Original Article

Biochemical Bone Markers in Greek Postmenopausal Women

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Introduction: Osteoporosis is a public health problem affecting more than 200 million people worldwide, especially women. The aims of this study were: 1) to measure the serum levels of three bone-specific proteins: osteocalcin (OC), osteopontin (OPN) and osteoprotegerin (OPG) in Greek postmenopausal women and 2) to evaluate correlations between these proteins with age, total serum alkaline phosphatase levels (ALP), serum calcium levels (Ca) and serum C-reactive protein levels (CRP).

Materials and Methods: During the study there were recruited 100 postmenopausal women, aged between 45 and 67 years. Samples were analyzed for ALP, Ca and CRP levels respectively on the Olympus AU640 fully automated analyzer and for OC, OPN and OPG with Luminex technology.

Results: Eleven patients had high ALP levels, four patients low Ca levels, four high Ca levels and forty seven high CRP levels. Eighty three samples presented OC levels within normal range, four presented lower levels and eleven increased OC levels. Increased OPN or OPG levels were not observed.

Conclusions: The lack of estrogen in postmenopausal women prevents the absorption and utilization of calcium and this constitutes a very important factor in the development of osteoporosis. The findings showed a low positive correlation of OPN with ALP, OPG with age, OPG with ALP and OPG with CRP. Correlations between any of the three bone-specific proteins and calcium levels were not observed. These preliminary results underline the requirement for large-scale population studies in Greek postmenopausal women in order to compare results of this population group with international reference ranges.

Key words: osteocalcin, osteopontin, osteoprotegerin, post menopause, osteoporosis

Declaration of Interest: All authors declare that they have no competing interests.

Introduction

Osteoporosis is a public health problem currently affecting more than 200 million people worldwide [1,2]. It is characterized by deterioration of the bone microstructure, reduction of bone mineral density and susceptibility to bone fractures. Lifetime risk for osteoporotic fractures is high, it ranges between 40% and 50% for women and between 13% and 22% for men [1,2]. The public health impact of osteoporotic fractures includes increased disability, diminished quality of life and mortality, which lead to increased physical, social and financial consequences [1,3]. In Greece in 2010 there were approximately 86,000 new fragility fractures, while

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640,000 people aged 50 years and older were affected. The economic burden of new and prior fractures is 680 million € each year, while a 20% increase is expected by 2025, due to the increased average life span [4]. Clinically, osteoporosis most often results from a combination of postmenopausal estrogen deficiency and age-related bone loss [1]. Other risk factors for osteoporosis are smoking, low physical activity, alcohol consumption, genetic background, hyperparathyroidism, rheumatic diseases, diabetes mellitus and the chronic use of some medications [5,6]. According to many studies [7-14], 20%–30% of postmenopausal women and over 50% of men have a second cause of osteoporosis [15]. The aims of the present study were 1) to measure the serum levels of three bone-specific proteins: osteocalcin (OC), osteopontin (OPN) and osteoprotegerin (OPG) in postmenopausal Greek women and 2) to evaluate correlations between these proteins with age, total serum alkaline phosphatase levels (ALP), serum calcium levels (Ca) and serum C-reactive protein levels (CRP).

Osteocalcin (OC) is synthesized by mature osteoblasts in the bone and has a molecular weight of 5.8 kD. It constitutes approximately 15% of non-collagenous bone matrix proteins and its serum concentrations increase with age. Serum OC levels constitute a sensitive marker of bone production and are associated with a high bone turnover rate [16,17].

Osteopontin (OPN) is a phosphorylated glycoprotein with diverse biological activities mediated by multiple cell-surface receptors. Interest in OPN has been focused on its roles in immunomodulation, inflammatory response, tissue mineralization and tissue remodeling [18-23]. In the bone, OPN is produced by osteoblasts, osteocytes, macrophages, and osteoclasts and it is a major non-collagenous protein [24-26] .

Osteoprotegerin (OPG) is a secreted soluble member of the tumor necrosis factor receptor family. It is expressed in osteoblasts as well as in many other tissues including heart, kidney, liver, spleen, and bone marrow. It exhibits a protective role to the bone and regulates bone mass by inhibiting osteoclast differentiation and activation [17,27]. Its deficiency eventually leads to decrease in total bone density and a high incidence of fractures [28,29]. Serum OPG levels increase with age and are higher in postmenopausal women who have an increased rate of bone loss, thus supporting the hypothesis of a counter-regulatory function of OPG in order to prevent further bone loss [30]. Correlation of OPG with age has also been reported by other authors [31-33]. Both OPG and OC are produced by the osteoblasts but are regulated by different mechanisms and enter different metabolic pathways in the body.

C-reactive protein (CRP) is a pentameric protein found in blood plasma, the levels of which rise in response to inflammation. CRP is an acute phase reactant that increases in response to tissue damage, inflammation and infection. This so-called acute phase response occurs as a result of a rise in the concentration of IL-6, which is produced by macrophages as well as adipocytes in response to a wide range of acute and chronic inflammatory conditions such as bacterial, viral, or fungal infections; rheumatic and other inflammatory diseases; malignancy; tissue injury and necrosis. These conditions cause release of interleukin-6 and other cytokines that trigger the synthesis of CRP and fibrinogen by the liver [34,35]. It is a known risk factor for heart disease because heart disease is largely an inflammatory disorder. Osteoporosis is also inflammatory in nature and this test is helpful in detecting unwanted inflammation, which may contribute to bone health problems [36]. However, CRP appears to act as a surrogate for other factors directly associated with osteoporosis. Further studies are needed to validate these findings [28] . High serum CRP levels have been associated with lower BMD in women but it is still argued whether this association is representative of the general population [37].

Materials and Methods

For the purposes of this study 100 postmenopausal women, aged between 45 and 67years (mean age 55.8 years), were recruited. A control group was not included in this study and the reference values for each bone biomarker were considered as normal values. Patients were recruited from the Internal Medicine Department (Naval and Veterans Hospital, Athens, Greece), during a two year period of time. The Biochemical markers (serum ALP, Ca and CRP levels) were measured in the Department of Biochemistry of the same hospital and serum levels of bone specific proteins (OC, OPN, and OPG) where measured at the Department of Cytopathology ("ATTIKON" University Hospital, Athens, Greece), experienced in Luminex technology. The study was approved by the local Medical Ethics Committee and was conformant to the ethical guidelines of the Declaration of Helsinki.

The post menopausal status was self reported. Twenty of the postmenopausal cases were within the first 5 years after the onset of menopause and the remaining cases were more than 5 years after the onset of menopause. Participants with medical history of meta-

bolic diseases, autoimmune diseases and nutritional disorders that could affect bone metabolism were excluded. All blood samples were obtained between 8 and 9 a.m because of the circardian rhythm that present the different bone biomarkers, according to the Evidence-Based Guidelines for the use of biochemical markers of bone turnover [38]. Venous blood samples were collected by venipuncture from all the subjects after 12 hours overnight fasting and were centrifuged at 2500 rpm for 10 minutes. These were analyzed on the day of collection for serum ALP, Ca and CRP levels on the Olympus AU640 fully automated analyzer, using the commercial kits P.N. 1418-0130, 1418-0200 and 1418-0520 respectively, by Medicon Hellas SA. Subsequently serum samples were stored at -20°C, and then levels of OC, OPN, and OPG were analyzed on the same day that the samples were thawed at room temperature. Levels of OC, OPN and OPG, were measured using the Human Bone Panel-1 kit (cat3 HBN1A-41k, Millipore Corporation, Billerica) exploiting Luminex technology.

The detection was made according to the manufacturer's instructions. Briefly, 200μl Assay Buffer was added on the filter plate and the latter was put on a plate shaker for 10 minutes at room temperature. After vacuum aspiration, 25μl of assay buffer, standards, controls, serum matrix and samples were added into the appropriate wells. 25μl of mixed beads were added and put to overnight (16-20 hours) incubation on a plate shaker at 4°C. Samples were washed three times with 200 μl wash buffer and then 50 μl of detection antibodies solution were added, followed by one hour incubation. In the next step 50 μl of streptavidin-phycoerythrin were added and incubated for 30 minutes. Samples were washed three times and 100μl of sheath fluid was added into each well. The plate was analyzed on the Luminex 200 platform. The statistical processing of our data was based on descriptive statistics, Pearson's correlation coefficients and linear regression analysis, performed with the statistical package SAS 9.2 for Windows (SAS Institute Inc. Cary, NC, USA).

Results

In our study we observed that the ALP levels ranged from 37.22 to 423.40 U/L (mean 85.80 U/L; normal range 30.00-120.00 U/L), Ca levels ranged from 7.60 to 11.42 mg/dl (mean 9.70 mg/dl; normal range 8.40-10.60 mg/dl) and CRP levels ranged from 0.15 to 241.20 mg/l (mean 12.65 mg/l; normal range 0.01-7.00 mg/l) (see Table 1 for the summarized statistics of the measured quantities) . In 11 patients the ALP levels were high. In 4 patients the Ca levels were low, while in 4 patients the Ca levels were high. Respectively, in 47 patients the CRP levels were high (Figure 1).

Table 1 Descriptive statistics (mean, minimum and maximum values) and ranges of normal values, for the measured quantities of the 100 patients involved in the study

	Mean	Minimum	Maximum	Normal range
OPG	6432.23	2857.13	11171.98	14000.00-64000.00 pg/ml
OC	7334.10	313.74	87708.25	1500.00-11000.00 pg/ml
OPN	10670.97	1547.75	63676.24	492.00-16560.00 pg/ml
ALP	85.80	37.22	423.40	30.00-120.00 U/L
Ca	9.70	7.60	11.42	8.40-10.60 mg/dl
CRP	12.65	0.15	241.20	0.01-7.00 mg/l

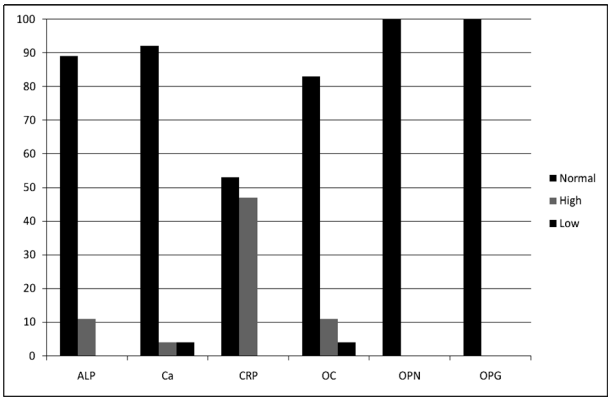

Fig.1 Summarized presentation of the results for the studied population. The bars for each parameter indicate the percentage of patients that their relevant measurements were in range of normal limits, higher or lower.

OC levels ranged from 313.74 to 87708.25 pg/ml (mean 7334.10 pg/ml; normal range1500.00-11000.00 pg/ml). In 83 patients OC levels presented within normal range, 4 patients presented lower levels and 11 patients increased OC levels (see Figure 1), note that for two patients the results were lower than the limit of detection of the technique, thus they are not included.

OPN levels ranged from 1547.75 to 63676.24 pg/ml, (mean 10670.97 pg/ml; normal range 14000.00-64000.00 pg/ml). Increased OPN levels were not observed in any patient (Figure 1).

OPG levels ranged from 2857.14 to 11171.99 pg/ml (mean 6432.23 pg/ml; normal range 492.00-16560.00 pg/ml). Increased OPG levels were not observed in any patient.

The results of the Pearson's correlation test showed a low positive correlation between OPN and ALP levels (r=0.215), OPG and patient age (r=0.243), OPG and ALP levels (r=0.281) and between OPG and CRP (r=0.220). Figure 2 presents the scatter plots of these four correlations respectively; in each image the measurement pairs for each patient are presented in the x and y axes along with the 95% confident limits of fitted ellipse. High correlations are indicated by elongated ellipses (such as the correlations of OPG with ALP and OPG with CRP) in contrast to lower correlations (such as the correlations of OPG with age and OPN with ALP). These correlations indicate that might be a linear relation between variable pairs, thus measurement of one variable may lead to calculation of the other. Additionally other conclusions may be extracted: patients with higher age tend to have higher levels of OPG, similarly low levels of OPG are related to lower levels of ALP, CRP and OPN. Correlations between any of the three bone-specific proteins and calcium levels were not observed, specifically the Pearson's correlation coefficient between serum Ca levels and OC, OPN and OPG was: 0.1272, -0.1486 and -0.1338 respectively ($p>0.05$ in all cases).

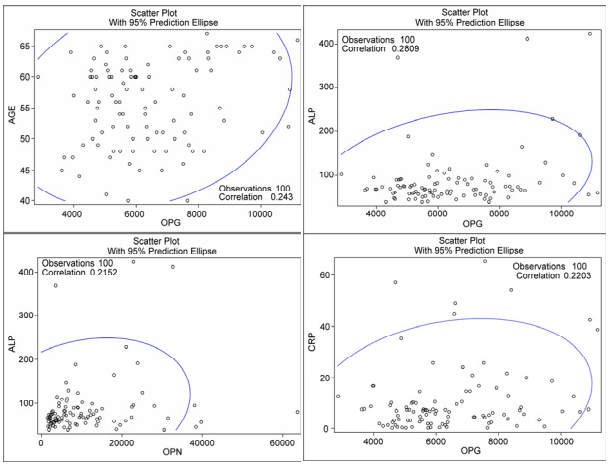

Fig.2 Scatter plots representative of the correlations between OPG with patient age (top left), OPG with ALP (top right), OPN with ALP (bottom left) and OPG with CRP (bottom right). In all cases there is observed low positive correlation (Pearson correlation coefficient r is in a range between 20% and 28%) as indicated by the non-elongated fitted ellipses.

Discussion

Osteoporosis constitutes a major health problem worldwide and is characterized by low bone mass and deterioration of bone structure. It is a health problem arising in women entering menopausal age and seriously affects their quality of life. Worldwide, the lifetime risk for women to have an osteoporotic fracture is very high [1,4,15]. Epidemiologic studies suggest that menopause and ageing are associated with accelerated loss of cortical bone. The lack of estrogen in postmenopausal women prevents the absorption and utilization of calcium and this constitutes a very important factor in the development of osteoporosis. Moreover, women are much more likely to develop osteoporosis than men, who do not experience a menopause equivalent condition [39].

The goal of our study was the measurement of serum levels for three bone-specific proteins, osteocalcin (OC), osteopontin (OPN) and osteoprotegerin (OPG) in a sample of 100 postmenopausal Greek women and to examine the correlations between any of these proteins with age, ALP, Ca and CRP.

The OC levels showed low positive correlation with patient age (r=0.229) and OPG (r=0.202). A positive correlation of OC with age has also been reported by other authors [40,41]. Moreover, 83 samples presented OC levels within normal range, 4 samples presented lower levels and 11 samples increased OC levels. These results are discordant with other studies [19,42]. This can be attributed to methodological differences because different assays detect either the intact OC molecule, which is unstable due to cleavage between amino acids 43 and 44, or the resulting big fragment which is more stable [43,44]. Furthermore, it has been suggested that exist discrete groups among post-menopause women with similar, lower or higher OC serum concentrations as compared to age-matched controls [45].

The OPN levels showed low positive correlation only with ALP levels (r=0.215). Moreover, our data did not demonstrate increased serum OPN levels in the sample under study. Previous studies have demonstrated that postmenopausal women with vertebral osteoporotic fracture show a significant increase in OPN levels compared to postmenopausal women without vertebral osteoporotic fracture [32,33].

OPG showed a low positive correlation with patient age (r=0.243) [30-33]. We also observed a low positive correlation of OPG with OC (r=0.202), ALP (r=0.281) and CRP (r=0.221), while many studies have shown increased levels of inflammatory markers, such as CRP and IL-6, with progressive bone loss, indicating a possible inflammatory mechanism for osteoporosis

[28,29,46].

Other inflammatory markers, such as IL-6, were not studied due to the restricted budget provided by the departments involved in the study. As a final conclusion it should be mentioned the requirement for a larger scale study that will include the measurement of cytokines, such as IL-6 and TNF –α , as well as detailed demographic data, nutritional, physical exercise and life style habits of the population group under study.

References

1. Dempster DW. Osteoporosis and the burden of osteoporosis-related fractures. Am J Manag Care 2011; 17 Suppl 6S164-9.
2. Masi L. Epidemiology of osteoporosis. Clin Cases Miner Bone Metab 2008; 5(1):11-3.
3. Becker DJ, Kilgore ML, Morrisey MA. The societal burden of osteoporosis. Curr Rheumatol Rep 2010; 12(3):186-91.
4. Svedbom A, Hernlund E, Ivergard M, et al. Osteoporosis in the European Union: a compendium of country-specific reports. Arch Osteoporos 2013; 8(1-2):137.
5. Zhu K, Prince RL. Lifestyle and osteoporosis. Curr Osteoporos Rep 2015; 13(1):52-9.
6. Painter SE, Kleerekoper M, Camacho PM. Secondary osteoporosis: a review of the recent evidence. Endocr Pract 2006; 12(4):436-45.
7. Kanis JA, Johansson H, Johnell O, et al. Alcohol intake as a risk factor for fracture. Osteoporos Int 2005; 16(7):737-42.
8. Hallstrom H, Wolk A, Glynn A, et al. Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. Osteoporos Int 2006; 17(7):1055-64.
9. Barr RJ, Stewart A, Torgerson DJ, et al. Population screening for osteoporosis risk: a randomised control trial of medication use and fracture risk. Osteoporos Int 2010; 21(4):561-8.
10. Allport J. Incidence and prevalence of medication-induced osteoporosis: evidence-based review. Curr Opin Rheumatol 2008; 20(4):435-41.
11. Nikander R, Sievanen H, Heinonen A, et al. Targeted exercise against osteoporosis: A systematic review and meta-analysis for optimising bone strength throughout life. BMC Med 2010; 847.
12. Leslie WD, Derksen S, Prior HJ, et al. The interaction of ethnicity and chronic disease as risk factors for osteoporotic fractures: a comparison in Canadian Aboriginals and non-Aboriginals. Osteoporos Int 2006; 17(9):1358-68.
13. Maurel DB, Boisseau N, Benhamou CL, et al. Alcohol and bone: review of dose effects and mechanisms. Osteoporos Int 2012; 23(1):1-16.
14. Andersen S, Laurberg P, Hvingel B, et al. Vitamin D status in Greenland is influenced by diet and ethnicity: a population-based survey in an Arctic society in transition. Br J Nutr 2013; 109(5):928-35.
15. Jagtap VR, Ganu JV, Nagane NS. BMD and Serum Intact Osteocalcin in Postmenopausal Osteoporosis Women. Indian J Clin Biochem 2011; 26(1):70-3.
16. Lee AJ, Hodges S, Eastell R. Measurement of osteocalcin. Ann Clin Biochem 2000; 37 (Pt 4)432-46.
17. Tanabe N, Wheal BD, Kwon J, et al. Osteopontin signals through calcium and nuclear factor of activated T cells (NFAT) in osteoclasts: a novel RGD-dependent pathway promoting cell survival. J Biol Chem 2011; 286(46):39871-81.
18. Diao H, Kon S, Iwabuchi K, et al. Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. Immunity 2004; 21(4):539-50.
19. Wang KX, Denhardt DT. Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev 2008; 19(5-6):333-45.
20. Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. Nat Rev Immunol 2009; 9(2):137-41.
21. Shinohara ML, Lu L, Bu J, et al. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. Nat Immunol 2006; 7(5):498-506.
22. Shinohara ML, Kim JH, Garcia VA, et al. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. Immunity 2008; 29(1):68-78.
23. Shinohara ML, Kim HJ, Kim JH, et al. Alternative translation of osteopontin generates intracellular and secreted isoforms that mediate distinct biological activities in dendritic cells. Proc Natl Acad Sci U S A 2008; 105(20):7235-9.
24. Mizuno A, Kanno T, Hoshi M, et al. Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. J Bone Miner Metab 2002; 20(6):337-44.
25. Nakamura M, Udagawa N, Matsuura S, et al. Osteoprotegerin regulates bone formation through a coupling mechanism with bone resorption. Endocrinology 2003; 144(12):5441-9.
26. Cretu A, Grigorie D, Neacsu E, et al. Implications of Osteoprotegerin in Postmenopausal Osteoporosis. Timisoara Medical Journal 2003; 53(3-4):226-229
27. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. Osteoporos Int 2009; 20(6):843-51.
28. Ganesan K, Teklehaimanot S, Tran TH, et al. Relationship of C-reactive protein and bone mineral density in community-dwelling elderly females. J Natl Med Assoc 2005; 97(3):329-33.
29. Ferrari SL, Ahn-Luong L, Garnero P, et al. Two promoter

polymorphisms regulating interleukin-6 gene expression are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women. J Clin Endocrinol Metab 2003; 88(1):255-9.
30. Ulivieri FM, Piodi LP, Marchelli D, et al. Osteoprotegerin: a valid new marker of bone turnover in post-menopausal osteoporosis? Journal of Orthopaedics and Traumatology 2005; 6(2):88-90.
31. Fahrleitner-Pammer A, Dobnig H, Piswanger-Soelkner C, et al. Osteoprotegerin serum levels in women: correlation with age, bone mass, bone turnover and fracture status. Wien Klin Wochenschr 2003; 115(9):291-7.
32. Kudlacek S, Schneider B, Woloszczuk W, et al. Serum levels of osteoprotegerin increase with age in a healthy adult population. Bone 2003; 32(6):681-6.
33. Hajialilo M, Rameshknia V, Ghorbanihaghjo A, et al. Dickkopf-1 levels and serum Osteoprotegerin in women with Osteoporosis. J Analyt Res Clin Med 2013; 1(2):68-74.
34. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. J Clin Invest 2003; 111(12):1805-12.
35. Lau DC, Dhillon B, Yan H, et al. Adipokines: molecular links between obesity and atheroslcerosis. Am J Physiol Heart Circ Physiol 2005; 288(5):14.
36. Ishii S, Cauley JA, Greendale GA, et al. C-reactive protein, bone strength, and nine-year fracture risk: data from the Study of Women's Health Across the Nation (SWAN). J Bone Miner Res 2013; 28(7):1688-98.
37. De Pablo P, Cooper MS, Buckley CD. Association between bone mineral density and C-reactive protein in a large population-based sample. Arthritis Rheum 2012; 64(8):2624-31.
38. Pietschmann P, *Principles of osteoimmunology : molecular mechanisms and clinical applications*.
39. Turki KM, Al-Osami MH, Mohammed NS. Evaluation of Serum Osteocalcin and Osteopontin Levels as Bone Biochemical Markers in Postmenopausal Women. Al-Kindy College Medical Journal 2013; 9(2):40-44.
40. Kalaiselvi Vs, Prabhu K, Mani Ramesh, et al. The association of serum osteocalcin with the bone mineral density in post menopausal women. J Clin Diagn Res 2013; 7(5):814-6.
41. Atalay S, Elci A, Kayadibi H, et al. Diagnostic utility of osteocalcin, undercarboxylated osteocalcin, and alkaline phosphatase for osteoporosis in premenopausal and postmenopausal women. Ann Lab Med 2012; 32(1):23-30.
42. Sodek J, Batista Da Silva AP, Zohar R. Osteopontin and mucosal protection. J Dent Res 2006; 85(5):404-15.
43. Chang IC, Chiang TI, Yeh KT, et al. Increased serum osteopontin is a risk factor for osteoporosis in menopausal women. Osteoporos Int 2010; 21(8):1401-9.
44. Gürsoy G, Acar Y, Alagöz S. Osteopontin:A multifunctional molecule. Journal of Medicine and Medical Sciences 2010; 1(3):055-060.
45. Fodor D, Bondor C, Albu A, et al. The value of osteopontin in the assessment of bone mineral density status in postmenopausal women. J Investig Med 2013; 61(1):15-21.
46. Naylor KE, Rogers A, Fraser RB, et al. Serum osteoprotegerin as a determinant of bone metabolism in a longitudinal study of human pregnancy and lactation. J Clin Endocrinol Metab 2003; 88(11):5361-5.