Anti-carbamylated protein antibodies in rheumatoid arthritis: their relationship to joint damage and disease activity

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Background
Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation, autoantibody production, and cartilage and bone destruction. The cause of RA is still unknown. Identification of disease-related biomarkers would be helpful in diagnosing and classifying RA. The two autoantibody systems most commonly used are rheumatoid factor and anticitrullinated protein antibodies (ACPA). Several RA-associated autoantibody systems have been identified such as anti-carbamylated protein antibodies (anti-CarP Ab), anti-acetylated protein antibodies, and others. Anti-CarP Abs are the second most vigorously researched anti-modified protein antibodies following ACPA. Research on anti-CarP Ab may provide novel insights into the pathology and etiology of RA.

Aim
To detect the sensitivity and specificity of serum anti-CarP in diagnosis of RA especially in seronegative ACPA.

Patients and method
This study included 60 patients with RA fulfilling the ACR/EULAR 2010 criteria. A total of 20 apparently healthy participants were taken as controls. Written consents were taken from the patients before enrollment in this study. IRB of Assiut Faculty of Medicine approved the study. IRB local approval number is 17100085.

Result
There was a statistically significant increase in the level of anti-CarP among patients with RA in comparison with the control group, but there is an insignificant increased level of anti-CarP in the ACPA seropositive group in comparison with the seronegative ACPA group.

Conclusion
The level of anti-CarP was significantly higher among patients with RA in comparison with the control group. Five (25%) patients from the healthy control group gave anti-CarP-positive result; this can be explained by that anti-CarP Abs may exist in healthy participants many years earlier than the onset of manifestation of RA.

Keywords:
Anti-carbmalated protein antibodies Anti-citrulinated protein antibodies, Anti-modified protein antibodies, cyclic citrulinated peptide antibody Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with progressive disability, systemic complications, and early death [1].

The presence of autoantibodies is a distinctive feature of RA. The two autoantibody systems most commonly used as an aid for diagnosing and classifying RA are rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPA) [2]. RF precedes the onset of disease symptoms and predicts a more severe disease course, indicating a pathogenic role in RA. Therefore, it helps in a more accurate prognosis and a better disease management. Its importance was recently emphasized by the inclusion of ACPA alongside the previously included RF in the ACR/EULAR 2010 RA diagnostic criteria. ACPA exhibits high sensitivity with the highest predictive value for RA development and severity. These autoantibodies enabled the stratification of RA regardless of different genetic and environmental contribution factors [2].

Significant differences between ACPA-positive and ACPA-negative disease have been reported; however, less information is available on ACPA-negative RA. This lack of information is owing to the absence of robust biomarkers characterizing this manifestation of RA [3]. So, despite the diagnostic value of RF and ACPA, more serological markers are needed to improve

early diagnosis and treatment of the patients as well as to lead to a better understanding of the molecular pathways involved in RA [2].

Several autoantibodies against proteins with post-translational modifications have been detected in RA. They are called anti-modified protein antibodies. Anti-carbamylated protein antibodies (anti-CarP Ab) are the second most strongly researched anti-modified protein antibodies following ACPA. Carbamylation means conversion of lysine to homocitrulline, which is irreversibly induced by cyanate [4]. Research on anti-CarP Ab may provide novel insights into the pathology and etiology of RA [4].

Patients and method

A total of 60 patients were selected from 120 patients with RA attending the Reumatology Clinic, Assiut University hospital, who fulfilled the ACR/EULAR 2010 criteria to be divided into two groups according to presence or absence ACPA. Moreover, 20 apparently healthy participants were taken as controls. IRB, Assiut Faculty of Medicine, approved the study. IRB local approval number is 17100085. Written consents were taken from the patients before enrollment in this study. These groups were as follows:

(1) Seropositive ACPA group (30 patients).
(2) Seronegative ACPA group (30 patients).

Sample collection, storage, and handling

(1) Blood specimen:

Overall, 8 ml of venous blood was collected under complete aseptic conditions and divided into the following:

(1) Two milliliters into EDTA tube for complete blood count.
(2) About 1.6 ml was added to a tube containing 0.4 ml sodium citrate for erythrocyte sedimentation rate (ESR) measurement.
(3) Four milliliters was collected into plain tube for liver function tests, kidney function tests, anti-cyclic citrullinated peptide (anti-CCP) level, and anti-CarP Ab level:
    (a) Blood was allowed to clot for 2 h in room temperature, and serum was separated by centrifugation at 1000 rpm for 15 min.
    (b) Separated serum was inspected to ensure it is clear and nonhemolyzed or lipemic.
    (c) Serum was divided into aliquots and were stored at −20°C till the time of assay.

Laboratory investigations

Laboratory investigations were done at the central laboratory of Clinical Pathology Department, Assiut University Hospital.

(1) Complete blood count was assessed by a cell counter Cell dyn Ruby (Abbott, USA).
(2) ESR was performed by Westergen tube.
(3) Chemical investigations were done by the automated chemistry analyzer Dimention RXL max (Siemens, USA) used for measuring liver function tests and kidney function tests.
(4) C-reactive protein and RF were done by latex enhanced immunoturbidimetric test for the quantitative determination on the ADVIA 1800 chemistry systems (Siemens Healthcare Diagnostics, USA).
(5) Anti-CCP test.

The anti-CCP assay is a chemiluminescent microparticle immune assay (CMIA) for the semiquantitative determination of the IgG class of autoantibodies specific to CCP in the human serum done by the Architect i1000 (Abbott Laboratories, USA).

(6) Anti-CarP Abs:

These autoantibodies are detected by sandwich enzyme-linked immunosorbent assay for *in vitro* quantitative determination of anti-CarP Ab concentration in the serum. The kit was supplied by SinoGeneClon Biotech, Hangzhou, China (Catalog No. SG 12390).

(7) Principle of the test:

Sandwich enzyme-linked immunosorbent assay was based on the formation of antibody-antigen-enzyme-antibody complex to identify IgM and IgG class antibodies against carbamylated peptides. Absorbance is measured spectrophotometrically at a wavelength of 450 nm.

Statistical analysis

Data were verified, coded, and analyzed using IBM-SPSS 21.0 (IBM-SPSS Inc., Chicago, Illinois, USA). Descriptive statistics were represented by means, SDs, and ranges. χ^2/Fisher's exact test was calculated to compare the frequencies among groups. For continuous variables, independent *t* test analysis was carried out to compare the means of normally distributed data, whereas Mann–Whitney *U* test was calculated to test the median differences of the data that do not follow normal distribution. *P* value was significant if less than or equal to 0.05.

Results

The study was conducted on 60 patients with RA, subdivided based on ACPA into the following:

(1) Seropositive ACPA group (30 patients).
(2) Seronegative ACPA group (30 patients).

Baseline data of patients with rheumatoid arthritis and control group

The groups had insignificant differences regarding age an BMI, and most of them were females, as shown in Table 1.

Routine laboratory data of enrolled groups

Table 2 shows that there were insignificant differences between enrolled groups regarding baseline laboratory data, with exception of the following:

ESR was significantly higher among patients with RA in comparison with the control group. Both seropositive and seronegative ACPA groups had insignificant differences regarding ESR.

Hemoglobin level was significantly lower among patients with RA in comparison with the control group. Both seropositive and seronegative ACPA groups had insignificant differences regarding hemoglobin level.

Level of anti-carbamylated protein in studied groups

Table 3 shows that level of anti-CarP was significantly higher among patients with seropositive ACPA ($P < 0.001$) and patients with seronegative ACPA ($P = 0.04$) in comparison with the control group (4.97 ± 0.11 μmol/l). However, there is insignificant increase in the level of anti-CarP in the ACPA seropositive group in comparison with the seronegative ACPA group ($P = 0.48$).

With anti-CarP cutoff point more than 7, 19 (63.3%) of ACPA seropositive group patients were above the cutoff value, and nine (30%) of ACPA seronegative group patients were above the cutoff value. On the contrary, five patients of the control group were positive anti-CarP.

Comparison of anti-carbamylated protein with other established laboratory tests in studied groups

In Tables 4–6, patients with RA are divided into two groups according to level of anti-CarP and compared according to other disease parameters (ESR, C-reactive protein, RF, and anti-CCP).

There is only a significant association between anti-CarP and anti-CCP, where 67.9% of

Table 1 Baseline data of enrolled groups based on anticitrullinated protein antibodies

	Seropositive ACPA (*n*=30)		Seronegative ACPA (*n*=30)		Control group (*n*=20)		Significance		
	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	*P*1	*P*2	*P*3
Age (years)	17-74	43.80±12.33	17.67	42.36±12.26	20-65	42.33±5.67	0.66	0.50	0.40
Female sex	25 (83.3)	22 (73.3)	15 (75)	0.22	0.12	0.91			
BMI (kg/m^2)	21.36-35	28.64±3.83	20.57-34	27.91±3.38	20.12-31.4	27.11±4.56	0.70	0.23	0.10

Data expressed as *n* (%), mean (SD). Age and BMI were compared with analysis of variance test followed by post-hoc test while sex was compared with χ^2 test. ACPA, anticitrullinated protein antibodies. *P* value was significant if < 0.05. *P*1 compares between seropositive and seronegative ACPA. *P*2 compares between seropositive ACPA and control group. *P*3 compares between seronegative ACPA and control group.

Table 2 Baseline laboratory data of enrolled groups based on anticitrullinated protein antibodies

Variables	Seropositive ACPA (*n*=30)		Seronegative ACPA (*n*=30)		Control group (*n*=20)		Significance		
	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	*P*1	*P*2	*P*3
WBCs (x10^9/l)	3.70-12	6.74±2.18	4-10	6.32±1.61	4.44-9.9	7.11±3.02	0.76	0.80	0.43
Hb (g %)	5.8-15	11.44±2.06	10-15	12.23±1.35	11.1-15.4	13.11±2.11	0.15	< 0.001	0.04
Platelets (x10^9/l)	145-453	282.56±80.98	86-430	264.7±81.18	178-405.1	245.5±55.6	0.64	0.80	0.45
Urea (mmol/l)	2.7-10	4.93±1.94	2.4-15	4.74±2.56	3.23-7.12	6.56±2.45	0.93	0.15	0.50
Creatinine (μmol/l)	35-100	58.94±1.94	28-100	60.40±17.15	63-121	66.1±16.11	0.94	0.10	0.22
ALT (U/l)	6-41	21.98±9.45	5-40	18.66±8.14	9.45-38	22.11±8.01	0.30	0.91	0.09
AST (U/l)	7.8-54	22.31±9.07	6.7-40	19.90±7.56	8.11-37.8	17.34±7.78	0.52	0.40	0.11
ESR (ml)	12-112	47.63±27.46	12-130	48.10±31.24	4-11	6.11±3.33	0.99	<0.001	<0.001
CRP (mg/dl)	0-120	32.13±10.45	0-102	28.77±10.45	3-5	3.56±1.01	0.33	<0.001	<0.001
Positive	21 (70)	–	23 (76.6)	–	–	–	0.09	–	–
RF (IU/ml)	3-509	79.41±12.45	3.2-600	55.74±13.56	2-8	3.10±2.45	0.99	<0.001	<0.001
Positive	24 (80)	–	23 (76.6)	–	–	–	0.40	–	–

Data expressed as *n* (%), mean (SD). ACPA, anticitrullinated protein antibodies; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; RBS, random blood sugar; RF, rheumatoid factor; WBCs, white blood cells. All data were compared with analysis of variance test followed by post-hoc analysis. *P* value was significant if <0.05. *P*1 compares between seropositive and seronegative ACPA. *P*2 compares between seropositive ACPA and control group. *P*3 compare between seronegative ACPA and control group.

Table 3 Level of anti-Carp in enrolled groups

Variables	Seropositive ACPA	Seronegative ACPA	Control group
Anti-CarP (μmol/l)	6.86±2.87	6.29±1.20	4.97±0.11
Range	2–19.50	3.35-10.50	4.50-7
Number	19	9	5
Significance	*P*1	*P*2	*P*3
Anti-CarP	0.48	< 0.001	0.04

Data expressed as mean (SD). Data compared with analysis of variance test followed by post-hoc analysis. ACPA, anticitrullinated protein antibodies; Anti-Carp, anti-carbamylated protein. *P* value was significant if less than 0.05. *P*1 compares between seropositive and seronegative ACPA. *P*2 compares between seropositive ACPA and control group. *P*3 compare between seronegative ACPA and control group.

Table 4 Relationship between Anti-CarP and Disease Markers

Parameters	Seronegative (*n*=32) [*n* (%)]	Seropositive (*n*=28) [*n* (%)]	*P*
ESR 1st hour			
Negative	6 (18.8)	5 (17.9)	0.589
Positive	26 (81.2)	23 (82.1)	
CRP			
Negative	9 (28.1)	7 (25)	0.509
Positive	23 (71.9)	21 (75)	
RF			
Negative	8 (25)	5 (17.9)	0.363
Positive	24 (75)	23 (82.1)	
Anti-CCP			
Seronegative	21 (65.6)	9 (32.1)	0.010*
Seropositive	11 (34.4)	19 (67.9)	

Anti-CCP, anti-cyclic citrullinated peptide; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor. **χ^2 test was used to compare proportions between groups. * is a mark to show significant difference, P value was significant if < 0.05.**Chi-square test was used to compare proportions between groups

positive anti-CarP patients are positive for anti-CCP (*P* = 0.01) (Fig. 1).

Relationship between disease activity and disease markers

In the corresponding table, patients were divided according to disease activity score (DAS).

DAS reflects the general activity of the disease on a continuous scale, DAS and its modification uses only 28-joint counts (DAS28) [5].

It was noticed that activity of RA is significantly associated positively with anti-CarP Ab (*P* = 0.046).

Disease activity is also significant associated positivity with RF and anti-CCP (Figs. 2 and 3).

Diagnostic performance of anti-carbamylated protein in diagnosing rheumatoid arthritis

It was noticed that anti-CarP at a cutoff point more than 7 ng/ml had 47% sensitivity and 75% specificity

Table 5 Level of anti-Carp in RA based on disease activity

	Mild (*n*=22) [*n* (%)]	Moderate (*n*=25) [*n* (%)]	Severe (*n*=13) [*n* (%)]	*P*
RF				
Negative	10 (45.5)	3 (12)	0	0.001*
Positive	12 (54.5)	22 (88)	13 (100)	
Anti-CCP				
Seronegative	16 (72.7)	9 (36)	5 (38.5)	0.025*
Seropositive	6 (27.3)	16 (64)	8 (61.5)	
Anti-CarP				
Seronegative	14 (63.7)	11 (44)	7 (54)	0.046*
Seropositive	8 (36.3)	14 (56)	6 (46)	

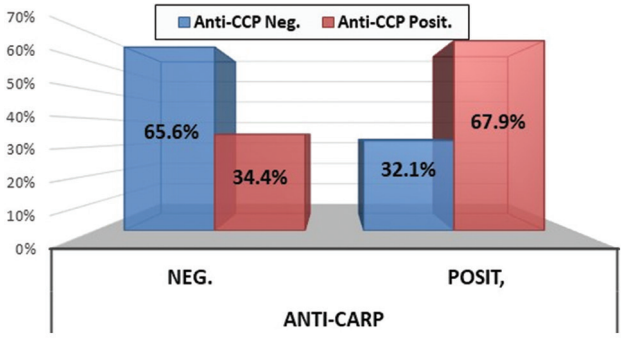
Anti-CCP, anti-cyclic citrullinated peptide; Anti-Carp, anti-carbamylated protein; RF, rheumatoid factor. *χ^2 test was used to compare the proportion difference between groups.

Table 6 Diagnostic criteria of rheumatoid arthritis markers for prediction of disease

	RF	Anti-CarP	Combined
Cut-off	>14	>7	14 and 7
Sensitivity %	80	47	82
Specificity %	100	75	100
PPV %	100	65	100
NPV %	83.5	59	85
Accuracy %	90	61	91
AUC	0.937	0.614	0.948

Anti-Carp, anti-carbamylated protein; AUC, area under the curve; NPV, negative predictive value; RF, rheumatoid factor; PPV, positive predictive value. Sensitivity (true positives/all diseased); specificity (true negatives/all nondiseased). NPV, true negatives/all test negatives; PPV, true positives/all test positives.

Figure 1



Comparison of anti-CarP with other established laboratory tests in studied groups. Anti-Carp, anti-carbamylated.

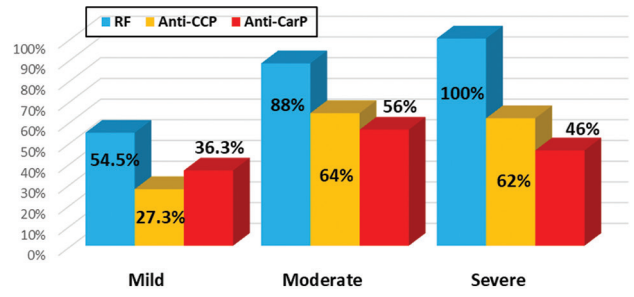
with overall diagnostic accuracy of 88.7% for diagnosing RA. The combination of RF and anti-CarP elevates sensitivity and specificity to 82 and 100%, respectively.

Discussion

RA is a chronic autoimmune disease characterized by chronic inflammation, leading to tissue damage, severe disability, and premature mortality [6].

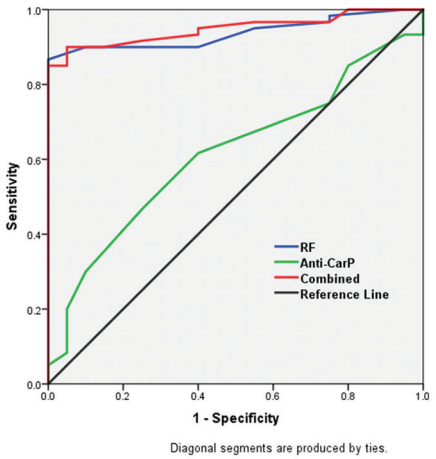
The present study included 60 patients diagnosed as having RA according to the ACR/EULAR2010

Figure 2



Relationship between disease severity and disease markers.

Figure 3



ROC curve for RA markers for prediction of disease. RA, rheumatoid arthritis; ROC, receiver operating characteristic.

criteria. A total of 47 (78.3%) patients were female and 13 (21.7%) were males, with an average age of 48 ± 12 years. This is in line with the result of Elsayed *et al.* [7], who reported that the female percent was higher than males, with 57 (81%) females and 13 (19%) males, respectively, with an average age of 47 ± 11 years. This may be owing to hormonal factors that affect women [8].

In our study, the level of anti-Carp was significantly higher among patients with RA in comparison with the control group (P = 0.001). Of 60 patients with RA, 28 (46.6%) had a high titer of anti-CarP Ab above the cutoff value (cut off >7 ng/ml), whereas five (25%) patients from the healthy control group gave anti-CarP-positive result. This was consistent with the findings of the study by Othman *et al.*[9] who reported that 41.9% of patients with RA were positive for anti-CarP and 11 (22%) in the healthy control group. It has been noted that anti-CarP Abs can exist in healthy participants many years before the onset of manifested RA [10]. Anti-CarP Ab could be detected in patients' sera long time preceding the clinical presentation of the disease [11].

In other studies, anti-CarP was found to be positive in 30 [12], 34.4 [13], 29.4 [14], and 44.9% [15]. However, the study by Mohamed *et al.*[16] reported that 77% of patients with RA were positive for anti-CarP Abs.

Anti-CarP Abs can predict development of RA independent of ACPA. In our study, nine (30%) patients were positive for anti-Carp Abs in ACPA-negative group. This result was supported by the study by El-Shorbagy *et al.* [17], who reported that anti-CarP Ab is a significant serological marker in seronegative patients with RA, which has the potential to differentiate patients with RA from the control group.

Based on disease activity, we found that majority of seropositive anti-CarP patients were of moderate disease activity (56%), and this is quite similar to the study by Othman *et al.* [9], who found that 47.4% of seropositive anti-CarP patients had moderate disease activity.

On studying the correlation between level of anti-Carp Abs and disease activity, this study revealed a significant correlation between DAS28 score and level of anti-Carp Abs (P = 0.046). This is supported by the findings of Elsayed *et al.* [7], who reported that there was a significant correlation between DAS28 score and anti-Carp Ab titer, and he explained the ongoing joint damage with the high serum titer of anti-CarP Abs, which was confirmed by Larsen score at baseline and after 1 year in the positive anti-CarP group.

In this study, anti-Carp at a cutoff of 7 ng/ml had 47% sensitivity and 75% specificity, with area under the curve of 0.61. On combing both RF and anti-CarP, sensitivity and specificity were elevated to 82 and 100%, respectively, so it is interesting that combing both anti-CarP and RF may help in early diagnosis of RA, mainly in ACPA seronegative patients.

Conclusion

The study confirmed the presence of anti-Carp Abs in patients with RA, and in spite of having low sensitivity, they were detected in patients negative for anti-CCP. A moderate positive correlation was found between anti-Carp level and disease activity (DAS28 score), which may help in predicting severity of the disease and joint damage. Although a correlation between anti-CarP level and other disease markers such as RF was not found, a combination between anti-CarP Abs and other disease markers may help in narrowing the serological gap in diagnosis of the disease. Anti-CarP Ab is a useful biomarker in the diagnosis of RA even

in ACPA seronegative patients; thus, it could have an additive diagnostic value besides RF and anti-CCP.

Recommendations

A prospective large study on healthy participants is required for accurate estimation of the predictive value of anti-CarP Abs before developing of the disease clinical criteria and follow-up for assessment of its relationship with severity of the disease and joint damage.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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