Anti-CCP2 testing

The Gold Standard in Diagnosis of Rheumatoid Arthritis
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1. Scope

This document is based on existing literature studies and data and its aim is to provide an overview of the characteristics of anti-CCP2 as diagnostic marker in rheumatoid arthritis (RA). In addition, the benefits of anti-CCP2 as compared to other commonly used serological markers for RA are discussed.

The CCP2 test, i.e. assay for the determination of antibodies to CCP2, was originally developed by Euro Diagnostica in collaboration with leading scientists. Ever since its introduction on the market in 2002, the test has been extensively investigated and used by laboratories all over the world. For several years, assays for the determination of anti-CCP2 have been considered the gold standard among the ACPA (anti-citrullinated protein antibodies) assays.

The amount of scientific publications that support the use of anti-CCP2 testing in the diagnosis of patients with RA is huge. On the other hand its use is strictly regulated by patents and license agreements. This situation has naturally led to the development of other tests, with claims to be alternatives to the CCP2 assays. In this document pros and cons of some of these more recently developed ACPA-tests will be discussed and their clinical performance, as compared to the CCP2 test, evaluated.

The conclusion is that despite intensive efforts to find even better peptides the CCP2 test still provides the best combination of sensitivity and specificity.

Further information about CCP2 and the Euro Diagnostica product portfolio within Rheumatoid Arthritis is found in the appendices (chapter 12). A glossary of terms is provided in chapter 11.

2. CCP2: Definition and Background

As the results of collaboration between leading scientists and Euro Diagnostica, CCP2 (cyclic citrullinated peptide 2) was found by screening synthetic peptide libraries of totally about 12 million peptides. Several peptides were isolated and those which performed best were selected for the CPP2 assay. This work resulted in a highly sensitive and the most specific autoantibody test for RA.

When the CCP2 assay was introduced on the market by Euro Diagnostica in 2002 it was also the first ACPA test that was commercially available for a broader market.

The introduction of testing for anti-CCP2 was a major breakthrough and today the presence, or absence, of ACPA is a crucial parameter for the diagnosis of RA. Since antibodies to CCP2 are often present early in the disease the test is also an important tool for the physician in treatment initiation decisions.

3. The market for CCP2 assays

The market for RA diagnostic markers is strongly related to the 2010 ACR/EULAR Rheumatoid Arthritis Classification Criteria\(^1\) of which serology is a key domain.

\[ \geq 6 \text{ points} = \text{RA} \]

\( Figure 1. \) Summary of the 2010 ACR/EULAR Rheumatoid Arthritis Classification Criteria. The serology domain, that gives up to 3 points, is an essential part of the criteria. Note that scores of 6 points or more are classified as RA.
The serology domain of the ACR criteria comprises testing of Rheumatoid Factor (RF) and/or ACPA. RF was first discovered in 1940 and has for decades been the first line screening test for RA. Today both RF and ACPA are commonly used in clinical practice and depending on study population these markers may complement each other (see chapter 7 for further details).

4. The Euro Diagnostica CCPlus® Immunoscan

Euro Diagnostica provides CCP2 tests in various formats (please see appendix I). The Euro Diagnostica CCPlus® Immunoscan is the leading anti-CCP2 ELISA kit on the market. Some basic product characteristics are provided below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CCPlus® Immunoscan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Released</td>
<td>2002 (new version 2008)</td>
</tr>
<tr>
<td>Product code</td>
<td>RA-96PLUS</td>
</tr>
<tr>
<td>Format</td>
<td>ELISA</td>
</tr>
<tr>
<td>Tests</td>
<td>96 wells</td>
</tr>
<tr>
<td>Calculation</td>
<td>Semi-quantitative</td>
</tr>
<tr>
<td>Antigen</td>
<td>CCP2</td>
</tr>
<tr>
<td>Units</td>
<td>U/mL</td>
</tr>
<tr>
<td>Calibrators</td>
<td>5</td>
</tr>
<tr>
<td>Range</td>
<td>25 - 3 200 U/mL</td>
</tr>
<tr>
<td>Incubation time</td>
<td>60+30+30 min</td>
</tr>
<tr>
<td>Detection system</td>
<td>HRP/TMB (450 nm)</td>
</tr>
</tbody>
</table>

5. Clinical Benefits of anti-CCP2 testing

The characteristics of anti-CCP2 as serological marker in RA have been extensively investigated during the latest decade. CCP2 has redefined how RA patients are categorized; into ACPA positive RA with more destructive disease versus ACPA negative RA with milder disease. A summary is provided in a literature survey (see appendix II) that concludes the CCP2 assay is a front line diagnostic test for RA, and especially early RA, since it is:

- Highly sensitive and has excellent specificity
- Present early in the disease
- Useful as a prognostic marker
- Useful for decision of treatment strategies
- Part of ACR/EULAR classification criteria

Altogether, this makes anti-CCP2 a superior tool in differentiating RA from other forms of inflammatory arthritis, degenerative diseases that damage the joints, or other autoimmune connective tissue diseases.

6. Clinical Performance of anti-CCP2 testing

5.1 Anti-CCP2 has been investigated in more than 160 peer reviewed articles

The CCP2 assay has been extensively used and investigated by laboratories all around the world. The overall clinical performance of the test in terms of sensitivity and specificity was summarized by van Venrooij et al² in 2011 when they published accumulated data from in total 164 studies, see table 1.
Table 1. Data compiled from 164 peer reviewed publications from 2002-2010².

| Specificity and sensitivity of the CCP2 test for RA |
|-----------------------------------|----------------|----------------|----------------|----------------|
| Patient group                     | Patients/Controls (n) | Positive CCP2 test (n) | Sensitivity (%) | Specificity (%) |
| RA total                          | 18 061          | 12 953          | 71.7           | N/A            |
| Early RA                          | 4 589           | 2 827           | 61.6           | N/A            |
| Established RA                    | 13 472          | 10 126          | 75.2           | N/A            |
| Controls                          | 20 908          | 1 010           | 4.8            | 95.2           |
| Non-RA (disease controls)        | 15 971          | 960             | 6.0            | 94.0           |
| Healthy                           | 4 937           | 50              | 1.0            | 99.0           |

The authors concluded that according to the literature anti-CCP2 is still recognized as the golden standard of testing for ACPA (anti-citrullinated protein antibodies.)

5.2 Outcome of anti-CCP2 testing with CCPlus® Immunoscan

The data in table 1 includes CCP2 tests by various manufacturers. Although the antigen is the same, slightly different results have been reported for CCP2 tests provided by different manufacturers.

When summarizing some of the most extensive individual studies including the CCPlus® Immunoscan from Euro Diagnostica, the outcome is well within the ranges reported by van Venrooij et al, with very high specificity (95-98%) as outstanding characteristic, see table 2.

Table 2. Outcome of anti-CCP2 testing with CCPlus®. Data published in the Instructions for Use and an in-house study from 2012 corresponds well with the outcome of peer reviewed publications.

<table>
<thead>
<tr>
<th>Euro Diagnostica CCP2 test (CCPlus® Immunoscan)</th>
<th>Patients RA/controls (n)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bizzaro 2007</td>
<td>100/202</td>
<td>75%</td>
<td>97%</td>
</tr>
<tr>
<td>Coenen 2007</td>
<td>101/197</td>
<td>76%</td>
<td>95%</td>
</tr>
<tr>
<td>Vander Cruyssen 2008</td>
<td>92/463</td>
<td>67%</td>
<td>98%</td>
</tr>
<tr>
<td>In house test 2012 (appendix III)</td>
<td>40/40</td>
<td>70%</td>
<td>98%</td>
</tr>
<tr>
<td>CCPlus IFU (appendix IV)</td>
<td>399/781</td>
<td>77%</td>
<td>98%</td>
</tr>
</tbody>
</table>

7. The roles of ACPA and RF in RA Diagnosis

The serology domain of the ACR criteria (see chapter 3) comprises testing of Rheumatoid Factor (RF) and/or ACPA. Rheumatoid Factor (RF) has traditionally been used as a first line screening test for RA. Biologically, RFs are antibodies against the Fc part of the IgG molecule. Assays for determination of RF are available in various formats.

RF is generally considered to have a relatively good sensitivity for RA. However, RF may also be present in related diseases, i.e. positive test results can be due to other causes than RA and the specificity is often poor. Hence, comparative studies consistently show that ACPA has a higher specificity than RF for RA, especially when it comes to early RA. Also the positive predictive values are strongly in favor of ACPA (Aggarwal et al⁶).

Given the characteristics and overlap of RF and ACPAs, it is reasonable to question the relevance of routine testing of both assays. A cost effectiveness analysis was performed by Konnopka et al⁷ with the aim to evaluate if the introduction of ACPA testing in the classification of RA is cost-effective. Based on their analysis up-front testing of ACPA was judged to be cost-effective, with savings in the range of 1 000 € per quality adjusted life year.
It is clear that ACPA has some benefits as compared to RF. However, these biomarkers are based on completely different biological systems and do not measure the same thing. In conclusion, many physicians prefer to use both markers and this approach may also increase the overall sensitivity for RA.

RF was directly compared to anti-CCP2 as part of an in-house study performed by Euro Diagnostica in 2012, please refer to chapter 9.3 and appendix III.

"Data indicate that ACPA has a higher specificity than RF for early RA, good predictive validity, high sensitivity, apparent cost-effectiveness and good stability and reproducibility. Given its superior performance characteristics and increasing availability, ACPA is emerging as the most useful single assay for the diagnosis of RA."  
Aggarwal et al 2009

8. Other ACPAs: CCP3/CCP3.1 and MCV

As previously discussed some other ACPAs have been introduced on the market in recent years. These include:

- CCP3: Commercial tests are QUANTA Lite® CCP3 IgG and QUANTA Lite® CCP3.1 IgG/IgA, (Inova, US)
- MCV: Commercial tests are anti-MCV® and anti-CCP hs (high sensitive)® (Orgentec, Germany)

8.1 CCP3 and CCP3.1

The peptides used in the CCP3 test were selected by screening a limited library of cyclic citrullinated peptides. Additional epitopes are included in an effort to increase sensitivity. The CCP3.1 test offers combined IgG/IgA detection, which the manufacturer claims will increase the sensitivity even further.

However, this claim has not yet been verified in peer reviewed literature. Instead there are several publications concluding that detection of both classes is not useful for routine diagnostic purposes, since IgA-ACPA have not been found without IgG-ACPA.

“There was no difference between the CCP-3.0 method, which uses an anti-IgG conjugate, and the CCP-3.1 method, which detects IgA as well as IgG class antibodies. In view of the results of this study, combining the determination of IgA with IgG antibodies does not improve the performance of the test and therefore does not seem useful.” Bizzaro et al 2007

Studies with the aim to investigate the relation between anti-CCP2 antibodies of various isotypes have been performed by Svärd et al8 and Verpoort et al9, among others. Svärd et al reported that all sera tested positive for IgA anti-CCP also were positive in the IgG assay, but only 47% of the sera tested positive for IgG anti-CCP had anti-CCP antibodies of the IgA class.

Neither Verpoort could identify further RA-patients by adding other isotypes of anti-CCP2; among 80 CCP2 IgG-negative RA patients no IgA-CCP2 or IgM-CCP2 patients were found, see figure 2.

Figure 2. Data from study by Verpoort et al. Left) Presence (%) of anti-CCP2 isotypes in 152 anti-CCP2 IgG positive RA patients. IgA-CCP2 was found in 62% and IgM-CCP2 in 61% of the IgG positive RA patients. Right) Presence (%) of anti-CCP2 isotypes in 80 anti-CCP2 IgG negative RA patients. No IgA-CCP2 or IgM-CCP2 was found among the anti-CCP2 IgG negative RA patients.
8.2 MCV (mutated citrullinated vimentin)
Both anti-MCV® and anti-CCP hs® are based on MCV. Studies investigating the role of anti-MCV testing in the diagnosis of RA often give contradictory results. High sensitivity is reported in some studies, when using the manufacturer's instructions for cut-off. However, under these conditions anti-MCV is often also found in patients with several other autoimmune and infectious diseases, resulting in poor specificity (Bartoloni et al¹⁰).

"The present study on a large cohort of RA patients showed that, at the recommended cut off value of 20 U/ml, anti-MCV can be detected not only in 15% of healthy subjects, but also in a number of patients with chronic inflammatory and autoimmune disorders and infectious diseases, thereby reducing the specificity to 65%."

Bartoloni et al 2012

9. Anti-CCP2 as compared to other RA markers

9.1 Literature surveys and meta analyses comparing ACPAs

Naturally, there are many studies with the aim to compare the role of various commercially available ACPAs in the diagnosis of RA. Several of them confirm the superiority of anti-CCP2 testing in the diagnosis of RA as compared to CCP3/CCP3.1 or anti-MCV testing. In fact, a significant loss in specificity for RA, as compared to anti-CCP2, has been reported repeatedly for other ACPAs.

As pointed out by van Venrooij et al² it is well known that diagnostic tests should be compared under stratified conditions only*. The authors conclude that when this approach is applied none of the presently available ACPA tests has a higher sensitivity than the CCP2 test:

"It is well known that diagnostic tests should be compared only under stratified conditions (for example, the comparisons of sensitivities at a fixed clinical specificity), so that correct positive and negative predictive values, as well as positive and negative likelihood ratios, can be calculated.

In several published articles, such a comparison between different commercial ACPA tests showed that, at a stratified specificity, none of the presently available ACPA tests has a higher sensitivity than the CCP2 test."

Van Venroij et al 2011

Also Pruijn et al¹¹ and Taylor et al¹², who have performed literature surveys with the aim to compare various ACPAs draw similar conclusions:

"The results shown that for diagnostic purposes the CCP2 test has the highest specificity, the highest sensitivity in stratified studies and the highest positive predictive value" Pruijn et al 2010

"In comparative studies using the same specificity values for the three biomarkers the sensitivities of the anti-CCP2 tests were equal to or better than those of the anti-CCP3 and anti-MCV tests across all studies."

Taylor et al 2011

* To obtain a relevant specificity it is essential to choose a proper control group. In RA this is of utmost importance since RA is only one of more than 100 different types of inflammatory or degenerative diseases that damage the joints. The physicians will in most cases use the test to distinguish between RA and other similar diseases. Therefore, the true specificity of RA diagnostic tests can only be determined by means of relevant disease controls.
9.2 Individual studies comparing CCP2 tests with tests for other ACPAs

Examples of individual studies showing significant loss in clinical specificity without improvement in sensitivity when comparing CCP3/CCP3.1 tests with CCP2 tests have been published by Bizzaro et al 2007\(^3\) and Vander Cruyssen et al 2008\(^5\), see figure 2 and 3.

![Figure 2. Comparative analysis of 22 early RA, 78 established RA and 202 disease control samples. Bizzaro et al 2007.](image)

![Figure 3. Comparative analysis of 555 consecutive patients with rheumatic symptoms; 92 diagnosed with early RA and 463 with a non-RA disease.](image)

One larger study investigating the diagnostic value of testing for anti-MCV as compared to anti-CCP2 has been performed by Dejaco et al\(^1\)\(^3\). The authors conclude that also the anti-MCV assays shows limited specificity as compared to CCP2 testing:

“In the high specificity range of both tests, which is clinically the most relevant, the anti-CCP2 ELISA appears to be superior to the anti-MCV assay.” Dejaco et al 2006

9.3 Euro Diagnostica In-House study 2012

The position of anti-CCP2 as the golden standard was further emphasized by a study presented during the 11th Dresden Symposium on Autoantibodies in September 2013. The diagnostic value of CCPlus\(^\circ\) was compared to tests for anti-CCP3.1, anti-MCV, anti-CCP hs and also to RF IgM and IgA. The results were compared to data presented in peer reviewed publications and confirm the position of anti-CCP2 as the ACPA test of highest diagnostic value, see table 3 and figure 5.
Table 3. Various ACPAs and RF were analysed in 80 samples; 40 from RA patients and 40 disease controls. The manufacturer’s instructions for cut-off were used.

<table>
<thead>
<tr>
<th></th>
<th>RA (n=40)</th>
<th>Disease Controls (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCPlus</td>
<td>RF IgM</td>
</tr>
<tr>
<td>Positive (n)</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>70%</td>
<td>80%</td>
</tr>
</tbody>
</table>

**Figure 5.** Comparison of sensitivity data in the RA group at stratified specificity; 95-98%.

Results from the study (Euro Diagnostica 2012) were compared to the outcome of three peer reviewed publications.

10. Conclusions

- There is extensive support in the literature that anti-CCP2, in spite of the challenges in recent years by other ACPAs, still is the golden standard among biomarkers for RA.

- In 2012 an in-house study was performed with the aim to further investigate the clinical value of various RA markers; CCP3/CCP3.1, anti-MCV and RF included. The data corresponded very well with previously published literature and verified that anti-CCP2 is the ACPA of highest diagnostic value.

- Anti-CCP2 has high sensitivity for RA and with very high specificity the test discriminates RA patients from other forms of autoimmune inflammatory diseases.

Further data on our anti-CC2 tests and their performances is provided in appendix IV, RA Products Presentation, appendix IV.
## 11. Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ACPA</td>
<td>Anti-citrullinated peptide antibodies. Key parameter of the ACR/EULAR classification criteria for RA as part of the serology domain.</td>
</tr>
<tr>
<td>CCP</td>
<td>Cyclic citrullinated peptides</td>
</tr>
<tr>
<td>CCP1</td>
<td>The first version of CCP, based on the filaggrin molecule. Developed in 2000 but not widely marketed.</td>
</tr>
<tr>
<td>CCP2</td>
<td>The second version of CCP with improved sensitivity and high specificity for RA. Originally developed by Euro Diagnostica in 2002 in collaboration with leading scientists and recognized as the golden standard. The access to CCP2 is strictly limited by license agreements.</td>
</tr>
<tr>
<td>CCP3/CCP3.1</td>
<td>The third version of CCP introduced on the market by Inova (US) in 2005. CCP3.1 contains a dual conjugate for IgG/IgA detection while CCP3 analyses IgG only.</td>
</tr>
<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin; antibodies are immunoglobulins</td>
</tr>
<tr>
<td>Immunoscan CCPlus®</td>
<td>ELISA kit for determination of antibodies against CCP2 provided by Euro Diagnostica. The Gold Standard for anti-CCP-testing according to several publications.</td>
</tr>
<tr>
<td>Anti-MCV and Anti-CCP hs</td>
<td>MCV = Mutated citrullinated vimentin. Antibodies targeting MCV (anti-MCVs) belong to the group of ACPAs. An anti-MCV ELISA is provided by Orgentec (Germany). Anti-CCP hs is another ELISA kit by Orgentec based on antigens that present numerous epitopes chosen from mutated citrullinated vimentin (MCV).</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis, autoimmune disease that results in a chronic, systemic inflammatory disorder. It may cause chronic inflammation of the joints but can affect many other tissues and organs. The incidence is approximately 1%.</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor, part of the ACR/EULAR classification criteria for RA as part of the serology domain.</td>
</tr>
</tbody>
</table>
12. Appendices


II. Anti-CCP2: An excellent diagnostic marker for RA. E-088-GB00, May 2013

III. Grenmyr E and Sommarin Y. Anti-CCP2 is the ACPA of highest diagnostic value in Rheumatoid Arthritis. Poster no 32, 11th Dresden Symposium on Autoantibodies, September 2013.

IV. RA Products Presentation, E-080-GB00, 2013-05.

V. Instructions for Use, RA96Plus, E-23-0182-89, 2012-01

13. References


