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Salivary Interleukin 6 is A Valid Biomarker for Diagnosis of Osteoporosis in Postmenopausal Women

Widad Farhan Jabber[1], Taghreed F. Zaidan[1], Faiq I. Gorial [2*], Ahmed S. Al-Naaimi[3]
1. Department of Oral Diagnosis, College of Dentistry, University of Baghdad, Iraq
2. Department of Medicine, College of Medicine, University of Baghdad, Baghdad, Iraq
3. Department of Community Medicine, College of Medicine, University of Baghdad, Baghdad, Iraq

Abstract
Background: Interleukin 6 (IL6) is pro-inflammatory cytokine associated with decline in ovarian function during menopause which is involved in the pathophysiology of postmenopausal bone loss.
Objective: To investigate validity of salivary IL6 as a biomarker to diagnose osteoporosis in post-menopausal women.
Patients and Methods: This cross sectional study was conducted on 75 postmenopausal women. Bone mineral density (BMD) was measured by dual energy x-ray absorptiometry (DXA) to diagnose osteoporosis. Salivary IL6 was measured for all postmenopausal women by using Human ELISA kit**.**
Results: Of a total 75 individual involved in the study, 25 were healthy postmenopausal women, 25 had osteopenia, and 25 had osteoporosis. There was no statistical significant differences between age and body mass index in all groups ($p>0.05$). Mean level of salivary IL6 was significantly different among healthy controls, osteopenic group, and osteoporotic group (11.66 ± 4.44 vs 14.51 ± 6.5 vs 23.9 ±5.52 pg/ml; $p<0.001$) respectively. The mean salivary IL-6 was significantly higher in osteoporosis group (23.9 pg/ml) compared to control group (11.66 pg/ml). Salivary IL-6 was a valid parameter to predict osteoporosis in postmenopausal woman (ROC area=0.91, $p<0.001$).Salivary IL-6 showed positive strong significant linear correlation with bone t- score ($r=0.62$, $P<0.001$). Salivary IL6 at the optimum cut off value ≥19.45 pg/ml has highest accuracy (81.3%) to diagnose osteoporosis in postmenopausal women with sensitivity was 84.%, specificity 80 %, positive predictive value (PPV) at pretest probability 50% was 80.8%, and PPV at pretest probability 90% was 97.4 %, and negative predictive value (NPV) at pretest probability 10% was 97.8 %.
Conclusions: Salivary IL6 was a simple, easy, and a valid biomarker to diagnose osteoporosis in postmenopausal women with high accuracy. This may indicate a hopeful measure for early diagnosis and treatment of osteoporosis.
Keywords: Salivary IL6, Menopause, Proinflammatory cytokines, Osteoporosis.

1. Introduction

Osteoporosis prevalence is consistently increasing due to increased life expectancy worldwide. It is known that one of the most common causes of hospitalization among elderly postmenopausal women is hip fractures due to osteoporosis [1–3]. Nearly half of all women are expected to suffer an osteoporotic fracture in their lifetime with subsequent functional dependent that requires long term nursing care, and increasing in mortality [4].

Thus, most relevant studies aimed at developing new measures and treatment modalities to reduce the burden of this health problem [5]. In recent years, saliva-based diagnostic tests have increased in popularity because of their non-invasive nature. Using saliva rather than serum has benefits: it is non-invasive, easy to obtain, painless and there is no need to employ specially trained personal for sample collection [6, 7]. Cytokines, the key regulators of the immune responses, are crucially involved in neuroendocrine immune interactions, whereas estrogen appears to regulate immune function [8-10]. IL6 is pro-inflammatory cytokines associated with the decline in ovarian function in menopause have been shown to act as bone-resorbing cytokines. The alteration in cytokines during menopausal transition is involved in the pathogenesis, development, and progression of postmenopausal osteoporosis [11]. This study was carried out to investigate salivary IL6 in postmenopausal women with osteoporosis.

2. Patients and Methods

2.1 Study design

This cross-sectional study was conducted in Institute of Radiology, Baghdad Medical City from 23rd of September 2014 till 10th of February 2015. Informed consent was taken from all participants in the study and ethical approval had been obtained from the Ethics Committee of Department of Oral Diagnosis, College of Dentistry, University of Baghdad.

2.2 Sample selection

Eligible individuals included in the study were postmenopausal women who had experienced at least 12 consecutive months of amenorrhea. The postmenopausal stage was defined as a stage beginning at the time of

the woman final menstrual period [12].Subjects were excluded from the study if they had: diabetes mellitus, thyroid and parathyroid disease, autoimmune diseases, history of periodontal therapy within the last 3 months; current use of medications such as corticosteroids or any immune suppressive within the previous 3 months, chemotherapy, ovarictomy; smokers, alcohol users, fracture and neoplastic diseases

2.3 Clinical, laboratory, and radiological evaluation

A Questionnaire paper consisted of age, weight, height, and body mass index (BMI) which was measured according the equation BMI=weight / height ^{2,} and duration of menopause.

About seven ml of unstimulated whole saliva were collected from each postmenopausal woman under standardized conditions, following the instruction cited by Tenovuo and Lagerloff [13]. Collection done at the morning from (9-12 am) the subjects were stopped drinking and eating at least one hour prior to the collection and asked to rinse their mouth out well (without drinking the water) to remove any possible debris or contaminating materials. Subjects were asked to spit in to the collection cup for up to 10 minutes. After collection of unstimulated saliva samples each one was centrifuged at 3000 rpm for 10 minutes, the clear supernatant was separated, divided in parts in sterile eppendorffs and stored frozen at 28^0C deep freeze until analysis on each tub a subject name was recorded by water resistance marker [13].

For cytokine determination saliva samples were stored at -80^0C. Salivary IL- 6 was analyzed by using Human enzyme-linked immunosorbent assay (ELISA) kit for IL6 (Abcam, USA).

The diagnosis of the osteoporotic patients was made by rheumatologist according to the results of BMD (WHO, 1994) using dual x-ray absorptiometry scan (DXA scan: central DXA type DEXXUM 3). All women were divided into three groups according to the results of BMD (WHO, 1994) [14]: Group one: twenty five post postmenopausal women with osteoporosis (T score $\leq$ -2.5), Group two: twenty five post postmenopausal women with osteopenia: T score between −1 and −2.5 standard deviations below the mean value of peak bone mass. Group three: 25 healthy postmenopausal women (T score $\geq$ -1.0).

2.4 Statistical analysis

Statistical software (SPSS version 22, IBM, USA) was used for data analysis. Kolmogorov-Semirnov test was done to assess the distribution of continuous variables (Age, BMI, duration of menopause and salivary IL6). The statistical significance of differences in mean of a normally distributed variable between 2 groups was assessed by independent samples t-test. The statistical significance of differences in mean of a normally distributed variable between more than 2 groups was assessed by ANOVA test. When ANOVA model detects a statistically significant difference, further exploration for statistical significance of difference in mean between all possible paired combinations of study groups was performed using LSD (least significant difference). The statistical significance, direction and strength of linear correlation between 2 quantitative variables, one of which being non-normally distributed variable was measured by Spearman's Rho linear correlation coefficient. A receiver operating characteristic (ROC) curve analysis was used to assess validity parameters and set optimum cut-off values for salivary IL6 when used to predict a diagnosis of osteoporosis P value less than the 0.05 level of significance was considered statistically significant.

3. Results

A total of 75 postmenopausal women involved in the study, of them 25 were healthy controls, 25 osteopenic patients, and 25 were osteoporotic patients. The mean age of patients with osteoporosis was (52.2±5.3) years, osteopenia (56.1±4.2) years; and control subjects (55±5.1).There was no statistical significant difference between the groups (P= 0.07).The mean duration of menopause was highest in osteoporosis group (9.2±6.3) and the lowest was in control group (4.4±2.8) years. The mean duration of menopause was significantly higher in osteoporosis group (9.2) years compared to control group (4.4) years ($p<0.001$.On the other hand the mean duration of menopause was obviously higher in osteopenia group (8.2±4.5) years compared to control group (4.4±2.8) years, P= 0.005, but the mean duration of menopause in osteoporosis was not significantly different from osteopenic patients. The (mean ±SD) of body mass index (BMI) in osteoporosis patients was (30.8±5.7)kg/m², while the (mean ±SD) of BMI in osteopenia patients and in control subjects was (31.4±5.5) kg/m² and (33.8±4.9) kg/m² respectively. The difference in mean between studied groups was not statistically significant (p= 0.46).

In table 1: The mean salivary IL-6 was highest in osteoporosis group (23.9 pg/ml) and lowest in control group (11.66 pg/ml) .The mean salivary IL-6 was significantly higher in osteoporosis group compared to control group (23.9 pg/ml vs11.66 pg/ml, $p<0.001$).The mean salivary IL-6 was obviously higher in osteopenia group (14.51 pg/ml) compared to control group (11.66 pg/ml), but the observed difference failed to reach the level of statistical significance($P>0.05$). The effect of osteoporosis on increasing salivary IL-6 was stronger (ROC area=0.95) than that of osteopenia (ROC area=0.64). In addition, salivary IL-6 showed a statistical significant difference between the study groups $p<0.001$) and statistical significant strong positive linear

correlation of salivary IL6 with bone t- score (r=0.62, P<0.001).

Salivary IL-6 was a valid parameter to predict osteoporosis in postmenopausal woman (ROC area=0.91, p<0.001) as shown in table 2.

The best cut-off value providing the best separation between postmenopausal women with osteoporosis and those without osteoporosis was salivary IL-6 concentration 19.45 pg/ml which had highest accuracy (81.3%) and was associated with a sensitivity of 84 % and specificity of 80 %. And Testing positive at this optimum cut-off will establish a diagnosis of osteoporosis with 80.8% confidence in clinical context where the pretest probability of having osteoporosis is 50% (equal odds for having osteoporosis versus not having it). However, when the clinical context of osteoporosis is of high probability based on clinical suspicion only (pretest probability =90%) then it will establish the diagnosis with 97.4% confidence (Table 3).

Table 1: The difference in mean salivary IL-6 between study groups

	Study group			
	Healthy controls	**Osteopenia**	**Osteoporosis**	**P**
Salivary IL-6 (pg/ml)				<0.001
Range	(5.85 to 20.5)	(6.98 to 31.16)	(13.28 to 31.94)	
Mean	11.66	14.51	23.9	
SD	4.44	6.5	5.52	
SE	0.888	1.3	1.105	
N	25	25	25	

P (LSD) for the difference in mean between:
Osteopenia x Healthy controls = 0.07[NS]
Osteoporosis x Healthy controls = <0.001
Osteoporosis x Osteopenia = 0.001
Effect size (ROC area P value)
Osteopenia X Healthy (ROC=0.65 P=0.06[NS])
Osteoporosis X Healthy (ROC=0.95 P<0.001)
Osteoporosis X Osteopenia (ROC=0.86 P<0.001)
Linear correlation with t-score:
r=0.62
P<0.001

Table 2: ROC area for salivary IL6 when used as test to predict osteoporosis among postmenopausal women

Predicting Osteoporosis	**ROC**	**P**
Salivary IL6	0.91	<0.001

ROC, receiver operating characteristics

Table 3: **Validity parameters of salivary IL6 level in predicting osteoporosis**

Positive if ≥ cut-off value	**Sensitivity**	**Specificity**	**Accuracy**	**PPV at pretest probability = 50%**	**PPV at pretest probability = 90%**	**NPV at pretest probability = 10%**
Salivary IL6 level, pg/ml						
13.15 (Highest sensitivity)	100.0	68.0	78.7	75.8	96.6	100.0
19.45 (optimum cutoff value)	84.0	80.0	81.3	80.8	97.4	97.8
31.33 Highest specificity)	8.0	100.0	69.3	100.0	100.0	90.7

PPV, positive predictive value; NPV, negative predictive value

4. Discussion

Postmenopausal osteoporosis is a very common metabolic disease that has no clinical features except when there is a fracture, so early diagnosis and treatment is very important. This study investigated salivary IL6 in postmenopausal women with osteoporosis and found that mean salivary IL-6 was significantly higher in osteoporosis group compared to control group. In addition. Salivary IL6 was a valid biomarker to diagnose osteoporosis in postmenopausal women with high accuracy.

The impact of IL6 in postmenopausal women may be related to the fact that IL6 is a pro-inflammatory cytokine and interacts in complex ways with the cells involved in bone remodeling. It may indirectly promote

osteoclastogenesis by increasing the release of RANK-L by osteoblasts, and it diminishes the proliferation of osteoblasts at late differentiation stages and subsequently led to osteoporosis. [15].

Interestingly, the current study reported that salivary IL6 was an excellent predictor for osteoporosis in postmenopausal woman with strong effect size (ROC area=0.91, <0.001). This is clinically relevant because it may help us to differentiate healthy women from post-menopausal women with osteoporosis and suggest early diagnosis and appropriate targeted treatment.

In addition, at optimum cut off value≥ 19.45 pg/ml, we got the highest accuracy (81.3%), with sensitivity 84%, specificity 80%,. And a positive result of salivary IL6 test established a diagnosis of postmenopausal women with osteoporosis with 80.8 % confidence in a clinical setting in which the odds of having the osteoporosis were equal between healthy and postmenopausal osteoporotic women, while in a clinical setting in which osteoporosis was highly suspected (90% pretest probability, based on clinical or other criteria apart from the test under question, The confidence was 97.4 %. A negative salivary IL6 result excluded a possible diagnosis of osteoporosis in postmenopausal women with 97.8 % confidence in a clinical setting in which the differential diagnosis of osteoporosis was considered to be of very low probability (10% pretest probability).This may suggest a valid easy test with high accuracy and confidence for early assessment and screening of post-menopausal women for osteoporosis that subsequently rapid diagnosis and treatment to prevent complications of osteoporotic fractures which has high morbidity and mortality.

Several studies have reported that proinflammatory cytokine IL6 have been implicated in the regulation of bone cells and play a critical role in bone remodeling. It acts both directly and indirectly to increase bone resorption, and/or inhibit bone formation. Al-Daghri et al [16] proved that the IL6 accelerate the bone loss in postmenopausal women. Zupan et al [17] demonstrated a higher expression of interleukin (IL)-6 in postmenopausal woman with osteoporosis. Desai et al [18] reported that IL6 correlates negatively with estrogens and BMD. Other studies demonstrated that IL6 negatively correlated with Bone mineral density and increased circulating levels of IL6 with high bone turnover and resorption in postmenopausal women [19, 20]

The limitation of this study was low number of patients that can be solved by a larger and longer prospective study. However the strength of the current study included strong inclusion and exclusion criteria in addition to being the first cross sectional study in Iraq that assessed salivary IL6 in postmenopausal women with osteoporosis.

5. Conclusion

Salivary IL6 was a simple, easy, and a valid biomarker to diagnose osteoporosis in postmenopausal women with high accuracy, sensitivity and specificity. This may indicate a hopeful measure for early diagnosis and targeted treatment of postmenopausal women with osteoporosis.

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