

THE DIAGNOSTIC VALUE OF ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES, ADENOSINE DEAMINASE ACTIVITY AND OTHER POTENTIAL BIOMARKERS FOR PREDICTING AND MONITORING RHEUMATOID ARTHRITIS

DIJAGNOSTIČKI ZNAČAJ ANTI-CIKLIČNIH CITRULINIRANIH PEPTIDNIH ANTITELA, AKTIVNOSTI ADENOZIN DEAMINAZE I DRUGIH POTENCIJALNIH BIOMARKERA U OTKRIVANJU I PRAĆENJU REUMATOIDNOG ARTRITISA

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Summary: The aim of this study was to investigate the presence of anti-cyclic citrullinated peptide antibodies (anti-CCP2) in RA and non-RA patients and to evaluate the combination of these auto-antibodies with some other markers such as RF and CRP. We examined the enzymatic activity of ADA in RA patients without therapy and RA patients treated with methotrexate. Therefore, the aim of this study was also to assess the possibility of introducing these biochemical parameters in the diagnosis and monitoring of the RA patients in this region. Serum antibodies directed to cyclic citrullinated peptide were analyzed using an anti-CCP2 antibody ELISA. Serum ADA activity was measured by a spectrophotometer using adenosine as substrate (Giusti method). Rheumatoid factor (IgG-RF) was analyzed using a latex agglutination test. Serum CRP was measured by an immunoturbidimetric assay. The measurement of anti-CCP2 by itself, is useful for the diagnosis of RA for its high sensitivity and specificity, however, a combined use of anti-CCP2 with RF is much more useful. Serum ADA activity can be used as a biochemical marker of inflammation in RA. Measuring CRP in the RA patients has no diagnostic value.

Keywords: anti-cyclic citrullinated peptide antibodies, adenosine deaminase, rheumatoid arthritis

Kratak sadržaj: Cilj rada je bio utvrđivanje prisustva anti-cikličnih citruliniranih peptidnih antitela (anti-CCP2) kod pacijenata sa i bez reumatoidnog artritisa (RA) i poređenje tih auto-antitela s drugim markerima kao što su reumatoidni faktor (RF) i C-reaktivni protein (CRP). Određivana je katalitička aktivnost adenzin deaminaze (ADA) u serumima pacijenata sa reumatoidnim artritisom, bez terapije i sa terapijom metotreksatom. Cilj rada bio je i utvrđivanje mogućnosti uvođenja navedenih biohemijskih parametara u dijagnostikovanje i praćenje reumatoidnog artritisa u našu regiju. Anti-CCP2 su analizirana ELISA testom, katalitička aktivnost ADA određivana je spektrofotometrijskom metodom uz adenzin kao supstrat. RF je određivan Latex testom. CRP je meren imunoturbidimetrijskom metodom. Određivanje anti-CCP2 je korisno u dijagnostikovanju RA zbog visoke osetljivosti i specifičnosti, međutim, kombinovano određivanje anti-CCP2 sa RF je mnogo korisnije. Katalitička aktivnost ADA u serumu može da se koristi kao marker upalnog procesa u RA. Određivanje CRP u serumu pacijenata sa RA nema dijagnostički značaj.

Ključne reči: anti-ciklična citrulinirana peptidna antitela, adenzin deaminaza, reumatoidni artritis

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, whose main characteristic is persistent joint inflammation that results in joint damage and loss of function. RA is the most common inflammatory joint disease, affecting 1–2% of the world population.

Despite many years of intensive research, the exact etiology of RA is still unknown. Besides environmental influences, like infectious agents, smoking and oral contraceptives, genetic factors are believed to be responsible for approximately 60% of the risk of developing RA (1).

Early diagnosis of RA is an important challenge for clinical rheumatologists. This is because there is substantial evidence that early treatment with disease-modifying antirheumatic drugs leads to a better disease outcome (2).

The diagnosis of RA can presently be made using as a tool the 1987 revised American College of Rheumatology (ACR) criteria for the classification of RA (3). There are seven such criteria with rheumatoid factor (RF) being the only serological reviewed criterion specified (4). Four of the seven must be present to make the classification (diagnosis) of RA. However, six of the seven criteria are frequently observed in diseases other than RA, such as infections, other rheumatic diseases, among many other conditions as reviewed by Pinals (5). The highly variable and unpredictable course of the disease suggests the need for highly sensitive and specific diagnostic tests.

As an autoimmune disease, RA is characterized by the production of autoantibodies specific for disease, such as the rheumatoid factor (RF), autoantibodies against cyclic citrullinated peptides (aCCP), and non-specific ones, e.g., antinuclear autoantibodies (ANA).

RF was the first autoantibody correlated with RA. The RF is an antibody directed to the Fc domain of IgG molecules and is present in approximately 75% of RA patients. However, this antibody can also be found in other autoimmune diseases, infectious diseases, and in 3–5% of the healthy population, which increases to 10–30% in the elderly, illustrating that these antibodies are not very specific for RA (2).

Anti-CCP autoantibodies represent a novel group of autoantibodies, currently under study, which show the highest specificity and also a rather high sensitivity for RA (6). The anti-CCP antibodies are produced locally in the inflamed synovium of RA patients (7, 8), and can be detected very early in the course of RA and can therefore be helpful in early diagnosis. Two independent studies using pre-disease serum samples of RA patients who were former blood donors have shown that both RF and anti-CCP antibodies can be detected years before manifestation of the first symptoms of the disease (9, 10). The presence of anti-CCP antibodies can also be predictive for the development of the disease (11, 12). Jansen et al. (13) reported that the combined presence of IgM-RF and anti-CCP1 (sensitivity of 55.4% and a specificity of close to 97%) is able to predict which patients with early arthritis ultimately develop RA.

Currently, some laboratory measures are established for monitoring RA disease activity (14). Although C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) correlate with the degree of joint inflamma-

tion and the development of erosions, they are indicators of inflammation in general that may be influenced by other stimuli of the acute phase response.

Adenosine deaminase (ADA; EC 3.5.4.4) is a key enzyme in purine metabolism that catalyzes irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. Elevated serum ADA activities have been reported in patients with diseases in which cellular immunity is stimulated. It is also known that total ADA activity is significantly higher in the synovial fluid (SF) of rheumatoid arthritis (RA) patients than in the SF of osteoarthritis (OA) patients (15). Serum adenosine deaminase (ADA) activity is closely associated with RA and this non-invasive investigation can be used as a biochemical marker for inflammation (16). This may provide additional information regarding disease activity along with the traditional indices such as ESR and CRP.

Methotrexate (MTX), one of the most effective antirheumatic drugs, reportedly increases extracellular adenosine concentrations at sites of inflammation and represses the infiltration of inflammatory cells (17). In addition, the nonselective adenosine receptor antagonists theophylline and caffeine are capable of reversing the antiinflammatory effects of MTX in rat adjuvant arthritis (18). These findings suggest that adenosine is an effector molecule mediating the antirheumatic effects of MTX via adenosine receptor signaling, and that reduction of the local concentration of adenosine by ADA may contribute to the joint inflammation of RA (19, 20).

The aim of this study was to investigate the presence of anti-CCP2 antibodies in RA and non-RA patients and to evaluate the combination of these auto-antibodies with some other markers such as RF and CRP. We examined the enzymatic activity of ADA in RA patients without therapy and RA patients treated with methotrexate (MTX).

In the clinical centers of Republika Srpska (BiH) the RA diagnosis is done only by using the revised ACR criteria as a tool. Therefore, the aim of this study was also to assess the possibility of introducing these biochemical parameters in the diagnosis and monitoring of the RA patients in this region.

Materials and Methods

Patients and controls

For the present study 165 patients from rheumatology departments of three clinics and healthy controls were selected and divided into 4 groups. The first group consisted of 44 patients with classical RA according to the ACR criteria (5 were male, 39 female, with ages ranging between 23 and 80 years). This group received no therapy. The second group consisted of 44 patients with classical RA according to the ACR criteria (5 were male, 39 female, with ages ranging between 29 and 76 years) treated with

MTX. To analyse the sensitivity and specificity of the tests, the third group of patients was used. These patients were affected by other rheumatic diseases, mostly degenerative or other inflammatory joint diseases, including psoriatic arthritis and osteoarthritis (the non-RA group consisted of 33 patients, 2 were male, 31 female, with ages ranging between 21 and 72 years). The fourth group consisted of 44 healthy controls (HC) (10 were male, 34 female, with ages ranging between 28 and 74 years).

Methods

Serum antibodies directed to cyclic citrullinated peptide were analyzed using an anti-CCP2 antibody ELISA (Immunoscan RA, Euro-Diagnostica, Arnhem, The Netherlands) according to the manufacturer’s instructions with the cut-off at 25 units/mL (sensitivity 0.74, specificity 0.97–0.99).

Serum ADA activity was measured by a spectrophotometer using adenosine as substrate (Giusti method) (21). The principle of this method is to measure the amount of ammonia liberated during the conversion of adenosine to inosine, using the Berthelot reaction. The results are expressed as U/L.

Rheumatoid factor (IgG-RF) was analyzed using a Latex agglutination test, DIALAB, Austria. Serum CRP was measured by an immunoturbidimetric assay (Roche Diagnostic, Mannheim, Germany) and values of more than 8 mg/L were considered positive.

Statistical analysis of the results was carried out using a Student’s t-test, sensitivity and specificity values of the tests were calculated by using the 2x2 table method (22).

Results

The laboratory characteristics of patients with RA with or without therapy are shown in *Table I*. When anti-CCP2, ADA and CRP were analyzed, in both groups of patients, a significant difference ($p < 0.001$) was found only in the case of catalytic activity of ADA.

When the catalytic activities of ADA (U/L) measured in the groups of patients with untreated RA (19.71 ± 7.86) and treated RA (12.96 ± 5.06) were compared with the values of ADA measured in the healthy controls (14.20 ± 5.13), a statistically significant difference was found ($p < 0.001$) in the patients who were not receiving any therapy for RA (*Figure 1*).

The diagnostic performances of anti-CCP2 antibodies and RF show very high sensitivity and specificity, with values of 0.90 and 0.94 for anti-CCP2 and 0.93 and 1.00 for RF. At the same time, CRP showed low sensitivity (0.31) and specificity (0.82), that was lower than the other two parameters (*Table II*).

In the group of patients with untreated RA, there were 40 anti-CCP2 positive patients and only one was found to be RF negative (2.5%). In the same

Table I Laboratory characteristics of patients with RA with or without therapy.

	RA* (n=44)	RA** (n=44)	p value
Anti-CCP2 (U/mL) ^a	353.88±647.71	285.52±448.29	NS
ADA (U/L) ^a	19.71±7.86	12.96±5.06	$p < 0.001$
CRP (mg/L) ^a	8.38±10.88	9.73±12.14	NS

*Patients with RA without therapy, **patients with RA with therapy, a mean ± standard deviation, NS not significant.

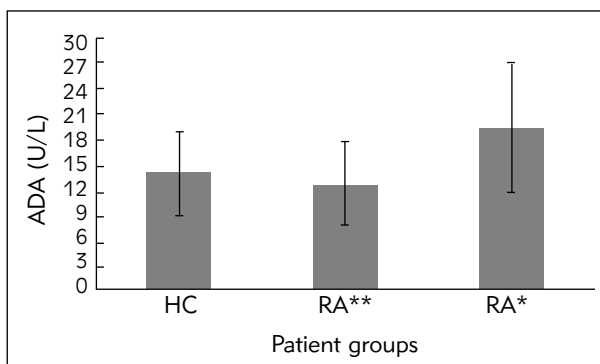


Figure 1 Serum ADA levels (U/L) in the healthy controls (HC), patients with RA without therapy (*), $p < 0.001$ when compared to HC and patients with RA with therapy (**), not significant when compared to HC.

Table II Diagnostic performance of anti-CCP2 antibodies, CRP and RF.

RA vs. non-RA	Anti-CCP2	CRP	RF
Sensitivity	0.90	0.31	0.93
Specificity	0.94	0.90	1.00
PPV	0.95	0.82	1.00
NPV	0.86	0.50	0.92

PPV – positive predictive value, NPV – negative predictive value

Table III Cross tabulation of anti-CCP2 antibodies and agglutinating RF results in RA patients without therapy.

	Anti-CCP2 (+)	Anti-CCP2 (-)	Total
RF (+)	39	1	40
RF (-)	1	3	4
Total	40	4	44

Results are shown as No.

group of patients, 4 of them resulted anti-CCP2 negative, while 3 patients were RF negative (75%). (*Table III*). All the healthy controls were negative for RF, while the RA patients who were receiving therapy were all RF positive.

Discussion

It has been noticed that in our clinical institutions the 'gold standard' for diagnosis of a rheumatic disease involves a clinical assessment, rather than laboratory tests. The rheumatology community might examine critically the use of autoantibodies and other laboratory tests, many of which cost more than a visit to a rheumatologist in usual clinical care (23). One more reason for this are different dilemmas regarding CRP and RF but also anti-CCP.

In recent years, it has been recognized that anti-CCP might improve its sensitivity over the measurement of RF to make a diagnosis of RA in a patient with early undifferentiated arthritis (24). Nonetheless, tests for anti-CCP, as well as for RF, have important limitations. More than 33%, and up to 40% of patients with RA are negative for anti-CCP and rheumatoid factor (25). Although these antibodies might be of great value in research settings for understanding further the pathogenesis and course of RA, negative results could be interpreted incorrectly by some physicians as indicating the absence of RA in individuals who in fact need treatment (25).

In addition to false-negative tests for ESR, CRP, RF and anti-CCP, false-positive tests can be seen (23). Indeed, in population surveys, about 1% of the normal population has a positive test for RF. Therefore, a positive test for RF is seen in as many people who do not have RA as in people who have this disease (22).

The first generation of anti-CCP tests, commercially available from the year 2000, had a diagnostical sensitivity for RA of around 50% and specificity of around 97%. Second generation tests (CCP2) using a mixture of synthetic cyclic peptides are now available, which have produced a further significant increase in sensitivity (around 80%), while maintaining very high specificity (98–99%) (26). But very few studies were specifically designed to compare the diagnostic accuracy of different commercial kits in the same serum. Still, one of these studies compares 11 commercially available kits, for testing 100 serum samples from patients with RA and 202 samples from healthy subjects or patients with other autoimmune, viral, or neoplastic diseases. The results of this study show a sensitivity that ranged from 60% (manufacturer Aesku) to 75% (manufacturer Euro-diagnostica). Herold et al. (25) found the sensitivity of the anti-CCP test to be up to 88%.

For the present anti-CCP2 study a kit from the manufacturer Euro-diagnostica was chosen. The results regarding the test sensitivity (90%) are higher, while those regarding specificity are in accordance with the cited authors (26, 27). Nevertheless, this study included a significantly lower number of patients and healthy controls, when compared to other published studies. Therefore, it is difficult to conclude what the cause of these results is. One of the possible explanations might be the fact that for the RA

diagnosis the ACR criteria were strictly followed. Another possible reason may be found in the test sensitivity assessment itself, where non-RA patients, carefully selected, were used.

The results of this study regarding RF in the patients with RA compared to the results for the same parameter but in the group of non-RA patients show a very high sensitivity (93%). Considering the fact that in the group of untreated RA patients among 40 anti-CCP2 positive there was only one RF negative patient (2.5%), while in the same group of patients among 4 anti-CCP2 negative there were 3 RF negative patients (75%), it is possible to conclude that the results of the present study are much better when compared to those of other published studies. Nevertheless, it is important to notice that both groups of patients, those with RA and without RA, were very small. In the non-RA group all the patients were RF negative. According to Visser (2), the diagnostic value of RF for the RA test decreases with increasing age.

For the result evaluation of this study it is important to consider that the RA group consisted of 44 patients with recently diagnosed RA (3 to 6 months prior to this study). They were all suspected to be RA positive also before giving the final RA diagnosis and no therapy was prescribed to these patients. Analysis of anti-CCP and RF seems to be very useful (28–31).

Measurement of CRP in the sera of the RA patients had a low diagnostic sensitivity (0.31%). This is in accordance with the results from other studies that suggest that CRP has higher importance in monitoring the inflammatory process connected with RA than for the diagnosis of RA itself (14).

There is a limited amount of published data that would confirm some possible changes in anti-CCP during therapy. Results of the present study show no statistically important difference in anti-CCP between RA patients receiving therapy and those who were receiving no therapy.

Serum adenosin deaminase (ADA) activity is closely associated with RA and this non-invasive investigation can be used as a biochemical marker for inflammation (16). In our study a statistically important increase in serum ADA in untreated RA patients was found, when compared to healthy controls. This is in accordance with Sari et al. (16), who consider ADA an additional marker in the RA diagnosis.

In the group of MTX treated RA patients a statistically important variation in serum ADA was found. An explanation may be found in the findings suggesting that adenosine is an effector molecule mediating the antirheumatic effects of MTX via adenosine receptor signaling, and that reduction of the local concentration of adenosine by ADA may contribute to the joint inflammation of RA (19, 20).

Conclusions

The measurement of anti-CCP2 by itself is useful for the diagnosis of RA for its high sensitivity and speci-

ficity, however, a combined use of anti-CCP2 with RF is much more useful. Serum ADA activity can be used as a biochemical marker of inflammation in RA. Measuring CRP in the RA patients has no diagnostic value.

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