Role Of IL-6, Atnf And Calprotectin In Acpas Positive & Negative Patients Of Rheumatoid Arthritis

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Keywords:

IL-6, αTNF, Calprotectin, ACPAs

ABSTRACT

Auto immune disease such as Rheumatoid Arthritis is the consequence of persistence imbalance between proinflammatory cytokines and anti-inflammatory immune mechanisms leading to chronic inflammation. In RA, many factors activate multiple pathways, all converging to enhance osteoclastogenesis, ultimately disturbing bone metabolism to bring about joint destruction. This study is aimed at finding levels of serum calprotectin, serum IL-6 and serum TNF-α in treatment naïve seropositive and seronegative RA patients. It also aimed at finding association of these parameters with RA disease in Pakistani population.

Subjects and Methods
A comparative cross-sectional study was carried out at RA patients of age 35-55 years, not yet started any treatment, divided into group 1 ACPA +ve (n=28) Group 2 ACPA -ve (n=28) and Group 3 healthy control (n=28). Blood samples were taken and processed for serum preparation and stored at -80˚C. The anthropometric profile (height, weight and body mass index) was recorded. ESR, CRP and serological record were taken from medical file of patient. Calprotectin, IL-6 and TNF-α were estimated by human ELISA kits. Data was analyzed by SPSS-21. Normality was checked and statistical tests were applied accordingly. A p value of $\leq$ 0.05 was considered significant.

Results
Level of Serum calprotectin, IL-6 and TNF-α were significantly elevated in both ACPA +ve and ACPA -ve RA patients as compared to healthy controls (p value < 0.001), (p value < 0.001), (p value < 0.001) respectively. However, no significant difference in levels of calprotectin, IL-6 and TNF-α was noticed in ACPA +ve and ACPA -ve groups (p value < 0.179), (p value < 0.725), (p value < 0.629) respectively. A notable association of these parameters with DAS-28-ESR was also found. Both seropositive and seronegative groups of our study reported high DAS -28.

Conclusion
This study found that levels of serum calprotectin, IL-6 and TNF-α were significantly elevated in RA patients as compared to healthy controls and these high levels were associated with RA disease activity. These findings suggest their possible role in disease

	pathology and these parameters show notable association with disease severity. Moreover, the study concluded that, severity of disease is equally high in both seropositive and seronegative RA patients. Hence, seronegative RA patients cannot be ignored and should be treated promptly. Also, the study concluded that calprotectin has discriminatory capacity to predict disease activity in both groups equally.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic and debilitating autoimmune disorder that affects approximately 1% of the global population (Alamanos et al., 2006). It is characterized by persistent inflammation, synovitis, and joint damage, leading to significant morbidity and mortality (Singh et al., 2020). The pathogenesis of RA involves a complex interplay of genetic, environmental, and immune system factors, including the production of pro-inflammatory cytokines such as Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α) (Chen et al., 2020). Elevated levels of these cytokines have been linked to disease activity, joint damage, and disability in RA patients (Klaasen et al., 2018).

Calprotectin, a protein complex released by neutrophils, has also been implicated in RA pathology, serving as a potential marker of disease activity and inflammation (Kataria et al., 2020). The presence of anti-citrullinated protein antibodies (ACPA) is a common feature of RA, although not all patients test positive for these antibodies (Lopez-Lopez et al., 2020). Studies have shown that ACPA-positive RA patients exhibit higher levels of IL-6 and TNF-α than ACPA-negative patients (van der Linden et al., 2019). However, the relationship between ACPA status and calprotectin levels in RA remains unclear, highlighting the need for further research (Lopez-Lopez et al., 2020).

This study aims to investigate IL-6, TNF-α, and calprotectin levels in the serum of ACPA-positive and ACPA-negative RA patients, compared to healthy controls. By exploring these markers, the research study sheds light on the underlying disease mechanisms and identifies potential biomarkers for RA diagnosis and monitoring.

MATERIALS AND METHODS

All the samples were collected from the samples were collected from Rheumatology Department, Central Park Teaching Hospital, Lahore. The study was performed in the Biochemistry Department, University of Health Sciences, Lahore.

This research was carried out in the Biochemistry Department at University of Health Sciences Lahore. All participants were individually informed about the purposes of the study, written informed consent was obtained. This cross-sectional comparative study was approved by the Advanced Studies & Review Board of University of Health Sciences, Lahore, Pakistan. The study was conducted in full accordance with the Declaration of Helsinki. Our study targeted 84 individuals (n=28) in 3 groups having 28 participants each. Group I, II & III had ACPA +ve RA, ACPA -ve RA & healthy controls respectively. All were in between 35-55 years of age both male & female with history of no treatment in past. Individuals having any kind of acute infection, autoimmune sickness (systemic lupus erythematosus). malignancy, Patient on HRT, Kidney, disease, Chronic liver disease, rheumatic disorders were excluded.

DAS-28 Calculating Formula

Global assessment of health was done by asking about pain and giving points from 0-10. DAS-28-ESR was then calculated with the help of formula by using above results. Results of DAS-28-ESR implies that DAS-28 > 5.1 (Active disease), DAS-28 > 3.2 < 5.1(Moderate disease), DAS-28 < 3.2 > 2.6(Low disease activity), DAS-28 < 2.6(Remission).

Sample preparation

A total 5ml of blood was drawn under aseptic conditions from the median cubital vein from anterior aspect of forearm. The blood was collected in gel containing serum separation tubes. After clotting the blood was centrifuged at 3000 rpm (revolutions per minute) for 10 minutes. The serum was transferred in properly labelled autoclaved Eppendorf tubes and was stored at -80 degree Celsius for subsequent biochemical analysis. Serum

Calprotectin, TNF-α and, CRP levels were measured by using commercially available kits on ELISA and plasm were used to measured ESR level by Westergren method**.**

Statistical analysis

SPSS (version 21.0) was used for analyzing statistics.,Normality was checked by Shapiro-Wilk test. If p-value ≥ 0.05, data was assmued to be normal. For normally distributed data, Mean ± SD was given while skewed data was presented in median and inter-quartile range (IQR),One way ANOVA test was applied for comparision ,Post hoc Tukey's test was applied for groupwise ,Kruskul Walis test was applied for comparision of medians and IQR of non normally distributed data,Post hoc Mann Whitney U test was applied to compare medians of groups,Pearson co-efficient of correlation was determined to find out association of ESR, CRP, serum IL 6, serum TNF-α and serum Calprotectin with DAS-28 in two groups of ACPA +ve and ACPA -ve RA patients .

RESULTS

Results are explained in terms of Mean + SD for BMI, Serum TNF-α and DAS-28 and in Median + IQR for Age, CRP, ESR Serum Calprotectin and Serum IL-6. A P-value < 0.05 has been considered statistically significant.

Demographic Characteristics of RA Patients and Healthy Control Group

Age and BMI did not show any significant difference in all groups as shown in Table 1.

Table 1: Demographic Characteristics of RA and Healthy Control Group

Variables	ACPA +ve RA patients (n = 28)	ACPA -ve RA patients (n = 28)	Healthy Controls (n = 28)	P Value
Age (years)	48.5 (41.25 - 55)	51 (43.25 - 55)	43.5 (40 - 51.75)	0.118
BMI	26.04 ± 5.9	26.7 ± 6.05	27.85 ± 3.99	0.489

Biochemical Estimation.

The level of Serum Calprotectin, TNF-α , IL-6, ESR, DAS-28 and CRP were significantly increased in ACPA +ve and ACPA -Ve in group as compared to control are shown in Table 2.

Table 2: Biochemical parameters of RA and Healthy Control Group

Variables	Seropositive RA patients (n = 28)	Seronegative RA patients (n = 28)	Healthy Controls (n = 28)	Kruskal Wallis test
	Median (IQR)	Median (IQR)	Median (IQR)	p-value
ESR (mm in 1st hour)	28 (24.25-32)	26 (24-28)	11 (10-11.75)	<.001
CRP (mg / L)	14.25 (12.53-16.05)	14.65 (12.43-15.6)	1.75(1.5-2)	<.001
DAS - 28	4.88 (4.13 - 5.93)	4.95 (4.6-5.88)	-	<.001
Serum IL 6 (pg / ml)	9.28 (7.86 - 11.01)	9.65 (7.69-14.01)	2.2 (1.58 - 3)	<.001
Serum TNF-α (pg / ml)	57.5 (43.2 - 61.31)	57 (47.25-68.5)	4.95 (3.43 -6.05)	<.001
Serum Calprotectin (pg / ml)	5081.62 (3946.43-8033.2)	6441.3 (4907.72-7339.09)	438.66 (346.7 - 497.9)	<.001

Groupwise Comparison of Biochemical Variables (Serum Calprotectin, IL-6 and TNF-α)

The levels of Calprotectin, TNF-α and IL-6 in both Groups of RA Patients were almost similar, and no significant difference was noticed in their values (p = 0.179, p = 0.629 and p = 0.725 respectively) (Table 3). A significant difference was found on the comparison of ACPA +ve RA patients with the healthy control group (p-value < 0.05). Similarly, a significant difference was found in the comparison of ACPA -ve RA patients with the healthy control group.

Table 3: Pairwise Comparison along with Percentage Increase of Biochemical Variables (Serum Calprotectin, IL-6 and TNF-α), According to Mann Whitney U test and Post HOC Tuckey.

Serum	Comparison		p value	% Increase
Serum Calprotectin *	ACPA +ve RA Group	ACPA -ve RA Group	0.179	-
	ACPA +ve RA Group	Control Group	< .001	1260.86 %
	ACPA -ve RA Group	Control Group	< .001	1374.50 %
Serum TNF-α **	ACPA +ve RA Group	ACPA -ve RA Group	0 .629	-
	ACPA +ve RA Group	Control Group	< .001	1006.25 %
	ACPA -ve RA Group	Control Group	< .001	1063.10 %
Serum IL-6 *	ACPA +ve RA Group	ACPA -ve RA Group	0 .725	-
	ACPA +ve RA Group	Control Group	< .001	352.27 %
	ACPA -ve RA Group	Control Group	< .001	409.09 %

***** Results generated by Mann-Whitney U test. (p value < 0.05 considered statistically significant)
** Results generated by Post HOC Tuckey. (p value < 0.05 considered statistically significant)

DISCUSSION

Results of our study show that demographic characteristics including age of patient and BMI do not affect the progression of disease (Table-1) and does not show any significant association with disease severity.

In our study levels of IL-6 in seropositive and seronegative RA patients were found to be significantly high as compared to healthy control group (Table 2). Results of our research shows that medians of IL-6 were meaningly increased in ACPA +ve RA and ACPA -ve RA patients as compared to healthy control group (352.46% and 408.91% increase respectively) (Table 2). Pairwise comparison between ACPA +ve RA group and healthy control confirmed significant difference between medians of IL-6 (p value < 0.05). Similarly, ACPA -ve RA group and healthy control also showed significant difference between medians of IL-6 (p value < 0.05). However, non-significant p value (p value = 0.725) at comparison of medians of IL-6 between ACPA +ve and ACPA -ve RA patients expressed no difference among these groups (Table 2). This result is of extraordinary importance as it display that levels of IL-6 raise equally in both groups of RA patients, giving rise to same levels of disease severity in seropositive and seronegative RA groups.

Other investigators had pointed out that percentage of IL-6 increase significantly in serum of rheumatoid patients as compared to healthy controls (Takeuchi et al., 2021) and our results were in accordance. In rheumatoid arthritis, IL-6 contributes in pathogenesis of disease by producing inflammation and is considered as a disease driving cytokine (Matsumoto et al., 2021). Raised levels of IL-6 in ACPA +ve and ACPA -ve RA subjects under our study confirmed the belief that IL-6 is the driving force.

IL-6 displays dual function on bone cells. Firstly, it facilitates RANK-L expression over osteoclasts hence, control bone metabolism by increasing osteoclast activity and depressing activity of osteoblast (Takeuchi et al., 2021). Secondly it induces expression of TNF-α which boost bone resorption. High IL-6 levels were reported to be directly associated to severity of disease in RA patient (Rajaei et al., 2020). Our study confirmed that patients with higher IL-6 levels displayed severe disease concluding IL-6 is a defining factor while estimating the severity of RA (Yang et al., 2021).

Our research shows significant increase in values of TNF-α in seropositive and seronegative RA patients is observed as compared to healthy control group (Table 2).

Results of our study confirm the observation of previous studies that there is clinically significant increase in TNF-α in ACPA +ve RA and ACPA -ve RA group as compared to healthy control (1006.25%, 1063.10% respectively) (Table 2). In Post Hock Tucky test, pairwise comparison showed clinically significant difference in means of TNF-α between control and ACPA +ve RA patients (p value < 0.05) and in means of TNF-α between control and ACPA -ve RA patients (p value < 0.05). However, means of ACPA +ve and ACPA -ve RA patients were almost same and this shows no difference between these groups.

Literature research on topic revealed that newly diagnosed and treatment naïve patient with less severe disease show lower levels of TNF-α as compared to group with the moderate and high disease activity proving that it has possible pathologic linkage with arthritis in RA patients (Samimi, 2020).

In our study, we also found high TNF-α levels in those patients who clinically had severe form of disease intensifying the results of other investigators who identified that patients with clinically severe disease had raised levels of TNF-α in their serum. (Buchari et al., 2021). We found direct correlation with diseases activity. (Table 4)

Table 4: Correlation of DAS-28 with Different Variables Among Healthy Control and RA with anti-CCP Positive & Negative Group.

Parameter	Healthy Controls (n = 28)	ACPA +ve RA patients (n = 28)		ACPA -ve RA patients (n = 28)	
		Rho (p)	p-value	Rho (p)	p-value
Calprotectin	-	0.296	0.063	0.034	0.432
TNF-α	-	0.228	0.122	0.212	0.139
IL-6	-	0.154	0.004	0.056	0.389
ESR	-	0.487	0.004	0.205	0.147
CRP	-	0.026	0.449	0.277	0.077

- Values generated according to Spearman Correlation.
- Correlation is significant at p value < 0.05
- This table describes correlation of various study parameters with DAS-28 in study groups.
- Moderately strong significant correlation found among ESR and DAS-28 in ACPA +ve RA patients.
- Weak correlation found amongst Calprotectin and DAS-28 in ACPA +ve RA patients.
- Direct correlation amongst TNF-α and DAS-28 was found in both ACPA +ve and -ve RA patients.
- Weak correlation amongst CRP and DAS-28 was found in ACPA -ve RA patients.

Our study results were in line with work of Arnigazina et al., who reported that clinical signs and symptoms of RA, such as pain in joints, swelling of joints and morning stiffness, correspond with blood levels of TNF-α and IL-6 (Aringazina et al., 2022). Our study participants have high disease activity shown by DAS-28-ESR > 5.1 and simultaneous raised levels of IL-6 and TNF-α showed that synergistic effect of both cytokines might be the cause of severe disease of our study participants.

Alarmins are early amplifiers of inflammation and are considered as early phase signal of tissue and cellular damage .Calprotectin had recently become prominent as indicator of inflammation. It was established that it was released in synovium during interaction between activated endothelium, monocytes and trans endothelial migrating leukocytes and then due to its small molecular weight 36.5kDa it easily get released into blood from inflamed joint. In our study we found, clinically significant increase in medians of calprotectin in both ACPA +ve and ACPA -ve groups of RA patients as compared to healthy control group on application of Kruskal Wallis test for comparison of groups it became clear that group of rheumatoid patients was clearly different from healthy control group as alarmin, (Table 2) Serum Calprotectin showed 1260.85% and 1374.50% rise in seropositive and seronegative RA patients respectively as compared to healthy control group (Table 3).

Pairwise comparison with Mann Whiteny-U Test showed clinically significant difference in medians of calprotectin amongst control and ACPA +ve RA patients (p value < 0.05) and in medians of calprotectin amongst control and ACPA -ve RA patients (p value < 0.05). However, the levels of calprotectin in ACPA +ve and ACPA -ve RA patients show no significant difference (p value = 0.179) (Table 3).

Our study confirmed association between clinical parameters , disease severity score (DAS-28) and levels of calprotectin (Table 4).

An important part of our study was to estimate the levels of IL-6, TNF-α and calprotectin and disease activity in both seronegative and seropositive RA patients simultaneously. We found that levels of these cytokines and

protein calprotectin were almost same in both groups of RA and above mentioned parameters show positive association with disease activity for 28 joints, DAS-28- ESR (Table 4) which concluded that equal progression of disease takes place in both groups. This was also explained by (Reed et al., 2020) who found equal disease activity in seropositive and seronegative patients duly explained that ACPA -ve patients are not truly seronegative but have subset of autoantibodies called ACPA fine specificities equally distributed in ACPA +ve and ACPA –ve groups. Occurrence of the subsets in seronegative group are responsible for high disease severity in these patients. They also suggested that Calprotectin is a good tool to measure disease severity in these patients for early and quick diagnosis (Reed et al., 2020).

Results of our study also show high levels of calprotectin and its association with disease activity in both groups. In our own study, most patients have severe disease status accessed by DAS-28-ESR and high levels of CRP (Table 2) and serum calprotectin irrespective of anti-ccp status.

Although high serum CRP levels signify inflammation and could further deepens inflammatory process but RA patients can have severe disease even with normal CRP levels and investigators have confirmed association between calprotectin levels and disease severity in spite of normal CRP in their research (Hurnakova et al., 2018).. Our results show that patients with high DAS-28 score have high CRP levels. Higher CRP levels enhance RA disease activity (Erre et al., 2022). Raised serum CRP with raised cytokines and serum calprotectin levels, enhance each other's function, resulting in high severity of disease. In our study, this could be the possible mechanism of severe disease status in both groups of RA patients.

In this study, we also found that patients having high disease severity shown by high DAS-28 values have increased ESR (Table 2) and IL-6 levels. Possibly, because IL-6 induces acute phase proteins.

In our study we investigated association of various parameters with disease severity which was calculated by DAS-28-ESR.

In our cohort we observed that raised levels of IL-6 positively associate with increased disease severity measured by DAS-28-ESR in RA patients, although we found weak correlation ($p < 0.004$, $\rho = 0.154$) (Table 4). Researchers disclosed that raised IL-6 levels have significant association and with DAS-28 (Matsumoto et al., 2021). Our results of weak correlation are attributed to small sample size and due to less increase in levels of IL-6. IN Pakistani population.

In our study, we assessed serum calprotectin in cohort of treatment naïve RA for finding association with DAS-28 to confirm its association with rheumatoid disease and find positive association although weak correlation (Table 4) was noticed in both RA groups. Our work is supported by many researchers who all demonstrated positive association of serum calprotectin with severe form of disease in RA patient. Severity was calculated by DAS-28 and CRP levels in those studies (Greenmyer et al., 2020).

our study we also found direct association of inflammatory markers TNF-α with DAS-28 in ACPA +ve and ACPA -ve group (Table 3) going in accordance with results of other studies who found significant relationship in newly diagnosed RA patients (Inam Illahi et al., 2021). TNF-α levels in our study show weak correlations with DAS-28 in both seronegative and seropositive patients.

An important part of our research is that we assessed levels of calprotectin and driving proinflammatory cytokines (IL-6 and TNF-α) simultaneously in seropositive and seronegative groups of RA. Disease severity is also calculated at the same time and it is concluded that both groups show no significant difference in levels of these parameters. This illustrates that seronegative group cannot be ignored

CONCLUSION

Hence, conclusion is drawn that increased levels of serum calprotectin in cohort of clinically active RA patients might be a predictor of disease activity and can be used for early diagnosis, irrespective ACPA status. Rheumatoid patients can be treated by its antagonist.

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