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Original Article

Evaluating of *VDR* Gene Variation and its Interaction with Immune Regulatory Molecules in Osteoporosis

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Abstract

Background: To evaluate *VDR* gene variation and its interaction with immune regulatory molecules in osteoporosis.
Methods: Totally 205 pre and postmenopausal women were recruited in the study. After an overnight fast, peripheral blood was taken and centrifuged to sprat serum for measurement of serum parathyroid hormone, 25 hydroxyvitamin D, osteocalcin and cross laps. The *Fok*I polymorphism in exon 2 of the *VDR* gene was detected by PCR-RFLP. Expression of osteoprotegrin, vitamin D receptor (*VDR*) and β-actin genes were quantified by quantitative real-time reverse transcriptase. To design the experimental model we randomly selected five participants of each genotype groups. PBMC were cultured and induced with vitamin D. At several times, cells were harvested and total RNA was extracted .Then expression of target genes evaluated by real time PCR.
Results: The frequencies of Ff, FF and ff genotypes were 34.2%, 56.5% and 9.2%. The mean of bone mineral density in FF genotype was higher than other genotypes. Also in this genotype, mean of serum inflammatory cytokines was lower than other genotypes. The expressions of the *VDR* and osteoprotegrin were up regulated by 1, $25(OH)_2D_3$ in PBMC from participants with FF genotype. PBMC from healthy control comparison to osteoporotic patients had a clearly better response to vitamin D_3 incubation.
Conclusion: Inflammation may important role in osteoporosis whereas osteoporotic patients have elevated pro-inflammatory profile. This cytokine profile and gene expressions of *VDR* and Osteoprotegrin were different in *VDR* genotype groups.

Keywords: *Vitamin D receptor, Polymorphism, Cytokines, Gene expression*

Introduction

Osteoporosis is a common multifactor disease that in its pathogenesis involves both genetics and environmental factors. In osteoporosis bone loss increased and strength of bone decreased thus fracture risk increased (1). Bone mineral density (BMD) and geometry are factors that establish bone strength (2). During the bone formation period suitable peak bone mass (PBM) can make good protection against osteoporosis. Age and quantity of PBM related to the race is widely different.
Up to 80% of the population variance for PBM explains by heritability and genetic factors (3, 4). However, the role of genetics in bone turnover in the aging is less clear (5). Several serum and urine biochemical markers indirectly can predicted bone turnover status (6).

In mineral homeostasis and bone metabolism completely related with Vitamin D. This vitamin plays his role through its receptor and vitamin D receptor gene (*VDR*) determine the structure of receptor thus *VDR* gene can be an important candidate gene of osteoporosis (7).
The *VDR* gene is located chromosome 12 (12q12–14) on the long arm. Ten exons and eight introns is determine on this gene structure that the first exon is not transcribed and totally coding exons are transcribed into the *VDR* messenger RNA (8).
The *Fok*I polymorphism in *VDR* gene was has been described by diallelic variant (ATG/ACG) in initiation transcription codon (9). The f allele indicated that existence of the restriction site. This gene variant let translate protein from the first ATG but presence of F allele cause that initi-

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ates translation move to second downstream ATG. Thus, a three amino acid difference may be detected in *VDR* length depend on polymorphism of this gene. This variation may changes the function of the *VDR* protein.

Morrison et al. first reported that association between *VDR* polymorphism and BMD. Other study later reported relationship between BMD and *Fok*I polymorphism in the *VDR* (9, 10). There are several studies in this relationship but only there are few studies in influence of genetic variation of *VDR* on bone turnover.

Bone turn over process is greatly complicated and several hormones involve it including growth factors and steroidal components also immune regulatory molecules like cytokines are important to this route (11). Several immune regulatory molecules are known to balance bone metabolism. Inflammatory cytokines like TNF-α inhibits the osteoblasts differentiation (12). IL-1, TNF-α, and IFN-γ inhibit collagen synthesis in osteoblasts (13–16). Moreover new specific cytokines are known that can modify intercross talk between bone cells. Among them Osteoprotegrin (OPG) was originally identified as a soluble decoy-like factor (17, 18). Osteoporosis induced in mice when OPG transgenically was overexpressed (18).

These documents regarding effect of *VDR* gene polymorphism and the role of immune system in osteoporosis and the immunomedulatory effect of vitamin D on immune system purpose this study to Evaluate of *VDR* gene variation and its interaction with immune regulatory molecules (IL1, IL6, TNFα, Osteoprotegerin) in osteoporosis.

Material and Methods

The subjects for the study were 205 women aged between 20-75 yr that randomly selected form endocrinology and metabolism research center outpatient clinics. Exclusion criteria included endocrinological disorders (such as hyperthyroidism, hypo- and hyperparathyroidism, diabetes mellitus), chronic disorders of liver and kidney, other skeletal diseases (Paget's disease, osteogenesis imperfecta and rheumatoid arthritis), use of medications that are known to affect bone density and metabolism (such as calcium supplements, corticosteroids, anticonvulsants and heparin), or unusual gynecological history like bilateral oophorectomy, early or late menarche or irregular cycles or premature menopause before the age of 40 yr.

All the subjects had undergone BMD measurements by dual energy X-ray absorptiometery (DEXA) at lumbar spine (vertebrae L2–L4) and hip. The coefficient of variation for longitudinal BMD measurements in the DEXA machine averaged at 1.04%. Normal bone mass was defined as BMD measurements at or above -1 standard deviation (S.D.) from the optimal peak bone density (T-score) of healthy young adult of the same sex. BMD measurement at or below -2.5 S.D. from the optimal peak bone density of healthy young adult of the same sex was osteoporotic, as per World Health Organization standard definitions.

Laboratory measurements

After an overnight fast, 6ml of peripheral blood was taken and centrifuged to sprat serum for measurement of serum parathyroid hormone, 25 hydroxyvitamin D, and osteocalcin and cross laps. Serum concentration of 25-hydroxy vitamin D3 was measured using by a Biosource kit (Biosource Europe S.A, Belgium); intra- and inter-assay coefficients of variation (CV) were 5.2% and 7.5%, respectively (normal range: 2.5-75 ng/ml). Serum PTH was also detected using a Biosource kit (Biosource Europe S.A, normal range: 13-66 pg/ml), with an intra- and inter-assay CV of 6.3% and 5.7%, respectively.

Osteocalcin was measured by immunoassay (ELISA) using a Bioscience kit (Nordic Bioscience Diagnostic A/S, Denmark). The intra- and inter-assay CV were 2.6% and 4.7%, respectively. serum Cross Laps was measured by immunoassay (ELISA) using a Bioscience kit (Nordic Bioscience Diagnostic A/S, Denmark), with intra- and inter-assay CV of 5.1% and 6.6%, respectively. Serum concentration of Interleukin1beta (IL-1β) was measured by immunoassay (ELISA) using a R&D system kit (R&D system, USA); intra and inter-assay coefficients of variation (CV) were 4.8% and 4.1%, respectively. Serum Inter-

leukin 6(IL-6) was also detected using a R&D system kit (R&D system, USA), with intra- and inter-assay CV of 2.4% and 4.7%, respectively. Serum TNFα was measured by immunoassay (ELISA) using a R&D system kit (R&D system, USA); intra- and inter-assay coefficients of variation (CV) were 4.8% and 6.1%, respectively.

About 4ml aliquots of peripheral blood samples were collected from the subjects and stored in EDTA coated vacutainers. Genomic DNA was isolated from peripheral blood leukocytes according to standard methods. The *Fok*I polymorphism in exon 2 of the *VDR* gene was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method previously described (9).

PCR was performed to amplify target region of DNA with length of 265 base pairs. PCR product was digested with *Fok*I. The digested PCR products were resolved on 2.0% agarose gels. When PCR product was digested two bands (69 and 196 base pairs) detected on gel electrophoresis that showed f allele and not able to digest indicated the T to C transition (F allele). Fig. 1 shows a sample of gel electrophoresis of PCR-RFLP related to *Fok*I polymorphism detection.

For gene expression evaluations in each genotype groups selected 20 participants (10 osteoporotic patients and 10 healthy). From fresh blood, RNA extraction performed by using the High Pure RNA isolation kit (Roche Diagnostics). Osteoprotegrin, vitamin D receptor (*VDR*) and b-actin genes were quantified by quantitative real-time reverse transcriptase (RT)-PCR.

In brief, total RNA was reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit (Fermentase, EU). PCR reactions were performed in a Light Cycler (Roche Applied Science).

To design of experimental model we randomly selected five participants of each genotype groups and from peripheral blood of participants, Peripheral blood mononuclear cells (PBMC's) were isolated through the Ficoll density method. The middle layer containing peripheral blood mononuclear cells was harvested and washed twice. PBMC were cultured in RPMI 1640 supplemented with Glutamax-I, 25mM HEPES (Cambrex Bio Science, Verviers, Belgium) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) plus 15% FCS for 24 h. In parallel experiments, 1, 25(OH) 2D3 (10-7 M) (Sigma-Aldrich, St. Louis, MO) were added in some experiments either at the beginning of culture.

At each time point, 106 cells were harvested and RNA extraction performed using the High Pure RNA isolation kit (Roche Diagnostics). Target genes including Osteoprotegrin, vitamin D receptor (*VDR*) and β-actin genes were quantified by quantitative real-time reverse transcriptase. β-Actin was used as a housekeeping gene control.

Statistical analysis

Data were analyzed using SPSS software, version 11.5. The student's *t*-test and analysis of variance were used to compare the differences between the means of variables. The chi-square test was used to compare the frequency of variables. Pearson correlation was used to investigate correlation between two variables. In all tests, the level of significance was 0.05.

Results

Totally 205 women (109 postmenopausal and 96 pre menopausal) participated in the study. The background characteristics of the study population are shown in Tables 1. Postmenopausal women had higher bone turn over and lower BMD comparing to pre menopausal group (Table1).

The frequencies of Ff, FF and ff genotypes were 34.2%, 56.5% and 9.2% in the study population. The observed proportions of *Fok*I genotypes were no significant difference in pre and postmenopausal women (P= 0.9).

The genotype grouping of did not differ significantly in age, Body mass index (BMI) (Table 2).

Menopause has been associated with an increase in osteocalcin and cross laps values, therefore the role of menopausal status was assessed by regression analysis and analysis of covariance including age, menopausal status, and *Fok*I genotype. Menopausal status was a weaker determinant of serum cross laps concentrations than *Fok*I polymorphism.

Analyzing pre menopausal and postmenopausal women separately did not alter the results, and genotype was a stronger predictor than menopausal status (Table 2).

Using analysis of covariance, we found that subjects with ff genotype exhibited a significantly (P= 0.004) lower hip bone mass, by dual-energy X-ray absorptiometry relative to those with Ff and FF genotypes. Conversely, Serum osteocalcin and cross laps were significantly higher in ff and Ff compared to FF genotype. Also serum concentrations of pro inflammatory cytokines in ff genotype were higher than FF genotype and serum osteoprotegrin concentration in FF genotype was higher than ff genotype (Table 3). Finally, *Fok*I genotype predicted serum cross laps after adjustment for age, menopause status, serum vitamin D (P< 0.001).

To assess the effect of vitamin D3 on gene expression, cells were exposed to 10-7M 1, 25(OH)2D3 for 0, 3,8, and 24 h. As can be seen in Fig. 3, PBMC from healthy control comparison to osteoporotic patients had a clearly different pattern of *VDR* and osteoprotegrin upon vitamin D3 incubation.

Of note is that mRNA expressions of *VDR* and osteoprotegrin in healthy control were clearly higher than osteoporotic patients (Fig. 2, 3). Also FF genotype had a higher expression in all time points, being significantly different from ff genotype in healthy control and osteoprotic patients. Fig. 2 shows that *VDR* mRNA levels were up regulated in cells with FF genotype from healthy control whereas mRNA levels of osteoporotic patients remained at a basal level.

Table 1: Characteristics data of study population

Characteristic	Pre menopausal	Postmenopausal	*P*
Age(yr)	50.35 ±4.79	56.13 ±5.13	0.001
BMI(Kg/m^2)	27.76±2.25	28±1.71	0.01
Hip BMD(gr/cm^2)	0.97±0.1	0.92±0.1	0.002
Spine BMD(gr/cm2)	1.15±0.14	1.1±0.21	0.05
Serum Vitamin D(nmol/L)	26.05 ± 8.22	29.02 ± 6.17	0.004
Serum PTH(pmol/L)	14.42 ± 8.98	14.07 ± 6.14	0.74
Serum Crosslaps (ng/mL)	0.18± 0.09	0.26± 0.14	0.001
Serum Osteocalcin (ng/mL)	6.44± 2.47	8.09±3.49	0.001

Values are expressed as mean ±SD, comparing of variables means in two groups performed by Student T test

Table 2: Background characteristics of participants with respect to *VDR* genotypes

Characteristic	Genotypes			*P* value ANOVA
	FF	Ff	ff	
Age (yr)	53.55(6,62)	53.40(3,63)	53.14(6.77)	0.9
BMI(Kg/m2)	27.80 (2.34)	27.03 (1.67)	27.22(1.89)	0.05
Serum 25-hydroxyvitamin D3 (nmol/dl)	32.59(4.79)	22.26(4.45)	25.27(8.80)	0.001
Serum PTH (pmol/L)	11.95(5.76)	11.36(4.68)	26.13(4.78)	0.001
Ostocalcin (ng/mL)	6.93(2.65)	7.93(3.81)	7.12(2.84)	0.11
Cross Laps (ng/mL)	0.19(0.12)	0.2(0.12)	0.33(0.10)	0.01
BMD (total hip, g/cm2)	0.89(0.11)	0.86(0.10)	0.84 (0.07)	0.004
BMD (L2-L4, g/cm2)	1.01(0.14)	0.99(0.15)	0.94(0.12)	0.045

Values are expressed as mean (SD)
ANOVA, analysis of variance

Table 3: Serum concentrations of cytokines with respect to *VDR* genotypes

Serum concentrations	Genotypes			*P* value ANOVA
	FF	Ff	ff	
Serum OPG (pmol/L)	6.05(2,05)	6.24(1,67)	5.78(0.9)	0.01
Serum IL1 (ng/mL)	0.63 (1)	0.9 (1.33)	0.9(1.89)	0.1
Serum IL6 (ng/mL)	1.73(2)	1.72(1.70)	2.83(2.80)	0.01
Serum TNFα (ng/mL)	0.25(0.34)	1.04(1.42)	0.91(1.36)	0.01

Values are expressed as mean (SD)
ANOVA, analysis of variance

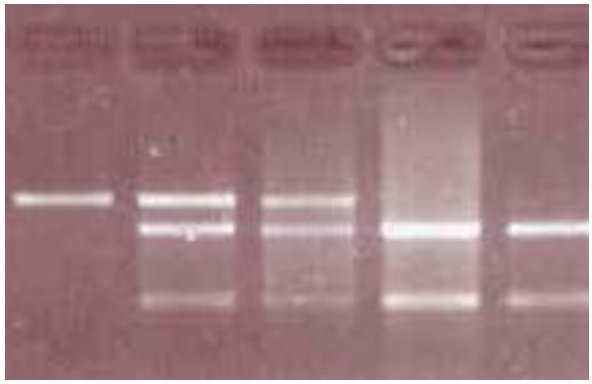

Fig. 1: Gel electrophoresis related to *Fok*I polymorphism shows different genotype groups. Lane 1 shows FF genotype and lane 2, 3 shows Ff genotype and lane 4, 5 shows ff genotype

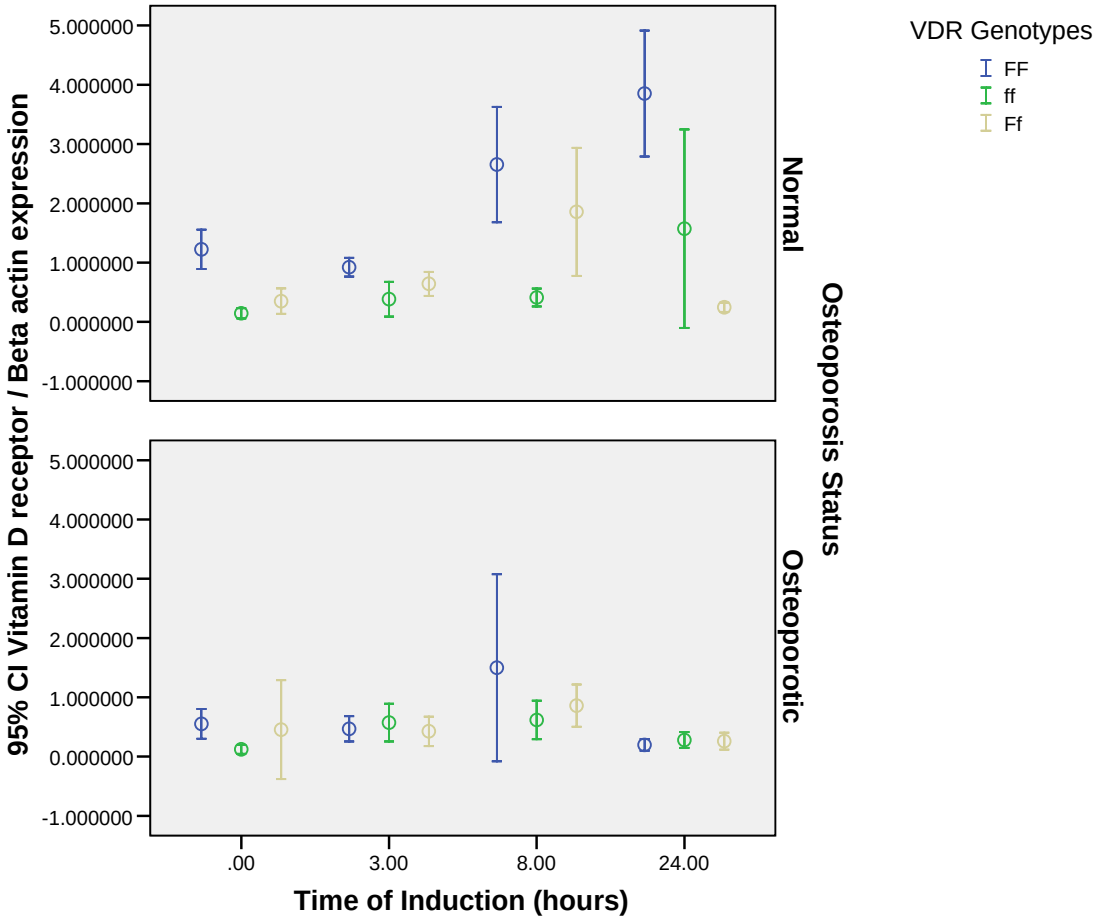

Fig. 2: Expression of Vitamin D receptor in Peripheral blood mononuclear cells of different genotype groups from healthy controls and osteoporotic patients. *VDR* mRNA expression levels in freshly isolated PBMC [in time 0] and in PBMC incubated with Vitamin D3 for 3,8,24 hours was quantified by real-time RT-PCR. Levels were normalized to beta-actin gene expression. Data are represented as 95% Confidence Interval of mean

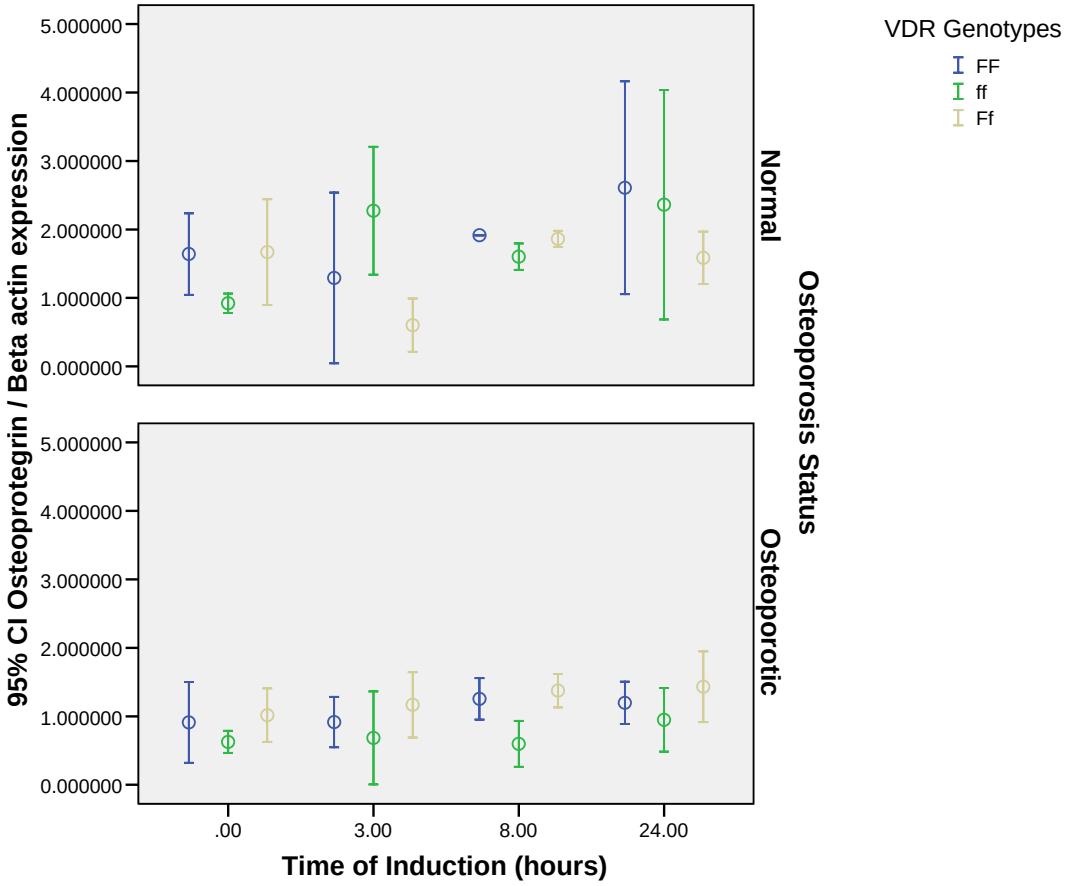

Fig. 3: Expression of Osteoprotegrin gene in Peripheral blood mononuclear cells of different genotype groups from healthy controls and osteoporotic patients. Osteoprotegrin mRNA expression levels in freshly isolated PBMC [in time 0] and in PBMC incubated with Vitamin D3 for 3,8,24 hours was quantified by real-time RT-PCR. Levels were normalized to beta-actin gene expression. Data are represented as 95% Confidence Interval of mean

Discussion

In regarding association between *Fok*I *VDR* polymorphism and bone mineral densities, several studies have reported lower bone mineral densities for ff genotype (9, 19-26), these findings consist with our results. Our data suggest that *Fok*I *VDR* polymorphism may associate with bone mass and turnover in both pre- and postmenopausal women in this geographically isolated population.

There are several studies that investigate this relationship between *Fok*I polymorphism and bone mass. Lower BMD reported in black and white women with ff genotype compared to FF women (27). Also in Mexican women there was greater bone loss and lower BMD in ff as compared to FF was found (9).

In old black women this polymorphism of *VDR* gene was not related with BMD or bone loss (28). The discrepancies between different studies could be explained by several reasons. The influence of the genetic component of BMD may change with age. If this is the case, some polymorphisms may be associated with peak bone mass and others with bone loss. Therefore, related to target population different result may be reported.

Similar distribution of *Fok*I polymorphism reported in other studies. The prevalence of the *VDR-Fok*I genotypes in American population was

51% *FF*, 42% *Ff*, and 8% *ff*.(29). In African-American women the distribution of *Fok*1 polymorphism was FF 60%, Ff 37% and ff 2% (30). In Finnish adolescent the observed proportions of *Fok*I genotypes were 40% for FF, 43% for Ff, and 17% for ff (31).

Our results suggest that the F allele could be more advantageous for bone health. This finding confirm by physiological mechanism that explaining why this genetic variant is associated with higher BMD.

Another study suggested that influence of *VDR* polymorphism on BMD may relate to interactions between calcium and vitamin D status and *VDR* genotypes, in fact there was a suggestion of a better response to calcium supplementation in FF genotype (32). In terms of the biological rationale for a differential response to vitamin D within each of the *Fok*1 polymorphisms, PTH levels have been reported to vary with *Fok*1 polymorphisms (33) and the FF polymorphism was reported to be the more active form with a greater efficiency in exerting 1,25(OH)2D effects (34). In consistent our result indicated that PTH level had significantly difference in respect of *FOK*I genotyping groups (Table 2, 3).

At another level, the responses by *VDR* genotype have been analyzed as differences in serum bone markers. Several different serum markers thought to be vitamin-D-specific, such as osteocalcin, cross laps and PTH. In particular, an osteocalcin and cross lap has been analyzed because this is a highly vitamin-D-responsive gene and it is frequently measured in clinical practice to monitor bone metabolism.

Our results show that *Fok*I genotype influence on bone turn over by improving bone metabolism. In this mechanism FF genotype associated with PTH suppression and decreased cross laps. In the other study was shown that In the FF group, there was greater PTH suppression and a significant decline in urinary FDPD with vitamin D as compared to placebo (30).

Kelly et al. (35) observed, in a study of twins, that serum osteocalcin was under strong genetic influence, with up to 80% of the variance in osteocalcin levels explained by genetic factors. Moreover, the difference in osteocalcin levels among dizygotic or nonidentical twin pairs predicted the difference in bone density, as measured by dual photon absorptiometry, in the same twins. This result supports the hypothesis that genetic effects on bone turnover relate to genetic effects on bone density (36).

The close relationship between the bone and immune system extends beyond the cytokines and transcription factors they share. Interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α are strong inductors of bone resorption (37). The estrogenic deficiency that occurs during menopause leads to an increase in the production of these cytokines (38). Major evidence points towards a link between inflammation and osteoporosis. Our results suggest that circulating PBMC are also likely to be contributing to this inflammatory milieu.

In this study we have shown that osteoporotic patients have elevated pro-inflammatory profile. This abnormal profile is mainly true in osteoporotic patients, confirming previous studies where osteoporotic patients presented higher plasma levels of cytokines such as TNF-α and IL-6.Gianni et al. reported that osteoporotic patients had high baseline expression of IL-6 and TNFα (39).

In the present study it is clear that 1,25(OH)2D3 is a modulator of cytokine expression by PBMC. Indeed, less effects were seen on cytokine expression when PBMC from osteoporotic patients were used, but important down-regulation of IL-6, TNF-a, IL-1 was seen when PBMC from healthy controls were induced with 1, 25(OH)2D3. Monocytes/macrophages are the cells of the innate immune system. Experimental study show that $1,25(OH)_2D3$ inhibited the production of both extra cellular and cell-associated immunoreactive IL1 alpha and IL1 beta and concluded that part of the ability of $1,25\text{-}(OH)_2D3$ to inhibit T cell proliferation may be due to direct effects on monocytes by down-regulating IL-1 production (40).

Osteoprotegerin (OPG) is a soluble member of the TNF receptor family that decoy receptor for RANKL that inhibits its interaction with RANK, thus preventing osteoclastogenesis (18). In our

study OPG expression in the osteoporotic patients were lower than healthy control. Also OPG gene expression induced by 1,25-$(OH)_2$D3. Mature osteoblasts play an inhibitory role in bone resorption, with active vitamin D metabolites acting through the *VDR* to increase OPG (41).

In previous reports OPG known as osteoclastogenesis inhibitory factor (17). OPG inhibits not only formation of osteoclasts-like cells in murine cultures *in vitro* but also bone resorption *in vitro* and *in vivo* (17,18). OPG knock-out mice exhibited severe osteopenia due to accelerated bone resorption (18).

In conclusion our results indicated that inflammation may critical role in osteoporosis whereas osteoporotic patients have elevated pro-inflammatory profile. Vitamin D and its receptor may immuno modulatory effect in osteoimmunology.

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The authors declare that they have no conflict of interests.

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