



Study of serum traditional and nontraditional biomarkers in Rheumatoid arthritis patients

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ABSTRACT

The study of biomarkers in Rheumatoid arthritis (RA) is highly indispensable to understand mechanisms of pathogenesis, diagnosis, prognosis, and treatment of disease. The role of traditional biomarkers like Anti-CCP, RF, and inflammatory markers like ESR and CRP is well established. In this study, we aimed to measure nontraditional biomarkers like Hyaluronic acid (HA), Cartilage oligomeric matrix protein (COMP), and Osteocalcin in the serum of RA patients and also to establish an association with traditional markers. It was a cross-sectional study involving 152 RA patients based on the 1987 ACR criteria for the diagnosis of RA and 68 age- and sex-matched healthy controls. After the clinical examination, traditional markers were assessed to measure the disease activity along with non traditional markers in RA patients. All the values were expressed as median (25th–75th percentile). In our study, there was a significant increase in serum HA levels in RA patients compared to healthy controls ($p < 0.03$), whereas no significant difference in serum COMP and osteocalcin levels. The traditional inflammatory markers were significantly increased in RA patients than controls with ($p < 0.001$). The serum HA levels were significantly correlated with traditional markers in RA patients. Conclusion: Significant increase in serum HA level in RA patients indicating synovial inflammation, but there were no notable changes in COMP and osteocalcin level in serum presuming the combination of these markers may be useful along with traditional markers in the different stages of RA.



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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disease of unknown etiopathogenesis. The course of the disease is variable among patients with RA. A substantial population of RA patients exhibits symptoms like poly-articular inflammation associated with synovitis, osteitis, periarticular osteopenia, and erosion of subchondral bone eventually leads to narrowing of joint space. These features commonly lead to progressive joint damage, impaired function, and permanent disability in these patients (Diarra *et al.*, 2007). Arthritis is established as a devastating disorder resulting in excruciating pain, impairment, and dysfunction among patients having an injury to the synovium,

cartilage, and bone of the affected joints. Based on a clinical presentation at the late stages of arthritis, many methods to diagnose and monitor the progression of the disease. So pertinent interventions in the early stages with effective treatment modalities can change the course and progression of the disease, minimize functional disability that can improve the standard of living. Therefore, efficient biomarkers are needed for the early diagnosis and to monitor the prognosis of rheumatoid arthritis (Schett *et al.*, 2005).

However, the majority of the chronic diseases have a standard test for diagnosis. On the contrary, quality, trustworthy laboratory test for RA diagnosis still elusive. The American College of Rheumatology (ACR) in 1987 and New Rheumatology criteria in 2010 used to define and classify / diagnosis RA internationally (Aletaha *et al.*, 2010). By estimating the biomarkers of cartilage damage and repair, it is possible to better understand the variations in joint remodelling and monitor RA disease progress. COMP is one such biomarker, which is also known as thrombospondin 5, belongs to the family of pentameric calcium-binding proteins. Even though the exact function is not known, it plays an important structural role by interacting with collagen fibrils and matrix components in endochondral ossification and stabilization of the extracellular matrix (Posey *et al.*, 2018). Studies proved that COMP is detected in serum as well as synovial fluid of RA and osteoarthritis (OA) patients (Morozzi *et al.*, 2007).

Hyaluronic acid (HA) is a glycosaminoglycan made up of repeated units of N-acetyl glucosamine and glucuronic acid. It is an important constituent of synovium and cartilage, rendering the lubricating mechanism of synovial fluid (Engstrom-Laurent and Hallgren, 1985). Many studies in the past proved that high volume of HA are produced locally from inflamed synovium of patients with different rheumatic diseases, including rheumatoid arthritis (Al-Dalaen *et al.*, 2016). One increasingly apparent thing is the bone loss at an accelerated rate in RA patients. Though the exact mechanisms are poorly understood, osteoporosis is part of clinical symptoms in the diagnosis of RA (Gevers *et al.*, 1986). Bone formation and resorption is a tightly coupled continuous process happening all through life for remodelling of bone. Osteocalcin, also called bone Gla protein, is present in non-collagenous bone matrix. It is produced by osteoblasts reflecting the bone formation status. It is an established biochemical marker that is specific for bone metabolism most likely speculate bone formation (Nagaya *et al.*, 1999).

Aim and Objectives

Thus, the aim of the study is to

1. To measure the serum concentrations of nontraditional biomarkers like HA, COMP, and Osteocalcin as cartilage and bone markers along with traditional in RA patients.
2. To compare the serum traditional and non-traditional biomarkers in RA and healthy individuals and assess the relationship between these markers.

MATERIALS AND METHODS

It is a case-control study, including a total of 152 RA patients (75 early RA with the duration of disease less than two years and 77 late RA with more than two years from the onset of initial symptoms), with ages between 23 and 70 years attending the Rheumatology Clinic at Sri Ramachandra Medical College and Research Institute, Chennai from October to December 2010. The primary inclusion criterion was definite RA fulfilling the 1987 American College of Rheumatology criteria and disease duration of 6 months onward. The control group comprised of 68 age and sex-matched healthy volunteers with no other forms of inflammatory rheumatic disease. The exclusion criteria for both the groups included physiological statuses such as pregnancy, lactation, and individuals with habitual smoking and alcohol consumption excluded from our study. We also excluded individuals with known infectious diseases and other diseases such as diabetes mellitus, hypertension, thyroid dysfunction, neurological disorders, cancer, secondary osteoarthritis, any other forms of autoimmune diseases, and types of arthritis from the study.

All these RA patients met the new 2010 RA classification criteria retrospectively. Under the guidance of a rheumatologist, we recorded the values of physical activity, Visual Analog Score (VAS), disease duration, number of swollen and tender joints obtained by the personal interview of all the patients along with relevant clinical data, and the treatment history was collected using the Health Assessment Questionnaire (HAQ). We made sure that by selecting patients, who were under uniform dosage of nonsteroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs as per treatment protocol without influencing our objective of the study. For the biochemical markers, 5 mL of fasting blood sample with and without anticoagulant were collected. The serum sample was collected and kept at a temperature of -70°C for further biochemical analysis. The patients and controls were

similar in ethnicity and nutritional habits. Other than Anti-CCP and RF autoantibodies, the conventional markers in RA consisted of the various Disease Activity Scores (DAS) which include composite parameters such as counts of tender and swollen joints and levels of serum CRP or ESR were included in our study. Traditional markers of inflammation, like ESR and CRP, were analyzed using standard laboratory methods. The biochemical markers analyzed were anti-CCP, RF, and HA, COMP, and Osteocalcin in both patients and controls by in vitro quantitative ELISA method. There is no involvement of any kind of in vivo experiments or clinical trials on humans or animals in our study. Our study protocol (MEC/06/51/23) was approved by the Research ethics committee of Sri Ramachandra medical college and research institute, Chennai. All patients gave written informed consent before their enrolment in this study.

Statistical analysis

Statistical analysis in this study was performed using the SPSS® statistical program version 16.0. Because most of the variables did not follow a normal distribution, all the statistical analyses had done using nonparametric tests. In the study group, non normally distributed quantitative variables were presented as median (interquartile range). Comparison of biochemical marker concentration between groups was calculated by nonparametric significant Kruskal–Wallis test, followed by Bonferroni-adjusted Mann–Whitney test for unpaired differences. Spearman’s Rho test and $P \leq 0.05$ were considered statistically significant when we calculated the correlation between different parameters.

RESULTS AND DISCUSSION

The demographic data, including disease activity measures and serum biomarker levels, were compared between the rheumatoid arthritis patient group and the control, as shown in Table 1, and comparison of serum levels of biochemical markers between RA cases and controls (Table 2). Correlation analysis was performed between serum non-traditional markers like HA, COMP, and Osteocalcin with traditional markers like RF and Anti-CCP, as shown in Table 3. Figures 1 and 2 shows a significant increase of ($p < 0.001$) Anti-CCP and RF between RA patients and normal control group respectively. A significant increase in HA between RA and normal is explained in Figure 3. We observed a significant positive correlation ($p < 0.01$) between serum HA and anti-CCP levels in RA patients (Figure 4), and another Figure 5 shows a significant negative correlation ($p < 0.05$) between COMP and anti-CCP level in

the serum of RA patients.

The synovial proliferation of cartilage and bone is a characteristic feature of chronic inflammatory rheumatoid arthritis (Harris, 1990). When RA is clinically suspected, immunological studies such as testing for the presence of traditional markers such as RF and Anti-CCP along with disease activity measures like ESR, CRP, and joint count should be performed. Based on these tests and clinical examinations, the physician would rule out the possibility of other autoimmune diseases (Narayan et al., 2019).

We have observed all these traditional markers of disease activity levels increased in RA patients compared with controls (Table 1). All the values are calculated as the median (25th–75th percentile). The age of the study participants for RA patients and healthy controls was 47 (39–53) and 50.5 (39–59.25) years, respectively, and patients had a disease duration of 2.6 (1–6.25) years. The inflammatory markers like ESR and CRP levels were significantly elevated ($p < 0.001$) in RA patients than healthy controls.

The cartilage and bone tissue turnover biomarkers, so-called non-traditional, reflected in the systemic circulation due to the changes in the extracellular matrix of these tissues in RA patients. On evaluation, we also noticed that synovial inflammation traditional markers like AntiCCP and RF levels are significantly increased in RA patients than normal control group whereas no such significant changes noticed between COMP and Osteocalcin levels (Table 2).

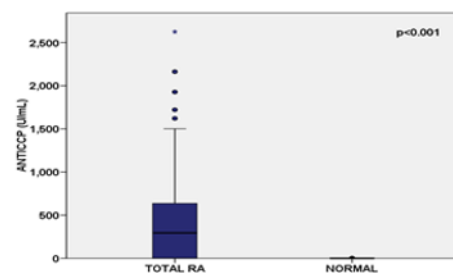


Figure 1: Serum levels of anti-CCP in RA patients and normal

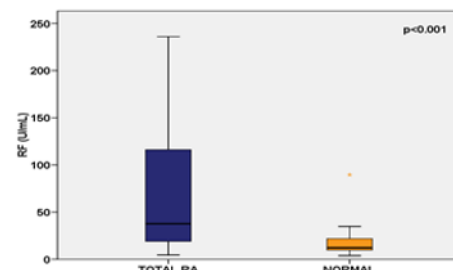


Figure 2: Serum levels of RF in RA patients and normal

Table 1: Demographic data of all subjects

Parameters	RA (N=152)	Normal (N=68)
Sex (male; %)	39 (27%)	18(26%)
(female; %)	113 (73%)	50(74%)
Age (yrs)	47 (39-53)	50.5 (39-59.25)
Duration of disease (yrs)	2.6 (1-6.25)	-
Number of swollen joints	4 (3-6)	-
Number of tender joints	3(1-5)	-
Physical activity (1-7 points)	4.5(3.2-5.8)	-
Visual Analog score (VAS) (0-100mm)	55(37-75)	-
Disease Activity Score 28 (DAS 28)	4.25 (3.86 -4.79)	-
Erythrocyte sedimentation rate ESR (mm/hr)	40.5 (32.75 -51.25)***	10 (6.75-15.25)
C- reactive protein CRP (mg/dL)	26.5 (8-55.5)***	2 (1-3)

***p<0.001. All the values expressed as Median (25th percentile – 75th percentile).

Table 2: Serum levels of various biochemical markers in RA and Normal

Parameters	RA (N=152)	Normal (N=68)	p-Value
AntiCCP (U/mL)	295.68 (2.38-640.54)	0.31 (0.0-0.71)	<0.001
RF (U/mL)	30.82 (16.32-65.07)	11.62 (7.75-17.78)	<0.001
Hyaluronic acid (ng/mL)	74.59 (29-176.4)	54.58 (35.63-81.68)	<0.03
COMP (ng/mL)	659.4 (496.9-842.83)	694.09 (543.75-941.68)	0.17
Osteocalcin (ng/mL)	6.92 (6.24-7.69)	6.56 (5.53-7.46)	0.11

All the data expressed as Median (25th percentile – 75th percentile).

Table 3: Correlations between the biomarkers in RA patients

Correlation between	The correlation coefficient (r)
HA and ESR	0.24*
HA and CRP	0.31**
HA and Anti - CCP	0.27**
COMP and ESR	0.02
COMP and CRP	0.04
COMP and Anti – CCP	-0.21
Osteocalcin and ESR	-0.07
Osteocalcin and CRP	-0.12
Osteocalcin and Anti - CCP	0.02

***p<0.001, **p<0.01, *p<0.05. Above table showing significant positive correlation between HA and inflammatory markers in RA.

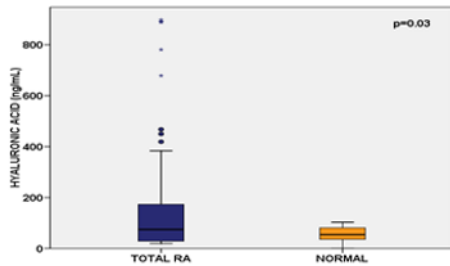
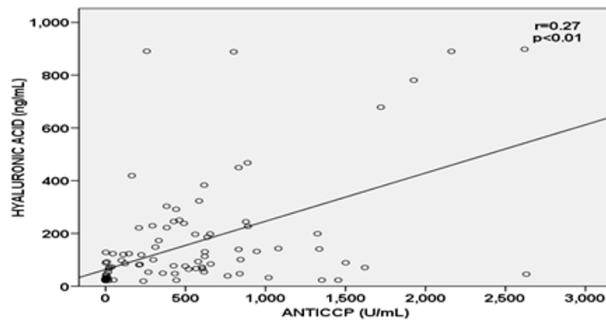
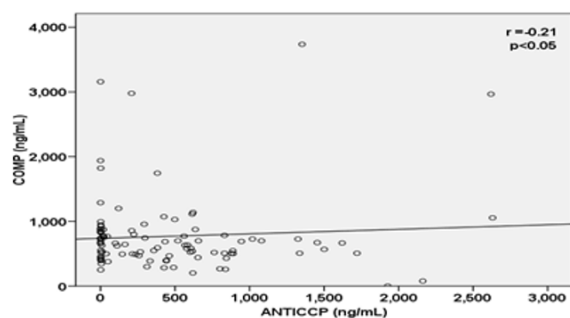


Figure 3: Serum levels of hyaluronic acid in RA patients and normal



Serum HA and Anti-CCP showed a significant positive correlation ($p < 0.01$) in RA patients.

Figure 4: Correlation between serum hyaluronic acid and anti-CCP levels in RA patients.



Serum COMP and Anti-CCP showed a significant negative correlation ($p < 0.05$) in RA patients.

Figure 5: Correlation between serum COMP and anti-CCP levels in RA patients

Hyaluronic acid

Synovial inflammation is a characteristic feature in rheumatoid arthritis, and HA is produced by synovial tissue and escapes out from inflamed synovium and raises its level in the blood (Yap et al., 2018). In results obtained from our study showed an increase in serum HA levels in RA patients significantly compared to the control group (Figure 3) suggesting an early indicator of structural damage and hence can be a useful prognostic marker of RA pathogenesis (Majeed et al., 2004). A study proved that serum hyaluronic acid level correlates with clinical and laboratory measures of disease activity like

ESR, CRP in RA patients (Al-Dalaen et al., 2016). In agreement with this study, we have also proved a significant correlation between ESR, CRP with levels of hyaluronic acid in serum (Table 3) in RA patients suggesting synovial inflammation leads to RA pathogenesis. The release of HA and its fragments into the systemic circulation is due to the degeneration and turnover of the extracellular matrix. It is confirmed that localized inflammation of the synovial arthritic joints is the root cause of increased production and release of HA than cartilage degradation (Gupta et al., 2019).

A study demonstrated a significant positive association between CRP and HA level in RA patients suggesting increased synthesis of HA may be linked to the intensity of the inflammation process (Petrey and de la Motte, 2014). In our study, we have observed a significant positive correlation between HA with the traditional markers of RA such as Anti-CCP suggesting the involvement of synovium in RA pathogenesis (Figure 4). From rheumatoid synovium, a substantial amount of Hyaluronic acid from the synovial joint enters systemic circulation by lymphatic drainage as demonstrated by different stages of cell culture studies (Sokolove and Lepus, 2013). We were able to establish a reasonable correlation between HA with ESR, CRP, and Anti-CCP, all of which are directly involved in RA pathogenesis. According to conventional thinking, HA and Anti-CCP are more associated with synovial degradation and ESR and CRP with inflammation it is clear that synovial inflammation is a distinguishing feature of RA pathogenesis suggesting the role of the above-mentioned markers in RA pathogenesis.

Cartilage oligomeric matrix protein (COMP)

The serum COMP is one of the most extensively studied cartilage markers so far and researchers have found an association between increased COMP & progression in rheumatoid arthritis. Increased cartilage turnover is reflected upon as raised serum COMP levels, therefore used as a prognostic marker of cartilage breakdown in confirmed RA patients (Bi, 2018). But different from these observations in our study, there is not much significant change in the amount of COMP in RA patients when compared with controls, shows the degree of uncertainty on the role of COMP in RA pathogenesis. Several previous studies provide contradicting reports about serum COMP-either decrease or increase or no change in RA patients. Although COMP being a biomarker of cartilage wear and tear in RA and rheumatic / joint diseases, we have found that it is less significant unlike other biomarkers in predicting the disease progression, and from our observa-

tions, it is clear that serum COMP values are synonymous in RA patients and normal healthy controls. In agreement with one such study, we also propose that there are no changes in serum COMP levels in RA patients signifying its limited role in RA pathogenesis (Tseng *et al.*, 2009). Simultaneously both COMP and Osteocalcin showing a weak correlation with traditional markers of disease activity, in the study group.

From our study, we were unable to establish any kind of significant relationship between COMP with traditional disease activity markers of RA such as ESR, CRP, swollen and tender joint count. The same observation was reported in another study that, serum COMP showed a weak correlation with ESR, CRP in RA patients and increased serum COMP levels might occur early in the course of rheumatoid arthritis as a sign of cartilage involvement (Lindqvist, 2005). We have observed, according to Figure 5, the serum COMP and AntiCCP showed a significant negative correlation in RA patients. At the same time, we have found no significant correlation between serum levels of COMP with RF in RA patients. This biomarker strongly correlates with joint cartilage degradation; however, the results of all studies are not consistent. Besides, some studies have indicated that in advanced stages of rheumatoid arthritis there is a decline in serum COMP level (Wisłowska and Jabłowska, 2005), whereas synovial inflammation may persist, indicating normal or slightly elevated Anti-CCP autoantibodies. The main influence of COMP in the RA pathogenesis is ambiguous, although there is proof about the role of COMP in turning on the complement system which contributes to disease development. In RA, there is a high turnover of cartilage matrix, eventually releasing COMP due to catabolic reaction, especially in late stages where repair mechanism cannot recompense joint destruction (Saghafi *et al.*, 2017).

Osteocalcin

Rheumatoid arthritis is associated with severe progressive joint destruction and erosion of bone. So, we have studied serum osteocalcin, a bone-specific protein and marker of bone formation in rheumatoid arthritis patients and control. In our study, there was no statistical significance in serum osteocalcin level between RA patients with normal controls, which we had discussed in our earlier study (Vinod *et al.*, 2011). Studies reported both increased and decreased serum osteocalcin concentrations in patients with RA (Shoji *et al.*, 2017; Liu *et al.*, 2018). Meanwhile, one study reported that there is inconsistency about the levels of markers

of bone formation in RA patients and as we evaluated osteocalcin level found there is no significant difference between RA and controls suggesting normal bone turnover rate in RA patients (Riaz *et al.*, 2020).

Normal osteocalcin levels in rheumatoid arthritis patients could be due to various reasons. A study reported that a higher amount of inactive osteocalcin and a low amount of active osteocalcin produced in the serum of chronic RA patients (Angela *et al.*, 1990). It is reported that improper sample collection, specimen handling, and using different laboratories for successive measurements can seriously affect the precision of serum osteocalcin assay (Singer and Eyre, 2008). In our study, these possibilities are minimal since we have strictly followed the protocol and procedures as per the instruction manual, despite we have observed no significant changes in the serum osteocalcin levels between RA and controls. From the results of our study, we are suggesting a conventional bone resorption activity in RA, more probably due to imbalance between osteoblastic and osteoclastic activity in these patients.

In our study, there was no correlation between osteocalcin with traditional markers of RA. A study reported that OC concentrations were significantly decreased in RA patients compared with the controls and not correlated with ESR and CRP, suggesting reduced bone formation and bone remodeling in RA (Kroger *et al.*, 1993). This is an important observation from our study on Indian patients with RA. In a broad and comprehensive nontraditional biomarkers are used to measure the disease severity in rheumatoid arthritis patients there was no single biomarker signified the biology of disease and helped in predicting the clinical course of the illness. Therefore, a blend of non-traditional biomarkers can help to evaluate disease activity better in the early stage of rheumatoid arthritis.

CONCLUSION

Based on all the findings it can be concluded that the serum non-traditional markers like COMP and osteocalcin, no doubt are good markers for RA disease activity but it would be premature to establish their involvement in RA pathogenesis as more future studies are required. However, Hyaluronic acid can be considered as a potential biomarker which in combination with traditional markers of RA can be useful for early diagnosis of rheumatoid arthritis.

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Conflict of Interest

The authors declare that they have no conflict of interest in this study.

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