

High diagnostic performance of ELISA detection of antibodies to citrullinated antigens in rheumatoid arthritis

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Objective: We investigated the rheumatoid arthritis (RA) diagnostic performances of anti-cyclic citrullinated peptide antibody (anti-CCP) and antifilaggrin antibody (AFA) in comparison with RF and matrix metalloproteinase-3 (MMP-3).

Methods: We used a second generation enzyme-linked immunosorbent assay (ELISA) kit for the detection of anti-CCP. We constructed recombinant human filaggrin, which was citrullinated *in vitro* by human peptidylarginine deiminase, and subsequently used it as the coating antigen for AFA-ELISA. A total of 549 RA patients and 208 other rheumatic disease patients were included in the study.

Results: The specificities of anti-CCP (88.9%) and AFA (94.7%) were superior to those of RF (81.7%) and MMP-3 (49.5%). The sensitivity of anti-CCP (87.6%) was superior to all others. However, the sensitivity of AFA (68.7%) was inferior to those of RF (69.8%) and MMP-3 (75.7%). Furthermore, receiver operating characteristic curves of anti-CCP and AFA passed closer to the upper left corner than those of RF and MMP-3, and the areas under the curves (AUC) of AFA and anti-CCP were significantly larger. In addition, the AUC of anti-CCP was significantly larger than that of AFA.

Conclusion: ELISA detection of antibodies to citrullinated antigens, especially a second generation anti-CCP, showed higher discriminative ability than other assays, including RF, and would be useful to aid the diagnosis of RA in clinical practice.

Key words: anti-cyclic citrullinated peptide antibody, antifilaggrin antibody, rheumatoid factor, matrix metalloproteinase-3, sensitivity, specificity, receiver operating characteristic curve, area under the curve

RA is a systemic autoimmune disease of unknown aetiology, characterized by chronic inflammation of joints resulting in tissue degradation and joint deformation. Rheumatoid factor (RF) has been widely used to diagnose RA in clinical practice since its initial description by Waaler in 1940 and Rose et al in 1948. RF can be detected in 60–80% of RA patients. However, its specificity is limited, since RF is also detected in other diseases, such as autoimmune and infectious diseases, as well as in healthy individuals — especially the elderly. Nevertheless, there have been no substitute assays that are reproducible, easy-to-use, and highly efficient for the diagnosis of RA.

Two highly RA-specific autoantibodies, antiperinuclear factor (APF) (1) and antikeratin antibody

(AKA) (2), as detected by indirect immunofluorescent methods, were discovered in 1964 and 1979, respectively. Their specificities were reported to be close to 100%. Since the target antigens of APF and AKA were revealed as filaggrin and related proteins (3–5), they were designated as antifilaggrin antibody (AFA). The identification of filaggrin as their target antigen led to the development of an AFA assay, based on immunoblotting (6, 7), in addition to ELISA using purified natural filaggrin (8–10). Although they were demonstrated to be as specific as APF and AKA, their sensitivities were lower than that of RF.

Recently, it was reported that citrulline, which is created through post-translational modification of arginine residue by peptidylarginine deiminase (PAD), is the essential constituent of the epitope recognized by APF and AKA (11, 12), and two ELISA systems using citrullinated antigens were developed. One is anti-cyclic citrullinated peptide antibody (anti-CCP) ELISA using artificial cyclic citrullinated peptide, whose sequence is derived from

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human filaggrin (13). The other is AFA-ELISA using recombinant filaggrin as the coating antigen, which is citrullinated (deiminated) *in vitro* by rabbit PAD (14). When the RA diagnostic performance of anti-CCP was compared with that of RF, anti-CCP presented higher specificity but lower sensitivity than RF (13, 15–18). Although AFA-ELISA using recombinant filaggrin was demonstrated to be as specific as AKA, the RA diagnostic performance of AFA was not compared with that of RF in previous studies (14, 19). Therefore, although the two ELISA systems are quantitative, reproducible, easy-to-use, and thus suitable for routine use in clinical practice, it remains unclear whether ELISA detection of antibodies to citrullinated antigens, i.e. anti-CCP and AFA, are superior to RF in terms of RA diagnostics.

In the present study, we measured anti-CCP using a commercially available second-generation ELISA kit. We also developed an in-house AFA-ELISA system using recombinant human filaggrin, which was citrullinated *in vitro* by human PAD. The RA diagnostic performances of these two assays were compared with that of RF. Serum matrix metalloproteinase 3 (MMP-3, stromelysin-1), which has been shown to be elevated in inflammatory diseases, including RA, and to be correlated with disease activities of RA (20–23), was also measured for comparison. Based on the findings obtained, we discuss the usefulness of measurement of autoantibodies to citrullinated antigens for the diagnosis of RA.

Patients and methods

Patients

Serum samples of patients with RA and other rheumatic diseases were obtained from the following five

hospitals in Japan: Juntendo University Hospital, Kyoto University Hospital, National Sagami Hospital, Niigata Prefectural Senami Hospital, and University of Tokyo Hospital. A total of 549 serum samples were obtained from RA patients diagnosed according to the revised criteria of the American College of Rheumatology (24) (Table 1). The median disease duration of RA patients was 9.4 years (range: 6 weeks to 56 years). A total of 208 serum samples were obtained from patients with other rheumatic diseases (Table 1).

Serum samples were also collected from normal individuals, to examine their titers of anti-CCP and AFA. A total of 320 serum samples were obtained from normal individuals who underwent a medical check-up. Beforehand, subjects were excluded whose blood chemistry findings of alanine aminotransferase or aspartate aminotransferase or blood urea nitrogen were beyond the reference ranges. Forty-one percent of subjects were male. The median age of the normal individuals was 48 years (range 19–79). The appropriate ethics committee approved this study, and all patients gave their written informed consent.

AFA ELISA

Subunits of human profilaggrin were amplified by reverse transcriptase-PCR (RT-PCR), using human skin cDNA as the template. The primer sequences utilized for RT-PCR were as follows: sense primer; 5'-CCCCATATGCAGGTGAGCACTCATGAACAGTCTG-3' (the *Nde* I site is underlined) and antisense primer; 5'-CCCCTCGAGTCCCTGTGACCGGCCGCGTGTGGACT-3' (the *Xho* I site is underlined). The amplified human filaggrin fragments were digested with *Nde* I/ *Xho* I and cloned into the *Nde* I - *Xho* I site of pET22b(+) vector, and the construct was introduced into *Escherichia coli*

Table 1. Characteristics of patients.

	Total	Male		Age	
	n	n	%	Median	Range
RA	549	99	18	60	19–83
Other rheumatic diseases	208	22	11	53	20–87
SLE	56	3	5	43	20–79
Systemic sclerosis	35	2	6	58	32–87
Sjögren's syndrome	30	0	0	57.5	24–80
Polymyositis/dermatomyositis	24	8	33	54	28–85
Mixed connective tissue diseases	16	0	0	46	25–69
Vasculitis	15	5	33	60	37–86
Osteoarthritis	15	0	0	75	67–82
Other diseases*	17	4	24	59	25–82

*Behçet's disease (n=5); Polymyalgia rheumatica (n=4); adult onset Still's disease (n=2); anti-phospholipid syndrome, CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, and telangiectasias), overlap syndrome, pustulosis palmaris et plantaris, ulcerative colitis, and Weber-Christian disease (n=1).

BL21(DE3), and the expression of recombinant flaggrin was induced by addition of isopropyl β -D-thiogalactopyranoside (IPTG). The expressed recombinant protein was purified using a cobalt-chelate column, and the purified recombinant flaggrin was subsequently citrullinated by recombinant human PAD type III (25).

The citrullinated recombinant human flaggrin was coupled covalently to 96-well CovaLink-NH plates for ELISA detection of antibodies (Nunc, Rocester, NY). Coupling was performed at 4°C for 16 hours. The wells were washed with phosphate-buffered saline (PBS), and the plates were blocked with 1% BSA for 1 hour at room temperature.

Patient and normal sera were diluted 1:100 with PBS containing 0.15% Tween-20 and 1% casein enzymatic hydrolysate. One hundred microliters of the diluted sera, positive standard containing AFA, or negative standard without AFA were added to each well. After incubation for 1 hour at room temperature, the wells were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). Then, 100 μ L of horseradish peroxidase-conjugated goat antibody to human γ -globulins (Medical and Biological Laboratories, Nagoya, Japan) diluted at 1:5000 in 20 mM HEPES pH 7.4, 135 mM NaCl, 0.1% p-hydroxyphenylacetic acid was added to each well and incubated for 1 hour at room temperature. After washing four times with PBS-Tween, the bound antibodies were detected with 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction (30 min) was stopped by addition of 100 μ L of 1N sulphuric acid per well.

Plates were read at absorbance of wavelength 450 nm (A450). The AFA titer was expressed by means of an index calculated as $([A450 \text{ of measured sample serum} - A450 \text{ of negative standard}] \text{ divided by } [A450 \text{ of positive standard} - A450 \text{ of negative standard}]) \times 100$. Intra-assay and inter-assay variations were <10%. The optimal cut-off value for AFA-ELISA was determined from the receiver operating characteristic (ROC) curve.

Other serum analyses

The second generation anti-CCP ELISA kit (DIA-STAT Anti-CCP) was purchased from Axis-Shield, Dundee, UK. The assay was conducted according to the manufacturer's instructions. The optimal cut-off value for anti-CCP ELISA was determined from the ROC curve. RF was measured by latex-enhanced immunonephelometric assay (Dede Behring, Marburg, Germany). The cut-off value for RF was 15 IU/ml. MMP-3 was measured by ELISA (The Binding Site Limited, Birmingham, UK). Cut-off values for MMP-3 were 45.3 ng/ml for males and 21.0 ng/ml

for females. C-reactive protein (CRP) measured by latex turbidimetric immunoassay (Medical and Biological Laboratories, Nagoya, Japan), and erythrocyte sedimentation rate (ESR) were examined as inflammatory markers for RA.

Statistical analyses

For the construction of ROC curves, relations between sensitivity (ordinate) and 1-specificity (abscissa) for various cut-off points were plotted. In general, a closer location of the ROC plot to the upper left corner indicates a higher diagnostic performance of the assay. The area under the ROC curve (AUC) provides an index of the overall discriminative ability of the test (26). Computation and comparison of AUC were performed using The Statistical Package for the Biosciences (COMWORKS, Saitama, Japan).

Comparison of the titer distributions between RA and other rheumatic diseases was made using the Mann-Whitney U test. Comparisons of sensitivity and specificity were made using McNemar's test. Differences in AUC were analyzed using the z-test. Spearman's rank correlation coefficient was used to assess the importance of the different variables. Differences were considered significant where $p < 0.05$.

Results

Serum levels of anti-CCP and AFA in normal individuals

We first examined the distributions of anti-CCP and AFA in normal individuals; these values were currently unknown for Japanese people. The values of anti-CCP and AFA were distributed in a non-Gaussian fashion. The median values of anti-CCP and AFA in normal individuals were 0.6 U/ml (range 0.3–118.3) and 3.4 (range 1.2–81.9), respectively. There were no significant associations between the titer of anti-CCP or AFA and patient gender or age (data not shown). To calculate the upper reference limit, defined here as the value located two standard deviations above the mean, we logarithmically transformed the data and subsequently transformed them back to the original data-scale. The upper reference limit of anti-CCP was thus determined to be 2.3 U/ml, and that of AFA was 9.9.

Serum levels of AFA, anti-CCP, RF, and MMP-3, in RA and other rheumatic diseases

We examined the titers of anti-CCP, AFA, RF, and MMP-3 in patients with RA and other rheumatic

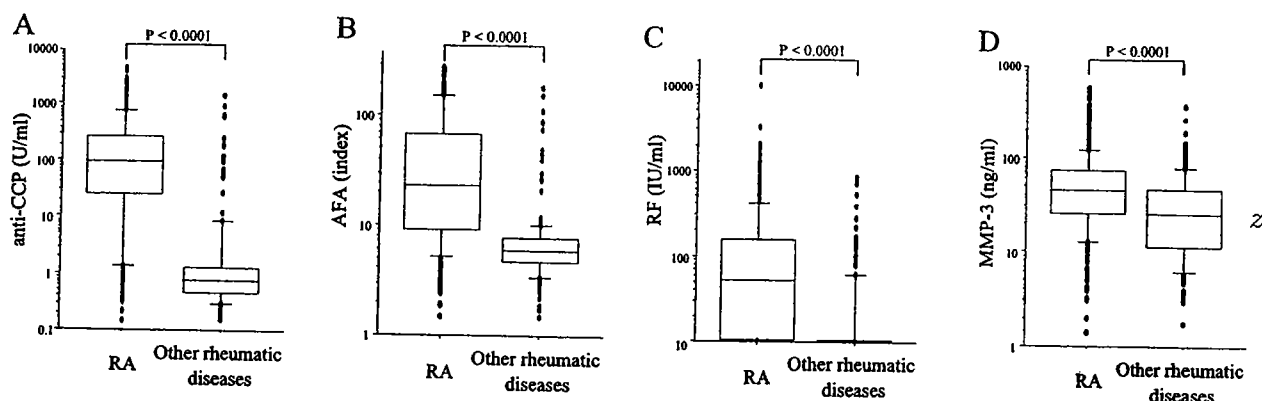


Figure 1. Serum levels of anti-CCP, AFA, RF, and MMP-3 in RA patients and in other rheumatic disease patients. Distributions of anti-CCP (A), AFA (B), RF (C), and MMP-3 (D) in sera from 549 RA patients in addition to 208 patients with other rheumatic diseases are shown. Comparisons of the titers of an assay between two patient groups were made using the Mann-Whitney U test. Lines in the boxes: median points, edges of the boxes show the lower and upper quartile points. Error bars=10% and 90% points, small circles show outlying data below the 10% and above the 90% points.

diseases. As shown in Fig. 1, anti-CCP separated the two distributions most, followed by AFA separation.

The titers of anti-CCP and AFA in RA patients were significantly higher than those in normal individuals ($p < 0.0001$ and $p < 0.0001$, respectively), and anti-CCP and AFA in other rheumatic disease patients were also significantly higher than those in normal individuals ($p = 0.0007$ and $p < 0.0001$, respectively).

Sensitivity and specificity of AFA, anti-CCP, RF, and MMP-3

From ROC curves we determined the optimal cut-off values for anti-CCP as 4.5 U/ml and for AFA as 12. We used predetermined cut-off values for RF and MMP-3, and calculated the sensitivity and specificity of each assay (Figure 2). Apparently, the RA diagnostic performance of anti-CCP was superior to those of RF and MMP-3, in terms of its significantly higher sensitivity and specificity. However, it was not clear whether anti-CCP was superior to AFA because of its significantly higher sensitivity and lower specificity. It was also difficult to clarify the differences between AFA and RF, and between AFA and MMP-3 from the comparisons of sensitivities and specificities.

When we examined the sensitivities of the tests in the population of 91 patients with early RA, which was defined here as being disease duration of < 1 year, anti-CCP still had the highest sensitivity value (83.5%) among AFA (70.3%), RF (78.0%), and MMP-3 (72.5%). However, only the difference between anti-CCP and AFA was statistically significant in this population ($p < 0.005$).

Among the 166 RF-negative RA patients, 69.3% were positive for anti-CCP, and 45.8% were positive for AFA.

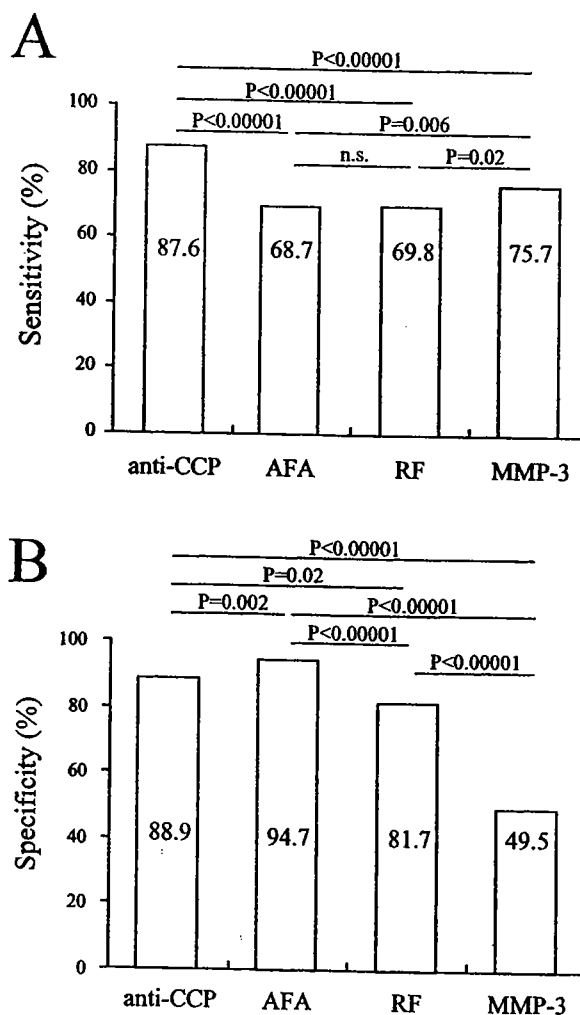


Figure 2. The sensitivity (A) and specificity (B) of anti-CCP, AFA, RF, and MMP-3. Comparison of the sensitivity and specificity data between assays was made using McNemar's test. Numbers shown within each bar are the values of sensitivity or specificity of respective assays, expressed as a percentage. n.s. = not significant.

ROC analyses

For further comparison of the diagnostic utilities of each assay, we constructed ROC curves and calculated the AUC (Figure 3). The ROC curves of anti-CCP and AFA passed closer to the upper left corner than those of RF and MMP-3. This means that when the sensitivities of each assay are compared at the same specificity value, the sensitivities of anti-CCP and AFA are higher than those of RF and MMP-3. As to the relative superiority between anti-CCP and AFA, the ROC curve of anti-CCP generally passed above that of AFA. Therefore, it appears that anti-CCP has a higher diagnostic performance than AFA. The superiority of anti-CCP to AFA was confirmed by comparing AUC, since AUC of anti-CCP was significantly larger than that of AFA ($p=0.001$). Furthermore, the superiority of anti-CCP and AFA to RF was also confirmed by AUC, since AUC of anti-CCP or AFA was significantly larger than that of RF ($p<0.001$ and $p=0.03$, respectively).

We could easily compare the sensitivity values of each assay at fixed specificity levels from the ROC curves. For example, when the specificity value was fixed to 90%, sensitivity values of anti-CCP, AFA, and RF were 84.2%, 72.5%, and 54.5%, respectively. Sensitivity of anti-CCP was significantly greater than that of AFA or RF ($p<0.00001$) and sensitivity of AFA was significantly greater than that of RF ($p<0.00001$).

It was previously demonstrated that the distribution of MMP-3 in normal individuals is different in males and females (20). Therefore, we constructed separate ROC curves of MMP-3 for male and female populations. As shown in Figure 3, the ROC curve of MMP-3 in female patients was generally below those of anti-CCP, AFA, and RF. Furthermore, the AUC of MMP-3 was significantly smaller than those of anti-CCP (0.902, $p<0.001$), AFA (0.843, $p<0.001$), and RF (0.782, $p=0.002$) in female patients, clearly indicating that anti-CCP, AFA, and RF are superior to MMP-3 for the diagnosis of RA.

Correlations of the titers of assays with inflammatory markers in RA

As shown in Table 2, the titers of anti-CCP, AFA, RF, and MMP-3 in RA patients were all significantly correlated with inflammatory markers, including CRP and ESR. The degrees of correlation of anti-CCP and AFA with inflammatory markers were similar to that of RF, and much weaker than that of MMP-3.

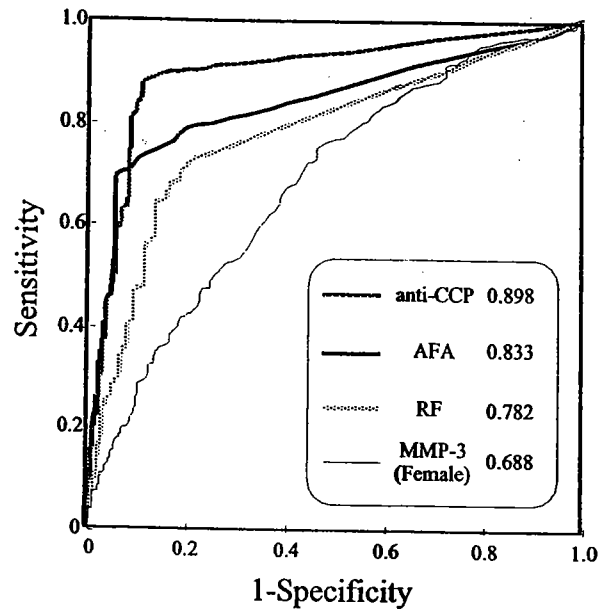


Figure 3. ROC curves of anti-CCP, AFA, RF, and MMP-3. ROC curves of anti-CCP, AFA, and RF were constructed, based on the data obtained from patients with RA ($n=549$) and other rheumatic diseases ($n=208$). Since the distribution of MMP-3 in normal individuals differs between female and male populations, the ROC curves of MMP-3 were constructed separately. The ROC curve of MMP-3 based on the data from female patients with RA ($n=450$) and other rheumatic disease patients ($n=186$) is shown. Numbers shown within the window at the lower right are the values of areas under the ROC curves.

Discussion

ELISA detection of antibodies to citrullinated antigens, anti-CCP, and AFA was previously demonstrated to be highly specific for RA. However, it remains unclear whether ELISA provides better RA diagnostic performance than RF assay. In the present study, we used a commercially available second-generation anti-CCP ELISA kit. For measuring AFA, we developed AFA-ELISA using recombinant human filaggrin citrullinated by human PAD. In addition, we examined RF and MMP-3, and compared the four assays in terms of RA diagnostic

Table 2. Spearman's rank correlation coefficients between each assay and inflammatory marker in RA patients.

	CRP		ESR	
	r	p	r	p
Anti-CCP	0.25	<0.0001	0.27	<0.0001
AFA	0.21	<0.0001	0.26	<0.0001
RF	0.27	<0.0001	0.28	<0.0001
MMP-3 (male)	0.58	<0.0001	0.54	<0.0001
MMP-3 (female)	0.61	<0.0001	0.48	<0.0001

performance. We demonstrated that ELISA detection of antibodies to citrullinated antigens shows better RA diagnostic performance than RF and MMP-3, based on the analyses of sensitivity and specificity, ROC curves, and AUC.

With regard to superiority of either anti-CCP or RF, previous studies have yielded conflicting results, suggesting that the specificity of anti-CCP was higher than RF but its sensitivity was lower (13, 15–18). Schellekens et al constructed ROC curves of anti-CCP and IgM-RF; however, the two curves appeared to be similar and the AUC were not calculated (13). Accordingly, previous studies failed to demonstrate which method is superior for the diagnosis of RA. We have clearly demonstrated the superiority of anti-CCP ELISA to RF in the present study. It should be noted that there exists a difference in anti-CCP assays.

Vossenaar et al, the developers of anti-CCP ELISA, recently reported that selection from their peptide libraries allowed the production of a second-generation anti-CCP kit (27). The sensitivity of the anti-CCP assay could be increased from 60–68% to 75–80%, whilst retaining its specificity of >98%. The anti-CCP ELISA utilized in the present study was a second-generation kit: the generation of anti-CCP was not described in previous studies in which the diagnostic performances of anti-CCP and RF were compared. Judging from the relatively low sensitivities of anti-CCP assays, the anti-CCP used in previous studies would probably be considered to be first generation. Therefore, it is suggested that discrepancy, in terms of the superiority of anti-CCP to RF, derives from generation differences of anti-CCP assay between the present and previous studies.

Another possibility for the superiority of anti-CCP shown in the present study could arise from racial differences. So far as we have investigated, previous studies were performed in Europe and the USA. We could not find any other studies examining the prevalence of antibodies to citrullinated antigens in Asian patients with RA. The role of racial differences in the production of antibodies to citrullinated antigens remains to be clarified.

There have been no head-to-head comparisons between AFA and RF in terms of RA diagnostic ability, but we compared them directly with each other in the present study. From the viewpoint of sensitivity and specificity, it was difficult to determine which was superior because AFA presented lower sensitivity but higher specificity than RF. Such a discrepancy can occur when the diagnostic performances of two assays are compared in terms of sensitivity and specificity. In such a situation, a comparison of sensitivity at the same specificity level

is often attempted. We therefore constructed ROC curves for comparison. The ROC curve of AFA entirely passed through the upper left side compared with that of RF. Accordingly, in a comparison of the sensitivities of AFA and RF at the same specificity value, the sensitivity of AFA is always higher. Comparison of each AUC significantly showed the superiority of AFA. Collectively, these findings suggest that AFA has a more efficient discriminative ability for the diagnosis of RA than RF.

In this study, the diagnostic value of anti-CCP was significantly superior to that of AFA. However, Vincent et al recently reported a contrasting result, demonstrating that the RA diagnostic performance of their in-house AFA assay based on recombinant rat filaggrin was superior to that of anti-CCP (19). One possibility for this discrepancy is that rat filaggrin is more suitable than human filaggrin for detecting autoantibodies, since AFA-ELISA based on recombinant human filaggrin, which they evaluated simultaneously, was inferior to not only anti-CCP assay (the same as our results), but also AFA-ELISA using recombinant rat filaggrin.

It is also possible that a species difference in PAD might influence the performance of AFA assays. The degree of citrullination of recombinant human filaggrin performed by human PAD type III was equal to that performed by human PAD type I or rabbit PAD type II, reaching approximately 40% (data not shown) in our preliminary experiments. Although this citrullination degree was similar to that of recombinant rat filaggrin performed by rabbit PAD type II reported in the study of Vincent et al, we cannot specify the differences with regard to the citrullination of particular arginine residues between human and rat filaggrin. The difference in the patient populations between these studies might have influenced the results.

Along with these factors, there appears to be another explanation for the discrepancy: the generation difference of anti-CCP. As mentioned above, we used a second-generation kit, however, the generation of anti-CCP used in Vincent et al's study was not described. Judging from the sensitivity data of anti-CCP, the assay used by them would be considered to be first generation. In our preliminary study consisting of 177 RA patients and 173 other rheumatic disease patients, sensitivities of the first-generation anti-CCP and AFA were 59% and 74%, and their specificities were 95% and 97%, respectively. These findings suggest that our in-house AFA assay was superior to the first-generation anti-CCP assay for the diagnosis of RA. In the present study, however, our assay was inferior to the second-generation anti-CCP: this situation may also have occurred in the case of the AFA assay used by

Vincent et al. To confirm this, a head-to-head comparison would be necessary between their AFA and the second-generation anti-CCP.

With regard to comparisons with MMP-3, ELISA detection of antibodies to citrullinated antigens showed superior diagnostic performance, which was clearly demonstrated by the comparisons of ROC curves and the AUC. Serum MMP-3 level was reported to be elevated, not only in RA patients but also in other diseases, especially in systemic lupus erythematosus (SLE) (22, 28). Indeed, 73% of SLE patients were positive for MMP-3 in the present study. It is suggested that relatively high positivity in other disease patients resulted in low specificity and, consequently, inferior RA diagnostic performance of MMP-3.

In addition, we examined whether titers of antibodies to citrullinated antigens correlated with inflammatory markers, including ESR and CRP. Although there were significant correlations of the titers with inflammatory markers, the correlation values were much weaker than those of MMP-3 with inflammatory markers. Therefore, it is suggested that, for the purpose of monitoring the disease activities of RA, ELISA detection of antibodies to citrullinated antigens may not be as efficient as MMP-3. Further studies examining the relation of the titers of antibodies to citrullinated antigens with disease activities of RA in detail, preferably in a prospective way, would be necessary to corroborate their usefulness as disease markers of RA.

Although filaggrin proteins are not expressed in the synovium, it has been demonstrated that B cells in the synovial tissue (10) and synovial fluid (29) from patients with RA can spontaneously produce antibodies to citrullinated filaggrin. It is reasonable, therefore, to assume that citrullinated antigens, other than citrullinated filaggrin, exist in the synovium, and that the reactivity of antibodies to citrullinated filaggrin is a cross-reaction. Recently, Masson-Bessiere et al demonstrated that citrullinated forms of fibrin are one of the targets of AFA in the synovium (30). They postulated that the use of citrullinated fibrin or peptide derived from fibrin would probably display a higher diagnostic value than those using filaggrin or filaggrin-related peptides.

We present here the superiority of ELISA detection of antibodies to citrullinated antigens, especially second-generation anti-CCP, for the diagnosis of RA. Some other citrullinated proteins have been suggested as targets of antibody present in RA patients' sera (31). The detection of autoantibodies to citrullinated antigens described in the present study, or developed in the near future, may become a major criterion for the diagnosis of RA, in addition to clinical and radiographic findings.

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References

1. Nienhuis RLF, Mandema E, Smids C. A new serum factor in patients with rheumatoid arthritis, the antiperinuclear factor. *Ann Rheum Dis* 1964;23:302-5.
2. Young BJJ, Mallya RK, Leslie RD, Clark CJM, Hamblin TJ. Anti-keratin antibodies in rheumatoid arthritis. *Br Med J* 1979;2:97-9.
3. Girbal E, Sebbag M, Gomès-Daudrix V, Simon M, Vincent C, Serre G. Characterisation of the rat oesophagus epithelium antigens defined by the so-called 'antikeratin antibodies', specific for rheumatoid arthritis. *Ann Rheum Dis* 1993;52:749-57.
4. Simon M, Girbal E, Sebbag M, Gomès-Daudrix V, Vincent C, Salama G, et al. The cytokeratin filament-aggregating protein filaggrin is the target of the so-called 'antikeratin antibodies', autoantibodies specific for rheumatoid arthritis. *J Clin Invest* 1993;92:1387-93.
5. Sebbag M, Simon M, Vincent C, Masson-Bessière C, Girbal E, Durieux J-J, et al. The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies. *J Clin Invest* 1995;95:2672-9.
6. Vincent C, Simon M, Sebbag M, Girbal-Neuhauser E, Durieux J-J, Cantagrel A, et al. Immunoblotting detection of autoantibodies to human epidermis filaggrin: a new diagnostic test for rheumatoid arthritis. *J Rheumatol* 1998;25: 838-46.
7. Vincent C, de Keyser F, Masson-Bessière C, Sebbag M, Veys EM, Serre G. Anti-perinuclear factor compared with the so called 'antikeratin' antibodies and antibodies to human epidermis filaggrin, in the diagnosis of arthritides. *Ann Rheum Dis* 1999;58:42-8.
8. Palosuo T, Lukka M, Alenius H, Kalkkinen N, Aho K, Kurki P, et al. Purification of filaggrin from human epidermis and measurement of antifilaggrin autoantibodies in sera from patients with rheumatoid arthritis by an enzyme-linked immunosorbent assay. *Int Arch Allergy Immunol* 1998;115: 294-302.
9. Aho K, Palosuo T, Lukka M, Kurki P, Isomäki H, Kautiainen H, et al. Antifilaggrin antibodies in recent-onset arthritis. *Scand J Rheumatol* 1999;28:113-6.
10. Masson-Bessière C, Sebbag M, Durieux J-J, Nogueira L, Vincent C, Girbal-Neuhauser E, et al. In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum. *Clin Exp Immunol* 2000;119: 544-52.
11. Schellekens GA, de Jong BAW, van den Hoogen FHJ, van de Putte LBA, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest* 1998;101: 273-81.
12. Girbal-Neuhauser E, Durieux J-J, Arnaud M, Dalbon P, Sebbag M, Vincent C, et al. The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues. *J Immunol* 1999; 162:585-94.
13. Schellekens GA, Visser H, de Jong BAW, van den Hoogen FHJ, Hazes JMW, Breedveld FC, et al. The diagnostic

- properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 2000;43:155-63.
14. Nogueira L, Sebbag M, Vincent C, Arnaud M, Fournié B, Cantagrel A, et al. Performance of two ELISAs for anti-flaggrin autoantibodies, using either affinity purified or deiminated recombinant human flaggrin, in the diagnosis of rheumatoid arthritis. *Ann Rheum Dis* 2001;60:882-7.
 15. Goldbach-Mansky R, Lee J, McCoy A, Hoxworth J, Yarburo C, Smolen JS, et al. Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset. *Arthritis Res* 2000;2:236-43.
 16. Bizzaro N, Mazzanti G, Tonutti E, Villalta D, Tozzoli R. Diagnostic accuracy of the anti-citrulline antibody assay for rheumatoid arthritis. *Clin Chem* 2001;47:1089-93.
 17. Bas S, Perneger TV, Seitz M, Tiercy J-M, Roux-Lombard P, Guerne PA. Diagnostic tests for rheumatoid arthritis: comparison of anti-cyclic citrullinated peptide antibodies, anti-keratin antibodies and IgM rheumatoid factors. *Rheumatology (Oxford)* 2002;41:809-14.
 18. Jansen AL, van der Horst-Bruinsma I, van Schaardenburg D, van de Stadt RJ, de Koning MH, Dijkmans BA. Rheumatoid factor and antibodies to cyclic citrullinated peptide differentiate rheumatoid arthritis from undifferentiated polyarthritis in patients with early arthritis. *J Rheumatol* 2002;29:2074-6.
 19. Vincent C, Nogueira L, Sebbag M, Chapuy-Regaud S, Arnaud M, Letourneur O, et al. Detection of antibodies to deiminated recombinant rat flaggrin by enzyme-linked immunosorbent assay: a highly effective test for the diagnosis of rheumatoid arthritis. *Arthritis Rheum* 2002;46:2051-8.
 20. Yoshihara Y, Obata K, Fujimoto N, Yamashita K, Hayakawa T, Shimmei M. Increased levels of stromelysin-1 and tissue inhibitor of metalloproteinases-1 in sera from patients with rheumatoid arthritis. *Arthritis Rheum* 1995;38:969-75.
 21. Manicourt DH, Fujimoto N, Obata K, Thonar EJ-MA. Levels of circulating collagenase, stromelysin-1, and tissue inhibitor of matrix metalloproteinases 1 in patients with rheumatoid arthritis. Relationship to serum levels of antigenic keratan sulfate and systemic parameters of inflammation. *Arthritis Rheum* 1995;38:1031-9.
 22. Keyszer G, Lambiri I, Nagel R, Keysser C, Keysser M, Gromnica-Ihle E, et al. Circulating levels of matrix metalloproteinases MMP-3 and MMP-1, tissue inhibitor of metalloproteinases 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers. *J Rheumatol* 1999;26:251-8.
 23. So A, Chamot AM, Péclat V, Gerster J-C. Serum MMP-3 in rheumatoid arthritis: correlation with systemic inflammation but not with erosive status. *Rheumatology (Oxford)* 1999;38:407-10.
 24. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
 25. Kanno T, Kawada A, Yamanouchi J, Yosida-Noro C, Yoshiki A, Shiraiwa M, et al. Human peptidylarginine deiminase type III: molecular cloning and nucleotide sequence of the cDNA, properties of the recombinant enzyme, and immunohistochemical localization in human skin. *J Invest Dermatol* 2000;115:813-23.
 26. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 1993;39:561-77.
 27. Vossenaar ER, van Venrooij WJ, Pruijn GJM. Anti-CCP antibodies in (early) rheumatoid arthritis. In: Conrad K, Fritzler M, Meurer M, Sack U, Shoenfeld Y, eds. *Proteomics to molecular epidemiology: relevance of autoantibodies*. Lengerich: Pabst Science Publishers, 2002:454-62.
 28. Zucker S, Mian N, Drews M, Conner C, Davidson A, Miller F, et al. Increased serum stromelysin-1 levels in systemic lupus erythematosus: lack of correlation with disease activity. *J Rheumatol* 1999;26:78-80.
 29. Reparón-Schuijt CC, van Esch WJE, van Kooten C, Schellekens GA, de Jong BAW, van Venrooij WJ, et al. Secretion of anti-citrulline-containing peptide antibody by B lymphocytes in rheumatoid arthritis. *Arthritis Rheum* 2001;44:41-7.
 30. Masson-Bessière C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, et al. The major synovial targets of the rheumatoid arthritis-specific anti-flaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 2001;166:4177-84.
 31. Lapointe E, Déry U, Vaillancourt F, Ménard HA, Senshu T. Rheumatoid sera potentially recognize all citrullinated proteins. *Arthritis Rheum* 1999;42 Suppl:S86.