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Significance of Hyaluronic acid as a biomarker with seropositive and seronegative autoantibodies in Rheumatoid arthritis patients

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ABSTRACT

Testing for autoantibodies is a flagship feature of Rheumatoid arthritis (RA), a chronic inflammatory autoimmune disorder affecting both the male and female population. Synovial inflammation followed by cartilage, bone, and joint destruction in the later stages of RA puts life in peril, especially for those with other comorbidities. In this study, we focused on to measure serum Hyaluronic acid (HA) along with seropositive and seronegative RF, AntiCCP autoantibodies to establish any association with these biomarkers. 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 clinical examination, the traditional markers were assessed to measure the disease activity, such as CRP, ESR, Anti -CCP, and RF in RA patients. The serum HA levels were measured using the ELISA method. All the values were expressed as median (25th–75th percentile). Based on seropositive and seronegative RF and AntiCCP autoantibodies, the patient group was divided into four groups- both seropositive, both seronegative, and the other two mixed groups. The traditional inflammatory markers were significantly increased in RA patients than in controls with ($p < 0.001$). In our study, there was a significant increase in serum HA levels in RA patients compared to healthy controls ($p < 0.03$). At the same time, serum HA level is increased in the group with seropositive for both antibodies showing statistical significance. Serum Hyaluronic acid is involved in synovial inflammation, manifesting a common triggering mechanism more with AntiCCP antibodies than RF, promising for better clinical utility in the early stages of rheumatoid arthritis.

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INTRODUCTION

Synovial inflammation, along with cartilage and bone destruction, is the characteristic feature of most rheumatic diseases, including Rheumatoid arthritis (RA), an autoimmune disorder effecting both male and females. Multiple reasons have been identified to contribute in the RA pathogenesis (Fujita *et al.*, 2020). Unfortunately, rheumatoid arthritis is considered as a long durable devastating disorder seen more frequently in females, primarily affecting the synovial joints associated with progressive disability ultimately lowering the qual-

ity of life (Mekic and Hadzigrahic, 2020) The disease puts life in peril, especially for those with comorbidities. According to the ACR/EULAR 2010 classification criteria, the definite diagnosis of RA is based on synovitis manifestations in at least one human joint. The diagnostic criteria include symptoms duration, serological and, abnormal levels of acute-phase response biomarkers and site of the affected joints (Aletaha *et al.*, 2010).

Testing for autoantibodies is a flagship feature of rheumatoid arthritis. The immunological parameter of RA includes Rheumatoid factor (RF), immune complexes, characteristic complement levels, anticyclic citrullinated peptide antibody (Anti-CCP) and other factors. If RA is suspected clinically, it is confirmed by RF and other serological tests, including Anti-CCP antibodies (Ingegnoli *et al.*, 2013). The Anti-CCP antibodies are of value for the severity of rheumatoid arthritis. They are produced and significantly present at the site of joint and synovial tissue inflammation playing an active role in the pathogenesis. The high levels of AntiCCP antibodies have been linked with the erosive nature of the disease (Ediz *et al.*, 2011) Several studies in the past proved both RF and Anti-CCP are said to have good sensitivity and specificity for rheumatoid arthritis. Sometimes despite RF being normal, with high suspicion of RA, test for AntiCCP will be the order of the day. RF is the cheapest test for screening for RA though it is not always a reliable marker, and 10-15 % of RA patients with joint deformities generally presented with seronegative RF antibodies (Singh *et al.*, 2020)

Existing methods for diagnosis and prognosis of RA are based on late clinical presentations. Screening tests should be used for evaluating the progression of the disease, convalescence rate and efficacy of treatment. The diagnosis in the early stage of RA needs specific, sensitive and suitable biomarkers that should be detected in blood or synovial fluid (Niu and Chen, 2014). Hyaluronic acid (HA) is a glycosaminoglycan that consists of repeated units of N-acetyl glucosamine and glucuronic acid. It is produced by synoviocytes and chondrocytes, essential for protecting cartilage structure (Das, 2008). Few studies have reported that increased serum HA levels demonstrate and showing correlation with indices of joint inflammation in RA patients indicating subsequent joint destruction in the early stages of rheumatoid arthritis (Garnero *et al.*, 2000; Sasaki *et al.*, 2011).

The absence of specific clinical manifestations in the early stage of RA has captivated laboratory diagnosis of rheumatoid arthritis. Since the traditional biomarkers of RA such as RF, AntiCCP can be detectable in patients with other pathological condition and a small percentage even in healthy donors, as yet, there is no single laboratory test with high sensitivity and specificity that can diagnose RA in the early stages. An alternative strategy of employing multiple specific biomarkers simultaneously is the need of the hour for RA diagnosis and for predicting the therapeutic effect of various combinations of drugs. According to conventional thinking, only a few sets of biomarker have been substantiated by various past studies and can be applied in clinical practice. Many studies established the role of Hyaluronic acid in osteoarthritis and other forms of rheumatic diseases. But there are no published studies on HA level and its association with RA patients with seronegative and seropositive RF and Anti CCP antibodies. The outcome of the study may stress upon the usefulness of Hyaluronic acid as a non-traditional biomarker in RA patients, specifically based on the seronegative and seropositive for other traditional autoantibodies.

RESULTS AND DISCUSSION

According to Table 1, in our study, we have observed a significant increase in measures of inflammation like ESR and CRP levels in RA patients compared with normal controls. These two are the most frequently used acute phase reactants in the diagnosis and prognosis of disease status sometimes show high values in chronic disease stages in RA patients (Bitik *et al.*, 2015). The traditional inflammatory biomarkers like AntiCCP and RF are also significantly increased in our RA patients when compared with controls, in agreement with several studies done in the past. A study concluded that investigation of AntiCCP and RF antibodies could be supportive in serological diagnosis and overseeing the patients with rheumatoid arthritis (Aridogan *et al.*, 2008). In our study group, we have observed 73% sensitivity and 97% specificity for AntiCCP, whereas 51% sensitivity and 90% specificity for RF antibodies in total RA patients indicating sensitivity and specificity are a little higher for AntiCCP test than RF (Table 2). Another Indian study is also showing an almost similar outcome in terms of sensitivity and specificity autoantibodies in rheumatoid arthritis (Manivelavan, 2012).

All the values expressed as Median (25^{th} percentile – 75^{th} percentile). Table 1 represents demographic data of total RA patients and normal group showing ESR, CRP, RF and Anti-CCP levels are significantly increased ($p<0.001$) in total RA patients compared with the normal group. A significant

Table 1: Demographic data of all subjects

Parameters	RA (N=152)	Normal (N=68)
Sex (male; %) (female; %)	39 (26%) 113 (74%)	18(26%) 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)
AntiCCP (U/mL)	295.68 (2.38-640.54) ***	0.31 (0.0-0.71)
RF (U/mL)	30.82 (16.32-65.07) ***	11.62 (7.75-17.78)

***p<0.001

Table 2: Comparison of AntiCCP and RF reactivity in RA patients and Normal controls

Anti-CCP and RF reactivity	RA n=152 (%)	Normal n=68 (%)
Anti- CCP +	110 (73%)	2(3%)
Anti- CCP -	42 (27%)	66(97%)
RF+	78 (51%)	7(10%)
RF-	74 (49%)	61(90%)
Anti- CCP and RF+	40(26%)	1 (1.5%)
Anti- CCP and RF-	04(03%)	60 (88%)
Anti- CCP +and RF-	70(46%)	1 (1.5%)
Anti- CCP-and RF+	38(25%)	6 (9%)

Table 3: Correlation between HA and RF, AntiCCP with traditional markers of RA

Correlation between	Correlation coefficient (R)
HA and Age	-0.10
HA and Duration of disease	-0.12
HA and joint count	-0.10
HA and DAS-28	-0.04
HA and ESR	0.24*
HA and CRP	0.31**
RF and CRP	0.25*
Anti-CCP and CRP	0.40***
Anti-CCP and ESR	0.36***
Anti-CCP and Hyaluronic acid	0.27**

*p<0.05, **p<0.01,***p<0.001

increase($p<0.03$) was also observed in serum HA in the RA group.

Table 2 represents the Anti-CCP and RF reactivity among the 152 RA patients and 68 normal healthy controls.

Table 3 represents the correlation between HA, Anti-CCP and RF with measures of disease activity in RA patients.

Figure 1 shows serum HA level is significantly increased in RA patients compared with normal healthy controls.

Figure 2 shows serum HA level is significantly increased in (RF + and Anti-CCP+) RA patients compared with (RF - and Anti-CCP-).

Figure 3 represents there was a significant increase ($p<0.001$) in serum HA level in (RF - and Anti-CCP+) RA patients compared with (RF - and Anti-CCP-).

Figure 4 shows serum HA level is significantly increased in (RF - and Anti-CCP+) RA patients compared with the other three groups.

Figure 5 shows a significant positive correlation ($p<0.01$) was observed between serum HA and anti-CCP level in RA patients.

Moreover, AntiCCP antibodies are more specific than RF, with the same sensitivity also most helpful in pointing out cases of earlier undiagnosed inflammatory arthritis when the conventional tests report negative for RA (Mekic and Hadzigrahic, 2020). It is interesting to note that 65 out of 110 RA patients with seropositive for AntiCCP antibodies belong to the early stages of the disease. But an exact number of seropositive and seronegative RF antibodies in these early RA patients could not be measured. Occasionally, RF is often not detected in early RA, and AntiCCP antibodies can impart further information enabling specific and early diagnosis (Bose and Calabrese, 2012).

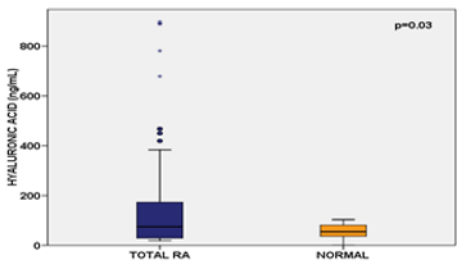

Figure 1: Serum levels of hyaluronic acid in RA patients and normal

We also noticed that 46% of our RA patients had a seropositive AntiCCP AntiCCP and seronegative RF antibodies. A study revealed that though RF is

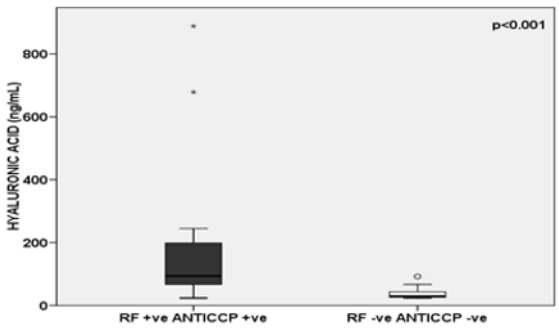

Figure 2: Comparisonof Serum HA levels in Total RA patients in with(RF + and Anti-CCP+) and (RF- andAnti-CCP-)

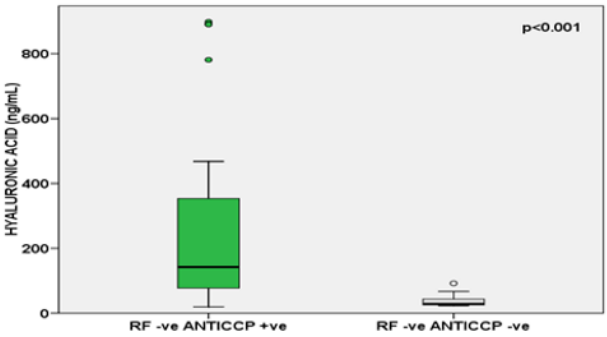

Figure 3: Serum HA levels in RA patients with (RF - and Anti-CCP+) and (RF- and Anti-CCP-)

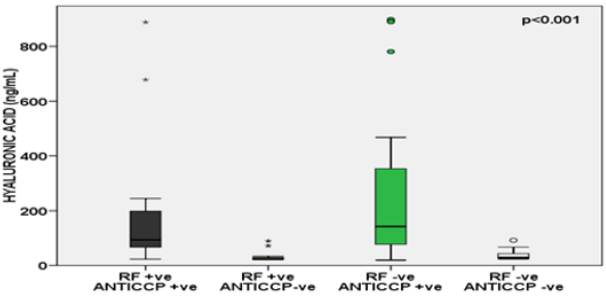

Figure 4: Comparison of Serum HA levels in Total RA patients with Four groups involving RF and Anti-CCP

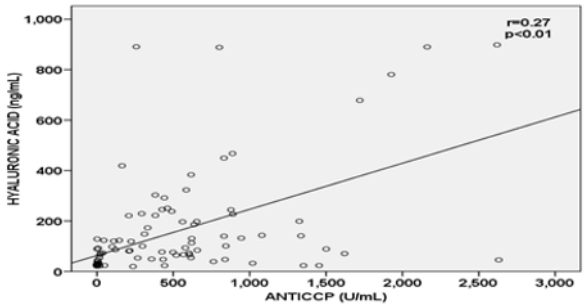

Figure 5: Correlation between serum hyaluronic acid and anti-CCP levels in RA patients in phase I

one of the screening tools, RA patients with positive RF are usually subjected to anti-CCP tests for confirmation of disease. This does not mean that patients with seronegative for RF antibodies cannot have seropositive AntiCCP antibodies (El-Banna and Jiman-Fatani, 2014). 26% of our total RA patients are seropositive for both antibodies, and barely 3% of RA patients showed seronegative for both antibodies. This is because, in the early stages of RA, both antibodies may be tested negative in these patients (Somers *et al.*, 2011). We are certain that these 4 patients are early RA with less than a year in the duration of disease.

Besides, 49%of of RA patients were RF negative, and 10% of healthy controls showed positive for RF antibodies in our study group. When we scrutinized further, most of these RF negative patients are in the early stages of RA. It is reported that 4% of the young, healthy Caucasian population tested positive for RF antibodies, but during the first year of the disease, RF is usually negative. Although RF tests are useful for differential diagnosis and prognosis of rheumatoid diseases, the high titer is perhaps associated with joint destruction and extra-articular manifestations (Simard and Holmqvist, 2012).

As reflected in Table 3, we have observed a significant positive correlation between serum Hyaluronic acid with conventional disease activity inflammatory markers like ESR and CRP levels in RA patients suggesting synovial inflammation leads to RA pathogenesis. Synovitis, a condition that develops due to irritated and inflamed synovial joint lining, plays a principal role in the pathophysiology of RA (Guo *et al.*, 2018). A couple of studies have reported that serum HA, a useful measure of disease activity, levels are elevated and correlated with serological markers of RA such as ESR, CRP, and joint count in RA patients (Majeed, 2004). Contrary to our observations, studies also reported no correlation between serum HA levels and disease severity of RA (Jiang *et al.*, 2007) We could not establish any statistical significance between HA and other disease activity measures like DAS 28 and joint count in RA patients.

The outcome from a study disclosed that, though the exact reason for high serum HA levels in RA patients is unspecified evidently, serum concentrations of RF or immune complexes do not have any impact on circulating HA levels. In the same study, a significant positive correlation with CRP suggesting increased synthesis of HA might be associated with the ferocity of the inflammatory process (Engstrom-Laurent and Hallgren, 1985). In agreement with another study, we could not establish any correlation between serum hyaluronic acid level with clinical and laboratory measures of disease activity like swollen and tender joint count, DAS-28, VAS scores among our RA patient groups (Majeed, 2004).

As per Figure 1, in our study, we have observed an increase in serum HA level [74.59 (29-176.4)ng/ml] in RA patients when compared to normal healthy controls [54.58 (35.63-81.68)ng/ml], which is statistically significant ($p<0.03$). A study reported a significant increase in serum levels of HA in RA patients compared to the control group, with higher sensitivity and specificity reflecting the erosive status in RA patients (Al-Dalaen *et al.*, 2016). Our results were in agreement with the other two studies, suggested that increased systemic HA level indicates early structural damage and useful prognostic marker in RA patients (Majeed, 2004; Santos *et al.*, 1994) A study put forward an increase production and release of HA from RA joints reflecting more of local synovial inflammation than cartilage breakdown in rheumatoid arthritis patients (Chubinskaya *et al.*, 2006)

As illustrated in Figure 2, the serum HA level is significantly increased in RA patients showing positivity for both RF and AntiCCP autoantibodies when compared with RA patients with both negative for autoantibodies. It is proved that increased production of HA in the local inflamed synovium of patients with RA and other rheumatic diseases (Kogan *et al.*, 2007). There are no published studies regarding the correlation between HA and seropositive/seronegative autoantibodies in serum samples. There may be some nonspecific mechanism common for the production of HA and AntiCCP in locally inflamed synovium tissue which triggers the local citrullination of intraarticular proteins as it is known that HA fragments increase the expression and protein production of several cytokines in rhythmic activation of immune cells in the synovium (Teder, 2002). It was reported that HA is produced locally by cells of the extracellular matrix, and the degeneration and turnover of the extracellular matrix result in the release of HA and HA fragments into the systemic circulation (Elliott *et al.*, 2005).

Clinical evidence from a study suggested increased oxidative stress followed by intensified oxidant activity in synovial fluid of RA patients with seropositive AntiCCP than seronegative antiCCP antibodies (Ediz *et al.*, 2011). So we have speculated that oxidative stress generated during rheumatoid arthritis is accountable for oxidative damage in synovial fluid pathophysiology, enhancing destruction of the joint structure. It may release HA fragments

into systemic circulation as a protective measure as HA exhibits anti-inflammatory activity. In general, another reason for increased serum HA level in RA patients could be physical activity as well as the production of a large volume of synovial fluid, thereby increases serum HA level. The RA patients with both seropositive AntiCCP and RF antibodies would have resorted to an additional physical activity/physiotherapy as part of the treatment plan may probably raise HA levels. An old study disclosed that the effect of physical activity based on recorded joint mobility pronounced high HA levels in RA patients than in healthy individuals (Engstrom-Laurent and Hallgren, 1985). Hence, we are postulating that since HA showing a significantly positive correlation with AntiCCP in our study, it plays a significant role in synovial inflammation in RA patients.

When we further subjected the same set of RA patients into two groups- (RF negative and AntiCCP positive) and (both RF and AntiCCP negative) as shown in Figure 3, we have found serum HA levels are significantly increased in 70 patients (46 %) with RF negative and AntiCCP positive group when compared with 4 patients (3%) both seronegative RA patients. The difference in the number of RA patients could be one reason for significantly decreased HA levels in both seronegative RA patients than in other groups. According to Figure 4, results from the compilation of all the four groups involving RA patients with seropositive and seronegative RF and AntiCCP antibodies, we have observed serum HA level is relatively increased in RF – and AntiCCP + groups when compared with others showing statistical significance. We have also noticed in RF+ and AntiCCP + group showing a moderate increase in HA level. As anticipated, the remaining two groups involving seronegative for AntiCCP antibodies HA levels are low, proving the prominence of AntiCCP antibodies in synovial inflammation.

The synovial inflammation is more aggressive and intrusive in nature in the RA patients with both antibodies positive and hence they need to be more cautious. This piece of information would be useful for physicians to decide a further course of action. It is also proved that HA shows anti-inflammatory properties by activating immune cells at the site of injury to manage rupture in tissue integrity. So, information on synovial fluid hyaluronic acid at this juncture will be crucial in disease diagnosis and treatment point of view. All these observations proved a strong correlation between HA with AntiCCP levels in serum in our study.

Our study defers from previous existing studies that serum HA level increased in RA patients who are seropositive for both RF and AntiCCP antibodies (26%) and seropositive for only AntiCCP in RA patients (46%). According to Figure 5 besides, it is showing a positive correlation with AntiCCP indicating its significance in the inflammatory process in the RA pathogenesis. It may be due to synovial oxidant activity and excessive scavenging of reactive species, a common factor for the raise in Hyaluronic acid and AntiCCP levels in chronic RA patients (Sato *et al.*, 1988).

Knowing the molecular weight (MW) of Hyaluronic acid helps to understand the status of inflammation, high MW HA fragments for restraining inflammation and lower MW form possesses a pro-inflammatory effect. These HA fragments stimulate the expression of inflammatory genes via heterogenic immune cells at the site of injury. It may be pertinent for AntiCCP as well as other antibodies also (Walimbe *et al.*, 2017). As we have discussed about the anti-inflammatory role of HA earlier, this could be another basis for incessant raise in both biomarker levels in RA patients. It has been reported that the interactions between the endogenous matrix component of hyaluronan and its signaling receptors may commence inflammatory reaction, preserve cell structure, and resurgence from tissue injury (Jiang *et al.*, 2007)

Another major finding from our study was that we could not establish any significant correlation between HA and RF in our study population. Though HA level is moderately raised in both seropositive antibody groups, it is evident that the presence of AntiCCP is more influential in escalating HA level in these sets of RA patients. As claimed by a study, intra-articular injections of Hyaluronic acid could be an efficient procedure for clinically controlling synovial joint inflammation in osteoarthritis patients (Ayhan *et al.*, 2014). However, the role of HA injections in rheumatoid arthritis is not yet known. Although more high-quality corroboration is needed on HA injections in soothing pain, improving knee function, and better quality of life in RA patients, the current study opens up the likely role of HA injections in clinical interventions, particularly in the early stages of rheumatoid arthritis. Since this was not the primary focus of our study, the outcome portrays the importance of Hyaluronic acid as a worthy biomarker along with both seropositive autoantibodies or at least seropositive for AntiCCP in the inflammatory process of RA patients.

Limitations of the study

1. Assessing biomarker levels in the knee joint or any other joint synovium would have given a

better understanding of the pathophysiological inflammatory process in RA patients.

2. We did not measure the role of these traditional inflammatory markers separately in a subclass of RA patients -early and late stages of the disease.
3. An additional liver function test for analysing the clearance of hyaluronic acid should have performed to rule out hepatic dysfunction in raised HA levels.

Taking into consideration of the pitfalls in our study would like to analyse separately in early and late RA patients from the existing research design in future. Our database also includes the follow-up information (after one year from the baseline study) of RA patients may be pursued as future investigation.

CONCLUSIONS

Based on all the findings, it can be concluded that serum Hyaluronic acid is involved in synovial inflammation, manifesting a common triggering mechanism more with AntiCCP antibodies than RF, promising for better clinical utility in the early stages 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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