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**Do all anti-citrullinated protein/peptide antibody (ACPA) tests measure the same?
Evaluation of discrepancy between ACPA tests in RA and non-RA patients.**

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Abstract

Background: Different methods exist to demonstrate anti-citrullinated protein/peptide antibodies (ACPA).

Aims: To evaluate discrepancy between 4 ACPA tests.

Patients and Methods: Population 1 consisted of patients with a new diagnostic problem including 86 RA and 450 non-RA patients. Population 2 consisted of 155 RA patients with longstanding disease. Population 3 consisted of 188 psoriatic arthritis (PsA) patients and population 4 consisted of 192 lupus (SLE) patients. Populations 1 and 2 were tested with the anti-human fibrinogen antibodies (AhFibA) test, anti-CCP2 from Eurodiagnostica (CCP2-euro), anti-CCP2 from Pharmacia (CCP2-phar) and anti-CCP3 test by Inova (CCP3). Samples were annotated as discrepant if positive in one and negative in at least one other test. Each discrepant sample was re-analyzed in a different run. Populations 3 and 4 were analyzed in the CCP2-euro and AhFibA test.

Results: In population 1, ACPA positivity was found in 17 of 450 (3.8%) non-RA patients. 14 (82%) of those 17 samples were discrepant. In contrast, 61 (70.9%) of 86 RA patients were ACPA positive of whom 18 (29.5%) out of 61 were discrepant (70.9% vs. 29.5%, $p < 0.001$). The discrepancies between tests could be partly attributed to borderline results, inter-assay discrepancy and inter-test variability. They were more prevalent in ACPA positive SLE patients than in ACPA positive PsA patients.

Conclusions: Discrepancy between different ACPA tests was observed attributable to the occurrence of borderline results, inter-assay variability and mainly to inter-test variability. The lowest inter-test discrepancy is observed between tests that use the same substrate.

Key words

ACPA

Rheumatoid arthritis

Anti-CCP

AhFibA

Introduction:

Irreversible joint erosion may occur early in the disease course in patients with rheumatoid arthritis (RA). Also, intensive therapy is most successful if applied early in the disease course (1). Therefore, it is important to diagnose RA correctly and fast. It is in this window of the disease, when often not all clinical manifestations are visible, that a sensitive and specific serological test is needed. Two important autoantibody systems have been described in this autoimmune disease: rheumatoid factor (RF), directed against the Fc part of an IgG molecule, and the anti-citrullinated protein/peptide antibodies (ACPA), which are significantly more specific for RA. ACPA are directed against various proteins which contain arginine residues that have been converted to citrulline by posttranslational modification, catalyzed by peptidylarginine deiminase (PAD) enzymes (2). Different substrates have been developed to detect ACPA. The best known and most widely used assay is the CCP2 assay. Numerous studies reported sensitivities ranging from 65% to 80% with a high specificity of more than 95%. (3). More recently, the presence of citrullinated fibrin in the synovial membrane of RA patients (4) led to the use of citrullinated fibrinogen to assay the serum antibodies against deiminated fibrinogen (AhFibA) (5-8). Other substrates include citrullinated vimentin (9), and a more recently developed cyclic citrullinated peptide based assay (anti-CCP3) (10, 11). Although most of those ACPA tests seem to have similar diagnostic properties, we aimed to evaluate whether the positive or negative result of one ACPA test can be reproduced by another test and how discrepancies between tests can be described.

Patients and methods

Patient population

The present analyses are based on 4 previously described cohorts of patients. **Population 1** consists of consecutive patients with rheumatic symptoms from whom serum samples were sent to our laboratory for ACPA determination within the context of a diagnostic investigation. In this population, 92 patients were classified as definite RA, and 463 patients were diagnosed as definite non-RA (6). The most frequent diseases diagnosed in the non-RA patients were osteoarthritis (31%), other mechanical disease (including peri-arthritis scapulohumeralis, tendinopathies,...) (20%), spondyloarthropathy (13%), systemic lupus erythematosus (9%), vasculitis (6%), polymyalgia rheumatica (5%) other connective tissue diseases (including scleroderma and Sjögren's syndrome) (2%), adult JIA (juvenile idiopathic arthritis) patients (1%), psoriatic arthritis (PsA) (5%), crystal arthritis (3%) and other diseases including infections, malignancies and neurological disorders (5%). **Population 2** consists of 180 consecutive RA patients with longstanding disease of at least 4 years duration (6, 12). **Population 3** consisted of 192 PsA patients and **population 4** consisted of 235 SLE (systemic lupus erythematosus) patients. Population 3 and 4 were previously used to describe the occurrence of ACPA in non-RA patients (13-15).

ACPA determination

ACPA were determined by the following 4 tests. Three commercially available ACPA tests were used: Anti-CCP2 EIA by Pharmacia (CCP2-phar), Immunoscan RA Anti-CCP mark 2 ELISA by Eurodiagnostica (CCP2-euro), Anti-CCP3 (CCP3) by Inova. Those three tests were conducted as instructed by the manufacturers. Anti-human fibrinogen antibodies (AhFibA) were tested by ELISA as previously described (5-8).

Populations 1 and 2 were analysed with all 4 ACPA tests; population 3 and 4 were analysed with the AhFibA and CCP2-euro tests.

Cut-offs were provided by the manufacturer or determined in independent populations corresponding to 98% specificity levels (6, 12). The cut-offs for positivity were 0.110 OD for the AhFibA test, 42 U for the CCP2-euro test, 7 U for the CCP2-phar test and 40 U for the CCP3 test. Based upon those cut-offs, ACPA positivity was annotated when a sample was positive for any of those 4 ACPA tests. An ACPA positive sample that was negative on one or more other tests was further called "discrepant".

For each test, additional cut-offs were used to describe borderline negative or borderline positive results at specificity levels of at least 95% or at least 98.5% respectively. A borderline negative result is one which is situated between the 95% and the 98% specific cut-off, a borderline positive result is situated between the 98% and 98.5% specific cut-off. To evaluate inter-assay discrepancy, discrepant samples from population 1 and 2 were re-analyzed with all 4 ACPA assays.

Statistics

Due to the skewed distributions and the difficulties to transform the data in order to obtain normality and standardization, results of ACPA tests were analyzed after dichotomization (negative/ positive) or categorization (negative, borderline negative, borderline positive, positive). Inter-assay and inter-test discrepancy were expressed in grades, with the highest difference defined as grade 3, which expressed a difference from negative to positive. Other steps were defined as grade 2 (e.g. negative to borderline positive), grade 1 (e.g. borderline negative to borderline positive) or grade 0 (no discrepancy). Inter-assay discrepancy applied to differences between runs of the same test, inter-test discrepancy applied to differences between kits. Comparison of proportions was evaluated by the chi-square statistic. Cluster

analysis for ordinal data was performed based on correlations calculated by Tau correlation coefficients. All analyses were performed using SPSS 12.0 (Chicago, Illinois, USA).

Results

Description of the populations

Sufficient serum for complete case analysis was available in 86 RA and 450 non-RA patients of population 1, in 155 RA patients of population 2, in 188 PsA patients of population 3 and in 192 SLE patients of population 4.

Performance of the different tests

Performing ROC analyses, no significant differences between the 4 ACPA assays could be observed in population 1. Sensitivities and specificities of the tests at the different cut-offs are given in table 1.

Cluster analysis of ACPA tests in RA and non-RA patients in population 1 and 2.

Based on the cut-offs as defined in table 1, cluster analysis was performed based on correlations calculated by Tau correlation coefficients. Similar results were obtained on the pooled dataset of population 1 and 2 and after split in RA and non-RA patients. This cluster analysis defines one cluster of the CCP2-euro and CCP2-phar test.

Occurrence of discrepant samples in RA and non-RA patients in population 1 and 2

In population 1, ACPA positivity was found in 17 of 450 (3.8%) non-RA patients. 14 (82%) of those 17 samples were discrepant. In contrast, 61 (70.9%) of 86 RA patients were ACPA positive of whom 18 (29.5%) were discrepant. Similarly, in population 2, 122 of 155 RA-patients were ACPA positive of whom 27 (22.1%) were discrepant. This suggests, that, when a sample tests ACPA positive, discrepant results are more prevalent in non-RA than in RA patients (82% vs. 29.5%, $p < 0.001$). Non-RA discrepant samples were positive in 1 ACPA test in 85.7% and positive in 2 ACPA tests in 14.3% of the cases. None of the non-RA discrepant samples were positive in ≥ 3 ACPA tests. RA discrepant samples were positive in 1 ACPA test in 20%, positive in 2 ACPA tests in 31.1% and positive in 3 ACPA tests in 48.9% of the cases.

Evaluation of ACPA positivity and discrepancy in PsA patients (population 3) and SLE patients (population 4).

In populations 3 and 4, sufficient serum was available from 188 PsA patients and 192 SLE patients for analysis with both AhFibA and CCP2-euro tests. 15 (8%) of the 188 PsA patients were ACPA positive (on at least one of the tests) of whom 13 out of 15 (87%) were positive in both tests. In contrast to the PsA patients, only 5 out of 17 (29%) ACPA positive SLE patients of population 4 were positive on both tests (29% vs. 87%, $p = 0.002$).

Can discrepant samples be explained by borderline results or inter-assay discrepancy?

Of the 59 discrepant samples in population 1 and 2, sufficient serum was available to be retested by the CCP2-euro, the CCP3 and the AhFibA assay in 48 cases. 37 of them were RA patients, 11 were non-RA patients. CCP2-phar could be retested in only 16 of 37 RA patients. Table 2 describes the number of discrepant samples that display a negative test result, a borderline negative, a borderline positive or a positive test result in function of the different ACPA tests. From this table, one can calculate that borderline results occur in up to 29% of the discrepant samples.

Inter-assay discrepancy was calculated by retesting the samples in a different run. The highest inter-assay discrepancy occurred for the CCP2-euro test with 23% of the samples showing a grade 3 discrepancy (Table 3).

Taking into account inter-assay discrepancy, overall grade 3 discrepancy between tests remained in more than 54.5% to 62.5% of the samples. The lowest inter-test discrepancy was observed between the CCP2-euro and CCP2-phar tests (Table 4).

Discussion

Although most ACPA tests have similar diagnostic properties, previous studies showed that there may be an imperfect correlation between ACPA results and that discrepancies between ACPA tests may occur (6, 10, 16, 17). The present analysis confirms these observations with additional ACPA tests and confirms the finding that, independent of the studied population, clusters of ACPA tests can be defined based on these discrepancies.

Although very specific for RA, a low prevalence of ACPA reactivity (5-10%) has previously been observed in different non-RA inflammatory diseases: psoriatic arthritis (14, 18-20), systemic lupus erythematosus (13, 21), and other diseases (22-24). In the present analysis, it is demonstrated that, if such ACPA reactivity occurs in non-RA patients, there is a higher probability that this ACPA reactivity is discrepant than in case of ACPA positivity in RA patients. Also, if such discrepancy occurs, a non-RA ACPA positive sample tends to be negative on a higher number of ACPA tests than a RA ACPA discrepant sample. Thus, a non-RA patient showing ACPA positivity has a higher probability that this result will not be confirmed by another ACPA test than a RA patient. Moreover, within non-RA diseases, SLE patients who show ACPA reactivity are more prone to show discrepancy between ACPA tests than PsA patients (10).

In order to evaluate whether discrepancies between ACPA tests could be attributed to borderline results or inter-assay discrepancies, 48 samples with discrepancy between the 4 ACPA tests were further evaluated: borderline (negative or positive) results occurred in up to 29% of the test results (Table 2) and grade 3 inter-assay discrepancy (evaluated by retesting the discrepant samples with the same ACPA tests at different runs) in up to 27.3 % (Table 3).

Finally, after minimizing the effect of inter assay discrepancy (by using the lowest discrepancy), it was shown that grade 3 inter test discrepancy could be observed in the majority of the samples (Table 4). This suggests that borderline results and inter assay discrepancy contribute less to the observed inter test discrepancy than the differences between the tests used.

In addition, the remaining inter-test discrepancy was the lowest for the CCP-euro and CCP-phar test, confirming the initially described cluster between those two tests which use the same substrate. Given the fact that those two tests use the same substrate, it can be hypothesized that the remaining small inter-test discrepancy (table 4) can be attributed to other components of the kits such as standards, secondary antibodies or the cut-offs used (15).

Higher discrepancy rates could be observed between tests that use different substrates. This might be attributed to differences in epitope recognition. Proteome analysis previously showed that different citrullinated autoantigens can be differentially recognized by different ACPA positive samples and that spots containing highly citrullinated peptides/proteins displayed higher reactivities (25). It thus could be hypothesized that RA samples recognize a higher number and more similar citrullinated epitopes than non-RA samples. In non-RA one could assume that reactivity against non-citrullinated epitopes may occur (6), however, it seems that most ACPA reactivities in patients with rheumatic diseases can be attributed to recognition of citrullinated epitopes (15).

These data may also be important for optimization of test strategies in case of borderline results suggesting that retesting the same sample with a different assay may be more informative than retesting the same test with the same test. Whether this strategy is inferior or superior on re-sampling the same patient at another time point remains to be investigated.

To conclude, discrepancies between ACPA tests may occur and can be observed more frequently in non-RA samples. Discrepancy between ACPA tests can be explained by borderline results and inter-assay discrepancies, but mostly by differences in the used substrates.

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Table 1: Diagnostic characteristics of the 4 different ACPA tests at the different cut-offs

	Cut-off	Specificity (population1)	Sensitivity (population1)	Sensitivity (population2)	Result
AhFibA Ab					Negative
	0.05	0.971	0.663	0.768	Borderline Negative
	0.11	0.985	0.581	0.697	Borderline positive
	0.15	0.987	0.581	0.677	Positive
CCP2-euro					Negative
	25	0.976	0.674	0.678	Borderline Negative
	42	0.985	0.628	0.652	Borderline positive
	50	0.987	0.616	0.652	Positive
CCP3					Negative
	20	0.953	0.628	0.761	Borderline Negative
	40	0.982	0.612	0.710	Borderline positive
	60	0.987	0.581	0.671	Positive
CCP2- phar					Negative
	5	0.976	0.686	0.774	Borderline Negative
	7	0.987	0.674	0.774	Borderline positive
	10	0.988	0.674	0.735	Positive

AhFibA : anti-human fibrinogen antibodies test, CCP2-euro: anti-CCP2 antibodies test (Eurodiagnostica), CCP2-phar: anti-CCP2 antibodies test (Pharmacia), CCP3: anti-CCP3 antibodies test (Inova). Population 1: RA and non-RA patients; Population 2 : longstanding RA patients. RA = rheumatoid arthritis; non-RA= RA excluded; CCP= cyclic citrullinated peptide.

A sample was considered positive if displaying a result equal or higher than the cut-off.

Table 2: Number of discrepant samples that display a negative test result, a borderline negative/positive test result or a positive test result in function of the different ACPA tests.

		RA (n=37)		Non-RA (n=11)	
CCP2- euro	Neg	14	37.8%	7	63.6%
	Bord Neg	6	16.2%	0	0.0%
	Bord Pos	0	0.0%	0	0.0%
	Pos	17	45.9%	4	36.4%
CCP2- phar	Neg	5	13.5%	8	72.7%
	Bord Neg	0	0.0%	1	9.1%
	Bord Pos	5	13.5%	0	0.0%
	Pos	27	73.0%	2	18.2%
AhFibA	Neg	8	21.6%	8	72.7%
	Bord Neg	10	27.0%	0	0.0%
	Bord Pos	1	2.7%	0	0.0%
	Pos	18	48.6%	3	27.3%
CCP3	Neg	8	21.6%	5	45.5%
	Bord Neg	6	16.2%	2	18.2%
	Bord Pos	5	13.5%	1	9.1%
	Pos	18	48.6%	3	27.3%

AhFibA : anti-human fibrinogen antibodies test, CCP2-euro: anti-CCP2 antibodies test (Eurodiagnostica), CCP2-phar: anti-CCP2 antibodies test (Pharmacia), CCP3: anti-CCP3 antibodies test (Inova). Population 1: RA and non-RA patients; Population 2 : longstanding RA patients.

RA = rheumatoid arthritis

non-RA= RA excluded

CCP= cyclic citrullinated peptide.

Pos= positive

Neg= negative

Bord= borderline

Table 3: Number of discrepant samples with different inter assay discrepancy gradings.

	Grading	RA (n=37)*		Non-RA (n=11)	
CCP2- euro	0	22	59.5%	8	72.7%
	1	5	13.5%	0	0.0%
	2	2	5.4%	0	0.0%
	3	8	21.6%	3	27.3%
CCP2- phar*	0	14	87.5%	11	100.0%
	1	1	6.3%	0	0.0%
	2	1	6.3%	0	0.0%
	3	0	0.0%	0	0.0%
AhFibA	0	28	75.7%	11	100.0%
	1	7	18.9%	0	0.0%
	2	2	5.4%	0	0.0%
	3	0	0.0%	0	0.0%
CCP3	0	35	94.6%	9	81.8%
	1	2	5.4%	2	18.2%
	2	0	0.0%	0	0.0%
	3	0	0.0%	0	0.0%

CCP2-euro: anti-CCP2 antibodies test (Eurodiagnostica), CCP2-phar: anti-CCP2 antibodies test (Pharmacia), AhFibA : anti-human fibrinogen antibodies test, CCP3: anti-CCP3 antibodies test (Inova). Results obtained on both Population 1 and Population 2

RA = rheumatoid arthritis

non-RA= RA excluded

CCP= cyclic citrullinated peptide.

Grading: 0: no discrepancy, 1: 1 step discrepant (ex. borderline neg -> borderline pos), 2: 2 steps discrepant (ex. Borderline neg -> pos), 3: 3 steps discrepant (neg. vs. pos.)

*: only 16 RA patients could be retested on the CCP phar test.

Table 4 Number of discrepant RA and non-RA samples with different gradings of inter test discrepancy after minimizing for inter assay discrepancy.

	Grading	RA (n=37)*		Non-RA (n=11)	
CCP2- phar/CCP3*	0	11	68.8%	4	36.4%
	1	2	12.5%	0	0.0%
	2	1	6.3%	4	36.4%
	3	2	12.5%	3	27.3%
CCP2- euro/AhFibA	0	18	48.6%	7	63.6%
	1	5	13.5%	0	0.0%
	2	8	21.6%	0	0.0%
	3	6	16.2%	4	36.4%
CCP3/AhFibA	0	12	32.4%	3	27.3%
	1	10	27.0%	1	9.1%
	2	7	18.9%	3	27.3%
	3	8	21.6%	4	36.4%
CCP2- euro/CCP3	0	16	43.2%	5	45.5%
	1	4	10.8%	0	0.0%
	2	7	18.9%	3	27.3%
	3	10	27.0%	3	27.3%
CCP2- phar/AhFibA*	0	8	50.0%	7	63.6%
	1	2	12.5%	1	9.1%
	2	2	12.5%	0	0.0%
	3	4	25.0%	3	27.3%
CCP2- euro/CCP- phar*	0	14	87.5%	9	81.8%
	1	0	0.0%	1	9.1%
	2	0	0.0%	0	0.0%
	3	2	12.5%	1	9.1%
Overall* (all 4 tests)	0	2	12.5%	2	18.2%
	1	1	6.3%	0	0%
	2	3	18.8%	3	27.3%
	3	10	62.5%	6	54.5%

CCP2-euro: anti-CCP2 antibodies test (Eurodiagnostica), CCP2-phar: anti-CCP2 antibodies test (Pharmacia), AhFibA : anti-human fibrinogen antibodies test, CCP3: anti-CCP3 antibodies test (Inova). Results obtained on both Population 1 and Population 2.

RA = rheumatoid arthritis

non-RA= RA excluded

CCP= cyclic citrullinated peptide.

RA = rheumatoid arthritis

non-RA= RA excluded

CCP= cyclic citrullinated peptide.

Grading : 0: no discrepancy, 1: 1 step discrepant (ex. borderline neg -> borderline pos), 2: 2 steps discrepant (ex. Borderline neg -> pos), 3: 3 steps discrepant (neg. vs. pos.)

*: only 16 RA patients could be retested on the CCP phar test.