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The Emerging Role of Calprotectin in Disease Activity and Response to Treatment Among Iraqi Patients with Rheumatoid Arthritis

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Abstract

Background Calprotectin is a heterodimeric compound belonging to the S100 family, it is mostly produced in leukocytes and is implicated in various human disorders, including tumors and autoimmune diseases.

Objective To evaluate the usefulness of calprotectin serum level as a valid target for monitoring progression of rheumatoid arthritis (RA) disease and response to tumor necrosis factor-alpha (TNF-α) inhibitor therapy (Infliximab).

Methods This case-control study comprised 150 participants, consisting of 50 healthy individuals and 100 patients with RA on anti-TNF-α (Infliximab) therapy (50 responders and 50 non-responders). The concentration of calprotectin in the serum was determined using the enzyme-linked immunosorbent assay.

Results The clinical disease activity index revealed a substantial distinction between patients, specifically responders and non-responders (P <0.001). The study's findings revealed 68% of the patients who responded to treatment were positive for rheumatoid factor (RF), whereas 88% of the non-responders were seropositive. Furthermore, non-responders' patients exhibited a greater presence of anti-citrullinated protein antibodies. According to this study, RA patients who test positive for anti-citrullinated protein antibodies exhibit a diminished response to anti-TNF-α therapy. The serum levels of calprotectin were markedly elevated in non-responder patients compared to both responders and the control group (P <0.001).

Conclusion Calprotectin serum level may reflect an effective target for monitoring progression of RA disease and response to anti-TNF-α therapy.

Keywords Calprotectin, rheumatoid arthritis, TNF inhibitor

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List of abbreviations: ACPA = Anti-citrullinated protein antibodies, ADAs = Anti-drug antibodies, BMI = Body mass index, CDAI = Clinical disease activity index, MTX = Methotrexate, RA = Rheumatoid arthritis, RF = Rheumatoid factor, TNF = Tumor necrosis factor

Introduction

Tumor necrosis factor (TNF)-α is the primary inflammatory cytokine and plays a major role in tissue destruction and the underlying mechanisms of rheumatoid arthritis (RA). Consequently, the discovery of anti-TNF-α agents has been essential for the

treatment of RA. Specific autoantibodies, including anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) [1], frequently indicate the onset of the disease years before to the onset of symptoms.

Based on clinical and experimental evidence, the use of antibodies to block TNF-α has been shown to result in the reduction of interleukin IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and other active molecules [2-4]. These molecules play a significant role in causing joint damage in patients with RA [5].

Calprotectin, also known as S100A8/A9 or myeloid-related protein (MRP) 8/14, enhances and triggers inflammation during infectious and inflammatory events by binding to the receptor for advanced glycation end-products and Toll-like receptor 4 [6].

Calprotectin is implicated in various human disorders, including autoimmune diseases; studies measuring calprotectin levels in serum or plasma have demonstrated a correlation between calprotectin and clinical disease activity, as measured by the disease activity score 28 (DAS28) [7]. Furthermore, it has been found that it is more strongly linked to disease activity compared to C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), as evidenced by previous studies [8-15]. In addition, it has been observed that calprotectin can be used as a predictive factor for the response to methotrexate and biologic disease-modifying anti-rheumatic drugs (b-DMARDs).

Infliximab (IFX) is a chimeric monoclonal antibody that specifically targets TNF-α, its mechanism of action involves inhibiting the activity of TNF-α on cell surface receptors [6].

The efficacy of anti-TNF-α therapy and its cost pose significant challenges and do not provide a solution for every patient [13].

So, before commencing therapy in individuals with RA, it is crucial to determine the markers that can accurately predict a favorable treatment outcome. Hence, this study elucidates the possibility of utilizing calprotectin as a biomarker to forecast the response to anti-TNF-α therapy in Iraqi patients with rheumatoid arthritis.

Methods

One hundred patients diagnosed with RA, who had received Infliximab infusion therapy for a duration of less than or equal to six months, were selected for this study. These patients were admitted to the Rheumatology Unit of Baghdad Teaching Hospital.

They were divided into two groups: 50 patients' responders and 50 non-responders. The study was conducted from November 2022 to August 2023. The Institutional Review Board (IRB), College of Medicine, Al-Nahrain University was responsible for overseeing research ethics and ensuring compliance with regulations (IRB/25 on 28/12/2022).

Inclusion criteria

Patients diagnosed with RA who have been receiving treatment with TNF-α inhibitors for 6 months or less, and are at least 18 years of age.

Exclusion criteria

Patients who have been diagnosed with RA at an early stage, pregnant women, and patients with other chronic illnesses.

Two ml of blood were collected from each patient (before the treatment infusion) and control group. The levels of calprotectin were measured by using the enzyme-linked immunosorbent assay (ELISA) kit provided by Cloud-Clone Corp.

This ELISA kit employs the Sandwich-ELISA principle.

Procedure

1. One hundred µL of standard or sample was added to each well. Incubated 1 hours at 37°C.
2. One hundred µL prepared Detection Reagent A was aspirated and added to each well. The plate was incubated 1 hour at 37°C.
3. The plate was aspirated and washed 3 times.

4. After that 100 µL prepared Detection Reagent B was added to each well. Incubated 30 minutes at 37°C.
5. The plate was aspirated and washed 5 times.
6. Ninety µL Substrate Solution was added to each well. Incubated 10-20 minutes at 37°C.
7. Fifty µL Stop Solution was added to each well. The plate was read at 450 nm immediately.

Detection of RF-IgG ELISA Kit

Assay procedure

1. One hundred µL of standards and serum were added to certain wells.
2. The plate was sealed off by sealer then incubated at 37°C for 90 minutes.
3. The plate was washed 3 times and immerse for 1 minute each time.
4. One hundred µL of conjugate was pipetted to each designated well, the plate was sealed off by sealer then incubated at 37°C for 30 minutes.
5. The plate was washed 5 times and immerse for 1 minute each time.
6. Ninety µL of TMB substrate solution was pipetted to each well then incubated 15 minutes at 37°C.
7. Fifty µL of stop solution was pipetted to each well.
8. The optical density was read at 450 nm.

Human CCP-Ab ELISA kit

Procedure

1. One hundred µL of standard or sample was added into the well. Incubated for 90 minutes at 37°C.
2. After discarding the liquid, 100 µL of Biotinylated Detection Ab working solution was immediately added to each well. Incubated for 60 minutes at 37°C.
3. Aspiration was performed and rinsed the plate 3 times.
4. A volume of 100 µL of the HRP conjugate working solution was introduced. The sample was placed in an incubator and kept at a temperature of 37°C for a duration of 30 minutes. Rinsed and cleaned the plate five times.
5. A volume of 90 microliters of substrate reagent was added. Incubated for 15 minutes at 37°C.
6. A volume of 50 µl of stop solution was transferred to each well using a pipette.
7. The plate was promptly analyzed at a wavelength of 450nm. Calculation of the data followed.

Statistical analysis

The statistical analysis involved the use of GraphPad Prism 8.4.3 software, employing ordinary one-way ANOVA and Newman-Keuls multiple comparisons post hoc test, as well as an unpaired t-test. The threshold for acceptable statistical significance is 0.05 or lower. The Spearman correlation coefficient was employed to assess the correlation between the variables under investigation.

Results

In this study, there was no statistically significant difference between the patients' group and the control group regarding the age, and this is in line with the specifications of the current study's quality, as it is case control study (the mean age of the healthy group was 46.82±12.01 years, and in the RA group it was 45.99± 11.08 years). There was a significant association between non responder RA patients and body mass index (BMI) with a P value 0.018. The incidence of RA was higher in females compared to males, with a ratio of 3.05 to 1. The current results showed that there was a total of 28 smokers, consisting of 10 responders, 12 non-responders, and 6 control individuals. On the other hand, there were 122 non-smokers, including 38 responders, 37 non-responders, and 47 control individuals (Table 1).

The study's findings revealed that 68% of the patients who responded to treatment were found to be RF positive, whereas 88% of the non-responders tested positive for seropositivity. Furthermore, the findings

indicated that non-responders to the treatment exhibited higher ACPA serum positivity, the serum level of calprotectin in RA patients was substantially different from that of the control group, with a P value <0.001 when comparing responders and non-responders (Table 1).

About the treatment for RA, there were a total of 70 patients who were administered Methotrexate (MTX), with 37 of them being responders and 33 being non-responders. Additionally, there were 30 patients who did not receive MTX, with 13 of them being responders and 17 being non-responders. The Clinical Disease Activity Index (CDAI) revealed a substantial disparity between patients (responders and non-responders), with a P value <0.001 (Table 1), (Figure 1).

Table 1. Demographic characteristics, rheumatoid factor RF, anti-citrullinated protein antibodies, clinical disease activity index and Calprotectin in studied groups

Parameter		Study groups			P value		
		Control	None responsive	Responsive	Control vs None responsive	Control vs Responsive	None responsive vs Responsive
Sex	Female	34 68.00%	41 82.00%	38 76.00%	0.165^{NS}	0.505 ^{NS}	0.624 ^{NS}
	Male	16 32.00%	9 18.00%	12 24.00%			
Body mass index		24.6 (23.9-25.5)	30 (29.4-31.1)	27.95 (25.9-29.4)	0.018*	0.442 ^{NS}	0.108 ^{NS}
RF	Negative	48 96.00%	3 6.00%	17 34.00%	<0.001**	<0.001**	<0.001**
	Positive	2 4.00%	47 94.00%	33 66.00%			
ACPA	Negative	50 100.00%	0 0.00%	26 52.00%	<0.001**	<0.001**	<0.001**
	Positive	0 0.00%	50 100.00%	24 48.00%			
Smoking habit	Yes	4 8.00%	12 24.00%	12 24.00%	0.056^{NS}	0.056^{NS}	0.999^{NS}
	No	46 92.00%	38 76.00%	38 76.00%			
MTX treatment	Yes		33 66.00%	38 76.00%			0.378^{NS}
	No		17 34.00%	12 24.00%			
CDAI			20 (18-23)	12 (11-15)			<0.001**
Calprotectin (pg/ml)		94.33 (87.28-98.86)	530.46 (480.52-596.19)	111.1 (104.37-115.45)	<0.001**	<0.001**	<0.001**

NS: non-significant, *: P < 0.05, **: P < 0.01, RF: Rheumatoid factor, ACPA: Anti-citrullinated protein antibodies, CDAI: Clinical disease activity index, MTX: Methotrexate

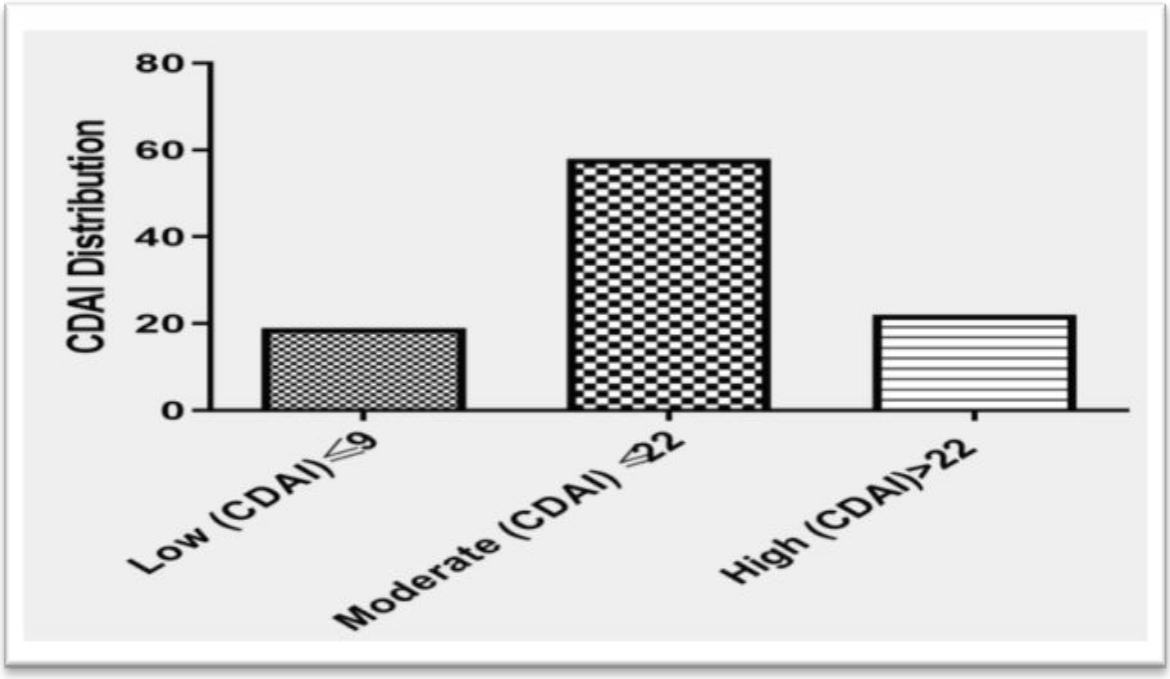

Figure 1. Clinical disease activity index distribution score in patients

As well as there was a significant correlation between calprotectin with CDAI, RF and ACPA and between CDAI and ACPA in RA group, as shown in (Table 2).

Table 2. Correlation among body mass index, clinical disease activity index, rheumatoid factor, anti-citrullinated protein antibodies and calprotectin in rheumatoid arthritis patients

Parameter		Clinical disease activity index	Rheumatoid factor	Anti-citrullinated protein antibodies	Calprotectin
Body mass index	r	0.088	0.088	0.074	0.140
	p	0.385	0.384	0.465	0.165
Clinical disease activity index	r	1.000	0.172	0.260**	0.395**
	p		0.087	0.009	0.000
Rheumatoid factor	r		1.000	0.046	0.281**
	p			0.652	0.005
Anti-citrullinated protein antibodies	r			1.000	0.582**
	p				0.000

**: P< 0.01

Discussion

The present study revealed a noteworthy disparity in the BMI between non-responder patients with RA and the healthy control group. Moreover, it was observed that obesity is linked to heightened arthritis activity and a diminished likelihood of responding to anti-TNF drugs. Conversely, weight loss enhances the likelihood of treatment success. Women have a higher incidence of RA compared to men due to many causes, including the influence of sex hormones such as estrogens

and their heightened susceptibility to infections [16]. Additionally, environmental triggers, such as stress and their unique reaction to external contaminants, as well as hereditary variables, contribute to this disparity [17]. The present findings indicate that there was a total of 28 smokers, consisting of 10 responders, 12 non-respondents, and 6 control subjects. In contrast, there were 122 non-smokers, comprising of 38 responders, 37 non-responders, and 47 control subjects. A study conducted by van Wesemael et al., (2016) demonstrated a correlation between cigarette smoking and the presence of numerous autoantibodies, specifically RF and ACPA [18]. Cigarette smoking may disrupt tolerance to autoantigens in RA, potentially serving as a trigger for the onset of RA in some patient subgroups [19].

The study included 70 patients who received MTX, with 37 being responders and 33 being non-responders. Additionally, 30 patients did not receive MTX, with 13 being responders and 17 being non-responders. MTX has been utilized in the management of RA, multiple studies have assessed the alterations in disease activity following the cessation of MTX in patients with RA who have achieved a favorable response to MTX in combination with biologic disease-modifying antirheumatic drugs (b-DMARDs) [20]. MTX exhibits anti-inflammatory effects by directly and indirectly modulating the function of many cell types implicated in inflammation, such as neutrophils, monocytes, T cells, B cells, endothelial cells, and fibroblast-like synoviocytes [21].

This study revealed that 68% of responder patients with RF positive, while there were 88% seropositive in non-responder patients. RFs are present in several conditions, not limited to RA, encompassing both autoimmune and non-autoimmune disorders. They have been detected in a prevalence of up to 4% among both young, healthy individuals and the elderly [22]. RFs likely arise from the immunological response to inflammation, influenced by hereditary factors, and can potentially regulate the generation of immunoglobulins by modulating B cell activation [23].

The findings of the current investigation indicate that all 50 individuals (100%) in the non-responder group tested positive for ACPA, while only 24 individuals (48%) in the responder group tested positive, suggesting a more pronounced autoimmune reaction to citrullinated proteins. RA patients who test positive for ACPA have a diminished response to anti-TNF-α therapy [24,25].

The levels of ACPA grew as the disease activity progressed, peaking at its maximum level in patients with severe status who did not react to anti-TNF-α (infliximab) treatment. Prior findings indicate that the elevation of ACPA in the blood of individuals with RA is linked to the onset and advancement of the disease [26]. Elevated levels of ACPA and RF are both linked to a greater likelihood of experiencing erosive joint injury. ACPA may provide a higher risk compared to RF [27].

Also, there was a significant higher level of serum calprotectin in patients in comparison to apparently healthy group. There is developing evidence in RA to suggest that calprotectin is a more accurate predictor of disease activity compared to CRP and ESR [28]. Moreover, past evidence indicates that measuring calprotectin serum levels can be used as a potential method for monitoring disease activity and assessing the effectiveness of biological therapy in patients. Calprotectin is a crucial factor in the activation of the innate immune system. It enhances the movement of immune cells towards the site of infection and triggers the activation of polymorphonuclear cells (PMN) [29]. Additionally, it stimulates the synthesis of inflammatory proteins called cytokines and chemokines, hence contributing to the development of RA [29]. There was a significant correlation between calprotectin with CDAI, RF and ACPA. Specifically, seropositive individuals with active illness exhibited elevated levels of calprotectin, which were related to higher levels of ACPA and RF. This suggests that calprotectin can serve as a biomarker for RA, both for prognosis and monitoring of disease progression, in conjunction with other biomarkers.

Inc conclusion, calprotectin serum level may be considered as a valid target for monitoring the

progression of RA disease and response to TNF-α inhibitor therapy.

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Author contribution

Jassim: Conducted the lab tests of the research and preparation the initial version of this manuscript. Dr. Abbas: Design and supervised the project with preparation of the final manuscript. Dr. Al-Osami: facilitated patient recruitment and aided in the collection of samples.

Conflict of interest

None.

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