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Osteocalcine concentration in saliva and blood serum conducted on postmenopausal patients

Badanie stężenia osteokalcyny w ślinie i krwi pacjentek w okresie pomenopauzalnym

INTRODUCTION

Bone metabolism and its dynamics depend on the stage of development of the organism. Balancing the processes of bone resorption and bone production results in an increase or a decrease of bone mass. This process is called internal reconstruction of bone tissue (remodelling). It runs in a specific way for the cortical and trabecular bone. The remodelling process is cyclical in nature and takes place in specific places of the skeleton, known as a bone remodelling units (BRU). One episode of apposition lasts from 3 to 6 months; the resorption phase ends after about three weeks. Histologically, the bone remodeling unit in compact bone is represented by osteon, and in the spongy bone by erosion lacuna (Howship's lacuna).

Biochemical markers of bone metabolism are helpful in monitoring the bone turnover. They represent fragments of the protein structural elements of bones or their degradation products. In addition, there are also enzymes and proteins released into the circulation during the metabolic activity of bone production cells (osteoblasts) and bone resorption cells (osteoclasts). Determination of the concentration or activity of the relevant indicators (markers) is therefore a measure of the rate of bone turnover. This information is useful in assessing the rate of decline of bone mineral density as well as in monitoring treatment and evaluation of fracture risk. Osteocalcine (bone GLA protein – BGP) is a non-collagen bone matrix protein formed by osteoblasts. Its carboxylated form combines with calcium, contributing to the process of bone mineralization. The osteocalcine found in blood serum is its uncarboxylated form (ucOC), derived from the newly synthesized protein that is not deposited in bone matrix. The level of osteocalcine concentration in blood indicates the limit of osteoid mineralization, activation of osteoclasts, inhibition of bone formation by osteoblasts and regulation of bone turnover. This fraction is about 15% of total BGP. Since the physical half-life of

BGP amounts to several minutes, its concentration shows considerable fluctuations over the day, sometimes reaching 10–30% in an osteoblastic circadian manner (maximum in the middle of the night, minimum in the morning).

Osteocalcine concentration analysis conducted on postmenopausal patients is justified because the extinction of ovarian function in this period leads to the development of estrogen deficiency and has a profound negative impact on bone tissue. When using osteocalcine levels diagnostically, it is important to note that excretion through the kidneys and kidney function (especially renal failure) may multiply its concentration [15]. Acceleration of bone turnover and predominance of the process of bone resorption over bone production in postmenopausal women leads to bone loss and development of systemic metabolic bone disease – post-menopausal osteoporosis and gradual weakening of mechanical strength of bone (OCG). Postmenopausal osteoporosis is now an essential clinical problem, whose etiology and pathogenesis have not been sufficiently explored.

The aim of this study was to identify and present the difference in the concentrations of osteocalcine – a biological marker of bone formation – in blood serum and saliva of patients in the menopausal period, and to reveal the correlation between the concentration of a biomarker and bone density.

MATERIAL AND METHODS

Clinical trials were conducted on a group of 30 postmenopausal women (studied group), whose last menstrual period occurred at least 12 months before the test, and who did not receive hormone replacement therapy (mean age 55.4 years). The comparative group consisted of 30 women at menopause, who underwent hormone replacement therapy (HRT) (mean age 53.0 years) All patients gave their consent to testing and testing procedures received the approval of the Local Ethics Committee in Lublin.

In order to obtain accurate information on the health status of the women, an individual survey card was populated, including the details of the interview and the examination of the oral cavity. Questions in the survey related to the age, type of a job, date of last menstrual period, height, weight, number of births, duration of the use of hormone replacement therapy, smoking, type of diet (including coffee and alcohol consumption), physical activity, medication and completed operations (gynecological). Examination of the oral cavity was undertaken to elucidate the condition of the teeth, particularly the number of teeth removed, the state of oral mucosa, periodontal status, the presence of dentures and the time of their use and any other possible oral health problems. The group of 60 women was divided into four subgroups according to established guidelines: M – a group of menopausal women, OV – a group of women after oophorectomy, OV + HRT – a group of women after surgical removal of the ovaries using HRT, M + HRT – a group of menopausal women using HRT.

After conducting an interview and a scrupulous clinical examination, samples of unstimulated saliva as well as venous blood from ulnar vein were collected from women on an empty stomach in the morning. After centrifugation, the obtained supernatant (saliva) and serum (blood) were stored at – 70 °C. Blood and saliva were subjected to laboratory examination by ELISA (an immunoenzymatic

method). Investigation of bone mineral density of the lumbar spine was performed at the Laboratory of Densitometry in The Institute of Agricultural Medicine in Lublin, by means of the DPX-A Luna equipment and absorptiometry of X-rays beams of two energies. Bone density was specified in g/cm^2.

RESULTS

The results obtained were statistically analyzed. The arithmetic mean (M) and the standard deviation (SD) were calculated. Significant differences among particular groups were determined on the basis of confidence intervals (NIR) determined from the analysis of variance (ANOVA). The results are presented in the tables (below), and the statistical means are illustrated in the figures through the 'box and whisker plot' type graph. The significance of differences was noted in the tables in the column 'p'. The same was marked at the drawings of the different graphs (means differ significantly if they are not marked with the same letter of the alphabet).

Results showing the level of osteocalcine in blood serum in the tested groups of women are shown in Table 1 and illustrated in Figure 1.

Table 1. The level of serum osteocalcine (ng/ml) in studied groups of women

Group	n	The level of serum osteocalcine (ng/ml) (M ± SD)	The relevance of differences (p)*
M	15	5.69 ± .34	b
OV	15	3.59 ± 0.85	a
M+HTZ	15	4.75 ± 0.45	b
OV+HTZ	15	5.80 ± 2.01	b

*Means differ significantly if they are not marked with the same letter of the alphabet

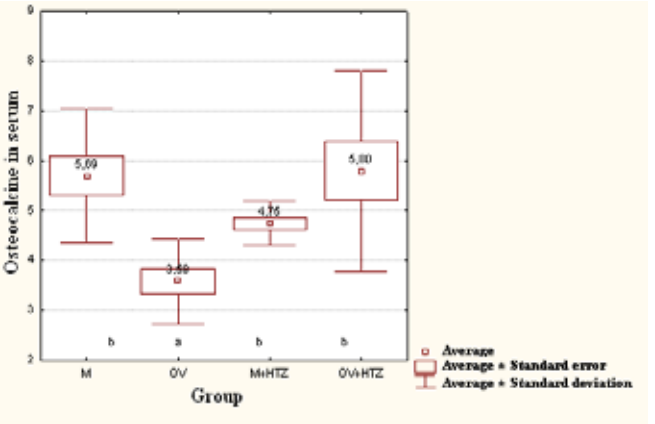

Fig 1. The level of serum osteocalcine (ng/ml) in the studied groups of women

Results showing the level of osteocalcine in the saliva of women in the studied groups are presented in Table 2 and illustrated in Figure 2.

Table 2. The level of osteocalcine in saliva (ng/ml) in studied groups of women

Group	n	The level of osteocalcine in saliva (ng/ml) (M ± SD)	The relevance of differences (p)
M	15	2.01 ± 0.11	ab
OV	15	1.96 ± 0.04	a
M+HTZ	15	2.03 ± 0.02	b
OV+HTZ	15	1.97 ± 0.06	a

*Means differ significantly if they are not marked with the same letter of the alphabet

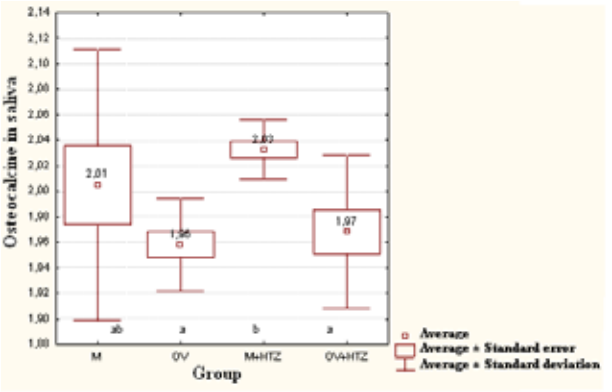

Fig. 2. The level of osteocalcine in saliva (ng /ml) in studied groups of women

Results of research on spinal bone density in studied groups are shown in Table 3 and illustrated in Figure 3.

Table 3. Spine bone density (BMD) in the studied groups of women (g/cm^2)

Group	n	Spine bone density (BMD) (g/cm^2)(M ± SD)	The relevance of differences (p*)
M	15	0.95 ± 0.04	a
OV	15	0.92 ± 0.08	a
M+HTZ	15	1.11 ± 0.02	b
OV+HTZ	15	1.09 ± 0.04	b

*Means differ significantly if they are not marked with the same letter of the alphabet.

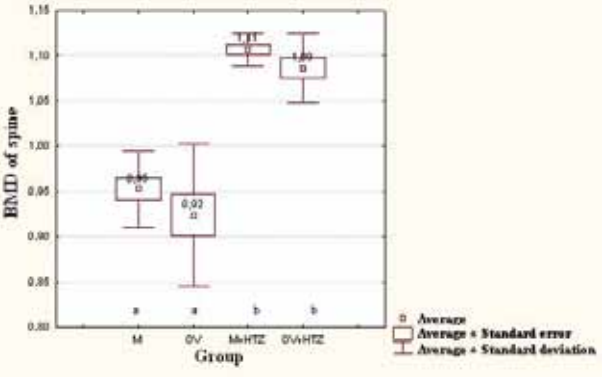

Fig. 3. Spine bone density (BMD) in the studied groups of women (g/cm^2)

OVERVIEW OF RESULTS

The content of osteocalcine in serum ranged on average from 3.59 ng/ml in women from group OV to 5.80 ng/ml in group OV + HRT. In group OV (after excision of ovaries) the recorded level of osteocalcine was the lowest. The highest level of this marker was found in group OV + HRT – patients after oophorectomy treated with hormone replacement therapy. Statistically significant changes in the level of osteocalcine occurred in group OV, because a significant decrease in the level of osteocalcine was reported in comparison with other treatment groups.

Saliva tests showed statistically significant differences in marker concentrations between the groups at menopause (M), and at the menopause, receiving hormone replacement therapy (M + HRT). Patients after oophorectomy had the lowest level of osteocalcine.

Bone density (BMD) of the spine in women from group M and OV was lower than BMD in the groups that used HRT. Among women from group M, spine bone density was on average 0.95 g/cm^2, whilst in group M + HRT equalled 1.11 g/cm^2 and the difference between the groups was statistically significant. The difference between spine BMD in groups OV and OV + HRT was also statistically significant.

DISCUSSION

The study, examining the changes in the level of osteocalcine in postmenopausal women with osteoporosis and after oophorectomy, highlights the relationship between the concentration of biomarker and the level of bone density. These results can be correlated with the conclusions obtained by other studies on osteocalcine levels in serum and saliva. Most of them support the assertion that there is an inversely proportional correlation between the biomarker concentration and the level of sex hormones, as well as the age of patients. With the loss of ovarian function at menopause many changes in the body emerge. These changes are caused by the drop in estrogen levels. One of these changes is the disorder of bone metabolism with the predominance of the resorption over the apposition of bone, which is reflected in the increased level of osteocalcine in serum.

However, not all authors agree with this assertion. The study conducted by Bogdan Lewandowski et al. [18], revealed that the markers of bone remodelling are not the authoritative source of knowledge about predicting, amongst other things, pathological bone fractures. This is because the bioindicators used are influenced by various factors, and the same biomarker levels fluctuate daily. Serum osteocalcine exists in the form of the whole molecule and several fragments of different sizes. This fact can cause discrepancies in the results using immunological methods to determine levels of osteocalcine. Lee [16] marked and investigated the changes in the level of osteocalcine in women in relation to the time that had elapsed since the last menstrual period. During the test, four subgroups were constructed: those whose menstrual period occurred during the period from 1) 12 to 48 months previously 2) 49 to 84 months previously, 3) 85 to 120 months previously and 4) above 121 months. The results were as follows 1) 9.26 + / -5.89 ng/ml, 2) 9.01 + / -2.75 ng/ml, 3) 8.36 + / -4.99 ng/ml 4) 9.88 + / -3.82 ng/ml. The results were not clear. The high level of osteocalcine in groups 1, 2 and 4 with a high margin of error prompted the authors to conclude that there is no significant and unequivocal link between the serum osteocalcine and the metabolic changes occurring in bone in

postmenopausal women. The concentration of each biomarker is not specific for particular diseases and reflects changes in bone metabolism, regardless of their cause. An increased level of ucOC occurs not only in osteoporosis, but also in many other diseases, for example in hyperparathyroidism, hyperthyroidism, Paget's disease or bone tumors. Difficulties in the practical use of biomarkers are also caused by the fact that most markers of bone metabolism may also come from other tissues. However, it is believed that they come to play an increasingly important role diagnostically.

Marked fluctuations in the levels of osteocalcine associated with the severity and duration of postmenopausal osteoporosis were presented in the work of Camelia Vidit Gurban and associates [9]. The researchers investigated the levels of estrogen and osteocalcine in postmenopausal women. In the studied group, whose ovulation had ceased more than 15 years before, the level of osteocalcine was 20.12 ± 0.87 ng/ml, whereas in the group with this period shorter than 15 years, the level was 15.12 ± 1.55 ng/ml. The control group of postmenopausal women without osteoporosis had osteocalcine levels in the range of 16.22 ± 1.62 ng/ml. It was stated that there is a link between the increase in osteocalcine content and the decreasing function of osteoblasts (thus slowing apposition of bone tissue) and bone-cell apoptosis due to lack of estrogen stimulation. There is a directly proportional relationship in the correlation between osteocalcine and the age of the patients. This is related to hormonal changes occurring in the body. A significant difference in the levels concerned pre- and postmenopausal women. This increase was linear and noticeable in a study conducted with three different techniques. Significant differences relate mainly to postmenopausal women. Studies conducted in women with normal function of ovaries do not give such clear results [6, 8, 21]. Additional confirmation of this finding can be found in the work of Bauer [1].

Osteoporosis is a serious, chronic disease leading to permanent defects in bone tissue. However, the process starts with the decalcification – a condition called osteopenia. A study conducted by Mechanovic-Nikolic et al. [20] showed the difference between the concentration of osteocalcine in patients suffering from osteoporosis and osteopenia. The concentration of the marker in patients with osteopenia was 29.26 ± 3.65 ng/ml and with osteoporosis – 32.07 ± 6.24 ng/ml. These studies are consistent with the work of Topic et al. [25]. On this basis, the relationship between the level of ucOC and osteoporosis can be demonstrated in a more obvious and clear way.

Charles et al. [24] after examining 52 patients with postmenopausal osteoporosis found osteocalcine content in the range of 18.2 ± 5.9 ng/ml vs. 14.7 ± 5.9 ng/ml. It was noted that there was a relationship between metabolism of calcium in the body and a relative decrease in the level of this marker after application of vitamin D and corticosteroids. People who did not undergo treatment had a constant level of osteocalcine. This enables us to draw a conclusion that osteocalcine is one of the most accurate markers that show the efficacy of treatment of the bone metabolism disorders. This statement undermines the thesis put forward in the work of Bogdan Lewandowski et al. [18].

An important factor which has an influence on the concentration of ucOC is the supply of vitamin K. This vitamin increases the production and secretion of osteocalcine by osteoblasts. In addition, menaquinone (vitamin K2), a cofactor for gamma-carboxylation of osteocalcine, leads to the reduction in the release of OC from bone to plasma [23]. Masataka Shiraki, and Akira Itabashi conducted a study on 109 postmenopausal women. The results obtained showed that in the group which had undergone a 6-month supplementation with vitamin K, the level of ucOC was 2.4 ± 0.2 ng/ml, whilst in the control group the concentration of ucOC was within the range of 4.2 ± 0.5 ng/ml. The authors demonstrated in their study that the deficiency of vitamin K may reduce the level of osteocalcine in bone tissue, which

leads to its demineralization and increased fragility.In the same work, the researchers found that the demand for vitamin K is higher in women at a more advanced age than in younger women. Vitamin D has a similar effect. Calcitriol (1.25 - (OH) 2D3), the active form of vitamin D3, also stimulates the production of OC and decreases the concentration of ucOC in serum [14]. High concentrations of ucOC can be considered as an indicator of deficiency of vitamin K and D in the bone.

The present study revealed that the level of osteocalcine in serum decreased in women M + HRT (4.75 ± 0.45), compared with the results for women from group M (5.69 ± 1.34), suggesting a positive influence of estrogen on bone metabolism. Spine bone mineral density tests showed the following values of BMD: for women of the group M 0.77 ± 0.12 and for the patients of group M + HRT 0.92 ± 0.05. The negative correlation between the level of serum osteocalcine and BMD of the spine in menopausal women confirms the results of previous studies (BMD decrease is accompanied by an increasing level of osteocalcine in blood) [21]. The same (significant) correlation can be found in studies of Holvik et al. conducted amongst Norwegian postmenopausal women [12].

Obesity is often perceived as a protective factor in osteoporosis [7]. In obese women, there is probably an increase of bone formation or a decrease of bone resorption, or both processes function simultaneously. A negative correlation between daily calcium excretion (expressed as creatinine) and body weight has been stated. The excretion of calcium in obese postmenopausal women was lower than in non-obese women [13]. An increase in the amount of body fat leads to the reduction of bone resorption without a concomitant reduction in bone formation (measured as levels of osteocalcine) [10]. In obese postmenopausal women, due to increased aromatization of androgens into estrogens in adipose tissue, the increased level of endogenous estrogens can be observed. Confirmation of this fact can be found in the work of Davidson et al. [5]. It is worth noting that leptin, produced by adipocytes, has an impact on bone metabolism. The action of leptin on bone remodelling may be twofold. This protein can inhibit bone formation by affecting RANKL (Receptor Activator for Nuclear Factor κ B ligand), or increase the expression of CART (Cocaine and Amphetamine-Regulated Transcript), which leads to a slowdown in the process of resorption [14]. Leptin has no direct effect on the production of osteocalcine, but it has impact on lowering the concentration of blood ucOC [11]. This fact could have had a significant impact on the obtained results in this study, because all the tested patients had a Body Mass Index higher than 25 (above normal).

It is believed that there is also a reversible dependence between fat and the bone tissue. The fact that both tissues are derived from mesenchyme forms a basis for consideration of this. ucOC, released from bone tissue affects the metabolism of the whole organism. Citing Lee et al.: increased serum levels of osteocalcine increase insulin secretion, stimulate the proliferation of mitochondria, accelerate fat metabolism and increase energy consumption of the body [17].

The researchers also examined the effectiveness of hormone replacement therapy on the prevention of pathological fractures in female patients suffering from obesity. Comparing obese and non-obese women, using and not using HRT, it turned out that the use of HRT slightly reduces the risk of fracture in the group of obese women. However, HRT had a strongly protective effect on the non-obese women [22], which correlates with the results of studies dealing with obesity as a protective factor for osteoporosis.

The bone loss in postmenopausal women is also associated with the loss of epidermal connection (CAL-clinical attachment level), increased gingival pocket depth (PD – probing depth) and the establishment of bone pockets. It has frequently beeen stated that there is a directly proportional relationship between the concentration of osteocalcine, both in serum and saliva, and the severity

of periodontal changes. Bullon et al. conducted tests confirming this thesis [3, 4]. Becker found that women who did not have sufficient HRT demonstrate a negative correlation between the time since last menstrual period and the number of preserved teeth [2].

A study by McGehee and colleagues revealed that the level of biomarkers of bone demineralization in saliva is as reliable as their concentration in serum [19]. Our results showed that the concentration of ucOC in the secretion of the salivary glands is slightly lower than that in serum; however, it also shows a statistically significant difference between various research groups. Both measurements imply much about the ongoing metabolic processes in bones. It is therefore likely that in the future these biomarkers may be used for screening tests of diseases associated with bone metabolism and as a way of monitoring drug therapies that affect bones. Examination of ucOC in saliva is much simpler and more convenient for patients than the study of blood serum or urine. For these reasons, further research confirming the conclusions presented in this paper should be carried out.

In most of the studies noted above, the researchers confirmed the relationship between the serum osteocalcine and bone mineral density levels in postmenopausal women with osteoporosis. Some of them gave more clear and unequivocal results, while some suggested the existing relationship, but did not show it explicitly. Measurements presented in this paper differed slightly, which made it difficult to evidence a clear thesis. It is therefore suggested that future studies on a larger study groups are required to help obtain more reliable results.

CONCLUSIONS

1. The level of uncarboxylated osteocalcine shows variability in different research groups.
2. There is a negative correlation between the changes of the serum osteocalcine and BMD level in menopausal women and menopausal women undergoing HRT.
3. Osteocalcine concentration in saliva is slightly lower than in serum and it differs between the study groups in a statistically significant way.
4. Further research on osteocalcine levels in saliva and serum carried out on a broad research group is required.

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SUMMARY

The aim of this study was to show the difference between the concentration of osteocalcine in serum and saliva of menopausal patients and to demonstrate the correlation between the concentration of this biomarker and bone density. The examination included 60 postmenopausal women, 30 of whom underwent oophorectomy. Half of the women were treated with hormone replacement therapy (HRT). Samples of blood and saliva were tested using ELISA. Densitometry was conducted on the lumbar vertebral column. Densitometry revealed a significant difference in BMD between patients not undergoing HRT and those receiving HRT. Concentration of osteocalcine in serum ranged from 3.59ng/ml to 580ng/ml. A statistically significant fluctuation of osteocalcine in serum was discovered in the post-oophorectomy group. Examination of saliva showed differences in marker concentration, which in a statistically significant way involved menopausal women and patients treated with HRT. There is a negative correlation between BMD and osteocalcine levels in the blood serum of menopausal women and women undergoing HRT. Osteocalcine concentration in saliva is slightly lower than in serum. Measurements presented in this study were subject to variations that make it difficult to derive a definitive thesis. It is recommended that future studies on larger cohorts are undertaken to vacilitate more reliable results.

Keywords: osteocalcine, BMD, osteoporosis, menopause

STRESZCZENIE

Celem pracy było ukazanie różnicy w stężeniu osteokalcyny – markera biologicznego kościotworzenia – w surowicy oraz ślinie pacjentek w okresie menopauzalnym oraz wykazanie korelacji między stężeniem tego biomarkera a gęstością kości. Badania kliniczne przeprowadzono w grupie 60 kobiet w okresie pomenopauzalnym, z czego 30 pacjentek przeszło zabieg owariektomii. Połowa z kobiet była leczona hormonoterapią zastępczą (HTZ). Od pacjentek pobierano na czczo ślinę niestymulowaną oraz krew żylną. Krew i ślinę poddano badaniu laboratoryjnemu metodą ELISA. Przeprowadzono również badania BMD kręgosłupa lędźwiowego. Badania densytometryczne wykazały, iż różnica w BMD między pacjentkami niepoddanymi hormonoterapii oraz przyjmującymi HTZ była statystycznie istotna. Istotne statystycznie wahania poziomu osteokalcyny w surowicy dotyczą kobiet z grupy po owariektomii. Badania śliny wykazały różnice w stężeniu markera, które w istotnie statystyczny sposób dotyczyły kobiet w wieku menopauzalnym oraz kobiet leczonych HTZ. Wystąpiła negatywna korelacja pomiędzy BMD oraz poziomem osteokalcyny w surowicy krwi w grupie kobiet w wieku menopauzalnym i grupie kobiet w wieku menopauzalnym poddanych HTZ. Wykazano, iż stężenie osteokalcyny w ślinie jest nieznacznie niższe niż w surowicy. Pomiary poziomu osteokalcyny przedstawione w niniejszej pracy różniły się w małym stopniu, co utrudniało postawienie jednoznacznej tezy. Zakłada się, że badania przeprowadzone w przyszłości na większej grupie badawczej pozwoliłyby na uzyskanie bardziej wiarygodnych wyników.

Słowa kluczowe: osteokalcyna, BMD, osteoporoza, menopauza