

The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis

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Short title: The diagnostic performance of ACPA tests

Abstract

The presence or absence of antibodies to citrullinated peptides/proteins (ACPA) is an important parameter that helps a clinician set a diagnosis of early rheumatoid arthritis and, hence, initiate treatment. There are several commercial tests available to measure ACPA levels, although it can be difficult to decide what the best test for a given clinical question is. We analyzed literature data in which the diagnostic and other properties of various ACPA tests are compared. The results show that for diagnostic purposes the CCP2 test has the highest specificity, the highest sensitivity in stratified studies and highest Positive Predictive Value. For the prediction of future joint destruction the CCP2, MCV, and CCP3 tests may be used. The ability to predict the likelihood of not achieving sustained DMARD-free remission was highest for the CCP2 test. Finally, the levels of anti-CCP2 and anti-CCP3 (and possibly anti-MCV) in RA patients are not significantly influenced by TNF α blocking agents.

Abbreviations

ACPA, anti-citrullinated protein antibodies; CCP, cyclic citrullinated peptide; cFil, citrullinated recombinant rat filaggrin; clgG, citrullinated immunoglobulin; DMARD, disease-modifying anti-rheumatic drug; EBV, Epstein Barr virus; MCV, mutated citrullinated vimentin; MTX, methotrexate; NPV, negative predictive value; PPV, positive predictive value; RA, rheumatoid arthritis; RF, rheumatoid factor; TNF α , tumor necrosis factor α ; UA, undifferentiated arthritis

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation of the joints, which can ultimately lead to cartilage and bone destruction. In the last decade it has become apparent that citrullinated proteins/peptides and in particular autoantibodies directed to them (ACPA: anti-citrullinated protein antibodies) are likely to be involved in the development of this disease in at least 70% of the patients (reviewed in [1]). In the clinical setting ACPA have mostly been detected by use of the anti-CCP (anti-cyclic citrullinated peptide) test, although more recently other tests using various citrullinated proteins have also been employed.

Recently it became clear that RA patients can be classified into two major subsets, namely those who have ACPA (here termed anti-CCP(+)) and those who are anti-CCP(-) [2]. Whilst in the early phase of the disease these two groups of patients show a very similar clinical presentation, the picture changes considerably as the disease develops further. The presence of ACPA at early diagnosis predicts more pronounced radiographic progression, as demonstrated by many studies showing a strong association between anti-CCP positivity with the development of bone erosions. Importantly, environmental risk factors e.g. tobacco smoking differ to a large extent between these two populations [3], and the risk of developing ischemic heart disease is clearly higher in anti-CCP(+) compared to anti-CCP(-) RA patients [4]. Furthermore, treatment response to, for example, synthetic disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate may differ between these groups of patients [5]. It is therefore important for a clinician to be able to accurately separate anti-CCP(+) from anti-CCP(-) patients. During such a decision making process it is important that both clinicians and laboratory specialists are fully aware of the advantages and disadvantages of the various ACPA tests that are commercially available. This review intends to critically review the literature on comparisons between the most frequently applied commercial tests in terms of specificity and sensitivity of ACPA detection.

ACPA and CCP

Several lines of evidence indicate that the ACPA response in RA patients is polyclonal and heterogeneous [6]. Thus, antibodies to a variety of citrullinated epitopes on different proteins can be detected and their production is likely to vary between individual patients. The commercial ACPA tests are all aimed at detecting most, if not all, ACPA epitope reactivities found in RA patients. The majority of published studies in which the presence of ACPA in RA patients was investigated have used the second generation CCP test (termed CCP2 test). Using this test, about 75% of RA patients with long-term established diagnosis and 61% of patients with established early RA patients were anti-CCP2 positive (Table 1).

Anti-CCP antibodies of these patients can be eluted from the CCP2 ELISA plate (by low pH or high salt) and the eluate can subsequently be used to stain western blots containing different citrullinated proteins, such as fibrinogen, histones or vimentin. The eluted antibodies react with all of these citrullinated proteins indicating broad cross-reactivity between anti-CCP and these various antigens (R. Toes, pers. commun.). These data have been complemented by studies of synovial exosomes from RA patients, which were shown to contain ACPA as well as a number of citrullinated constituents e.g. citrullinated fibrinogen peptides and citrullinated Sp α (a CD5 antigen-like protein) [7]. Moreover, the number of anti-CCP(-) sera that show reactivity with other citrullinated antigens is very small [8,9]. Taken together, these data indicate that the vast majority of ACPA can be detected by the CCP2-test.

Anti-CCP(+) RA and anti-CCP(-) RA

Early diagnosis of RA coupled with rational use of DMARDs has been shown to have a favorable effect on the course of the disease. Therefore, early and accurate diagnosis has become increasingly important. For several reasons the presence of anti-CCP antibodies is a great help to clinicians in deciding which patient needs early treatment:

First, anti-CCP antibodies are very specific for RA, and they are produced at significant levels very early in disease. The specificity of anti-CCP antibodies for the diagnosis of RA is high, as shown in Table 1. In addition it is known that anti-CCP antibodies can be present many years before the first visit to the clinic (up to 18 years) [10-12].

Second, studies of early arthritis cohorts have shown that a large number of these early arthritis patients can not be accurately diagnosed at their first visit, and hence are often referred as undifferentiated arthritis (UA) patients. However, if patients are found to be anti-CCP(+) when referred to the clinician, more than 90% develop RA within 3 years in contrast to only 30% of the anti-CCP(-) patients. Thus, the presence of anti-CCP antibodies in UA accurately predicts development of RA [13,14].

Third, the presence of anti-CCP antibodies at the first visit to the clinician predicts radiographic progression, as demonstrated by many studies that have shown a strong association of anti-CCP positivity with the development of bone erosions [1,15,16]. In the past IgM RF positivity was assumed to predict radiographic progression, but a recent report clearly indicates that the radiographic progression seen is actually associated with ACPA(+)/RF(+) and ACPA(+)/RF(-) RA, but not with ACPA(-)/RF(+) and ACPA(-)/RF(-) RA [17]. The conclusion therefore seems to be that presence of ACPA as such is associated with an erosive course and the presence of IgM RF is just a co-expressed autoantibody as has been known for a long time.

Fourth, in anti-CCP(+) RA patients more germinal centers in synovial tissue infiltrates are found [18]. It is known that germinal centers contribute to RA pathogenesis by supporting autoantibody production [19]. In the same report distinct synovial features such as increased fibrosis in the synovial tissue and a thicker synovial lining layer were found in anti-CCP(-) RA patients [18].

In conclusion, although at baseline the clinical features of both RA subsets are very similar, anti-CCP(+) RA is more strongly associated with poor outcome than anti-CCP(-) RA (reviewed in [20]).

This conclusion may also have important implications for treatment. Synthetic DMARDs, such as methotrexate (MTX) are often used in treating RA, frequently in combination with TNF α blockers to enhance the treatment response. In a large Dutch study, the so-called PROMPT study (methotrexate versus placebo treatment), Van Dongen and coworkers found that anti-CCP(+) patients responded well to MTX treatment, while a parallel anti-CCP(-) group did not [5]. In a subsequent study it was shown that MTX treatment resulted in a more favourable response in patients with a low or intermediate pre-treatment level of ACPA [21]. These data not only suggest that the effectiveness of a drug can be different in anti-CCP(+) as compared to anti-CCP(-) arthritis, but also that very early treatment, even in patients not yet fulfilling ACR criteria for RA, can be beneficial if used in a selective way [20].

CCP and other ACPA tests

In the last three years about 25 articles have appeared in which performances of different ACPA tests were compared. However, only 9 of these studies followed the essential principle that any such comparisons can only be made when the test results are properly stratified. Stratification means that sensitivity values have to be calculated at a predefined specificity (mostly 98% or more) using the same cohort of RA patients and disease control sera. The chosen specificity should be as high as possible without compromising essential sensitivity and thus the overall diagnostic efficiency. Another important point is the cohort of patients selected for such studies.

The main advantage of ACPA lies in their proven ability to predict the development of RA and their potential use as a criterion for RA even at baseline [22]. Therefore, the ideal cohort to evaluate the clinical value of ACPA tests is the mixed population of patients visiting an early arthritis clinic where some patients eventually will develop RA [6]. The use of a cohort of established RA patients with longstanding disease is clearly less useful. In the studies reviewed in this article all sorts of RA patient cohorts and control groups have been used, and

this, at least in part, explains some of the differences in sensitivity and specificity of ACPA tests between different studies.

The tests

It is primarily the antigen (substrate) that decides how specific or sensitive a test will be. In 2002 the CCP2 test was launched, and this test is still the golden standard and most frequently used test in clinical practice. Table 1 gives the accumulated data from 154 publications using the CCP2 test.

There are at least 6 tests available using the CCP2 peptides as antigen (Axis-Shield, Euro-Diagnostica, Euroimmun, Inova, Phadia, Abbott). Despite using the same set of CCP2 peptides, these assays tend to show small differences in their diagnostic profiles [23,24]. The main reason is that although the antigen is the same, the solid support materials might be different, and the added variables (conjugate, buffers, incubation time, etcetera) are also different and these may contribute to the small differences reported. Aside from the CCP2 test, several other ACPA tests using different substrates have more recently been made commercially available. Some of these newer tests have rarely been used in published studies and are therefore not included in our calculations. Assays that have been used in published data include a test based on in vitro citrullinated mutated human vimentin as antigen (MCV from Orgentec), the Inova CCP3 test (cyclic citrullinated peptides) and its variant Inova CCP3.1, the Genesis' citrullinated recombinant rat filaggrin (cFil), the Aesku citrullinated IgG peptide (clgG) and the Astra citrullinated EBV nuclear antigen derived peptide. In publications in which (some of) these tests are compared, the RF-IgM test is also often included. However, the RF tests are mostly used without a clinically useful cut-off based on predefined specificity, and thus true comparisons of results obtained with this test in different studies are not possible.

The diagnostic performance of ACPA tests

The data extracted from recent literature and tabulated in Tables 2 and 3 show that the CCP2 test still performs best when compared to other ACPA tests.

Comparison with the classical IgM-RF test confirms previous reports that the CCP2 test has a superior specificity and, in stratified studies, a much higher sensitivity (Table 2). Recent data from Van der Linden and co-workers [17], who showed that the rate of joint destruction in RA was not affected by the presence or absence of IgM RF but rather by the presence or absence of ACPA, corroborate the idea that ACPA positivity should be included as a criterion for the diagnosis of RA in clinical studies [25]. These authors also advocated the inclusion of ACPA into any revision of the current ACR criteria for RA. Interestingly the European League against Rheumatism (EULAR) has already recommended that in all new cases of RA the measurement of anti-CCP should be considered [26], and at the latest ACR meeting in Philadelphia an ACR/EULAR panel of specialists included ACPA testing into the New Rheumatoid Arthritis Criteria.

Several studies have addressed the diagnostic performance of the MCV assay. In stratified studies this test shows a lower sensitivity (see Table 2) and in non-stratified studies a lower specificity than the CCP2 test [27-30]. A similar conclusion was reached in a recent review on the diagnostic and prognostic properties of the MCV assay [31]. There are a few reports indicating that anti-MCV is present in a significant number of anti-CCP(-) sera [21,32-34] and this subgroup of patients appears to have a higher rate of radiographic destruction than seronegative patients. Anti-MCV positivity therefore seems to indicate poor radiographic prognosis in a larger group of RA patients than does anti-CCP positivity [34]. However, data from the study of Van der Linden and collaborators indicate that the presence of anti-MCV antibody with negative anti-CCP does not strongly affect the level of joint damage in RA [17]. It is clear that further evaluation of the MCV antibody in clinically well characterized cohorts is needed. It would also be useful to introduce a “control” mutated vimentin antigen (not citrullinated) in order to test whether this CCP-negative population of anti-MCV antibody is directed to the vimentin protein or to the citrulline moiety of mutated vimentin [9,35].

Only the CCP3 test appears in some studies to be comparable to the CCP2 test [36-38], although in the majority of published studies the CCP3 test shows a somewhat lower specificity and/or sensitivity [23,24,39-43]. Based upon the combined results of these studies it can be concluded that the CCP3 test has no apparent diagnostic advantage compared to the CCP2 test. Inova has also recently introduced the CCP 3.1 test that additionally includes measurement of IgA antibodies. In general this test does not appear to be better than the CCP3 test [23,41] and would appear to have a limited usage in a routine laboratory set up.

Citrullinated peptide antigens have also been derived from proteins like EBV nuclear antigen (Astra) and IgG (Aesku). The tests with these peptides were included in the stratified study of Bizzaro and collaborators [23], but scored rather low in sensitivity (Aesku 44%, Astra 47%). In a single study, the Genesis test (cFil) shows a PPV that is lower than that of CCP2, but higher than the PPV of CCP3 and MCV [44]. Finally, Lutteri and co-workers also measured the diagnostic abilities of a new test using synthetic citrullinated peptides (RA/CP from Triturus). The specificity, sensitivity, PPV and NPV of this test were all much lower than those of CCP2 and CCP3 [24].

What test is best for the clinician?

As outlined above, ACPA are not only important predictors of RA development but are also among the most potent predictors of the outcome of RA, as measured by the rate of radiographic joint destruction. There are at least four clinically important reasons to perform and compare ACPA tests.

- (1) The ability to confirm or predict the development of RA with the highest reliability.
- (2) The ability to predict radiographic progression.
- (3) The ability to predict remission.
- (4) The ability to predict response to anti-TNF α treatment.

Ad (1). From Tables 1-3 it is clear that although most ACPA tests are perfectly able to predict or confirm a diagnosis of RA, none of the tests has a better diagnostic record than the CCP2 test. In addition it was reported recently that the PPV for predicting progression from UA to RA was highest for CCP2 (67.1%). Combinations of two or more ACPA tests appear to give no additive value [17]. A somewhat different situation may exist for the additional testing of RF, since RF testing may carry additional clinical value beyond testing for anti-CCP alone [45]. It should be noted that RF positivity still is a criterion for RA, and that RF positive/anti-CCP negative RA patients display different environmental risk factors than those that are only anti-CCP-positive [3].

Ad (2). A positive test for anti-CCP2, anti-CCP3, or anti-MCV was associated with a higher Sharp/van der Heijde score at all time points except baseline and was also associated with a higher rate of joint destruction over a period of 7 years [17]. There was no difference between these tests with regard to their ability to predict radiographic progression. The use of a second or third autoantibody test did not increase the predictive accuracy for the rate of joint destruction [17]. Similar results were reported by a.o. Dejaco [36], Majka [46], Syversen [47] and collaborators. It is also interesting to note that not only in the presence of ACPA, but also in the absence of these antibodies, RF did not significantly correlate with increased rates of joint destruction. This indicates that RF, in contrast to ACPA, does not by itself contribute to disease progression. In some studies MCV was reported to have a somewhat higher sensitivity (mostly accompanied by a lower specificity) than CCP2 (e.g. [27,32-34]). However, these studies also showed that the rate of joint destruction in MCV(+)/CCP(-) patients was comparable to that in patients lacking ACPA, indicating that the presence of anti-MCV antibody alone does not affect the level of joint damage in RA [17,33,48].

Ad (3). The test's ability to predict the likelihood of not achieving sustained DMARD-free remission was highest (11.6%) for anti-CCP2 and varied between

4.7-6.0% for anti-CCP3, anti-MCV and RF [17]. Again, performing 2 ACPA tests had no additional value compared to the anti-CCP2 test alone. It is however clear that we need more data on this aspect of ACPA testing.

Ad (4). Treatment of RA mostly is assumed to combat important disease mechanisms and thus lower the inflammation. As a consequence one might also expect a reduction of the activation of autoreactive B-cells followed by reduced ACPA levels, which would allow monitoring the effect of treatment. In the initial studies no significant effect of infliximab on anti-CCP levels was observed (reviewed by Zendman and collaborators [49]). Also in later studies neither anti-CCP3 nor anti-CCP2 levels were found to be influenced by TNF α blocking agents, and the test results failed to predict responses to anti-TNF α treatment [36]. A possible reason for these observations may be that although the inflammation may decrease, the citrullinated antigens (and consequently the production of autoantibodies and immune complexes) are still there and this is reflected in the antibody levels (see [1]). However, in other studies significant decreases of anti-CCP2 and anti-MCV titers at 18 and/or 24 months of infliximab treatment have been reported [27]. At the moment we have to conclude that none of the available ACPA tests unequivocally shows the ability to predict response to treatment.

Perspectives

Reference serum

In most of the commercially available tests the cut-off values used to define a positive result varied significantly, even when the antigenic substrate was provided by the same manufacturer [23]. Therefore, there is an urgent need for reference material that can help investigators to harmonize data obtained with the various commercially available tests. The use of International Units, based on the reactivity of a reference serum or antibody will hopefully help laboratory experts and clinicians to decide which serum is ACPA-positive and which is not.

At the request of the Committee for the Standardization of autoantibodies in Rheumatic and Related Diseases (referred to as The Committee), the Center for Disease Control and Prevention (CDC, Atlanta, Georgia, USA) has prepared a lyophilized reference material, obtained from an RF and ACPA positive patient, which is available on request as an international ACPA reference reagent. This reference reagent has already been tested by some laboratories using several commercial tests and was found to improve considerably the comparison of quantitative results between different commercial tests (N. Bizzaro, pers. commun.). The reference serum has also been tested by laboratories of several members of The Committee by using the same substrate, using kits commercially available in Europe and is now available at the CDC for the scientific community (P.L. Meroni, pers. commun.).

Universal serum collection

A second important development that will help to compare the specificity and sensitivity of ACPA (and other) tests is the initiative of the European AutoCure consortium to generate a large depository of sera from patients with RA and other rheumatic diseases that will become available for comparative diagnostic studies. The use of a universal set of RA patients and control sera will allow a direct comparison of the diagnostic performance of current and yet to be developed tests.

Potential test improvements

From several recent studies it became clear that a positive reaction in an ACPA test for non-RA sera is frequently due to the presence of antibodies that recognize the target molecule in a non-citrulline dependent fashion, because the same molecule containing arginine instead of citrulline was bound by the antibodies at least as efficiently as the citrullinated antigen [9,35]. This indicates that the inclusion of a non-citrullinated control antigen in the test is likely to improve the specificity of the test for RA. Currently it is not clear whether the manufacturers of ACPA tests are considering such a modification of the test.

Finally, since each of the target molecules used for ACPA detection might have its specific utility in the identification of a particular subset of RA patients, the development of multiplex tests combining all of these target molecules in a single analysis may be a significant step forward in the detailed analysis of autoantibody reactivities in sera of this heterogeneous disease. Several experimental platforms can be envisaged to achieve this, including fluorescent secondary antibody-based microarrays, imaging surface plasmon resonance-based microarrays and Luminex addressable beads or nanotechnology-based systems.

Competing interests

GP and WvV are scientific advisors for Axis-Shield Diagnostics Ltd and Euro-Diagnostica AB.

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Tables and captions

Table 1. Sensitivity and specificity of the CCP2 test.

Patient group	n	anti-CCP2(+)	Sensitivity (%)	Specificity (%)
RA total	16828	12043	71.5	
early	4379	2677	61.1	
established	11528	8671	75.2	
Controls	19448	925	4.8	95.2
non-RA	14994	882	6.0	94.0
healthy	4454	43	1.0	99.0

The cumulative sensitivity and specificity of the CCP2 test is based on the results of 154 independent studies published between 2002 and June 2009. Separation into early and established RA was adapted from the original studies.

Table 2. Comparison of the sensitivity of various ACPA and RF tests at stratified specificity.

Reference	No. of patients (RA / control*)	Stratified specificity (%)	Sensitivity at stratified specificity (%)				
			CCP2	CCP3	MCV ⁱ	other	RF
Bizzaro et al. [23]	100 / 202	98.5	64-74 ^{b,c,d,e,f}	67 ^{g,h}	62	41-47 ^{j,k,l}	17
Coenen et al. [44]; Bossuyt, pers. comm.	102 / 196	95.0	76.2-77.0 ^{b,d,e}	75.5 ^g	65.7	69.3 ^k	n.d.
Damjanovska et al. [50]; Thabet, pers. comm.	566 / 351	93.4	56.9 ^e	56.2 ^h	52.5	n.d.	n.d.
Dejaco et al. [51]	164 / 303	98.7	70.1 ^d	n.d.	53.7	n.d.	n.d.
Innala et al. [41]	210 / 102	98.0	80.4 ^c	78.5-79.0 ^{g,h}	69.0	n.d.	n.d.
Mutlu et al. [37]	93 / 83	98.8	57.0-60.2 ^{a,t}	60.2 ^g	29	n.d.	48.4
Soos et al. [52]	119 / 118	95	74.8	n.d.	69.7	n.d.	33.6
Vander Cruyssen et al. [53]	272 / 463	98.5	67.4-68.0 ^e	n.d.	n.d.	n.d.	16.3-24.4
Vander Cruyssen et al. [42] (early arthritis)	92 / 463	98.7	61.6-67.4 ^{d,e}	58.1 ^g	n.d.	n.d.	n.d.
Vander Cruyssen et al. [42] (established RA)	180 / 463	98.7	65.2-77.4 ^{d,e}	67.1 ^g	n.d.	n.d.	n.d.
Average		97.3	69.2	66.1	57.4		29.9

* Note: control groups are not identical

^a AxSYM anti-CCP (Abbott)

^b CCP IgG (Euroimmun)

^c Diastat anti-CCP (Axis-Shield)

^d EliA CCP (Phadia)

^e Immunoscan-RA Mark 2 (Euro-Diagnostica)

^f Quanta Lite CCP IgG (Inova)

^g Quanta Lite CCP 3.0 IgG (Inova)

^h Quanta Lite CCP 3.1 IgG-IgA (Inova)

ⁱ Anti-MCV (Orgentec)

^j Aeskulisa RA CP-Detect (Aesku)

^k CPA (Genesis)

^l VCP IgG (Astra)

Table 3. Comparison of ACPA and RF tests in terms of positive and negative predictive values.

Reference	No. of patients (RA / control*)	CCP2		CCP3		MCV		RF	
		PPV** (%)	NPV** (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)	PPV (%)	NPV (%)
Caro-Oleas et al. [54]	124 / 158	95.2	73.1	92.3	70.5			90.9	73.6
Coenen et al. [44] ^d	133 / 165	89.7- 91.4 ^a	87.1- 89.1 ^a	76.7	88.2	80.0	87.2		
Correia et al. [39]	86 / 90	92.6- 96.7 ^b	72.9- 78.7 ^b	91.9- 94.9 ^b	74.4- 74.6 ^b			80.3	72.4
Dos Anjos et al. [40]	70 / 88	91.7	84.7	90.6	87.2				
Liu et al. [33]	170 / 136	95.5	66.8			93.7	77.4	82.0	69.9
Lutteri et al. [24]	120 / 170	87.7- 96.2 ^c	76.6- 78.3 ^c	92.6	78.1			77.9	79.5
Sghiri et al. [29]	170 / 309	91.1	86.3			66.0	84.7	58.6	79.9
Soos et al. [52]	119 / 118	97.6	74.4			90.0	78.8	80.2	74.0
Ursum et al. [48]	123 / 39	95.8				96.1			
Van der Linden et al. [17]	201 / 424	67.1	79.0	64.0	80.0	56.3	79.2	61.7	77.8
Average		91.2	78.4	84.9	79.8	80.4	81.5	75.9	75.3

* Note: control groups are not identical

** PPV: positive predictive value; NPV: negative predictive value

^a Three CCP2 tests used

^b Two CCP2 tests and two cut-off values used

^c Six CCP2 tests used

^d Coenen and collaborators also used the citrullinated filaggrin test (CPA, Genesis): PPV: 85.5, NPV 85.6)

