Vitamin D receptor gene polymorphism in rheumatoid arthritis and its association with atherosclerosis
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Introduction
Rheumatoid arthritis (RA) is an autoimmune disease that causes systemic inflammatory disorder and affects many tissues and organs, mainly the synovial joints [1]. People with RA are susceptible to cardiovascular disease (CVD), which is the cause of 40–50% of the deaths in this population [2].

The patients with subclinical CVD have a higher risk for atherosclerotic plaques, increased intima-media thickness (IMT) of the carotid arteries [3].

The presence of vitamin D receptor (VDR) and the production of vitamin D hormone from activated dendritic cells may suggest the immunoregulatory properties of the vitamin D [4].

Vitamin D has antiproliferative, antiangiogenic, and antioxidant properties, which have a significant protective effect on the cardiovascular system. It is well known that cardiomyopathy is secondary to nutritional rickets [5].

Aim of the work
Determine vitamin D receptor gene BsmI, FokI polymorphism and 25-hydroxyvitamin D in early Egyptian rheumatoid patients and its association to subclinical atherosclerosis.

Patients and methods
This study included forty early rheumatoid arthritis patients and forty healthy controls.

Disease activity score 28 (DAS-28), Modified Health Assessment Questionnaire (MHAQ), Carotid intima-media thickness (cIMT) were assessed using B-mode ultrasound, Erythrocyte sedimentation rate (ESR), C reactive protein (CRP), Lipid profile, anti cyclic citrullinated PolyPeptid (anti-CCP), serum interleukin-6, Total serum vitamin D and genotype determination of BsmI, FokI polymorphism and allel frequency were measured.

Results
Vitamin D deficiency was observed in 25% of patients. There was no significant difference between RA patients and controls regarding the distribution of BsmI genotype frequencies and allele. However, a significant difference between rheumatoid arthritis patients and controls regarding the distribution of FokI genotype and allele frequencies was found. In addition, FokI polymorphism and the F allele was significantly associated with RA, anti-CCP, interleukin-6 levels, (cIMT) and vitamin D deficiency were significantly higher in the presence of bb homozygote of BsmI genotypes and FF homozygote of FokI genotypes. A significant negative correlation between 25 hydroxy vitamin D levels with (DAS-28), ESR, (CRP), and IL-6 ($p < 0.001$). However, there was positive correlation between 25 hydroxyvitamin D levels and HDL-C ($p < 0.001$).

Keywords:
atherosclerosis, vitamin D, rheumatoid arthritis

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of the Rheumatology and Rehabilitation department, Tanta University Hospitals (Egypt). They fulfilled the 2010 American College of Rheumatology/European League against Rheumatism classification criteria for RA [8]. All patients had a disease duration of less than 2 years, without prior use of disease modifying antirheumatic drugs and/or systemic steroids. Most of them were misdiagnosed or delayed diagnosed and were on NSAIDS, and a small number of them were diagnosed for the first time and they were not on any treatment. In addition, 40 healthy volunteers matched in age and sex were included as healthy controls. All female patients and controls were premenopausal, and all participants (including male participants) were subjected to the same amount of sunlight exposure (all patients were recruited in the same month in summer, and all of them were farmers and workers and were exposed to the same amount of sunlight). The patients were recruited over 2 months, May and June, from our department and from the orthopedic department.

Exclusion criteria
(1) RA patients who had hypertension (systolic blood pressure >150 mmHg and/or diastolic blood pressure >90 mm Hg).
(2) Patients with conditions that affect the lipid profile, such as diabetes mellitus, thyroid dysfunction, liver or kidney disease, Cushing syndrome, current smokers, obesity (BMI >30), and a history of familial dyslipidemia.
(3) Patients receiving medications affecting lipid metabolism (lipid-lowering drugs, β-blockers, oral contraceptives, estrogen, progestin, thyroxin, and vitamin E).
(4) Patients with a history of myocardial infarction during the last 6 months.
(5) Pregnant women and those taking vitamin D replacement therapy.

Approval for the study protocol was obtained from the local research ethics committee, and written informed consent was obtained from each participant, including controls.

Clinical assessment
Disease activity in RA patients was assessed by measuring the disease activity for the 28 joint indices score (DAS-28) [9]. The components of DAS-28 are erythrocyte sedimentation rate (ESR), patient-assessed global score (0–100), and swollen and tender joint counts (0–28). The Modified Health Assessment Questionnaire (MHAQ) [10], a standard eight-question instrument, was used to assess functional capacity based on difficulty in performing activities of daily living. This questionnaire is scored from 0 to 3, with higher scores indicating lower functional capacity.

Common carotid artery evaluation
Common carotid arteries were assessed using B-mode ultrasound (Siemens G60S, Seimens, Mountain view, Ca, USA) with a linear transducer (midfrequency, 10 MHz). The radiologist for this study was blinded to other data on RA patients and controls. Patients and controls were examined in the supine position, with the neck extended and the chin turned away from the side. Measurement of carotid intima-media thickness (cIMT) was always performed at the same arterial wall 1 cm proximal to the carotid bifurcation (the same area of the wall of right carotid artery only) according the protocol in the reference.

Images were obtained in longitudinal and axial projections. In longitudinal projection, the sound beam was placed perpendicular to the far wall of the common carotid artery, obtaining two parallel echogenic lines corresponding to the lumen/intima and media/adventitia interfaces. The distance between these two parallel lines corresponded to the cIMT. Values were expressed in millimeters [11].

Sampling
After 12 h of overnight fasting, venous blood samples (7 ml) were taken from the controls and RA patients; 1.6 ml of blood was transferred into a vacutainer tube containing 0.4 ml sodium citrate for ESR measurement. The remainder of the blood was delivered into a plain glass tube, allowed to clot at room temperature, and centrifuged at 2000 rpm for 10 min, and serum was separated. Lipid profile was determined immediately, and aliquots of the serum were stored at –70°C until analysis.

Laboratory investigations
Routine laboratory investigations
ESR, serum C-reactive protein (CRP) (cutoff value 2.19 g/l), lipid profile [normal low-density lipoprotein (LDL), 60–130 mg/dl; high-density lipoprotein (HDL), > 40 mg/dl; total cholesterol <200 mg/dl; triglyceride 10–150 mg/dl], and anti-cyclic citrullinated polypeptide (anti-CCP) (cutoff value >5 U/ml) were evaluated.

Specific investigations
(1) Total vitamin D was measured using 25(OH)-vitamin D direct ELISA Kit for quantitative determination of 1, 25-dihydroxy vitamin D in serum. The optimal concentration of 25(OH) vitamin D was defined as at least 30 ng/ml and vitamin D deficiency as a 25(OH)D of less than 30 ng/ml. [DDRG 25-OH vitamin D (total) ELISA (EIA-5396); DRG International Inc., Springfield, Newjersy, USA] [12]
(2) Interleukin 6 (IL-6) concentrations were determined using ELISA (Roche Diagnostics GmbH, Mannheim, Germany).
(3) Genotype determination: the genotype was determined in all patients and controls. We used Miller’s technique (1988) to extract genomic DNA from peripheral blood samples [13]. The DNA amplified using the PCR technology was subjected to restriction fragment length polymorphism analysis. PCR was performed on a total volume of 25 µl containing 50 ng DNA, 3 mmol/l MgCl₂, 0.1 mmol/l of each deoxyribonucleotide triphosphate, 1.25 U Taq DNA polymerase, and 5 µmol/l of each oligonucleotide. After denaturation for 4 min at 94°C, 35 cycles were performed, each consisting of denaturation for 40 s at 94°C, hybridization for 30 s at 62°C, and elongation for 60 s at 72°C. The final step was elongation for 2 min at 72°C. The probe oligonucleotide sequences were as follows: FokI: (sense) 5′- AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′ and (antisense) 5′-ATG GAA ACA CCT TGC TTC TTC TCC and BsmI: (sense) 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′ and (antisense) 5′-AAC CAG CGG GAA GAG GTC AAG GG-3′. The PCR products were visualized on 3% agarose gel. The amplicons were 265 bp for FokI and 825 bp for BsmI. In the presence of the FokI restriction site, digestion with the FokI restriction enzyme generated two fragments of 196 and 69 bp. In the presence of the BsmI restriction site, digestion with the BsmI restriction enzyme generated two fragments of 175 and 650 bp [13].

Statistical analysis
All data were analyzed using software (version 11; SPSS Inc., Chicago, Illinois, USA).

Baseline characteristics are presented as mean ± SD for the continuous variables and as frequency and percentage for the discrete ones. Comparisons between groups were made using the Student t-test. Allele and genotype frequencies were compared between the patient group and controls using the χ²-test. The strength of associations was assessed by computing the odds ratio (OR). For both the FokI and the BsmI polymorphisms, the Hardy–Weinberg principle was met in all groups and no bias occurred. Associations between clinical, laboratory markers and genotypes were determined with analysis of variance. Correlation between variables was examined using the Pearson’s correlation coefficient. P values less than 0.05 indicated statistical significance.

Results
The demographic, clinical, laboratory, and radiographic data for patients with RA and controls are summarized in Table 1. Thirty-seven patients were female and three were male, with a mean age of 41.15 ± 5.85 years. Meanwhile, 38 controls were female and two were male, with a mean age of 42.7 ± 4.67 years. The mean disease duration was 14.6 ± 6.5 months (<2 years). There were no significant differences in age, sex, and mean BMI values between RA patients and controls. There were 35 patients positive for anti-CCP, of them, 12 patients were positive for RA. Patients with early RA exhibited mild dyslipidemia characterized by significantly higher baseline of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) compared with controls. In addition, high-density lipoprotein cholesterol (HDL-C) levels were significantly lower compared with controls. As a consequence, the atherogenic ratio of total cholesterol/HDL-C, as well as that of LDL-C/HDL-C, was significantly higher in RA patients compared with controls.

In RA patients, laboratory parameters, including ESR, CRP, IL-6, anti-CCP levels, were significantly higher compared with controls. Moreover, cIMT was significantly higher in RA patients compared with controls (P < 0.001), as shown in Figs 1–4. Total serum vitamin D (25[OH]D) levels were significantly lower in RA patients compared with controls. Moreover, vitamin D deficiency (<30 ng/ml) was observed in 10 (25%) patients.

Table 1 The demographics, clinical, laboratory characteristics and carotid intima media thickness in rheumatoid arthritis patients and controls

|                         | RA patients (n = 40) | Controls (n = 40) |
|-------------------------|---------------------|-------------------|
| Age (years)             | 41.15 ± 5.85        | 42.7 ± 4.67       |
| Sex (male/female)       | 3/37                | 2/38              |
| Body mass index (kg/m²) | 25.7 ± 3.6          | 25.5 ± 3.5        |
| Disease duration (months)| 14.6 ± 6.5         |                   |
| MHAQ                    | 5.09 ± 0.72         |                   |
| ESR 1st hour (mm/h)     | 49.23 ± 19.54*      | 8.56 ± 3.97       |
| CRP (mg/L)              | 34.26 ± 15.13*      | 3.25 ± 1.42       |
| TC (mg/dl)              | 226.5 ± 30.22*      | 176.20 ± 15.28    |
| TG (mg/dl)              | 134.13 ± 45.78*     | 90.23 ± 20.60     |
| LDL-C (mg/dl)           | 139.03 ± 23.02*     | 121.7 ± 16.56     |
| HDL-C (mg/dl)           | 42.07 ± 9.12*       | 55.10 ± 10.88     |
| TC/HDL-C                | 5.6 ± 1.27*         | 3.5 ± 0.37        |
| LDL-C/HDL-C             | 3.6 ± 1.03*         | 2.4 ± 0.76        |
| IL-6 (P/g/ml)           | 35.3 ± 20.2*        | 5.5 ± 4.0         |
| Anti-CCP(U/ml)          | 94 ± 83*            | 13.11 ± 6.45      |
| 25(OH)D (ng/ml)         | 20.6 ± 11.5*        | 31.5 ± 4.69       |
| cIMT(mm)                | 0.84 ± 0.27*        | 0.54 ± 0.11       |

Values represent the mean ± standard deviation; *P < 0.001 compared to the control group; DAS-28, disease activity for 28 joint indices score; MHAQ, Modified Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; IL-6, interleukin-6; Anti-CCP, anti–cyclic citrullinated peptide; 25OHD, 25-hydroxyvitamin D; cIMT, carotid intima-media thickness.
The distribution of VDR gene BsmI and FokI genotypes and allele frequency among Egyptian RA patients and controls are summarized in Table 2. In our study, there were no significant differences between RA patients and controls as regards the distribution of BsmI genotype frequencies and allele (P = 0.50 and 0.56, respectively; OR = 0.81; 95% confidence interval = 0.27–2.41).
However, a significant difference between RA patients and controls as regards the distribution of FokI genotype and allele frequencies was found. In addition, FokI polymorphism and the F allele was significantly associated with RA (P = 0.009 and 0.04, respectively; OR = 1.91; 95% confidence interval = 0.63–5.80).

As regards the association of the BsmI and FokI genotypes with clinical, laboratory, and radiological assessment in RA patients, it was found that anti-CCP, IL-6 levels, and cIMT were significantly higher in the presence of bb homozygote of BsmI genotypes in comparison with Bb heterozygote and BB homozygote (P < 0.001). Moreover, their levels were significantly higher in the presence of FF homozygote of FokI genotypes in comparison with Ff heterozygote and ff homozygote (P < 0.001) (Figs 1 and 5).

Vitamin D levels were significantly lower in the presence of bb homozygote of BsmI genotypes in comparison with Bb heterozygote and BB homozygote (P < 0.001). However, vitamin D levels were significantly lower in the presence of FF homozygote of FokI genotypes in comparison with Ff heterozygote and ff homozygote (P < 0.001) (Fig. 6).

In our study, there was no significant association of DAS-28 (r = 0.123, P = 0.09), MHAQ (r = −0.324, P = 0.075), ESR (r = −0.324, P = 0.219), CRP (r = −0.027, P = 0.92), and lipid profile CRP (r = −0.026, P = 0.49) with BsmI and FokI genotypes.

As regards the correlation matrix, it was found that there was a significant negative correlation between 25(OH) D levels with DAS-28 score (r = −0.57, P < 0.001), ESR (r = −0.53, P < 0.001), CRP (r = −0.61, P < 0.0001), TG (r = −0.52, P < 0.001), and IL-6 (r = −0.57, P < 0.001). However, there was a positive correlation between 25(OH)D levels and HDL-C (r = 0.49, P < 0.001).

There was a positive correlation between cIMT and IL-6 (r = 0.49, P < 0.01) and a negative correlation between cIMT and 25(OH)D (r = −0.60, P < 0.001).

No significant correlation between cIMT and ESR and anti-CCP was found.

### Discussion

In our study early RA patients exhibited dyslipidemia and increased IL-6 levels compared with controls. Sattar et al. [14] summarized the implications of the systemic inflammatory response in the development of accelerated atherosclerosis in RA patients. According to these authors, proinflammatory cytokines such as tumor necrosis factor-α, IL-1β, and IL-6, generated in the synovial tissue, can be released into the systemic circulation. These circulating cytokines are capable of altering the function of distant organs, including adipose, skeletal muscle, liver, and vascular endothelium, to generate a spectrum of proatherogenic changes that include endothelial dysfunction, insulin resistance, a characteristic dyslipidemia, prothrombotic effects, and pro-oxidative stress. cIMT is a widely accepted surrogate marker of atherosclerosis [15]. Our study revealed that the mean cIMT in patients with early RA was significantly greater compared with controls.

We have found that vitamin D deficiency [25(OH)D values <30 ng/ml] affects 25% of our early Egyptian RA patients. Rossini et al. [16] and others [17,18] have reported that the prevalence of vitamin D deficiency ranges from 30 to 63% in RA. The hypothesis that vitamin D relates to autoimmune disorders emerged from the observation that people living near the equator were at a decreased risk of developing common autoimmune diseases [19]. Furthermore, several surveys on rheumatology populations found reduced levels of vitamin D with different autoimmune disorders, including SLE, RA, systemic sclerosis, polymyositis, multiple sclerosis, autoimmune thyroid diseases, and antiphospholipid syndrome [20]. These results were due to the seminal roles of vitamin D in the metabolism of calcium absorption; however, it is less acknowledged that vitamin D has many other functions, most importantly its contribution to the regulation and differentiation of the immune cells [21]. However, a serum-bank case–control study from the Netherlands found no correlation between serum 25(OH)D levels and the development of RA [22], and in an evaluation of the Nurse’s Health Study cohort including 190 patients with SLE and 722 with RA,

### Table 2 Distribution of VDR gene BsmI and FokI genotypes and allele frequency among RA patients and controls

| Genotype frequency n (%) | Allele frequency n (%) | OR (95%CI) | HWE X² (p value) |
|--------------------------|-----------------------|------------|-----------------|
| BsmI                     |                       |            |                 |
| RA (n = 40)              | 8 (20)                | 16 (40)    | 1.96 (0.50)*    |
| Control (n = 40)         | 10 (25)               | 22 (55)    | 0.74 (0.45)     |
| FokI                     |                       |            |                 |
| RA (n = 40)              | 18 (45)               | 28 (70)    | 1.91 (0.63–5.80) |
| Control (n = 40)         | 12 (30)               | 22 (55)    | 0.19 (0.53)     |

*Comparisons of genotype frequencies polymorphism in the rheumatoid arthritis group and controls; **Comparisons of allele frequencies in the rheumatoid arthritis group and controls; OR, Odds ratio; 95%CI, 95% confidence interval; HWE, Hardy-Weinberg equilibrium.
there was no correlation between vitamin D intake and the risk for SLE or RA [23].

In this study, inverse relationships between vitamin D levels and disease activity were observed. Similar results have been found by others as well. Rossini et al. [16] and Cutolo et al. [24] found that vitamin deficiency in patients with established RA is lower than that in patients on diseases remission. Moreover, Patel et al. [25] found an inverse relationship between 25(OH)D levels and tender joint count; DAS-28 was evaluated only at disease onset, but not in patients with a disease duration longer than 1–2 years.

We studied the association of VDR polymorphisms (FokI and BsmI) in Egyptian RA patients, and we found that FokI polymorphism and the F allele were significantly associated with susceptibility of Egyptian RA. No association was found between BsmI alleles with RA. Garcia-Lozano et al. [26] suggested that polymorphisms in the VDR gene could have some effects on RA etiopathology, such as affecting disease onset, and that the clinical course of RA is characterized by bone and joint destruction and it is therefore conceivable that polymorphic genes whose products have a direct effect on calcium/vitamin D metabolism may play a role in its pathogenesis. In contrast, Provvedini et al. [27] and Bhalla et al. [28] reported that the discovery of VDR in monocytes and activated, but not resting, lymphocytes suggested a role in immunoregulation and possibly that joint inflammation could be influenced by VDR polymorphisms.

The discovery of VDR expression in most cell VDR types of the immune system, in particular in antigen-presenting cells such as macrophages and dendritic cells, as well as in both CD4+ and CD8+ T lymphocytes, prompted a number of studies investigating the capacity of VDR agonists to modulate T-cell responses. VDR agonists were subsequently found to be selective inhibitors of Th1 cell development and were found to directly inhibit Th1-type cytokines such as IL-2 and VDR-α. Collectively, direct T-cell targeting by VDR agonists could contribute to account for their beneficial effects in the treatment of autoimmune diseases [29].

Studies on the genetic background of RA patients provide a first indication that VDR polymorphisms are linked to RA. As such, BsmI polymorphism of VDR gene is involved in the pathogenesis of osteoporosis in RA [30], and the F allele and F/F VDR FokI polymorphism are associated with RA in Europeans. [31,32]. They demonstrated that the F-associated allele in a VDR protein has three amino acids less than the f variant. FokI alleles differ functionally because of altered VDR affinity and transactivation of VDR elements containing promoter construct in HeLa and COS-7 cells. Moreover, an in-vitro study [33] demonstrated an increased transcription rate (1.7-fold) of the VDR gene in cells with the F/F genotype. An overexpression of VDR gene may affect the expression of genes containing such VDR response element. This could dysregulate the Th1/Th2 balance and therefore could cause the development of autoimmune process of RA. These results were in contrast with a case–control study conducted in the German population, which showed no evidence of RA association with VDR [23]. This could be due to heterogeneity between populations.

In the present study, we have shown that the bb homozygote of BsmI genotypes and the FF homozygote of FokI genotypes were significantly associated with vitamin D deficiency (vitamin D <30 ng/ml), and that they are also associated with anti-CCP, which is associated with severe disease [34] and cardiovascular risk (IL-6 and cIMT). Moreover, there was a significant negative correlation of 25(OH)D levels with DAS-28 score (r = −0.57, P < 0.001), ESR (r = −0.53, P < 0.001), CRP (r = −0.61, P < 0.0001), TG (r = −0.52, P < 0.001), IL-6 (r = −0.57, P < 0.001), and cIMT(r = 0.60, P < 0.001). However, there was a positive correlation between 25(OH)D levels and HDL-C (r = 0.49, P < 0.001). These findings clarify that vitamin D may have clinical relevance in identifying patients with early atherosclerotic changes in early RA.

Kendricka et al. [35] have shown that 25(OH)D deficiency was associated with a composite of self-reported angina, myocardial infarction, and stroke, independent of several established risk factors, in a nationally representative sample of the USA adult population.

Wang et al. [36] have recently shown that low 25(OH) D levels (i.e. <15 ng/ml) are independently associated with incident CVD in 1739 Framingham offspring study participants followed up for 5.4 years. Similarly, other European studies in type 2 diabetic patients have shown that 25(OH)D levels are inversely associated with prevalent CVD [33] and carotid intima-media thickening [37]. Moreover, 25(OH)D deficiency has also been found to be associated with all-cause and cardiovascular mortality [38,39].

Finally, some studies documented that vitamin D can inhibit various aspects of inflammation [40,41]. Long-term vitamin D supplementation in vitamin D-deficient individuals markedly reduced plasma levels of CRP, tissue matrix-metalloproteinase, and its inhibitors [42,43], and had beneficial effects on the elastic properties of the common carotid artery in postmenopausal women [44]. Recently, Gupta et al. [45] observed significantly decreased expression of adiponectin in the epicardial adipose tissue of vitamin D-deficient swine. These findings imply vitamin D deficiency as a contributory factor in
the activation of inflammatory adipokines in epicardial adipose tissue, suggesting the immunomodulatory role of 25(OH)D in the pathogenesis of CAD. Vanga et al. [46] documented that serum levels of 1,25(OH)2 vitamin D are inversely correlated with very LDL and triglyceride levels. Vitamin D deficiency may cause an abnormal lipid profile by increasing peripheral insulin resistance and contributing to metabolic syndrome. However, oral supplements of vitamin D3 in postmenopausal women did not improve total cholesterol, LDL, or HDL levels over 12 months [47]. Studies have suggested that statin therapy may increase vitamin D levels, a finding that may account for some of the nonlipid pleiotropic actions of statins [48,49]. It is postulated that the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase enzyme by statins results in increased 7-dehydrocholesterol. This excess 7-dehydrocholesterol is then converted to 25-hydroxycholecalciferol by sunlight or the CYP11A1 enzyme, thereby increasing vitamin D levels [49]. Lastly, a study that examined reductions in VDR signaling in patients with diabetes found increased foam cell formation in macrophages, an early sign of atherosclerosis [49].

**Conclusion**

In summary, our study has shown that 25(OH)D deficiency is associated with early sign of atherosclerosis in Egyptian patients with early RA. This association suggests that 25(OH)D may be a risk factor for CVD.

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**Conflicts of interest**

There are no conflicts of interest.

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