Original Article

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Comparative analyses of immune marker levels in seronegative and seropositive Iraqi rheumatoid arthritis patients

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ABSTRACT

Background: Rheumatoid arthritis (RA) comprises an inflammation-based condition with a clinical phenotype that is mainly reliant on the occurrence of rheumatoid factor and anti-citrullinated protein antibodies. Literature indicates that seronegativity might be accompanied by higher severity of clinical symptoms, while higher disease activity, based on the recent classification criteria should also be associated with seronegativity. **Aim:** This investigation targets the identification of more efficient immune markers of RA seropositivity as well as examining the possibility that the latter is associated with lower severity. **Methods**: Serum samples from 128 RA patients, 106 seropositive and 22 seronegative, were analyzed using ELISA to determine the levels of four biomarkers: Wnt-1-induced secreted protein-1 CCN4, vascular cell adhesion molecule-1 (VCAM-1), matrix melloprotenase-3 (MMP-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Additional clinical parameters were also investigated through a special questionnaire form that also covered demographic data. As a measure of RA activity, the parameter of joint disease activity score 28 (DAS28) was adopted. **Results**: Patients with seronegativity demonstrated higher RA activity, i.e., DAS28ESR score, in comparison with seropositive ones. Their sera also contained higher levels of the four tested immune markers in comparison with both seropositive patients and healthy controls. Das-28 ESR levels exhibited significant direct proportional correlations to CCN4, VCAM1, MMP3, GM-CSF, and Das-28CRP values. **Conclusions**: The tested four immune markers are considered biomarkers with high reliability and effectiveness to discriminate between seronegative and seropositive patients, as well as for the monitoring and prediction of RA activity and joint and bone damage in seronegative patients.

Keywords: rheumatoid arthritis, seronegative, seropositive, CCN4, VCAM-1, MMP-3

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INTRODUCTION

Rheumatoid arthritis (RA) comprises a condition of autoimmunity with characteristic inflammatory reactions in the synovium. These reactions result in irreversible damage to the bone and cartilage tissues.[1] One major phenotypic feature of the disease is seronegativity to rheumatoid factor (RF) and anti-

citrullinated protein antibodies (ACPAs).[2] The literature suggests that symptoms with higher severity at baseline accompany seronegativity; this phenomenon is required by the recent criteria for classifying the disease stages.[3] Moreover, RF and ACPA are not detectable in the sera of about 25% of the patients,[4] .Mouterde et al. described RA progression with the absence of these two serum markers in RA patients showing early inflammatory reactions.[4] Therefore, these two markers are considered to providing indicators a poor prognosis of the disease; they are utilized as a justification to intensify the treatment in patients with seropositivity.[5] Hence, it is uncertain at present whether seronegative patients have a better disease course compared to seropositive ones when the parameters of RA activity and radiological examination results are considered.[6] Information related to variations in the levels of systemic inflammatory markers among seropositive and seronegative patients is scarce.[7] In spite of the acceptability of antibodies against Anti-Cyclic Citrullinated Peptide (anti-CCPs)and RFs in the diagnosis of the disease, novel markers need to be discovered for better amelioration of the disease diagnosis. Despite the fact that many serum markers have been screened in RA, extensive investigations have been directed toward certain markers as better tools to reflect RA activity and monitor progress and responsiveness to treatment; however, the level of attention paid to these markers by specialists in this field has been insufficient.[8] Recently, researchers have been focused on collecting evidence on the role of the CCN family in RA. It is essential to comprehend this role in the pathophysiology of both seropositive and seronegative RA to improve the therapeutic approaches targeting molecular pathways, hopefully reducing pain in patients. Wnt-1-induced secreted protein-1 (WISP1/CCN4) is a member of the CCN family of matricellular proteins.[9] CCN4 displays an autocrine action of accelerating cell growth, inducing morphological transformation, increasing saturation density, and promoting tumorigenesis.[10] Despite uncertainty in RA pathogenesis, previous studies have proven the contribution of mononuclear cell migration involving vascular cell adhesion molecule-1 VCAM-1 in the inflammatory reactions in the synovium.[11] Preclinical results related to flares of RA that occur after treating the patients with granulocyte-macrophage colony-stimulating factor (GM-CSF) confirm this essential role in the pathogenesis.[12] Moreover, despite a bulk of research that targeted the role of the enzyme matrix metalloproteinase-3 MMP-3 in reflecting RA activity and in following up progress and therapeutic responsiveness, consideration of the results by specialists is minimal. Fibroblasts in the synovium synthesize and secrete MMP-3.Joint chondrocytes are implicated in joint injury. associated with RA.[13] This is a novel study aimed at assessing the possibility of higher severity in RA patients with seronegativity to the common markers by measuring serum levels of CCN4, VCAM-1, MMP-3, and GM-CSF in the RA seropositive and seronegative patients and their correlation to disease activity.

MATERIALS AND METHODS

The retrospective study was conducted from October 2022 to July 2023. The cohort comprised 250 adult males and females in the age range of 20–73 years. Among them, 128 participants met the 2010 American College of Rheumatology/European Alliance of Association for Rheumatology (ACR/EULAR) classification criteria.[14] They were selected from rheumatology consulting clinics (Al-Fayhaa, Al-Basrah, and Al-Sadder teaching hospitals; Basrah, South Iraq) and subjected to clinical diagnosis by a rheumatologist.

The 128 patients were divided into two subgroups, namely seropositive (SPRA; n = 106) and seronegative (SNRA; n = 22), based on the antibody levels against RF and anti-CCP. The patients were under different treatment protocols, including disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), steroids, and biological treatments. Hospital staff and other healthy subjects from different locations comprised the control group of 60 individuals, sex- and age (24–71 years)-matched, as shown in Figure 1. Questionnaire-based interviews were employed to collect historical data on the clinical and physical tests. Parameters assessed in the first examination covered demographic data, clinical findings, disease activity, drug history, presence of articular and extra-articular symptoms, presence of concomitant comorbid diseases, and outcomes of laboratory examinations. Information encompassed the demography and anthropometry of participants: age, body weight, height, family history, duration of the disease, and clinical symptoms. Data related to physical tests involved joint pain, joint swelling, morning stiffness, and spindle shape. The methodology of the World Health Organization (WHO) was adopted for body mass index (BMI) extraction through the division of height (m^2) by weight (kg). The WHO classification system was utilized

for the categorization of the patients into underweight (< 18.5 kg/m^2), normal (18.5–25 kg/m^2), overweight (25–<30 kg/m^2), or obese (> 30 kg/m^2). Those excluded were advanced cases with severe joint deformity, age < 20 years, refusal to participate, pregnancy, other types of autoimmunity, acute severe infection, acute cardiovascular disease, diabetes mellitus, hypertension, malignancy, and smoking, treatment with angiotensin-converting enzyme inhibitors, oral contraceptives, and statins. Laboratory tests included blood sampling and measuring the erythrocyte sedimentation rate (ESR) test, cyclic citrullinated peptides (anti-CCP), C-reactive protein (CRP), and RF levels. The CCN4, VACM-1, MMP3 proteins and GM-CSF levels in the sera of all participants were assessed using the enzyme-linked immune-sorbent assay (ELISA) kits, following the steps defined by the manufacturer (Elabscience,USA). Disease activity (DAS28- ESR) was calculated after the values of swollen joint count and tender joint count in 28 joints (TJC28 and SJC28, respectively) were determined. Patient's global assessment was also termed the general visual analog scale (VAS), which follows specific weighing scores (zero = best, 100 = worst). The RA activity was determined based on the following scores: remission (DAS28 ≤ 2.6), low activity (2.6 < DAS28 ≤ 3.2), moderate activity (3.2 < DAS28 ≤ 5.1), and high activity (DAS28 > 5.1).

Ethics approval for the study was obtained from the Research Committee of the Training and Human Development Center, Basra Health Department, Ministry of Health, according to resolution no. 270/2022, dated October 26, 2022. In addition, written informed consent was obtained from all the participants.

The data were statistically analyzed using SPSS, version 26 (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk and Kolmogorov-Smirnov tests were adopted to decide if the quantitative results were normally distributed. The mean ± standard deviation was adopted to reflect the parametric results. Student's t-test was adopted to decide if the parametric results between groups were significant, whereas Mann-Whitney *U* test was adopted for non-parametric results. The non-parametric results of more than two groups were evaluated using the Kruskal-Wallis test. Spearman's non-parametric correlation test was adopted to determine the correlation between the groups with quantitative results. A threshold of statistical significance of differences was set at $p < 0.05$.

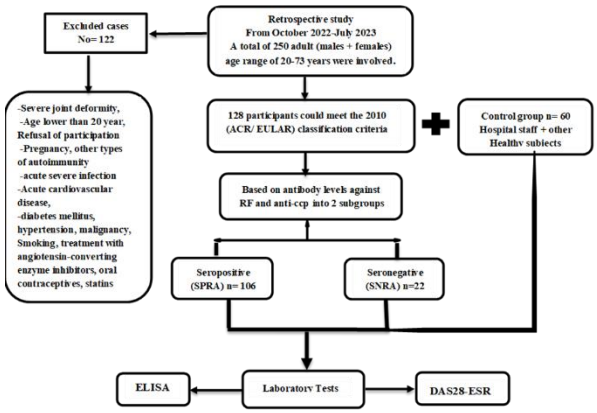

Figure 1: Flow chart of the study

RESULTS

Tested variables were significantly different ($p \leq 0.05$) between seropositive and seronegative RA in terms of sex, age, BMI, duration of disease, anti-CCP, CRP, RF, ESR, DAS28 ESR, and DAS28 CRP (Table 1). Surprisingly, seronegative patients had significantly higher (p = 0.0001) serum levels of CCN4, VCAM-1, MMP3, and GM-CSF (4954.4 ± 1871.9 pg/mL, 2278.7 ± 1572.2 ng/mL, 326.3 ± 192.0 ng/mL, and 128.1 ± 73.5 ng /mL, respectively) than the seropositive patients (4330.5 ± 1684.5 pg/mL, 1632.1 ± 1073.7 ng/mL, 241.4 ± 142.4 ng/mL, and 126.2 ± 47.7 ng/mL, respectively) and healthy controls (439.5 ± 205.8 pg/mL, 302.5 ± 65.4 ng/mL, 52.5 ± 22.2 ng/mL, and 3.02 ± 1.93 ng/mL, respectively), as shown in Table 1.

Table 2 clarifies the directly proportional significant correlations between DAS28 ESR levels and CCN4, CAM1, MMP3, GM-CSF, and DAS28 CRP levels in RA patients.

Table 1: Comparison of serum levels of CCN4, VCAM-1, MMP3, and GM-CSF in seropositive and seronegative RA patients compared to healthy controls

Parameter	Total RA patients N=128		Healthy Control (N = 60)	*p*-value
	Seropositive RA	Seronegative RA		
	N = 106	N = 22		
Sex				
Female, N (%)	90 (67.7%)	16 (12%)	27 (20.3%)	0.0001
Male, N (%)	16 (29.1%)	6 (10.9%)	33 (60.0%)	
Age, year	49.04 ±11.16	48.64 ± 13.35	49.65 ± 12.01	0.1
Range	(20–73)	(21–72)	(24–71)	
BMI (kg/m²)	30.38 ± 4.42	29.37 ± 5.19	26.16 ± 7.17	0.0001
Duration of disease	18.42 ± 7.15	8.31 ± 2.71	-	0.0001
Range, month	(0.5–240)	(0.5–72)	-	
Anti-CCP (IU/mL)	30.38 ± 9.94	14.70 ± 10.49	4.00 ± 1.52	0.0001
CRP (mg/dL)	15.42 ± 3.90	12.94 ± 5.12	3.11 ± 0.82	0.0001
RF (IU/mL)	21.39 ± 10.92	13.42 ± 3.88	7.51 ± 1.87	0.0001
ESR (mm/h)	38.92 ± 12.84	36.09 ± 10.02	14.30 ± 5.13	0.0001
DAS28 ESR	4.92 ± 0.91	5.13 ± 1.17		0.0001
DAS28 CRP	4.24 ± 0.78	4.39 ± 0.78		0.0001
CCN4 (pg/mL)	4330.5 ± 1684.5	4954.4 ± 1871.9	439.5 ± 205.8	0.0001
VCAM-1 (ng/mL)	1632.1 ± 1073.7	2278.7 ± 1572.2	302.5 ± 65.4	0.0001
MMP3 (ng/mL)	241.4 ± 142.4	326.3 ± 192.0	52.5 ± 22.2	0.0001
GM-CSF (pg/mL)	126.2 ± 47.7	128.1 ± 73.5	3.02 ± 1.93	0.0001

Data represented as mean ± Std. *p value is significant at 0.05.
BMI: Body Mass Index; **Anti-CCP:** Anti-Cyclic Citrullinated Peptide; **CRP:** C-Reactive Protein; **RF:** Rheumatoid Factor; **ESR:** Erythrocyte Sedimentation Rate; **DAS28 ESR:** Disease Activity Score 28 ESR; **DAS28 CRP:** Disease Activity Score28 CRP; **CCN4:** Wnt-1-induced secreted protein-1 (WISP1/CCN4); **VCAM-1:** Vascular Cell Adhesion Molecule 1; **MMP-3:** Matrix Metalloproteinase-3; **GM-CSF:** Granulocyte-Macrophage Colony-Stimulating Factor

Table 2: Assessment Correlation among Serum Levels of CCN4, VCAM-1, MMP-3, GM-CSF and DAS 28 ESR, DAS 28CRP

Parameter	PC	CCN4	VCAM-1	MMP3	GM-CSF	DAS 28 ESR
CCN4	R	1	0.906**	0.890**	0.939**	0.948**
	P		0.0001	0.0001	0.0001	0.0001
Das 28 ESR	R	0.948**	0.953**	0.926**	0.976**	1
	P	0.0001	0.0001	0.0001	0.0001	
Das -28 CRP	R	0.779**	0.763**	0.753**	0.817**	0.851**
	P	0.0001	0.0001	0.0001	0.0001	0.0001
ESR	R	0.485**	0.451**	0.342**	0.458**	0.459**
	P	0.0001	0.0001	0.004	0.0001	0.0001

*Correlation is significant at the 0.05 level (two-tailed).
** Correlation is significant at the 0.01 level (two-tailed

DISCUSSION

The present study aimed to identify new immunological markers that may accurately predict the development of RA in individuals with seronegative RA (SNRA). The literature has proposed that systemic inflammatory reactions might occur at the joint site.[15] This proposal was supported by multiple retrospective investigations showing a systemic increase in different inflammatory proteins in the pre-RA stage.[16] However, no research has so far addressed the levels of systemic inflammation markers in patients at risk of RA development but also with seronegativity to traditional RA markers.[17] seronegative RA patients are still controversially regarded as either having higher or lower severity compared to seropositive RA (SPRA)ones.[18]

The present study found significant variations with respect to extra-articular manifestations and clinical activity scales. However, variations related to the age of RA onset among SPRA and SNRA cases were insignificant. The difference between a female: male ratio in RA patients was much higher compared to that in Western populations,[19,20] and more comparable to the value in several developing countries and Brazil.[21] Our finding is aligned with that demonstrated earlier[22] about a possible relationship between female preponderance and levels of sex hormones.[23] Another possibility of this finding might be the easy access to clinical data of female patients since they are more frequently attending rheumatologic clinics. Nevertheless, such sex-related differences in disease susceptibility are still not fully explained.[24] Severe manifestations and high levels of inflammatory markers are frequently reported in obese

SNRA and SRRA patients.[25] An earlier work revealed higher synovitis scores in obese patients without RA, while an opposite correlation was found in RA patients.[26] Therefore, raising awareness of RA obese patients about the advantageous impact of decreasing weight on improved RA activity could secure another means for managing the disease.[27]

The outcome data also reported a significant variation in the average duration of disease in patients with seropositivity compared to those with seronegativity, which disagrees with the results of a previous study that found insignificant variations.[28] The mean ESR value in our sample of SNRA was decreased gradually, reflecting a slower response to treatment associated with prolonged underlying inflammatory activity. In addition, DAS 28 score in our sample showed a significantly higher value in SNRA patients, which is attributable to shorter RA duration. It is well documented that the earlier the RA is diagnosed and treated, the better the treatment outcome. This result is consistent with previous findings.[29] We also recorded higher disease activity at baseline in our SRNA sample of patients. The 2010 ACR/EULAR criteria assigned a bulk of weight to serological markers for early detection of RA. Hence, the involvement of only one or two joints in SPRA patients can be sufficient for a positive diagnosis with RA. Consistent with our findings, Nordberg et al. demonstrated higher inflammatory activity in patients with seronegativity, mirroring the need for the involvement of a higher number of joints in these patients to meet the 2010 ACR/EULAR criteria.[7] Barra et al. also demonstrated higher severity in SRNA cases.[30] Nevertheless, other researchers revealed similar or worse diseases in SPRA cases. This could be partly explained by the variations in the characteristics of the study sample.[5,31,32] The significant variations in the percentage of seropositive and seronegative RA patients fulfilling each classification criteria (1987 ACR criteria and 2010 ACR/EULAR criteria) underline the importance of these criteria in disease diagnosis, particularly in SNRA cases. Despite the notion that the 2010 ACR/EULAR criteria provide better tools for the early detection and diagnosis of RA, these criteria are largely dependent on serological tests. Hence, there is a possibility of failure of detection of some SNRA cases, as shown in this study. Such findings could also encourage primary physicians to check SNRA cases with severe disease more frequently than SPRA cases, regardless of disease severity.[33] A previous study proposed that variations between SPRA and SNRA patients with arthralgia in terms of pathogenesis and prognosis resulted from variable pathological events at the site of inflammation.[34]

Our study is the first to describe specific differences in serum immune markers (CCN4, VCAM-1, MMP-3, and GM-CSF) in SPRA and SNRA. Deane et al. showed lower frequency of pre-diagnosis samples that show positivity for cytokines in patients who later developed SNRA in comparison with those who later developed SPRA.[7] An in vitro study showed that ACPA/RF-containing immune complexes trigger cytokine release through a reaction mediated by FcγR-crosslinking.[35] The current study brings out the hypothesis of the responsibility of this mechanism in the higher expression of these markers in SPRA patients. The qualitative variations between SPRA and SNRA demonstrate the significance of the classification of RA patients based on their autoantibody status.

The role of CCN4 in RA has not yet been fully unraveled. CCN4 is a member of the CCN family of matricellular proteins. It exerts autocrine actions of accelerating cell growth, inducing morphological transformation, increasing saturation density, and promoting tumorigenesis.[36] It can also promote osteoblastic differentiation.[37] Furthermore, the significant functions of CCN4 during several cellular processes, such as cell proliferation, adhesion, migration, differentiation, along with the regulation of extracellular matrix differentiation, are well documented.[37]

The CCN4 implication in RA pathogenesis has also been frequently reported.[38,39] A previous study that focused on human synovial fluid assessed the signaling pathway responsible for the CCN4-induced VCAM-1 expression. The authors revealed that CCN4 molecule binds to αvβ5/α6β1 integrin receptor and activates Spleen tyrosine kinase (Syk), Protein kinase Cδ (PKCδ), and Jun N-terminal kinase (JNK), leading to the enhancement of AP-1 binding, trans-activation of VCAM-1 expression, and ultimately elevated VCAM-1 secretion. Such reactions promote the monocytes to adhere to Osteoarthritis synovial fibroblasts (OASFs) in humans.[40] The current study also observed an association between the levels of CCN4, VCAM-1, MMP-3, and GM-CSF in the circulation, as well as the values of DAS28-ESR, DAS28-CRP, ESR, CRP anti-CCP and RF. Such complex associations could explain the elevated levels of these serum markers. Complex associations also indicate the results of various pathophysiological reactions at the site

of inflammation. Hence, this investigation hypothesizes that these markers make the central difference between SNRA and SPRA, which might be due to the high rate of increase in their levels in seronegative patients compared to seropositive ones. This is especially true for CCN4, which is the catalyst for producing the remaining proteins.

The limitations of this study were the small sample size and that the other CCN families were not assessed. Relying on serums instead of knee synovial fluid and geographical limitations prompt the need for further studies into the intracellular signaling pathways and genetic variations in the *WISP1* gene in a large number of RA patients.

CONCLUSIONS

At baseline, RA patients with seronegativity showed a disease with higher activity in comparison to their seropositive counterparts. In addition, the elevated high levels of CCN4 VCAM-1, MMP-3, and GM-CSF in SNRA patients proved their value as markers for the prediction of disease stages as well as for the diagnosis-based classification of subjects at risk of RA development. Our findings emphasized the clinical implications of these biomarkers in managing RA more strongly. Eventually, the four immune molecules examined in the present study proved their reliability and effectiveness as biological markers that can be utilized for the monitoring and prediction of RA activity, as well as for joint and bone damage in seronegative patients with RA.

Authors' contributions

ATY, ETA: conceptualization, methodology. ATY, ETA, FHS: project administration. ATY, ETA, ANM: validation, diagnosis. ATY, ETA, investigation. ATY, ETA, FHS: resources. ATY, ETA: data curation. ATY, ETA: writing-original draft preparation. ATY, ETA: writing-review and editing. ETA. FHS: supervision. All authors have reviewed the final draft and agreed to publish this version of the manuscript.

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