An Evaluation of Vitamin D and Bone Turnover Markers Levels in Postmenopausal Women in Albania

Vitamin D is a lipophilic prohormone that is synthesized in the skin in response to sunlight, although diet may be a source of much lower amounts of Vitamin D. Receptors of the active form of Vitamin D (VDR), have been identified in the cells of the intestinal epithelium, renal tubules, bone and other tissues and organs, which indicates a broad spectrum of 25(OH)D_3.[1,2] Besides its role in intestinal calcium absorption, calcitriol may also affect bone health directly, as its receptors are expressed by osteoblasts.[3] The consequences of vitamin D deficiency are secondary hyperparathyroidism and bone loss, leading to osteoporosis and fractures, mineralization defects, which may lead to osteomalacia in the long term, and muscle weakness, causing falls and fractures.[4] Therefore, we aimed to investigate the association between serum levels of 25(OH)D and bone turnover markers in postmenopausal women, and their impact on osteoporosis. In this two – years study (2020-2022), we described the epidemiology of vitamin D status across women population in Albania and its potential associations with bone biomarkers (OC, PTH, ALP). Our study showed a clear seasonal variation of bone turnover markers and a negative Pearson correlation between serum 25(OH)D and osteocalcin (OC) ($r= -0.37$, $p<0.05$). Osteoporosis leads to decreased hydroxyapatite crystal formation and hence results in increase in serum osteocalcin levels. [4,5] We found reduced 25(OH)D concentrations in postmenopausal women (21.71 ng/mL) and showed that a deficient 25(OH)D concentration is associated with significantly increased markers of bone resorption and decreased bone mineral density (BMD) values.

Keywords: *25-Hydroxyvitamin D, osteocalcin, bone biomarkers, bone density.*

Introduction

Disorders of bone metabolism, most notably osteoporosis, are highly prevalent and predispose to fractures, causing high patient morbidity and mortality. Osteoporosis is a skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue, which leads to increased bone fragility and susceptibility to fractures. In adults the most common types are age-related (senile) osteoporosis and postmenopausal osteoporosis.[5]

The burden of osteoporosis increases with advancing age, and it is estimated that worldwide one in three women and one in five men above the age of 50 years will experience an osteoporotic fracture (International Osteoporosis Foundation, 2017). Also, the pain, the deformation, the disability, and the loss of proper function of the skeleton, as a consequence of osteoporosis, significantly affect the psychological cost of the patients. Diagnosis, treatment and monitoring of treatment for osteoporosis are of critical importance. Osteoporosis may be defined quantitatively using diagnostic thresholds based on measurements performed at the spine, hip and forearm using photon absorptiometry dual-energy X-ray (DXA). Bone Mineral Density (BMD) is considered the gold standard of the bone status

assessment in osteoporosis; however it does not offer the timely response desirable for monitoring. As osteoporosis emerges directly from alternations in the number or activities of osteoblasts and osteoclasts, it follows those biomarkers of the activity of these cells reflects current levels of bone turnover.[3,5]

Literature Review

Bone biomarkers are produced from the bone remodeling process included bone formation biomarkers, bone resorption biomarkers and regulators of bone turnover. Bone Turnover Markers (BTMs) can be categorized first as reflection either bone resorption or formation, and then further categorized into matrix products that are liberated during bone resorption or formation. In an individual bone remodeling unite, specific cells as osteoblasts, osteoclasts and osteocytes work together in a well synchronized cycle way. First the osteoclasts due to their morphology and their metabolic products, they cauterize an indention called lacuna. After this lacuna has reached a certain size (*approx. 200 µm in diameter and 50 µm in depth*) the osteoclasts stop their activity and another family cells, the osteoblasts, settles down on the bottom of the indention. They fill the lacuna with osteoid, which is a mixture of proteins, mainly non-carboxylated osteocalcin and precursors of collagen 1. After carboxylation of the osteocalcin and formation of collagen 1, calcification of the osteoid takes place with the contribution of Vitamin D and eventually the bone returns to its initial situation.[13]

There are two major forms of vitamin D, vitamin D2 (*ergocalciferol*) and vitamin D3 (*cholecalciferol*). Vitamin D2 is found in plants and can be consumed in fortified food products or as a supplement. Vitamin D3 is obtained from either dietary sources or through the conversion of 7- dehydrocholesterol in the skin upon exposure to ultraviolet B (UVB) radiation. Vitamin D3 from the skin is bound to the vitamin D-binding protein, whereas vitamin D2 and vitamin D3 from diet are bound to vitamin D-binding protein and lipoproteins. Both forms are hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D; D represents D2 or D3]. However, 25(OH)D is inactive and requires hydroxylation in the kidney to form 1,25-dihydroxyvitaminD/ [1,25(OH)2 D, calcitriol]. Calcitriol [1,25(OH)2 D] maintains calcium in the blood and has an array of effects on the body's organs. Calcitriol acts in an endocrine manner to regulate calcium metabolism by enhancing intestinal calcium absorption and mobilizing calcium from the skeleton.[8, 9, 10] Receptors of the active form of Vitamin D (VDR), have been identified in the cells of the intestinal epithelium, renal tubules, bone and other tissues and organs, which indicates a broad spectrum of $25(OH)D_3$. Besides its role in intestinal calcium absorption, calcitriol may also affect bone health directly, as its receptors are expressed by osteoblasts.[1] On the other hand, there are reports that $1\alpha,25(OH)_2D_3$ produces rapid biological responses that involve opening of chloride and calcium channels to activate exocytosis of bone matrix proteins such as osteocalcin.[6, 7] Osteocalcin is an osteoblast-specific secreted non-collagenous, vitamin K-dependent large protein synthesized by osteoblasts, odontoblasts, and some chondrocytes, and secreted into the general circulation. It binds to

hydroxyapatite, and it is deposited in the bone matrix. As osteocalcin fragments are released from the bone matrix during resorption, assays for circulation osteocalcin and its fragments reflect both bone formation and resorption.[11,12]

Materials and Methods

In each patient fasting venous blood samples were collected in the morning. The following serum indicators of bone and mineral metabolism were measured: two bone formation markers (osteocalcin and bone alkalin phosphatase), one bone resorption marker beta-collagen 1 C-terminal cross linked telopeptides (β-CTx serum), parathyroid hormone (iPTH), 25 hydroxyvitamin D [25(OH)D], calcium (Ca), phosphorus (P), alkalin phosphatase (ALP), bone specific alkaline phosphatase (BAP). The serum concentrations of OC, 25OHD, PTH, calcium, phosphor and alkaline phosphatase (ALP) were measured using an electro-chemiluminescent immunoassay (Cobas 6000 analyzer, Roche Diagnostics).

BMD was measured by dual-energy x-ray absorptiometry (DXA) scan (StreeMcare) at the lumbar spine – LS (L1-L4). According to WHO criteria, osteoporosis is defined as the T-score of less or equal to 2.5 and osteopenia as the T-score between 1.0 and 2.5. Vitamin D status was defined as deficient for circulating 25(OH)D concentration <20 ng/mL, as insufficient for 20-30 ng/mL and as optimal >30 ng/mL.

This study was performed from January 2020 to January 2022. We used data from private laboratory INTERMEDICA Center and radiology clinic MedXray, in Albania. Serum bone biomarkers levels were assessed in 50 females. The female patients were divided into two groups: the pre-menopausal (n=24) and the post-menopausal (n=26). Women were also grouped according to the seasons of 25(OH)D measurements: Spring, Summer, Autumn and Winter.

Statistical Analyses

Most of statistical analyses were performed using Excel 2013 and SPSS 20 statistical package. Multiple comparison test and Post hoc tests was used to identify which categories were significantly different from each other at a level of 5%. The correlation was analyzed by the Pearson linear regression test. Values of $p < 0.05$ were taken as statistically significant.

Results

The whole study group consisted of 50 adult women (mean age 48.1 ±18.5SD years). Twenty-four of them were premenopausal women and twenty-six were postmenopausal women. The mean serum 25(OH)D concentrations (21.4 ±12.0 ng/mL) of the whole participants was above the vitamin D insufficiency borderline (20 ng/mL). However, the majority of the patients 44% (n= 22) showed deficiency of vitamin D, followed by insufficiency detected in 34 % (n=17) and normal

vitamin D levels in 22 % (n=11). Mean β-CTx (0.283 ±0.15 ng/ml) and osteocalcin (22.9 ±18.5 ng/ml) levels were within the normal reference range.

Increased bone loss was also observed by this study. The average value of bone measurement density (BMD) of the participants was T-score = -1.81 g/cm^3, which indicates low bone mass (osteopenia) of women. The T-score of the lumbar spine in postmenopausal women ≥ 48 years in Albania is at the level of osteopenia (-1.96 g/cm^3). Subjects were grouped into normal (27 %), osteopenic (39.5 %), and osteoporotic (33.5 %) based on the t-scores.

In the investigated period, there were statistically significant differences in the mean T-score results of bone density in all seasons. Also, Albanian women had a clear seasonal dependency within the measurements of 25OHD, with significant differences between winter and autumn groups (p= 0.027). There were no significant seasonal differences in the values of OC, β –CTx, BAP, Ca and phosphor during this two years period study.

Figure 1. *Prevalence of Vitamin D deficiency status among female patients*

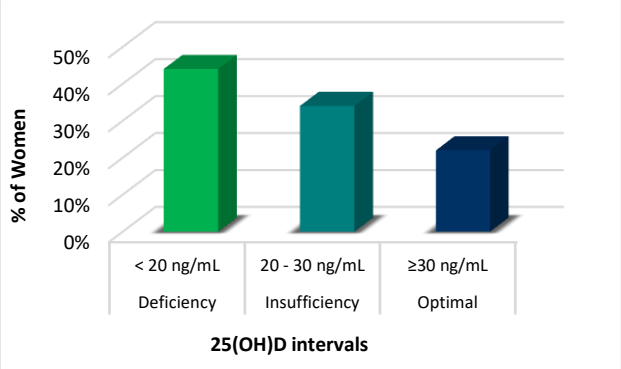


Figure 2. *Age relation of Vitamin D and Osteocalcin*

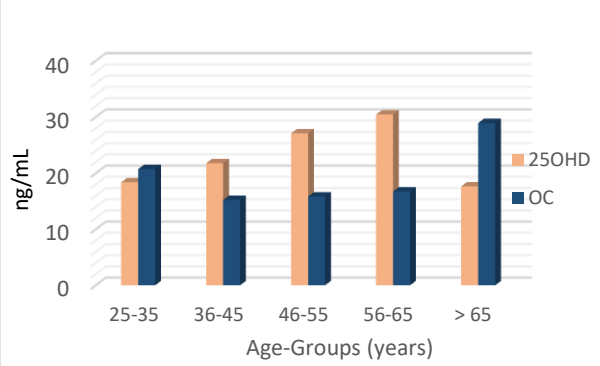


Figure 2 shows the mean value Vitamin D and Osteocalcin in different age groups, in the age group of 25 – 35 years 25OHD was 20.4 ng/mL and OC 20.75 ng/mL. In the age group of 46 – 55 years the mean vale 25OHD was 28.1 and OC 15.85 ng/mL. The age group 56 – 65 years was the only group that had 25OHD concentrations at the borderline of the Optimal Vitamin D level 30.46 ng/mL, but OC had the lowest concentrations 16.76 ng/mL. Women over 65 years showed the

lowest 25OHD concentrations 17.67 ng/mL and the highest OC concentrations 28.95 ng/mL.

The association between serological and BMD parameters was assessed using the Pearson's correlation coefficient. The level of 25OHD in Postmenopausal women group indicated a strong significant positive correlation with BMD T-score (r=0.586, *p=0.003*), and negative correlation with bone formation marker Osteocalcin (r= -0.37, *p=0.043*) and bone resorption marker β -CTx (r= -0.512, *p=0.036*). Furthermore, phosphor concentrations showed strong negative correlation with T-score of bone density measurement (r=-0.556, *p=0.048*) and PTH (r= -0.709, *p=0.010*), and strong positive correlation with OC (r=0.592, p=0.033). Also, there was observed a positive correlation between OC and β-CTx concentrations (r=0.591, p=0.012). On the other hand, serum Ca levels showed no significant associations with any of bone formation or bone resorption markers.

In contrast, in Premenopausal women group, there was not any significant correlation between 25OHD concentrations and T-score (p=0.537) and OC levels (p=0.367). Also, it was noticed a strong association between β -CTx and BAP concentrations (r=0.974, p=0.06), and a positive association between Vitamin D and serum β-CTx (r=0.610), but with lower significance (*p=0.081*).

Figure 3. *Scatterplot that shows exactly the association of Vitamin D with BMD and OC*

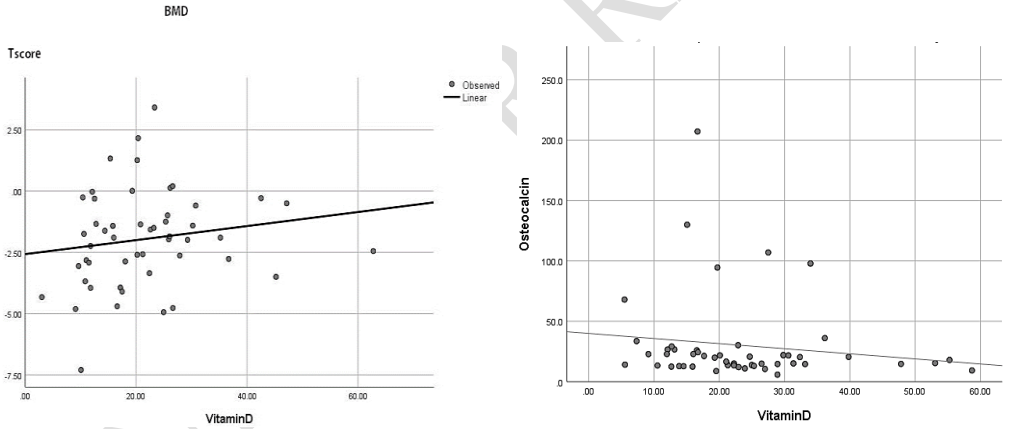
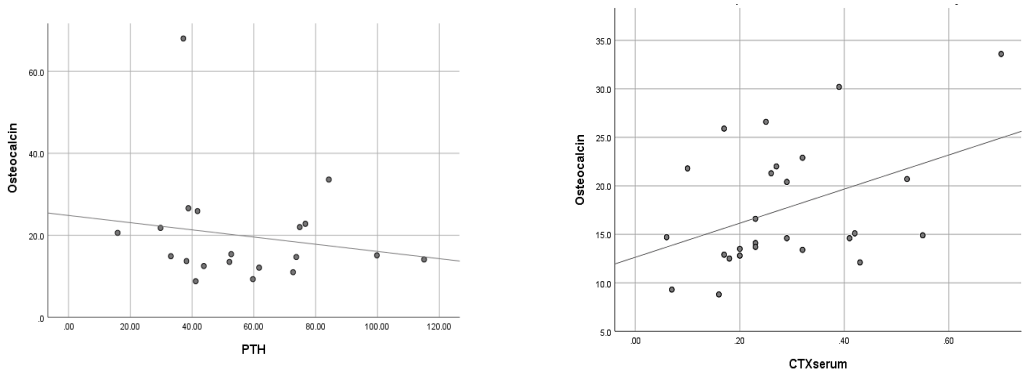


Figure 4. *Correlation between bone formation marker OC with bone resorption β-CTx and iPTH*



Using Multiple Comparisons, serum levels of Osteocalcin between normal, osteopenic, and osteoporotic groups were significantly different between normal and osteopenic groups (p= 0.017), normal and osteoporotic group (p=0.012). There were no significand differences of Osteocalcin levels between osteopenic and osteoporotic groups (p=0.728). We also found significant differences of Ca levels only among the patients with osteopenia and osteoporosis (p=0.056).

When the whole group was subdivided according to BMD parameters, there were changes in levels of BTMs. In particular, levels of 25OHD, β –CTx, ALP and phosphorus were lower in the osteoporotic group than in the normal group. In contrast, Osteocalcin levels were expressed at higher concentrations in the osteoporotic group compared with the normal group.

Figure 5. *a), b), c), d) Bone biomarkers levels for each BMD category*

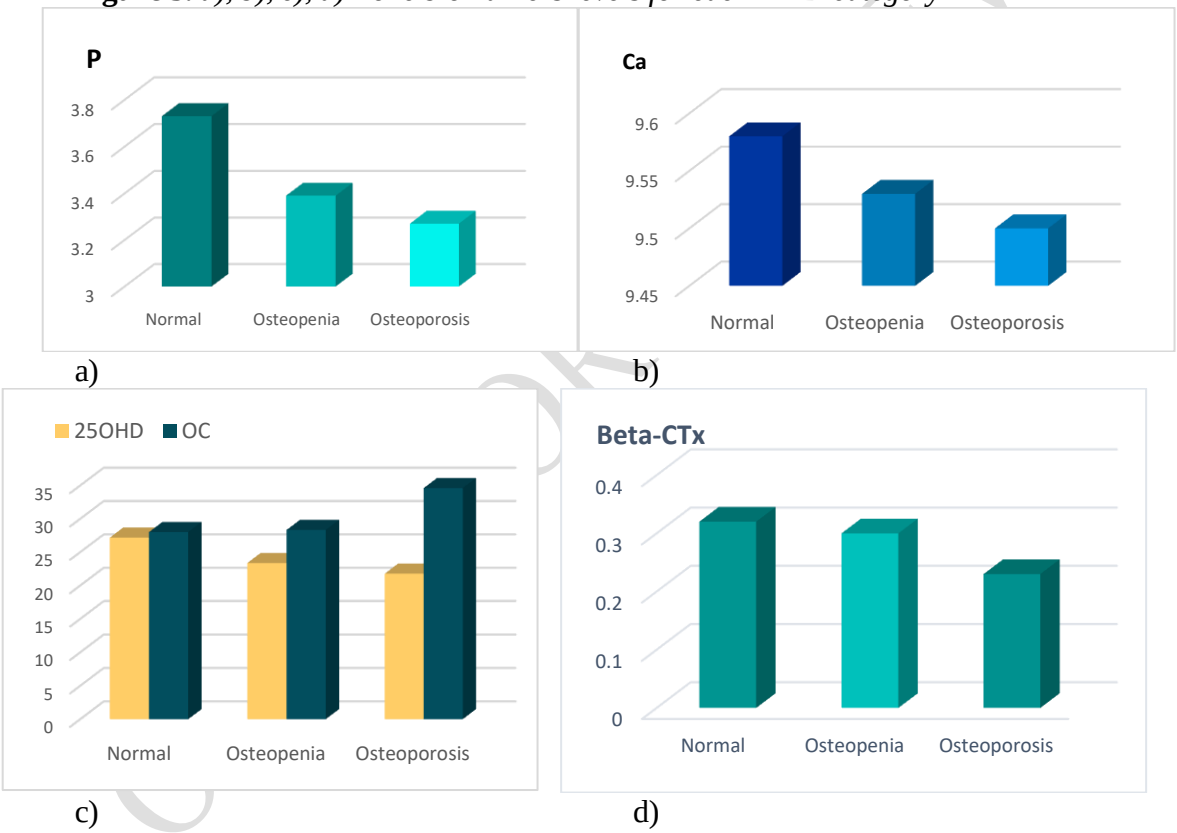


Table 1. *Comparison of BTM between BMD categories*

		Women		
BTMs	**Reference Range**	**Normal**	**Osteopenic**	**Osteoporotic**
25OHD (ng/mL)	< 20 ng/mL Deficiency 20 – 30 ng/mL Insufficiency > 30 ng/mL Normal	22.48 ±15.6	19.25 ±10.7	22.34 ±12.2
OC (ng/mL)	11 – 43 ng/mL	16.5 ±4.5	24.26 ±22.08	24.0 ±20.2
ALP (ng/mL)	35 – 105 U/L	76.6 ±20.5	62.4 ±15.2	69.2 ±14.4

BAP (%)	40 – 75 %	63.5 ±3.9	60.25 ±3.59	62.4 ±5.7
Ca (mg/dL)	8.6 – 10 mg/dL	9.59 ±0.32	9.54 ±0.43	9.53 ±0.11
Phosphor	2.6 – 4.5 mg/mL	3.82 ±0.30	3.48 ±0.52	3.09 ±0.60
β-CTx (ng/mL)	< 0.6 ng/mL < 1 ng/mL Post-Menopausal	0.32 ±0.11	0.30 ±0.10	0.23 ±0.16

We found no certain significant differences in 25OHD parameters between women menopausal groups, F= 3.17, *p=0.088*. Although there was detected a difference in osteocalcin levels, which was significantly decreased in postmenopausal women.

Table 2. *Study Population Characteristics*

		Pre-Menopausal Women			**Post-Menopausal Women**		
BTMs	**Reference Range**	**Normal**	**Osteopenic**	**Osteoporotic**	**Normal**	**Osteopenic**	**Osteoporotic**
25OHD	< 20 ng/mL Deficiency 20 – 30 ng/mL Insufficiency > 30 ng/mL Normal	22.26 ±6.9	22.7 ±9.5	18.58 ±8.9	22.67 ±18.2	15.8 ±8.4	**26.1** ±13.0
OC	11 – 43 ng/mL	17.4 ±6.3	22.63 ±20.2	**35.31** ±30.4	16.92 ±6.98	**31.2** ±25.0	**14.5** ±4.9
ALP	35 – 105 U/L	53.0 ±0.10	57.8 ±12.4	72.5 ±12.0	85.0 ±4.2	65.50 ±2.12	64.6 ±21.4
BAP (%)	40 – 75 %	62.5 ±3.53	59.5 ±2.6	**74.4** ±8.7	64.7±3.59	62.2 ±2.4	62.4 ±5.77
Ca	8.6 – 10 mg/dL	9.4 ±0.46	9.5 ±0.52	9.42 ±0.11	9.57 ±0.31	9.77 ±0.33	9.5 ±0.10
P	2.6 – 4.5 mg/mL	3.80 ±0.40	3.3 ±0.26	3.2 ±0.60	3.58 ±0.50	4.01 ±1.04	**2.9** ±0.65
PTH	15 – 65 ng/L	50.25 ±10.19	57.5 ±38.4	60.75 ±28.08	55.5 ±27.36	54.93 ±25.1	**57.17** ±25.9
β-CTx	< 0.6 ng/mL < 1 ng/mL Post-Menopausal	0.33 ±0.10	0.22 ±0.03	0.17 ±0.01	0.322 ±0.22	**0.39** ±0.01	**0.30** ±0.17

Discussion

The present study examined bone metabolic markers and DEXA parameters in Albanian females. Bone density changes in two stages after birth, first reaching the peak bone mass in the early adult and gradually progressing to the bone-loss process. T-score parameters of BMD decreased significantly as the age of the participant increases. The average value of bone measurement density (BMD) of the participants was T-score = -1.81 g/cm^3, which indicates low bone mass (osteopenia) of Albanian women.

In contrast, Vitamin D levels were not significantly associated with age group. Overall, 44 % of the participants exhibited vitamin D deficiency (25OHD <20 ng/mL). Our results showed a high prevalence of hypovitaminosis D among women in Albania. Different from our study, serum 25(OH)D levels are higher in Northern countries. The indicators of poor income (lowest levels of income, food

fortification insufficiency, use of supplements policy) are known to be associated with lower dietary intakes among Southern countries, including Albania.[23]

Our results of seasonal variation among postmenopausal women in Albania confirmed difference in 25(OH)D levels, with lowest levels measured in Summer months. This findings could be attributed to seasonal differences of dietary intake and nutritional status. Owing to its fat-soluble nature, dietary vitamin D (either D2 or D3) is absorbed with other dietary fats in the small intestine. Importantly, while a fraction of newly absorbed intestinal vitamin D is also transported along with amino acids and carbohydrates into the portal system to reach the liver directly, where the first step of activation begin.[24,25] According to data publish in literature, the intake of saturated fats, protein, carbohydrate and dietary fibers by women aged between 51and 86 years was lower during summer compered to winter. Considering the differences in intake of minerals and vitamins between seasons, there was a higher intake of calcium, magnesium, phosphorus, thiamin and pyridoxine during winter by women. [26]

The synthesis of osteocalcin or bone gla protein (BGP) by osteoblasts is markedly stimulated by 1,25-(OH)2D, a key hormone in the regulation of bone mineralization. The circulating levels of osteocalcin have been shown to reflect both the osteoid matrix production and the formation rate of mineralized bone in several metabolic bone diseases (osteoporosis, thyrotoxicosis, primary hyperparathyroidism) in which both mechanisms are tightly coupled because of the absence of mineralization defect.[14] In accordance with this, in our study the level of 25OHD in the whole group indicated a strong significant negative correlation with bone formation marker Osteocalcin (r= -0.37, *p=0.043*). In postmenopausal women, the mean value of osteocalcin is lower compared to premenopausal women (respectively 20.82 and 25.11 ng / ml), while 25OHD levels were higher in postmenopausal women.

Bone collagen degradation products have been the focus of laboratory procedures used to reflect bone degradation (Demers et al., 2000). An important type of degradation of type I collagen is beta crosslap (β-CTX). The level of this marker in our study appears higher in postmenopausal women compared to premenopausal women (0.336 and 0.24 ng / ml). Also, Postmenopausal women are characterized by higher levels of alkaline phosphatase (71.7 ng/mL) compared to women in premenopause (61.1 ng / mL). These findings are similar with another study in Albania (Hysi L. et al., 2014), in which higher values of β-CTX show a greater bone resorption in postmenopausal women.[16]

When postmenopausal women were subdivided based on BMD parameters, Osteocalcin, BAP, ALP and phosphorus levels decreased from normal to osteoporotic group, meanwhile β-CTx increased from normal into osteopenic, but decreased again into osteoporotic group. Also, concentrations of 25OHD and PTH increased from normal into osteoporotic group. In the other hand, Osteocalcin concentrations were lower and β-CTx concentrations higher in postmenopausal osteoporotic women compared with premenopausal osteoporotic. In accordance with Garniero et al. (1996), this suggests that in postmenopausal group bone resorption occurs most actively than bone formation.[17]

The findings that serum β-CTx and Osteocalcin were significant higher in the osteopenic group of postmenopausal women, consistent with Chavassieux et al. 2015 that reported a strong correlation between s-CTX level and histologic features in bone biopsy , suggested that these BTMs are good markers during the early stage of bone loss, when bone resorption begin to increase.[22]

Another observation of note in the present study was that phosphorus showed strong negative correlation with PTH (r= -0.709, p=0.010) and BMD (r= -0.556, p= 0.048), and positive association with osteocalcin (r= 0.592, p= 0.033). Phosphate plays several essential roles in our body.[18] Phosphate is necessary for proper mineralization of bone as a constituent of hydroxyapatite crystal. Serum phosphate level is regulated by several hormones including parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) and fibroblast growth factor 23 (FGF23).[19]

In our study decreased serum phosphor levels are associated with low bone density and low OC levels, and higher levels of PTH and Vitamin D. Low levels of phosphorus may stimulate the secretion of PTH, which activates reactions leading to the formation of 25OHD which may in turn increase phosphorus absorption from the intestine, decrease renal elimination and increase bone resorption, accompanied by low osteocalcin levels. These findings are consistent with those of Galan et al. (2012).[15] We did not, however observed any correlation between PTH and 25OHD levels.

Another observation of note in the present study was that mean ALP level was significantly higher in postmenopausal women with low BMD than those of normal BMD. Mean salivary ALP was numerically more in women with low BMD than those with normal BMD but statistically not significant. The finding of high ALP in low BMD group may be related to increase of bone turn over in patients with low BMD rather than bone formation alone. Also periodontal disease may significantly increase the activity of salivary ALP [Dabra et al]

Conclusion

In this 2-years follow-up study in female individuals, 25(OH)D levels were 21.1 ng/mL, below optimal reference range30 ng/mL. Our results of seasonal variation among postmenopausal women in Albania confirmed difference in 25(OH)D levels, with lowest levels measured in summer months. These findings could be attributed to seasonal differences of dietary intake and nutritional status.

Concentrations of β-CTx appears higher in postmenopausal women compared to premenopausal women (0.336 and 0.24 ng / mL), in contrast osteocalcin concentrations were lower in postmenopausal women (20.8 and 25.1 ng/mL). These findings are similar with another study in Albania (Hysi L. et al., 2014), in which higher values of β-CTX and low values of OC showed a greater bone resorption in postmenopausal women.

Here we observed significant strong correlation of 25OHD with Osteocalcin and β-CTx in postmenopausal osteoporotic women. Also, higher levels of β-CTx and OC in osteopenic group, may indicate that the loss of bone mass occurs as a

result of increased bone turnover, and lower levels of this OC and phosphorus in osteoporotic group may indicate slow bone remodeling, followed by an inability of the forming process to fill the resorbed spaces.

These data indicate that the overall rates of both bone formation and bone resorption remain high in postmenopausal women in Albania. The rate of bone turnover appears to play an increasing role as a determinant of bone mass. Thus, assessing bone marker levels may be useful in the evaluation of osteoporosis risk.

In conclusion, osteocalcin, β-CTx, ALP and phosphorus were valid biomarkers to diagnose postmenopausal women with low BMD. This may suggest a new promising measure to early diagnose patients at high risk of low BMD and subsequently giving early appropriate treatment.

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