

SHORT COMMUNICATION

Are antibodies to fine specificities of citrullinated peptides/proteins useful for stratification of rheumatoid arthritis patients?

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Abstract

Background. In rheumatoid arthritis (RA), antibodies to citrullinated protein (ACPA) are believed to be heterogeneous and patient stratification by antibody profiling raised clinical interest for patient management. However, heterogeneity might be partially artificial because of the use of heterogeneous methods for ACPA detection. In recent work instead, we found that ACPA were mainly directed towards a single fibrin-derived peptide, β 60-74BiotNt, but a comparative analysis with the presence of other ACPA specificities is still lacking. **Objectives.** To present an overview of RA patients' stratification based on the detection of the main ACPA fine specificities with the same method as compared to that of anti- β 60-74BiotNt antibodies. **Methods.** Over 4500 measurements were performed with more than 22 standardised ELISAs, sera from 180 RA patients and 200 to 436 non-RA rheumatic disease controls. **Results.** Four to 81% of RA patients had ACPA towards various targets, confirming the heterogeneity of ACPA specificities. However, the subgroups of patients overlapped up to 97% with ACPA levels of correlation coefficients up to 0.8, showing redundancy of some targets. Multiplexing decreased diagnostic specificity from 95% to 64%. Instead, anti- β 60-74BiotNt detection identified almost all ACPA-positive patients. **Conclusions.** Antibodies to citrullinated protein multiplexing shows some degree of redundancy and is not suitable for diagnostic purposes. ACPA fine specificities might be less heterogeneous than perceived by sera testing on multiple peptides. Patient stratification largely depends on detection methods and requires standardisation.

Keywords: antibodies to anti-citrullinated protein (ACPA), citrullinated peptides, B-cell epitopes, citrullinated fibrin, rheumatoid arthritis, fine specificities, vimentin, enolase

INTRODUCTION

In the diagnosis of rheumatoid arthritis (RA), antibodies towards citrullinated proteins/peptides (ACPA) are commonly detected by various commercial assays using undisclosed citrullinated peptides (anti-CCP, cyclic citrullinated peptides). However, citrullinated proteins targeted *in vivo* such as fibrin, vimentin and α -enolase may also be tested for ACPA detection.¹⁻³ Thus, the antigen specificity of ACPA and their associated repertoire are considered as heterogeneous, based on ACPA capture in serum with various citrulline-containing proteins/peptides. Consequently, studies of the so-called ACPA 'fine specificities' or 'ACPA profiles' emerged, using multiplex ACPA detection, tentatively defining subgroups of patients with distinct clinical characteristics or outcomes.⁴⁻⁶ Unfortunately, despite the high number of studies, the stratification of patients according to ACPA profiles failed to translate into clinical improvement.⁷⁻¹¹ We previously showed that most sera containing antibodies towards *in vitro* citrullinated human fibrinogen (AhFibA) or anti-CCP2 antibodies recognise a single immunodominant fibrin-derived β 60-74 peptide.¹² Moreover, we demonstrated that perceived levels of ACPA in serum towards a given peptide are dramatically impacted by the features of the peptide.¹³ Thus, results can highly differ depending on tests used for ACPA profiling, and impact diagnostic sensitivities, correlations of antibody levels and patients' stratification. In the present study, we tackle these questions by testing RA and controls patients with the most commonly tested peptides, analysing their relationships in patients' stratification. We suggest that stratification according to ACPA heterogeneity might be overestimated.

RESULTS

Proportions of ACPA fine specificities in RA and control sera

Diagnostic performances of the detection of ACPA in RA and control sera (details in Supplementary tables 1 and 2) with the different peptides are depicted in Figure 1a. The β 60-74Cit and CEP-1CitCyclic peptides allow to obtain high diagnostic values close to those of the reference tests, AhFibA-ELISA and anti-CCP2, with AUC ranging from 0.92 to 0.78 (Figure 1b upper right

part), while the diagnostic values obtained with most other peptides are lower (AUC < 0.75), particularly with the Vim and α 501-515Cit peptides (AUC < 0.60).

In Figure 1b lower left part, proportions of RA sera reactive with each peptide were compared at an equal diagnostic specificity of 95%. It shows that 80% and 69% of RA sera contain AhFibA and anti-CCP2 antibodies, respectively, while antibodies towards most peptides are less frequent. Sera positivity percentages towards non-biotinylated fibrin-derived peptides (β 60-74Cit, α 36-50Cit, α 621-635Cit, β 36-52Cit and α 501-515Cit) ranged from 68% to 10%. Anti-Vim59-74Cit and anti-Vim2-17Cit antibodies were detected in 22% and 4% of RA patients, respectively. With the α -enolase-CEP-1Cit peptide in its linear form, 33% of RA sera were positive. With the same peptide in a cyclic form, 62% were reactive, whereas 78% were positive with the biotinylated form of the β 60-74Cit fibrin-derived peptide. These results underline the huge impact of peptide structure on antibody detection. Thus, although ACPA fine specificities offer opportunities to stratify RA patients into subgroups, subgroups may change depending on peptide chemistry, making stratification unreliable.

ACPA profiles in RA and control patients

Antibodies to citrullinated protein reactivity of each individual sera is depicted in Figure 2a. Concerning RA sera (upper panels), 162/180 (90%) were positive towards one (25/180: 14%) or several (137/180: 76%) peptides including 18/36 (50%) of the AhFibA-negative and 17/56 (70%) of the anti-CCP2-negative sera (Supplementary figure 1). Concerning control sera, 72/200 (36%) were also reactive towards one or several peptides (lower panels). Whereas anti-CCP2 antibodies and AhFibA were more frequent in inflammatory diseases, anti-peptide antibodies were similarly distributed in all disease groups (Supplementary table 3).

When reactivity towards at least one peptide was considered to assert ACPA positivity, a dramatic decrease in diagnostic specificity from 95% to 64% occurred. If reactivity to at least 3 peptides was required, a specificity of 96% could be reached, but sensitivity decreased to 74%. Higher specificity thresholds for each separate test allowed to keep a final specificity over 95%. With 97.5% specificity thresholds, the best association

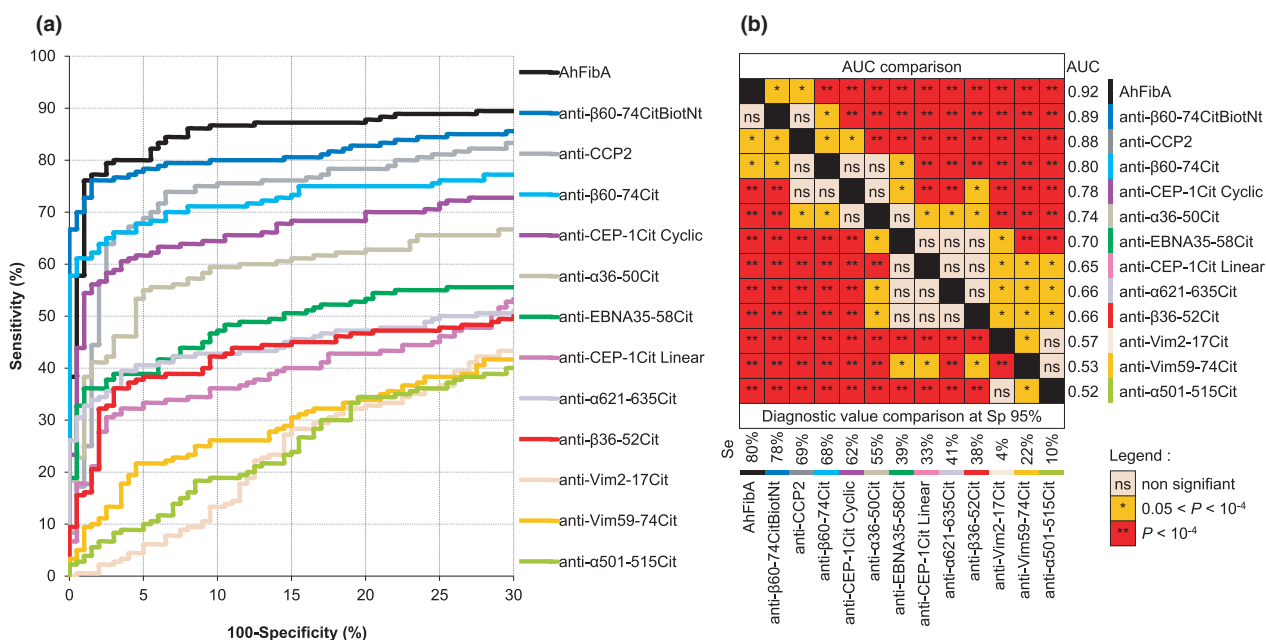


Figure 1. Diagnostic sensitivities and specificities of ACPA fine specificities. **(a)** Receiver operating characteristic (ROC) curves obtained with ACPA fine specificities compared to anti-CCP2 antibodies and to anti-human citrullinated fibrinogen antibodies (AhFibA) tested on 180 RA sera and 200 non-RA control sera. Citrullinated peptides are derived from the α (α 36-50, α 621-635, α 501-515) or β (β 60-74, β 36-52) chain of human fibrin, α -enolase (CEP), vimentin (Vim) or Epstein-Barr virus nuclear antigen 1 (EBNA). **(b)** Statistical analysis of all possible paired comparisons of the diagnostic sensitivities (Se) of the different ACPA at 95% specificity (Sp) thresholds and areas under curves (AUCs).

was β 60-74CitBiotNt + α 36-50Cit (82% sensitivity; 95.5% specificity). With 99% specificity thresholds: β 60-74CitBiotNt + α 36-50Cit + EBNA35-58Cit allowed reaching 82% sensitivity and 97.5% specificity, and adding CEP-1CitCyclic and α 621-635Cit, 83% sensitivity and 95.5% specificity.

Redundancy of seropositivity in ACPA multiplexing and patients' stratification

Figure 2b shows how the various fine specificities associate with ACPA-positive sera and reveals several layers of redundancy. For instance, among 111 patients seropositive for anti-CEP-1CitCyclic, the vast majority were also seropositive for anti- β 60-74Cit (88%) and *vice versa*. Moreover, the antibody levels were highly correlated (Figure 2c). More generally, three groups of ACPA fine specificities were observed: (i) a first group composed of anti- β 60-74CitBiotNt, anti- β 60-74Cit and anti-CEP-1CitCyclic antibodies almost always present in RA sera and highly correlated between each other; (ii) a second group composed of anti- β 36-52Cit, anti- α 621-635Cit, anti-EBNA35-58Cit and anti-CEP-1CitLinear antibodies, associated

with the first group in sera but with moderate correlation coefficients; and (iii) a third group composed of anti-Vim2-17Cit, anti-Vim59-74Cit, anti- α 501-515Cit and anti- α 36-50Cit antibodies, which do not correlate with other subfamilies.

Interestingly, almost all positive sera for AhFibA (92%) and anti-CCP2 (94%) antibodies were also positive for anti- β 60-74CitBiotNt and *vice versa*. Moreover, anti- β 60-74CitBiotNt levels highly correlated with both anti-CCP2 and AhFibA (Figure 2d and e). As observed for CEP-1 cyclisation, β 60-74Cit biotinylation enhanced the analytical sensitivity of its detection (Figure 2f and g). Moreover, the number of ACPA specificities detected in sera was associated with the levels of anti- β 60-74CitBiotNt similarly to those of anti-CCP2 and AhFibA (Figure 2h). Finally, the high diagnostic performance of the detection of anti- β 60-74CitBiotNt was confirmed with an extended cohort of patients (Figure 2i, Supplementary table 2).

Altogether, RA immune response appears to be almost always directed towards a single fibrin epitope associated or not with other fine specificities and ACPA multiplexing reveals a large part of redundancy.

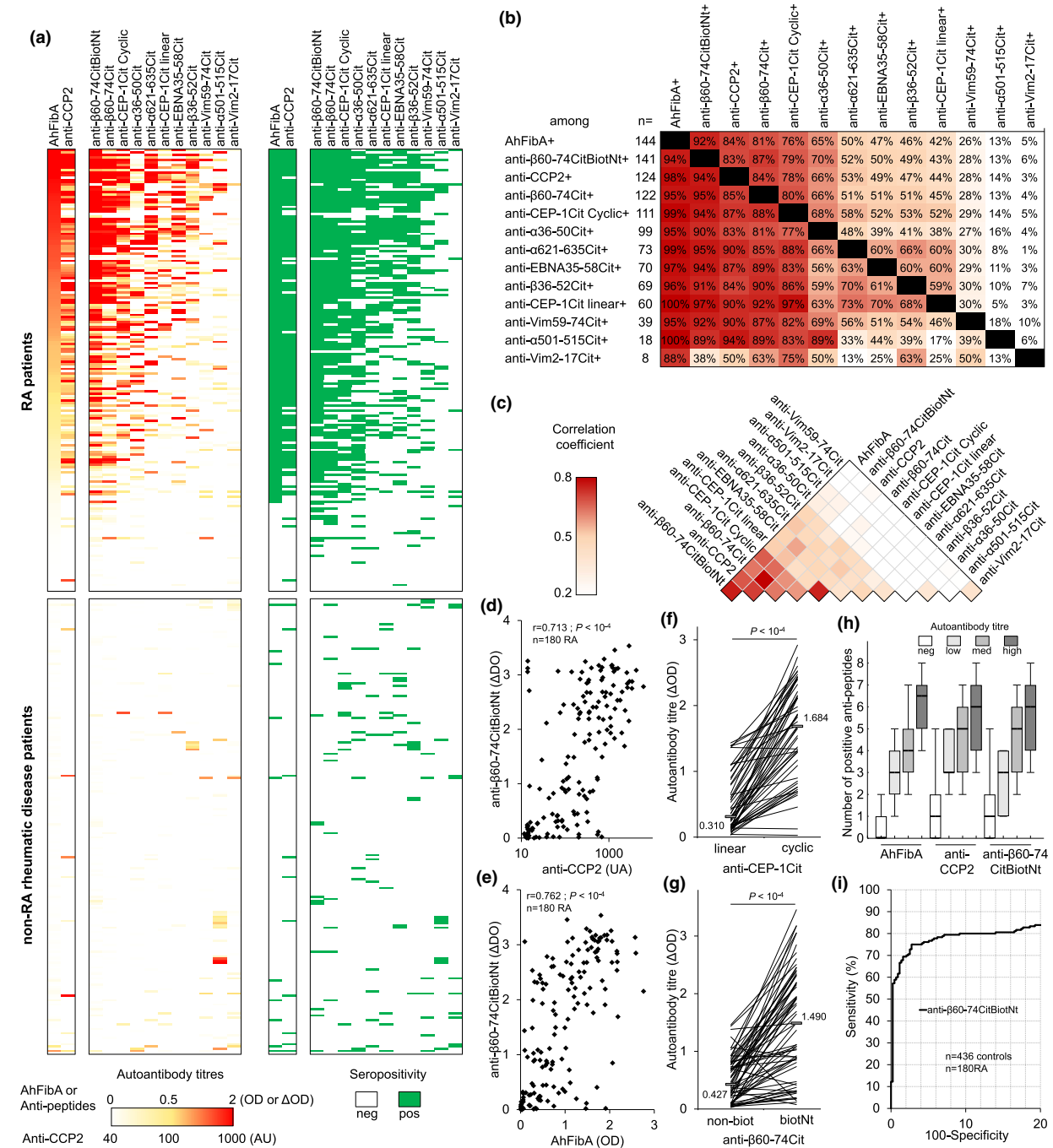


Figure 2. Co-‘detection’ of ACPA fine specificities: redundancy in ACPA multiplexing and patient stratification. **(a)** Left panel: Levels of antibodies towards citrullinated fibrinogen (AhFibA), CCP2 and citrullinated peptides in 180 RA sera and 200 non-RA control sera. Each line represents a serum tested on the protein/peptides indicated on column heading. Right panel: the corresponding seropositivity status (neg/pos) obtained at the 95% specificity threshold. **(b)** Percentage of patients seropositive for ACPA specificities indicated on column heading among those seropositive for the specificity indicated on each line. **(c)** Correlation coefficients between ACPA fine specificities, anti-CCP2 antibodies and anti-human citrullinated fibrinogen antibodies (AhFibA). **(d)** Correlation between anti-β60-74CitBiotNt antibodies and anti-CCP2 antibody levels. **(e)** Correlation between anti-β60-74CitBiotNt antibodies and AhFibA levels. **(f)** Effect of cyclisation of CEP on perceived antibody levels. **(g)** Effect of biotinylation of β60-74Cit on perceived antibody levels. **(h)** Association between AhFibA, anti-CCP2, anti-β60-74CitBiotNt antibody levels and the number of detected fine specificities at the 95% specificity threshold. **(i)** Receiver operating characteristic (ROC) curves of anti-β60-74CitBiotNt antibodies obtained with 180 RA and 436 non-RA controls.

DISCUSSION

Distinction of epitope specificities among polyclonal autoantibodies might constitute a breakthrough in disease monitoring and therapeutic strategies if specific autoantibody profiles are associated with distinct form of the diseases.¹⁴ However, studies have failed so far to consensually associate ACPA fine specificities with specific RA phenotypes.⁷⁻¹¹ Our work aimed to clarify subgrouping of patients according to the most studied ACPA fine specificities. It pointed out differences with published data, concerning the vimentin-derived and β 36-52Cit fibrin-derived peptides, which showed low diagnostic sensitivities in our work probably because of differences in technical protocol or peptide features. Indeed, we showed that minor changes in peptide structure dramatically impacted ELISA results.¹³ This is confirmed in the present work showing that the Nt-biotinylation of β 60-74Cit and the cyclisation of CEP-1 considerably enhance ACPA capture. This underlines the difficulty to clearly define ACPA fine specificities and thus highlights the relativity of final conclusions concerning ACPA profiles.

Antibodies to citrullinated protein response appears heterogeneous. Indeed, most (137/162) seropositive RA sera were multi-reactive. This can indicate either cross-reactivity or co-occurrence of several ACPA specificities in sera. In addition, the number of peptides recognised was linked to ACPA levels. This again may indicate either that low-affinity cross-reactive ACPA are not detected when concentrations are low or that the number of co-occurring fine specificities indeed increases with ACPA levels. Several groups analysed possible cross-reactivities of ACPA fine specificities leading to conflicting results.¹⁵⁻¹⁸ Some suggested that structural rather than sequence homology might be responsible for ACPA binding to different peptides.¹⁹ In our study, although large overlapping of patient subgroups suggests ACPA cross-reactivity, the limitation is that it is impossible to analyse each individual specificity by testing sera that are *de facto* polyclonal. ACPA issues are now being explained with monoclonal antibodies derived from RA patients' B cells, demonstrating that all ACPA display multi-reactivity to several citrullinated peptides that extend beyond the peptides used for B-cell capture.^{20,21} However, although ACPA subsets can be defined by distinct consensus motifs, with most often citrulline adjacent to glycine or serine motifs,²² the patterns of monoclonal antibody cross-

reactivity are still very heterogeneous and uneasily predictable.²⁰ This may be explained by somatic hypermutation accumulated during affinity maturation that may mediate epitope spreading and highly variable polyreactive patterns.²³

Nonetheless, in the present study, we observed several groups of patients defined by distinct patterns of ACPA reactivity and we and others also previously described low level or absence of cross-reactivity among some ACPA subgroups.^{12,17} Thus, even though ACPA heterogeneity might be smaller than expected, subgroups defined by sera reactivity towards peptides still exist. However, standardised methods to study ACPA fine specificities are lacking for reliable patient classification. For this purpose, chip multiplexing might be an option.⁶

For a diagnostic purpose on the contrary, chip multiplexing should not be used. Hence, depending on ACPA assays, seropositive sera are not perfectly overlapping and false-positive control sera are usually different from one test to another. This is also true for fine specificities and explains the drop of diagnostic specificity when the number of peptides increases (false-positive sera observed with each peptide are usually different sera).²³ This also partly explains the observed high percentages of anti-CCP2 (70%) and AhFibA (50%) negative RA sera, reactive towards at least one peptide, each negative serum being possibly reactive towards one or several peptides. In addition, these high percentages might be because of the 95% threshold used for positivity²⁴ but also because of the high diagnostic sensitivities of AhFibA and anti- β 60-74CitBiotNt antibodies (tested with the optimised assay) in this specific cohort. Excluding these antigens, 24% of anti-CCP2-negative RA sera were positive on peptide(s), which is in line with the results from Reed *et al.* in which 34.5% of anti-CCP2-negative RA sera contained ACPA fine specificities.²⁴

None of the peptides analysed are so far used in a routine setting to measure the presence of ACPA although we confirmed the high diagnostic performance of the detection of anti- β 60-74CitBiotNt antibodies, equivalent to that of AhFibA and anti-CCP2, almost all AhFibA- or anti-CCP2-positive patients being anti- β 60-74CitBiotNt-positive.¹²

Altogether, almost all ACPA-positive RA sera contain antibodies towards the single β 60-74CitBiotNt fibrin-derived peptide, associated or not with other fine specificities. In addition,

Table 1. Amino acid sequences of citrullinated peptides used to detect ACPA fine specificities

Name	Protein-derived from	Sequence
β60-74Cit	β-chain of human fibrin	CitPAPPISGGGYCitACit
β60-74CitBiotNt	β-chain of human fibrin	Biot-CitPAPPISGGGYCitACit
β36-52Cit	β-chain of human fibrin	NEEGFFSACitGHRPLDKK
α36-50Cit	α-chain of human fibrin	GPCitVVECitHQSACKDS
α621-635Cit	α-chain of human fibrin	CitGHAKSCitPVCitGIHTS
α501-515Cit	α-chain of human fibrin	SGIGTLDFGFCitHCitHPD
Vim2-17Cit	Human vimentin	STCitSVSSSYCitCitMFGG
Vim59-74Cit	Human vimentin	VYATCitSSAVCitLCSVSP
CEP-1Cit Linear	Human alpha enolase	KIHACitEIFDSCitGNPTVE
CEP-1Cit Cyclic	Human alpha enolase	CKIHACitEIFDSCitGNPTVEC
EBNA35-58Cit	Epstein-Barr nuclear antigen 1	GPAGPCitGGGCitGCitGCitGCitGHNDGG

Non-citrullinated counterparts have been used as controls.

subgroups of patients defined by ACPA fine specificities depend on the feature of the peptides used for testing and are largely overlapping. Thus, multiplexing contains a large part of redundancy and stratification of patients for improved clinical management might be challenging.

METHODS

Patients and serum samples

We used a series of 180 patients with established RA classified according to the 1987 American College of Rheumatology (ACR) criteria, and 200 control patients with non-RA rheumatic diseases (Supplementary table 1).²⁵ All sera were collected from patients attending the Rheumatology Centre of the Toulouse University Hospital and stored at -80°C until assayed in research protocols in accordance with national ethical requirements. For Figure 2i, the control group was enlarged to 436 patients (Supplementary table 2).

Peptide and antibody measurements

Ten peptides derived from the major proteins targeted by ACPA (Table 1) were tested in duplicate with a previously described single ELISA protocol to quantify ACPA fine specificity.^{12,25} Delta optical density (ΔOD) corresponding to the reactivity towards arginine-containing antigen, subtracted from that towards the citrulline-containing ones, was calculated to reflect ACPA level. Antibodies towards the biotinylated form of the β60-74 peptide (β60-74CitBiotNt), AhFibA and anti-CCP2 antibodies (Immunoscan RA; Euro Diagnostica, Arnhem, The Netherlands) were tested as previously described.^{12,13}

Statistical analyses

Data analyses were performed using MedCalc software for Windows® (Broekstraat 52 B-9030 Mariakerke, Belgium).

The Kruskal–Wallis or Mann–Whitney *U*-tests were used to compare the median differences. Correlations of antibody levels were assessed by the Spearman's rank test. Diagnostic performances were compared using McNemar's chi-square test. For multiple comparisons, *P*-values were adjusted using the Holm–Bonferroni method. *P*-values $\alpha \leq 0.05$ were considered significant.

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CONFLICT OF INTERESTS

We declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Leonor Nogueira: Conceptualization; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. **Emilie Parra:** Investigation; Methodology; Writing-review & editing. **Margaux Larrieu:** Investigation; Methodology; Writing-review & editing. **Evelyne Verrouil:** Data curation; Investigation; Methodology; Writing-review & editing. **Martin Cornillet:** Conceptualization; Formal analysis; Investigation; Methodology; Writing-original draft; Writing review & editing.

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Supporting Information

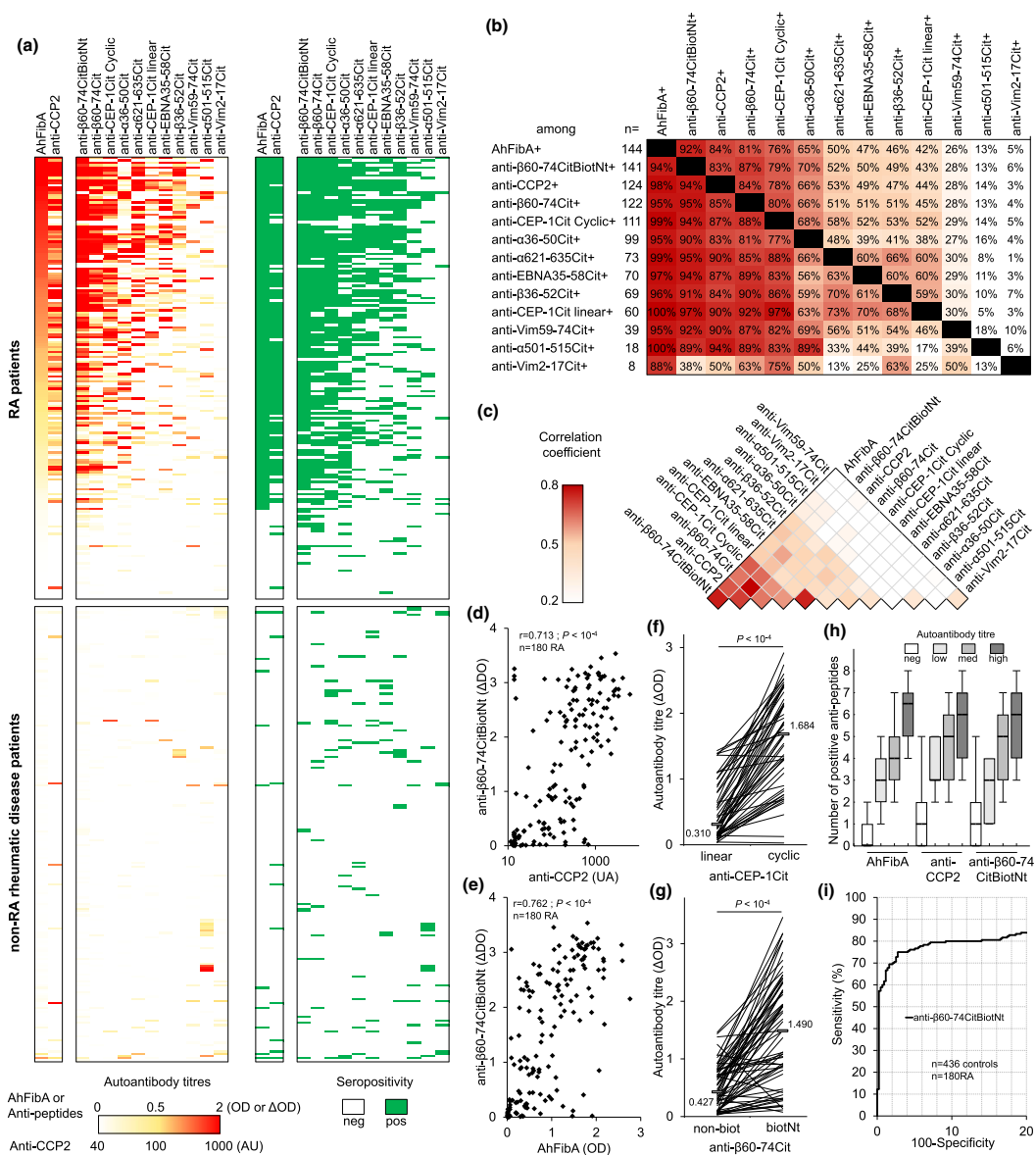
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Graphical Abstract

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In rheumatoid arthritis (RA), antibodies to citrullinated protein (ACPA) are believed to be heterogeneous and patient stratification by antibody profiling raised clinical interest. However, heterogeneity might be partially artificial. We show that ACPA multiplexing contains a degree of redundancy and that patient stratification largely depends on detection methods and requires standardisation.