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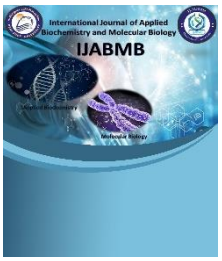


# The Interplay between Circulating CCR7, MMP9, and Vitamin D in Rheumatoid Arthritis

**Naglaa F. Abozeid [1*], Olfat G. Shaker[1], Eman M. Ezzat[2], Ghada Ayeldeen[1],**

1 Medical Biochemistry and Molecular Biology Department, Faculty of Medicine Department, Cairo University, Cairo 11956, Egypt.

2 Internal Medicine Department, Faculty of Medicine, Fayoum University, Egypt.

***Corresponding author**

Tel. of the corresponding author: +20 1003624844, E-mail: naglaafathy@kasralainy.edu.eg

**Running Title**: *CCR7, MMP9, and Vitamin D in Rheumatoid Arthritis*

## Abstract

**Background:** Vitamin D affects the lncRNA chemokine receptor 7 (CCR7) and matrix metalloproteinase 9 (MMP9) expression levels in many inflammatory conditions. The collaboration between CCR7 and MMP9 has been studied in many cancers but not in patients with rheumatoid arthritis (RA).

**Objective:** To dissect the potential effect of 25-OH vitamin D deficiency on CCR7 and MMP9 in RA patients.

**Subjects and Methods:** This study included 120 participants, 60 RA patients, and 60 healthy volunteers. History, clinical examination, and laboratory investigations were performed. The molecular analysis includes quantitative real-time PCR (qPCR) for revealing CCR7 levels, while the ELISA technique was used to measure 25-OH vitamin D and MMP9 levels.

**Results:** The present study revealed that the significant decrease in total 25-OH Vitamin D levels in RA patients was significantly correlated with the increased serum levels of the lncRNA CCR7 with fair sensitivity and high specificity. Combining 25-OH vitamin D with CCR7 to predict RA seems to have little effect. Similar findings were noted with MMP9.

**Conclusions:** We promote serum CCR7 and MMP9 to be widely investigated as possible noninvasive biomarkers for RA. We suggested their expression could be modulated by controlling 25-OH vitamin D levels.

**Keywords:** RA ,lncRNA, CCR7, Vitamin D, MMP9.

## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects up to 1% of the population worldwide (1). It affects women three times more frequently than men (2).

Dendritic cells (DCs) play crucial roles in the development of RA, including antigen presentation and enhanced pro-inflammatory cytokine production (3). Trafficking of the immune cells to the synovium is helped by the binding of many ligands named chemotactic cytokines or chemokines to their chemokine receptors (4) as the central chemokine (C-C motif) receptor 7 (CCR7) **(Supplementary Material, SM_1),** which links the innate and adaptive immunities (5). Vitamin D is a known multifactorial effector that regulates immune homeostasis by affecting many immune cells (6). DCs could be considered the primary immune cell target of vitamin D to dampen autoimmunity and inflammation (7). Vitamin D increases the regulatory T (Treg) cells (8) and affects DCs by converting them into a tolerogenic phenotype, which is characterized by low expression of cytokines needed for antigen presentation and lymphocyte activation (9). A wide range of data supports that vitamin D3, in inflammatory conditions, decreases the surface CCR7 expression of DCs (10). On the other hand, less recent studies supported that exogenous vitamin D caused enhanced maturation of DCs as well as up-regulation of CCR7 (11). The matrix metalloproteinase 9 (MMP9) was identified as a macrophage-related biomarker in RA (12). The dis-coordinate regulation of MMP9 by vitamin D was anciently reported in human mononuclear phagocytes (13) and in rat articular cartilage with osteoarthritis (14). The cooperative effect of CCR7 and MMP9 was studied in cancers (15–17) but not in RA.

As vitamin D modulates the production of chemokines and metalloproteinases, the present study investigates its effects on CCR7 and MMP9 production to provide a deeper understanding of these biomarkers that may help the exploration of new biological therapies.

## Subjects and Methods

### Study population:

All methods were performed in accordance with the guidelines and regulations approved by the Research Ethics Committee, Faculty of Medicine, Fayoum University (approval number: R578). This case-control study enrolled 60 RA patients and 60 healthy volunteers from the Internal Medicine Department, Faculty of Medicine, Fayoum University, Egypt.

Inclusion criteria for RA patients: individuals with a proven diagnosis of RA, based on the American College of Rheumatology (ACR 1987) criteria for RA. Exclusion criteria for RA patients: include patients aged less than 18 years or with concurrent autoimmune diseases or malignancy, pregnancy, or lactating patients. Inclusion criteria for the volunteers: a minimum of 18 years old. Exclusion criteria for the volunteers (by questionnaire in Arabic) comprise history of RA, including morning joint stiffness, the findings of rheumatoid nodules, or previous treatment for arthritis, heart or renal failure, and other autoimmune diseases or inflammatory disorders.

All participants gave written informed consent preceding the study to give a history, undergo a clinical examination, and undergo blood sampling. The separated serums were divided into portions for routine laboratory investigations, namely complete blood count (CBC), alanine aminotransferase (ALT), and creatinine. Serum samples stored at −80°C were used for RNA extraction and protein level determination for CCR7 and MMP9 levels.

In addition, for RA patients, history-taking included receiving disease-modifying anti-rheumatic drugs (DMARDs). Disease severity was assessed by measuring the disease activity score for 28 joints (DAS28) (18), which defines remission as a score below 2.6, 2.6-3.2 to be mild, 3.3-5.1 to be moderate that may require a change in medication, and > 5.1 to be severe that requires careful monitoring and adjustment to medication. More laboratory analyses were performed,

including measuring erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), and antinuclear antibody (ANA).

**RNA extraction and quantitative real-time PCR (qPCR):**

RNA was extracted from serum using Qiagen (Valencia, CA, USA). RNA quantification and purity evaluation were performed on RNA samples utilizing the NanoDrop®(ND)-1000spectro-photometer (NanoDrop Technologies Inc., Wilmington, USA).

Reverse transcription (RT) was performed using the miScript II RT kit (Qiagen, Valencia, CA, USA) on the whole RNA in final volumes of 20 uL RT reactions. The Rotor-gene thermocycler (Qiagen, USA) was used for qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (with the primer sequence F: 5′-CCCTTCATTGACCTCAACTA-3′, R 5′-TGGAAGATGGTGATGGGATT-3) was used for relative quantification of the target CCR7 (Hs-CCR7 cat No. QT00045507 Quanti Tect.primer assay, Qiagen, USA) while using the ΔCt method.

**Detection of serum levels of total 25-OH vitamin D and MMP9:**

The level of vitamin D was measured in serum, consuming a total 25-OH vitamin D EIA Kit Enzyme Immunoassay (Epitope Diagnostic Inc., San Diego, USA). Serum MMP9 levels were evaluated using a human ELISA kit (Koma Biotech Inc., Seoul, Korea). Both were performed according to the instructions of the manufacturer.

**Calculation of results and statistical analyses:**

The data were coded, and the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA) was used for the entry of the available information. Data was presented using mean± standard deviation (SD) in quantitative data and using positive count and percentage (%) for categorical data. For the quantitative variables, the unpaired t-test was used for comparisons between groups with normally distributed variables, while the non-parametric Mann-

Whitney test was used for non-normally distributed variables (19), and the Spearman correlation coefficient was used to perform correlations (20). For comparing categorical data, the Chi-square ($\chi 2$) test was performed, but when the expected frequency was <5, the Exact test was used instead (21).

## Results

The laboratory findings for the RA patients and the control groups are tabulated in Table 1. The RA patients and the control volunteers were age-matched (P-value is 0.948). 80% of the RA patients were females, while 66.7% of the control subjects were males (P-value is <0.001). Further data can be obtained from Table 2.

**Table 1: Laboratory investigations of the studied groups**

| Laboratory investigations | RA patients (n=60) | Control (n=60) | P-value |
|---|---|---|---|
| **RF** | 43(71.7%) | ----- | ----- |
| **ANA** | 8(13.3%) | ----- | ----- |
| **ESR (mm/h)** | 40.65±23.15 | ----- | ----- |
| **Hb (g/dl)** | 11.77±1.64 | 12.12 ±1.26 | 0.193 |
| **TLC ($\times 10^3/mm^3$)** | 7.49±2.36 | 7.64±2.29 | 0.718 |
| **PLT ($\times 10^3/mm^3$)** | 283.32±75.92 | 278.50±57.31 | 0.696 |
| **ALT (U/L)** | 28.70±21.91 | 13.80±5.02 | <0.001* |
| **Creatinine (mg/dl)** | 0.92±0.77 | 0.65±0.12 | <0.001* |

RF: Rheumatoid factor, ANA: Antinuclear Antibody, ESR: Erythrocyte sedimentation rate, Hb: hemoglobin, TLC: total leucocyte count, PLT: Platelet count, ALT: Alanine transaminase. RF and ANA are represented as the number of positive cases (%). ESR, Hb, TLC, PLT, ALT, and creatinine were represented as mean± SD. A P-value is considered significant (*) if < 0.05.

**Table 2: The demographic and clinical data of the RA patients**

| | |
|---|---|
| **Age (years)** | 39.85±10.96 |
| **Disease duration (years)** | 6.94±4.99 |
| **Disease severity** | 3.03±1.67 |
| **Gender** | |
| **Female** | 48 (80% |
| **Male** | 12 (20%) |
| **Clinical Manifestations** | Morning stiffness (min) 25.68±25<br>Arthritis 47(78.3%)<br>Deformity 40(%66.7)<br>Fever 11(18.3%)<br>Rheumatoid Nodule 10(16.7%)<br>Extra-articular manifestations 13(21.7%) |
| **Disease activity** | Remission 27(45%)<br>Low 5 (8.3%)<br>Moderate 20 (33%)<br>High 8 (13.3%) |
| **Treatments**<br>**(received at least one dose)** | TC 60(100%)<br>MTX 48 (80%)<br>Steroids 21(35%)<br>HQN 15(25%)<br>leflunomide 10(16.7%)<br>Others 14(23.3%) |

TC: Taxotere and cyclophosphamide, MTX: Methotrexate (MTX), HQN: Hydroxychloroquine. Age, disease duration, morning stiffness, and disease severity were represented as mean± SD. Gender, clinical manifestations, activity phase, and treatments are represented as the number of positive cases/ frequency (%).

**Expression profile for CCR7 in the study population**

The expression levels for lncRNA CCR7 in the RA patients compared to the control group showed a statistical increase (P< 0.001) (Fig.1a).

**Serum levels for MMP9 protein levels in the study population**

The mean serum levels for MMP9 protein in the RA patients against the control group showed a statistical increase (P< 0.001) (Fig.1b).

**Serum levels for total 25-OH vitamin D in the study population**

The mean serum levels for 25-OH vitamin D showed a statistical decrease (P< 0.001) between the two groups, as shown in Fig.1c.

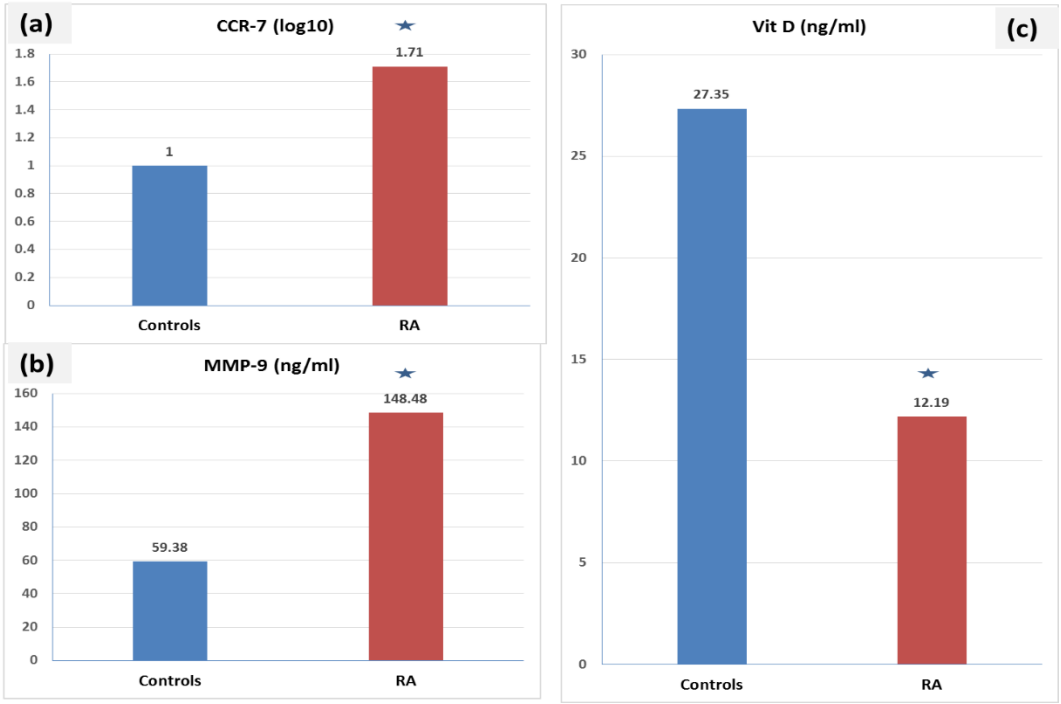


**Fig.1 Serum levels for the measured biomarkers between the RA group and the control group: a;** serum lncRNA CCR7 gene expression levels; **b;** serum mean levels for MMP9 protein; **c;** serum mean levels for 25-OH vitamin D. * means the P-value is significant (< 0.05) alongside the control group.

**Correlations between the serum biomarkers in the study group**

For further study of the measured biomarkers, we performed correlations between the RA group and the control group and found that 25-OH vitamin D in RA patients was negatively correlated with CCR7 (correlation coefficient -0.558 and P-value <0.001) (Fig.2a) and with MMP9 (correlation coefficient -0.366 and P-value <0.001) (Fig.2b). In addition, both CCR7 and MMP9 were significantly positively correlated (correlation coefficient 0.521 and P-value <0.001) (Fig.2c). In addition, we performed correlations between the measured biomarkers and the duration and the disease severity in the RA group and found that only MMP9 had a significant positive correlation with the duration of the disease (correlation coefficient 0.337 -and Pvalue0.008 ) (Fig.2d). No other significant correlations existed.

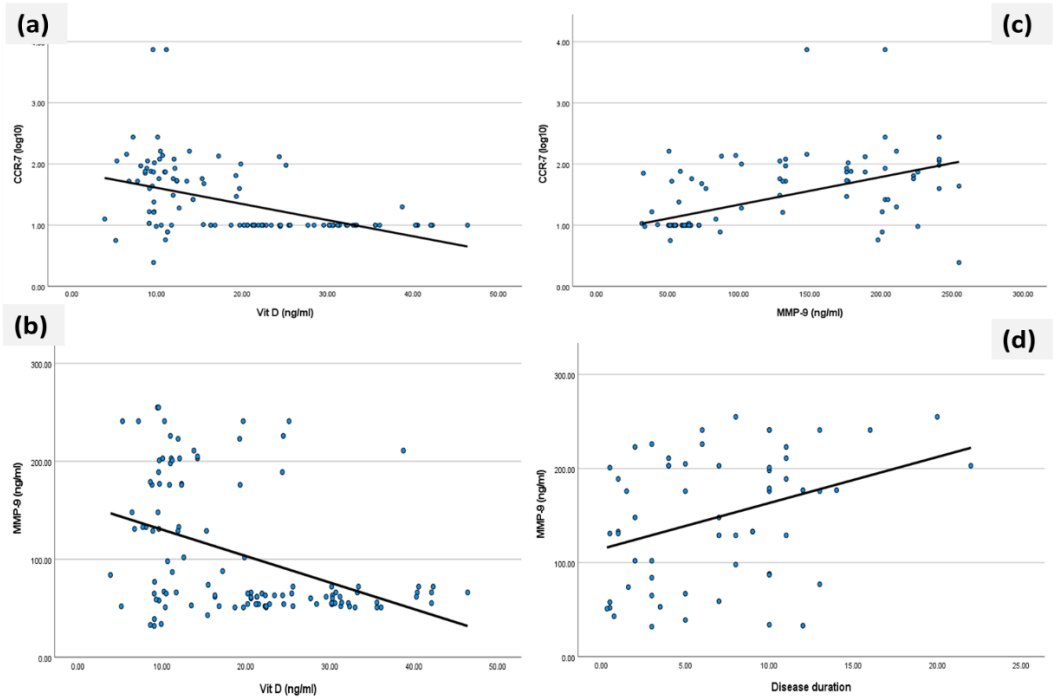


**Fig.2 Correlations between the serum biomarkers in RA patients and the control subjects: a;** correlation between serum 25-OH vitamin D levels and the expression levels of serum CCR7; **b;** correlation between 25-OH vitamin D and MMP9 serum protein levels; **c;** correlation between the expression levels of CCR7 and serum MMP9 levels; **d;** correlation between the serum MMP9 levels and the duration of the disease.

**Evaluating the predated probability of CCR7 alone and after combination with 25-OH vitamin D for diagnosis of RA**

The ROC (receiver operating characteristic) curve was employed to assess the predictiveness of CCR7 and MMP9 in RA patients, where CCR7 could distinguish them from control volunteers with a cut-off level of 1.005, 88.3% sensitivity, and exhibited the highest specificity (P-value <0.001) (Fig.3a). On combination with 25-OH vitamin D, sensitivity was enhanced (95%) with slightly decreased specificity (96.7%) (P-value <0.001) (Fig.3b).

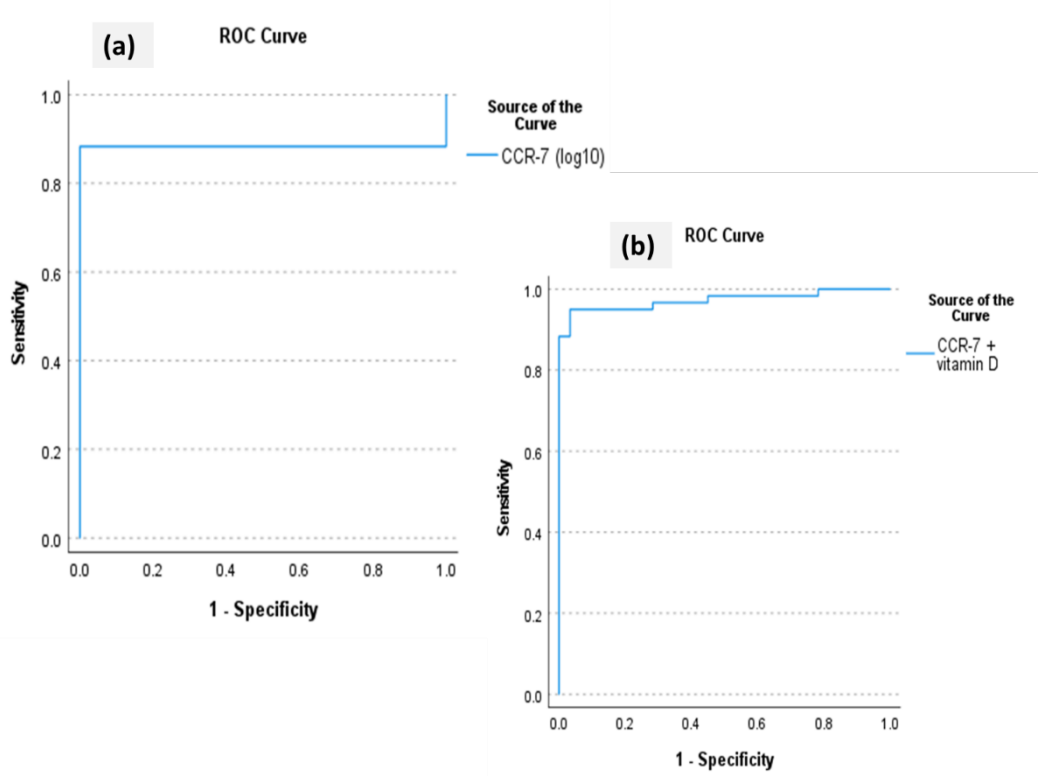


**Fig.3 ROC curve for predated probability of using CCR7: a;** CCR7 only; **b;** after combination of CCR7 with 25-OH vitamin D. P-value<0.05 reflected a threshold for significance.

**Evaluating the predated probability of MMP9 alone and after combination with 25-OH vitamin D for diagnosis of RA**

Using ROC curve analysis in RA patients, MMP9 levels exhibited a high prediction accuracy (sensitivity 80% and 100% specificity) in predicting RA (P-value <0.001) at a cut-off level of 73.05 (Fig.4a). In combination with 25-OH vitamin D, sensitivity was markedly enhanced (98.3%) with slightly decreased specificity (96.7%) (P-value <0.001) (Fig.4b).

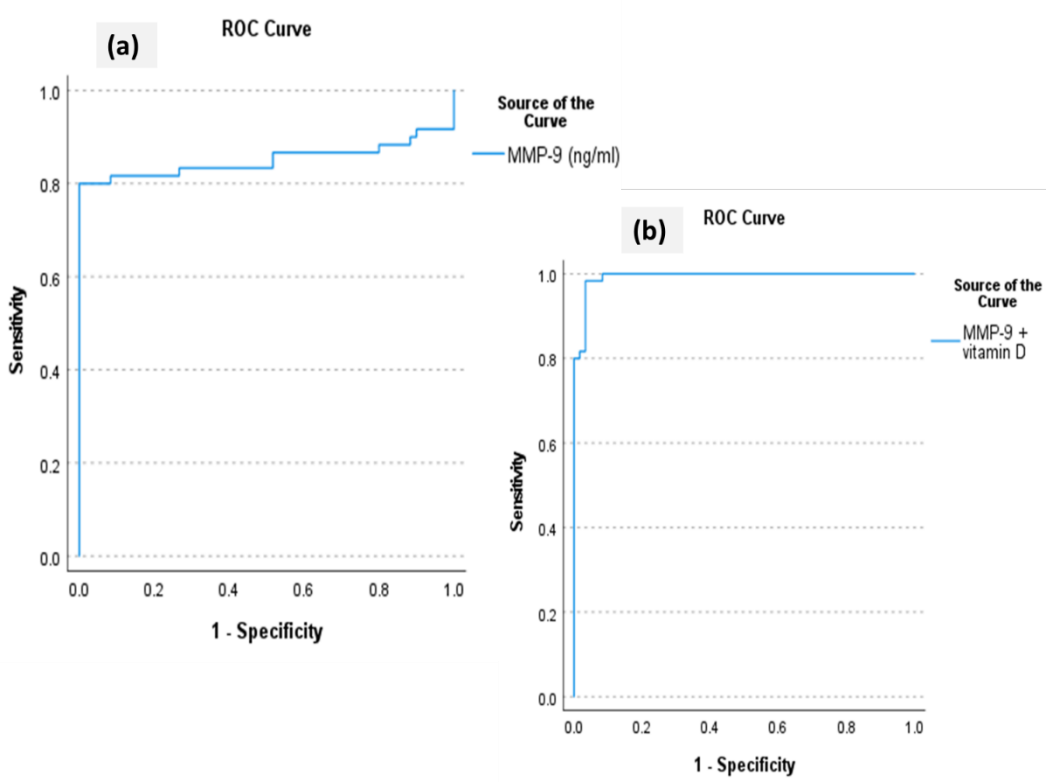


**Fig.4 ROC curve for predated probability of using MMP9: a;** MMP9 only; **b;** after combination of MMP9 with 25-OH vitamin D. P-value<0.05 reflected a threshold for significance.

## Discussion

The immunomodulatory and anti-inflammatory actions of vitamin D have been extensively studied in RA **(22-26).** The relationship between vitamin D and CCR7 expression in humans has been studied in some autoimmune diseases but not in RA. For instance, in systemic

lupus erythematosus (SLE), CCR7-Tregs were found to be enhanced after an intensive course of vitamin D3 for two years **(27).**

We and others **(28, 29)** have demonstrated that CCR7 showed a statistical increase in RA patients than in healthy subjects. We, therefore, took a comprehensive approach to investigate the effect of 25-OH vitamin D deficiency on CCR7 increased levels in RA, and we found it significantly correlated and that combining vitamin D with CCR7 may enhance its sensitivity for RA predictiveness but not specificity.

The same issue is facing the uncelebrated MMP9 in RA, as its altered expression caused by vitamin D was studied in many human diseases such as cardiovascular diseases **(30-36),** with scarce studies in patients with RA that involved invasive arthroplasty procedures **(37)** or cell lines **(13).**

Our study showed a significant correlation between 25-OH vitamin D deficiency and increased MMP9 serum levels. Generally, vitamin D may inhibit the synthesis of MMP9 by blocking the TNF-JNK pathway (38)**.**

Combining 25-OH vitamin D with MMP9 may enhance its sensitivity for RA predictiveness but not its specificity.

Also, CCR7 and MMP9 were significantly correlated to each other. Although up to that time not earlier studied in RA, this result was previously reported in many cancer types (15-17). Adding to that, we document that MMP9 was the only biomarker to correlate with the duration of the RA disease.

## Conclusions

Serum CCR7 and MMP9 are mounting biomarkers that could be further investigated as non-invasive, diagnostic biomarkers in RA. Optimizing 25-OH vitamin D levels may modulate their activity, reversing the pathogenesis of RA. Future large-scale investigations are mandatory to substantiate these results.

## Statements & Declarations

**Funding**

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

**Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

**Ethics approval and consent to participate**

The Scientific Research Ethics Committee, Faculty of Medicine, Fayoum University, Egypt, has permitted this work, numbered R578 (registration number: RHDIRB-NA-101023-01UC-GU-No.1023), which comes following the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all participants.

**Data availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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