RESEARCH ARTICLE

DIAGNOSTIC VALUE OF AUTO ANTIBODIES IN PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

Arthritis, an autoimmune disease, is a form of joint disorder which involves inflammation of one or more joints causing many complications. Early diagnosis of rheumatoid arthritis (RA) is important in order to prevent crippling and also helps the accurate interpretation of medical history and clinical examination. A study was conducted to find out the involvement of CCP Antibodies in RA suspected individuals. The level of CCP was determined in clinically suspected RA patients. In addition, the level of Rheumatoid Arthritis Factor and CRP level of the patients were also determined. A total of 110 patients with clinically suspected RA are included in the study. Anti CCP and RA measured and CCP positivity was compared in RAF positive and negative individuals. CRP level was measured in CCP positive patients. 40% of the clinically suspected RA patients were positive for RF. Thus it is concluded that among clinically suspected RA patients, measurements of CCP antibody is more specific for diagnosis. Substantial proportions have elevated CRP levels which are associated with high risk for future cardiovascular events.

Key Words: Rheumatoid arthritis, CCP antibodies, CRP level, fillagrin, ELISA.

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Introduction

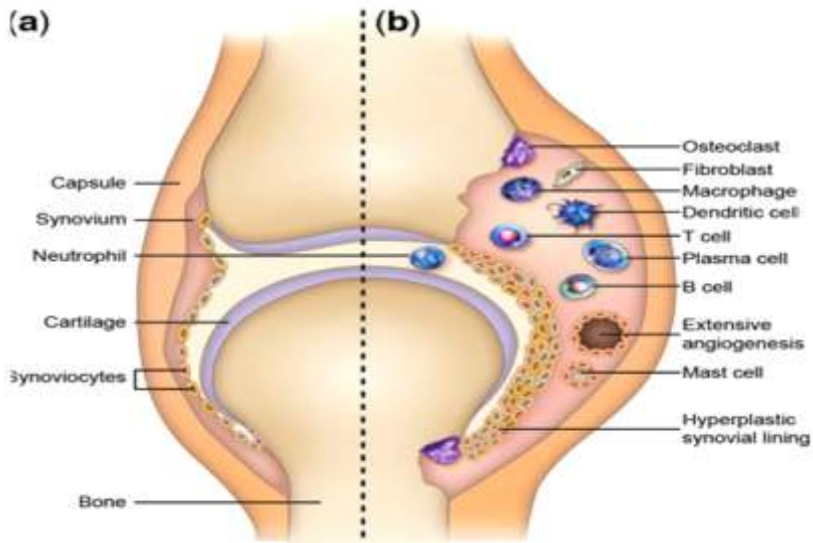
Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder primarily affecting the joints resulting in deformed and painful joints that can lead to loss of function. It is an autoimmune disorder which have signs and symptoms in organs other than joints also [1]. It affects almost 1% of the world's population and can lead to severe disability. The disease has been associated with a higher risk of mortality, higher risk of heart disease, and also a higher risk of lymphoma than the general population. Another point of interest is that smoking has been identified as a risk factor for developing rheumatoid arthritis[2].

The primary symptoms associated with rheumatoid arthritis include: joint pain, joint swelling or effusion, joint stiffness, redness and/or warmth near the joint, restricted range of motion, Rheumatoid nodules (firm lumps under the skin), found on elbows and hands of about one-fifth of rheumatoid arthritis patients, Fatigue and noticeable loss of energy, low grade fever and sometimes flu-like symptoms, loss of appetite, weight loss, anemia associated with chronic diseases, depression, dry eyes and dry mouth associated with a secondary condition Sjogren's syndrome, joint deformity and instability from damage to cartilage, tendons, ligaments, and bone, limited range of motion in affected joints etc [3].

Chronic inflammation of RA results in thickening of the normally thin synovium and also makes the joints swollen and puffy. Individuals with the disease produce a group of auto antibodies called Rheumatoid factor. These auto antibodies are reactive with the determinants in the Fc region of IgG. The classic rheumatoid factor is an IgM antibody with that reactivity. They bind with normal circulating IgG, forming IgM-IgG complex and gets deposited in the joints. These immune complexes can activate the complement cascade, resulting in a type III hypersensitivity reaction, which leads to chronic inflammation of the joints.

Auto antibodies such as rheumatoid factor (RF) and anti-cyclic Citrullinated peptide (ACCP) antibodies have important diagnostic value for the disease. Both these antibodies belong to a family of autoantibodies directed against citrullinated fillagrin, an epithelial cell protein. Citrullination is a posttranslational modification of the aminoacid, arginine to citrulline by the action of the enzyme peptidyl arginine deaminase (PAD). This process occurs naturally during inflammation, apoptosis and keratinization. When fillagrin is found absent in synovium, several citrullinated proteins present in RA synovium like fibrinogen and fibronectin, other citrullinated epitopes etc have been identified as targets of highly RA-specific autoantibodies.

RF has been widely used as a screening test for patients with arthritis. RF constitutes one of the classification criteria proposed by the American College of Rheumatology (ACR). Conventionally, the serology test routinely used in RA for the determination of serum Rheumatoid Factor (RF), which possess acceptable sensitivity, but modest specificity, particularly in the early course of the disease[4]. In addition, RF is present in patients with other autoimmune and infectious diseases, and even in a noticeable propor-



Schematic view of a normal joint (a) and a joint affected by RA (b) (Smolen and Steiner, 2003).

tion of normal healthy subjects, particularly in old individuals[5].

More recently determined auto antibodies for the diagnosis of RA are anti-cyclic citrullinated peptide antibodies (anti-CCP antibodies). A new serologic test, (Anti–cyclic citrullinated peptide [anti-CCP] enzyme-linked immunosorbent assay [ELISA]) was developed to determine the presence of antibodies directed towards citrullinated peptides, using a synthetic peptide designed for this purpose. The synthetic peptide used in this assay represents a relatively small set of antigenic determinants that do not entirely encompass the antigenic determinants present on the as yet unknown antigenic molecule in the joint[6]. In patients with early arthritis, the correlation with anti-CCP was significant, thus indicating that this assay may be used even in the early phases of the disease. Anti-CCP test is particularly useful in the diagnosis of RA and it is able to predict the severity of the disease and the irreversible damage[7].

Earlier studies have shown that the anti- CCP antibodies are moderately sensitive but highly specific for the diagnosis of RA, and their specificity is higher than RF[8]. It is claimed that, the presence of anti-CCP antibody in a patient could be the sign of RA with a rate of 90-95%[9]. About 35-40% of the RF-negative patients are anti-CCP antibody positive. Although negative RF results are consistent with conditions other than RA, they do not rule out RA[10]. The goal of this prospective study is to analyze the value and prognostic significance of anti-CCP titer quantification in RA subjects.

Materials and Methods

Almost 110 clinically suspected RA patients participated in the study with informed consent. 25 healthy subjects having no known health disorders formed the control group. Blood was drawn from the participants in a clot activator tube (Red tube), waited for 10 minutes and serum was separated by centrifugation at a speed of 1000 rpm for 15 minutes. Serum was used as the specimen for investigation.

Parameters estimated in patients with elevated CCP levels and also in control subjects:

1. Anti-CCP:

Enzyme-linked immunosorbent assay (ELISA) was used for qualitative determination of IgG antibodies to Cyclic Citrullinated Peptides (CCP) in human sera[11].

Principle: Anti-CCP antibody kit is based on an ELISA method. The test utilizes microtiter plate wells coated with citrullinated synthetic peptides (antigen). Diluted serum of the patient was applied to the wells and were kept for incubation. If specific antibodies are present, they will bind to the antigen in the wells. Unbound materials are washed away and any bound antibody is detected by adding horse radish peroxidase (HRP) labeled anti-human IgG, followed by a second washing step and an incubation with substrate. The presence of reaching antibodies will result in the development of color, which is proportional to the quantity of bound antibody which is determined photometrically.

Specimen collection: Collect venous blood specimens using acceptable medical techniques. Allow the blood to clot and separate the serum by centrifugation. Test serum should be clear and non-hemolyzed. Specimen may be refrigerated at 4-80^{0}C for maximum 48 hrs and for prolonged storage, freeze at -200 C. Avoid repetitive freezing and thawing of serum samples which may result in variable loss of autoantibody activity. Testing of heat-inactivated sera is not recommended.

Procedure: Prepare a sufficient number of microplate modules to accommodate control and prediluted patient samples [Mix 10µL sample in a tube with 490µL dilution buffer]. Pipette 100µL of calibrators, controls and prediluted patient samples in duplicate in to the wells. Incubate for 60min at room temperature (18-25^{0}c). Discard the contents of the microwells and wash 3 times with 300µL of wash solution. Dispense 100µL of the enzyme conjugate to each well. Incubate for 30min at room temperature. Discard the content of the microwells and wash 3 times with 300µL of wash solution. Dispense 100µL of TMB substrate solution to each well. Incubate for 30min at room temperature. Add 100µl of stop solution to each well of the module and incubate for 10min at room temperature. Read the optical density at 450nm and calculate the result. Biochromatic measurement with a reference at 600-690nm is recommended. The color developed will be stable for at least 30min. Read the optical density during this time. Calculate the absorbance (optical density) ratio for the control and for each sample using the equation.

$$\text{Absorbance Ratio} = \frac{\text{Control or Sample OD}}{\text{Reference control OD}}$$

In a normal range study with serum samples from healthy blood donors the following ranges have been established with anti-CCP test:

Samples with result <25 U/ML are defined as negative.

Samples ≥25 U/ML are defined as positive.

2. C-reactive protein

Principle: CRP is a classic acute phase protein of human serum, synthesized by hepatocytes. Normally, it will be present only in trace amounts in serum, but it can increase as much as 1,000 fold, in response to injuries or infections. Clinical measurement of CRP in serum, therefore, appears to be a valuable screening test for organic diseases and is a sensitive index of disease activity in inflammatory, infections and ischemic conditions [12].

Specimen collection: Collect venous blood specimens using acceptable medical techniques. Allow the blood to clot and separate the serum by centrifugation. Test serum should be clear and non-hemolyzed. Specimen may be refrigerated at 4-80 C for a maximum 48 hrs and for prolonged storage, freeze at -200 C. Avoid repetitive freezing and thawing of serum samples which may result in variable loss of autoantibody activity. Testing of heat- inactivated sera is not recommended.

Procedure: Place 50µL diluted saline buffer to each of five circles of the slide. Using a 50µL micro pipette, add 50µL serum sample to the drop of saline buffer in 1stcircle. Using the same micro pipette, mix the sample with saline by aspirating back and forth several times. Aspirate 50µL from 1st circle and transfer to 2nd circle. Repeat the same operation up to 5th circle and discard. Dilutions obtained are ½, ¼, 1/8, 1/16 & 1/32 etc. Then add 1 drop of CRP latex reagent to the above circles. Mix and rock the slide gently to and fro for 2 minutes, observe the agglutination under good source of light. Concentration of CRP in serum can be calculated as:

CRP Conc. (mg/L) = sensitivity × titre (highest dilution serum showing agglutination), Where, CRP sensitivity = 6 mg/L.

RHEUMATIOD FACTOR (RF)

Principle: Rheumatoid factors are a group of antibodies directed to the determinants in the Fc portion of the immunoglobulin G molecule (IGg). Although rheumatoid factors are found in a number of rheumatoid disorders, such as systemic lupus erythematosus (SLE) and sjogrens syndrome as well as in non rheumatic conditions, its central role in clinics lies in its utility as an aid in the diagnosis of rheumatoid factors (RA) [12].

Specimen collection: Collect venous blood specimens using acceptable techniques. Allow the blood to clot and separate the serum by centrifugation. Test serum should be clear and non-hemolyzed. Specimen may be refrigerated at 4-80^{0}C for maximum 48hrs and for prolonged storage, freeze at -20^{0}C. Avoid repetitive freezing and thawing of serum samples. Testing of heat- inactivated sera is not recommended.

Procedure: Place 50µL diluted saline buffer on to each of five circles of the slide. Using a 50µL micro pipette, add 50µL of the serum sample to the drop of saline buffer in 1stcircle. Using the same micro pipette, mix the sample with saline by aspirating back & forth several times. Aspirate 50µL from 1st circle and transfer to 2nd circle. Repeat the same operation up to 5th circle. Aspirate 50µL from 5th circle and discard. Dilution obtained as ½, ¼, 1/8, 1/16,1/32. Then add 1 drop of RF latex reagent to the above circle, mix and rock the slide gently to and fro for 2 minutes; observe the agglutination under good source of light. The Rheumatoid factor (RF) level in serum can be calculated as :

RF Conc. (IU/mL) = sensitivity titre (highest dilution of serum showing agglutination), Where, RF Sensitivity = 8.0IU/mL.

Results

Specificity of the data's are given below: Out of the 110 cases,

- Patients positive for RF is 40%.
- Patients positive for both RF and CCP is 88%.
- Patients negative for CCP but positive for RF is 12%.
- 6% of the clinically suspected RA patients are positive for CCP antibodies and negative for RF.
- 25% of the CCP positive individuals have elevated CRP level.

These data's determine the various factors that are intended for the cause of Rheumatoid arthritis.

Discussions

Present study focussed on the various factors that are involved in the causes of disease condition, Rheumatoid arthritis. The study was conducted on patient's serum sample with informed consent. For the study, about 110 clinically suspected RA patients were involved. 25 healthy individuals having no known health disorders were taken as control group. Blood samples were drawn from each individual for study; serum was separated by centrifugation and used as the specimen for investigation. The RF titre of the participants were measured by turbidometry, the level of CCP antibodies were determined by ELISA technology and CRP level were measured by turbidometry.

The study was done mainly on RA and Anti-CCP. And also, the CCP positivity was compared in case of RAF positive and negative individuals. CRP level was measured in patients having CCP positive. The findings in our data shows that the RA positive patients were positive for both RF and CCP and in some other cases negative for CCP but positive for RF. From these results, we can observe that CCP antibody is more specific for RA diagnosis. One of the substantial proportions of the elevated CRP level in RA is associated with high risk of cardiovascular events in future.

Few studies have empirically assessed the prevalence of CVD's in RA. Several traditional risk factors such as obesity, dyslipidemia, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), hypertension, physical inactivity, advanced age, male gender, family history of CVD, hyperhomocysteinemia, and tobacco have been associated with CVD in RA patients [13,14]. In fact, seropositive RA may, like diabetes, act as an independent risk factor for CVD [15]. A proinflammatory state, insulin resistance[16], hyperhomocysteinemia [17] and oxidative stress [18] are common characteristics of both RA and atherogenesis. The relative frequency shows that there is doubly the risk of developing CVD in patients possessing RA than non- RA population [18,19]. In fact, IHD secondary to artherosclerosis is the most prevalent cause of death associated with CVD in patients with RA[20]. Some other studies show that Methotrexate use is associated with a reduced risk of CVD in patients with RA. This suggests that, reducing the inflammation in RA using MTX not only improves disease-specific outcomes but may also reduce collateral damage such as arterosclerosis. The Anticyclic citrullinated protein antibodies are an implication for development of RA in CVD. The Anti-CCPs are key players in the inflammatory and proatherogenic status of RA patients. The effects are specific of the immune cell targeted, promoting over expression of thrombotic, inflammatory, and pro-oxidative markers in monocytes, pro-oxidative status in neutrophils and proinflammatory profile in lymphocytes. Targeting these autoantibodies would be an excellent strategy to prevent the development of cardiovascular disease in RA[20,21].

From our study on rheumatoid arthritis we can observe that Anti-CCP and RF are associated with the cause of this systemic autoimmune disease with chronic joint inflammation. Any elevated level of these factors in serum would also be associated with the risk of formation of CVD and other disease conditions.

Conclusion

The study conducted was mainly focussed on the biochemical parameters elevated in Rheumatoid Arthritis. The biochemical parameters checked are Anti-CCP antibodies, RF and CRP. The Anti-CCP antibodies were measured by ELISA technology and RF and CRP were measured by nephlometry and turbidometrically. Out of 110 cases, the patients positive for RF is 40%, positive for both CCP and RF is 88%, positive for RF and negative for CCP is about12% and CCP positive with elevated CRP is 25%.

From these results, it can be concluded that the Anti-CCP is a superior marker than RF for the disease condition, Rheumatoid arthritis. Elevated CRP level in arthritis increases the incidence of cardio vascular diseases in patients with rheumatoid arthritis.

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TABLE- 1:

Subject	Number	Percentage (%)
No: of patients with clinically suspected RA	110	-
No: of patients positive for RF	44	40
No: of patients positive for RF and CCP	39	88
No: of patients negative for CCP but positive for	5	12

FIGURE -1:

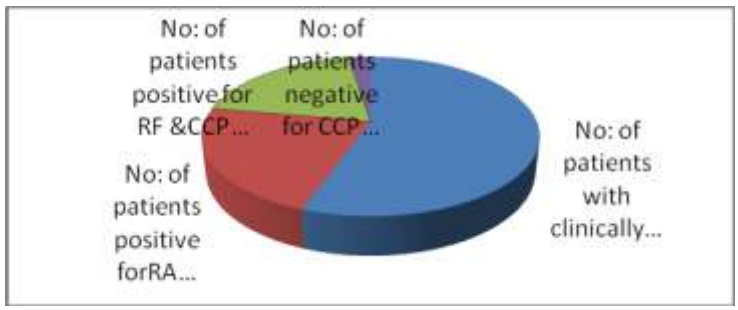

TABLE –II:

Subject	Number	Percentage (%)
No: of patients	110	-
No: of patients with RF negative and CCP positive	7	6

TABLE III:-

Subject	Number	Percentage (%)
No: of patients	110	-
No: of patients with CCP positive	46	51
No: of patients with CCP positive with elevated CRF	12	25

FIGURE-III:

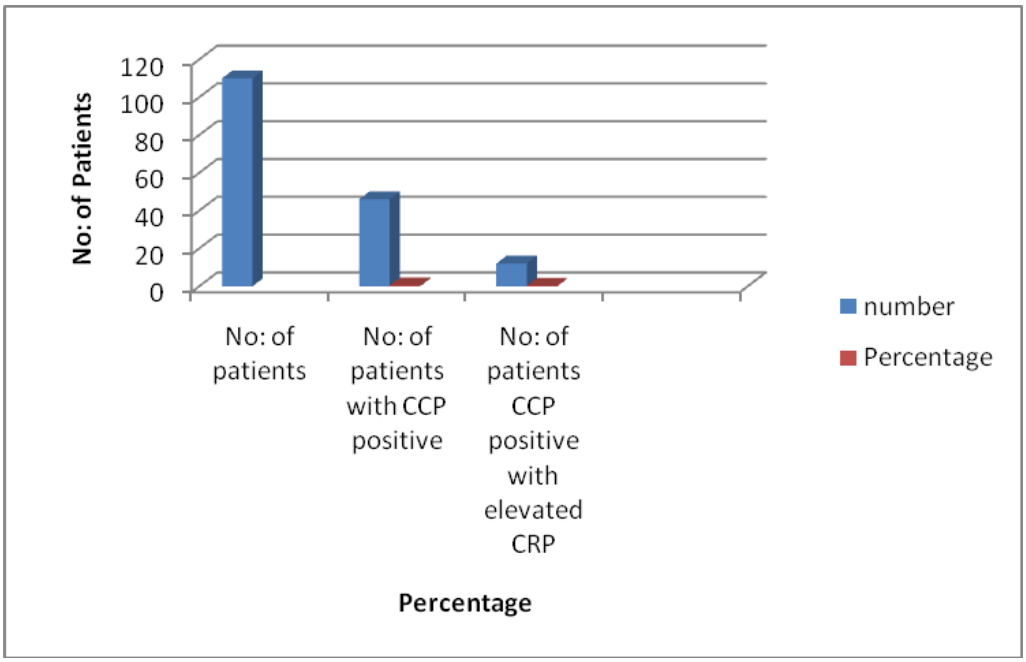

TABLE- 4: RF titre of clinically suspected patients with that of healthy controls.

Subject	Number	Mean	SD	t	P
Control	25	2.5	0.65	17.46	<0.01
RA	110	35.8	9.5		

FIGURE-III.A:

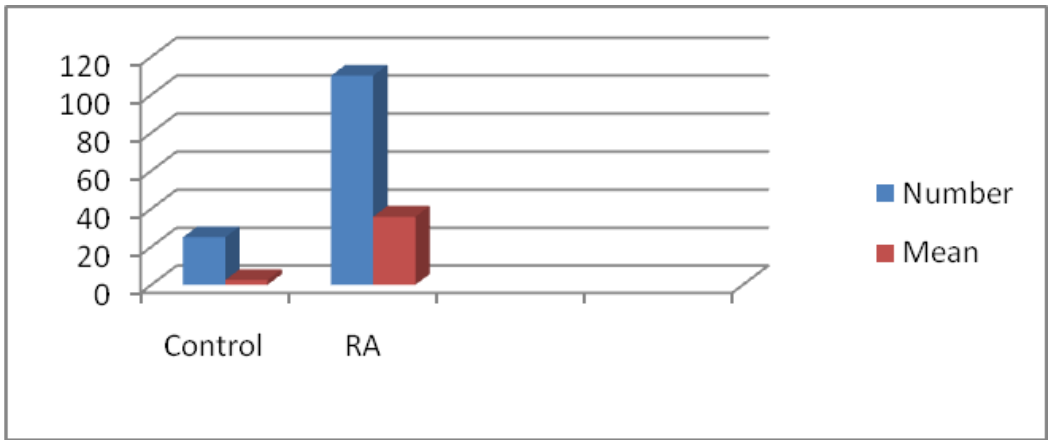

TABLE -5: Level of CCP antibodies of clinically suspected patients with that of healthy controls.

Subject	Number	Mean	SD	t	P
Control	25	2.9	0.9	15.62	>0.01
RA	110	20.5	5.6		

FIGURE-IV.A:

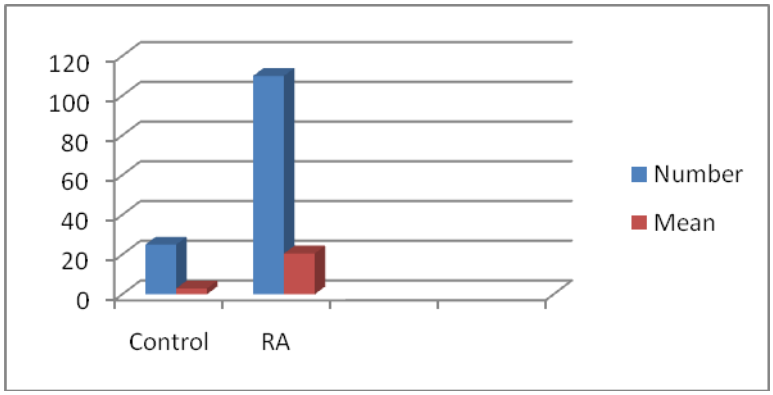

TABLE- 6: CRP titre of clinically suspected patients with that of healthy controls.

Subject	Number	Mean	SD	t	P
Control	25	1.5	0.25	10.68	>0.01
CCPpositive	46	5.7	1.95		

FIGURE-V.A: