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Anti-Carbamylated Protein Antibody Test Accuracy In Some Iraqi Patients With Rheumatoid Arthritis

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ABSTRACT

Background: *Rheumatoid arthritis is a persistent, immune-mediated disorder that causes pain, edema, and inflammation in the joints. A genetic history that is high in risk, when paired with genomic markers and environmental exposures, triggers a series of actions that not only results in synovitis and arthritis, but also affects a great number of organs that are not related to the joints. The identification of novel autoantibodies playing key roles in different stages of disease remains an issue of interest for RA. Therefore, autoantibodies are necessary to assist in making a diagnosis and prediction more quickly in RA. Anti-Carbamylated Protein antibodies are a different class of anti- transformed protein antibody that is often found in the blood of RA patients.* ***Objective:*** *Rheumatoid arthritis has a recently discovered biomarker, which is an antibody called anti-carbamylated protein (CarP). Our aim is to evaluate the usefulness of anti-CarP as a diagnostic tool for RA.* ***Participants and methods****: The study conducted on 60 people diagnosed with RA as well as 60 people who served as healthy controls. Both the disease activity score (DAS28) and the health assessment questionnaire (HAQ) were evaluated. Estimates were made based on laboratory examinations such as ESR and rheumatoid factor (RF). Anti-CarP and Anti-CCP antibodies were measured by enzyme-linked immune-sorbent assay.* ***Results:****Anti-CarP levels were considerably higher in the patients with RA compared to the group that provided as a control ($p < 0.000$). Therefore, the sensitivity and specificity for anti-CarP antibodies were 39% and 98% respectively, while the sensitivity and specificity for anti-CCP antibodies were 83% and 95% respectively, and for RF were 68% and 83% respectively. The area under the curve (AUC) for anti-CarP antibodies was 0.67.*

Conclusions:

Anti-CarP antibodies had comparatively lower sensitivity and slightly higher specificity than Anti-CCP.These findings suggest that anti-CarP antibodies works as an additional role in the diagnosis of RA.

Keywords: Rheumatoid Arthritis, Anti-Carbamylated Protein, Anti-Citrullinated Peptide, Rheumatoid Factor.

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INTRODUCTION

Rheumatoid arthritis is a chronic autoimmune disease that causes systemic inflammation. A disorder affects both small and big synovial joints in a symmetrical manner. This condition typically does not directly cause death, but if left untreated, it drastically diminishes the average of life and life expectancy of patients (Yap *et al*., 2018). The clinical symptoms of joint inflammation is the result of a close relationship among neighboring cells (like fibroblast-like synoviocytes) and cells of the natural (like macrophages, dendritic cells and neutrophils) and adaptive immune response(like B &T lymphocytes) (Angelotti *et al*.,2017). Several additional organ systems, including the

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pulmonary, cardiovascular, ocular, and cutaneous systems, are also known to be implicated (Deane *et al*., 2018). RA affects about 1% of the world's population (Li et al., 2016). The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis can be utilized to categorize RA patients. Within this quantitative approach, points are provided for the following factors: joint involvement, autoantibodies, acute phase reactants, and the length of time the symptoms have been present (Shi et al., 2015). Moreover, Prompt and accurate diagnosis of rheumatoid arthritis (RA) is essential for successful treatment. When a patient develops classic symptoms of RA, a prompt diagnosis is now possible. However, determining a correct diagnosis is often difficult and fraught with uncertainty (Allam *et al*., 2019).The identification of novel biomarkers playing key roles in different stages of disease remains an issue of interest for RA. Therefore, biomarkers are necessary to assist in making a diagnosis and prediction more quickly in RA (Verheul *et al*., 2015).

Autoantibodies obtained from patient serum and synovial fluid are both extremely important components of the development of RA. According to numerous studies, between (70 and 80 percent)of RA patients show positive for autoantibodies (auto-Abs)(Trouw *et al*.,2017,Smolen *et al*.,2018).Especially in the initial stages of the illness, rheumatoid factor and Anti-Cyclic Citrullinated Peptide antibodies are utilized to confirm a diagnosis of rheumatoid arthritis (D.A.Hussein *et al*.,2018). In contrast to citrullination, which changes arginine residues, homocitrullination (also called carbamylation) happens when lysine residues are altered. Therefore, even though homocitrulline residues are structurally similar to citrulline, they are in different places in the protein, have different amino acids beside them, and are, by definition, different antigens (M. Brink *et al*., 2015). Anti-Carbamylated Protein antibodies are a different class of anti-transformed protein antibody that recognizes homo-citrullinated antigens. They are a new antibody family that is often found in the blood of RA patients. They have a similar specificity to ACPA but less sensitivity (Castellanos-Moreira *et al*., 2020). Anti-CarP antibodies in RA patient sera may have diagnostic and prognostic value, particularly in RF and ACPA negative patients, due to their association with disease activity and joint erosions (Elsayed *et al*., 2019)

MATERIALS AND METHODS

Subjects: The study was done on 60 Rheumatoid Arthritis patients who were chosen at random from Al-Sadr Teaching City in Al-Najaf province, Iraq. There were 14 men and 46 women in the study. Between November 2021 and March 2022, it was done and it was a case-control study. Patients and controls range in age from 20 to 70 years.The consulting physicians determined that the patient had rheumatoid arthritis. Patients' names, ages, genders, diabetes, hypertension, and weights have all been collected by questionnaire. Sixty people, 19 males and 41 females, who appeared to be in good health served as the control group. A majority of the patients' ages matched up with theirs, too. Exclusion criteria included people with a history of severe allergies, other autoimmune illnesses, or malignant tumors will not be allow participating. The ethical committee at the University of Kufa's School of Medicine gave their blessing to this study before it even got started.

Blood Sample Collection: Each patient and control participant used to have five to ten milliliters of blood taken from a vein using a needle and syringes made of plastic. After allowing the blood to coagulate for ten minutes at room temperature, it was spun at four

thousand rotations per minute for fifteen minutes. The serum sample from each patient was place in an Eppendorf tube and then divided into three equal pieces. The tubes were kept at -20 to -45 °C until they were used. During the ESR test, blood is put into a Westergren tube until it reaches the 200 mm mark.

Anti-CarP Assay: This kit utilizes a highly accurate reverse phase enzyme immunoassay test as its foundation. On the micro-titer plate, an antigen specific to the target has been pre-coated. Incubate the wells with either samples or positive and negative controls. The antigen on the plate binds to the antibodies present in the samples. During the washing step, unbound antibody is removed using the rinsing step. Following this step, an antibody for detection that has been conjugated to horseradish peroxidase (HRP) is added and then allowed to incubate. Unbound HRP is removed from the system during a step known as washing. Next, the TMB substrate is applied, and while that is happening, the color develops .The reaction was stopped when an acidic stop solution was placed into the mixture, which resulted in the color changing to yellow at 450 nm (the unit of measurement). It is thus possible to detect whether or not anti-carbamylated protein is present by comparing the optical density of an unidentified sample to the optical density of both the (positive and the negative) control groups.

Statistical analysis: The SPSS program version 20 was used to look at the findings. Categorical parameters were shown as frequencies and percentages, and continuous variables were shown as the mean and SD. Mann Whitney U test was used to compare groups studied. The Chi-square test and bar graphs were utilized to examine and show the correlations between Anti -CarP groups. The P value was considered significant if it was =<0.05. The probable utility of anti-CarP antibodies as RA diagnostic indicators in comparison to RF and anti-CCP antibodies were measured by performance ROC curve.

RESULTS

1-Positivity of biomarker in studied groups.

The table 1 below, shows the correlation among Anti-Carp,Anti-CCP and RF serum levels in healthy controls and RA patients .There is a total of 22 (36.7%) RA patients, who have a positive Anti-Carp, while only one (1.7%) of the controls have a positive Anti-Carp. On the other hand, there is 55 (91.7%) RA patients who have a positive Anti-CCP, compared to only 2(3.3%) of controls.43 (71.7%) RA patients had a positive RF, compared to only 7(11.7%) of controls. There is a significant alteration between the study groups (controls and patients) when were compared with biomarkers at p value=0.000.

Table 1: Comparison positivity of autoantibodies in RA patients and control.

+ve autoAbs n (%)	RA patients (n = 60)	Controls (n = 60)	P. value
Anti-CarP	22(36.7%)	1(1.7%)	0.000
Anti-CCP	55(91.7%)	2(3.3%)	0.000
RF	43(71.7%)	7(11.7%)	0.000

2-Comparison between Anti-CarP groups and study parameters.

Anti-CarP in this study are divided into two groups: those with positive Anti-CarP and those with negative Anti-CarP. Table (4.4) shows that there is a highly significant difference between the average of the ACCP in positive Anti-CarP (105.4 ±19.1) and negative Anti-CarP (55.4 ±3.6), with the corresponding (P value =0.005).

The mean ESR for positive anti-CarP was 31.6 ±3.4 and the mean ESR for negative anti-CarP was 34.6 ±3.2 at (P value =0.9); the mean DAS28-ESR for positive anti-CarP was 4.6 ±0.2 and the mean DAS28-ESR for negative anti-CarP was 4.5 ±0.2 at (P value =0.9). While the mean CDAI of positive Anti-CarP is 18.7±1.8 and the mean CDAI of negative Anti-CarP is 20.6 ±1.5 at (P value = 0.4), respectively. Positive Anti-CarP patients had a mean disease duration of 59.8±17.1 days, while negative Anti-CarP patients had a mean disease duration of 41.3±12.5 days (P value =0.2). As can be seen in this table, the study parameters for Anti-CarP groups, with the exception of ACCP, do not differ significantly from one another.

Table 2: Investigation of the correlation between anti-carbamylated protein levels, clinical and laboratory markers in patients with RA.

Parameters	Anti-CarP in RA patients, n=60		P. value
	Positive (n=22) Mean± SE	negative (n=38) Mean± SE	
ACCP	105.4±19.1	55.4±3.6	0.005
ESR	31.6±3.4	34.6±3.2	0.9
DAS	4.6±0.2	4.5±0.2	0.9
CDAI	18.7±1.8	20.6±1.5	0.4
Disease duration	59.8±17.1	41.3±12.5	0.2

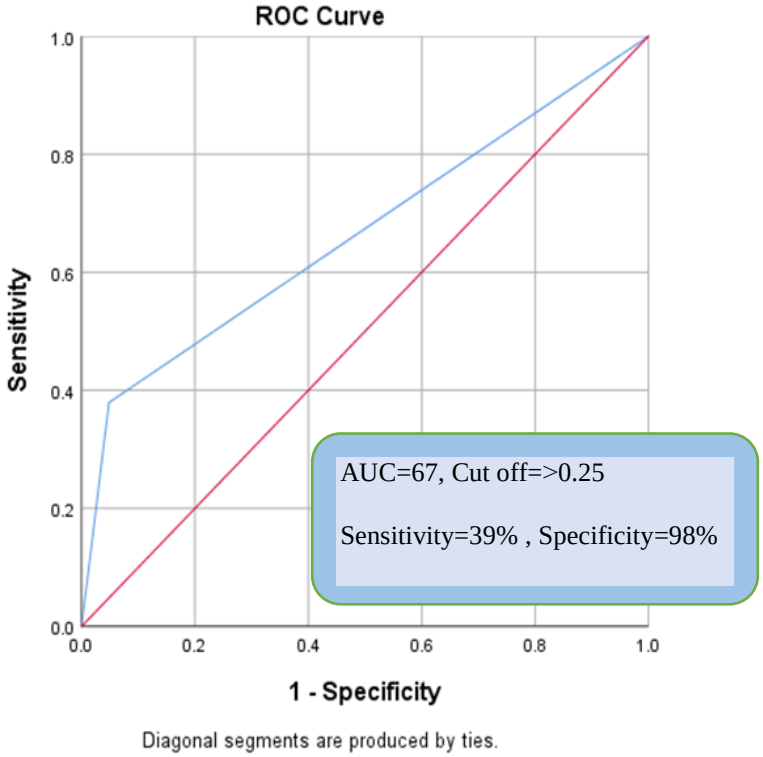
3-Comparison of the accuracies of CXCL13and anti-CarP in diagnosing RA by Receiver Operator Characteristic (ROC) Curve analysis.

ROC curve is the plotting of sensitivity versus 1-specifity and plays an important role When analyzing the diagnostic abilities of tests to distinguish between the real and false states of a disease, determining the values of the ideal cut off points, and comparing two different diagnostic tests when each test is carried out on the same disease. As an efficient method of measuring accuracy, the receiver operating characteristic curve (ROC) and the area under the curve (AUC) are utilized. In addition, anti-CCP had the biggest area under the curve (0.94%), which was significantly higher than anti-Carp (0.67%) and RF (0.76%). The overall significance level was 0.000. Anti-Carp antibodies only showed a 39% sensitivity of diagnostic potential, which was significantly lower than anti-CCP antibodies (83%), and RF antibodies (68%). Anti-Carp antibodies had the highest specificity at 98%, which was nearly level with anti-CCP antibodies (95%), while the specificity of RF antibodies was 83%. PPV and NPV of anti-Carp were 94%, 84% respectively. The highest tests for diagnostic accuracy were anti-CCP (88%) antibody compared to both anti-Carp (69%) and RF (76%).

Table 3:- Characteristic of Receiver Operator Characteristic (ROC) Curve in RA patients.

Characteristic	Anti-CarP	ACCP	RF
Sig	0.001	0.000	0.000
SE	0.052	0.025	0.046
AUC	0.67	0.94	0.76
Sensitivity (%)	39	83	68
Specificity (%)	98	95	83
PPV (%)	95	94	78
NPV (%)	64	84	74
Diagnostic Effectiveness(Accuracy) (%)	69	88	76

A:Anti-CarP ROC Curve



B:Anti-CCP ROC Curve.

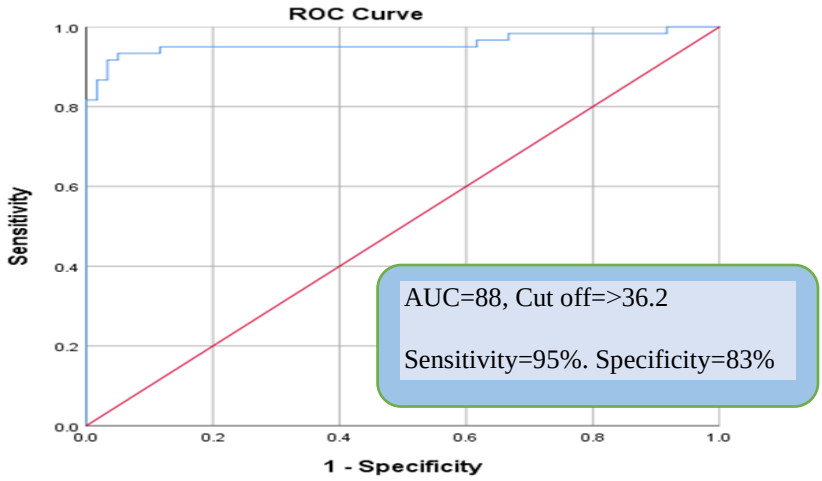


Figure 1: Summary receiver operating characteristic (ROC) curves of anti-Carp(A) and anti-CCP(B) in diagnosing rheumatoid arthritis.The curve shown is a regression line that summarizes overall diagnostic accuracy.

DISCUSSION

When diagnosing rheumatoid arthritis, ACPAs and RF are two diagnostic tools that are frequently utilized. Despite the fact that RA patients are more likely to have these autoantibodies, the specificity of these antibodies is not ideal. The existence of anti-carbamylated protein (anti-CarP) antibodies is related with joint damage in RA patients and is also correlated with the future development of

RA in people who are now experiencing arthralgia, therefore these antibodies have both diagnostic and prognostic importance. (Verheul *et al*., 2018). Anti-CarP antibodies are appear years before the onset of disease and show a progressive increase shortly before to illness onset, two data that suggest a role for anti-CarP directed immunity in the pathogenesis of RA (van & Huizinga, 2020). Anti-CarP levels were significantly elevated in the RA patients compared to the control group ($p< 0.000$). In accordance with the findings of Bell et al. (2017), which showed a high frequency of occurrence of anti-CarP antibodies in RA patients compared to healthy people and other rheumatic disorders.

The outcomes of the current study indicate that 36.7% of RA patients were anti-CarP positive. This is similar to the results of another study (Pecani et al., 2016) which revealed that 34.4% of RA patients were positive for anti-CarP Ab. In additional research, Verheul *et al*. (2016) discovered anti-carbamylated protein (anti-CarP) antibodies recognizing homocitrulline in approximately 44.9% of RA patients. According to the results of our research, control groups had a lower percentage of anti-CarP antibodies (1.7%). This is consistent with the results of another study, which found that 2.9% of control groups had a positive anti-CarP Ab test (Erre *et al*., 2019).

As was previously noted, the majority of anti-citrullinated and anti-homocitrullinated protein antibodies do not react cross-reactively with one another. The primary advantage of detecting numerous disease biomarkers either concurrently or alternatively is the ability to acquire a more comprehensive classification of RA subtypes, which results in an increase in the accuracy of RA diagnosis (Shi *et al*., 2013; Shaker *et al*., 2016). When compared to RF (68%) and anti-CCP (83%), the sensitivity of diagnostic potential displayed by anti-Carp antibodies was significantly lower (39%). Anti-Carp antibodies had a specificity that was nearly higher to that of anti-CCP antibodies, which was 95%. Anti-Carp antibodies had a specificity of 98%. In addition, anti-CCP exhibited a higher area under the curve (0.94%) compared to anti-Carp (0.67%) and RF (0.76%).Similarly, in an Italian RA cohort, anti-Carp sensitivity was 46.8% and specificity 91.95%, while the AUC was highest for the anti-CCP as in this work (Pecani *et al*., 2016). The PPV, NPV, and Accuracy of anti-Carp antibody were 95%, 64% and 69% respectively and those of ACCP antibody were 95%, 64% and 88% respectively. In another study(Shi *et al*., 2015), anti-Carp sensitivity and specificity were 44% and 89% respectively with an AUC of 0.67, which was close to the our present results.

In a recent meta-analysis, it was observed that the sensitivity and specificity of anti-CarP were 42% and 96% respectively when compared to control (Li *et al*., 2016). On the other hand, in an Iraqi study, the sensitivity and specificity of anti-CarP for the diagnosis of RA were 46% and 97.1% respectively (Shi *et al*., 2015). The findings was showed that the PPV and NPV of Anti-Carp were 95% and 64% respectively. These results were agreed with study conducted by Pecani *et al*., (2016)that observed the PPV NPV of Anti-CarP were 88% and 60% respectively.

CONCLUSION

Anti-CarP,Anti-CCP, and RF levels high significantly differ among rheumatoid arthritis patients and healthy groups. Anti-CarP antibodies demonstrated low sensitivity and higher specificity compared to ACPA and RF. On the other hand, the AUC for anti-CarP antibodies was 0.67; So Anti-CarP antibody has a moderate value in the diagnosis of RA.

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