Int J Clin Exp Med 2017;10(1):1179-1185
www.ijcem.com /ISSN:1940-5901/IJCEM0038056

Original Article

Changes in serum cytokines and vitamin D in Saudi postmenopausal women with osteoporosis

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Received August 17, 2016; Accepted October 24, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Accelerated bone loss has been observed in patients with inflammatory disorders, exacerbated with Vitamin D deficiency, whilst the impact of cytokines in the etiology of osteoporosis is unclear. However, the elucidation of this potential relationship could provide new insights to identify patients at early risk of osteoporosis as well as support the use of cytokine-based antibody therapies as potential interventions to reduce bone loss. The aim of the study was to determine the relationship between pro-and anti-inflammatory cytokines with bone loss in Saudi post-menopausal women with and without osteoporosis. Further to understand the relative importance of Vitamin D on changes in inflammatory cytokine status. For this study post-menopausal women with (n=101) and without osteoporosis (n=120) were recruited. Anthropometric was taken along with fasted blood to measure 25-hydroxyvitamin D [25(OH)D] and cytokines (TNF-α, TGF-β, IL-1β, IL-4, IL6, Leptin, adiponectin, resistin, PAI-1, Lipocalin). Data shows a significantly lower plasma TGF-β ($P<0.001$) and serum IL-4 ($P<0.001$) and a significantly higher serum resistin ($P<0.001$) in the osteoporosis patients compared with control. Vitamin D showed a significant negative association with resistin ($P=0.024$). The osteoporosis group displayed a pro-inflammatory state with elevated serum levels of IL-6, leptin, IL-1. Other Inflammatory biomarkers were not associated with vitamin in postmenopausal women. In conclusions, the present study showed that inflammatory factors, such as resistin, TGF-β and IL-4 may play an important role in bone metabolism in postmenopausal women with osteoporosis. It is important to understand the balance between pro and anti-inflammatory cytokines as the impact to lower anti-inflammatory cytokines may allow pro-inflammatory cytokines to have more of an impact, coupled with elevated resistin levels.

Keywords: Cytokines, vitamin D, postmenopausal, osteoporosis

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. Currently it is estimated that over 200 million people worldwide suffer from this disease. About 80% of those affected by osteoporosis are women, most of whom are postmenopausal women [1].

In Saudi Arabia, prevalence of osteoporosis (≥50 years) is as high as 44.5% in Saudi women and 33.2% in Saudi men according to Saudi reference data [2]. Moreover, the incidence of fragility fractures jumped from 2.9/1000 in 1999 [3] to 6/1000 in 2007 at an annual cost of SR 4.27 billion [4]. In the eastern province of Saudi Arabia, the annual cost of osteoporosis-related proximal femoral fractures management is US$ 12. 78 million [4] and due to increased life expectancy, the burden of fractures are expected to increase.

A number of risk factors for osteoporosis are well recognized, including vitamin D status, age, gender, smoking, physical inactivity and

estrogen deficiency. In recent years, inflammation has been also implicated. Certain pro-inflammatory cytokines play potential critical roles both in the normal bone re-modelling process and in the pathogenesis of peri-menopausal and late-life osteoporosis [5]. For example, interleukin (IL)-6 promotes osteoclast differentiation and activation [6]. This cytokine is involved in the pathogenesis of various metabolic bone diseases, including post-menopausal osteoporosis, Paget's disease and osteoporosis associated with hematologic malignancies [7]. IL-1 is another potent stimulator of bone resorption [8] that has been linked to the accelerated bone loss seen in idiopathic and postmenopausal osteoporosis [9]. The production of IL-1, IL-6, and/or TNF-α by peripheral blood monocytes has been positively correlated with bone resorption or spinal bone loss in healthy pre- and postmenopausal women [10]. Moreover, resistin is an adipocytokine that was discovered recently and seems to play a role in glucose homeostasis, insulin resistance and inflammation [11]. It has been shown recently, that plasma resistin levels correlate significantly with inflammatory markers [12, 13]. These findings may provide a novel link between elevated resistin levels and associated inflammatory processes. All these molecules, which include resistin, leptin, and IL-6, affect human energy homeostasis and may well be involved in bone metabolism. The other cytokine like TGF-β and IL4 have been shown to influence the osteoclastic and osteoblastic cell types.

Primary studies on the relations between cytokine levels and bone mineral density (BMD) or bone loss in postmenopausal women have provided inconsistent or even contradictory results [14]. The reasons for inconsistencies could be related to methodological differences, cytokines assessed as well as how osteoporosis has been determined in different populations.

Beyond the cytokines Vitamin D is known to play an important role in calcium regulation and bone metabolism [15, 16]. Yet people in Saudi, have vitamin D deficiency, despite the levels of sunshine. Furthermore it's known that 64% of patient with osteoporosis also have vitamin D deficiency [17, 18].

Therefore, this present study addressed the relationship between cytokines correlate with vitamin D in a cohort of Saudi post-menopausal women with or without osteoporosis.

Materials and methods

Patients

This study included a total of 221 Saudi post-menopausal women aged ≥50 years old [N= 101 with osteoporosis and N=120 without osteoporosis] recruited from the Primary Care Centers (PCCs), King Salman Hospital and King Fahd Medical City, Riyadh, Saudi Arabia. A written informed consent was obtained from all the participants before study enrolment. The Participant's history was recorded from a generalized questionnaire including age, age of menarche, age of menopause, age at first full term pregnancy, number of full term pregnancies, lactation, family history for osteoporosis, medical history; disease status. Ethics approval was granted by The Ethics Committee of The College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA). 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).

Bone mineral density BMD (g/cm^2) was measured at the femoral neck by dual-energy X-ray absorptiometry DEXA (Hologic QDR 2000 Inc., Woltham, MA, USA) for all Participants. The diagnostic criteria of osteoporosis was based on the T-score for BMD established according to WHO definitions that uses T score assessment, T-score value of -2.5 SD or below the mean for a young healthy adult woman 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.

Anthropometry and blood collection

Subjects anthropometry included height and weight were determined using standardized conventional methods in light clothes and without shoes, waist and hip circumference were obtained using a standardized non-stretchable fiber measuring tape, Waist-to-hip ratio (WHR) was calculated as the ratio of waist and hip circumferences, mean blood pressure (systolic and diastolic in mmHg) were measured. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2).

Table 1. Clinical Characteristics of the subjects

Parameter	Normal	Osteoporosis	*P*-Value
N	100	100	
Age (years)	55.03±4.29	56.10±5.84	0.269
Body Mass Index (kg/m^2)	32.01±3.86	30.87±5.56	0.126
Waist circumference (cm)	103.53±13.53	98.72±14.21	0.013
Hip circumference (cm)	114.48±12.36	108.01±14.6	0.001
Menarche Age (Years)	13.08±1.47	13.54±1.76	0.040
Menopause (Years)#	4.00 (2.00-6.00)	10.00 (5.00-15.00)	<0.001
First Pregnancy Age (Years)	18.91±3.23	19.38±3.84	0.368
Systolic Blood Pressure (mmHg)	124.16±18.24	126.48±17.78	0.178
Diastolic Blood Pressure (mmHg)	74.88±10.88	75.27±10.34	0.799
BMD Femoral neck volume#	0.29±0.73	-0.97±0.95	<0.001
Glucose (mmol/l)	7.86±3.34	8.16±3.46	0.151
Albumin (mmol/l)	39.43±4.61	39.59±4.46	0.803
Total Cholesterol (mmol/l)	4.84±0.97	4.94±1.12	0.533
HDL-Cholesterol (mmol/l)	0.99±0.20	1.02±0.24	0.314
Triglycerides (mmol/l)	1.54±0.58	1.65±0.57	0.171
Phosphate (mmol/l)	1.06±0.32	1.06±0.31	0.963
Calcium (mmol/l)	2.27±0.17	2.29±0.17	0.245
25(OH) Vitamin D (nmol/l)	57.74±26.99	55.26±27.40	0.721

Note: Data presented as N (%) for frequencies; mean ± standard deviation for normal continuous variables; #denotes continuous variables with non-Gaussian distribution presented Median (Ist quartile-3rd Quartile) and log transform; *P* value significance at 0.05 and 0.01.

Fasted blood samples were collected in tubes without anticoagulant (serum separator tubes). Samples were then left to clot at room temperature for 30 minutes, and then were centrifuged at 5000 RPM for 10 minutes. Serum samples were stored at -80°C until analysis.

Sample analyses

Fasting glucose, lipid profile, calcium, and phosphorous were measured using a chemical analyzer (Konelab, Espoo, Finland). 25-OH vitamin D were determined by electrochemiluminescence immunoassay, kit purchased from (Roche Diagnostics, Mannheim, Germany). Intra- and inter-assay CV were 4% and 6.5% respectively. BRP is an accredited laboratory by the 25(OH) vitamin D External Quality Assessment Scheme (DEQAS). PTH (intra-assay CV 2.7, and inter-assay CV 6.5%) were measured by COBAS e 411 Analyzer (Roche Diagnostics). Serum cytokines level was determined using Luminex Multiplex Assay System (Luminex Inc.) with intra-assay for all cytokines less than 7%, inter-assay less than 12%.

Statistical analysis

Data were entered and analyzed using Statistical Package for the Social Sciences (SPSS) version 16.5 (Chicago, IL, USA). Continuous data were represented by mean ± SD for variables following Gaussian distribution and Non-Gaussian variables. Categorical data were represented by frequencies and percentages. Each continuous variable was checked for normality by Kolmogorov-Smirnov test. Differences between groups (cases and control) were done using Student t test. For non-Gaussian variables and Mann-Whitney U test were determined to compare groups. Relationships among variables were sought by Spearman's correlation coefficient. Univariate and multivariate linear regression analysis were performed to identify independent factors affecting endotoxin. A *p*-value <0.05 was considered as statistically significant.

Results

The clinical, anthropometric, demographic and biochemical characteristics of the case-control study subjects are summarized in **Table 1**. The 100 Saudi postmenopausal women without osteoporosis were 52.62±5.65 years old, and 100 with osteoporosis were 57.35±4.29 years old participated in the study. There was a significant difference with respect to BMI, menopause, menarche age, waist and hip circumference. The BMD lumbar volume was significantly lower in patients with osteoporosis than controls. There were no significant differences between the two groups with respect to glucose, total cholesterol, high density lipoprotein cholesterol (HDL-C), and triglycerides.

Table 2 Shows a significantly lower plasma TGF-β (P<0.001) and serum IL-4 (P<0.001) and a significantly higher serum resistin (P<0.001) in the osteoporosis patients compared with case control subjects. TGF-β and serum IL-4 decreases by 15% and 53% respectively and

Table 2. Cytokines in cases and control

Parameter	Normal	Osteoporosis	*P*-Value
N	100	100	
TGF-β (μg/ml)	40.25 (34.69-52.37)	34.55 (17.81-46.33)	0.014
IL-4 (Pg/ml)	7.23 (4.56-10.36)	3.44 (2.41-7.21)	<0.001
IL6	8.64 (4.23-25.58)	11.60 (6.02-25.39)	0.119
Leptin (ng/ml)	16.71 (8.36-30.95)	18.28 (6.20-32.8)	0.706
TNF-αlpha	1.57 (1.05-2.06)	1.57 (1.00-2.58)	0.672
IL-1B	2.11 (1.64-2.83)	2.44 (1.79-2.91)	0.232
Adiponectin mg/dl	12.47 (9.07-15.03)	12.71 (8.15-15.89)	0.794
Resistin ng/ml	723.47 (236.48-1289.34)	1014.16 (492.43-1381.16)	0.024
PAI-1 (μg/ml)	89.61 (65.82-119.51)	82.87 (65.96-114.61)	0.441
Lipocalin (μg/ml)	65.84 (41.58-97.40)	61.37 (45.54-77.34)	0.321

Note: #denotes continuous variables with non-Gaussian distribution data presented in Median (1st quartile-3rd Quartile); *P* Value significance at 0.05 and 0.01.

Table 3. Associations between 25(OH) Vitamin D (nmol/l)# and cytokines

Parameters	Coefficient (R)		
	All	Normal	Osteoporosis
N	200	100	100
TGF-β (pg/ml)	-0.063	-0.237	-0.101
IL-4 (Pg/ml)	-0.008	0.085	-0.180
IL6	-0.048	-0.080	0.031
Leptin	0.044	-0.032	0.173
TNF-αlpha	-0.063	-0.001	-0.096
IL-1B	0.027	0.041	0.064
Adiponectin ng/ml	-0.149	-0.118	-0.137
Resistin ng/ml	-0.283**	-0.214*	-0.412**
PAI-1 (Pg/ml)	-0.038	-0.079	-0.007

Note: Data presented as coefficient (R); #denotes log transform; *denotes significance at 0.05 level; **denotes significance at 0.01 level.

resistin increases by 40% in the osteoporosis patients related to healthy ones.

The bivariate associations of vitamin D with inflammatory cytokine of all subject, controls and cases cohort studied are summarized in **Table 3**. In the univariate correlation analysis, vitamin D showed a significant negative association with resistin. A linearly correlation was found between vitamin D and resistin in all subject (**Figure 1**).

Discussion

To the best of our knowledge, this is the first study to prospectively evaluate the various serum cytokines, chemokines and vitamin D in Saudi postmenopausal with and without osteoporosis.

The present study clearly demonstrated that vitamin D deficiency was common in Saudi Arabia. Approximately 50% of the whole study population exhibited a serum 25(OH)D level less than that commonly considered to represent deficiency (<50 nmol/L). Indeed, poor vitamin D status has also been reported previously in Saudis cross-sectional studies [19, 20]. It is assumed that populations living in sunny locations, such as Saudi Arabia, would be less likely to be vitamin D deficient because of abundant sunshine throughout the year. However, the results of the present study challenge this assumption. In Saudi Arabia where there is year round sunlight the prevalence of vitamin D deficiency is high; being mainly attributed to reduced outdoor activity and lack of vitamin D-fortification in common foods [21].

The osteoporosis group displayed a pro-inflammatory state with higher serum levels of IL-6, leptin, IL-1, and resistin. These findings suggest that a progress of inflammation is a major component of osteoporosis. This observation highlights the importance of early intervention in osteoporosis state to prevent its progression. It was be observed a strong positive correlation between vitamin D and resistin (**Figure 1**). Although osteoporosis is not typically considered an immunological disorder, recent data have indicated over lapping pathways between bone biology and biology of inflammation [22-24]. There are multiple mechanisms and interactions by which cytokines regulate bone

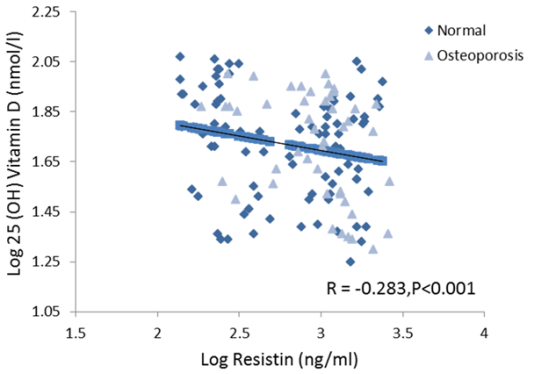

Figure 1. Significant inverse association between Vitamin D and Resistin (Normal and Osteoporosis).

resorption. IL-6 contributes to RANKL upregulation in osteoblastic cells. IL-1 may not only promote osteoclast generation, but they also appear to stimulate mature osteoclasts to perform more resorption cycles through modulation of RANKL activity. IL-1 is further involved in bone metabolism as an osteoblast activator: osteoblasts secrete RANKL which promotes survival and differentiation of the osteoclast precursors to mature osteoclasts through RANK. IL-1 and IL-6 also directly enhance osteoclast activity by RANKL-independent mechanisms. They may directly extend the lifespan of the osteoclasts by inhibiting osteoclast apoptosis. IL-1 inhibit collagen synthesis in osteoblasts and enhance degradation of the extracellular matrix [25]. In inflammatory or autoimmune disease states, activated T cells produce RANKL and pro-inflammatory cytokines, all of which can induce RANKL expression in osteoblasts [26]. In our study, there is significance increase of resistin in postmenopausal osteoporosis compared to healthy ones. Resistin, has proinflammatory properties by strongly upregulating IL-6 and TNF-α [27]. This adipocyte-derived protein is also detectable in inflamed joints of patients with RA and in peripheral blood mononuclear cells suggesting its possible role in inflammatory processes [28]. It plays an important role in bone metabolism by stimulating osteoblast and osteoclast differentiation, possibly through the nuclear factor kappa B (NF-jB) pathway [29]. Also, Oh et al. [30] showed that serum resistin level showed a significant negative correlation with lumbar spine BMD in middle-aged men.

Vitamin D levels were found to have a significant negative correlation with resistin levels but not with any other cytokines (in the group of all patients: r=-0.24, P<0.05, **Figure 1**. Resistin, shares several features with proinflammatory cytokines and can trigger a proinflammatory state in vitro as well as in vivo [31]. Therefore, we hypothesized that if resistin was a marker of inflammation, then it could be inversely related to vitamin D concentrations. This relationship between vitamin d and resistin levels confirm some previous findings regarding the inhibitory influence of inflammation on the rate of bone formation [32]. Thommesen et al. [33] have shown that resistin may play a role in bone remodeling, and Forsblad d'Elia et al. [34] have observed moderate correlations between resistin and a marker of increased osteoclast activity.

Furthermore, elevated levels of resistin have been shown in patients with rheumatoid arthritis and also correlated strongly with inflammatory markers. 11 these data support the hypothesis of resistin being an important member of the cytokine family with potent regulatory functions that might be involved in the pathogenesis of osteoporosis diseases in postmenopausal women

Blood glucose level was higher in the osteoporosis group (8.2 mmol/l) than controls (7.9 mmol/l) but this difference was not significant, Several recent lines of evidence in both humans and rodents have corroborated that T2DM is indeed detrimental to bone, leading to impaired osteoblast-mediated bone formation, accelerated bone resorption, microstructural defect, and poor bone quality.

The authors acknowledge several limitations. The cross-sectional study design cannot suggest any causal and temporal correlations. Further investigations are needed and separate studies done in men, since some of these cytokines are expressed differently by sex [35], to improve our understanding of vitamin d in the implication in inflammation in large scale to estimate association vitamin d with cytokines.

Conclusion

The present study provided evidence that resistin, leptin IL-1, IL-4, IL-6 and TGF-β play important role in bone metabolism in postmenopausal women. These diagnostic markers may be able to identify patients at risk for osteoporosis

and therefore predict fracture risks. Thus, early interventions to preserve bone health, for example, by anti-cytokine therapy, could be more effective and efficient. Vitamin D and cytokines not only act on bone independently, but also are linked by complex relationships.

Acknowledgements

This project was funded by the national plan for science for science, Technology and Innovation (MAARIFAH), King Abdulaziz City for science and Technology, Kingdom of Saudi Arabia, Award number (11-MED-2113-02). The authors thank Malak Nawaz Khan Khattak and Syed Danish Hussain for the statistical analyses.

Disclosure of conflict of interest

None.

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