

Original Article

Inflammatory and bone turnover markers in relation to PTH and vitamin D status among Saudi postmenopausal women with and without osteoporosis

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Abstract: Postmenopausal osteoporosis is characterized by rapid bone loss occurring in the post-menopausal period. The bone loss predominantly involves the trabecular bone and is brought about by an imbalance between the bone remodeling process which can be influenced by factors that could cause or contribute to osteoporosis. Pro-inflammatory cytokines (IL-1 β , IL-6, IL-8 and TNF- α) have been implicated in the regulation of bone cells and play a critical role in bone remodeling. They act both directly and indirectly to increase bone resorption, and/or inhibit bone formation. The aim of the study is to determine whether pro-inflammatory cytokines correlate with bone turnover markers (BTM) in a cohort of Saudi post-menopausal women with or without osteoporosis and which BTMs will correlate with PTH and Vitamin D for use in osteoporosis diagnosis. The study is composed of 100 post-menopausal patients and 100 controls aged 50 years and above. Serum concentrations of pro-inflammatory and BTMs as well as PTH and vitamin D were determined by ELISA, Luminex and electrochemiluminescence. Serum calcium, phosphorus, glucose, and lipid profile were measured by using a chemical analyzer. There was a significant increase in the levels of pro-inflammatory cytokines, PTH, CTx, and glucose. A significantly lower vitamin D and osteocalcin levels were observed in subjects with osteoporosis than those without. No significant differences were recorded in the circulating lipid profile between groups. The present study proved that the pro-inflammatory cytokines accelerate the bone loss in postmenopausal women.

Keywords: PTH, osteoporosis, Saudi women, vitamin D, bone biomarkers

Introduction

Osteoporosis is a common age-related systemic skeletal bone disease characterized by low bone mass, micro-architectural deterioration of bone tissue, and enhanced bone fragility. Fragility fractures are the most important disabling consequences of osteoporosis and result in loss of functional ability, serious morbidity associated with up to a three-fold increase in mortality, and high socio-economic burden [1]. The osteoporosis prevalence in the Saudi population is within 35-48% [2]. In the eastern province of Saudi Arabia, the annual cost of osteoporosis-related proximal femoral fractures management is US\$ 12.78 million [3]

and due to increased life expectancy, the burden of fractures are expected to increase.

Women are more susceptible to osteoporosis than men. They present approximately 80% of all patients with osteoporosis. Generally, women have smaller and thinner bones than men and there is a sharp decline in production of estrogen which increases the bone formation after menopause [4].

Osteoclast-mediated osteoporosis (post-menopausal osteoporosis) is characterized by rapid bone loss occurring in the post-menopausal period which is consistent with high bone turnover. There is a rapid phase of bone loss pre-

dominantly involving trabecular bone, vertebral and distal radial fractures [5]. Osteoporosis is a multi-factorial disorder and its etiology is attributed to various endocrine, metabolic and mechanical factors [6]. The early identification of individuals at risk for osteoporosis is fundamental for effective strategies of screening, diagnosis and/or treatment of osteoporosis.

A growing understanding of the bone remodeling process suggests that factors involved in inflammation are critically linked to bone remodeling which may contribute to the etiopathogenesis of postmenopausal osteoporosis [5]. Pro-inflammatory cytokines (IL-1 β , IL-6, IL-8 and TNF- α) are important regulators of bone resorption and may play an important role in estrogen deficiency-related bone loss in postmenopausal women. These pro-inflammatory mediators act both directly and indirectly to increase bone resorption, prevent, and/or inhibit bone formation [7]. In the present study, we aim to determine whether pro-inflammatory cytokines correlate with BTMs in a cohort of Saudi post-menopausal women with or without osteoporosis.

Subjects & methods

A total of 200 hundred Saudi postmenopausal women were recruited from Primary Care Centers, Prince Salman Hospital and King Fahd Medical City, Riyadh, Saudi Arabia. A written informed consent was obtained from all the participants before study enrolment. Participants were recruited with the following criteria: did not use hormone replacement therapy, calcium or vitamin D supplement for 6 months prior to study, had no history of any other bone disease or on drug therapy which could affect bone turnover and bone mineral density (BMD). A generalized questionnaire for personal history was taken from all participants including age, age of menarche, age at first full term pregnancy, number of full term pregnancies, lactation, and age of menopause, family history for osteoporosis and medical history; hypertension, obesity, diabetes mellitus, coronary heart disease (CHD) and any inflammatory diseases. Ethics approval was granted by The Ethics Committee of The College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA).

BMD for all participants was measured by DEXA (Hologic QDR 2000 Inc., Woltham, MA, USA) and evaluated by a radiologist. BMD values were given as g/cm² and the results were reported as T-scores (standard deviation above or below values for young healthy population). Participants were diagnosed as having osteoporosis according to World Health Organization (WHO) definitions that uses T score assessment. T-score value of -2.5 standard deviation (SD) or less indicate osteoporosis, T-score value between -1.0 and -2.5 SD indicate osteopenia and T- score value of -1.0 SD or more as normal. The 200 participants were divided into two groups based on the presence or absence of osteoporosis; 100 postmenopausal women with osteoporosis and a control group including 100 postmenopausal women.

Blood samples collection

Over-night fasting blood samples were obtained, 2 ml were collected in EDTA tubes and the other 4 ml collected in serum separator tubes. Samples were allowed to clot for 30 mins at room temperature then centrifuged at 2500 g for 10 mins. Plasma and serum samples were stored at -75°C until analysis.

Anthropometry and blood collection

Subjects anthropometry included height (rounded off to the nearest 0.5 cm), weight (rounded off to the nearest 0.1 kg), waist and hip circumference (centimeters), and mean blood pressure (systolic and diastolic in mmHg) (average of 2 readings) were determined. Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. Fasting blood samples were collected and transferred immediately to a no heparinized tube for centrifugation. Collected serum was then transferred to pre labeled plain tubes; stored in ice; and delivered to The Biomarkers Research Program (BRP) in King Saud University, Riyadh, KSA, for immediate storage at -20°C.

Sample analyses

Fasting glucose, lipid profile, calcium, and phosphorous were measured using a chemical analyzer (Konelab, Espoo, Finland). Serum 25 (OH) D was measured by using commercially available kits using Roche Elecsys Modular

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Table 1. Demographic characteristics of cases and control

	Cases	Control	P value
N	100	100	
Age (years)	50.6 ± 8.2	48.6 ± 7.3	0.08
BMI (kg/m ²)	32.6 ± 6.0	32.6 ± 5.4	0.94
Systolic BP (mmHg)	124.0 ± 13.8	121.1 ± 9.9	0.32
Diastolic BP (mmHg)	78.8 ± 8.2	78.5 ± 7.3	0.83
Waist (cm)	104.5 ± 17.6	102.6 ± 10.3	0.57
Hips (cm)	113.2 ± 19.0	115.1 ± 12.5	0.61
BMD (g/cm ²)	0.78 ± 0.13	1.1 ± 0.16	< 0.001
Glucose (mmol/l)	7.2 ± 3.6	6.5 ± 3.2	0.14
Total Cholesterol (mmol/l)	5.11 ± 1.06	5.12 ± 0.97	0.97
HDL-Cholesterol (mmol/l)	1.0 ± 0.32	1.1 ± 0.24	0.24
Triglycerides (mmol/l)	1.9 ± 0.11	1.68 ± 0.15	0.31

Note: Data presented as mean ± standard deviation; *p*-value significant at < 0.05.

Table 2. Biochemical bone markers and Pro-inflammatory parameters in cases and control

	Cases	Control	P value
N	100	100	
Vitamin D (nmol/l)	26.5 ± 1.5	33.5 ± 1.6	< 0.001
Osteocalcin (ng/ml)	6.0 (3.5, 8.5)	12.4 (9.4, 17.8)	< 0.001
CTX (ng/ml)	0.52 ± 0.26	0.33 ± 0.13	< 0.001
PTH (pg/ml)	38.0 (32.0, 44.3)	13.4 (8.6, 19.6)	< 0.001
Pi (mmol/l)	1.0 ± 0.24	1.2 ± 0.25	0.002
Calcium (mmol/l)	2.1 ± 0.21	2.2 ± 0.28	0.02
IL-6 (pg/ml)	17.1 ± 6.3	14.1 ± 5.2	0.01
IL-8 (pg/ml)	27.8 ± 6.2	23.9 ± 5.3	0.04
TNF-α (pg/ml)	7.6 ± 3.7	5.8 ± 3.4	0.03
IL-1β (pg/ml)	5.2 ± 2.3	2.1 ± 1.4	0.01

Note: Data presented as mean ± standard deviation; *p*-value significant at < 0.05.

Analytics Cobase411 utilizing electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). BRP is an accredited laboratory by The Vitamin D External Quality Assessment Scheme (DEQAS). Serum Cross-link C terminal peptides (intra-assay CV 4.6%, inter-assay CV 4.7%); osteocalcin (intra-assay CV 4%, inter-assay CV 6.5%); PTH (intra-assay CV 2.7, and inter-assay CV 6.5%) were measured by COBAS e 411 Analyzer (Roche Diagnostics). Serum Interleukin-1β level was determined using Luminex Multiplex Assay System (Luminex Inc.) intra-assay CV 7%, inter-assay CV 12%. Serum IL-6 and TNF-α levels were assayed using a solid-phase enzyme-linked immunosorbent assay (IL-6 Intra-assay CV3.1%, inter-assay 2.7%; TNF-α intra-assay CV 6.7%, inter-assay CV 11%).

Statistical analysis

Data were analyzed using SPSS version 16.0 (Chicago, IL, USA). Continuous data were represented by mean ± standard deviation for variables following Gaussian distribution and Median and interquartile ranges for Non-Gaussian variables. Categorical data were represented by frequencies and percentages. Each continuous variable was checked for normality by Kolmogorov-Smirnov test. Prior to analysis, Non Gaussian variables were transformed to log or square root. Differences between groups (control and cases) were determined by two tailed independent sample Student t-test. For non-Gaussian variables, Mann-Whitney U test was done to compare groups. Correlation between variables was tested by Pearson's correlation analysis with a level of significance was at $p \leq 0.05$.

Results

The results were presented as mean ± standard deviation, and group comparison was done using independent sample T-test. All non-Gaussian variables were represented as median and inter quartile range; group comparisons were done using Mann Whitney U-test and $p < 0.05$ was considered significant. **Table 1** shows the demographic characteristics of both groups. There was no significant difference between patients with osteoporosis and controls with respect to age, BMI, waist, hips, and blood pressure. The BMD was significantly lower in patients with osteoporosis as compared to control subjects ($p < 0.001$). There were no significant differences between the two groups with respect to glucose, total cholesterol, high density lipoprotein cholesterol (HDL-C), and triglycerides. Glucose was slightly increased in patients with osteoporosis than control group.

Table 2 shows a significantly higher plasma PTH ($p < 0.001$) and serum CTx ($p < 0.001$) and a significantly lower serum vitamin D ($p < 0.001$), osteocalcin ($p < 0.001$), and Pi ($p < 0.003$) in patients with osteoporosis than con-

Table 3. Associations between BTMs and pro-inflammatory markers

	Vit D	PTH	OCN	CTx	IL-6	TNF- α	IL-1 β
Vit D							
PTH	-0.18*						
OCN	0.24*	-0.15*					
CTx	-0.19*	0.23**	-0.18*				
IL-6	-0.12	0.11	0.13	0.10			
IL-8	-0.17	0.17	0.16	-0.21*	0.34**		
TNF- α	-0.13	0.30**	-0.21*	-0.11	0.31**	0.37**	
IL-1 β	0.08	0.32**	-0.18*	-0.16	0.42**	0.11	0.09

Note: Data presented as coefficient (R); *denotes significance at < 0.05; **denotes significance at < 0.01.

trols. Serum Ca was significantly lower in the osteoporosis group than in controls. A significantly higher serum pro-inflammatory parameters (IL-8, IL-1 β , IL-6, TNF- α (p < 0.05)) in the osteoporosis group compared to controls were found.

Table 3 shows the correlations between the studied BTMs and pro-inflammatory parameters in patients with osteoporosis. There was a significant correlation between serum levels of vitamin D and osteocalcin (r = 0.24, p < 0.05), CTx (r = -0.19, p < 0.05), and PTH (r = -0.18, p < 0.05). There was also a significant correlation between serum PTH and osteocalcin (r = -0.15, p < 0.05), CTx (r = 0.23, p < 0.05), TNF- α (r = -0.30, p < 0.05), and IL-1 β (r = 0.32, p < 0.05). Furthermore, there was a significant correlation between osteocalcin and CTx (r = -0.18, p < 0.05), TNF- α (r = -0.21, p < 0.05), and IL-1 β (r = -0.18, p < 0.05); between CTx and IL-8 (r = -0.21, p < 0.05); between IL-6 and IL-8 (r = 0.34, p < 0.001), TNF- α (r = 0.31, p < 0.001), and (r = 0.42, p < 0.001); between IL-8 and TNF- α (r = 0.37, p < 0.001).

Discussion

The present study showed no significant relationship between BMI and osteoporosis which is consistent with De Laet C et al., [8]. Other researchers however have found low BMI to be significantly associated with osteoporosis (Reid IR, 2002) [9]. Earlier epidemiologic studies investigating the relationship between obesity and osteoporosis centered on phenotypic correlations between body weight and bone mass, and produced the generally accepted view that increased mechanical loading associated with increased body weight, contributes to increased

bone mass [10, 11]. There was no significant difference in systolic and diastolic blood pressure between patients and controls. Popović MR observed that the rennin-angiotensin system, which plays a main role in blood pressure control, has an influence on bone density. High blood pressure is associated with calcium metabolism disorder, which affects increased excretion of calcium [12]. A significant correlation between hypertension and osteoporosis, thought to be through a link between cardiovascular diseases and osteoporosis, was demonstrat-

ed [13]. Blood glucose level was higher in the osteoporosis group than controls but this difference was not significant. The average level of blood glucose in the patients was 7.2 mmol/l, indicating possible diabetes. Reduced bone mass and increased fracture rates are common in type 1 diabetes and are linked to PPAR γ 2 and preferential adipogenesis over osteoblast-cell development [14]. Patients with diabetes have reduced late-stage differentiation of osteoblasts and a decreased osteoblast function [15]. Advanced glycated-end products (AGEs) have also been linked to abnormal development of osteoblasts and are thought to enhance bone resorption and induce apoptosis of mMSCs [16]. It is known that the enzymatic cross-linkage of collagen fibers gives strength to bone and AGE affect this process through induction of collagen cross-linkage which leads to increased fracture risk [17].

Osteocalcin and CTx levels as BTMs were evaluated in both groups. The level of osteocalcin was significantly lower in the osteoporosis group as compared to controls, and the level of CTx was also significantly higher than controls. This results are consistent with studies done on osteocalcin and CTx [18]. The exact mechanism of osteocalcin in bone is still unclear, but some studies suggested that osteocalcin influences energy metabolism by modulating the production and action of insulin [18]. Karsenty G, discovered that osteocalcin can regulate glucose levels through insulin production and improves the ability of other cells to take in glucose. Our results are consistent with this finding since glucose levels in patients with osteoporosis were slightly higher than controls. Bone resorption involves breakdown of type I collagen and the rate of bone resorption can be estimated by

measuring collagen degradation products as Carboxy-terminal telopeptides of collagen type I (CTx), which is used as a marker for bone resorption [19]. This is consistent with our finding since CTx was higher in the osteoporosis group than controls, indicating a high rate of bone resorption in women with osteoporosis.

A significant positive association between osteocalcin and vitamin D and a significant negative association between CTx and vitamin D were observed in osteoporotic women. These results support that vitamin D up-regulates osteocalcin expression and increases the production of osteocalcin *in vivo*, which in some osteoblastic cells is totally dependent on the presence of 1, 25(OH)2D3. On the other hand, the expression of type I collagen is down-regulated by 1, 25 (OH) 2D3 [19]. A significantly higher PTH in the osteoporosis group as compared to controls was detected. PTH indirectly stimulates bone resorption by osteoclasts as its receptors are located on osteoblasts. When PTH binds to its receptor, the PTH1R activates a Gs protein to increase PKA activity and thereby cAMP-mediated transcriptional activity. IGF-1 is essential for this activity. PTH1R also activates the Ca²⁺/PKC pathway and subsequently MAPK, which determines the proliferative response to PTH. Together, PKA, IGF-1 and PKC, as well as other factors, result in an increase in RANKL and CSF, and a decrease in the decoy receptor osteoprotegerin, which stimulate the recruitment of osteoclast precursors to form osteoclasts in a controlled manner. The effect of parathyroid hormone, on the kidney is to stimulate loss of phosphate ions and suppression of calcium loss in urine, this effect is mediated by stimulating tubular reabsorption of calcium [20]. In present study, the mean calcium and phosphorus levels were within the normal range (2.1-2.6 mmol/l and 1-1.5 mmol/l respectively). A significant negative correlation was found between serum intact parathyroid hormone (iPTH) and serum 25(OH) D values ($p = 0.04$) in patients and controls. Same observations were seen in French elderly people living in an environment with a lack of direct exposure to sunshine, diet failed to provide an adequate amount of vitamin D [21]. Vitamin D deficiency is a frequent finding in the postmenopausal women and when the 25 (OH) D concentrations became equal to or lower than 11.3 nmol/l, the PTH val-

ues reached the upper limit of normal values 55 pg/ml [22].

Pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and TNF- α) serum levels were significantly elevated in patients than controls. This is in agreement with another study that investigated the relationship between osteoporosis and inflammation [23]. Bone marrow cells of the monocyte/macrophage lineage are believed to be the major source of the postmenopausal increases in TNF- α and IL-1 secretion in bone tissue. However, in the past few years it has been increasingly recognized that activated T cells are also an important source of increased TNF- α production in the bone marrow after menopause [24]. In contrast, stromal cells/osteoblasts are considered to be the major producers of IL-6 in bone tissue [25]. Pro-inflammatory cytokines are among the most powerful stimulants of bone resorption, through directly and indirectly stimulation of other local factors intervene with every single step in osteoclastogenesis that determines the rate of bone resorption, from the proliferation and differentiation of the early osteoclast precursor cell to the resorption capacity and the lifespan of the mature osteoclast [26].

The first step in osteoclastogenesis that determines the rate of bone resorption is the proliferation of osteoclast precursor cells. In fact, a major consequence of estrogen deficiency, loss of ovarian function is permissive for the expression of the major cytokines that directly stimulate early osteoclast precursor proliferation, i.e., M-CSF, GM-CSF, and IL-6 [27]. Spontaneous increases in these cytokines may be further enhanced by the parallel increases in IL-1 and TNF- α with menopause, which are potent stimulators of M-CSF, GM-CSF [28], and IL-6 [29]. Estrogen is a potent stimulator of OPG production [30] and suppresses M-CSF production, loss of estrogen would also be expected to promote the signaling and gene expression cascade that leads to the differentiation of osteoclast progenitors to mature osteoclasts. A decrease in OPG production with estrogen deficiency is bound to increase the ratio of RANKL/OPG activity. It thus facilitates the binding of RANKL-expressing osteoblast/stromal cells and lymphocytes to osteoclast progenitors as the key signal for initiating osteoclast differentiation [31].

Transforming growth factor-beta (TGF- β) has recently been observed to have essential role in the differentiation of osteoclast precursor cells. TGF increases when estrogen is deficient. The increased levels of TGF- β stimulate pro-inflammatory cytokine production IL-1, TNF- α [32]. Prostaglandin E2 (PGE2) may not only promote osteoclast generation, but they also appear to stimulate mature osteoclasts to perform more resorption cycles via modulation of RANKL activity [33]. IL-1 [34] and IL-6 [35] also directly enhance osteoclast activity by RANKL-independent mechanisms. Finally, increases in IL-1, M-CSF, RANKL, and a relative decrease in TGF- β may directly extend the lifespan of the osteoclast by inhibiting osteoclast apoptosis [36]. In pre-menopause, variations in bone resorption are usually compensated by appropriate changes in bone formation. This is thought to be the result of locally released anabolic growth factors, such as TGF- β and IGFs. Efforts to compensate the increased bone resorption are also evident with estrogen deficiency but obviously do not suffice to achieve a neutral bone balance. Thus, depending on the perspective, postmenopausal bone loss may also be viewed as resulting from inadequate bone formation. It is well known that pro-inflammatory cytokines have potent effects on osteoblast function. Both TNF- α , and IL-1 inhibit collagen synthesis in osteoblasts and stimulate or inhibit bone cell proliferation, depending on the experimental systems employed. Both TGF- β and PGE2 are potent stimulators of bone formation. However, the relevance of postmenopausal changes in these cytokines with respect to bone formation has been much less explored compared with bone resorption [37].

Conclusions

In summary, our data indicate that in postmenopausal women an increased bone resorption associated with significantly higher plasma PTH ($p < 0.001$) and increased of serum pro-inflammatory parameters (IL-8, IL-1 β , IL-6, TNF- α ($p < 0.05$)), and with a significantly down regulation of serum vitamin D ($p < 0.001$), osteocalcin ($p < 0.001$), and Pi ($p < 0.003$) resulting in decreased bone formation. The present study proved that the pro-inflammatory cytokines accelerate the bone loss in postmenopausal women. However, because of the relatively small number of participants further

studies are necessary to establish on cytokine pattern and bone metabolism.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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