Investigating the Potential Correlation between Vitamin D with Rheumatoid Factor, Anticyclic Citrullinated Peptides Antibody, and Interleukin-12 Levels in Rheumatoid Arthritis Women in Erbil, Iraq

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Abstract

Background: Rheumatoid arthritis (RA) is an autoimmune disorder involving the synovial joints of humans. Recent research has demonstrated that vitamin D (VD3) can influence a person's susceptibility to RA, and a proinflammatory mediator affects the pathogenesis of RA. **Objectives:** This work aimed to investigate the correlation of VD3 with autoantibodies and interleukin-12 (IL-12) in RA patients. **Materials and Methods:** Eighty women were included in this case–control study, 60 confirmed RA and 20 healthy controls (HC) of the age range 29–71 years. The sera of study subjects were examined for anticyclic citrullinated peptides (anti-CCP), vitamin D3, and IL-12 by enzyme-linked immunosorbent assay. Rheumatoid factor (RF-IgG) was tested by latex agglutination technique. **Results:** The present findings revealed a significantly high concentration of anti-CCP, RF-IgG, and IL-12 in RA patients in comparison to HC. The anti-CCP expressed high sensitivity and specificity at 80% and 100% compared to RF-IgG at 76.6% and 90%, respectively. Both RA patients and HC groups showed lower levels of VD3 with a nonsignificant difference, 50% of RA patients and 55% of the HC had vitamin D deficiency (<20 ng/mL). In RA patients, negative associations were observed between VD3 and anti-CCP and IL-12 levels. In contrast, a positive correlation was observed between anti-CCP and IL-12 in RA women. **Conclusion:** Anti-CCP had a better diagnostic value than RF. Low vitamin D is prevalent in RA patients and HC. Also, IL-12 may possess a vital role in RA's pathophysiology and inflammatory activity, along with IL-12 inhibition may be beneficial in treating this disease.

Keywords: Anti-CCP, Rh factor, rheumatoid arthritis, vitamin D3, IL-12

Introduction

Rheumatoid arthritis (RA), a systemic autoimmune illness, primarily affects the joints with variable severity among patients. Organokines in this disease can have either positive or negative effects. Organokines are linked to raised inflammation and cartilage damage in RA patients because they increase the production of cytokines and metalloproteinases.[1] Approximately 1% of the general population is affected by this autoimmune illness. It has a female-to-male ratio of around 3:1.[2] In RA, the action of proliferative synovial tissue fibroblasts, which are supplemented with neutrophils, monocytes, and T and B lymphocytes infiltrating to articular synovium, is what primarily drives joint damage. Even though genetic and environmental factors have been identified as the primary causes of RA, the factors causing its pathogenesis are still poorly understood.[3] In addition to inflammation, oxidative stress is crucial for the pathogenicity and development of RA impairments.[4]

In the last 65 years, rheumatoid factor (RF), as well as anticitrullinated protein/peptide antibodies (ACPA) or

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anticyclic citrullinated peptides (anti-CCP) measurements, have become more important in diagnosing and classifying RA.[5] The occurrence of these autoantibodies is a defining characteristic of the illness. These autoantibodies might create immune complexes in the joint, which would draw immune cells there. RA patients can be divided into autoantibodies—positive and negative groups based on their presence.[6]

Vitamin D is a steroid hormone precursor that is chemically converted in the liver and kidney. The effect of vitamin D deficiency in the etiopathogenesis of rheumatoid disease is gaining attention, similar to that of other autoimmune diseases.[7] The powerful immunomodulatory properties of vitamin D's active form, 1,25-dihydroxy vitamin D3 [1,25$(OH)_2$D3], have made it more likely that it will be used to treat and prevent autoimmune diseases and infectious diseases. Numerous immune cells, such as dendritic cells, activated T cells, and macrophages, express intracellular vitamin D receptors and are responsive to 1,25$(OH)_2$D3.[8] Vitamin D's active form has anti-inflammatory and immunomodulatory properties. The pathogenesis of RA is thought to involve vitamin D, and a lack of vitamin D causes more inflammation. In addition, levels of calcium, phosphorus, and parathyroid hormone are necessary for its production.[9]

Emerging research in the last 20 years has demonstrated that the pathogenesis of RA is significantly influenced by the innate immune system. Monocytes, macrophages, and dendritic cells, among other innate immune cells, are participating in the inflammatory reactions in RA cases along with activating the acquired immune response, which is crucial in the late stages of the disease.[3,10] The novelty of this work is the first in our society to correlate IL-12 with vitamin D3 (VD3), anti-CCP, and RF levels as markers for rheumatoid disease.

Materials and Methods

Study design and subjects

Confirmed RA women aged from 29 to 71 years were included in this study, in comparison to age and sex-matched volunteers as a healthy control group. The women with confirmed cases of RA who were referred to the rheumatology department and the medical rehabilitation center for biological therapy for rheumatic diseases at Rzgary Hospital, Erbil, between January 2022 and May 2022 served as the subjects for this hospital-based case–control study.

Sample selection and collection

The patient's demographic information, including age, period of the disease, height, weight, and supplement use, was collected by filling questionnaire through a direct interview. Moreover, a physical examination and an evaluation of the RA patient's status were performed by the rheumatologist.

Five milliliters of peripheral blood samples were collected from 60 confirmed RA women patients and another 20 healthy women as a control group and kept in serum separation gel tubes. After blood clotting, the sera were prepared by centrifugation for 15 min at 3000 rpm.[11] Four aliquots were made for every serum sample by keeping 0.7 mL of it in Eppendorf tubes. Then prepared sera were stored at −20°C for additional experiments of assessing RF-IgG, anti-CCP, vitamin D, and IL12.

Estimation of the serological parameters

The cyclic citrullinated peptide IgG antibody (anti-CCP) evaluation

The assessment of anti-CCP was performed by the enzyme-linked immunosorbent assay (ELISA) method by the CCP-specific kit (AESKULISA/Germany) depending on the manufacturer's instructions. It is prepared for evaluating human autoantibodies against the cyclic citrullinated peptide. The antibody level >18 U/mL was considered positive. The levels of RA patients and control samples were determined by plotting optical density on the *y*-axis and standard concentrations on the *x*-axis to create a standard curve.

Estimation of rheumatoid factor IgG

RF-IgG was measured by the quality latex agglutination technique and conducted on all serum samples of study subjects.

Estimation of vitamin D3

The 25-hydroxy vitamin D ELISA kit (M67L2C2, MonoKit, Iran) was used to assess the concentration of VD3, based on the competitive ELISA technique, as per the manufacturer's guidelines for the kit. In order to assess the VD3 concentration of RA patients and control samples, the optical density (OD) values were plotted on the *Y*-axis and calibration concentrations (pg/mL) on the *X*-axis, respectively.

Interleukin-12 assessment

The concentrations of interleukin-12 (IL-12) in RA patients and control women sera were evaluated by ELISA using a commercial kit (Karmanya Pars Gene/Iran), depending on the manufacturer's instructions for the kit. The levels of the study's tested samples were determined by plotting the OD values on the *Y*-axis and the calibration concentrations on the *X*-axis to create the ELISA standard curve.

Statistical analysis

All statistics and graphics were created using Statistical Package for the Social Sciences version 26 (SPSS 26; IBM Corporation, Armonk, NY, USA), and GraphPad Prism version 9.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to present all the statistics and graphs. For the quantitative variables, mean value (M ± SD) was used. Otherwise, median interquartile range was

calculated. Statistical significance was defined as a *P*-value <0.05. The correlation between the tested parameters was calculated using Pearson's correlation. The diagnostic accuracy of each value in predicting the presence of IL-12 in RA women was assessed using receiver operating characteristic (ROC) curves and the area under the curve (AUC).

Ethical approval

This work was carried out following the ethical regulations found in the Declaration of Helsinki. It was performed with the patient's verbal and analytical approval before a sample was taken. A scientific ethics committee reviewed and approved the study proposal in the Faculty of Science, and the official form numbered 1/1/178 with the date of 15/11/2021.

Results

Study subject demography

Demographic data, including age and body mass index (BMI) (kg/m^2), are presented in Table 1. The results showed that the mean ages of the RA women and control group were 49.37 ± 10.40 years and 45.35 ± 11.04 years, respectively. The age distribution of the female RA patients revealed that 31.7% of them were in the second interval (41–47 years), with the highest number of participants of 19 patients, then the fourth interval (≥57), as 28.3 with 17 patients. In contrast, the smallest number of RA patients was noticed in the first and third groups including 12 (20%) patients for each of them. Regarding BMI, the findings revealed that, in comparison to the healthy control group, 6.7% (24) of RA patients fell into the normal weight range (18.5–24.9 kg/m^2), while 33.3% of patients, including 20, were overweight. Additionally, 16.7% (10) of RA women had severe obesity, and 43.3% (26) of RA were obese (between 30 and 34.9 kg/m^2). According to the findings, no significant differences between the body mass indices of the participants investigated under $P < 0.05$ was determined. Moreover, mean disease duration of the rheumatoid women was 7.43 ± 4.24 years, and most of them (71.66%) suffered from the disease for more than 4 years, 78.3% of women have a family history, as shown in Table 1.

Serological analysis

Evaluation of RF-IgG and anticyclic citrullinated peptide

For RF-IgG antibodies, among the RA women patients, 14 patients (23.3%) were negative, while 46 of them (76.7%) were positive. Also, 2 healthy control women were positive, and the other 18 were negative for RF-IgG antibodies against RA disease ($P < 0.001$).

Concerning anti-CCP antibodies, 12 RA patients (20%) were negative, while 48 RA patients (80%) were positive

Table 1: Baseline characteristics of rheumatoid arthritis women patients and healthy controls

	Rheumatoid arthritis	Healthy control
	No (%)	No (%)
Age		
≤40 years	12 (20)	8 (40)
41–47 years	19 (31.7)	5 (25)
48–56 years	12 (20)	4 (20)
≥57 years	17 (28.3)	3 (15)
Total (n = 80)	60 (100)	20 (100)
	χ^2 = 3.689; P = 0.297	
Mean age (years)	49.37 ± 10.40	45.35 ± 11.04
BMI (kg/m^2)		
Normal weight, 18.5–24.9	4 (6.7)	1 (5)
Overweight, 25–29.9	20 (33.3)	10 (50)
Obese, 30–34.9	26 (43.3)	6 (30)
Severe obesity, 35–39.9	10 (16.7)	3 (15)
Total (n = 80)	60 (100)	20 (100)
	χ^2 = 1.87; P = 0.6	
Disease duration (years)		
Mean disease duration	7.43 ± 4.24	–
1–2 years	5 (8.33%)	–
3–4 years	12 (20%)	–
>4 years	43 (71.66%)	–
Total (n = 60)	60 (100%)	–
Family history of RA		
Yes	47 (78.3%)	–
No	13 (21.6%)	–

($P < 0.001$, $\chi^2 = 40$). Also, none of the healthy control group gave positive results. The average level of anti-CCP in women with RA was significantly higher than the control group, at 49.97 ± 10.47 µm/mL and 9.27 ± 0.57 µm/mL, respectively ($P < 0.001$), as shown in Figure 1. The degree of sensitivity, specificity, and positive and negative predictive value of RF and anti-CCP tests in the diagnosis of RA was shown in Figure 2A and B [Table 2]. The sensitivity and specificity of the RA-IgG test were 76.7 and 90, respectively. Whereas the anti-CCP was superior to the RH-IgG in the diagnosis of RA disease, it showed 80% sensitivity and 100% specificity, as depicted in Table 2.

Estimation of vitamin D3 levels

The mean ± SE of vitamin D level in the RA patients was 20.16 ± 1.758 ng/mL, compared to its mean in the healthy control women as 16.923 ± 2.638, with no significant differences between them. Also, the median value of VD3 concentrations was presented in Figure 3, as 20.189 and 17.793 in the rheumatoid group and the control one, respectively.

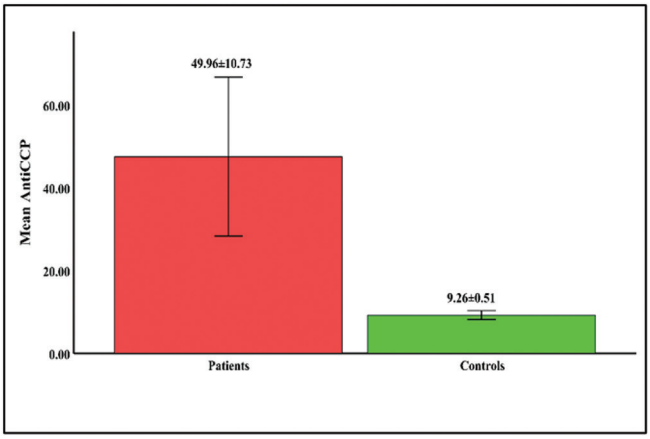

Figure 1: Anti-CCP levels in RA patients and HC. The anti-CCP levels were measured by ELISA. The means of anti-CCP was 49.96 ± 10.73 ng/mL in RA and 9.26 ± 0.51 ng/mL in HC

Among the rheumatoid women, 30 women (50%) had deficient vitamin D serum levels, 14 women (23.3%) had insufficient vitamin D serum levels, and 16 patients (26.7%) showed sufficient vitamin D levels as confirmed in Figure 4. Statistical analysis was conducted between four age groups of RA patients, nonsignificant differences in vitamin D serum levels were observed ($P = 0.041$) [Figure 4].

Estimation of interleukin-12

The present investigation displayed that the IL-12 was significantly amplified in the RA group in comparison to the control one; the median values were 106.69 pg/mL and 33.3 pg/mL, respectively, as depicted in Figure 5 ($P < 0.001$).

The best serum IL-12 cutoff value that can accurately predict an RA diagnosis was determined using the ROC curve. The

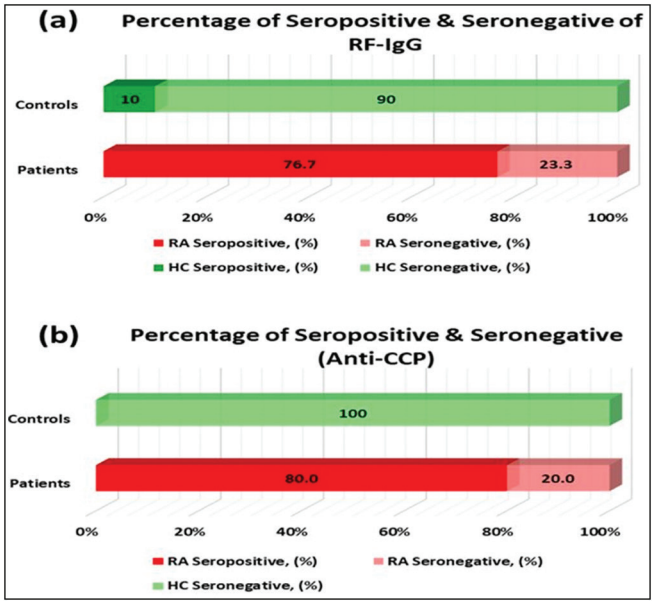

Figure 2: Percentage of anti-CCP and RF-IgG seropositivity among patients with RA and healthy control

Table 2: The diagnostic value of RF-IgG (a) and anti-CCP (b) in rheumatoid arthritis

	Value	95% CI	
		Lower bound	Upper bound
(a)			
Sensitivity	76.7	64	86.6
Specificity	90	68.3	98.8
Positive predictive value	95.8	86.7	99.5
Negative predictive value	56.3	37.7	73.6
(b)			
Sensitivity	80	67.7	89.2
Specificity	100	83.2	100
Positive predictive value	100	92.6	100
Negative predictive value	62.5	43.7	78.9

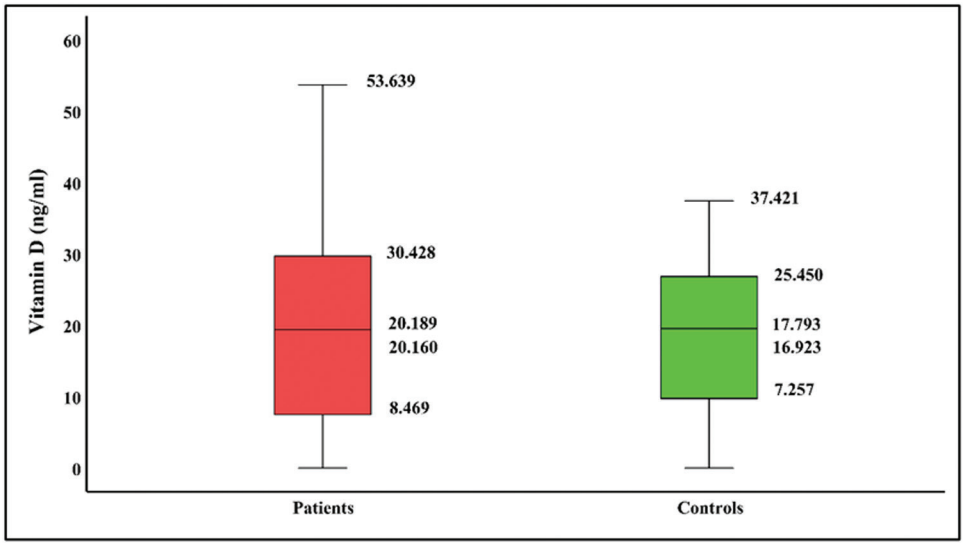

Figure 3: Box plot represents serum concentration of VD3 in RA and control group. Median values are represented by the horizontal lines with maximum and minimum values. The mean values are also indicated

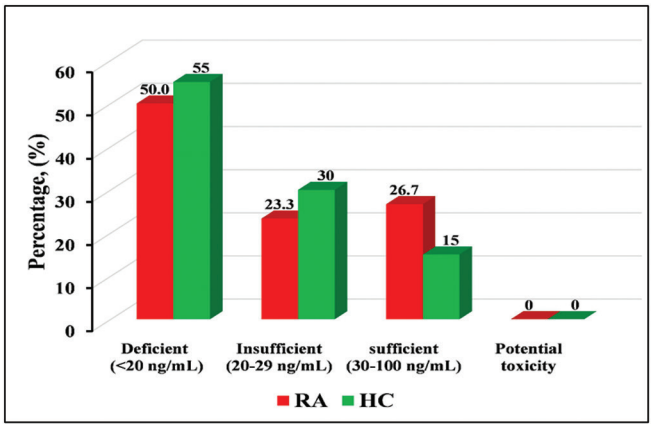

Figure 4: The distribution of RA patients and control according to VD3 concentration grading. The first group was VD3 deficiency (<20 ng/mL), the second group was VD3 insufficient (20–29 ng/mL), and the third group was VD3 sufficient (30–100 ng/mL)

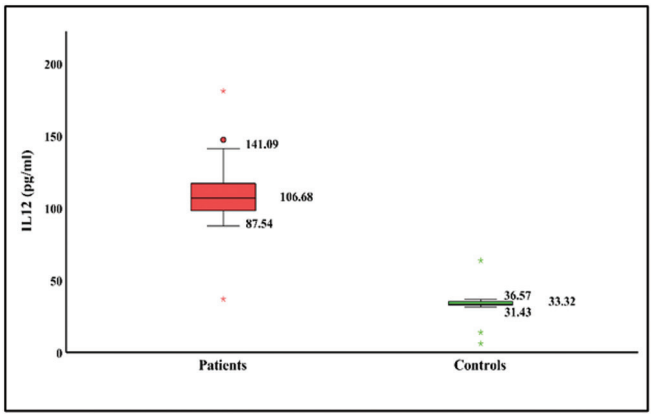

Figure 5: Il-12 levels in RA patients and the healthy control. The median and the Interquartile Range for the RA and the controls were 106.69 (19.07) and 33.33 (2.60), respectively, Z = −6.656; $P < 0.001$ by Mann–Whitney *U* test

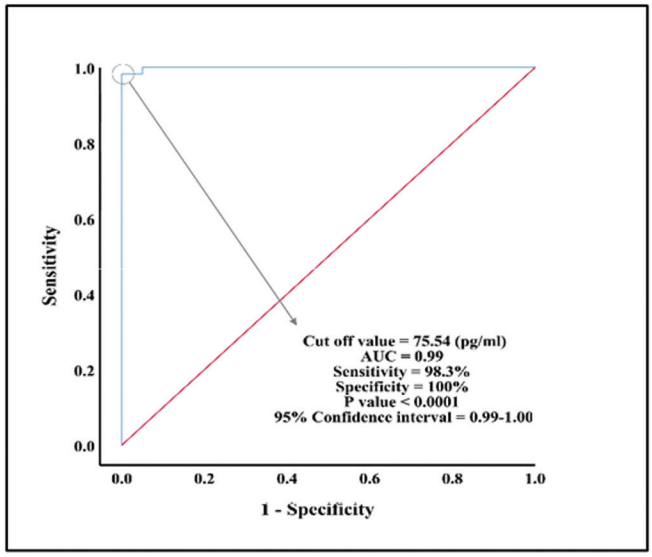

Figure 6: Receiver operator characteristics (ROC) curves for IL-12. ROC curve analysis revealed the optimal cutoff value, sensitivity, and specificity values, as 75.54%, 98.3%, and 100%, respectively. The area under the curve was 0.9992, 95% confidence interval: 0.9964–1.00, $P < 0.0001$). The ROC revealed a significant area under the curve (AUC)

cutoff value was 75.54 (pg/mL), the sensitivity was 98.3%, and the specificity was 100%, as predicted in Figure 6.

Pearson's correlation among vitamin D3 and anticyclic citrullinated peptide and interleukin-12

A negative and nonsignificant relationship was detected among VD3 concentrations of RA patients and their anti-CCP values, with a Pearson's correlation of −0.025, as shown in Figure 7A. The present findings detected a nonsignificant negative correlation between VD3 concentrations of RA patients and their serum IL-12

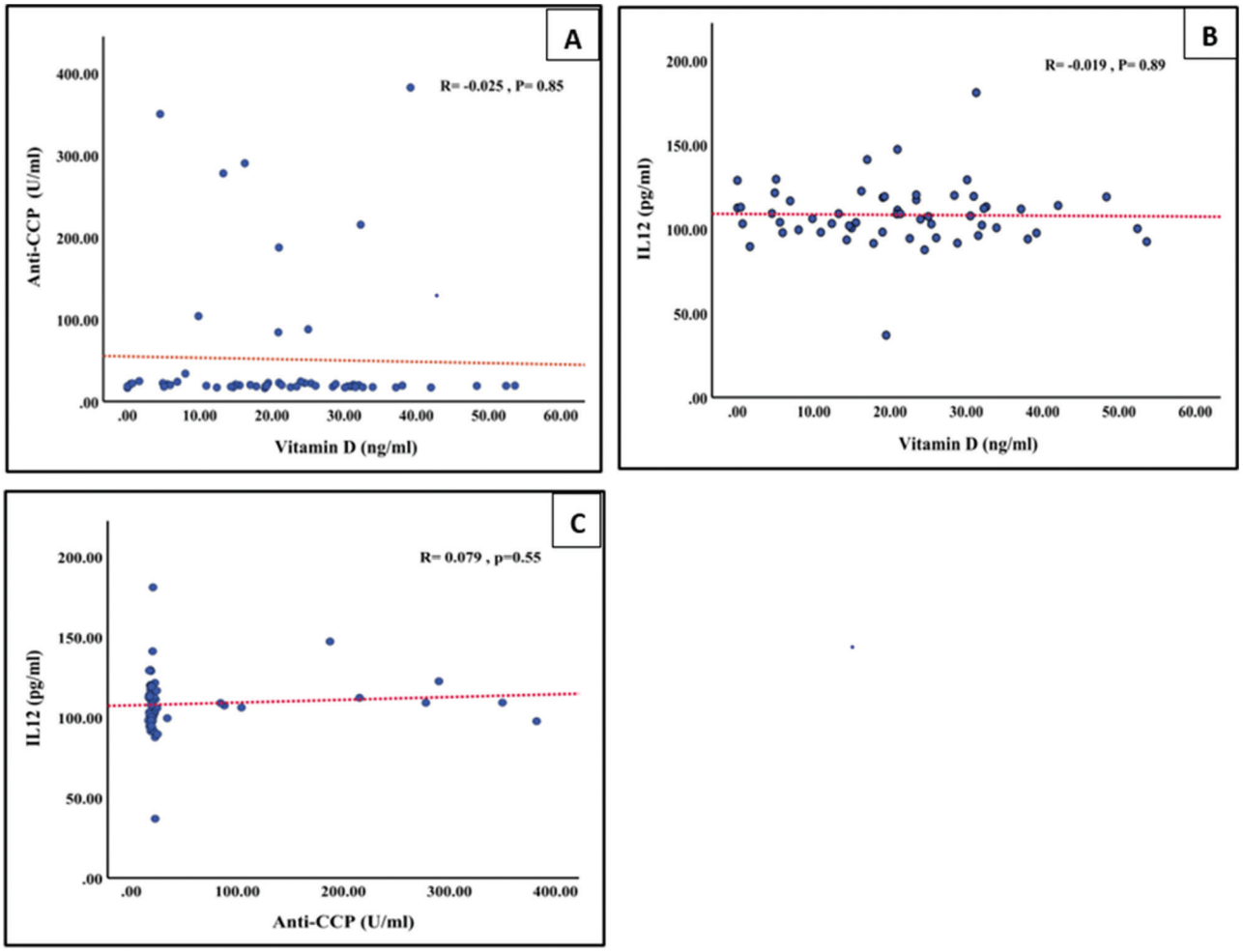

Figure 7: (A) Pearson's negative correlation between VD3 and anti-CCP of RA patients. (B) Pearson's correlation between VD3 and IL-12 levels. (C) Pearson's correlation between IL-12 and anti-CCP in RA patients

values, with a Pearson's correlation of −0.019, as shown in Figure 7B. A positive and nonsignificant correlation was found among IL-12 serum concentrations in RA patients with their anti-CCP levels. The Pearson correlation was 0.079 at $P = 0.55$, as shown in Figure 7C.

Discussion

According to the current study, the mean age of the female RA patients was 49.37 ± 10.40 years, with a mean disease duration of 7.43 ± 4.24 years. The second group had the highest percentage of RA women patients (31.7%), followed by the fourth group in terms of age distribution. The findings of Khadim and Al-Fartusie[12] who discovered that the mean age of RA patients in Iraqi women was 48.56 supported our outcomes. Additionally, a current retrospective investigation of RA cases in Iraq revealed that 82.7% of them were women, with a mean age of 48.34 ± 11.7.[13] Also, the mean age of RA patients was observed as 49.9 ± 11.9 in another study conducted in Iraq.[14] According to Hussein *et al.*,[15] the majority of patients were aged between 40 and 59. In Iraq, the propensity of RA to affect older people is well documented. Moreover, this disease is highly prevalent among Iraqi women, in a study of RA cases during the period from 2014 to 2019, there were 1814 patients overall. Women patients were 1577, and men patients were 237.[16]

Based on our research, 80% of RA women gave positive results for the anti-CCP antibody, while 76.7% of them showed positive results for RF-IgG. According to another study, anti-CCP antibodies were present in 70.4% of the RA samples.[17] Another recent work found that 42% of rheumatoid patients exhibited positive anti-CCP antibodies.[18] Depending on the available evidence, ACPA was advised in the 2009 RA diagnostic standards.[19] Anti-CCP may be able to identify RA earlier in the disease's course because it is more sensitive than RF. According to prior investigations, RA patients who are seropositive for RF or anti-CCP antibodies exhibit more severe symptoms than those who are seronegative.[13,20]

The present findings showed that the specificity and sensitivity of anti-CCP for RA were 100% and 80%,

respectively. Whereas the specificity was 90% and the sensitivity was 76.9% for RF-IgG. Anti-CCP would be favored from the RF because it closely correlates with disease activity. Nevertheless, it is unsuccessful in managing the extra-articular symptoms of the disease.[21] In this regard, a recent study discovered that patients' serum concentrations of anti-CCP were significantly high compared to those of controls. Anti-CCP gave a sensitivity in addition to the specificity of 93.5% and 100%, respectively, for rheumatoid disease diagnosis.[22] Additionally, previous research has proven 93% sensitivity and 96% specificity of the anti-CCP test for rheumatoid diagnosis.[23] Using ELISA kits for anti-CCP, one of the earliest studies revealed a specificity of 98% and a sensitivity of 68%.[24] Moreover, Braschi *et al.*[25] have observed similar sensitivity of anti-CCP as well as RF tests, but lower specificity was found in the RF.

The results of our study confirmed that vitamin D insufficiency in RA women was comparable to that of healthy controls with nonsignificant differences. It is commonly considered that a 25(OH)D serum amount of less than 20 ng/mL should constitute vitamin D insufficiency. In our research, vitamin D deficiency was discovered in 50% of RA patients, and it was close to that reported in control women (55%), who are typical of the general population, indicating that vitamin D deficiency is a widespread dilemma, at least in Iraq. In an earlier study conducted in Erbil, Iraq, they discovered VD3 deficiency in 70% of the studied RA cases, although 46% of the healthy control expressed VD3 deficiency.[26] In light of the fact that, as was already mentioned, VD3 is in charge of regulating immune response and enhancing the immune system, a recent study proposed that hypovitaminosis D is a common risk factor for many autoimmune diseases, including RA.[27] Vitamin D deficiency is common in rheumatoid patients and may contribute to the onset or aggravation of signs, according to Ref. [28]. The risk of RA was negatively correlated with vitamin D intake at least in postmenopausal women. The 1,25-dihydroxy vitamin D, which is the active structure of vitamin D, interacts with the vitamin D receptor to exert a variety of effects on both innate and acquired immune systems responses, including the inhibition of inflammation and the stimulation of tolerogenic reactions.[28]

Recent studies proved that there is strong evidence that VD3 deficiency contributes to obesity in breast cancer postmenopausal women. VD3 levels are also negatively related to patients' ages.[29] Furthermore, in children suffering from type 1 diabetes mellitus, VD deficiency is associated with a higher risk of diabetic nephropathy.[30]

The heterodimeric cytokines IL-12, IL-23, IL-27, and IL-35, which are members of the IL-12 family, play significant roles in autoimmunity. The cytokines in the IL-12 group have similar structures, but they work in different ways. Leukocyte migration, bone destruction, and angiogenesis are regulated in RA by an IL-23-IL-17, which can be partially blocked by the activity of IL-12, IL-27, as well as IL-35.[31]

Our findings revealed that IL-12 recorded a significantly high serum concentration in women with RA in comparison to healthy ones ($P < 0.001$). This outcome was comparable to earlier findings, Paradowska-Gorycka *et al.*[32] as well as Shen *et al.*,[33] observed that serum IL-12 was considerably elevated in patients with RA. The researchers concluded that serum IL-12 may be a more essential and predictive component in RA progression. In contrast to healthy, the synovium fluid from RA patients has higher concentrations of IFN-gamma, IL-12, and TRAIL. This finding suggests that the predominant cytokine pattern in the course of RA is Th1, which is more prevalent in synovial fluid than serum, they demonstrated that rheumatoid patients were distinguished by a disturbance of Th1/Th2 equilibrium to Th1.[34,35]

The results of the ROC analysis indicated that sera IL-12 with the greatest specificity (100%) at a cutoff of >75.54 pg/mL could differentiate patients from controls considerably. However, a few studies demonstrate that IL-12 correlates with other inflammatory indicators in RA. In addition, Paradowska-Gorycka[32] discovered no association between serum IL-12 and inflammatory markers like RF and anti-CCP. This difference between these studies may be the result of various populations, sample numbers, and illness criteria. In the present investigation, there was a nonsignificant correlation between IL-12 levels in the sera and anti-CCP levels.

Conclusion

According to the current results, and as a screening approach for women with RA, anti-CCP had a superior diagnostic value from RF. Also, low vitamin D is common among rheumatoid women and healthy control and a large percentage of them were characterized as deficient. IL-12 concentrations were elevated in all RA females in comparison to the control group. IL-12 may perform a crucial function in RA's pathophysiology and inflammatory activity, and its inhibition may be beneficial in treating this disease. Moreover, anti-CCP antibodies and IL-12 levels were positively related in RA patients. This proposes that IL-12 can be utilized as an index to evaluate the severity of illness. The small levels of VD3 noticed in female RA patients in this study and their relationship to the amounts of RF, anti-CCP, and the pro-inflammatory IL-12 provide evidence that VD3 deficiency contributes to the pathophysiology experienced during RA. The ROC revealed a significant AUC. Our results depended on a moderate-sized sample, to validate these findings. It is necessary to conduct more research with a bigger sample size.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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