for autoantibodies during the time of sampling and observation, their corresponding 57 samples were excluded from analyses to prevent an artifactual extension of the period of preclinical positivity. Hence, we utilized the remaining 231 samples from 64 unique patients for all analyses

except only those corresponding to Figure 4. Of the remaining 231 samples, 42% were classified as double-positive, 12% were anti-CCP+/RF–, 16% were anti-CCP–/RF+, and 30% were double-negative.

3.2. Concurrence of ACPAs and RF is linked to RA-associated inflammation

We used multiplex bead-based assays to measure the levels of 48 different cytokines and chemokines in 231 serial serum samples collected from the 83 patients, all of whom developed clinical RA at some point during the longitudinal sample collection. We then compared these levels between groups of samples stratified according to their individual autoantibody positivity status: the double-negative group, the anti-CCP+/RF- group, the anti-CCP-/RF+ group, and the double-positive group. Four-way multiclass group comparisons were performed with SAM, and the output was sorted on the basis of FDRs. The analysis indicated that levels of 20 of the 48 cytokines and chemokines were significantly (significance was defined as a q-value less than 5%) higher in the double-positive group compared to the other three groups (Figure 1).

To ensure that the relationships observed are not an indirect result of being closer to seropositive RA disease onset, the analysis was restricted to a similar time interval, between 1 and 5 years prior to disease diagnosis, between the four groups. Four-way multiclass group comparisons were performed with SAM, and the output was sorted on the basis of FDRs. The analysis indicated that levels of 16 of the 48 cytokines and chemokines were significantly (significance was defined as a q-value less than 5%) higher in the double-positive samples compared to samples in the other three groups.

When compared solely to the double-negative group, the double-positive group exhibited significantly higher levels of 26 of the 48 inflammatory cytokines and chemokines as determined by an unpaired t-test using Welch's correction. Of the 26 elevated cytokines, results for 6 are presented in Figure 2a, including tumor necrosis factor alpha (TNF-α), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin 12 subunit p70 (IL-12p70), granulocyte-colony stimulating factor (G-CSF), and interleukin 15 (IL-15). The double-positive group exhibited significantly elevated levels of these cytokines, compared to the CCP+/RF- and CCP-/RF+ groups as well. Additionally, we found that the double-positive group had significantly lower levels of a single chemokine, RANTES (CCL5), when compared to the other three groups (Figure 2b). Although many of the cytokines analyzed, including those most upregulated in

the double-positive group, are highly related and thus at reduced risk for Type I error that can occur in multiplex cytokine analysis, stringent implementation of Bonferroni's correction for the 26 potentially independent cytokines would require a *P*-value less than 0.002, and this was achieved for all the cytokines shown in Figure 2. Hence, we conclude that the differences in cytokine and chemokine levels identified in our analysis are indeed significant and not merely a by-product of the increased probability of identifying at least one significant result due to chance as more hypotheses are tested.

A smaller, but in most cases statistically significant, increase in cytokine and chemokine reactivity levels was observed in the anti-CCP-/RF+ group compared to the double-negative and anti-CCP+/RF- groups (Figure 2a). Though potentially related to the activity of RF as a heterophilic antibody [44], we have previously suggested [32] that modest upregulation of cytokines and chemokines in the anti-CCP-/RF+ group may be explained by the presence of some anti-CCP-/RF+ patients possessing ACPAs that either do not react with the synthetic CCP in the commercial anti-CCP2 assay or are present at a level that is below the detection range of this assay. To further investigate this possibility, we used a multiplex antigen array containing 34 putative citrullinated autoantigens to identify what, if any, ACPA specificities were elevated in the anti-CCP-/RF+ group compared to the double-negative group (Figure 3). The level of ACPA reactivity was elevated in the anti-CCP-/RF+ group compared to the double negative group for 29 of the 34 citrullinated autoantigens when tested with an unpaired t-test (Figure 4). These results suggest that increased levels of ACPAs, like cytokine and chemokine levels, are associated with the presence of RF, as indicated by the significant increase in ACPA reactivity in the anti-CCP-/RF+ group when compared to the double-negative group (Figure 4).

3.3. Concurrence of ACPAs and RF indicates imminent onset of clinical RA

We next examined the relationship between a sample's anti-CCP/RF autoantibody status and the time between sample collection and the transition to RA as determined by clinical diagnosis by a board-certified rheumatologist. The mean interval between sample collection and diagnosis of clinical RA was calculated by taking the difference in time between the sample collection date and the RA clinical diagnosis date, and then calculating an average of the time for each of the autoantibody groups. The mean interval was 1.3 years in the double-positive group, 2.0 years in the anti-CCP-/RF+ group, 3.5 years in the anti-CCP+/RF- group, and 5.8 years in the double-negative group (Figure 5a). Statistical analysis indicated that the samples in the double-positive group were significantly closer to developing clinically apparent RA than the samples in the other three groups

(P<0.01 by unpaired t-test with Welch's correction). This finding suggests that the concurrence of ACPAs and RF reflects a preclinical disease state that is closer to the disease severity observed at clinical diagnosis.

Because we found that serum samples designated anti-CCP-/RF+ have, in some cases, have positive ACPA reactivities (Figure 3) and have higher levels of cytokines and chemokines than the serum samples designated anti-CCP-/RF- (Figure 2), we examined how the number of ACPA reactivities in a sample relates to the length of the interval between the sample's collection and the corresponding patient's diagnosis with RA. The 37 samples in the anti-CCP-/RF+ group, were divided into three groups based on the number of ACPA reactivities that were enriched above the mean reactivity for the given antigen in the entire sample set: ACPA number >1, ACPA number >2, and ACPA number >3. The mean interval between sample collection and diagnosis of clinical RA was calculated by taking the difference in time between the sample collection date and the RA clinical diagnosis date, and then calculating an average of the time for each of the ACPA reactivity number groups. The mean interval was 5.6 years in the ACPA number >1 group, 4.2 years in the ACPA number >2 group, and 1.1 years in the ACPA number >3 group. We found that the higher number of ACPA specificities a sample contained, the shorter the interval between the sample's collection and the subsequent diagnosis of clinical RA (Figure 5b). This suggests that ACPAs that are not detected by the commercially available anti-CCP2 assay synergize with RF in promoting inflammation and the pathogenesis of RA, such that the concurrence of RF and ACPAs reflects a relatively advanced disease state that is closer to the onset of clinical RA. This is consistent with the anti-CCP+/RF+ group exhibiting the highest number of ACPA reactivities in a multiplex RA autoantigen array analysis when compared to the single-positive and double-negative groups (Figure 6).

4. Discussion

In this study, we examined RA-related autoimmunity and inflammation in the preclinical period through multiplex analyses of autoantibodies and cytokines in serial serum samples collected at serial time points during the period leading-up to the diagnosis of clinical RA. We detected the presence of ACPAs, RF, and certain inflammatory chemokines and cytokines in the circulation years before the clinical diagnosis of RA. Further, we demonstrated that the concurrence of ACPAs and RF in the preclinical phase is indicative of a higher inflammatory and more advanced disease state that is temporally closer to the onset of clinical RA.

Several previous studies showed that ACPAs and RF synergize in mediating RA-associated inflammation and disease activity [19, 32, 45, 46] and can be associated with the onset of RA [18, 19]. Our present findings support this conclusion in an independent patient cohort with preclinical RA. We further show that, among patients who later developed clinical RA, serum levels of inflammatory cytokines associated with RA pathophysiology [4] are significantly higher in samples that are positive for both ACPA and RF autoantibodies as compared to samples that have neither autoantibody or only one of the autoantibodies, with the exception of one chemokine that was decreased in the double-positive group. Several inflammatory cytokines were also elevated, albeit to a lesser extent, in the anti-CCP-/RF+ samples. Interestingly, we show that anti-CCP-/RF+ samples can show reactivity to citrullinated autoantigens when analyzed by antigen array, indicating that they contain ACPAs that were not detected by the CCP2 assay. This suggests that certain individuals within the anti-CCP-/RF+ classification may in fact represent a sub-class that is a more advanced preclinical disease state than those who did not show reactivity to the citrullinated autoantigens. The increased inflammation seen in the double-positive group supports a role for anti-CCP and RF autoantibodies synergizing to promote inflammation and progression from preclinical to clinical RA.

The study has several limitations. First, our samples derived from a military population that has a lower mean age at diagnosis and higher percentage of males than other RA populations [47]. Hence, caution should be exercised before generalizing these results. Second, in this study, we did not perform longitudinal comparisons within individual patients because sufficient numbers of serial samples were not available from the majority of patients. Since each sample was tested individually, and not in regard to the subject it was taken from, samples obtained over time from the same subject may be categorized into different, and in certain cases more than one, ACPA and RF positivity group(s). When performing statistical analyses this represents a potential confounding factor. In the future, it will be important to further validate the findings using large cohorts with more comprehensive sets of serial samples that will enable longitudinal comparisons. Third, classification of each study subject was based on their most characteristic autoantibody profile for all visits. Thus, individuals that were anti-CCP+ on one visit only, which was then followed by subsequent negative samples, are classified as anti-CCP-. Conversely, if the anti-CCP+ test result reappears on a subsequent visit, this individual is deemed to be anti-CCP+. Although this was only relevant to a small number of individuals studied, such may lose some of the nuances of how the ACPA response evolves over time in individuals as previously demonstrated by our group

[39]. Finally, our methods minimize the possibility of RF resulting in a falsely elevated signal in sandwich immunoassays. The fact that the signal observed in the single-positive RF+ group is relatively low or absent suggests that artifacts due to heterophilic antibodies are not a significant confounding factor in our study; however, this possibility is not completely eliminated.

5. Conclusions

In summary, our findings show that samples that are positive for both RF and ACPA autoantibodies during the preclinical phase of RA have higher levels of systemic inflammation than samples which have neither or only one of these autoantibodies. Furthermore, our data suggest that concurrence of RF and ACPAs is indicative of a more advanced preclinical disease state that is temporally closer to transition into clinical RA. This suggests a pathogenic process in which these two types of autoantibodies are present and driving the transition from asymptomatic to symptomatic RA. Our results yield insight into the ACPA/RF profile in the preclinical phase of RA and its relation to the onset of clinical RA, and this information that could be useful in developing better algorithms for predicting the imminent onset of clinical RA and thus providing for primary prevention, earlier treatment, and/or more effective treatment.

Funding

The research is supported by NIH NIAMS R01 AR063676, NIH NIAMS AR051394, NIH NIAID AI061479, NIH NIAID U01 AI101981, NIH NIAID U01 AI057229, NIH NIAID U19 AI110491, NIH NIAMS/NIAID/FNIH AMP Program UH2 AR067681, and the Northern California Chapter of the Arthritis Foundation (NCCAF) Center of Excellence.

Competing interests

The authors declare no competing interests.

Authors' contributions

All authors were involved in drafting the article or revising it critically for intellectual content, and all authors approved the final version to be published. Study conception and design: Sokolove, Holers, Edison, Deane, Robinson. Acquisition of data: Lingampalli, Sokolove, Lahey. Analysis and interpretation of data: Lingampalli, Sokolove, Holers, Deane, Robinson.

Acknowledgements

The authors would like to thank Steve Binder and Michelle Delanoy of Bio-Rad Laboratories (Hercules, CA) for their provision of antigen-coated beads, technical support, and technical guidance, and Michelle Bloom for editing and proofreading.

The views expressed herein are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense, or the US government.

Table 1. Clinical characteristics of the patient cohort stratified by RF and anti-CCP autoantibody status at the time of initial sample collection*

Characteristic	Total Patient Cohort (n=83)	Anti-CCP-/RF- (n=55)	Anti-CCP+/RF- (n=6)	Anti-CCP-/RF+ (n=6)	Anti-CCP-/RF- (n=16)
Sociodemographics and comorbidity					
Male Sex	59.0	58.2	66.7	33.3	68.8
Race/ethnicity					
White	68.7	69.1	83.3	33.3	75.0
African-American	25.3	23.6	16.7	66.7	18.8
Other	6.0	7.3	0	0	6.2
RA Factors					
Age at diagnosis, mean ± SD years	49.9 ± 10.0	40.3 ± 10.2	36.3 ± 10.0	31.3 ± 7.9	43.2 ± 8.8
Erosions					
Positive	50.6	45.5	83.3	33.3	62.5
Negative	41.0	47.3	0	50.0	31.3
Not done	8.4	7.2	16.7	16.7	6.2

References

1. Scublinsky D, Gonzalez CD: **Quantifying Disease in Challenging Conditions: Incidence and Prevalence of Rheumatoid Arthritis**. *The Journal of rheumatology* 2016, **43**(7):1263-1264.
2. Neovius M, Simard JF, Askling J, group As: **Nationwide prevalence of rheumatoid arthritis and penetration of disease-modifying drugs in Sweden**. *Annals of the rheumatic diseases* 2011, **70**(4):624-629.
3. Firestein GS: **Evolving concepts of rheumatoid arthritis**. *Nature* 2003, **423**(6937):356-361.
4. McInnes IB, Schett G: **The pathogenesis of rheumatoid arthritis**. *The New England journal of medicine* 2011, **365**(23):2205-2219.
5. van Nies JA, Krabben A, Schoones JW, Huizinga TW, Kloppenburg M, van der Helm-van Mil AH: **What is the evidence for the presence of a therapeutic window of opportunity in rheumatoid arthritis? A systematic literature review**. *Annals of the rheumatic diseases* 2014, **73**(5):861-870.
6. Mankia K, Emery P: **Preclinical Rheumatoid Arthritis: Progress Toward Prevention**. *Arthritis & rheumatology* 2016, **68**(4):779-788.
7. Majka DS, Holers VM: **Can we accurately predict the development of rheumatoid arthritis in the preclinical phase?** *Arthritis and rheumatism* 2003, **48**(10):2701-2705.
8. Majka DS, Deane KD, Parrish LA, Lazar AA, Baron AE, Walker CW, Rubertone MV, Gilliland WR, Norris JM, Holers VM: **Duration of preclinical rheumatoid arthritis-related autoantibody positivity increases in subjects with older age at time of disease diagnosis**. *Annals of the rheumatic diseases* 2008, **67**(6):801-807.
9. Deane KD, O'Donnell CI, Hueber W, Majka DS, Lazar AA, Derber LA, Gilliland WR, Edison JD, Norris JM, Robinson WH *et al*: **The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner**. *Arthritis and rheumatism* 2010, **62**(11):3161-3172.
10. Aho K, Heliovaara M, Maatela J, Tuomi T, Palosuo T: **Rheumatoid factors antedating clinical rheumatoid arthritis**. *The Journal of rheumatology* 1991, **18**(9):1282-1284.
11. del Puente A, Knowler WC, Pettitt DJ, Bennett PH: **The incidence of rheumatoid arthritis is predicted by rheumatoid factor titer in a longitudinal population study**. *Arthritis and rheumatism* 1988, **31**(10):1239-1244.
12. Halldorsdottir HD, Jonsson T, Thorsteinsson J, Valdimarsson H: **A prospective study on the incidence of rheumatoid arthritis among people with persistent increase of rheumatoid factor**. *Annals of the rheumatic diseases* 2000, **59**(2):149-151.
13. Kurki P, Aho K, Palosuo T, Heliovaara M: **Immunopathology of rheumatoid arthritis. Antikeratin antibodies precede the clinical disease**. *Arthritis and rheumatism* 1992, **35**(8):914-917.
14. Silman AJ, Hennessy E, Ollier B: **Incidence of rheumatoid arthritis in a genetically predisposed population**. *British journal of rheumatology* 1992, **31**(6):365-368.
15. Walker DJ, Pound JD, Griffiths ID, Powell RJ: **Rheumatoid factor tests in the diagnosis and prediction of rheumatoid arthritis**. *Annals of the rheumatic diseases* 1986, **45**(8):684-690.
16. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, Habibuw MR, Vandenbroucke JP, Dijkmans BA: **Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors**. *Arthritis and rheumatism* 2004, **50**(2):380-386.

17. Kokkonen H, Soderstrom I, Rocklov J, Hallmans G, Lejon K, Rantapaa Dahlqvist S: **Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis**. *Arthritis and rheumatism* 2010, **62**(2):383-391.
18. Brink M, Hansson M, Mathsson-Alm L, Wijayatunga P, Verheul MK, Trouw LA, Holmdahl R, Ronnelid J, Klareskog L, Rantapaa-Dahlqvist S: **Rheumatoid factor isotypes in relation to antibodies against citrullinated peptides and carbamylated proteins before the onset of rheumatoid arthritis**. *Arthritis Res Ther* 2016, **18**:43.
19. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ: **Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis**. *Arthritis and rheumatism* 2003, **48**(10):2741-2749.
20. Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Ronnelid J, Rantapaa-Dahlqvist S: **Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis**. *Arthritis Res Ther* 2011, **13**(1):R13.
21. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ: **Citrulline is an Essential Constituent of Antigenic Determinants Recognized by Rheumatoid Arthritis-specific Autoantibodies. 1998**. *Journal of immunology* 2015, **195**(1):8-16.
22. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ: **The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide**. *Arthritis and rheumatism* 2000, **43**(1):155-163.
23. Lee DM, Schur PH: **Clinical utility of the anti-CCP assay in patients with rheumatic diseases**. *Annals of the rheumatic diseases* 2003, **62**(9):870-874.
24. Demoruelle MK, Parish MC, Derber LA, Kolfenbach JR, Hughes-Austin JM, Weisman MH, Gilliland W, Edison JD, Buckner JH, Mikuls TR *et al*: **Performance of anti-cyclic citrullinated Peptide assays differs in subjects at increased risk of rheumatoid arthritis and subjects with established disease**. *Arthritis and rheumatism* 2013, **65**(9):2243-2252.
25. Ioan-Facsinay A, el-Bannoudi H, Scherer HU, van der Woude D, Menard HA, Lora M, Trouw LA, Huizinga TW, Toes RE: **Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities**. *Annals of the rheumatic diseases* 2011, **70**(1):188-193.
26. Nell VP, Machold KP, Stamm TA, Eberl G, Heinzl H, Uffmann M, Smolen JS, Steiner G: **Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis**. *Annals of the rheumatic diseases* 2005, **64**(12):1731-1736.
27. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, Saigo K, Morinobu A, Koshiba M, Kuntz KM *et al*: **Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis**. *Annals of internal medicine* 2007, **146**(11):797-808.
28. Greiner A, Plischke H, Kellner H, Gruber R: **Association of anti-cyclic citrullinated peptide antibodies, anti-citrullin antibodies, and IgM and IgA rheumatoid factors with serological parameters of disease activity in rheumatoid arthritis**. *Annals of the New York Academy of Sciences* 2005, **1050**:295-303.
29. Silveira IG, Burlingame RW, von Muhlen CA, Bender AL, Staub HL: **Anti-CCP antibodies have more diagnostic impact than rheumatoid factor (RF) in a population tested for RF**. *Clinical rheumatology* 2007, **26**(11):1883-1889.
30. Hueber W, Utz PJ, Robinson WH: **Autoantibodies in early arthritis: advances in diagnosis and prognostication**. *Clinical and experimental rheumatology* 2003, **21**(5 Suppl 31):S59-64.

31. Miriovsky BJ, Michaud K, Thiele GM, O'Dell JR, Cannon GW, Kerr G, Richards JS, Johnson D, Caplan L, Reimold A *et al*: **Anti-CCP antibody and rheumatoid factor concentrations predict greater disease activity in men with rheumatoid arthritis**. *Annals of the rheumatic diseases* 2010, **69**(7):1292-1297.
32. Sokolove J, Johnson DS, Lahey LJ, Wagner CA, Cheng D, Thiele GM, Michaud K, Sayles H, Reimold AM, Caplan L *et al*: **Rheumatoid factor as a potentiator of anti-citrullinated protein antibody-mediated inflammation in rheumatoid arthritis**. *Arthritis & rheumatology* 2014, **66**(4):813-821.
33. Gerlag DM, Norris JM, Tak PP: **Towards prevention of autoantibody-positive rheumatoid arthritis: from lifestyle modification to preventive treatment**. *Rheumatology* 2016, **55**(4):607-614.
34. **World Medical Association declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects**. *Jama* 1997, **277**(11):925-926.
35. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS *et al*: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis**. *Arthritis and rheumatism* 1988, **31**(3):315-324.
36. Sokolove J, Lindstrom TM, Robinson WH: **Development and deployment of antigen arrays for investigation of B-cell fine specificity in autoimmune disease**. *Frontiers in bioscience* 2012, **4**:320-330.
37. Hueber W, Tomooka BH, Batliwalla F, Li W, Monach PA, Tibshirani RJ, Van Vollenhoven RF, Lampa J, Saito K, Tanaka Y *et al*: **Blood autoantibody and cytokine profiles predict response to anti-tumor necrosis factor therapy in rheumatoid arthritis**. *Arthritis Res Ther* 2009, **11**(3):R76.
38. Hueber W, Tomooka BH, Zhao X, Kidd BA, Drijfhout JW, Fries JF, van Venrooij WJ, Metzger AL, Genovese MC, Robinson WH: **Proteomic analysis of secreted proteins in early rheumatoid arthritis: anti-citrulline autoreactivity is associated with up regulation of proinflammatory cytokines**. *Annals of the rheumatic diseases* 2007, **66**(6):712-719.
39. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, Edison JD, Gilliland WR, Tibshirani RJ, Norris JM *et al*: **Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis**. *PloS one* 2012, **7**(5):e35296.
40. Mikuls TR, Padala PR, Sayles HR, Yu F, Michaud K, Caplan L, Kerr GS, Reimold A, Cannon GW, Richards JS *et al*: **Prospective study of posttraumatic stress disorder and disease activity outcomes in US veterans with rheumatoid arthritis**. *Arthritis care & research* 2013, **65**(2):227-234.
41. Tusher VG, Tibshirani R, Chu G: **Significance analysis of microarrays applied to the ionizing radiation response**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**(9):5116-5121.
42. de Hoon MJ, Imoto S, Nolan J, Miyano S: **Open source clustering software**. *Bioinformatics* 2004, **20**(9):1453-1454.
43. Saldanha AJ: **Java Treeview--extensible visualization of microarray data**. *Bioinformatics* 2004, **20**(17):3246-3248.
44. Todd DJ, Knowlton N, Amato M, Frank MB, Schur PH, Izmailova ES, Roubenoff R, Shadick NA, Weinblatt ME, Centola M *et al*: **Erroneous augmentation of multiplex assay measurements in patients with rheumatoid arthritis due to heterophilic binding by serum rheumatoid factor**. *Arthritis and rheumatism* 2011, **63**(4):894-903.
45. Laurent L, Anquetil F, Clavel C, Ndongo-Thiam N, Offer G, Miossec P, Pasquali JL, Sebbag M, Serre G: **IgM rheumatoid factor amplifies the inflammatory response of macrophages induced by the rheumatoid arthritis-specific immune complexes containing**

anticitrullinated protein antibodies. *Annals of the rheumatic diseases* 2015, **74**(7):1425-1431.

46. Anquetil F, Clavel C, Offer G, Serre G, Sebbag M: **IgM and IgA rheumatoid factors purified from rheumatoid arthritis sera boost the Fc receptor- and complement-dependent effector functions of the disease-specific anti-citrullinated protein autoantibodies**. *Journal of immunology* 2015, **194**(8):3664-3674.
47. Doran MF, Pond GR, Crowson CS, O'Fallon WM, Gabriel SE: **Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period**. *Arthritis and rheumatism* 2002, **46**(3):625-631.