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Research Article

Diagnostic And Prognostic Utility of Anti-Modified Albumin Autoantibodies in Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of synovial joints. Anti-citrullinated protein autoantibodies are detected by the CCP test in early disease and predict the development of erosive disease as well as extra-articular manifestations. We recently reported that many RA patients have anti-citrullinated albumin (ACA) autoantibodies, which only recognize albumin in its citrullinated form.

Methods: Anti-modified albumin autoantibodies, predominantly ACA, were quantitated using a rapid point-of-care test and the data analyzed by ACA and CCP status for correlations with clinical and laboratory parameters and medication.

Results: In two cohorts of RA patients (n=168), 55% were positive for anti-modified albumin autoantibodies (here referred to as ACA+) while healthy donors (n=37) were negative ($p<0.0001$). Patients with other rheumatologic diseases were generally negative for ACA with a few exceptions, such as those with RA overlap syndromes. ACA values correlated with titers of rheumatoid factor. The ACA test segregated seronegative RA patients into two distinct categories, while seropositive RA patients with a positive ACA had higher disease activity, more erosions on x-rays, and a higher proportion of TNF inhibitor non-responders and combination therapy than ACA-negative patients.

Conclusions: The ACA test could help identify seronegative RA patients with a citrullination-associated disease similar to seropositive RA (despite a negative CCP test), as well as to recognize seropositive RA patients with a worse prognosis and in need of more aggressive therapy. The rapid point-of-care test could prove useful for screening and in settings where a clinical laboratory is not readily available. 250 words

Keywords: Rheumatoid arthritis; Citrullination; Anti-citrullinated protein antibodies; Albumin

Introduction

Some 70% of all patients with rheumatoid arthritis (RA) have autoantibodies against citrullinated epitopes on a range of intracellular or extracellular proteins, termed anti-citrullinated protein antibodies (ACPA) [1]. Because these autoantibodies are very rarely detectable in patients with other diseases, and only occasionally in first-degree relatives of RA patients or in individuals who subsequently develop RA, they have become widely used in the diagnosis of RA [2, 3] using the Cyclic Citrullinated Peptide (CCP) test [4]. Patients with a positive CCP test typically also test positive for rheumatoid factor (RF) and are then referred to as having 'seropositive' RA. Patients who are 'seronegative' tend to have a more variable disease presentation, with many patients ultimately receiving a different diagnosis [5]. It has been suggested that seronegative RA, in fact, is an artificial assemblage of several distinct forms of arthritis, including unspecified inflammatory arthritis due to crystal deposition, or atypical psoriatic arthritis, as well as rheumatoid arthritis with a 'cryptic' ACPA that does not yield a positive CCP test. These latter patients could likely be identified by another test for more specific ACPA.

The role of citrullination in the pathogenesis of RA remains unresolved, but genetic evidence links two of the five citrullinating enzymes encoded for by the human genome, protein arginine deiminase (PAD) 2 and 4, to the development of RA [6-10]. There is also evidence that the citrullination of many proteins is qualitatively and quantitatively altered in patients with RA. [11-14]. While very few proteins are known to be citrullinated in healthy individuals, RA patients have numerous citrullinated proteins in synovial fluid, synovial tissue, and in circulation, including histones [15], fibrinogen [16, 17], vimentin [18, 19], α -enolase, immunoglobulins [20], complement proteins [20] and human serum albumin [20, 21]. Interestingly, the citrullinated proteins include both classical extracellular proteins made by the liver (e.g., albumin), as well as proteins with an intracellular location and function in immune cells, particularly neutrophils [22, 23].

We recently discovered that nearly half of all RA patients have IgG autoantibodies that recognize albumin citrullinated in vitro by PAD4, but not unmodified albumin [24]. These autoantibodies were more prevalent in seropositive RA patients and showed a trend towards association with bone erosions and biologic plus DMARD combination treatment regimens. We have now refined this analysis with a calibrated and standardized rapid point-of-care test suitable for bedside use that detects albumin bound to autoantibodies. We find that ACA, particularly in conjunction with CCP status, can refine the diagnosis of RA and assist in assessing its prognosis and management.

Materials and Methods

Cohorts of RA and patients with other rheumatologic disease

Cohort 1 (Table 1) consisted of serum samples from RA patients (n=105) and cohort 2 (Table 2) consisted of plasma samples from RA patients (n=63). Healthy donors (n=37) and disease controls included serum samples from 40 systemic lupus erythematosus (SLE), 3 patients with RA/SLE overlap syndrome, as well as 2 RA patients with secondary Sjögren's syndrome (RA/SjS), 13 with primary Sjögren's syndrome (SjS), 12 with anti-neutrophil cytosolic antibody-associated vasculitis (AAV), 11 with large vessel vasculitis (giant cell arteritis and Takayasu arteritis), and 8 with psoriatic arthritis. All samples were from the UW Rheumatology Rheumatic Disease Biorepository and were stored at -20°C until use. IRB approval for our study was obtained from the University of Washington ethics board (STUDY00006196) and informed written consent was obtained from all participants according to the Declaration of Helsinki.

Table 1: Characteristics of RA patient cohort 1 (serum).

Characteristic	Total (n=105)	Male (n=42)	Female (n=61)
Age	53.1 ± 15.3	54.0 ± 14.3	52.6 ± 16.1
Ethnicity			
European	70	29	41
Hispanic	19	7	12
Asian	1	0	1
Black	8	4	4
Native American	3	1	2
Native Hawaiian	1	1	0
Unknown/mixed	3	1	2
Laboratory measures			
RF-positive	62	24	38
RF-negative	26	11	15
RF not recorded	17	8	9
ACPA-positive	56	19	37
ACPA-negative	31	15	16
ACPA not recorded	26	10	16
C-reactive protein (CRP)	8.3 ± 11.6	9.0 ± 11.8	7.9 ± 11.7
Disease activity			
High (CDAI >22)	11	3	8
Moderate (CDAI >10–22)	13	3	10
Low (CDAI >2.8–10)	12	5	7
Remission (CDAI ≤2.8)	18	7	11
Current CDAI not recorded	51	25	26

Radiographic erosions	45	20	25
Current treatment			
NSAID/prednisone only	3	1	2
DMARD(s) only	44	21	23
Biologic only	17	6	11
Biologic + DMARD(s)	33	12	21
JAK inhibitor only	1	0	1
JAK inhibitor + DMARD(s)	7	3	4

Table 2: Characteristics of RA patient cohort 2 (plasma).

Characteristic	Total (n=63)	Male (n=18)	Female (n=43)
Age	50.3 ± 15.3	56.3 ± 13.4	47.7 ± 15.4
Ethnicity			
European	36	11	25
Hispanic	10	2	8
Asian	3	0	3
Black	7	5	2
Native American	3	0	3
Unknown/mixed	2	0	0
Laboratory measures			
RF-positive	45	14	13
RF-negative	14	4	10
RF not recorded	2	-	-
ACPA-positive	44	15	29
ACPA-negative	13	3	11
ACPA not recorded	2	-	-
C-reactive protein (CRP)	11.2 ± 18.8	11.2 ± 18.8	11.2 ± 18.8
Disease activity			
High (CDAI >22)	6	1	5
Moderate (CDAI >10–22)	6	1	5
Low (CDAI >2.8–10)	11	3	8
Remission (CDAI ≤2.8)	13	7	6
Current CDAI not recorded	25	6	19
Radiographic erosions	31	12	19
Current treatment			
NSAID/prednisone only	2	0	2
DMARD(s) only	29	10	19
Biologic only	9	2	7
Biologic + DMARD(s)	21	6	15

Point-of-care test and its use

Serum or plasma samples were measured using an ACA point-of-care test system (Figure 1A) according to the manufacturer's instructions (Diabetomics, Inc, Hillsboro, OR 97006). Similar to other tests by this supplier [25, 26], the ACA test system consists of an immunochromatography test strip in a cassette and a portable reader for quantification. The test is based on antibodies against human IgG, IgM, IgA and detection of bound albumin with anti-albumin antibodies with colloidal gold. Briefly, 150 µl of 1:80 diluted serum or plasma is added to the test strip and inserted

into the reader. The ACA concentration is displayed on the reader at the end of 15 minutes. Calibration information is supplied by the manufacturer as a lot specific QR code tag on each test kit. The dynamic range of the ACA assay is 15-4,000 U/mL. The intra- and inter-assay coefficients of variation were 6.2 and 9.8%, respectively. The assay cut-off is 25 U/ml as per vendor document (98th percentile of healthy donor cohorts of serum (n=334) and plasma (n=150) is less than 25 U/ml).

Statistical analyses

All data sets were considered non-paired with a non-Gaussian distribution and were analyzed using the Mann-Whitney U test. Correlations between two variables were tested by a simple linear regression analysis. We defined the upper limit of 'normal' was as the average plus two standard deviations of the healthy control group. Values above this cut-off were termed 'positive'. GraphPad Prism 9.2.0 was used for the analyses, which were considered statistically significant at $p<0.05$.

Results

ACA autoantibodies are highly selective for RA

Serum samples from healthy controls gave very low readings, half of them 0, the other half between 1 and 22, compared to control line readings of 2455.9 ± 96.4. The average of all tested healthy controls was 3.57 ± 5.48 (n=37) (Figure 1B). Taking this average plus two standard deviations as a cut-off (i.e., 14.53), only 2 healthy controls were considered positive (readings of 21 and 22). The vendor has established 25 U/ml as a cut-off point.

In contrast, our test cohort 1 consisting of serum samples from 105 RA patients, was 53.9% positive with an average of 411.9 ± 707.7 (Figure 1B). A second cohort of plasma samples from RA patients (n=63) were 60.3% positive with an average reading of 542.1 ± 775.0 (Figure 1C). The difference between the two cohorts was not statistically significant and is likely due to the somewhat different clinical characteristics of the two cohorts. Notably, cohort 2 has a lower average age, especially among the female patients (47.7 versus 52.6 years) and a higher average CRP (12.7 versus 7.9).

Combining the two cohorts (n=168), 55.8% of all RA patients were positive with an average reading of 427.23 ± 706.0 (Figure 1D) and a control line reading of 2435.8 ± 108.2. This control line reading is essentially identical to that in the healthy control measurements, but the ACA readings are on average 120-fold higher ($p<0.0001$ by the Mann-Whitney t-test).

ACA autoantibodies in other diseases and in RA overlap syndromes

We also tested a number of other rheumatological diseases for ACA autoantibodies. In a cohort of SLE patients (n=40), 10 patients were positive for ACA, 3 of them strongly positive (readings >1,000) (Figure 1D). Despite being 75% ACA-negative, the difference between SLE and HC reached statistical significance ($p=0.046$). Serum from patients with other rheumatologic diseases were mostly negative (Figure 1E) but had a few positive readings: 4 in Sjögren's syndrome (n=13), 2 in ANCA-associated vasculitis (n=12), 1 in large-vessel vasculitis (n=11), and 1 in psoriatic arthritis (n=8). The positive readings were modest (all <500, most <150). In contrast, 2 of 3 patients with RA/SLE overlap syndrome were positive, while 2 patients with RA and secondary Sjögren's syndrome were negative.

Longitudinal ACA values

A small number of patients in our patient cohorts, had samples taken at more than one time point 3 to 36 months apart. Figure 1F shows one SLE patient, who stayed negative at three time points over a year. Four RA patients with positive readings in their most recent serum sample (included in Figure 1B) were also positive in earlier samples. Three additional RA patients with negative readings in their most recent serum samples (included in Figure 1B) were also negative in earlier samples. Two RA/SLE overlap patients also stayed positive at all time points. However, in all these positive patients, the readings increased or decreased somewhat between blood draws. These longitudinal data are too limited for any conclusions other than suggesting some consistency in individuals over time.

ACA values versus disease duration and sex

Our RA cohorts included some newly diagnosed patients, as well as patients with a near 40-year history of RA (average 9.1 ± 8.3 years, range 0-38 years). There was a weak trend towards higher ACA values in patients with long-standing disease, but this did not reach statistical significance (data not shown). Many patients with recently diagnosed RA were ACA-positive, suggesting that ACA do not appear later in the disease, but can be present at the time of diagnosis. Segregating the ACA+ readings by sex revealed a trend towards higher values in female patients than in male patients (Figure 1G). However, this difference did not reach statistical significance.

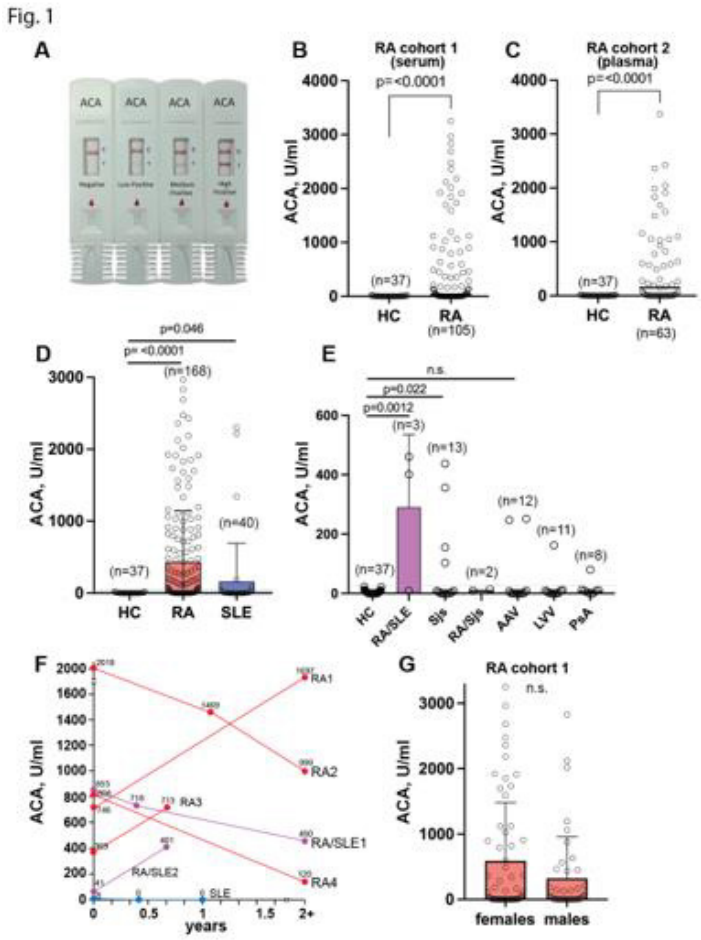

Figure 1: Anti-citrullinated albumin (ACA) autoantibodies in RA patients
Figure 1A: Four representative ACA point-of-care tests with visual results.
Figure 1B: ACA values (arbitrary units/ml) from cohort 1 consisting of RA patient serum samples. The median is indicated with a horizontal bar in A and B.
Figure 1C: Values from cohort 2 (plasma samples).
Figure 1D: RA cohorts 1 and 2 together (red column) and SLE (blue bar) shown with mean and standard deviation.
Figure 1E: data from 6 different indications: primary Sjögren's syndrome (Sjs), anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), large vessel vasculitis (LVV), and psoriatic arthritis (PsA).
Figure 1F: longitudinal data from 4 RA patients, 2 RA/SLE patients, and 1 SLE patient.
Figure 1G: ACA values from females and males in RA cohort 1. The mean (red bars) and standard deviation are shown.

Correlations between ACA and other RA serology

Patients with positive ACA readings had higher ACPA titers (i.e., CCP test values) (Figure 2A) and ACA values correlated with CCP test values in a significant manner (p=0.0003) (Figure 2B). Similarly, rheumatoid factor (RF) titers were higher in ACA-positive patients than in ACA-negative patients (Figure 2C) and ACA values correlated with RF values (p<0.0001) (Figure 2D). There was also a trend towards a positive correlation with CRP values at the time of blood draw (Figure 2E), but this did not reach statistical significance.

Smoking and ACA values

Smoking is a known risk factor for RA (27). ACA values were, on average, somewhat higher in active smokers than in never smokers (p=0.027), while former smokers were intermediate (Figure 2F). However, the impact of smoking must be considered minimal despite statistical significance, particularly when some of the highest ACA readings were seen in never smokers.

Fig. 2

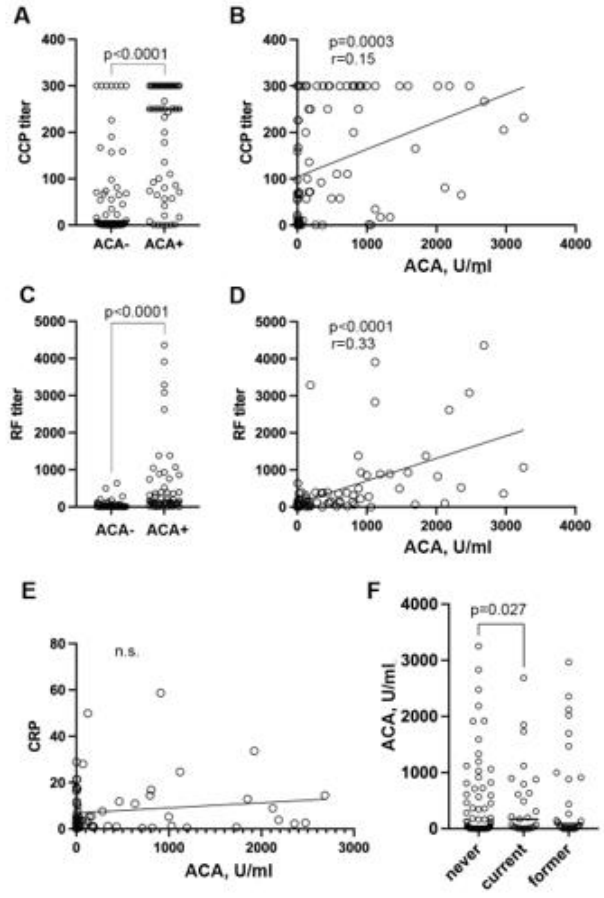

Figure 2: Correlation of ACA with laboratory measures.
Figure 2A: the CCP value in ACA negative (ACA-) or positive (ACA+) RA patients.
Figure 2B: Correlation between ACA and CCP values.
Figure 2C: the RF value in ACA- and ACA+ RA patients.
Figure 2D: Correlation between ACA and RF values.
Figure 2E: Trend towards correlation between ACA and CRP values at the time of blood draw.
Figure 2F: ACA values in RA patients who never smoked, currently smoke, or smoked in the past.

Analyses of RA subpopulations segregated by ACA and CCP

Based on the above results, we decided to segregate the patients in the combined two cohorts into four populations by CCP and ACA status, i.e., the CCP-ACA-, CCP-ACA+, CCP+ACA-, and CCP+ACA+ populations (n=117) (Figure 3A). Figure 3B shows how RF titer differs between these 4 groups. The difference between the double-negative (CCP-ACA-) and double-positive (CCP+ACA+) groups was statistically highly significant ($p<0.0001$). The 4 groups also differed in the proportion of patients who had documented radiographic erosions (Figure 3C), clinical disease activity (CDAI) at time of blood draw (Figure 3D), and average age (Figure 3E).

The four populations also differed by having somewhat different treatment regimens (Figure 4). The CCP-ACA- group was particularly different from the others in having fewer anti-TNF treated patients (Figure 4B), but more DMARD use (Figure 4A), particularly in combinations without biologics. The double-positive (CCP+ACA+) group had more TNFi-non-responders (Figure 4C) and more exposure to non-TNF biologics than CCP-ACA+ or CCP+ACA- patients (Figure 4A). A higher portion of them had biologics of JAK inhibitors plus combinations of several DMARDs (e.g., leflunomide plus sulfasalazine) (not shown). In contrast, the CCP-ACA+ group had no TNF non-responders (Figure 4C), and instead more TNF inhibitor alone or with only methotrexate (Figure 4A).

Fig. 3

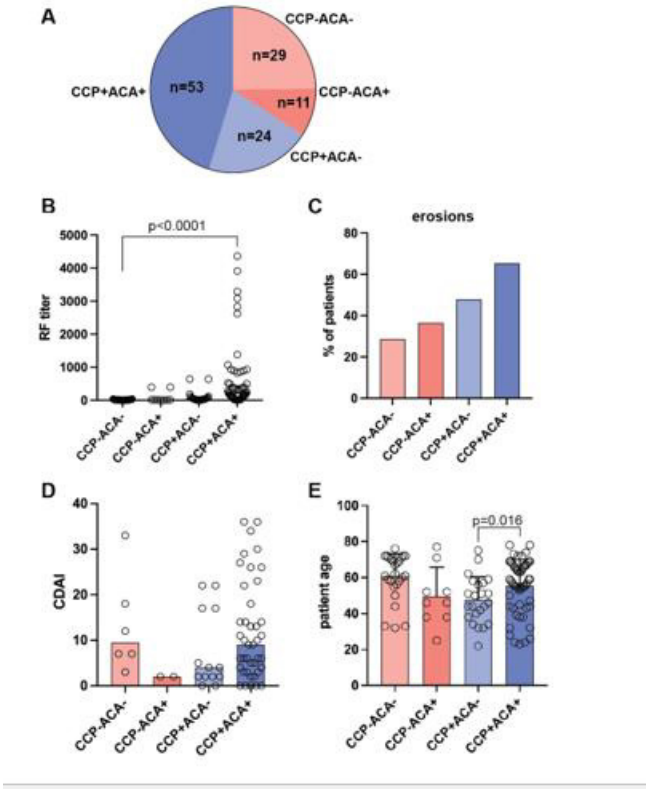

Figure 3: Analysis of patients segregated by CCP and ACA status.
Figure 3A: Numbers of RA patients in the four categories by known CCP and recorded ACA values (n=117).
Figure 3B: RF values in the four groups.
Figure 3C: Portion of patients with radiographic erosions in each of the four groups.
Figure 3D: Clinical disease activity index (CDAI) of individual patients in the four groups (n=62).
Figure 3E: The age of patients in the four groups.

Fig. 4

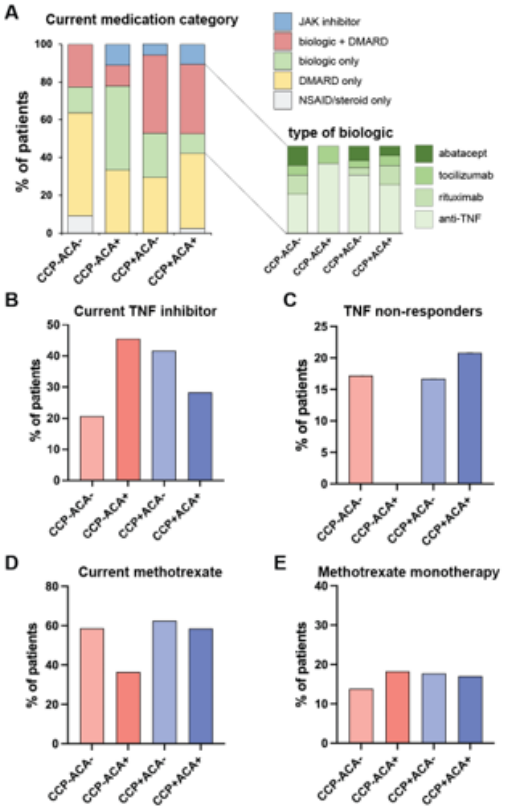

Figure 4: Medication regimens of patients segregated by CCP and ACA status.
Figure 4A: Current medication categories of patients in the four groups, with the type of biologics (alone or in combination) indicated on the right side. Steroid therapy refers to doses less than the equivalent of prednisone 15 mg daily
Figure 4B: Portion of patients with a current TNF antagonist.
Figure 4C: Portion of patients with a history of TNF discontinuation for lack or loss of efficacy.
Figure 4D: Portion of patients with current methotrexate.
Figure 4E: Portion of patients on methotrexate monotherapy.

Discussion

Diagnostic point-of-care tests are not often employed in the field of rheumatology yet have the potential to assist physicians in resource-limited clinical settings. The point-of-care test utilized in this study allowed for the determination of ACA status in 15 minutes. Since only 2µl is required, the test could be performed on a small drop of blood. We also surmise that the utility of the test is not dependent on quantitation of the test line using a reader, since a visible test line is sufficient to denote a positive ACA. Hence, even without a CCP test, the RA diagnosis could be tentatively made. In combination with a CCP test, the ACA test would further support treatment decisions and overall prognosis.

The rapid ACA test developed by Diabetomics Inc. has the advantage of measuring albumin present in immune complexes, reducing the risk of detecting broadly reactive ACPA unless they indeed bind citrullinated albumin in the patient. This helps explain why a portion of CCP-negative RA patients are ACA-positive; they have autoantibodies that selectively bind albumin but lack the broadly reactive ACPA [28] captured by the cyclic peptides in the CCP test. On the other hand, in theory, other types of autoantibodies that recognize modified albumin could yield a positive ACA test. While unmodified albumin is not recognized by autoantibodies in RA [24, 29], other diseases, or healthy individuals [24], carbamylated (homocitrullinated) [30] albumin could theoretically yield a positive test if both the carbamoylated albumin and autoantibodies specific for it are present in the patient's circulation. Carbamoylation is reportedly increased in RA [29, 31], but is not specific for RA and present in other inflammatory diseases [32], particularly in chronic kidney disease [33, 34]. Autoantibodies against carbamoylated proteins tend to be broadly reactive [29] and can be detected by ELISA using in vitro carbamoylated albumin. Such autoantibodies have been reported to be high in RA patients with interstitial lung disease (ILD) [31]. However, of the 4 patients in our cohorts with an ILD diagnosis, 3 were ACA-negative and the fourth just above the cut-off for positivity. Nevertheless, we cannot entirely exclude the possibility that anti-carbamoylated albumin antibodies contribute to ACA positivity. Other modifications of albumin that result in immunogenicity [30] could theoretically also do so. For this reason, we refer to the test as 'anti-modified albumin', while assuming that the predominant signal comes from autoantibodies specific for citrullinated albumin.

Albumin is the most abundant protein in circulation with a normal concentration range of 35 – 55 mg/ml and has 15 surface-exposed arginine residues reported to be citrullinated in RA patients [20, 21]. Intriguingly, several of these residues are directly involved in the binding of biomolecules and drugs transported by albumin [35, 36], including methotrexate. It is possible that citrullination of these arginines alters the transport function of albumin and perhaps the pharmacology of affected drugs. It is also possible that the loss of the positive charge of the deiminated arginine(s) alter the overall isoelectric point of albumin, which may affect its properties in maintaining the oncotic pressure of the extracellular fluid. The binding of autoantibodies to citrullinated epitopes on albumin may also create multivalent immune complexes, which could activate Fc γ receptors on immune cells and thereby promote inflammation [37]. These questions will require further investigations.

A positive CCP test provides strong support for a RA diagnosis and generally signifies the potential for a more serious clinical disease course with erosive joint damage, extra-articular manifestations, and cardiovascular complications [38]. However, many CCP-positive patients experience a milder disease that can be well managed by methotrexate monotherapy or a TNF inhibitor alone. In addition, some patients with a CCP-negative arthritis appear to have a very similar disease course, suggesting that classical RA can be accompanied by ACPA that elude the CCP test. Our data fit this view: the presence of ACA in CCP-negative patients [24] indicates that autoantibodies with selectivity for citrullinated epitopes (i.e., ACPA) indeed exist in these seronegative RA patients even if they lack the broader spectrum of ACPA detected by the CCP test [39]. Our data also indicate that CCP-ACA- and CCP-ACA+ patients differ from each other in many respects, including in the presence of radiographic erosions, CDAI, and medications. On average, CCP+ACA+, a larger proportion of whom require advanced biologic therapies, have higher CDAI scores, and radiographic erosive disease. Hence, we propose that ACA could be used for a more refined subclassification of both CCP-positive and -negative disease. ACA detection could assist with difficult treatment choices particularly in the seronegative RA population.

Conclusion

We report that 55% of RA patients have a positive ACA test, which can help identify seronegative RA patients with a citrullination-associated disease similar to seropositive RA (despite a negative CCP test), as well as to recognize seropositive RA patients with a worse prognosis and in need of more aggressive therapy. The rapid point-of-care test could prove useful for screening and in settings where a clinical laboratory is not readily available.

Declarations

Ethics approval and consent to participate

Institutional Review Board approval for our study was obtained from the University of Washington ethics board (STUDY00006196) and informed written consent was obtained from all participants according to the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The ACA test system was supplied by Diabetomics Inc. T.M. has received consultant fees from Cugene, ROME, QiLu Pharma, Applied Molecular Transport Inc, and MiroBio unrelated to the present work.

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Authors' Contributions

All four authors carried out the acquisition, analysis, and interpretation of data, contributed to writing, and approved the submitted version and agree both to be personally accountable for their own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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