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Research Article

Nephroblastoma-Overexpressed Protein as a Prognostic Marker for High Disease Activity in Iraqi Patients with Rheumatoid Arthritis

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Abstract

Background: Many data indicate a strong relationship between CCN3 and the intensity and length of RA symptoms. Furthermore, RA patients' bone deterioration, joint degeneration, and impaired functional status strongly correlate with serum CCN3 levels. ***Objective***: To evaluate the levels of CCN3 protein, IL6, and anti-MCV biomarkers with the activity of RA disease and determine if there is any relationship between CCN3, anti-MCV, and IL6 in RA patients. ***Methods***: In this prospective case-control study, 60 patients with RA were selected and subdivided according to disease activity, and 60 healthy individuals were served as controls. An enzyme-linked immunosorbent test (ELISA) was used to evaluate blood levels of CCN3, RF, anti-CCP, anti-MCV, and IL-6. ***Results***: A significant correlation was reported for CCN3 with high and moderate disease activity and ulnar deformity but not with IL6, anti-MCV, anti-CCP, sex, or duration of disease. There is a strong positive correlation between anti-MCV and disease duration, as well as anti-MCV and RF. ***Conclusions***: CCN3 has a significant association with disease activity and joint deformity with high sensitivity and specificity compared to other parameters, making it a good prognostic biomarker for RA.

Keywords: Anti-MCV, Disease activity, CCN3, IL-6, Rheumatoid arthritis, Rheumatoid factor.

البروتين المفرط التعبير عن الورم الأرومي الكلوي كعلامة إنذار لنشاط المرض العالي في المرضى العراقيين المصابين بالتهاب المفاصل الرثوي

الخلاصة

الخلفية: البروتين NOV/CCN3 هو عضو في عائلة CCN. لا يزال دور CCN3 في التهاب المفاصل الرثوي غير واضح. أشارت الأبحاث إلى وجود علاقة قوية بين CCN3 ومدة فترة الإصابة و شدة أعراض التهاب المفاصل الرثوي. بالإضافة إلى ذلك، ترتبط مستويات CCN3 في المصل بقوة في الحالات التي تصل الى مرحلة تدهور العظام، و في حالة انحلال المفاصل، وضعف الحالة الوظيفية لدى مرضى التهاب المفاصل الرثوي. **الهدف**: تقييم مستوى المؤشرات الحيوية لبروتين CCN3 و IL6 و Anti MCV مع نشاط المرض، وتحديد ما إذا كانت هناك علاقة بين CCN3 و Anti-MCV و IL6 في مرضى التهاب المفاصل الرثوي ومقارنة علاقة Anti MCV و CCN3 مع مؤشرات الالتهابات المختلفة مثل CRP و ESR و Anti CCP و RF و IL6. **الطرق:** شملت الدراسة عينة من 120 فردًا، تتألف من و60 فرد سليم و 60 فرد تم تشخيصهم بالتهاب المفاصل الرثوي، و الذين تم تقسيمهم الى مجاميع حسب نشاط المرض. وتراوحت أعمار افراد العينة بين 20 الى79 عاماً. تم استخدام اختبار (ELISA) لتقييم مستوى CCN3 في مصل الدم. Anti-CCP، و anti-MCV و IL6. **النتائج:** تم العثور على ارتباط كبير بين نشاط المرض المرتفع والمعتدل و CCN3 وكذلك CCN3 والتشوه الزندي و لم يكن هناك ارتباط ثابت بين CCN3 و IL6، Anti-MCV، Anti-CCP، RF، العمر، الجنس، ومدة العلاج. وجود علاقة إحصائية بين ESR و CRP مع نشاط المرض، كما توجد علاقة إيجابية عالية بين مضادات MCV ومدة المرض ومضادات MCV مع RF . **الاستنتاجات**: إن الارتباط بين CCN3 ونشاط المرض وكذلك CCN3 مع التشوه بالإضافة إلى الحساسية العالية والنوعية لـ CCN3 مقارنة بمعلمات أخرى تجعل هذا البروتين علامة حيوية جيدة ونذيرًا جيدًا لالتهاب المفاصل الرثوي.

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INTRODUCTION

Rheumatoid arthritis (RA) is a heterogeneous autoimmune-inflammatory illness that is characterized by the destruction of bone and cartilage in the synovium. This damage causes joint dysfunction and increases the death rate [1]. The prevalence of RA varies across the globe, with a typically higher incidence in industrialized countries. This may be explained by exposures to environmental risk factors, but it may also be explained by genetic variables, different demography, and under-reporting in other areas of the world [2]. Previous estimates indicated that RA was most common between the ages of 30 and 50, but there has been an uptick in instances reported in people's late 60s in recent years [3]. Research has shown that the immune system plays a part in developing RA, and many inflammatory cytokines and enzymes that tear down cartilage and bone are released when immune cells infiltrate synovial joints, which is the first step in developing autoimmune disease [4]. Once autoreactive B cells undergo plasma cell differentiation, they primarily

secrete and generate autoantibodies. Cross-reactivity of some post-translationally altered proteins with external antigens [5] is one way that autoreactive B cells might multiply in RA. There is debate about whether rheumatoid factor (RF) levels are associated with disease activity in the clinic; RF values could change or go backward when the illness first starts to progress. Changes in RF also have little bearing on the clinical evaluation results [6]. However, RF is a practical diagnostic indicator for RA that is used in everyday clinical practice [7]. To identify early RA and start the first treatment, rheumatologists rely on anti-citrullinated protein antibodies (ACPA) [8]. It has been suggested that ACPA activates immune cells and upregulates the production of inflammatory cytokines, which might explain why inflammation is a key factor in the development of RA [7]. Researchers have shown that measuring serum anti-MCV levels may be utilized to diagnose RA. Increasing anti-MCV levels in the blood without taking into account serum RF and anti-CCP levels highlights the importance of anti-MCV as a serum marker for predicting RA diagnosis on its own. One possible specialized technique to help in the early diagnosis of RA patients is serum anti-MCV [9]. The CCN family, of which nephroblastoma-overexpressed (NOV/CCN3) is a member, contains six matricellular proteins. All of these proteins are useful in the treatment and follow-up of renal cell carcinoma. It is pivotal in inflammation, wound healing, and angiogenesis, three crucial pathophysiological processes [10]. The levels of CCN3 were positively correlated with the titer of the RA-specific anti-cyclic citrulline peptide antibody (anti-CCPAb) and with the expression of CCN3 and IL-6. However, no such association was found between CCN3 and RF or TNF-α [11]. The prototypical cytokine interleukin (IL)-6 is one of several cytokine family members exhibiting pleiotropic and redundant functional activity [12]. IL-6 contributes significantly to the pathophysiology of RA by leading to synovitis, joint degeneration, and pannus formation [13]. The current study aims to assess the levels of CCN3 protein, IL-6, and anti-MCV biomarkers with RA disease activity to determine if there is any relationship between CCN3, anti-MCV, and IL-6 in RA patients.

METHODS

Study design and participants

From the end of August 2023 to the beginning of October, a prospective case-control study was conducted at the Department of Rheumatology. The study was conducted at the Baghdad Teaching Hospital in Baghdad, Iraq, with 120 participants who provided their consent to participate. The sample consists of 60 Iraqi patients diagnosed with RA according to either the criteria established by the American College of Rheumatology (ACR) in 1987 or the criteria established by the European Alliance of Associations for Rheumatology (ACR-EULAR) in 2010. This group was subdivided into three subgroups according to the disease activity (high, moderate and low disease activity). The other group consists of 60 healthy subjects with no family history of autoimmune disease, and matched age and sex with the patient served as the control group.

Inclusion criteria

Inclusion criteria for the study were (i) patients with RA diagnosed according to ACR/EULAR 2010 criteria; (ii) adults, aged over 18 years; and (iii) male or female sex.

Exclusion criteria

Patients were excluded from the study if (i) they exhibited other overlapping autoimmune diseases; (ii) they had evidence of infection; and (iii) they were pregnant.

Ethical approval

The study, part of an MSc dissertation in microbiology, received approval from the Ethical Committee of the Research and Ethics Committee of the Immunology, Department of Microbiology, College of Medicine at Baghdad University, Baghdad, Iraq (October 2023).

Outcome measurements

Each subject provides 5.0 ml of venous blood. The samples were placed in a gel tube and then centrifuged at a speed of 3000 rpm for 10 minutes. The resulting sera was then separated and stored in separate tubes at a temperature of -20 ºC for future processing. The immunological investigations are performed using enzyme-linked immunosorbent assay (ELISA) tests conducted at the laboratory of higher studies, which is part of the Microbiology Department in the College of Medicine at Baghdad University. The CCN3, anti-MCV, anti-CCP, RF, and IL-6 enzyme-linked immunosorbent assay (ELISA) was performed. The objective of this test was to measure the serum level of CCN3, as instructed by MyBioSource, USA, and anti-MCV, as instructed by the manufacturer in Shanghai, China. Additionally, the concentration of IL6 was determined using the instructions provided by the manufacturer, Cloud-clone Corp., USA; anti-CCP was determined using Fine Test, China; and RF was determined using a kit manufactured by Human, Germany.

Statistical analysis

For numerical variables that follow a normal distribution, the descriptive statistical analysis used the mean ± SD, whereas for variables that do not, the median (interquartile range) was employed. For the categorical data, percentages and rates were computed. We used the Chi-square test to determine the correlation between CCN3 and various socio-demographic variables. The independent t-test to compare the means of the samples, the area under the curve (AUC), was done using the ROC curve. We conducted a multiple linear regression analysis using the enter approach to identify the factors that influence or correlate with other parameters. One-way analysis of variance (ANOVA) was used to evaluate the

difference in mean of numeric variables among more than two groups, provided that these numeric variables were normally distributed. All statistical analyses were conducted using SPSS version 26. A *p*-value <0.05 was considered for significant differences.

RESULTS

There were no differences in demographic parameters between the patients with RA and the controls. Females were predominant in both studied groups (*p*= 0.850). The mean age ± standard deviation for the patient group was (50.02±12.90) and for the control group was (50.03±12.577). The study included 53 females (88.30%) and 7 males (11.70%) within the patient group, with 53 females (88.30%) and 7 males (11.70%) within the control group. The disease was more prevalent in females than males, with a ratio of 4:1. Table 1 shows the correlation between disease activity and other parameters.

Table 1: Distributions of parameters within disease activity patient groups

Variables	Disease activity	Results	*p*-value (ANOVA)
BMI (kg/m^2)	Low activity	28.83±4.99	0.121
	Moderate Activity	28.23±6.28	
	High activity	30.16±7.27	
Duration(year)	Low activity	7.23±5.38	0.604
	Moderate activity	10.37±7.72	
	High activity	11.15±9.84	
ESR (mm/hr)	Low activity	27.55±10.71	0.013
	Moderate activity	33.35±15.78	
	High activity	45.73±28.89	
CRP	Low activity	25.91±26.3	0.025
	Moderate activity	29.48±32.35	
	High activity	31.15±31.85	
Anti-CCP	Low activity	83.9±65.15	0.981
	Moderate activity	112.4±179.58	
	High activity	63.88±72.15	
RF	Low activity	105.91±78.75	0.858
	Moderate activity	102.86±82.33	
	High activity	114.77±69.87	
CCN3	Low activity	29.29±10.06	0.006
	Moderate activity	18.15±8.45	
	High activity	24.68±9.44	
Anti-MCV	Low activity	31.87±54.14	0.374
	Moderate activity	23.01±44.48	
	High activity	26.76±56.74	
IL-6	Low activity	301.51±78.53	0.792
	Moderate activity	306.54±59.45	
	High activity	294.28±86.16	

We used ANOVA to compare the variables concerning disease activity. RA patients with low disease activity (*p*= 0.107) had higher mean CCN3 values in their blood compared to those with high disease activity (*p*= 0.031) or moderate disease activity (*p*= 0.010). We observed a statistical correlation between CCN3 and disease activity, indicating moderate disease activity (*p*= 0.010) and high disease activity (*p*= 0.031). RA patients with low disease activity had an increase in mean anti-MCV values in their blood (*p*= 0.424). Anti-MCV and disease activity showed no statistical correlation (*p*-value= 0.374). RA patients with high disease activity had increased mean RF values in their blood (*p*= 0.999). We observed no statistical relationship between RF and disease activity (*p*-value= 0.858). The mean anti-CCP values were increased in the blood of RA patients with moderate disease activity, i.e., *p*= 0.863; there was no statistical relation observed between anti-CCP and disease activity (*p*= 0.981). The mean IL-6 values were increased in the blood of RA patients with moderate disease activity (*p*= 0.508), compared to those with low disease activity (*p*= 0.552) or high disease activity (*p*= 0.936). there was no statistical relationship observed between IL6 and disease activity (*p*= 0.792). The mean ESR values were higher in RA patients with high disease activity (*p*= 0.507) compared to those with moderate activity. We found a statistical relationship (*p*= 0.013) between ESR and moderate and low disease activity (*p*= 0.015). A statistical relationship was found between ESR and moderate and low disease activity (*p*= 0.571), compared to those with moderate activity (*p*=0.328) or low disease activity (*p*= 0.560). No statistical relation was observed between the duration of the disease and the disease activity (*p*= 0.604). The disease's high activity led to an increase in the mean BMI, but there was no statistical correlation between p = 0.328 disease activity (*p*= 0.121). Table 2 presents the results of the correlation study between IL-6 levels and other RA patient parameters. There were no significant relationships identified between IL-6 and another parameter like age (*r*= -0.032, *p*= 0.405), BMI (*r*= 0.030, *p*= 0.409), duration (*r*= -0.058, *p*= 0.330), ESR (*r*= 0.034, *p*= 0.397), CRP (*r*= -0.087, *p*= 0.254), anti-CCP (*r*= 0.051, *p*= 0.350), RF (*r*= -0.117, *p*= 0.187), CCN3 (*r*= -0.174, *p*= 0.187), anti-MCV (*r*= -0.150, *p*= 0.126), smoking (*r*= -0.049, *p*= 0.354), and treatment type (*r*= -0.110, *p*= 0.201). Table 3 shows the correlation between the anti-MCV and another patient parameter. The study discovered that Anti-MCV was positively related to RF (*r*= 0.229, *p*= 0.039), strongly related to the length of the disease (*r*= 0.381, *p*= 0.001), negatively related to ESR (*r*= -0.228, *p*= 0.040), and not significantly related to CCN3 (*r*= 0.161, *p*= 0.109), Anti-CCP (*r*= -.0.099, *p*=

0.226), CRP (*r*= -0.152, *p*= 0.123), or smoking (*r*= 0.047, *p*= 0.361).

Table 2: The correlation between IL-6 and RA parameter (n=60)

Parameters		IL6
Age	*r*	-0.032
	p-value	0.405
BMI	*r*	0.030
	p-value	0.409
Duration (yrs)	*r*	-0.058
	p-value	0.330
ESR	*r*	0.034
	p-value	0.397
CRP	*r*	-0.087
	p-value	0.254
Anti-CCP	*r*	0.051
	p-value	0.350
RF	*r*	-0.117
	p-value	0.187
CCN3	*r*	-0.174
	p-value	0.092
Anti-MCV	*r*	-0.150
	p-value	0.126
Smoking	*r*	-0.049
	p-value	0.354
Treatment type	*r*	-0.110
	p-value	0.201

Table 3: Correlation between Anti-MCV and RA parameter (n=60)

Parameters		Anti-MCV
CNN3	*r*	0.161
	p-value	0.109
RF	*r*	0.229
	p-value	0.039
Anti-CCP	*r*	-0.099
	p-value	0.226
CRP	*r*	-0.152
	p-value	0.123
ESR	*r*	-0.228
	p-value	0.040
Smoking	*r*	0.047
	p-value	0.361
Duration	*r*	0.381
	p-value	0.001
BMI	*r*	-0.144
	p-value	0.137
Treatment Type	*r*	0.154
	p-value	0.121
Ulnar deviation	*r*	0.075
	p-value	0.283

Table 4 presents the results of the correlation study between CCN3 levels and the other RA patient parameters. The study identified inverse significant relationships between CCN3 and Ulnar deviation (*r*= -0.292, *p*= 0.011), while it found no significant relationships between CCN3 and other parameters such as BMI (*r*= -0.024, *p*= 0.428), Duration (*r*= 0.085, *p*= 0.258), ESR (*r*= 0.008, *p*= 0.474), CRP (*r*= 0.144, *p*= 0.137), Anti-CCP (*r*= 0.097, *p*= 0.230), RF (*r*= 0.035, *p*= 0.397), Smoking (*r*= -0.156, *p*= 0.117), and Treatment type (*r*= 0.119, *p*= 0.001).

Table 4: Correlation between CCN3 and RA parameter (n=60)

Parameters		CCN3
RF	*r*	0.035
	p-value	0.397
Anti-CCP	*r*	0.097
	p-value	0.230
CRP	*r*	0.144
	p-value	0.137
ESR	*r*	0.008
	p-value	0.474
Smoking	*r*	-0.156
	p-value	0.117
Duration	*r*	0085
	p-value	0.258
BMI	*r*	-0.024
	p-value	0.428
Treatment Type	*r*	0.119
	p-value	0.183
Ulnae deviation	*r*	-0.292
	p-value	0.011

DISCUSSION

This study discovered that the standard deviation of the age of patients diagnosed with RA was 12.907 years, with an average age of 50.02 years. Previous studies on Iraqi RA patients conducted by Al-Rawi *et al*., Al-Ubaidi *et al*., Al-Karkhi *et al*., and Mohammed *et al*. [14-17] support this finding.

According to these studies, the development of RA usually begins in the middle years of life, and the disease primarily affects those over the age of 40. According to the current study, females are more likely than males to develop RA, with a 4:1 sex difference in susceptibility. Abdul-Wahid *et al*. did a local investigation in 2013 [18], which yielded results similar to this. This study discovered that high BMI in RA patients did not associate with disease activity or other measures. The current investigation found no significant association between sex, age, disease duration, BMI, and CCN3 therapy type. Furthermore, the current conclusion is consistent with a study that found no relationship between CCN3 and disease duration [19] but differs from another study that reported a strong link between CCN3 and age and BMI [17]. However, CCN3 showed a significant association with moderate to high disease activity, which is consistent with the findings of Wei *et a*l., who found a positive correlation between CCN3 and disease activity [11]. The current study's findings reveal no significant link with IL-6, which contradicts previous research that found a favorable correlation with IL-6 [11,17] since all patients were taking different anti-inflammatory medicines. This study also found an inverse link with ulnar deviation deformity in RA patients. The anti-CCP and CCN3 relationships were not statistically significant, contrary to a previous study that found a substantial positive association between CCN3 and anti-CCP [19]. We discovered no significant association between CCN3 and RF, C-reactive protein, ESR, or smoking. This is comparable to the study that reported no significant link between CCN3 and RF [11], but differs from the study that found a substantial relationship between CCN3 and ESR [19]. Furthermore, we discovered a very clear cut-off value for the ROC test for CNN3 markers, with 92.6% sensitivity and 89.1% specificity. This shows that CNN3 is a useful diagnostic marker (see Table 5 and Figure 1). This study suggests that even when RA patients get various types of treatment, the biomarker anti-MCV remains detectable in their sera. This anti-MCV autoantibody had high sensitivity but lower specificity (82.7%-72.1%).

Table 5: Validity data for serum CCN3 levels using ROC test with respect to patients with RA and controls

Validity test	CCN3
Sensitivity (%)	92.6
Specificity (%)	89.1
Positive predictive value (%)	87
Negative predictive value (%)	86.7
Accuracy (%)	88%
Area under curve	0.857
Cut-off value	≥15.9
p-value	<0.001

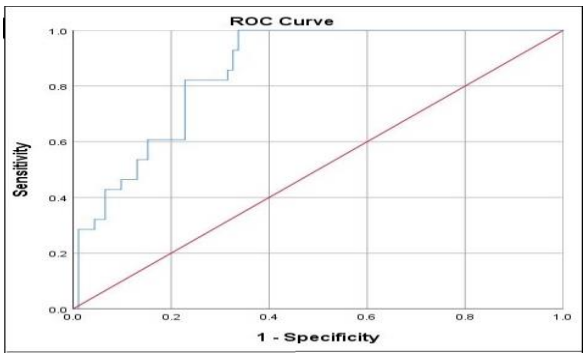

Figure 1: Validity tests for serum CCN3 levels using ROC testing in patients with RA and controls

Lee *et al*. found that anti-MCV is more sensitive, but less specific (Table 6 and Figure 2), and has lower diagnostic accuracy than anti-CCP in RA patients [20]. Based on these findings, Sghiri *et al*. (2008) concluded that anti-MCV antibodies are ineffective for diagnosing RA since they are less specific than anti-CCP antibodies but equally sensitive [21]. Furthermore, there is no significant relationship between anti-MCV and CCN3, anti-MCV and IL-6, anti-CCP, CRP, disease activity, smoking, BMI, or type of therapy. This finding contradicts earlier studies that found a strong connection between anti-MCV and anti-CCP [22,23].

Table 6: Validity data for serum Anti-MCV levels using ROC test with respect to patients with RA and controls.

Validity test	Anti-MCV
Sensitivity (%)	82.7
Specificity (%)	72.1
Positive predictive value (%)	83
Negative predictive value (%)	62.9
Accuracy (%)	72
Area under curve	0.507
Cut-off value	≥17.1
p-value	<0.001

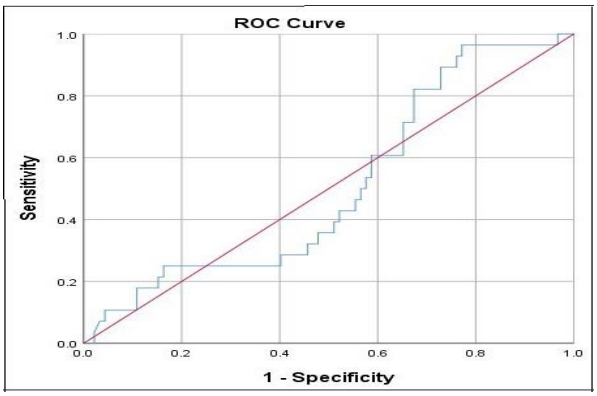

Figure 2: Validity tests for serum Anti-MCV levels using ROC testing in patients with RA and controls.

The current findings are similar to those of others who found no link between anti-MCV and disease activity [9,22], but differ from other studies that reported a correlation between anti-MCV and disease activity [24]. Nigm *et al*. observed no relationship between anti-MCV and CRP. According to this study, there was a significant relationship between anti-MCV, ESR, and RF, as well as a strong correlation with disease duration. In line with the findings of Al-Shukaili *et al*., who found a significant positive correlation between anti-MCV and RF [25], another study similar to ours found a significant link between anti-MCV, ESR, and disease duration [26]. This study suggests that even when RA patients used different types of treatment, the biomarker RF is still detected in their serum. This RF autoantibody exhibited a sensitivity of 87.4% and a specificity of 82.1%, which is similar to RF testing in RA patients, which has a sensitivity of 60% to 90% and a specificity of 85% [27,28] (Table 7), but others found no significant association between RF and disease activity [29].

Table 7: Validity data for serum RF levels using ROC test with respect to patients with RA and controls

Validity test	RF
Sensitivity (%)	87.4
Specificity (%)	82.1
Positive predictive value (%)	86
Negative predictive value (%)	81.7
Accuracy (%)	84
Area under curve	0.674
Cut-off value	≥16.8
p-value	<0.001

RF does not have a substantial role in RA pathogenesis, and the disease is primarily cellular. The current study suggests that even when RA patients are treated with various types of medication, anti-CCP is still detectable in their sera. The anti-CCP autoantibody demonstrated good sensitivity and specificity (91.4% and 88.1%, respectively) (Table 8).

Table 8: Validity data for serum Anti-CCP levels using ROC test in patients with RA and controls

Validity test	Anti-CCP
Sensitivity (%)	91.4
Specificity (%)	88.1
Positive predictive value (%)	87
Negative predictive value (%)	85.8
Accuracy (%)	89
Area under curve	0.770
Cut-off value	≥16.2
p-value	<0.001

The current study's findings were consistent with those of Abdullah *et al*. (2012) and Al-Ubaidi *et al*. (2012), who discovered that this test was very effective for diagnosing RA [30,31]. In keeping with what Porto *et al*. discovered, there is no strong relationship between anti-CCP and disease activity. For example, they found no statistically significant relationship between anti-CCP levels and the DAS28, SDAI, or CDAI activity indices in RA patients who previously had the disease [32]. Our study found a strong link between disease activity and radiocarpal and ulnar styloid abnormalities, but not with another type of deformity. Yoshii *et al*. published a study that

found a link between disease activity and joint abnormalities [33]. Our investigation found a highly significant association between ulnar deviation and CCN3, but no correlation with anti-MCV. Ulnar deviation, like other deformities associated with high disease activity, had a high association with disease activity, and CCN3 had a high correlation with ulnar deviation, although autoantibodies played no meaningful impact in disease activity.

Study limitations

Sample sizes will always be a challenge in such studies, but it is recommended that, where possible, future studies elucidate the CCN3 protein's respective functions, targets, and contributions to immunopathogenic mechanisms, as these may enhance disease diagnosis, management, and therapeutic options to confirm the present findings.

Conclusion

This study underscores the significant association between CCN3 levels, disease activity, and joint deformity in RA patients, with higher sensitivity and specificity relative to other parameters. This robust correlation highlights the potential of CCN3 as a reliable biomarker and a valuable prognostic tool for rheumatoid arthritis, suggesting its utility in both diagnostic and therapeutic settings.

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Conflict of interests

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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