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RESEARCH ARTICLE

Serum calreticulin in rheumatoid arthritis and its relation with disease activity and severity.

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Abstract

Objectives: To assess the serum level of CRT in RA patients and its association with disease activity and severity.

Methods: this case-control study was carried out at Rheumatology and Rehabilitation Department, Zagazig University Hospitals on 80 subjects. The subjects were divided into two groups: RA group: Forty patients with rheumatoid arthritis who were randomly selected. The patients fulfilled the 2010 American Collage of Rheumatology (ACR) / European League Against Rheumatism Classification (EULAR) criteria for rheumatoid arthritis. Control group: Forty apparently healthy subjects age and sex matched with the patients. Assessment of disease activity in RA patients was done by Disease Activity Score 28 (DAS 28). Also VAS of pain was recorded, Modified Sharp Score and Hochberg Functional status were also assessed . Assessment of disease severity in RA patients was done by Rheumatoid Arthritis Severity Scale (RASS). The concentration of CRT in all collected serum was detected by sandwich ELISA.

Results: There was a highly significant difference between RA group and control group as regards serum CRT (MW=49.5, $p < 0.001$). The correlations of serum CRT with DAS 28 and RASS were highly significant ($P< 0.001$), and correlations with disease duration, VAS, and Modified Sharp Score were significant ($p < 0.01$), while correlations with age & other activity parameters were non significant ($p > 0.05$).

Conclusion: we concluded that serum CRT levels are elevated in RA patients, providing further evidence for a role of CRT in RA. Furthermore, serum CRT levels parallel the degree of activity and severity of the disease.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown cause. In most patients, RA has an insidious onset. The pathogenesis of RA is not completely understood. An external trigger (eg, cigarette smoking, infection, or trauma) that triggers an autoimmune reaction, leading to synovial hypertrophy and

chronic joint inflammation along with the potential for extra-articular manifestations, is theorized to occur in genetically susceptible individuals. Synovial cell hyperplasia and endothelial cell activation are early events in the pathologic process that progresses to uncontrolled inflammation and consequent cartilage and bone destruction [1].

Calreticulin (CRT) was discovered in skeletal muscle cells in 1974. Thomas Ostwald and David MacLennan were characterizing proteins that bind to calcium in the sarcoplasmic reticulum; a specialized type of endoplasmic reticulum found in muscle cells. Ostwald and MacLennan isolated seven proteins from an extract of rabbit skeletal muscle sarcoplsamic reticulum, and when they measured the calcium binding activity, only one protein showed a high affinity for the element. They gave this newly identified protein the name "High Affinity calcium Binding Protein" (HABP) [2]. The protein was then named "calreticulin", reflecting its calcium binding capacity and its localization in the sarcoplasmic/endoplasmic reticulum. Next, scientists compared the human CRT amino acid sequence with other known sequences from different organisms using phylogenetic analyses. So far, CRT has been found in all eukaryotic cells, except erythrocytes (these cells lack an ER) and yeast [3].

The full-length CRT is of 46 Kda [4]. CRT has three domains: an amino (N) terminal domain, a central or (P) domain, and a Carboxyl (C) domain [5]. CRT is expressed on the surface of various types of cells such as T and B lympohcytes, and macrophages, and acts as an important innate immune system receptor. It has been shown that the intracellular CRT can be secreted from cells [6]. CRT has been identified as an autoantigen of RA [4].

Holoshitz et al. in **2010** have revealed that CRT is implicated in a number of autoimmune processes, including molecular mimicry, epitope spreading, complement inactivation and stimulation of inflammatory mediators, such as nitric oxide production. They suggested that CRT is not just an autoantigen, but plays an active role in the pathology of various autoimmune diseases. They indicated that cell surface CRT (csCRT) acted as a signal transducing receptor for the RA-shared epitope [7].

Ling et al. (2013) have identified SE as a ligand that interacts with csCRT and activates immune dysregulation, determining the effect of CRT citrullination on shared epitope (SE) signaling. They documented that citrullinated CRT is over-abundant in the RA synovium, and potentiates SE-activated signaling *in vitro [8]*. Furthermore, **Tarr et al. (2010)** determined which factors might inhibit FasL-Fas binding in vivo and those that would inhibit apoptosis of T lymphocytes in an in vitro model system. They demonstrated that extracellular CRT was present in the joints of patients with RA and inhibited FasL-mediated apoptosis of T cells [9].

Although CRT had been shown previously to be associated with RA, the serum concentration of CRT has seldom been reported, and its association with disease activity and individual clinical manifestations has not been established. Only in **2013, Ni et al.** evaluated the serum levels of CRT in RA and the correlations between CRT levels and RA disease activity, various clinical and laboratory parameters and concluded that the elevated serum CRT levels in RA patients parallel the degree of disease activity [10]. Moreover, to our best knowledge, no previous studies evaluated the relation between serum CRT and disease severity. So the aim of our study was to assess the serum level of CRT in RA patients and whether it is associated with disease activity and severity or not..

2. Subjects and methods

2.1. Subjects

After review and approval by the Institutional Review Board (IRB) Committee, this case-control study was carried out at the outpatient and inpatient clinics of Rheumatology and Rehabilitation Department, Zagazig University Hospitals on 80 subjects after taking a written consent from them for ethical consideration. The subjects were divided into two groups: Group 1 (RA group): Forty patients with rheumatoid arthritis who were randomly selected. The patients fulfilled the 2010 American Collage of Rheumatology (ACR) / European League Against Rheumatism Classification (EULAR) criteria for rheumatoid arthritis [11]. Group 2 (control group): Forty apparently healthy subjects age and sex matched with the patients.

2.2. Clinical and radiological assessment:

All patients were subjected to detailed history taking, general & local examination. Assessment of disease activity in RA patients was done by Disease Activity Score 28 (DAS 28) [12]. Also Visual Analogue Scale (VAS) of pain was recorded [13], Modified Sharp Score [14] and Hochberg Functional status [15] were also

assessed . Assessment of disease severity in RA patients was done by Rheumatoid Arthritis Severity Scale (RASS) [16].

2.3. Laboratory investigations which included complete blood picture (done on Sysmex cell counter), liver & kidney functions tests, total calcium, C-reactive protein and rheumatoid factor (these parameters were done on Cobas Integra 400 analyzer), antibodies to cyclic citrullinated peptide (Anti-CCP) (done on Cobas e 411 analyzer), these two analyzers were provided by (Roche diagnostics). Some of these data were taken from the patients' files at rheumatology & rehabilitation department.

Venous blood samples (5 mL) were drawn from subjects, these samples were divided into EDTA-containing tubes and tubes without anticoagulant. Serum samples of all subjects were collected after centrifugation of blood samples without anticoagulants at 4,000 rpm/min for 10 min , for CRT determination stored samples at -80 °C until measurements .

Measurement of serum CRT was done by quantitative sandwich enzyme immunoassay technique (ELISA kit produced by SunRed Co). According to the standard protocol of manufacturer, forty microliters of sample or standards, 10 µl of rabbit anti-human CRT and 50 µl of HRP-labeled streptavidin were added to the wells. After that, the plate was left to incubate at 37 °C for 1 h. Wells were washed for five times for 30 s for each rinse. The chromogenic agent was applied and the reaction was developed for 10 minutes at 37 °C in the dark. After adding the stop solution, the wells were assessed by ELISA plate reader at 450 nm.

2.4. Statistical methods:

Data collected throughout history, basic clinical examination, and outcome measures coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the social sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis. According to the type of data, the following tests were used to test differences for significance, differences between frequencies (qualitative variables) and percentages in groups were compared by Chi-square test (X^2), and differences between means (quantitative variables) in two parametric groups were compared by Student's t test (t). Non parametric data were analyzed by Mann Whitney U (MW) test. Correlations were used to study the relationship between resistin and studied parameters. Pearson correlation was used. The p value was assumed to be significant at 0.05 or less and highly significant at 0.001 or less.

3. Results:

3.1. Demographic, clinical & laboratory characteristics of SLE patients

Table (1) shows the demographic, clinical and laboratory characteristics of RA patients. The mean age of our patients was 48.3 ± 9(21-66), and mean disease duration was 11 ± 8.9(1-35). Thirty three percent of our patients were females. The most prominent extraarticular feature was dry mouth (35%). All patients were seropositive (100%).

Table (1): Demographic, clinical and laboratory characteristics of RA patients.

RA patients' characteristics (n=40)	
Demographic Data	
Age in yrs *(Mean ± SD)*	48.3 ± 9(21-66)
Duration *(Mean ± SD)*	11 ± 8.9(1-35)
Gender: Males *No(%)*	7 (17.5%)
Females *No(%)*	33 (82.5%)
Clinical features *No(%)*	
Morning stiffness	35(87.5)
Articular involvement	40(100)
Dry eye	6(15)
Dry mouth	14(35)

Lymphadenopathy	1(2.5)
Subcutaneous nodules	7(17.5)
Pulmonary	7(17.5)
Heart	4(10)
Gastrointestinal	7(17.5)
Renal	0(0)
Laboratory investigations *Mean ± SD(Range)*	
ESR (m.m./h.)	37.4 ± 21.3(5-95)
CRP (mg/L)	17.16 ± 18.8(1-87.5)
Hemoglobin (g/dl)	11.9 ± 1.6(6-14.5)
WBCs count ($x10^3$/ul%)	7.7 ± 2.2(2.3-12.9)
Platelets count($x10^3$/ul%)	219.7 ± 82(69-450)
ALT (U/L)	29.5 ± 21(6-117)
AST (U/L)	24.1 ± 12(8-53)
Serum Urea(mg/dl)	19.9 ± 8.5(4-42)
Serum Creatinine (mg/dl)	0.7 ± 0.2(0.2-1.2)
Serum Calcium (mg/dl)	9.2 ± 1(7-12)
Serology *No(%)*	
Positive Rheumatoid factor	40(100)
Positive Anti-CCP	30(75)
Medications *No (%)*	
Corticosteroids	18(45)
Methotrexate	29(72.5)
Leflunamide	13(32.5)
Salazopyrine	12(30)
Antimalarial	33(82.5)

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, WBCs: white blood cell, ALT: alanine transaminase, AST: aspartate transaminase, Anti-CCP: Anti-cyclic citrullinated peptide.

Table (2) shows disease activity and severity assessments in our RA patients. Twenty eight percent of our patients were highly active having DAS 28 > 5.1. Fifty percent of our patients were in class III Hochberg functional status. Many of our patients had significant physical damage on radiography represented as joint space narrowing and erosions.

Table (2): Disease assessments in RA patients including activity, severity, and functional status.

Disease Assessments	
VAS *Mean ± SD(Range)*	4.8 ± 2.1 (2-9)
DAS28 *Mean ± SD(Range)*	5.7 ± 1.6 (2-8)
Mild activity *No(%)*	7(7.5)
Moderate activity *No(%)*	5(12.5)
High activity *No(%)*	28(70)
Hochberg Functional Status *No(%)*	
Class I	2(5)

Class II Class III Class IV	17(42.5) 20(50) 1(2.5)
Modified Sharp Score *Mean ± SD(Range)* Erosion score Space narrowing	 6.1 ± 7.1(0-35) 47.3 ± 20(7-103)
RASS *Mean ± SD(Range)* Disease activity Functional impairment Physical damage Total	 59 ± 19(20-80) 52.2 ± 14.5(20-80) 36.7 ± 15.5(10-70) 144 ± 39.3(50-230)

RA: Rheumatoid arthritis, SD: standard deviation, No(%): number and percentage of cases. VAS: Visual analogue scale [13], DAS28: Disease activity score [12], RASS: Rheumatoid arthritis severity scale [16]. Modified Sharp Score [14] and Hochberg Functional status [15].

3.2. The comparison between serum CRT levels among patients & controls

Figure (1) shows that there was a highly significant difference between RA group and control group as regards serum CRT in ng/ml (MW=49.5, $p < 0.001$).

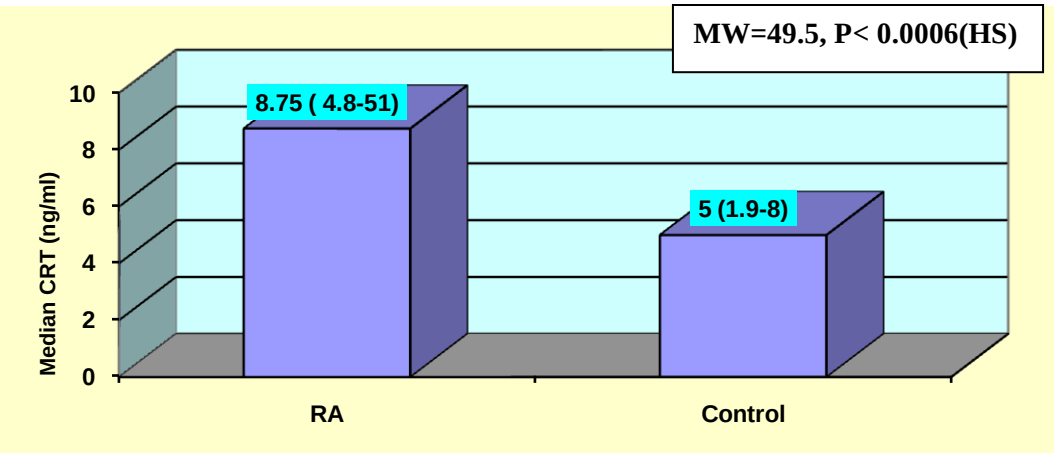

Figure (1): Serum calreticulin measured in ng/ml in RA group (n=40) and control group (n=40). The figure shows that serum calreticulin was higher in RA group compared to control group and the analysis was done by Mann Whitney U test (MW=49.5, $p < 0.001$). RA: Rheumatoid arthritis, CRT: calreticulin.

3.3. Correlations between different patient characteristics and serum CRT

Table (3) shows that correlations of serum CRT with DAS 28 and RASS were highly significant ($P< 0.001$), and correlations with disease duration, VAS, and Modified Sharp Score were significant ($p < 0.01$), while correlations with age & other activity parameters were non significant ($p > 0.05$).

Table (3): Correlations between different patient characteristics and serum CRT

Patient Characteristics	r	P
Age	0.02	0.83
Disease duration	0.4	0.05 (S)
Disease Activity		
VAS	0.31	0.04 (S)
DAS 28	0.4	< 0.001 (HS)
Morning Stiffness	-0.06	0.7
Hemoglobin	-0.1	0.8
CRP	0.14	0.15
Albumin	0.04	0.6
Modified Sharp score		
Erosion	0.36	0.04 (S)
Space narrowing	0.4	0.037 (S)
RASS		
Disease activity	0.39	0.0037 (HS)
Functional impairment	0.34	0.0072 (HS)
Physical damage	0.34	0.0044 (HS)
Total score	0.45	0.0032 (HS)

r: correlation coefficient, VAS: visual analogue scale, DAS 28: disease activity score, CRP: C-reactive protein, RASS: rheumatoid arthritis severity scale, (S): significant, (HS): Highly significant.

4. Discussion:

Rheumatoid arthritis is a systemic inflammatory disease characterized by a chronic inflammatory process, based primarily on synovial membrane that is typically associated with serological evidence of systemic autoimmunity and characterized by persistant joint inflammation that results in joint damage and loss of function [17]**.**

Calreticulin (CRT) has been identified as an autoantigen of RA. The full-length CRT is of 46 kDa, an endoplasmic reticulum (ER) chaperone responsible for Ca2+ homeostasis and glycoprotein folding. CRT, expressed on the surface of various types of cells such as T and B lymphocytes, and macrophages, acts an important innate immune system receptor. It has been shown that the intracellular CRT can be secreted from cells [6]**.**

Although CRT had been shown to be associated with RA, the serum concentration of CRT has seldom been reported, and its association with disease activity and individual clinical manifestations has not been established. Also, its association with disease severity has not been studied before. So, we aimed to determine the serum level of calreticulin in rheumatoid arthritis patients and to assess the relation between serum levels of calreticulin and disease activity and severity of rheumatoid arthritis patients.

RA is a disease of middle age, in which females are affected 3 times more than males [18]**.** Our controls were age and sex matched to the patients and there was no significant difference between the 2 groups regarding age and sex ($p > 0.05$).

Regarding CRT level, it was higher in RA group than in control group and the difference was highly significant ($p < 0.001$), agreeing with the fact that CRT interactions produce immune dysregulation denoting its pathophysiological role in RA [8]**.** These results were similar to the results of **Hong et al.** in **2010** who reported that serum CRT levels were highly in patients with RA compared with control group [6]. Moreover, **Tarr et al.** in **2010** [9] and **Ni et al.** in **2013** [10] also described increased plasma levels of CRT in RA patients.

In our study, as regards to disease duration, there was a significant correlation between serum CRT and disease duration ($p < 0.05$), while correlation with age was non-significant ($p > 0.05$). Serum CRT level in female

RA patients was higher than male RA patients and the difference was significant ($p < 0.05$). These results are in agreement with results reported by **Ni et al.** in **2013** [10].

DAS28 is now the most commonly used index for assessing the level of disease activity in RA patients [19]**.** In our study, there were a highly significant correlation between CRT and DAS-28 ($p < 0.001$) and a significant correlation with VAS ($p < 0.05$). This matches the fact that CRT stimulates the release of inflammatory mediators such as NO production [7].

These results were in agreement with the study of **Ni et al.** in **2013** who evaluated the serum levels of CRT in RA patients and its correlation with RA disease activity, various clinical and laboratory parameters [10]. This study found a significant correlation between serum CRT levels and disease activity in RA.

Autoantibodies in RA are useful not only for diagnosis but also prognosis. It is reported that IgM RF, are associated with severe erosive disease. It is also accepted that the presence of anti-CCP antibodies is associated with presence of deformities [20]**. Ling et al.** in **2013** stated that calreticulin potentiates RA-shared epitope signaling and found that citrullinated CRT is overabundant in RA synovium [8]. This is in agreement with our study in which there was a significant correlation between erosion and joint space narrowing (modified Sharp score) and serum CRT level.

In our study we were limited to assess the difference between serum CRT in seropositive and seronegative patients since all our patients were seropositive.

Also, there was a highly significant correlation between serum calreticulin and all 3 dimensions of RA Severity Scale and the total RA severity scale ($p < 0.001$). These findings indicated that not only RF and anti-CCP denote severe disease but also serum CRT may be considered as a novel marker for assessment of disease severity in RA patients. But we did not find any previous study assessing the relation between CRT and disease severity, making this a field for further research.

We concluded that serum CRT levels are elevated in RA patients, providing further evidence for a role of CRT in RA pathophysiology. Furthermore, serum CRT levels parallel the degree of activity and severity of the disease which may identify patients with more active and severe disease, who might benefit from more aggressive treatment. Finally, we suggest that serum CRT is a promising marker for disease activity and severity in RA.

Conflict of interest:

There is no conflict of interest to declare.

References :

1. Komano Y, Harigai M, Koike R, et al. (2009): Pneumocystis jiroveci pneumonia in patients with rheumatoid arthritis treated with infliximab: a retrospective review and case-control study of 21 patients. Arthritis Rheum; 61(3):305-12.

2. Fliegel L, Burns K, MacLennan DH, et al. (1989): Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J Biol Chem; 264 (36):21522-8.

3. Michalak M., Corbett,E.F., Mesaeli, et al. (1999): Calreticulin: one protein, one gene, many functions. Biochem. J., 344, 281–292.

4. Goeb V, Thomas-L'Otellier M, Daveau R, et al. (2009): Candidate autoantigens identified by mass spectrometry in early rheumatoid arthritis are chaperones and citrullinated glycolytic enzymes. Arthritis Res Ther 11: R38.

5. Waisman DM, Salimath BP, Anderson MJ. (1985): Isolation and characterization of CAB-63, a novel calcium-binding protein. J Biol Chem. Feb 10;260(3):1652–1660.

6. Hong C, Qiu X, Li Y, et al. (2010): Functional analysis of recombinant calreticulin fragment 39–272: implications for immunobiological activities of calreticulin in health and disease. J Immunol; 185:4561–9.

7. Holoshitz J, De Almeida DE, Ling S (2010): A role for calreticulin in the pathogenesis of rheumatoid arthritis. Ann N Y Acad Sci; 1209: 91–8.

8. Ling S, Cline EN, Hang TS, et al. (2013): Citrullinated calreticulin potentiates rheumatoid arthritis shared epitope signaling. Arthritis Rheum; 65(3): 618-626.

9. Tarr JM, Winyard PG, Ryan B, et al. (2010): Extracellular calreticulin is present in the joints of patients with rheumatoid arthritis and inhibits FasL (CD95L)-mediated apoptosis of T cells. Arthritis Rheum; 62:2919–29.

10. Ni M, Wei W, Wang Y, et al. (2013): Serum levels of calreticulin in correlation with disease activity in patients with rheumatoid arthritis. J Clin Immunol.

11. Aletaha D, Neogi T, Silman AJ, et al. (2010): Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum; 62(9):2569-81.

12. Prevoo ML, van 't Hof MA, Kuper HH, et al., (1990): Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arth Rheum; 38(1):44-8.

13. Dixon JS and Bird HA (1981): Reproducibilty along 10 cm. vertical viscual analogue scale. Ann Rheum.Dis.(40):87-9.

14. Van der Heijde D (1999): How to read radiographs according to the Sharp/van der Heijde method. J Rheumatol; 26: 743-5.

15. Hochberg MC, Chang RW, Dwosh I, et al. (1991): The American College of Rheumatology revised criteria for the classification of global functional status in rheumatoid arthritis. Arthritis Rheum; 39(5): 498-502.

16. Bardwell WA, Nicassio PM, Weisman MH, et al. (2002): Rheumatoid arthritis severity scale: A brief, physician-completed scale not confounded by patient self-report of psychological functioning. Rheumatology; 41: 38-45.

17. EL Gabalawy HS and Wilkins JA. (2004): Anti-Sa antibodies prognostic and pathogenic significance to rheumatoid arthritis: Arthritis Rem Ther; 6:86-9.

18. Areskoug-Josefsson K and Oberg U (2009): A literature review of the sexual health of women with rheumatoid arthritis. Musculoskeletal Care; 7(4):219-26.

19. Farheen K and Agarwal SK (2011): Assessment of disease activity and treatment outcomes in rheumatoid arthritis. J Manag Care Pharm; 17: S09–13.

20. Agrawal S, Misra R, Aggarwal A (2007): Autoantibodies in rheumatoid arthritis: association with severity of disease in established RA. Clin Rheumatol; 26:201–4.