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Serum Interleukin-17 and its Correlation with Anti-CCP antibodies, Vitamin D3, and Obesity in Rheumatoid Arthritis women Patients

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease. Members of the IL-17 cytokine family have various biological functions, including enhancing defense against multiple pathogens while promoting inflammatory pathology during autoimmune disease. Additionally, it has been suggested that vitamin D may be connected to the etiology of RA. Therefore, this study aimed to assess serum IL-17 and vitamin D3 concentrations in RA women and determine how these levels relate to anti-CCP, obesity, and age.

Methods: This work included eighty women aged between (22-60) years. Sixty were afflicted with RA and attended the department of rheumatology and medical rehabilitation centre for rheumatic diseases at Rzgary teaching hospital-Erbil, Iraq, compared to twenty healthy women. The concentrations of Anti-CCP antibodies as a diagnostic test, Vitamin D3 and IL-17, were assessed by ELISA. The Mann-Whitney U test and normality were applied for the statistical analysis of the results. Also, the relationship between the study parameters was examined using Pearson's correlation. The ideal cutoff value for IL-17 with the highest sensitivity and specificity was detected using a Receiver Operating Characteristic (ROC) Curve.

Results: This study revealed that serum IL-17 levels were significantly higher in RA women than in the control group (p < 0.001) and that serum VD3 levels were significantly lower in RA women than in healthy controls. Seropositive anti-CCP Ab was found in about 42% of RA patients. The pro-inflammatory IL-17 serum level was positively correlated with vitamin D, age, and obesity in RA women. The best IL-17 cutoff for predicting the presence of RA was 69.5 pg/ML, with a sensitivity of 96% and a specificity of 75%.

Conclusion: In patients with RA, vitamin D deficiency was common and correlated with age. Consequently, a high level of suspicion is needed when assessing at-risk patients, particularly women who complain of vitamin D deficiency.

Introduction

Guo et al. [1] stated that rheumatoid arthritis (RA) is a chronic systemic autoimmune joint condition that mainly affects the lining of synovial joints and is linked to socioeconomic issues, premature death, and progressive disability. Additionally, it is categorized as an erosive disease that causes joint deformity and the loss of physiological functions [2]. RA disease affects around 1% of the population, with a women-to-men ratio of about 2.5–1. According to Smolen et al. [3], RA is more common in older people and primarily affects women between the ages of 40 and 60. Several risk factors, including a family history of RA or other autoimmune diseases, smoking, environmental factors, ageing, and viral infections, are linked to an increased risk for RA [4,5].

Along with the production of autoantibodies, which harms the synovial membrane and joints, a strong inflammatory immune response causes RA to develop. Activation of inflammation increases cytokines, chemokines, and inflammatory reactants such as C-reactive protein (CRP). Also, an increase in inflammation causes a series of immune responses. Consequently, autoantibodies are overproduced, elevating immunoglobulin M (RF) and anti-CCP. Hence, CRP, RF, and anti-CCP represent RA's inflammatory and immune response products and are known diagnostic blood biomarkers [6-9]. The global prevalence of RA from 1980 to 2019 reached 460 per 100,000 people, with differences according to geographical site and study approach [10]. Based on the production of antibodies to rheumatoid factor and citrullinated protein antigens (ACPA), rheumatoid arthritis patients can be classified into two types. Approximately two-thirds of RA patients are seropositive for the antibody, and these individuals typically exhibit more severe symptoms. ACPAs and RF are frequently found in the blood long before any indications of joint inflammation, suggesting that autoimmunity triggering may happen somewhere other than the joints, for instance, in the lung [11]. Numerous immunomodulatory molecules, mostly cytokines produced by the immune cells, facilitate RA's pathogenesis, resulting in disease treatment and management complications. Even though the exact aetiology of RA is still unknown, current publications indicate that T-helper cells (Th17) may have a crucial role in its pathogenesis. IL-17 is a cytokine produced by Th17 cells and may also have a considerable role in the development of RA [12]. (TH17) is a group of CD4+ effector T cells dissimilar to TH1 and TH2 cells due to their capability to produce interleukin IL-17. Some human autoimmune diseases, primarily TH1- or TH17-mediated, such as rheumatoid arthritis and psoriasis, have been linked to the pathogenic effects of TH17 lymphocytes. Scientific facts suggest that TH1 and TH17 cells are shared in the pathogenesis of RA [13]. The high expression of IL-10 in colorectal cancer patients' sera proves its usefulness for detecting patients' prognoses [14].

Vitamin D is a significant endocrine mediator with immunomodulatory characteristics. In the last 20 years, it has been found to have a close relationship with immune responses. It is understood that vitamin D possesses a pleiotropic anti-inflammatory action and directly contributes to the expression of vitamin D receptors on various immune cells. This conclusion, along with the discovery that low vitamin D levels and vitamin D deficiency are frequent in RA patients, proposes that vitamin D can act as a preventative factor against the disease [15-17]. Vitamin D possesses an immunomodulatory and anti-inflammatory role, and its deficiency has been correlated with many autoimmune syndromes, including RA. Its deficiency is more prevalent in RA patients and may be one of the reasons for the disease's development [18].

This work aimed to evaluate the serum levels of IL-17 and Vitamin D3 in RA-affected women and explore their relationships with anti-CCP, obesity, and patient age in Iraqi RA women. This study is the first investigation on IL-17 levels in female RA patients in Kurdistan, Iraq.

Methods

Study Subject

Female rheumatoid arthritis patients aged between (22-60 years) were eligible for this study compared to the age and sex-matching healthy control women.

Samples selection and collection

Peripheral blood samples were taken from sixty post-diagnosed female patients afflicted with RA and attending the rheumatology department and medical rehabilitation center of biological therapy for the rheumatic disease at Rzgary teaching hospital-Erbil from November 2021 to January 2022. Associated hospital staff, a rheumatologist, and patient permission were obtained before sample collection, and the patient signed a consent form. For healthy controls, peripheral blood samples from twenty age and sex-matching individuals were randomly selected from healthy women. Three ml of peripheral blood was drawn from patients and healthy controls and put in a serum separation tube (gel tube). After allowing the blood specimens to clot for 30 minutes at room temperature, the sera were separated by centrifuging the samples for 15 minutes at 3000 rpm [19]. Three aliquots of serum were prepared for each sample by transferring 0.7 mL of serum into 1.5 mL Eppendorf tubes. The serum samples were kept at -20°C till further analysis. Enzyme-linked immunosorbent assay (ELISA) was used to detect anti-CCP, interleukin -17, and vitamin D3 in the sera of RA

patients as well as healthy control (HC) groups. To confirm their diagnosis, all participants completed a questionnaire about their health status. The questionnaire asked about age, BMI, drug type, time since diagnosis of the disease, and family history.

Estimation of serum cyclic citrullinated peptide IgG antibody (CCPA)

The serum level of IgG antibodies to specific cyclic citrullinated peptides was measured by ELISA (DIAGNOSTIC AESKULIZA CCP, REF 3166, 55234 Wendelsheim- Germany55234) following the manufacturer's instructions. The ELISA standard curve was created to estimate the tested sample by plotting the OD values on the Y-axis and calibration concentrations (U/ml) on the X-axis.

Estimation of Vitamin D3

The serum level of human Vitamin D3 was assessed by the Human Vitamin D Direct AccuBind ELISA kit (Monobind Inc, Lake Forest, CA 92630, USA, product code: 9425-300) based on the competitive ELISA method following the kit's manufacturer's instructions. The ELISA standard curve was drawn by plotting the OD values on the Y-axis and calibration concentrations(pg/ml) on the X-axis to calculate the VD3 concentration of tested samples using EXCEL 2016.

Estimation of Interleukin-17

The serum level of interleukin 17 (IL-17) in the study subjects was estimated by ELISA. A human IL-17 ELISA Kit was used based on the Sandwich method (SUN LONG BIOTECH/CHINA, Catalogue number: SL0978Hu) following the directions provided by the kit's manufacturer. The ELISA standard curve was drawn by plotting the OD values on the Y-axis and calibration concentrations(pg/ml) on the X-axis to assess the concentration of tested samples.

Statistical Analysis

The initial normality and non-parametric test (Mann-Whitney U test) were performed to analyze the data statistically. Pearson's correlation was applied to determine the tested parameters' correlation. The receiver operating characteristic (ROC) curve was established. The area under the curve (AUC) was evaluated to compare the diagnostic efficacy of each value to predict the presence of RA. All statistics and graphics were created using Excel 2016 and the GraphPad Prism 8.05 program.

Results

Study subject demography and clinical characteristics

Demographic and clinical data, including age, Body Mass Index (BMI), family history of patients, and patient treatment usage, were presented in Table1. The findings

revealed that the mean age for the RA and HC groups was 47.27 ± 1.187 and 44.15±2.27, respectively. The distribution of age among RA patients showed that (41.7%) of RA patients were within the third group (42-51 years), which had the highest number, including 25 RA women, followed by the fourth group (52-61) with 21 patients, whereas a low number of patients were observed in the first group (22-31 years), including 3 (5%) patients.

The present outcomes demonstrated that 45.0% (27) of RA women were within the normal weight category, including (18.5-24.9), as well as 36% of RA patients, which included (22) were overweight compared to the healthy control group, where the majority of them tended to be normal weight. A person's BMI was determined using the equation (BMI = kg /m2), where kg is their weight in kilograms and m2 is their height in meters squared. The duration of the disease in patients was 12 ± 216 years and ranged between (1-39) years.

The present study showed that 51.7% (31) of RA patients had a family history of the disease, while 48.3% (29) of RA patients showed no family history of RA disease. Also, 51.7% (31) of RA patients have a family history of the disease, while 48.3% (29) do not. Patients with first-degree relatives with RA (mother, sister, or brother) made up 46.7% of the total (28), while patients with second-degree relatives (grandparents, aunts, and uncles) with RA made up only 5% of the total (3).

According to their doctor's diagnosis and tests, 45% (27 RA patients out of 60) of the study's RA women took vitamin D3 due to vitamin D deficiency. While 33 RA patients, or 55% of the study's patients, did not receive vitamin D3. Moreover, present results revealed that 68.3% (45) of RA patients took biological therapy while 31.7% (19) did not. As well as the majority of RA patients, 86.7% (52) received Anti-Rheumatoid drugs, while only 13.3% (8) did not. Biological therapy was an Enbrel injection. Physicians also recommended antirheumatic medications, such as methotrexate and hydroxychloroquine, as Disease-Modifying Antirheumatic Drugs (DMARD).

Serological study

For the serological study, sixty RA female patients and twenty HC were enrolled to estimate the serum Anti-CCP level, serum Vitamin D3, and IL- 17 (table 1).

Estimation of cyclic citrullinated peptide antibody IgG (Anti-CCP)

As shown in Figure 1, A, the current findings showed that Anti-CCP levels were significantly higher in the RA group compared to the HC group (p< 00001), means and ranges were 72.77 ± 14.08 (0.9768- 320.2) and 1.629 ± 0.1440 (0.9819- 3.421) pg/ml, respectively). The mean CCP level in the -ve cases was 2.719 ± 0.3276 U/ml. The study results of the seropositivity and sero-negativity of

RA women concerning CCP levels revealed that 41.7% of RA patients were positive for CCP, and the mean level was 170.9 ± 21.94 U/ml, leaving 58.3% of RA patients as negative cases. The mean concentration for HC was 1.629 ± 0.144 U/ml, and all samples tested negative for CCP, which is 100%, as shown in Figure1 B.

The fourth age group expressed high levels of anti-CCP, followed by the second and third age groups of RA women. Also, the Anti-CCP elevated with advancing with age significantly, as represented in Figure2- A.

Age groups	Rheumatoid arthritis group		Healthy control group	
	Number	Percentage	Number	Percentage
Group 1 (22-31) year	3	5	3	15
Group 2 (32-41) year	11	18.3	4	20
Group 3 (42-51) year	25	41.7	7	35
Group 4 (52-61) year	21	35	6	30
Total	60	100	20	100
BMI (Kg/ m2)	No.	%	No.	%
Underweight<18.5	7	11.7	5	25
Normal weight, 18.5-24.9	27	45	13	65
Overweight 25-29.9	22	36.7	2	10
Obese 30-34.9	3	5	0	0
Severe obese 35-39.9	1	1.7	0	0
Total	60	100	20	100
RA patients with Positive family history				
First-degree relatives	28	46.7	-	-
Second-degree relatives	3	5	-	-
No family history of RA	29	48.3	-	-
Total	60	100	-	-
Biological therapy	41	68.3	-	-
Anti-rheumatoid drugs	52	86.7	-	-
Vitamin D3 supplement	27	45	-	-

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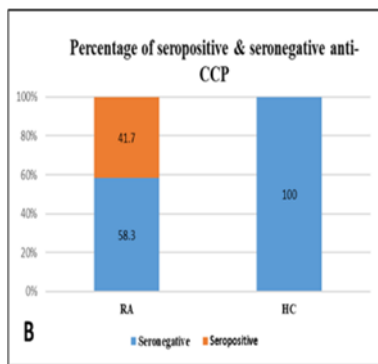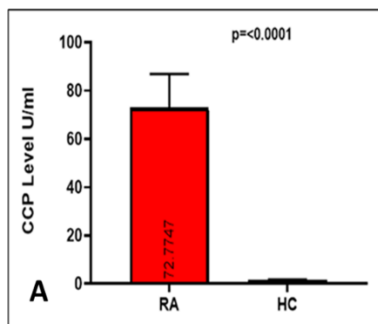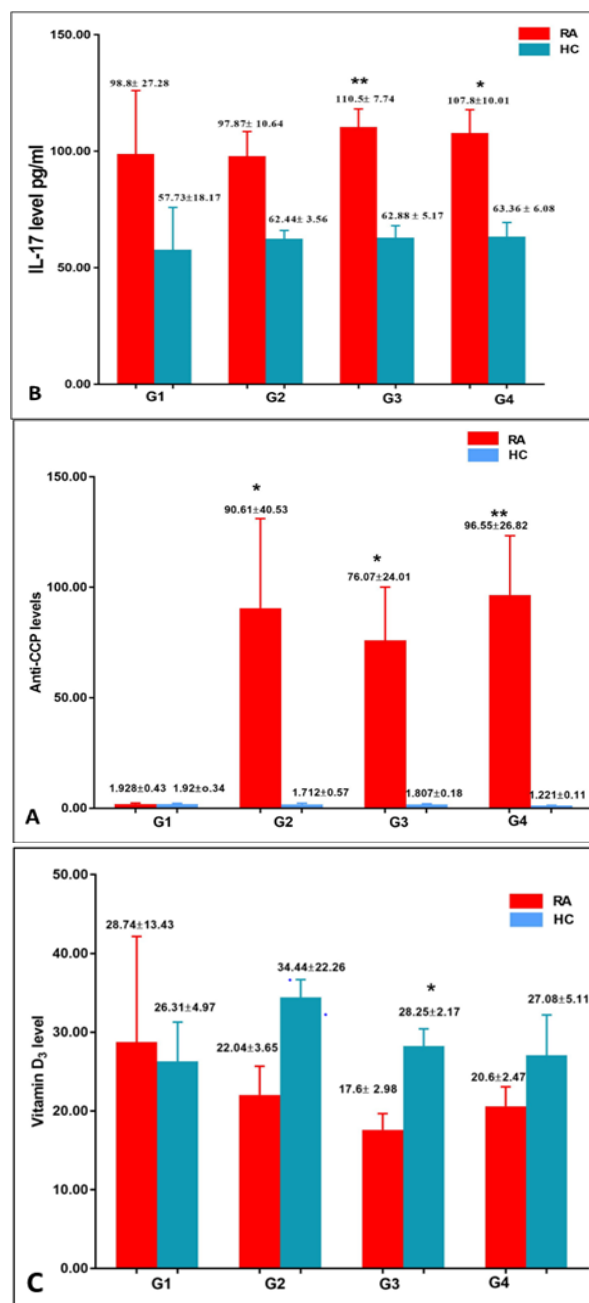

Figure 1: A. Anti-CCP level in RA and HC women by ELISA. **B.** The percentage of seropositive and negative anti- CCP cases in RA and HC women. RA: Rheumatoid Arthritis, HC: Healthy Controls

Figure 2: **A**. Anti – CCP according to age groups of RA and HC groups. Anti-CCP titer in the RA group increased significantly with age (p<0.0001). **B.** Interleukin – 1 according to age groups of RA and HC groups. A significant increase was detected in RA patients' third and fourth age groups (P<0.0001). **C.** Vitamin D3 levels according to age groups of RA and HC groups. A significantly low value of VD3 in the third group (P<0.0001).
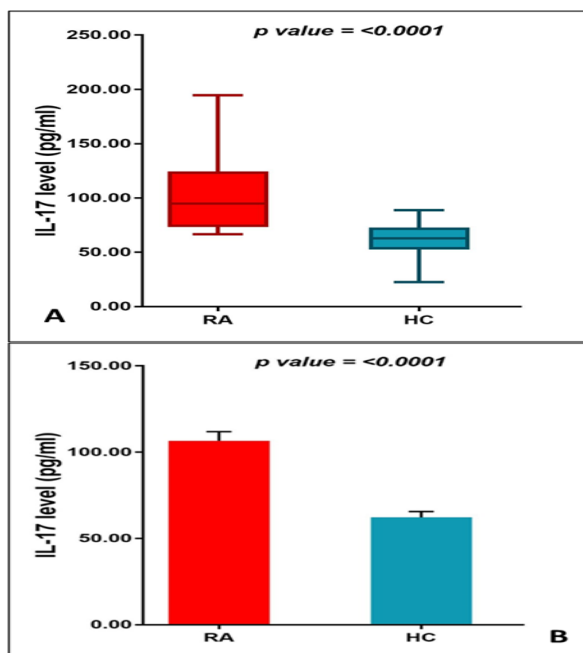
Figure 3: A. Box plot represents serum concentration of IL-17 in RA and control groups. Median values are represented by the horizontal lines with maximum and minimum values. **B.** The levels of IL-17 were significantly increased in RA patients compared to HC measured by the ELISA test (P<0.0001). RA: Rheumatoid Arthritis, HC: Healthy Controls, (pg/ml) picograms per milliliter.
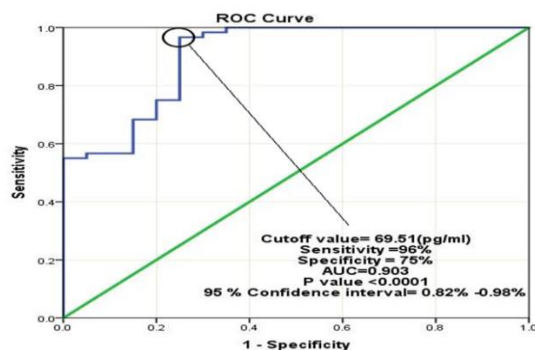

Figure 4: The receiver operating characteristic (ROC) curve of serum IL-17 concentrations for RA. The area under the curve (AUC) was 0.903. Standard Error of AUC =0.041, P value <0.0001, 95 % Confidence interval= 0.82-0.98%. Cutoff value= 69.51pg/mL. Sensitivity was 96%, and specificity was 75%.

Estimation of serum Vitamin D3 concentrations in RA patients

The present results demonstrated that the level of vitamin D3 was significantly decreased (p =0.0002) in RA women compared to HC. The means and range were 20.02 ±1.514 ng/ml (2.304-54.84 ng/ml) and 28.85 ±1.890 ng/ml (3.113 -39.54ng/ml) ng/ml, respectively. Furthermore, the Vitamin D3 levels of study subjects were divided into four groups depending on the kit concentration range. The first group was VD3 deficiency (< 20 ng/ml), the second group was VD3 suboptimal (20-30 ng/ml), the third group was VD3 optimal (30 - 50 ng/ml) and the fourth group of upper normal ($\geq$ 50 ng/ml). Most RA patients suffered from VD3 deficiency (VD group 1), which comprised 56.9% counts 34 of all patients, and the mean VD3 level was 12.10 ± 0.9216 ng/ml. RA patients with suboptimal VD3 levels (VD group 2) included 26.7 %, which counts 16 patients with a VD3 level mean of 24.23±0.7050 ng/ml. 15.0%, which measures nine patients with an optimal level of VD3 (38.57± 2.083 ng/ml), and finally, just one of them (1.7 %) had an overdose of VD3 level with a mean of 54.84 ± 0.000. In healthy control, about half of the HC individuals who were 45% counted nine in the third group with a VD3 mean of 35.29 ± 1.136 ng/ml, while 40 % of those who counted eight had a suboptimal level of VD3 (27.23±0.5558 ng/ml). Only 15% of HC had VD3 deficiency levels with a mean of (13.80± 5.368 ng/ml). In contrast to the other age groups, the third group of RA women displayed a low significant value of V D3 (P<0.0001).

Serum IL-17 levels in RA and HC subjects

The current findings showed a significant rise in IL-17 levels in the RA group in comparison to HC (p<0.0001). The means and ranges were 106.6 ±5.221 (66.620-194.7) and 62.16 ± 3.419 (22.48-88.80) pg/ml, respectively, as illustrated in Figures 3-A and B. Patients in the third and fourth age groups had levels of Il-17 that were significantly higher than those in other age groups, as shown in Figure 2- B.
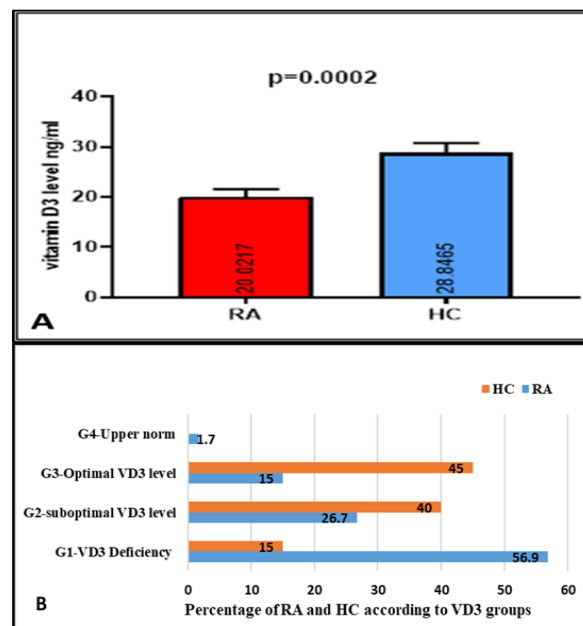

Figure 5: A. The VD3 levels in RA women and HC by ELISA. The mean of VD3 was 20.02 ±1.514, significantly lower in RA women than in the HC group, which was 28.85 ±1.890 ng/ml. **B.** Percentages of RA and healthy control women within VD3 grading. The first group was VD3 deficiency (<20 ng/ml), the second group was VD3 insufficient (20-30 ng/ml), the third group was VD3 optimal (30-50 ng/ml), and VDR3 the fourth group represents upper normal (≥ 50 ng/ml)

The ROC curve was performed to evaluate the best serum IL-17 cutoff value that can predict a diagnosis of RA. As depicted in Figure 4, the Cutoff value was 69.51 (pg/mL), sensitivity was 96%, and specificity was 75%.

Pearson's correlation between Vitamin D3 level and age groups

Figures 6- A and B depict the relationship between the age of the RA group and HC and their vitamin D3 levels. A non-significant negative correlation was observed between Vitamin D3 serum levels and the age of RA and HC women. Pearson's correlation was -0.1146 and -0.0325, respectively.

Pearson's correlation between IL-17 levels and age groups

Figures 6- C and D depict the relationships between IL-17 levels, RA patient ages, and HC. A non-significant weak positive association was detected between IL-17 serum levels and ages of RA and HC groups; the Pearson's correlations were 0.062 and 0.206, respectively.

Pearson's correlation between IL-17 levels and Vitamin D3

The correlation between the study subject's vitamin D3 level and IL-17 level revealed a non-significant weak positive relationship between these parameters in the RA group, in which Pearson's correlation was 0.038. In contrast, the HC group's Pearson's correlation value was negative - 0.2328 between the two parameters, as shown in Figures 7- A and 7- B. Figure 7- C & D depicts the interaction between the IL-17 concentration and BMI of the RA and HC groups. In the group of RA women, there was a non-significant weak positive association between IL-17 level and BMI. In contrast, a non-significant weak negative relationship in the HC group was found. Pearson's correlation was 0.2231 and - 0.05756, respectively.

Discussion

This study found that in Kurdistan, Iraq, the mean age of female RA patients was 47.27 ± 1.187. Most patients were in the third age group (42-51) and the fourth age group (52-61). This result is consistent with Khadim and Al-Fartusie's finding [20] that RA patients in Iraqi women had a mean age of 48.56. In addition, a recent retrospective study of RA patients in Iraq found that their average age was 48.34 ± 11.7 years (range: 23-95 years) and that they were females (82.7 %). Seventy-six per cent of those patients demonstrated seropositivity for ACCP antibodies [21]. The tendency of rheumatoid arthritis disease to affect older adults is well-described in Iraq. Hussein et al. [22] also found that most patients were between 40 and 59 years old.
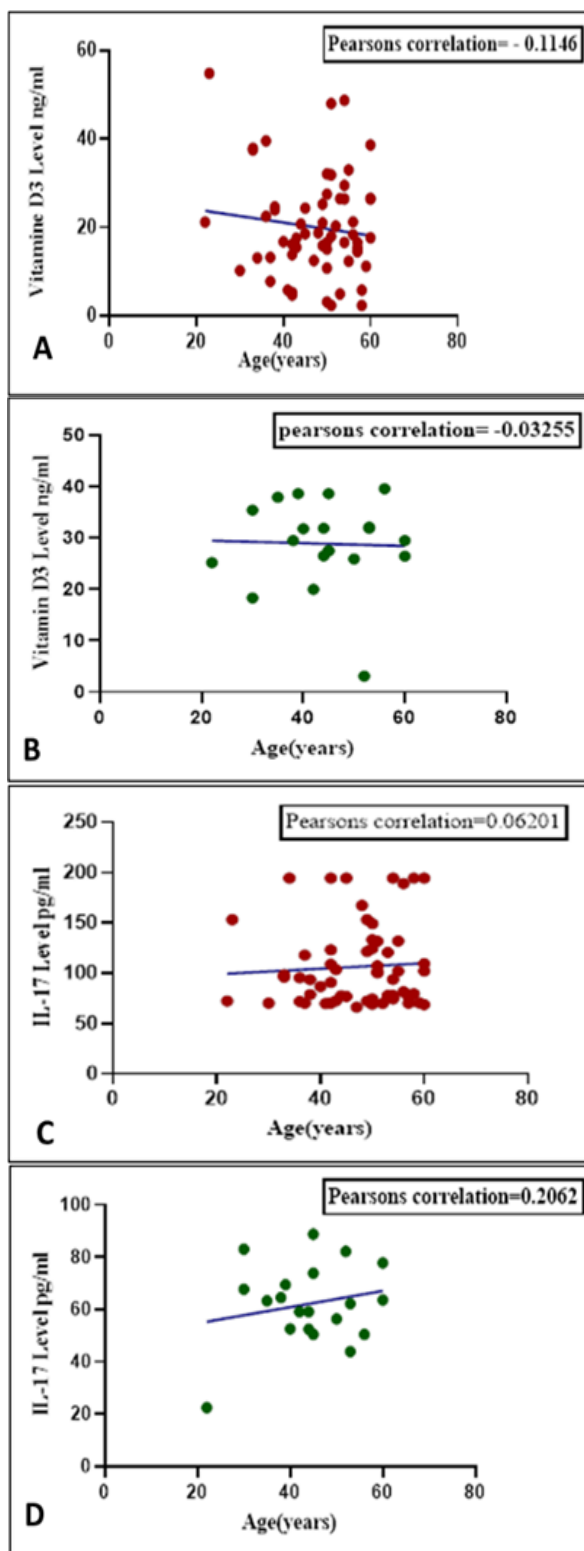

Figure 6: **A.** Pearson's correlation between vitamin D3 and age in the RA group. **B**, Pearson's correlation between vitamin D3 and age in the HC group. **C**, Pearson's correlation between IL 17 and age in the RA group. **D**, Pearson's correlation between IL-17 and age in the HC group.
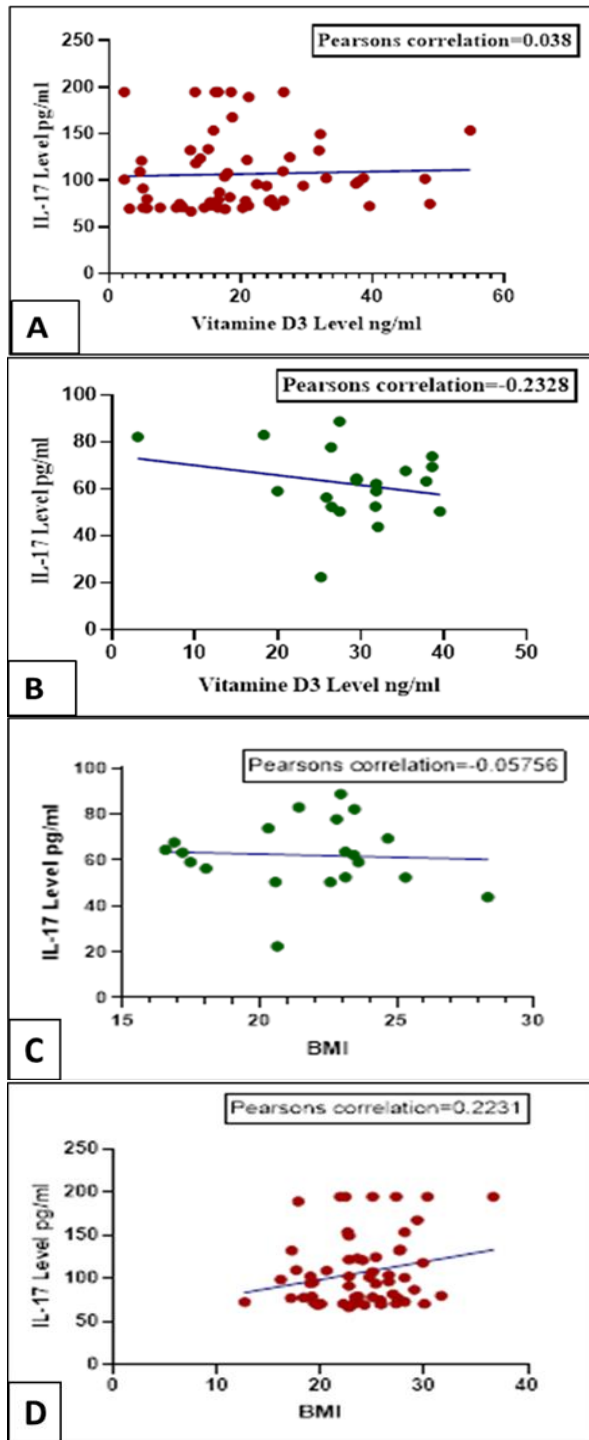
Figure 7: A. Shows a non-significant weak positive Pearson's correlation between the RA group's IL-17 and VD3 levels. **B,** Negative Pearson's correlation between IL-17 and VD3 in the HC group. **C,** Pearson's correlation between IL-17 and BMI of RA patients. **D.** Pearson's correlation between IL-17 and BMI of HC.

The present study showed that 51.7% (31) of RA patients have a family history of this disease, while 48.3% (29) of RA patients showed no family history. Patients with a first-degree relative who had RA

represented 46.7% (28) and patients with second-degree relatives with RA comprised only 5% (3). Recently, Gr et al. [23] concluded that higher seropositive of Anti-CCP in RA patients' asymptomatic first-degree relatives (AFDR) might increase the risk of developing RA soon. Therefore, all AFDRs should be screened for early detection, and positive cases must be followed up.

Sera negative and positive Anti- CCP cases were described and mentioned in many studies; according to our findings, 41.7% of RA patients tested positive for anti-CCP, while 58% of RA patients tested negative. Another study mentioned that 81 (70.4%) of the RA samples had anti-CCP antibodies [24]. Previous investigations proved that ACPA-positive individuals experienced more active symptoms during follow-up than ACPA-negative patients [25]. As a result, ACPA was recommended in the 2009 RA diagnostic standards based on the available evidence [26]. Anti-CCP is more sensitive than RF and may detect RA disease earlier in its progression. Since it correlates well with disease activity, anti-CCP should be chosen over RF. However, it is ineffective in controlling the disease's extra-articular manifestation (EAM) [23]. According to recent studies [21,27,28], (RA patients who are seropositive for RF or anti-CCP antibodies are thought to have more aggressive symptoms than seronegative patients.

Women with RA frequently lack adequate amounts of vitamin D. Our research found that the mean levels of vitamin D3 in RA patients were significantly lower than those in healthy controls; these values were 20.02 ± 1.514 ng/ml and 28.85 ± 1.890 ng/ml, respectively. This study revealed that most RA patients suffered from VD3 deficiency, which includes 56.9% of RA cases. Previously, in research conducted in Erbil, Iraq [29]. They found that 70% of the studied RA cases had Vitamin D3 deficiency. Another study suggested that hypovitaminosis D is a common risk factor for many autoimmune diseases, including RA, because VD3 is responsible for regulating immune response and boosting the immune system, as mentioned [30]. Meena et al. [18] concluded that Vitamin D deficiency is more prevalent in RA patients and may be one of the reasons for developing or worsening symptoms. Women with RA had lower basal serum levels of 25(OH)D than healthy controls, which is consistent with our observations, and also seems to be an environmental risk factor for RA [31]. Similarly, at least in postmenopausal women, vitamin D intake and the risk of RA were negatively correlated [32,33].

Our results showed a significant increase in IL-17 levels in the RA group compared to HC, with a mean of 106.6 ±5.221and 62.16 ± 3.419, respectively. This result agrees with [34,35], Who documented a significant difference between the serum IL-17 levels in the RA patients and the control group. They proposed that

serum IL-17 levels might be a key indicator of RA disease activity. Moreover, the present findings point out that the AUC of IL-17 for diagnosing RA was 0.903. The 95% confidence interval of 0.82- 0.98%, with high sensitivity of 96% and specificity of 75%, suggests that the serum levels of IL-17A can potentially be a biomarker for RA. Similarly, Qu et al. [34] concluded that IL-17 has better sensitivity and specificity than IL-10 for diagnosing RA. In addition, a recent study proved that IL-17A has potential efficiency as a biomarker for disease activity in RA [36]. Rheumatoid arthritis is one autoimmune disease for which IL-17 has been linked to its pathogenesis.

Nevertheless, in vivo and in vitro results depict that IL-17A is one of the causes that spread local inflammation in the synovium, particularly in RA [37]. Suppression of interleukins is beneficial in controlling some diseases. It has been believed that suppression of IL-31 may be a helpful way to treat inflammatory cases [38]. New strategies are required to precisely control IL-17-mediated immunopathology in a chronic autoimmune disorder [39].

This work revealed that serum IL-17 levels were significantly higher in RA women than in the control group (p < 0.001). Vitamin D deficiency was prevalent in RA patients and correlated positively with anti-CCP and inversely with the age. Consequently, a high level of suspicion is needed when assessing at-risk patients, particularly women who complain of vitamin D deficiency. Moreover, IL-17 has good sensitivity and specificity, which helps to raise the diagnostic bar for RA and serves as a guide for the disease's pathogenesis, diagnosis, and treatment. Further studies with more cases of RA and analyzing the IL-17 level in the synovial fluid are needed to validate the study's findings.

Competing Interest

The authors declare that there is no conflict of interest in this work.

Author Contributions

RR Qadir carried out the experimental work, BH Shnawa designed and supervised this study, and AG Shlimon revised the draught of the paper. All authors have read and agreed to the published version of the manuscript.

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