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A STUDY OF THE INFLUENCE OF VITAMIN D AND VITAMIN K2 STATUS ON BONE TURNOVER IN WOMEN WITH POSTMENOPAUSAL OSTEOPOROSIS

ABSTRACT

of a dissertation for acquiring PhD degree

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The dissertation contains 182 pages and is illustrated with 12 tables and 70 figures.

The list of references includes 450 literary sources, of which 6 are in Cyrillic and 444 are in Latin.

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The dissertation work was approved and directed for defense by the extended departmental council of the department of "Physiology and Pathophysiology" at the Medical University – Pleven.

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The defense materials are available in the Scientific Department and are published on the website of the MU – Pleven – www.mu-pleven.bg

ABBREVIATIONS

ALP – alkaline phosphatase
BTM – bone turnover markers
BMI – body mass index
BMD – bone mineral density
DXA – dual energy X-ray absorptiometry
OP – osteoporosis
PTH – parathormone
WHO – World Health Organization
EFSA – European Food Safety Authority
FRAX-Hfr – hip fracture
FRAX-MO – major osteoporotic fracture
IFCC – International Federation of Clinical Chemistry and Laboratory Medicine
IOF – International Osteoporosis Foundation
MGP – Matrix Gla-protein
OC – osteocalcin
RANKL – ligand receptor activator nuclear factor κappa B
SD – standard deviation
ucOC – undercarboxylated osteocalcin

CONTENTS

INTRODUCTION

Osteoporosis (OP) is "silent epidemic." Today, the disease is defined as a primary metabolic skeletal disorder with a complex pathogenesis and a strong causal relationship between low bone mineral density (BMD) and increased fracture risk. Its asymptomatic or uncharacteristic clinical course is a reason for underestimation of the problem by patients and medical professionals and late diagnosis, often after the occurrence of a spontaneous fracture. Complications are severe, associated with disability, prolonged care, and often lethal outcomes, which requires the scientific community to concentrate its efforts on enriching the knowledge of the disease in different countries, as well as the creation and implementation of strategies to prevent its development and the occurrence of complications.

Adequate nutrition plays a major role in preventing and treating OP. Calcium and vitamin D are the most important of the nutrients. In many countries, calcium supplementation alone or combined with vitamin D is recommended in prevention and treatment regimens because of their presumed ability to maintain bone health and increase BMD. However, new scientific evidence links calcium and vitamin D supplementation with an increased risk of cardiovascular disease. The simultaneous development of OP and arterial calcification has been termed the "calcium paradox" and is common in postmenopausal women. Studies suggest that the two diseases may be interrelated. Women who receive calcium supplements are at higher risk for cardiovascular disease, and publications in the scientific literature support the possibility that vitamin K2 and possible deficiency are the link between calcium supplementation and increased cardiovascular risk.

It is now known that vitamins D and K2 play a central role in calcium metabolism and interact synergistically in maintaining bone and cardiovascular health. Vitamin D stimulates protein synthesis, which is activated by vitamin K2-dependent carboxylation. The vitamin K2-dependent proteins osteocalcin (OC) and matrix Gla-protein (MGP) in bone and blood vessels regulate calcium homeostasis. Osteoblasts synthesize osteocalcin and maintain calcium binding to the bone mineral matrix, and MGP is synthesized by vascular smooth muscle cells and prevents ectopic calcification. Until recently, vitamin K2 deficiency was considered very rare and only found in patients of certain risk groups. In the current scientific literature, there is increasing evidence of poor vitamin K2 status and associated adverse effects on bone and vascular metabolism through

increased levels of the uncarboxylated functionally inactive fractions of these proteins.

Vitamin D deficiency is the most common nutritional deficiency worldwide and is still considered a risk factor in the development of OPs and fractures.

To date, it is still not fully understood whether optimal levels of vitamin D increase BMD and reduce fracture risk or whether it exerts additional musculoskeletal effects, as some recent meta-analyses of randomized clinical trials have questioned these previously proposed effects of vitamin D, and data obtained in different geographical regions are conflicting.

There is also conflicting evidence in the global scientific literature on the relationship between vitamin D deficiency, BMD, and fracture risk, and the likelihood of fracture varies substantially between geographical regions.

Vitamin D status in patients with postmenopausal OP is an important element of the comprehensive evaluation because optimal levels are essential to maximize the effect of antiresorptive therapy.

Supplementation with vitamin D and calcium is recommended in many guidelines as an element in the complex treatment of OP. However, a growing body of scientific evidence links calcium supplementation with accelerated onset of arterial calcification and increased cardiovascular risk in patients with vitamin K2 deficiency.

Vitamin K2 is involved in regulating many vital processes. Still, its physiological effects and mechanisms for influencing calcium metabolism and its potential role in maintaining bone health and the development of OP need to be fully understood.

Bone is a living tissue that is constantly being rebuilt and renewed due to microscopic damage that occurs during daily physical activity. This process is called bone turnover, and its objective assessment is performed using biochemical markers. Biochemical bone turnover markers (BTM) are new tools that detect the dynamics of bone remodeling in terms of bone formation and resorption. They are easily measured in blood and urine samples. They should become the method of choice in monitoring bone metabolic diseases because radiographic measurements of bone mass respond slowly to changes in bone physiology. Therefore, current efforts aim to standardize their use for both scientific and clinical purposes. The availability of reliable, cost-effective, sensitive, and specific assays can complement BMD measurement in the management of OP, particularly in the follow-up of patients who have been on antiresorptive or bone formation therapies.

Monitoring the early effects of OP treatment is a difficult task. Serial measurement of BMD by DXA is currently the "gold" standard for assessment. Such is recommended at the earliest one year after treatment initiation. However, BMD increases, and fracture incidence decreases as an expected therapeutic effect is commonly observed after the second year of treatment.

Biochemical markers of bone turnover may be a useful tool in this regard. Still, their widespread implementation in clinical practice is hampered by the lack of universally valid reference values worldwide, especially for postmenopausal women, and as highlighted, more research is needed. Serum concentrations of some of the markers show geographical variations, but data is still lacking, and the one published in scientific literature is mixed.

In Bulgaria, the problem of OP is highly underestimated. There is a lack of good organisation in patient identification, diagnosis, treatment and follow-up.

Worldwide, published data on vitamin D and vitamin K2 levels in postmenopausal women and women with OP are partially contradictory.

In Bulgaria there is no published data on vitamin D and K2 levels, as well as BTM levels in postmenopausal women and OP patients. These markers are recommended in OP guidelines as a possibility for early detection of the effect of the treatment. Still, there is no experience in their clinical application in our country.

Based on these unresolved issues, the aim and objectives of the thesis were formulated.

AIM

The aim of the present research study was to determine the vitamin D and vitamin K2 status of women with postmenopausal osteoporosis, investigate the effects of the two vitamins on bone turnover, and evaluate the feasibility of their use in the management of osteoporosis.

TASKS

1. To assess the bone mineral density of the participants by dual-energy X-ray absorptiometry measurements to inform the groupings and calculate body mass index and fracture risk as elements of the initial assessment.
2. To investigate calcium, phosphorus, parathyroid hormone, and biochemical markers of bone turnover alkaline phosphatase, osteocalcin, and beta CrossLaps.
3. To assess vitamin D status by testing plasma concentrations.
4. To assess vitamin K2 status indirectly by examining plasma ucOC concentrations and ucOC/OC ratio.
5. To look for correlations between vitamin D and K2 levels, calcium-phosphorus metabolism parameters, bone turnover, and bone mineral density.
6. To investigate the dynamics of serum levels of beta CrossLaps and alkaline phosphatase in patients with osteoporosis before and six months after initiation of complex treatment and to study the usefulness of their follow-up for early monitoring of the treatment effect.

CLINICAL CONTINGENT, MATERIALS AND METHODS

Clinical contingent

To achieve the aim of the thesis and to fulfill the set tasks, a selection of postmenopausal women meeting certain criteria was made for inclusion in the treatment and control groups.

Criteria for inclusion of participants in the study group

- Age ≥ 45 years.
- Onset of menopause.
- Anamnestic and clinical evidence of absence of diseases leading to secondary OP.
- Demonstrated postmenopausal OP by BMD examination with DXA.
- Lack of osteoporosis treatment until diagnosis, including supplementation with vitamin D, K2, and calcium preparations.

Criteria for inclusion of participants in the control group

- Age ≥ 45 years.
- Onset of menopause.
- Demonstrated absence of OP by DXA BMD testing.
- Supplementation with vitamin D, K2, and calcium preparations was not performed until the time of the study.

Formation of the study and control groups

From October 2018 to May 2021, within the framework of Project No.10/2018 and Project No.8/2020, funded by MU-Pleven, the selection of the participants, forming the groups, creating the necessary documentation, as well as conducting the planned immunological and biochemical tests were carried out.

One hundred (100) menopausal women were studied. All the participants signed informed consent. The diagnosis was made after measurement of BMD of the lumbar spine vertebrae by DXA. Grouping was done according to WHO standards, and BMD was determined as follows:

- Normal bone mineral density: T-score ≥ -1.0 SD;
- Osteopenia: T-score between -1.0 SD and -2.5 SD;
- Osteoporosis: T-score ≤ -2.5 SD;
- Severe osteoporosis: T-score ≤ -2.5 SD + fracture.

The participants were divided into two groups:
Study group – untreated women with newly diagnosed osteoporosis.
Control group – women without osteoporosis, with normal bone mineral density or osteopenia

Materials and Methods

1. Clinical methods: history and physical examination.
2. Anthropometry – measurement of body mass according to an established standard methodology using a calibrated scale and height using a standard height gauge. From the body weight in kilograms and height in square meters, calculate BMI using the formula BMI = kg/m^2.
The WHO norms for BMI adopted in Bulgaria are as follows:

- Underweight: < 18.5 kg/m^2
- Normal weight: 18.5 – 24.99 kg/m^2
- Overweight: 25.0 – 29.99 kg/m^2
- Obesity: ≥ 30 kg/m^2

3. Sociological methods:

- **Questionnaire method** – Direct individual questionnaire to investigate the risk factors for osteoporosis by completing the "One-minute osteoporosis risk assessment test" questionnaire, which Prof. Michail Boyanov first designed. In it, individual risk factors receive different numbers of points depending on their severity. WHO data and large epidemiological studies on the so-called significant (major) risk factors were used to determine the relative weight of the risk factors. The sum of all scores assesses the overall risk of osteoporosis, and if present, the person is referred for a BMD measurement.
- **Documentary method** – Questionnaires were developed for each group of subjects, in which data on age, results of anthropometric measurements and bone mineral density, history of the disease, concomitant diseases, and medication intake were filled in.

4. Fracture risk assessment:

The FRAX® Fracture Risk Assessment Tool has been included in the WHO recommendations for fracture risk assessment since 2008. It is a web-based algorithm for quick and easy evaluation of individual fracture risk – https://www.sheffield.ac.uk/FRAX/. It estimates the ten-year probability of a significant osteoporotic fracture – hip, vertebrae, humerus, or forearm in percentages based on the examined BMD and some clinical risk factors. Two fracture indices are calculated using the algorithm:

- **FRAX-MO (Major osteoporotic)** – general risk of fracture.
- **FRAX-Hfr (Hip fracture)** – the risk of femoral neck fracture.

The indicators used to calculate FRAX include:

- DXA scan measured bone mineral density in g/cm^2 or T-score of the spine or femoral neck.
- Clinical risk factors: age, sex, height, body weight, BMI, history of fracture, family history of femoral fracture, smoking, alcohol use, glucocorticoid intake, rheumatoid arthritis, and other forms of secondary osteoporosis. Data on these risk factors were obtained from the history and completed questionnaires.

The current 2008 International Osteoporosis Foundation recommendations for threshold values above which fracture risk is considered elevated are:

- **FRAX-MO ≥ 20%,**
- **FRAX-Hfr ≥ 3%.**

At the end of 2020, the FRAX® calculator was validated for use in Bulgaria. However, it should be noted that the national consensus for good clinical practice still lacks recommendations for its use in fracture risk assessment.

5. Laboratory tests:

Venous blood was used to investigate the biochemical and immunological parameters provided in the study, i.e., calcium, phosphorus, alkaline phosphatase, parathormone, vitamin D, osteocalcin, non-carboxylated osteocalcin, beta CrossLaps. Blood tests were conducted between October and March in 2018 and 2020. Samples were collected in the morning between 8.00 and 9.30 am to avoid circadian variability and after a 12-hour fasting period. They were tested in a certified medical diagnostic clinical laboratory with an identical methodology to minimize variation due to technical errors.

- **Biochemical studies:**
  - **Calcium, phosphorus, alkaline phosphatase** – 5 ml of venous blood taken in sterile vacutainers for serum separated by centrifugation was used. Samples were analyzed on the day of collection with a Cobas C 311 biochemical analyzer, Roche Diagnostics.
- **Immunological tests:**
  - **Parathyroid hormone, vitamin D, osteocalcin, beta CrossLaps** – venous blood 5 ml taken in sterile serum vacutainers separated by centrifugation was used. Samples were examined on the day of collection by electrochemiluminescence immunoassay on a Cobas E

411 immunology analyzer, Roche Diagnostics. Elecsys PTH with a sensitivity of 1.20 – 5.00 pg/ml or 0.127 – 530 pmol/ and Elecsys Vitamin D total Roche Diagnostics kits with a sensitivity of 3.00 – 100 ng/ml or 7.50 – 250 nmol/l, Elecsys N-MID Osteocalcin Roche Diagnostics kit with a sensitivity of 0.500 – 300 ng/ml and Elecsys β-Crosslaps Roche Diagnostics kit with a sensitivity of 0.010 – 6.00 ng/ml were used, respectively. Reference values of indicators according to the analyses used are presented in Table 1:

Table 1: Reference values of indicators according to the analyses used

Parameter	**Reference values**
Calcium	2.15 – 2.55 mmol/l
Phosphorus	0.87 – 1.45 mmol/l
Alkaline phosphatase	≤ 128 U/l
Parathyroid hormone	15 – 65 pg/ml
Vitamin D	≥ 50 – 120 nmol/l – норма 25 – 50 nmol/l – недостатъчност ≤ 25 nmol/l – дефицит
Osteocalcin	жени – 11 – 43 ng/ml; менопауза 15 – 46 ng/ml
Beta Cross Laps	0 – 1.008 ng/ml

- **Undercarboxylated osteocalcin:** 3 ml venous blood in sterile hematology vacutainers was used. Samples for analysis after centrifugation and plasma separation were frozen and stored at -80°C. Concentrations were measured using the Human Undercarboxylated Osteocalcin (ucOC) ELISA Kit (Abbexa, UK), with a test range of 6.25 pg/ml - 400 pg/ml and a sensitivity of 3.75 pg/ml. Samples were diluted 1:100, and the assay was performed according to the manufacturer's instructions.
- **Vitamin K2 status was** assessed indirectly by the percentage ucOC/OC ratio. In a 2017 paper, EFSA accepted ucOC/OC values ≥ 20% as an indicator of dietary vitamin K2 deficiency

6. Statistical methods - the data were entered and processed using the statistical software SPSS v23.0 and Microsoft Office Excel 2003.

- The results are described by tables, graphs, and numerical values (structure indicators – relative shares, averages, correlation coefficients, etc.). Continuous variables are expressed as mean (X) ± standard deviation (SD). Categorical variables are expressed as absolute/relative frequencies. Data were tested for normality of distribution using Kolmogorov-Smirnov and

Shapiro-Wilk tests. Statistical treatment was tailored to the type of data (nominal, ordinal, interval) and the distribution of variables. Values of $p < 0.05$ were assumed to be statistically significant in the different analyses.

- Descriptive, comparative, correlation, and regression analysis methods were used.

Descriptive analysis:

- Frequency analysis of qualitative variables that included absolute and relative frequencies in percentages.
- Analysis of variance of quantitative variables was used to find the mean and standard deviation, standard error of the mean and median.

Comparative analysis: when comparing groups, the mean of the variables (X) ± standard deviation (SD) was most often used to indicate the variability of the factor under study or the values of the medians depending on the type of distribution.

- With normally distributed data, the significance of differences between groups was determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test, t-test for independent and correlated samples.
- For data deviating from the normal distribution, the Kruskal-Wallis nonparametric tests for more than two samples and Mann-Whitney tests for two independent samples were used for comparison.

Correlation analysis:

- Pearson correlation analysis was made to examine the relationship between two continuous, normally distributed quantities.
- Spearman's rank correlation was used to determine the relationship between two quantities with an irregular distribution and/or measured on an ordinal scale.
- The degree of correlations was assessed according to the correlation coefficient (r) on a 5-point scale as weak ($r < 0.3$), moderate ($0.3 < r < 0.5$), significant ($0.5 < r < 0.7$), high ($0.7 < r < 0.9$) and very high ($0.9 < r < 1$).

Univariate linear regression analysis: to examine the relationship between one or more independent variables

RESULTS

Bone mineral density

After DXA measurement of the BMD of the lumbar spine, participants with postmenopausal OP and T-score equal to or lower than -2.5 SD were included in the working group (n = 62). The participants without OP and T-score greater than -2.5 SD were included in the control group (n = 38).

The T-score data showed a skewed distribution and nonparametric tests were used for comparison. The Mann-Whitney test showed a statistically significantly lower BMD (-2.85 ± 0.54 SD) in the OP group compared with the BMD (-0.10 ± 0.67 SD) of women in the control group (U = 0.00; p < 0.001) (Fig. 1).

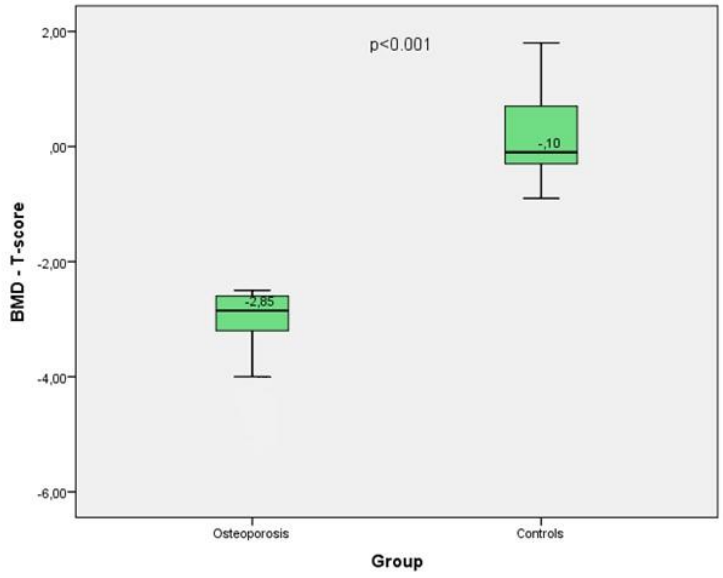


***Fig. 1**. Bone mineral density in the lumbar spine in the work and control groups. Data are presented as median, minimum, and maximum T-score values. The difference between the groups was statistically significant, p < 0.001.*

Study group

This group included 62 women with a mean age of 66.05 ± 8.70 years and a mean BMD expressed by a T-score of -2.85 ± 0.54 SD. To determine the severity of OP fracture data from medical records were obtained. There were 39 patients with OP without fracture, aged 65.21 ± 7.28 years, and T-score of -2.8 ± 0.62 SD, and 23 patients with severe OP, mean age 67.48 ± 10.71 yrs, T-score (-2.9 ± 0.53 SD), and proven fractures in different regions – forearm and spine. Comparison between the two groups with the Mann-Whitney test showed no statistically significant difference in age (U = 383.50; p = 0.343) and BMD (U = 417.50; p = 0.650).

Control group

This group included 38 postmenopausal, non-OP women with a mean age of 64.89 ± 8.42 years and a T-score of -0.10 ± 0.67 SD. In the control group, 11 women with a mean age of 68.82 ± 8.8 years and T-score (-0.09 ± 0.64) had proven forearm fractures. The Mann-Whitney test comparing women with and without fracture showed no statistically significant difference in age (U = 105.50; p = 0.165) and BMD (U = 106.50; p = 0.175).

The mean age of patients with OP was higher (66.06 ± 8.7) compared to controls (64.89 ± 8.42), but the results of a t-test performed to compare independent samples showed no statistically significant differences (t = 0.657; p = 0.513).

The measured BMD in the treatment and control groups stratified by decade is shown in Figure 2. In the OP group, BMD showed a gradual decrease each successive decade, lowest in women aged 70 – 79 y. Comparison using the Mann-Whitney test results by decades showed a statistically significant difference in BMP only in the OP group. Women aged 70 – 79 had statistically significantly lower BMD (U = 44.50; p = 0.022) than those aged 50 – 59.

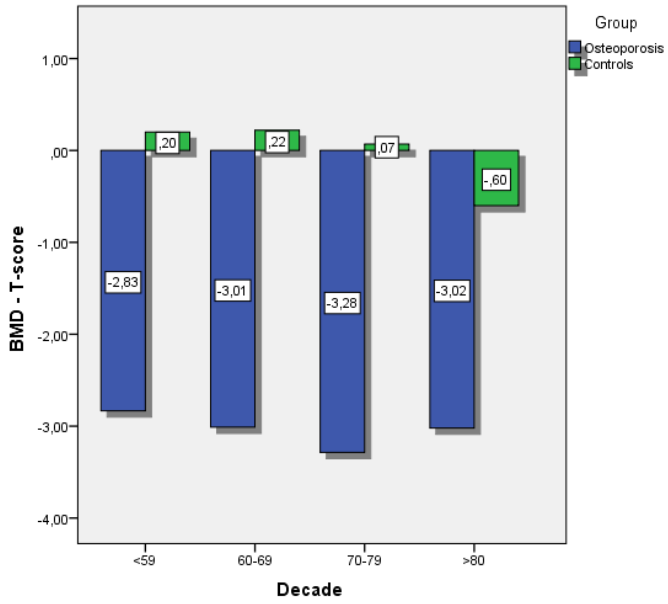


***Fig. 2**. Mean BMD expressed by T-score in women of the work and control groups distributed by decade. The difference in BMD of women in the OP group aged 50 – 59 and 70 – 79 was statistically significant, p < 0.05.*

Body mass index

An important parameter in the comprehensive evaluation of OP patients. It was calculated using a standardized methodology and interpreted following the norms accepted by WHO. Nonparametric tests were used for comparison due to data

maldistribution. In the OP group, the mean BMI was 24.08 ± 4.09 kg/m^2; in the control group, the BMI was 29.57 ± 5.01 kg/m^2. Between-group comparison using the Mann-Whitney test showed that the BMI of women with OP was statistically significantly lower compared with the BMI of controls (U = 446.0, p < 0.001).
The frequency distribution by BMI of participants in the work and control groups is shown in Figure 3. In controls, the shift of BMI values towards overweight and obese is obvious.

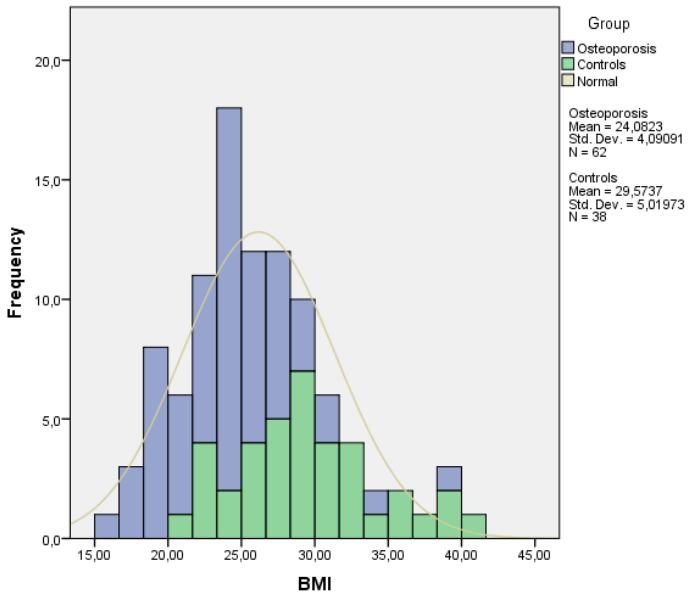


Fig. 3. *Composite histogram of frequency distribution by BMI in kg/m^2 of participants in the work and control groups. The difference in mean BMI values in the two groups was statistically significant, p < 0.001.*

In the OP group, the majority of the women, 58.1%, were normal weight, 29% were overweight, 6.5% were obese, and 6.5% were underweight. In the control group, 18.4% were of normal body weight, 42.1% were overweight, and 39.5% were obese. The Kruskal-Wallis nonparametric test showed statistically significant inequality in BMI distributions by group, both in women with OP (χ^2 = 47.56; p < 0.001) and in the control group (χ^2 = 31.798; p < 0.001).
BMD in the OP and control groups with different BMI are presented in Table 2.

Table 2. Bone mineral density of women in the osteoporosis and control groups with different BMI

	BMI kg/m^2				
BMD – T-score	**Underweight < 18.5**	**Normal weight 18.5 - 24.99**	**Overweight 25.0 - 29.99**	**Obesity > 30**	**Statistical significance**
Osteoporosis	-3.63±1.11	-3.06±0.61	-2.86±0.32	-3.15±0.38	p=0.323
Controls	–	0.37±0.79	0.05±0.72	0.17±0.57	p=0.589

BMD was expressed by T-score in SD and standard deviation.

In the OP group, there was a gradual decrease in BMD with a reduction in BMI in the overweight, normal weight, and underweight groups, but the results of Kruskal-Wallis tests showed no statistically significant differences ($\chi^2 = 3.483$; $p = 0.323$). The women in the control group also had no statistically significant differences in BMD at different BMIs ($\chi^2 = 1.060$; $p = 0.589$).

Fracture risk

The estimated risks of major osteoporotic and hip fractures for women in the work and control groups calculated using our nationally validated FRAX ® Fracture Risk Assessment Tool are presented in Table 3. The mean FRAX-MO values in both groups were less than 20%, and the FRAX-Hfr values were elevated only in the OP group.

Table 3. Ten-year fracture risk calculated using the FRAX ® Fracture Risk Assessment Tool in the work and control groups

Parameter	Working group	Control group	Statistical significance
FRAX-MO	18.29±10.33 (6.8–38)	5.26±2.93 (2.10–16.00)	p<0.001***
FRAX-Hfr	9.92±8.51 (2.5–38)	0.61±1.42 (0–8.8)	p<0.001***

FRAX-MO /major osteoporotic fracture/ and FRAX-Hfr /hip fracture/ values are presented as percentages ±SD, minimum, and maximum in parentheses. A statistically significant difference, $p \leq 0.001$***.

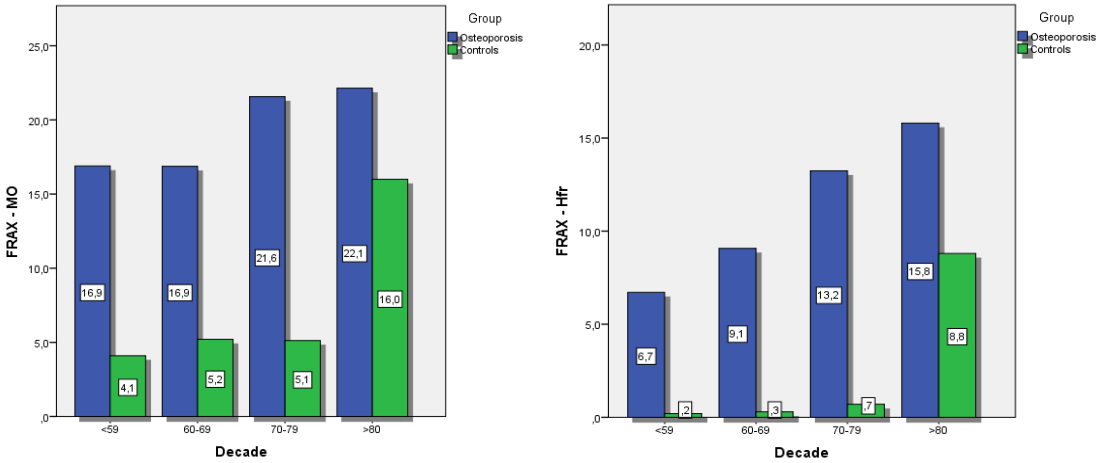
The Mann-Whitney test showed FRAX-MO ($U = 76.50$; $p < 0.001$) and FRAX-Hfr ($U = 41.00$; $p < 0.001$) statistically significantly higher in OP patients compared with controls.

In the OP group, the calculated FRAX-MO for women with and without prior fracture was 22.53 ± 10.76% and 15.79 ± 9.33%, respectively, and FRAX-Hfr was 11.86 ± 9.17% and 8.77 ± 8%, respectively. The FRAX-MO and FRAX-Hfr values in women with prior fractures were elevated above the threshold values. In women without a previous fracture, only FRAX-Hfr was elevated. The Mann-Whitney test showed that women with a fracture earlier had a statistically significantly higher ten-year fracture risk compared to women without a fracture for FRAX-MO ($U = 238.50$; $p = 0.002$) and FRAX-Hfr ($U = 286.5$; $p = 0.018$), respectively. The measured mean BMD in women with and without prior fractures was a T-score of -2.98 ± 0.53 SD and -3.05 ± 0.55 SD, respectively, and the difference was not statistically significant ($U = 417.50$; $p = 0.650$).

In the control group, FRAX-MO for women with and without prior fracture was 7.12 ± 4.23% and 4.51 ± 1.78%, respectively, and FRAX-Hfr was 1.24 ± 2.52% and 0.35 ± 0.41%, respectively, and were below the thresholds for increased risk. FRAX-MO in women with a prior fracture was statistically

significantly higher than those without a fracture (U = 84.00; p = 0.038). Differences in FRAX-Hfr in women with and without prior fractures were not statistically significant (U = 90.00; p = 0.057). The measured mean BMD in women with and without prior fractures had T-scores of -0.09 ± 0.64 SD and 0.26 ± 0.67 SD, respectively, and the difference was not statistically significant (U = 106.50; p = 0.175).

The decade-wise calculated FRAX-MO and FRAX-Hfr values of women in the work and control groups are presented in Figures 4 and 5. FRAX-MO increased unequally with each successive decade, but the difference did not reach statistical significance in both the OP group ($\chi^2 = 5.061$; p = 0.281) and the control group ($\chi^2 = 7.091$; p = 0.131) and the group of women with OP aged 70 years and older were elevated. FRAX-Hfr also increased progressively with age, with differences statistically significant in the control group ($\chi^2 = 12.672$; p = 0.013) but not in the OP group ($\chi^2 = 7.477$; p = 0.113). In the OP group, the risk was higher in all age groups. In the controls, the risk was higher only in those over 80.



Figs. 4 and 5. *Ten-year risk of major osteoporotic – FRAX-MO and femoral – FRAX-Hfr fractures expressed as percentages in women in the work and control groups distributed by decade. A statistically significant difference in FRAX-Hfr across ages was observed only in the control group, p < 0.05.*

Biochemical and immunological studies of calcium, phosphorus, ALP, osteocalcin, beta CrossLaps, PTH, vitamin D and vitamin K2

Calcium and phosphorus

Mean plasma concentrations of calcium of 2.43 ± 0.13 mmol/l and 2.46 ± 0.10 mmol/l and phosphorus of 1.23 ± 0.18 mmol/l and 1.20 ± 0.14 mmol/l were measured in the study and control groups, respectively. In both groups, calcium and phosphorus concentrations were within reference values. Calcium concentrations were lower in the OP group, but one-way analysis of variance

showed no significant difference between the two groups (F = 1.92; p = 0.169). Phosphorus concentrations also showed no statistically significant differences between the two groups (F = 0.65; p = 0.421).

Alkaline phosphatase

Mean concentrations of 85.84 ± 33.5 U/l and 82.08 ± 39.9 U/l were measured in the study and control groups, respectively. Data were non-normally distributed, and nonparametric tests were used for comparison. The enzyme concentration was higher in the working group, but the Mann-Whitney test showed no statistically significant difference between the two groups (U = 976.50; p = 0.152).

The mean concentrations of alkaline phosphatase distributed by decade in the working and control groups are shown in Table 4. The Kruskal-Wallis test showed no statistically significant difference in parameter concentrations in the OP group ($\chi^2 = 0.272$; p = 0.873) and the control group ($\chi^2 = 3.876$; p = 0.144). The Mann-Whitney test for comparison by decade between the group of women with OP and controls showed a statistically significant difference in concentrations only in the age group up to 59 years (U = 20.00; p = 0.021).

Table 4. Mean concentrations of alkaline phosphatase in U/l in women in the work and control groups distributed by decade

Age	≤ 59	60 – 69	70 – 79	≥ 80	Statistical significance
Osteoporosis	93.62±54.1	82.07±19.31	82.69±29.47	92.6±35.25	p=0.873
Controls	64.71±9.0	84.55±45.11	76.8±13.51	207±0	p=0.144

Data are presented as mean ± SD

Parathyroid hormone

Serum PTH concentrations in the study and control groups were 67.89 ± 39.56 pg/ml and 64.99 ± 25.12 pg/ml, respectively. The mean PTH levels were elevated in the working group and within reference values in the controls. Due to the skewed data distribution, nonparametric tests were used for comparison. The Mann-Whitney test showed no statistically significant difference in PTH concentrations between the OP and control group (U = 454.00; p = 0.911).

In the OP group, those with normal PTH levels below 65 pg/ml were 54.55%, mean age 63.7 ± 7.3 years, mean PTH concentrations of 43.55 ± 14.34 pg/ml and T-score (-3.04 ± 0.61 SD), and those with elevated levels above 65 pg/ml were 45.45%, mean age 68.6 ± 9.8 years, mean PTH concentrations of 97.13 ± 40.53 pg/ml and T-score (-3.04 ± 0.44 SD). Women with elevated PTH levels were older than women with normal levels, but the differences were not statistically significant (U = 168.00; p = 0.089). In the control group, 61.9%, mean age 64.7 ±

5.4 yr, PTH 48.63 ± 10.63 pg/ml, T-score (0.08 ± 0.62 SD), had normal PTH levels and 38.1%, mean age 64.1 ± 11.1 yr, PTH 89.09 ± 24.09 pg/ml, T-score (0.09 ± 0.75 SD) had elevated levels. The between-group comparison showed no statistically significant differences in BMD in women with elevated and normal PTH levels, both in the OP group (U = 211.50; p = 0.499) and in the control group (U = 52.00; p = 0.815).

The mean PTH concentrations in the OP and control groups distributed by decade are shown in Table 5. PTH values increased gradually with increasing age. Kruskal-Wallis tests showed a statistically significant difference in PTH concentrations across age groups in the OP group ($\chi^2 = 8.907$; p = 0.031) but not in controls ($\chi^2 = 2.437$; p = 0.296). In post hoc Mann-Whitney tests conducted to compare age groups by pairs in women in the workgroup, PTH showed a statistically significant increase in women aged 80 years and older compared with women aged up to 59 years (U = 1.00; p = 0.003).

Table 5. Mean PTH concentrations in U/l in women in the work and control groups distributed by decade

Age	**≤ 59**	**60 - 69**	**70 - 79**	**≥ 80**	**Statistical significance**
Osteoporosis	46.37±21.16	64.07±25.21	87.91±62.13	107.13±52.6	p=0.031*
Controls	60.72±21.47	60.24±23.67	76.61±29.65	–	p=0.398

Data are presented as mean ± SD; statistically significant difference, p ≤ 0.05*.

Vitamin D

Plasma vitamin D concentrations were measured in the working and control groups at 47.77 ± 21.9 nmol/l and 45.12 ± 20.27 nmol/l, respectively, and no statistically significant difference was found (F = 0.364; p = 0.548).

Comparison by decade in the working and control groups showed a statistically significant decrease in vitamin D with each successive decade only in the control group (F = 5.707; p = 0.003) but not in the working group (F = 1.984; p = 0.126) (Fig. 6).

Comparison between women with OP and controls across decades showed a statistically significant difference in vitamin D concentrations only in women aged 70 – 79 (t = 2.276; p = 0.036).

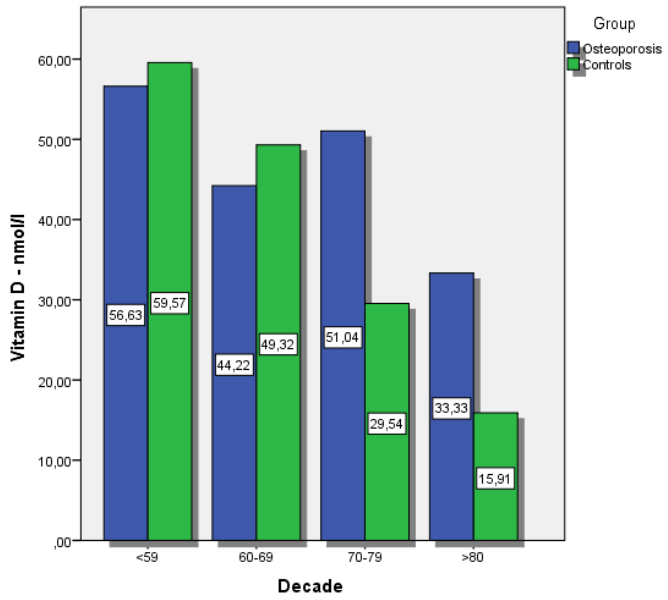
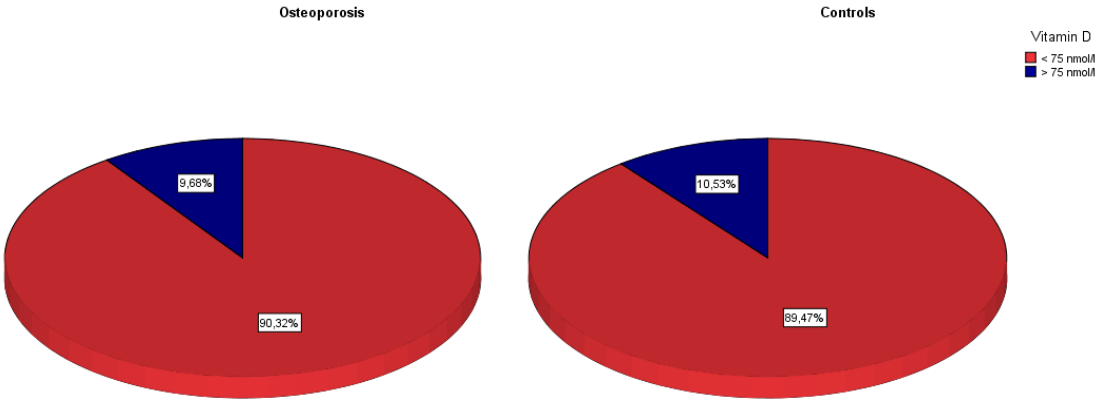


Fig. 6*. Mean serum vitamin D concentrations in nmol/l in the working and control groups distributed by decade. A statistically significant difference was observed in the control group, p < 0.05. In the age group 70 – 79, the difference between women with OP and controls was significant, p < 0.05.*

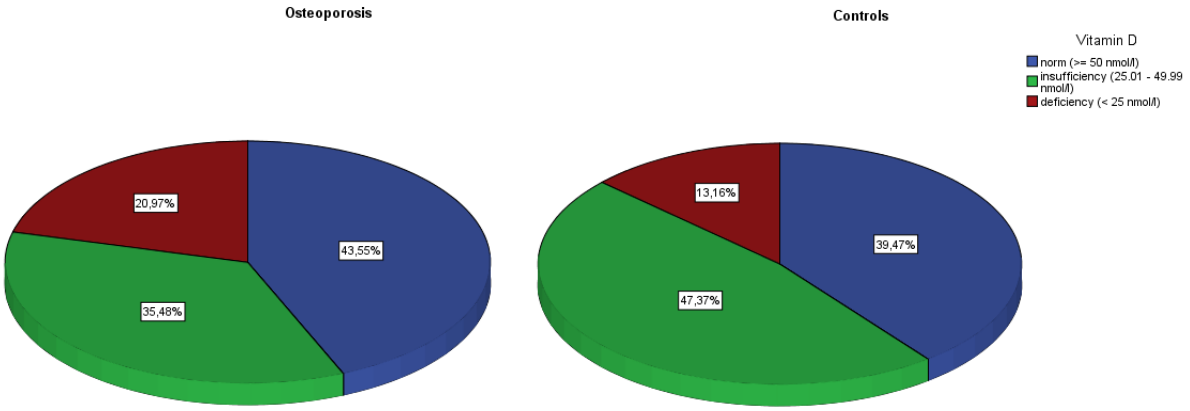
Serum concentrations of vitamin D above 75 nmol/l, which are considered optimal for maintaining BMD, normal function of the musculoskeletal system and lower limbs with minimal risk of falls and fractures, had only 9.7% (n = 6) of women in working and 10.5% (n = 4) of women in the control group. Suboptimal vitamin D levels below 75 nmol/l were found in 90.3% of the OP group (n = 56) and 89.5% of the controls (n = 34), respectively (Figs. 7 and 8).



Figs. 7 and 8. *Relative percentages of participants in the working and control groups with optimal (> 75 nmol/l) and suboptimal levels (< 75 nmol/l) of vitamin D.*

The relative proportions of participants in the two groups with different vitamin D levels are presented in Figures 9 and 10. In the OP group, 43.55% (n = 27) had normal vitamin D levels, 35.48% (n = 22) were vitamin D deficient, and 20.97% (n = 13) were vitamin D deficient. In the control group, 39.47% (n = 15) had normal vitamin D levels, 47.37% (n = 18) were deficient, and 13.16%

(n = 5) were vitamin D deficient. In other words, 56.45% of OP patients and 60.53% of controls had poor vitamin D status.



Figs. 9 and 10. *Relative percentages of participants in the OP and control groups with different vitamin D concentrations.*

According to the vitamin D levels we found, the study and control group participants were divided into three subgroups. The calcium-phosphorus metabolism parameters (calcium, phosphorus, ALP, and PTH), vitamin D, BMI, and BMD, and the results of the comparative analyses of the working group participants are presented in Table 6.

Table 6. Calcium-phosphorus metabolism parameters, BMI, and BMD of the participants of the working group with different Vitamin D levels

Working group	**Vitamin D levels**			
	Norm > 50 nmol/l	**Insufficiency 25 – 50 nmol/l**	**Deficiency < 25 nmol/l**	**Statistical significance**
Age - years	64.3±8.9	64.4±6.9	72.5±8.4	p=0.009**
Calcium – mmol/l	2.44±0,14	2.43±0.1	2.39±0.14	p=0.522
Phosphorus – mmol/l	1.26±0,2	1.21±0.15	1.18±0.18	p=0.481
ALP – U/l	89.37±41.83	82.7±20.87	83.85±33.19	p=0.890
PTH – pg/ml	60.07±46.43	65.4±24.26	86.51±41.52	p=0.037*
Vitamin D – nmol/l	67.87±4.07	40.6±5.97	18.16±4.71	p<0.001***
BMI – kg/m^2	23.43±3.93	24.85±4.71	24.13±3.27	p=0.412
BMD – T - score	- 3.01±0.52	- 3.03±0.57	- 3.05±0.56	p=0.941

Results are presented as mean ± SD; Statistically significant difference, p < 0.05*; p < 0.01**; p < 0.001***.

A statistically significant difference in the subgroups with different vitamin D levels was observed in the mean age (F = 5.068; p = 0.009). Tukey's post hoc test showed that the mean age of patients with vitamin D deficiency (M = 72.5) was significantly higher than the mean age of the patients with normal vitmin D levels (M=64.4; p = 0.017). One-way analysis of variance comparing groups with different vitamin D levels showed no statistically significant difference in mean

calcium (F = 0.657; p = 0.522) and phosphorus (F = 0.742; p = 0.481) concentrations. The statistically significant difference in mean vitamin D concentrations (F = 118.45; p < 0.001) was as expected.

Nonparametric Kruskal-Wallis tests showed no statistically significant differences in mean alkaline phosphatase ($\chi^2 = 0.232$; p = 0.890), BMI ($\chi^2 = 1.775$; p = 0.412), and BMP ($\chi^2 = 0.121$; p = 0.941) values. PTH concentrations increased progressively as vitamin D levels decreased and the differences were statistically significant (χ^2 = 6.587; p = 0.037). Post-hoc Mann-Whitney tests showed statistically significantly lower PTH concentrations in the group with normal vitamin D levels compared with the group with deficiency (p = 0.024).

The parameters of calcium-phosphorus metabolism (calcium, phosphorus, ALP, and PTH), vitamin D, BMI and BMD and the results of the comparative analyses of the control group patients are presented in Table 7.

Table 7. Calcium-phosphorus metabolism, BMI and BMD of the control group participants with different levels of Vitamin D

Control group	**Vitamin D levels**			
	Norm > 50 nmol/l	**Insufficiency 25 – 50 nmol/l**	**Deficiency < 25 nmol/l**	**Statistical significance**
Age - years	62.3±5.3	64.5±9.4	74.2±7.1	p=0.018*
Calcium – mmol/l	2.47±0,13	2.45±0.08	2.51±0.1	p=0.495
Phosphorus – mmol/l	1.21±0,12	1.23±0.13	1.02±0.1	p=0.004**
ALP – U/l	86.2±51.87	71.89±13.85	106.4±56.78	p=0.197
PTH – pg/ml	45.26±10.40	76.2±26.5	66.72±5.24	p=0.003**
Vitamin D – nmol/l	65.33±12.55	36.55±7.62	15.38±5.98	p<0.001***
BMI – kg/m^2	27.77±4.08	30.66±5.5	31.08±5.07	p=0.163
BMD – T - score	0.08±0.62	0.22±0.66	0.12±0.94	p=0.770

Results are presented as mean ± SD; Statistically significant difference, p < 0.05*; p < 0.01**; p < 0.001***

One-way analysis of variance to compare groups with different vitamin D levels showed no statistically significant difference in mean calcium concentrations (F = 0.718; p = 0.495). Mean phosphorus concentrations were statistically significantly different (F = 6.571; p = 0.004). Tukey's post-hoc test showed that participants with normal and deficient vitamin D levels had statistically significantly higher phosphorus concentrations (M = 1.21) than participants with deficiency (M = 1.23; p = 0.010), respectively (M = 1.02; p = 0.003). A statistically significant difference was observed in mean age (F = 4.529; p = 0.018), and post-hoc tests showed a statistically significantly higher mean age of women with vitamin D deficiency (M = 72.2) compared to the age of women with normal levels (M = 62.3; p = 0.014) and with vitamin D insufficiency (M = 64.5; p = 0.046), respectively. There was an expected

statistically significant difference in mean vitamin D concentrations (F = 62.319; p < 0.001).

Nonparametric Kruskal-Wallis tests showed no statistically significant differences in mean alkaline phosphatase ($\chi^2 = 1.250$; p = 0.197), BMI ($\chi^2 = 3.630$; p = 0.163), and BMP ($\chi^2 = 0.524$; p = 0.770) values.

Statistically significant differences were found in mean PTH concentrations ($\chi^2 = 11.78$; p = 0.003). Mann-Whitney post-hoc tests showed significantly lower mean PTH concentrations in the normal vitamin D levels group compared with the deficiency group (p < 0.001).

PTH concentrations at different vitamin D levels in women with OP and controls are shown in Figure 11.

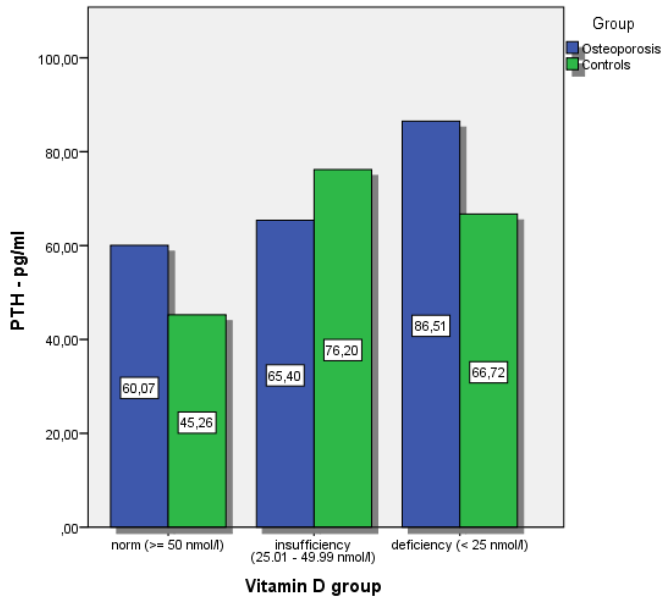


Fig. 11. *Mean plasma PTH concentration in pg/ml of participants from the working and control groups with different vitamin D concentrations. The mean PTH concentrations in the group with normal vitamin D levels were statistically significantly lower than the mean PTH concentrations in the deficient and OP groups, p < 0.05, and in the control group, p < 0.01.*

Comparison between women in the work and control groups with different vitamin D levels revealed no statistically significant differences in mean age, calcium, phosphorus, ALP, PTH, and vitamin D concentrations (p > 0.05).

Patients in the OP group showed statistically significantly lower BMI compared with controls at all vitamin D levels: normal vitamin D levels (U = 88.50; p = 0.003), insufficiency (U = 74.00; p < 0.001), and deficiency (U = 1.00; p < 0.001) (Fig. 12). The figure shows that only in the control group BMI increase progressively with worsening vitamin D status, but comparison of BMI between subgroups with different vitamin D levels showed no statistically

significant differences in either the OP group ($\chi^2 = 1.775$; $p = 0.412$) or the control group ($\chi^2 = 3.63$; $p = 0.163$).

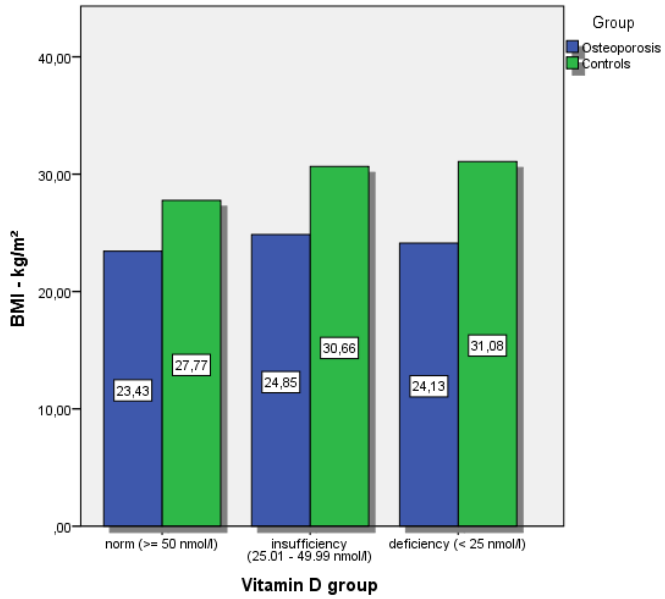


Fig. 12. *Mean BMI in kg/m² of participants in the treatment and control groups with different vitamin D concentrations. At all vitamin D levels, differences in BMI between women in the work and control groups were statistically significant $p < 0.01$.*

Comparison of BMD between subgroups with different vitamin D levels showed no statistically significant differences in either the OP group ($\chi^2 = 0.121$; $p = 0.941$) or the control group ($\chi^2 = 0.524$; $p = 0.770$).

Analysis of the data on fractures in the participants at the time of inclusion showed that 17.7% (n = 11) of the women in the study group with a mean age of 68.82 ± 12.07 years had vertebral fractures and 19.4% (n = 12) with a mean age of 64.42 ± 8.43 years had forearm fractures. In the control group, 28.9% (n = 11) aged 68.91 ± 8.71 years had forearm fractures. 62.9% of women with OP (65.77 ± 7.71 yrs) and 71.1% of controls (63.26 ± 7.89 yrs) were fracture-free at the time of inclusion. A comparison of vitamin D levels in the OP group showed statistically significantly lower levels in women with vertebral fractures than those with forearm fractures ($t = 2.265$; $p = 0.032$). The difference in vitamin D levels between women with forearm fractures and those without fractures did not reach statistical significance in neither the OP group ($t = -1.620$; $p = 0.123$) nor in the control group ($t = -0.069$; $p = 0.946$) (Fig. 13).

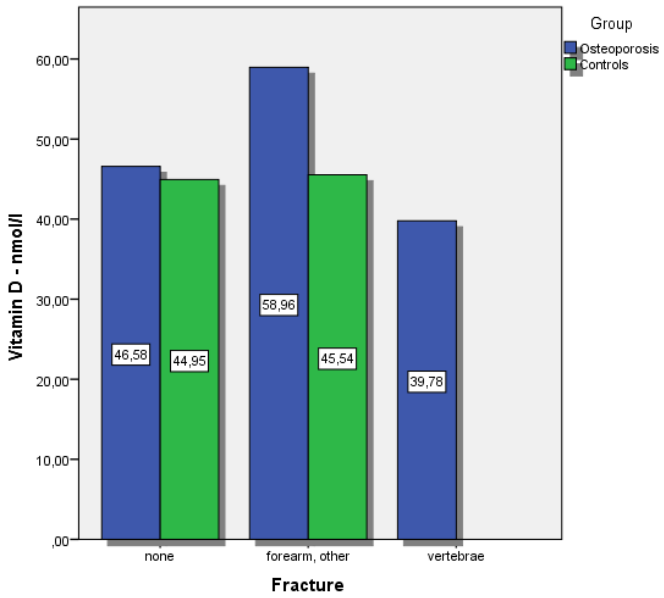


Fig. 13. *Mean vitamin D concentrations in nmol/l in the working and control groups in fractures of different localization. In the OP group, vitamin D levels in women with vertebral fractures were statistically significantly lower than in women with fractures of other localization, $p < 0.05$.*

Vitamin K2

Vitamin K2 status was determined indirectly by serum osteocalcin and undercarboxylated osteocalcin concentrations and the ucOC/OC ratio. We studied 48 postmenopausal women. After BMD measurement, participants were assigned to the study group of women with OP (n = 26) and the control group (n = 22). Mean age, OC concentrations, ucOC, ucOC/OC, vitamin D, BMI, and BMD are presented in Table 8.

Table 8: Concentrations of OC, ucOC, ucOC/OC, vitamin D, BMI, and BMD expressed by T-score in the working and control groups

	Osteoporosis	**Controls**	**Ниво на значимост**
Age	65.62±9.2	63.55±8.7	p=0.670
OC – ng/ml	23.41±10.08	23.09±6.94	p=0.605
ucOC – ng/ml	17.33±3.38	17.69±3.92	p=0.700
ucOC/OC – %	77.36±22.27	83.63±29.27	p=0.709
ALP – U/l	86.77±48.78	95.5±50.48	p=0.383
Vitamin D – nmol/l	48.09±19.41	48.34±23.1	p=0.943
BMI – kg/m^2	24.56±3.88	29.84±5.45	p<0.001***
BMD – T - score	-2.98±0.52	-0.21±0.85	p<0.001***

Results are presented as mean ± SD. Statistically significant difference p<0.001***.

OC levels in the treatment and control groups were within the reference range of the commercial kit used. The parametric t-test for independent samples and its

nonparametric alternative, the Mann-Whitney test, were used for comparison, depending on the data distribution. There were no statistically significant differences in mean age, vitamin D levels, and OC, ucOC, and ALP concentrations.

The ucOC/OC ratio showed values above the accepted norm of 20% in all women studied, with the lowest value in the OP group being 30.04% and in the control group 36.31%. The results showed poor vitamin K2 status in both groups; no statistically significant difference was found between them. Mean vitamin D levels showed insufficiency in both groups. OP patients had normal body weight compared to controls who were overweight, and the differences in BMI of the two groups were statistically significant, as expected ($t = -4.416$; $p < 0.001$). An expected statistically significant difference was also observed in mean BMD ($U = 0.00$; $p < 0.001$).

The concentrations of OC, ucOC, and ucOC/OC of women in the study and control groups distributed by decade are shown in Table 9.

Table 9. Concentrations of OC, ucOC, and percentage ucOC/OC of women in the treatment and control groups distributed by decade

Age	**OC – ng/ml**		**ucOC – ng/ml**		**ucOC/OC – %**	
	Osteoporosis	**Controls**	**Osteoporosis**	**Controls**	**Osteoporosis**	**Controls**
≤ 59	29.7±13.55	27.8±5.23	17.5±2,74	16.14±3.32	66.5±22.11	59.1±11.71
60 – 69	20.58±3.03	20.85±7.01	15.15±2.87	16.7±2.69	73.95±10.6	88.61±30.62
70 – 79	26.49±9.85	24.57±4,67	16.94±2.46	23.17±5.77	68.24±16.01	96.6±29.9
≥ 80	16.92±0	21.92±0	16.82±0	15.46±0	99.4±0	70.5±0

Results are presented as mean ± SD.

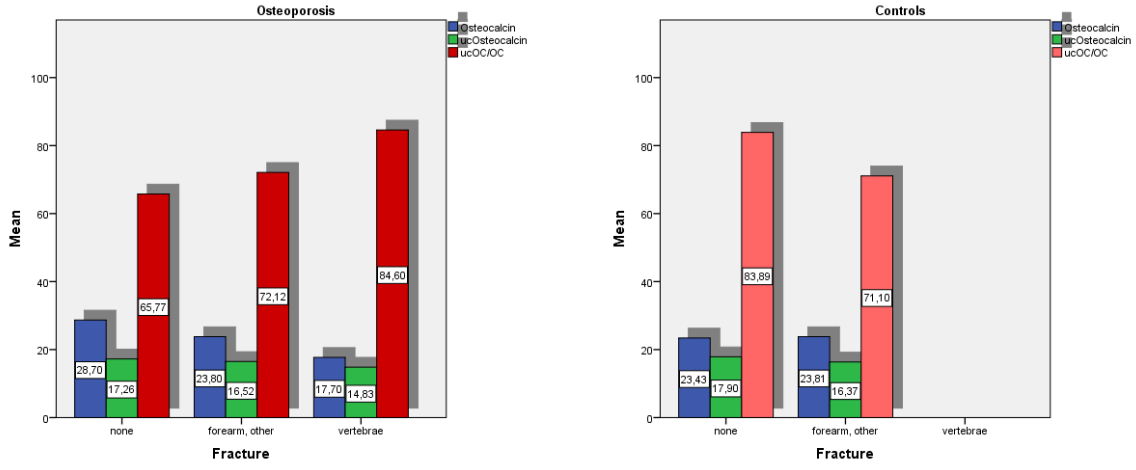
Comparison of parameters by decade with the Kruskal-Wallis nonparametric test in the OP group showed statistically significant differences in OC concentrations ($\chi^2 = 11.379$; $p = 0.01$) and no statistically significant differences in ucOC ($\chi^2 = 0.558$; $p = 0.906$) concentrations and ucOC/OC ($\chi^2 = 3.105$; $p = 0.376$). Mann-Whitney post hoc tests showed statistically significantly higher OC concentrations in the women up to 59, compared to women aged 60 – 69 years ($U = 69.00$; $p = 0.006$) and over 80 ($U = 3.00$; $p = 0.017$). In the controls, no statistically significant differences were found in OC concentrations ($\chi^2 = 4.993$; $p = 0.172$) and ucOC ($\chi^2 = 6.618$; $p = 0.085$) in women across age groups.

A gradual increase in the ucOC/OC ratio with advancing age was also observed in the working and control groups, but the differences were statistically significant only in the control group. Women aged up to 59 years had a statistically

significantly lower ucOC/OC ratio than women aged 60 – 69 (U = 10.00; p = 0.031) and 70 – 79 (U = 1.00; p = 0.048).

Comparison across age groups between women in the study group and the controls did not reveal statistically significant differences in the concentrations of OC, ucOC, and ucOC/OC (p > 0.05).

The mean serum concentrations of OC, ucOC, and ucOC/OC in women in the working and control groups without fractures and with fractures of different localization are shown in Figures 14 and 15. In the OP group, the OC and ucOC values gradually decreased, and the ucOC/OC ratio increased in women without fractures and with forearm and vertebral fractures. The Kruskal-Wallis tests showed a statistically significant difference in OC concentrations in the OP group (χ^2 = 8.534; p = 0.014) only. The post-hoc tests showed OC concentrations statistically significantly lower in women with vertebral fractures than those without fractures (U = 10.00; p = 0.006). Concentrations of ucOC (χ^2 = 2.683; p = 0.261) and the ucOC/OC ratio (χ^2 = 3.081; p = 0.214) showed no statistically significant differences in women without fractures and with fractures of different localization. In the control group, comparison with the Mann-Whitney test results in women with and without fractures showed no statistically significant differences in the concentrations of OC (U = 41.00; p = 0.934) and ucOC (U = 35.00; p = 0.564) and the ucOC/OC ratio (U = 35.00; p = 0.564).

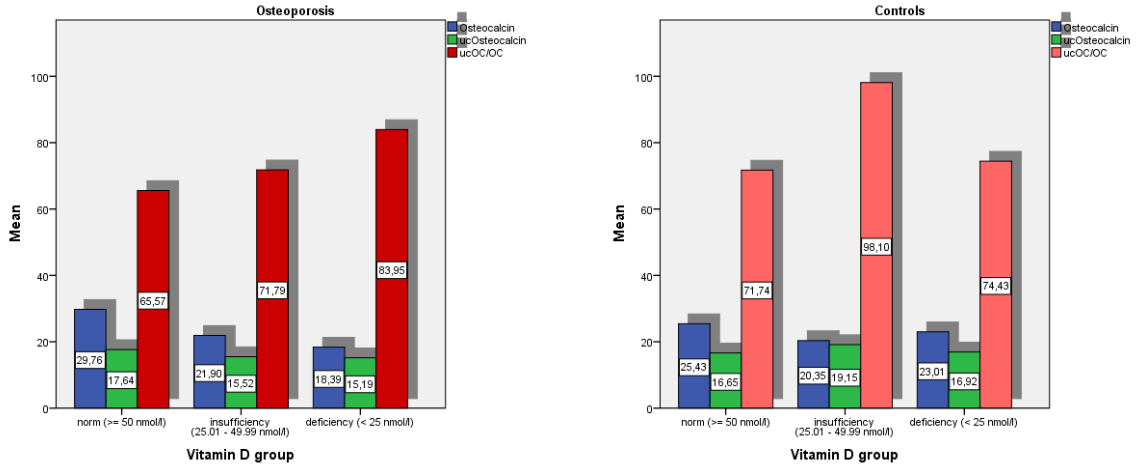


Figs. 14 and 15. *Mean values of serum OC, ucOC, and ucOC/OC in women of the working and control groups with fractures of different localization. In the OP group, OC concentrations in women with vertebral fractures were statistically significantly lower compared to women without fractures, p < 0.05.*

The mean concentrations of OC, ucOC, and the ucOC/OC ratio at different vitamin D levels in the OP group and controls are shown in Figures 16 and 17, respectively. In the OP group, a gradual decrease in OC concentrations was seen at different vitamin D levels, and the difference found by the Kruskal-Wallis test was statistically significant (χ^2 = 6.637; p = 0.036). The Mann-Whitney post-hoc

test showed statistically significantly higher OC concentrations in women with normal vitamin D levels than those with deficiency (U = 5.00; p = 0.015). Mean concentrations of ucOC decreased gradually, and the ucOC/OC ratio increased, but the tests showed no statistically significant differences ($\chi^2 = 3.841$; p = 0.147), respectively ($\chi^2 = 1.276$; p = 0.528) at different vitamin D levels.

In the control group, Kruskal-Wallis tests showed no statistically significant differences in OC ($\chi^2 = 3.127$; p = 0.209) and ucOC ($\chi^2 = 0.651$; p = 0.722) concentrations at different vitamin D levels. Statistically significant differences were observed in the ucOC/OC ratio ($\chi^2 = 6.117$; p = 0.047). Mann-Whitney post-hoc tests showed statistically significantly lower levels in the ratio (U = 10.00; p = 0.020) in women with norm compared to those with vitamin D insufficiency.



Figs. 16 and 17. *Mean serum OC, ucOC and ucOC/OC values in women of the work and control groups with different vitamin D levels. In the OP group, statistically significant differences in OC concentrations were observed in women with normal and vitamin D deficiency, p < 0.05. In the control group, statistically substantial ucOC/OC ratio differences were observed in women with normal and vitamin D deficiency, p < 0.05.*

Beta CrossLaps

The bone resorption marker was tested only in the women of the study group. Measured serum concentrations were 0.589 ± 0.251 ng/ml /0.06 – 1.20/ and were within reference values. Beta CrossLaps concentrations by decade are shown in Figure 18, and the one-way analysis of variance performed showed no statistically significant differences in concentrations between age groups (F = 0.045; p = 0.987).

The mean concentrations of beta CrossLaps in the normal vitamin D group were 0.555 ± 0.227 ng/ml, and in the deficient and insufficient groups, 0.649 ± 0.278 ng/ml and 0.560 ± 0.260 ng/ml, respectively. One-way analysis of variance showed no statistically significant differences in marker concentration between groups with different vitamin D levels (F = 0.645; p = 0.530). Beta CrossLaps

concentrations in women without fracture were 0.620 ± 0.284 ng/ml, and in women with forearm and vertebral fractures were 0.539 ± 0.187 ng/ml and 0.545 ± 0.206 ng/ml, respectively. We found no statistically significant differences in beta CrossLaps concentrations between women without fractures and those with fractures of different localization (F = 0.487; p = 0.618).

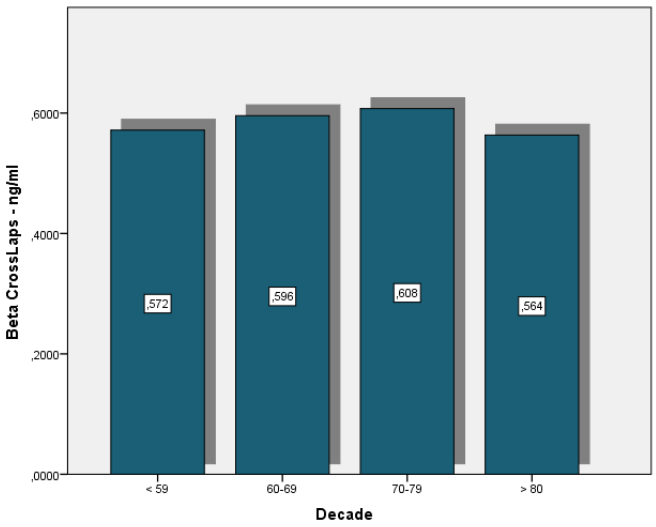
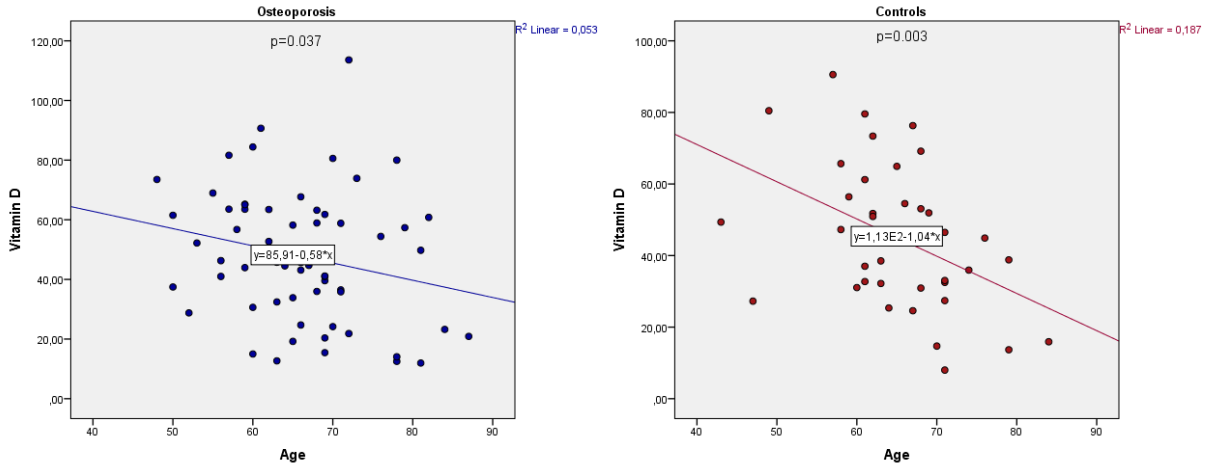


Fig. 18. *Mean serum concentrations of Beta CrossLaps in women with OP distributed by decade. Differences are not statistically significant, p > 0.05.*

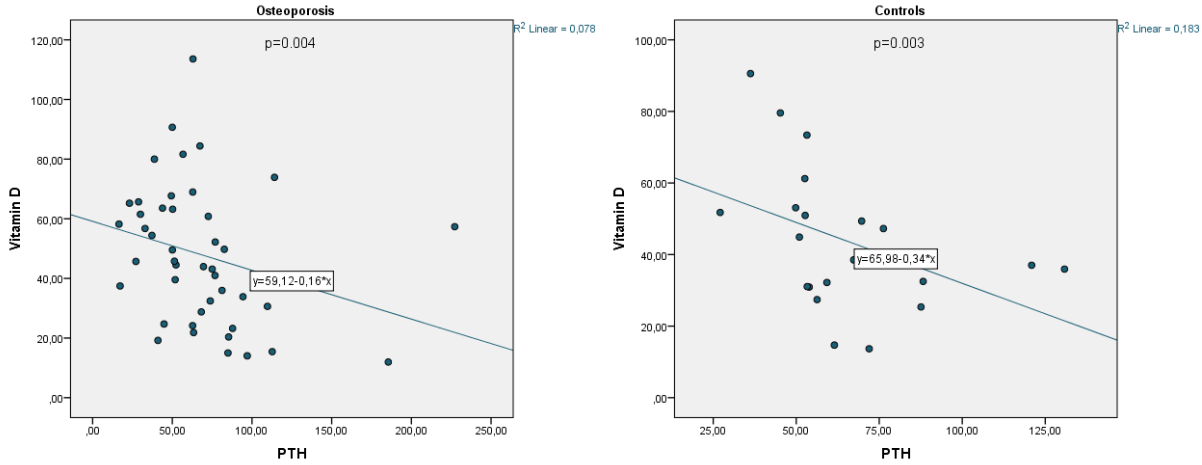
Correlations between vitamin D and indices of calcium-phosphorus metabolism, BTM, BMD, BMI, fracture risk and vitamin K2

Analyses showed a negative correlation between vitamin D levels and age, weak in strength (r = -0.229; p = 0.037) in the OP group and moderate in strength (r = -0.432; p = 0.003) in the control group (Figs. 19 and 20).



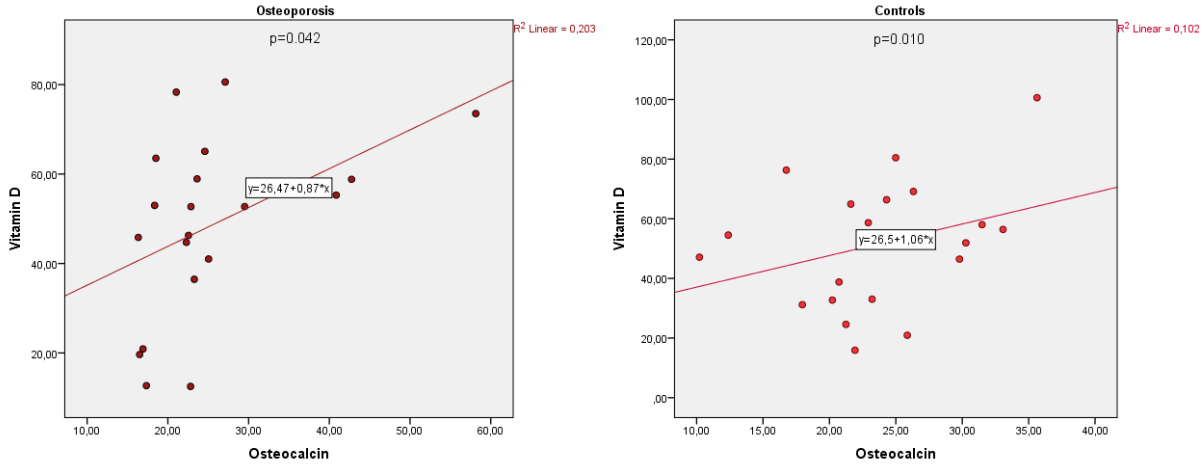
Figs. 19 and 20. *Regression model of negative correlation between serum vitamin D concentrations and mean age in the working and control groups.*

We found a moderate strength negative correlation between vitamin D and PTH in the OP group (r = -0.421; p = 0.004) and a significant strength in the control group (r = -0.618; p = 0.003) (Figs. 21 and 22).



Figs. 21 and 22. *Regression models of negative correlation between serum vitamin D concentrations and PTH in women in the work and control groups.*

Vitamin D and OC levels showed a positive correlation, moderate in strength in the OP group (r = 0.489; p = 0.029) and significantly strong in the control group (r = 0.535; p = 0.010) (Figs. 23 and 24).



Figs. 23 and 24. *Regression models of positive correlation between serum vitamin D concentrations and OC in the treatment and control groups.*

In the OP group, vitamin D levels also showed a moderate positive correlation with ucOC concentrations (r = 0.478; p = 0.033) (Fig. 25).

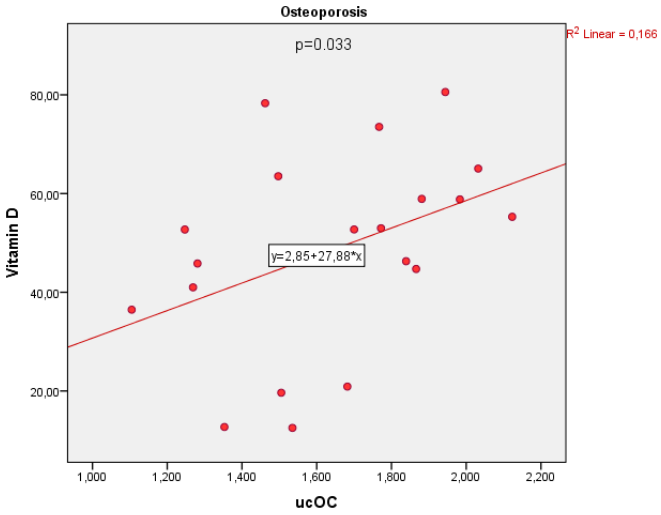
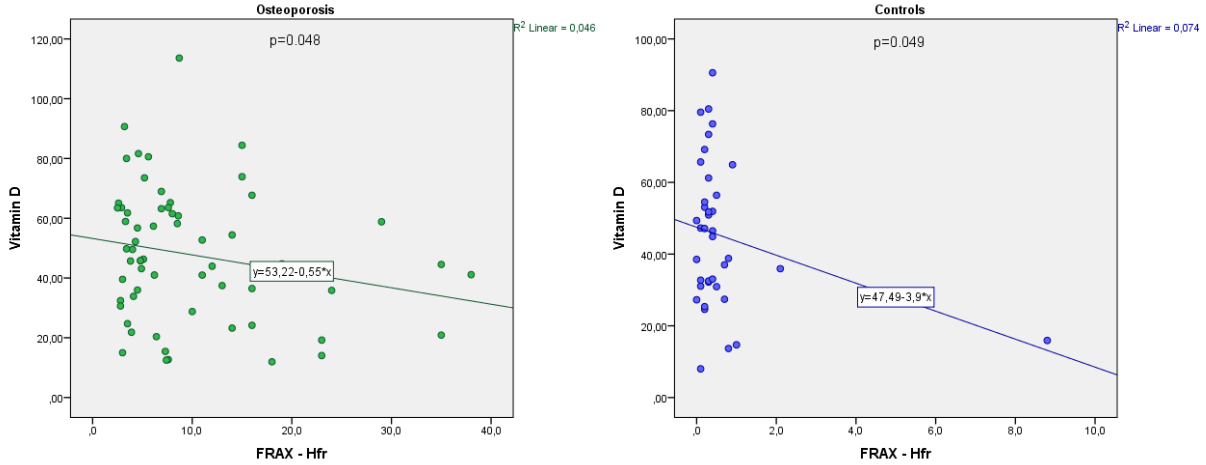


Fig. 25. *Regression model of moderate positive correlation between serum vitamin D concentrations and ucOC in the study group.*

We found a weak negative correlation between vitamin D and FRAX-Hfr (r = -0.214; p = 0.048) in women with OP (Fig. 26) and in controls (r = -0.273; p = 0.049) (Fig. 27).



Figs. 26 and 27. *Regression model of negative correlation between vitamin D levels and FRAX-Hfr in women in the study group and the controls.*

No significant correlations were found between vitamin D and BMI, BMD, calcium, phosphorus, ALP, ucOC/OC, and beta CrossLaps in the study group (p > 0.05). Also, we found no significant correlations between ucOC/OC and BMD, FRAX-MO, and FRAX-Hfr (p > 0.05). In the control group, we also found no significant correlations between vitamin D and BMI, BMD, calcium, phosphorus, ALP, ucOC, ucOC/OC (p > 0.05), and also between ucOC/OC and BMD, FRAX-MO and FRAX-Hfr (p > 0.05).

A moderate strength positive correlation with increasing PTH concentrations with increasing mean age was found in the OP group (r = 0.422; p = 0.004) (Fig. 28), but not in the control group (r = 0.244; p = 0.287).

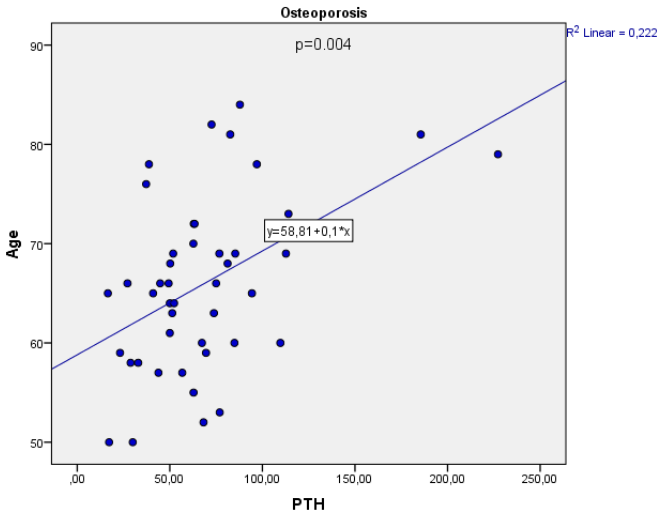


Fig. 28. *Regression model of positive correlation between mean age and serum PTH concentrations in study group women.*

In the OP group, we also found negative correlations between BMP and FRAX-MO, significant in strength (r = -0.621; p < 0.001), and FRAX-Hfr, very strong (r = 0.938; p < 0.001) (Fig. 29).

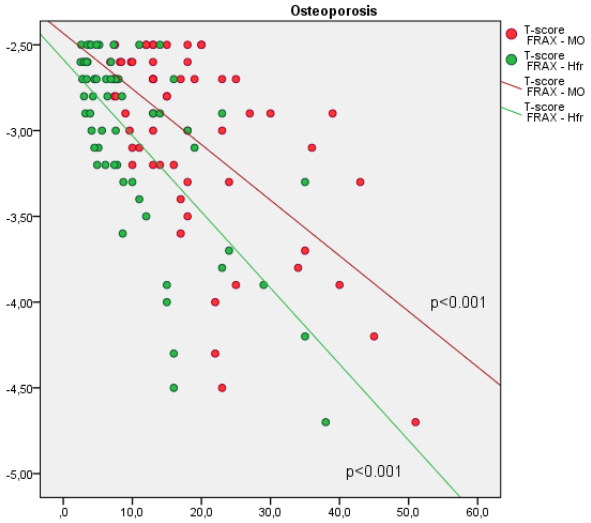


Fig. 29. *Regression model of negative correlation between BMD expressed by T-score and fracture risk FRAX-MO and FRAX-Hfr in percentage in the study group.*

There was a positive moderate strength correlation between age and FRAX-Hfr in the study group (r = 0.315; p = 0.003) (Fig. 30). With advancing age, the risk of hip fracture in women with OP increases.

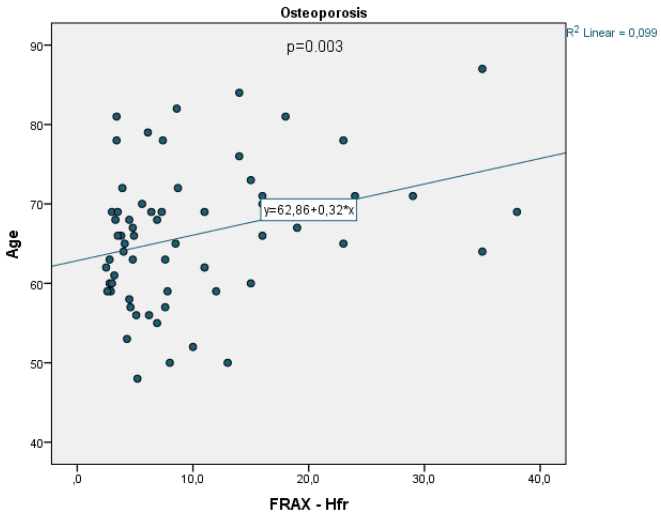
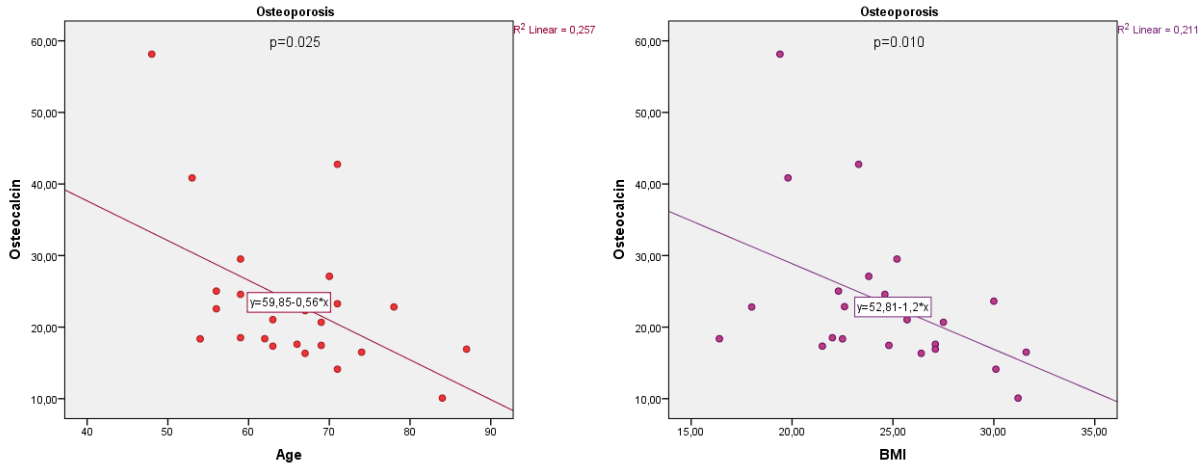


Fig. 30. *Regression model of positive correlation between mean age and FRAX-Hfr fracture risk in percentage in the working group.*

In the OP group, serum OC concentrations showed moderate negative correlations in strength with decreasing concentrations with increasing age (r = -0.440; p = 0.025) and BMI (r = -0.496; p = 0.010) (Figs. 31 and 32).



Figs. 31 and 32. *Regression models of negative correlations between OC concentrations and age and BMI in the women with osteoporosis.*

In this group, we found a moderate positive correlation between OC and ALP (r = 0.457; p = 0.025) and a significant negative correlation in strength between ucOC/OC and ALP (r = -0.574; p = 0.003) (Fig. 33).

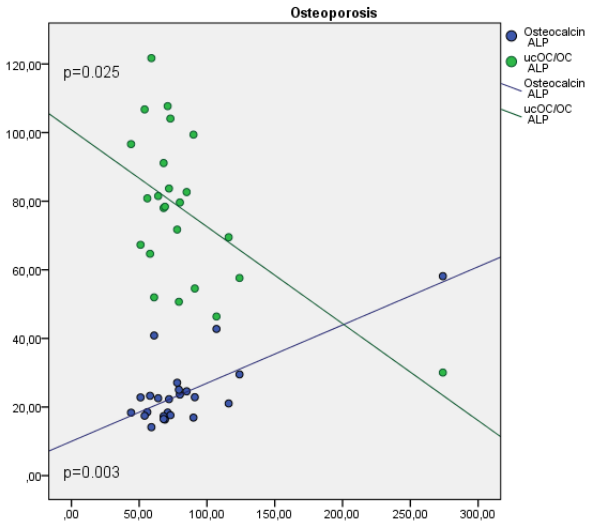


Fig. 33. *Regression models of moderate positive correlation between OC and ALP and moderate negative correlation between ucOC/OC and ALP in the women with OP.*

In women with OP, serum OC showed a moderate strength negative correlation with decreasing concentrations with increasing fractures (r = -0.460; p = 0.041) (Fig. 34). The results of single linear regression analysis showed that OC concentrations could statistically significantly predict the occurrence of fractures in OP patients, (F = 4.60; p = 0.042) with a moderate effect size. The regression constant **a** = 1.44; p = 0.001, and the regression coefficient **b** = -0.03, p = 0.042 were also statistically significant, and the value of the adjusted coefficient of determination (adjusted R2) was 0.13.

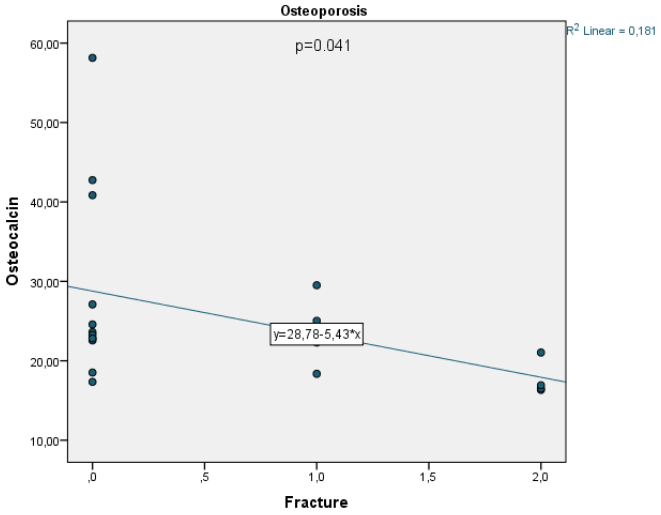


Fig. 34. *Regression model of negative correlation between serum OC concentrations and fractures in the study group.*

A positive correlation of significant strength was also found between the ucOC/OC ratio and BMI (r = -0.557; p = 0.011) (Fig. 35).

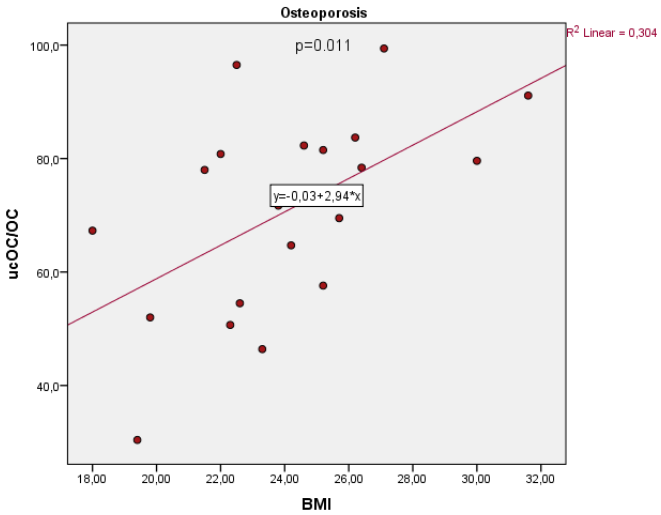
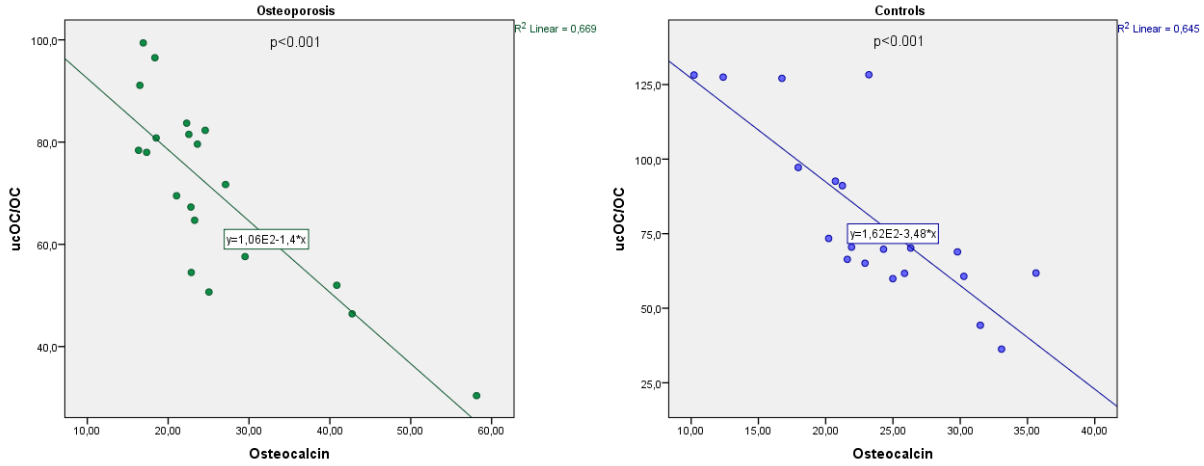


Fig. 35. *Regression model of positive correlation between ucOC/OC ratio and BMI in the study group.*

The ucOC/OC ratio showed a high negative correlation with OC in both the OP group (r = -0.772; p < 0.001) and the control group (r = -0.839; p < 0.001) (Figs. 36 and 37).



Figs. 36 and 37. *Regression model of negative correlation between ucOC/OS ratio and OC in the study and control groups.*

In the OP group, a moderate negative correlation was found with a decrease in beta CrossLaps concentrations as BMP increased (r = -0.326; p = 0.033) (Fig. 38).

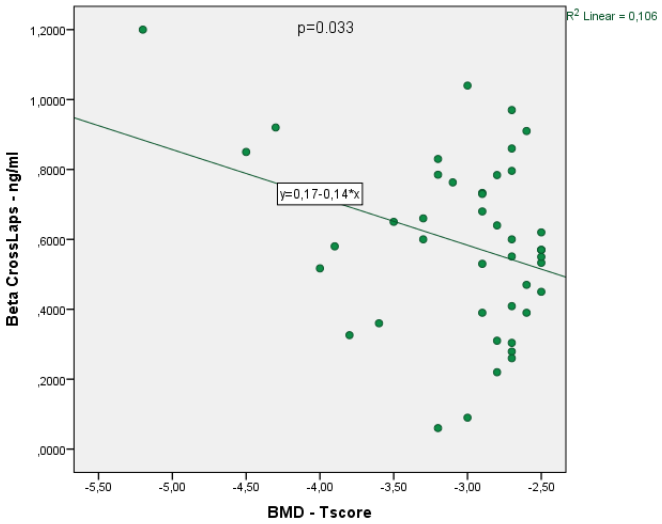
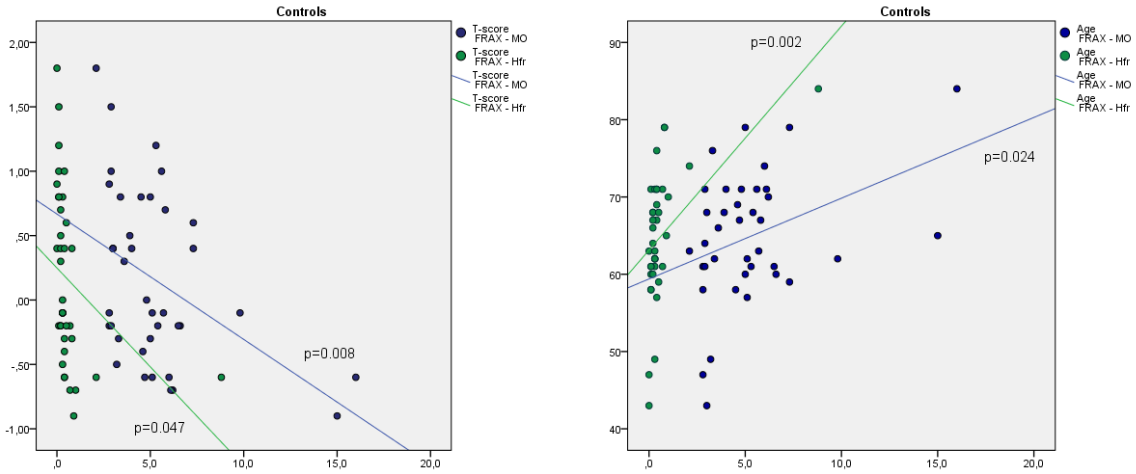


Fig. 38. *Regression model of negative correlation between serum beta CrossLaps concentrations and BMD expressed by T-score of the lumbar spine.*

In the control group, we found a negative correlation between BMD and fracture risk, moderate in strength for FRAX-MO (r = -0.427; p = 0.008) and significant for FRAX-Hfr (r = -0.325; p = 0.047) (Fig. 39). FRAX-MO (r = 0.365; p = 0.024) and FRAX-Hfr (r = 0.488; p = 0.002) showed a moderate positive correlation with mean age (Fig. 40).

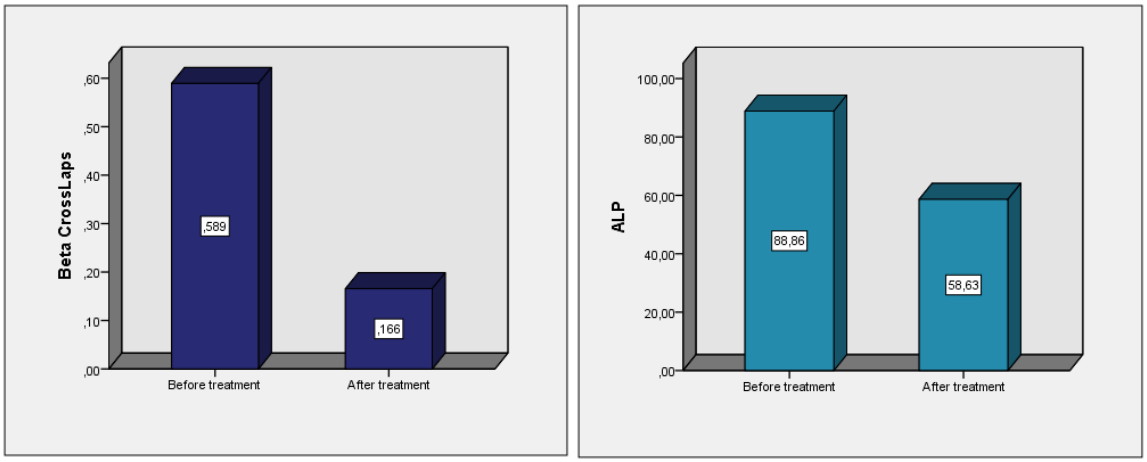


Figs. 39 and 40. *Regression models of negative correlation between BMD expressed by T-score, FRAX-MO, and FRAX-Hfr and positive correlation between mean age, FRAX-MO, and FRAX-Hfr in the control group.*

Dynamics in serum concentrations of beta CrossLaps and alkaline phosphatase after six months of treatment with Denosumab

Beta CrossLaps and ALP concentrations were measured before and after treatment with Denosumab at a dose of 60 mg subcutaneously once every 6 months. The parametric t-test for correlated samples was used for comparison between groups. Pre- and post-treatment Beta CrossLaps cancentrations were

within reference values, 0.589 ± 0.266 ng/ml and 0.166 ± 0.139 ng/ml, respectively (Fig. 41), and the decrease of more than 72% from baseline levels was statistically significant (t = 7.351; p < 0.001). According to the Bulgarian Society of Endocrinology consensus, a decrease of more than 56% from baseline marker values between the 3rd and 6th month of treatment is considered a success. Alkaline phosphatase pre- and post-treatment values were within reference values, 88.86 ± 22.21 U/l and 58.63 ± 18.34 U/l, respectively (Fig. 42), and the decrease was statistically significant (t = 6.372; p < 0.001).



Figs. 41 and 42. *Bone resorption marker beta CrossLaps concentrations in ng/ml and ALP in U/l before and after six months of treatment. For both parameters, the difference was statistically significant p < 0.001*

DISCUSSION

Osteoporosis is a metabolic syndrome in which bones become fragile due to loss of minerals and bone matrix in approximately equal proportions. Postmenopausal OP is the most common type. Estrogen deficiency results in increased bone turnover due to effects on all types of bone cells, and bone formation and resorption imbalances affect both trabecular and cortical bone. Bone mineral density in the L1 – L4 vertebral region in middle-aged and elderly individuals decreases with age. With the onset of menopause, the rate and extent of bone loss vary according to its duration and the different skeletal sites to be measured. Hormonal imbalance, aging, environmental factors, lifestyle, and genetic predispositions are responsible for about 50 – 80% of individual BMD loss. In this period, the cause of poor bone quality is the gradual loss of minerals from bone associated with aging and other age-related conditions such as impaired muscle function, vitamin D deficiency, and secondary hyperparathyroidism. In a longitudinal BMD study of 366 healthy postmenopausal women, Warming et al. found a small degree of bone loss in the femoral neck and vertebral column before the onset of menopause and a threefold increase in the first ten years after menopause. After this period, bone loss in the spine decreases to zero. In the control group of women we studied, BMD in the spine decreased gradually with advancing age, being lowest at age 80 years and older. However, differences across decades of menopause did not reach statistical significance. Similar results were reported by Ohta et al. They compared BMD in the lumbar spine of women with early-onset and normal menopause. They reported no decrease in BMD with advancing age in women with normal onset menopause. Results from a five-year multicentre longitudinal cohort study of 9423 Canadian women and men over the age of 25 showed that bone loss in women begins after the age of 40 years and occurs to varying degrees and at different rates in the femoral neck and lumbar spine. In the spinal region, it is most pronounced in the perimenopausal period and the first years after the onset of menopause. After age 60, BMD in the lumbar spine remains almost constant relative to the hip joint sites. This pattern probably reflects differences in bone composition in the lumbar spine, which has a greater content of trabecular rather than cortical bone compared with the femoral neck, as well as differences in mechanical stresses in the lumbar spine and hips or graded differences in the development of osteophytosis and other degenerative changes that more markedly affect the measurement of vertebral bone density, masking the loss of trabecular bone.

Osteoporosis is characterized by decreased BMD and bone strength, creating an increased risk of low-energy fractures. Women with OP show significantly lower BMP measured at all skeletal sites, which is a criterion for diagnosis. In our study, BMD measured in the lumbar spine by DXA in the OP group was statistically significantly lower than BMD in the control group. The rate of bone loss depends on many factors, such as gender, hormonal status, calcium status, body weight, degree of physical activity, genetic makeup, and regional characteristics of bone metabolism, and is not evenly distributed throughout the skeleton. Many experimental studies of bone loss rates are limited to specific bony sites, with the trabecular vertebral bone loss rate being significantly faster than that of cortical long bone. In the early stages of postmenopausal OP, estrogen deficiency causes increased RANKL expression, increased osteoclast number and activity, and concomitant suppression of osteoblast function. Subsequently, increased net bone resorption outpacing bone formation causes rapid loss of mainly trabecular bone mass. We found that women with OP aged 70 – 79 years had statistically significantly lower BMD in the lumbar spine compared with women in the 50 – 59 age group but not those in the 60 – 69 age group. There is insufficient data in the literature on the extent and rate of bone loss in women with postmenopausal OP. Studies have mainly focused on premenopausal and early postmenopausal women, and BMD in older women is very important for the risk of osteoporotic fractures. In women with OP, there is undoubtedly an imbalance in bone remodeling processes. The rate of bone resorption is increased, with an unchanged or delayed bone formation rate, and the accelerated loss affects the spongiosed bones more. These processes may explain the appearance of the statistically significant difference in spine BMD with advancing age. In women with OP, the decrease in BMD with advancing age appears to be more pronounced and persists to a greater extent compared with healthy controls. In women with OP, musculoskeletal health appears to be particularly compromised by immobilization and by low levels of exercise. Limited physical activity and various health problems in older women reduce dynamic loads on bone and further increase bone loss. Impaired musculoskeletal health with muscle and bone soreness in women with OP may further limit their daily activity, increase the incidence of falls, and consequently increase the risk of fractures.

Osteoporotic fractures increase with age, but modern medicine has a wide variety of pharmacological and non-pharmacological therapies that favorably affect bone mass and thus reduce the risk of fractures. Their use by healthcare practitioners is aided by tools that assess individual fracture risk. The world’s

most widely used web-based tool, FRAX®, calculates the ten-year probability of low-energy bone fractures, considering several common clinical risk factors and optional BMD measurement results, and can facilitate treatment decision-making. At the end of 2020, thanks to the efforts of Kirilova et al., the FRAX® calculator was validated for our country. The estimated 10-year probability of major osteoporotic and femoral fractures in women with a prior fracture in Bulgaria is consistently higher than in Serbia and Romania, lower than in Turkey, and similar to Greece. The probability of Bulgarian women aged over 50 years to sustain a femur fracture by the end of their lifetime is 11.2%, which is midway between Romania and Serbia (7.1 and 7.7%, respectively) and Greece and Turkey (15.4 and 15.9%, respectively).

Due to regional differences in fracture epidemiology and clinical risk factors, it is reasonable to compare our results with those obtained in Bulgaria and the region, which is difficult due to still insufficient experience with applying the algorithm. The analysis of fracture risk in the working and control groups showed a statistically significant difference for both FRAX-MO and FRAX-Hfr between the group of women with OP and controls. In both groups studied, fracture risk increased with age. We also found a positive correlation of moderate strength with an increase in FRAX-Hfr with an increase in mean age in postmenopausal women, which persisted in the OP and control groups. In women with osteoporosis, FRAX-Hfr was increased by over 3% in all age groups, and FRAX-MO was increased by over 20% only in women over 70. There is ample published epidemiological evidence of an increase in fracture incidence with advancing age, reflecting the progressive decline in BMD, particularly at the femoral neck, and the increased risk of falls in patients with OP. Our results confirm that the risk of osteoporotic fracture is higher in women with OP and increases with advancing age. We also found a statistically significantly higher risk of major osteoporotic fracture in women with prior fractures in both the OP and control groups, with no statistically significant differences in BMD between women with and without prior fractures. Kirilova and Vladeva Similar reported results in a study of risk factors contributing to fracture risk in 101 postmenopausal women aged 65 ± 11, divided into an elevated fracture risk group and a normal fracture risk group. They found that women with increased fracture risk were statistically significantly older and had lower BMD. They also reported a statistically significant difference and increased fracture risk among women with prior fractures and those without fractures. Results from an analytical cross-sectional study of 364 Romanian postmenopausal women, 228 of whom were diagnosed with primary OP by DHA,

showed a statistically significant difference in FRAX® calculated ten-year fracture risk between the OP group and healthy controls. The values they found for FRAX-MO and FRAX-Hfr in the OP group were 8.04 ± 4.68 and 2.76 ± 2.97, respectively, and were significantly lower and below the threshold values for increased fracture risk compared with the values we obtained of 18.29 ± 10.33 and 9.92 ± 8.51. The FRAX-MO and FRAX-Hfr values of 4.34 ± 2.47 and 0.82 ± 1.47 in the control group, respectively, were comparable to ours of 5.26 ± 2.93 and 0.61 ± 1.42 and below the threshold values.

The results of our study demonstrate a significant correlation between BMD and fracture risk. We found a strong negative correlation with an increase in FRAX-MO and FRAX-Hfr with a decrease in spinal BMD in both the OP group and controls. Similar results were reported by Liu et al., who studied 189 participants with OP aged 50 – 86 years and found a moderate negative correlation between BMD expressed by T-score in the lumbar spine and fracture risk calculated by FRAX®. Our findings of increased fracture risk in women with OP and the negative correlation with BMD in the spine are consistent with published results from epidemiological and observational studies by other authors. The FRAX® calculator is straightforward, and results for individual fracture risk are quickly calculated. This calculator offers a good opportunity to use these results to form the basis of non-pharmacological and pharmacological strategies to prevent osteoporotic fractures. It should stimulate its wider use by medical professionals in our country.

Body weight and BMI are important elements of assessing patients with OP because they are risk factors for developing the disease. It is now accepted that women with a high BMI traditionally have a higher BMD and are protected from OP. Menopause is characterized by the cessation of estrogen production and is associated with increased bone loss and weight gain at the expense of fat. Adipose tissue of premenopausal women is a relatively small source of estrogens, but in postmenopausal women, aromatization of estrogen precursors in adipose tissue becomes their major source. In postmenopausal obesity, increased synthesis of estrogens in adipose tissue is thought to be one potential mechanism for the protective effect of adiposity on bone. A low BMI ≤ 19 kg/m^2 is considered a risk factor for the development of OP. A low BMI predisposes postmenopausal women to rapid bone loss and low bone mass, which is critical in the pathogenesis of OP. However, a specific chart of BMI values to accurately predict OP has yet to be fully established. We found significant differences in the BMI of the groups of women studied. The women with OP had a statistically significantly lower

mean BMI than the control group. Female OP patients had a mean BMI within the normal body weight range of 24.08 kg/m^2, while controls had a mean BMI within the overweight range of 29.57 kg/m^2. Similar results were reported by Gurban et al. in a study of 149 Romanian postmenopausal women. The authors found BMI significantly lower in the OP group (25.1 ± 4.5 kg/m^2) than in the controls (29.2 ± 5.1 kg/m^2). A retrospective study of the association between body composition and BMI in 111 postmenopausal women published by Nikolov et al. reported similar statistically significant differences. Patients with lumbar spine T-scores ≤ -2.5 had significantly lower BMI than those with T-scores > -2.5.

Extensive data show that in healthy premenopausal and postmenopausal women, BMI correlates positively with BMD and negatively with fracture risk. The association between increasing body weight and decreased osteoporotic fracture incidence has long been statistically demonstrated in epidemiological studies. Overweight individuals have a higher BMD compared with individuals of lower body weight and are more protected against bone fractures than those who are underweight. Our results support the evidence that a higher BMI is a protective factor against the development of OP. However, they are somewhat at odds with the conventional wisdom that BMI correlates positively with BMD because analyses in the study and control groups did not confirm correlations between BMI and BMD. Women with OP showed a gradual decrease in BMD with a decrease in BMI in the overweight, normal-weight, and underweight groups, but the differences did not reach statistical significance. The women in the control group also lacked statistically significant differences in BMD at different BMIs. Evidence in the scientific literature indicates that BMI cannot be a valid predictor of BMD because of weak correlations. Lack of correlations between BMI and BMD was also reported by Gurban et al. in the previously cited study of 149 Romanian postmenopausal women. Published results from another study conducted among 140 postmenopausal Iranian women also showed no correlation between BMI and BMD in the spine. Partially similar to our results are those published by Dytfeld et al. and Głogowska-Szeląg in two studies of the correlation between BMI and BMD in Polish women with postmenopausal OP. In both studies, the participants were divided into three groups – normal weight, overweight and obese. Dytfeld et al. studied 92 women with postmenopausal OP and found no correlation between BMI and BMD in the lumbar spine but reported a positive correlation with body weight and fat mass. Głogowska-Szeląg studied 120 women and found no statistically significant differences in BMD of the lumbar spine in normal-weight and overweight women but reported a positive

correlation between parameters. The reasons for the conflicting data on the correlations between BMI and BMI are partly related to the number of individuals studied, differences in the definition and ethnicity of the analysis groups, the site of measurement of BMI and the pattern of body fat distribution, and the relationship between lean body mass, amount of adipose tissue, and BMI.

Bone strength is determined by their basic composition and structure and can be expressed in terms of two main characteristics: bone quality and bone density. The measurement of BMD only provides information on the amount of minerals in the bone, which is one of the characteristics. Bone quality describes other aspects of bone composition and structure contributing to bone strength independent of BMD. These include bone turnover, microarchitecture, mineralization, microdamage, and bone matrix composition. Calcium and phosphorus are deposited in bone, and calcium metabolism is intricately linked to hormone metabolism and bone aging. Bones provide a homeostatic mineral reservoir, primarily for calcium but also for other minerals, especially phosphorus and magnesium. The metabolic function of bone predominates over its structural function, as calcium and other minerals are removed from and replaced in bone to serve systemic homeostatic needs despite the loss of structural integrity of the skeleton. Adequate calcium intake is, therefore, essential for normal growth and development of the skeleton and teeth and adequate bone mineralisation. Calcium deficiency is one of the major risk factors for OP. Low serum concentrations involve homeostatic mechanisms affecting bone remodeling and inducing enhanced bone resorption.

Phosphorus is the second major component of bone tissue after calcium. In the human body, 85% of macronutrients, such as phosphoproteins and hydroxyapatite crystals, are found in bones and teeth. Adequate levels of inorganic phosphorus are important for the activity of osteoblasts and osteocytes in the process of matrix mineralization. It has been suggested that both deficiency and excess calcium and phosphorus may represent a risk factor for developing bone diseases such as OP. Changes in serum calcium and phosphorus concentrations in OP have been studied in humans and animals, but the results have been inconsistent. Various literature sources suggest that postmenopausal OP most often does not show changes in their serum concentrations, but the data are inconclusive. Several studies have shown that determining serum calcium and phosphorus concentrations has no clinical relevance in diagnosing OP, as their results are within reference values. Our results showed serum calcium and phosphorus concentrations within reference values for the laboratory assays used in the study

and control groups. Calcium concentrations were lower, and phosphorus concentrations were higher in the OP group compared with the control group, but the differences were not statistically significant.

The normal serum calcium and phosphorus concentrations in both postmenopausal women with OP and healthy postmenopausal women can be explained by decreased total bone mass. OP causes a decrease in the total amount of mineralized bone without a decrease in the ratio of bone mineral to organic matrix. Our results in both the OP group and controls showed relatively constant calcium levels and a gradual decrease in phosphate within reference values with advancing age. However, we found no statistically significant difference in the concentrations of the two elements when comparing the groups by decade. This partially agrees with published data from several studies of calcium-phosphorus metabolism parameters in postmenopausal OP patients and healthy postmenopausal women. Al-khakani et al. found serum calcium and phosphorus concentrations within reference ranges in women with postmenopausal OP and healthy postmenopausal women. In the OP group, the values were lower compared to the control group of healthy postmenopausal women. However, they reported statistically significant differences within the reference values for calcium and phosphorus concentrations in both groups. Sunithapriya et al. reported a statistically significant decrease in calcium concentrations in women with OP compared with a control group of healthy women. The authors found a statistically significant increase in the OP group regarding phosphorus concentration. Kadhim Ali reported finding normal calcium and phosphorus levels in women with OP and controls, but their concentrations were significantly higher in the OP group. Tariq et al. reported significantly high serum calcium levels in postmenopausal women with osteopenia, suggesting that bone decalcification begins during menopause so that serum calcium levels rise rapidly but decline 2 – 5 years after menopause.

Maintenance of calcium-phosphate homeostasis is based on a complex, tightly regulated system involving many ions and hormones. The main hormonal regulators of calcium levels are PTH and vitamin D, which act on the bones, kidneys, and gastrointestinal tract to increase plasma calcium and, to a lesser extent, calcitonin, which reduces bone resorption but appears to have little effect on calcium under normal circumstances. It is known that PTH levels often rise in old age. This may be explained by an age-related gradual decline in serum calcium levels, and the increase in PTH in the elderly may be termed secondary hyperparathyroidism. When serum calcium levels are low, PTH and vitamin D

exert physiological effects at various sites in the body to mobilize calcium stores and increase calcium absorption and reabsorption. Furthermore, calcium and vitamin D regulate the secretion of PTH by the negative feedback mechanism.

Mean plasma PTH concentrations in the study group of women with OP were elevated relative to reference limits compared with women in the control group, where concentrations were normal. Maintenance of normal serum calcium concentrations with advancing age appears to be associated with increased PTH secretion. PTH concentrations in the OP group increased statistically significantly with age, and PTH was positively correlated with age. Elevated serum PTH concentrations are observed in women with OP, but their clinical impact and the reasons for this are not yet fully understood. A study conducted by Cerdà et al. analyzed the prevalence and conditions associated with elevated serum PTH levels in 204 postmenopausal women with OP, as well as their clinical characteristics. They found elevated PTH levels > 65 pg/ml in 35% of subjects, and women with elevated serum PTH levels were older (67 ± 9 years) than those with normal PTH levels (63 ± 11 years). When conditions causing hyperparathyroidism, such as decreased calcium intake, poor vitamin D status, renal failure, and hypercalciuria, were analysed, they found a prevalence similar to that in women with normal PTH levels.

Parathyroid hormone is essential for maintaining calcium homeostasis in part by regulating bone remodeling. Although we found no statistically significant differences in the study and control groups, serum calcium concentrations were lower, while PTH concentrations were higher in women with OP. The parathyroid hormone stimulates bone formation and resorption, and the duration and periodicity of its effects govern the net effect on bone mass, i.e., these effects are catabolic or anabolic. Prolonged exposure to PTH results in catabolic effects on the skeleton, whereas intermittent low doses of PTH have osteoanabolic effects. Low dietary calcium and/or vitamin D intake, via ionized calcium, stimulate PTH release. With the persistence of nutritional deficiencies, prolonged exposure to elevated levels of PTH increases bone remodeling, leading to significant bone loss and increased risk of fractures. Postmenopausal women should receive adequate amounts of calcium to suppress the increased PTH secretion that may underlie the development of OP. However, the causal effect of PTH on bone BMD remains unclear. We found 45% of women in the OP group and 38% of women in the control group with PTH concentrations above 65 pg/ml. However, we found no statistically significant differences in spinal BMD between women with normal and elevated hormone levels in both study groups. Therefore, the question of

whether physiological changes in PTH secretion after menopause and with advancing age contribute to the pathogenesis of OP continues to be a focus of considerable scientific interest. Further studies are needed to define better the contribution of elevated PTH concentrations to bone loss in adults. In a large cohort study of 29155 participants of European ancestry, Qu et al. evaluated and described the potential causal effects of serum PTH levels on BMD and fracture risk at four skeletal sites: the wrist, femoral neck, lumbar spine, and heel, as well as the BMD characteristics of 5 age groups. The authors found that PTH concentrations were negatively associated with BMD, with the magnitude of association and causality being strongly pronounced in people aged 45 to 60. They conclude that serum PTH plays a role in the development of OP, and this causal effect is specific to certain skeletal sites and age. So, more studies of the potential mechanisms by which PTH regulates BMD are needed. We found no correlation between PTH levels and BMD at the lumbar spine in either of our groups. Similar results were reported by Sigurdsson et al., who investigated PTH, vitamin D status and BMD in 308 Caucasian women aged 70 years and older, with 33% of participants having OP. They found no correlation between PTH and vitamin D status and BMD at the vertebral column in either group, suggesting that other previously unexplained factors, in addition to PTH, likely contribute to age-related bone remodeling and OP.

Vitamin D and PTH regulate mineral metabolism in a tightly controlled feedback loop, with PTH being a major stimulator of vitamin D synthesis in the kidney and vitamin D exerting negative feedback on PTH secretion. Our studies showed statistically significantly lower PTH concentrations at normal vitamin D levels than concentrations at deficiency in both the OP and control groups. At vitamin D levels above 50 nmol/l, PTH concentrations in the OP group were higher (60.07 ± 46.43 pg/ml) than those in the control group (45.26 ± 10.40 pg/ml), though the differences did not reach statistical significance. Maintenance of normal plasma calcium levels in women with OP is associated with chronically increased PTH secretion, adversely affecting bone resorption. We confirmed the negative correlation between vitamin D levels and PTH. In our OP patients and female controls, vitamin D depletion was associated with increased PTH values. Vitamin D deficiency via low plasma calcium levels is associated with the development of secondary hyperparathyroidism, a risk factor for the development of OP and subsequent fractures. To normalize serum calcium levels in hypocalcemic conditions, vitamin D induces strong expression of RANKL, which

increases bone resorption. Mobilization of calcium from bones decreases mineral density, increasing the likelihood of developing OP.

Epidemiological data suggest that suboptimal vitamin D status is becoming a worldwide phenomenon. Vitamin D deficiency is the most common nutritional deficiency worldwide. Poor nutrition with reduced intake, limited sun exposure, absorption disorders, liver and kidney failure, and obesity may explain this high prevalence. It is estimated that more than 50% of the world's population is vitamin D deficient. Today, standardized laboratory analyses are used for population studies and comparisons in different countries, including Bulgaria. Data published by Lips et al. on vitamin D concentrations in the population range from adequate vitamin D status in Scandinavian countries to severe deficiency in Middle Eastern countries. Vitamin D deficiency is reported in less than 20% of the population in Northern Europe, 30 – 60% in Western, Southern, and Eastern Europe, and up to 80% in Middle Eastern countries. Severe deficiency is found in less than 10% of Europeans, which is a particularly worrying fact, especially in certain risk groups. The authors draw attention to improving vitamin D status and creating targeted high-priority strategies, such as reasonable sun exposure, adequate nutrition, food fortification policies, and vitamin D supplementation for high-risk groups.

There is enough published evidence in the scientific literature on the presence of low vitamin D levels in OP patients as well. At the time of our study in Bulgaria, there was no data on vitamin D status in patients with postmenopausal OP. The levels we found indicated poor vitamin D status. The mean concentrations of the vitamin in the study and control groups showed insufficiency, and there was no statistically significant difference in concentrations in the two groups: 56.45% of the OP patients and 60.53% of controls had poor vitamin D status Suboptimal vitamin D levels had 90.3% of OP patients and 89.5% of controls.

Data on vitamin D status in patients with OP in Southeast Europe countries are insufficient. Some published data cover a small number of participants but also show poor vitamin D status. Vitamin D levels were assessed in a study of various metabolic markers in bone health assessment by Dimitrova et al. They studied 84 Bulgarian women with normal BMD, osteopenia, and OP. They found no significant difference in vitamin D levels between the groups analyzed for BMD but noted that vitamin D deficient and insufficient individuals predominated in all three groups. The authors reported suboptimal vitamin D levels in 86.6% of healthy controls, 76.9% of women with osteopenia, and 76.7% of OP patients. A study team reported suboptimal vitamin D levels in Romanian postmenopausal

women. Based on the team's accepted criteria, 82.5% of women with OP and 72.7% of women in the control group had suboptimal levels. A population-based study of 596 Greek female OP patients with a mean age of 65.3 years on seasonal variations of vitamin D levels showed suboptimal values of 92.2% in winter. Similar results were reported by Anetakis et al., who investigated seasonal variations of vitamin D in a heterogeneous population of individuals from northern Greece. They found suboptimal levels in the winter period in 97.83% of the 35 women with OP studied at a mean age of 66. The same as in Bulgaria values below 75 nmol/l were considered suboptimal in both countries.

The risk factors for vitamin D deficiency vary across latitudes and ethnic groups. In Bulgaria, there has been no research to date on which of these contributes most significantly to poor vitamin D status in postmenopausal and OP women. It can be assumed that the causes are complex. Vitamin D levels decline with age, and this is a multifactorial issue. We also found a gradual decline in vitamin D with advancing age in both the study and control groups. Vitamin D concentrations showed a statistically significant decrease with each successive decade and a negative correlation with age. Women with vitamin D deficiency were statistically significantly older than women with insufficiency and normal levels.

Vitamin D is synthesized in the skin under the influence of ultraviolet rays and is taken with food, supplements, or vitamin D-fortified foods. It is also found in many animal products, but not many foods are rich in the vitamin. It is now accepted that an adequate intake of 15 μg/day of the vitamin, as defined by the European Food Safety Authority, is difficult to achieve through diet alone. Therefore, vitamin D supplements are recommended. In Bulgaria, fortifying foods with vitamin D is not compulsory, and practically none are on the market. Fish consumption is also low, suggesting that for the majority of the population, dietary intakes of vitamin D are generally poor. Increasing sun exposure may compensate somewhat for low dietary intake, but in the elderly, this is debatable. The ability of the skin to form the vitamin in people over 65 years of age decreases by 25% compared with 20 – 30-year-olds at the same sun exposure and is probably related to reduced amounts of 7-dehydrocholesterol. Other indirect factors reducing sun exposure are related to wearing clothes covering most of the body, using sunscreen, reduced physical activity, and insufficient time spent outdoors. Time spent outdoors in postmenopausal older women and OP patients may be further limited by muscle and joint soreness, anxiety related to possible falls and fractures, or reduced mobility due to osteoporotic fractures already sustained.

Today, the interrelationships between vitamin D status, BMD, and BMI are not yet fully understood and continue to be the subject of much debate. Our study did not show that vitamin D status was statistically significantly associated with BMI in either OP patients or healthy controls. We found no statistically significant difference in BMI in the groups with different vitamin D levels. We also found no significant correlations between the two parameters. A similar lack of correlation was reported by Głogowska-Szeląg et al. in a recent study of vitamin D levels in a small group of 69 women with OP.

Vitamin D status and its interrelationship with BMD at different skeletal sites continue to be the subject of numerous studies, and the data in the scientific literature is quite controversial. Poor vitamin D status today is still accepted as a risk factor for developing OPs and fractures due to bone demineralization that can reduce BMD. Bone loss and reduced bone strength are the main causes of osteoporotic fractures in the elderly. Some studies suggest vitamin D deficiency is associated with low BMD of the spine and femoral neck in postmenopausal women. Other studies do not support this association. In our study group of postmenopausal women, we evaluated vitamin D levels and their correlation with BMD in the lumbar spine and fractures of different localization. In addition, we examined the correlation between vitamin D status and fracture risk calculated with the FRAX® calculator, which data are lacking in our country. Our results showed no statistically significant correlation between vitamin D status and measured BMD of the lumbar spine in both OP patients and healthy controls. The fact that vitamin D levels can be low in people with normal bone mineral density suggests that other compensatory mechanisms prevent this deficiency from affecting bone density. Results from a recently published study of variation in vitamin D genes support this thesis. Tang et al. examined genetic polymorphisms in 417 580 participants of European descent to find a causal relationship between vitamin D levels and BMD at different skeletal sites: forearm, femoral neck, lumbar spine, and whole body in different age groups. The investigators deny such an association at all skeletal sites measured across the lifespan in the general population but recommend large-scale randomised controlled trials to investigate the role of vitamin D supplementation in preventing cardiovascular diseases in high-risk populations.

Results from observational and epidemiological studies indicate a correlation between vitamin D deficiency and increased risk of osteoporotic fractures. Low vitamin D levels are common in older people, and deficiency can lead to bone loss, increased bone resorption, fractures, and falls. However, studies evaluating

the effects of vitamin D on BMD have yielded conflicting results. Thus, it remains unclear whether low vitamin D levels predispose individuals to fragility fractures. Low serum vitamin D levels have been implicated as an independent risk factor for low-energy fragility fractures in the elderly. A 2017 meta-analysis of the association between serum vitamin D and fracture risk demonstrated that serum vitamin D was inversely related to fracture incidence, but these findings are still controversial. Some studies have demonstrated low vitamin D levels in patients with femoral neck fractures. We found a weak negative correlation with an increase in the ten-year risk of femoral neck fracture when vitamin D levels decreased in both the OP group and controls. No publications in the scientific literature examine the relationship between vitamin D levels and estimated fracture risk in patients with OP. However, the evidence that low vitamin D levels theoretically increase the possibility of femoral neck fractures requires attention and confirmation. Results from a meta-analysis published in 2020 suggest that high serum vitamin D levels reduce the risk of femoral fracture in OP patients aged 60 years and older but may not directly affect fracture risk.

Vitamin D insufficiency and deficiency have also been reported as a risk factor for vertebral fractures in both men and women. Results from other studies have shown no significant association between serum vitamin D levels and osteoporotic vertebral fractures. The relationship between serum vitamin D levels and osteoporotic thoracolumbar vertebral fractures remains controversial. However, vitamin D deficiency may be an independent predictor of fractures in women with OP. In the study group of women with OP, we found statistically significantly lower vitamin D levels (39.78 nmol/l) in patients with vertebral fracture compared with patients with forearm fracture (58.96 nmol/l) and without fractures (46.58 nmol/l). Results similar to ours were reported by Milenković et al., who investigated vitamin D levels, BMD, and BMP of 58 Serbian postmenopausal women with a mean age of 60.46 ± 6.55 years and newly diagnosed OP. The authors reported mean vitamin D concentrations of 46.45 ± 14.68 nmol/l, deficiency in 89.76% of the subjects, and statistically significantly lower concentrations in women with previous fractures compared to those without fractures (37.57 ± 13.08 versus 51.22 ± 17.26 nmol/l). They conclude that suboptimal vitamin D status in women with postmenopausal OP is an important risk factor for bone fractures. Also, results from an extensive study conducted among Chinese patients did not confirm a correlation between vitamin D status and BMD. Zhang et al. examined vitamin D levels and BMD in 534 patients with vertebral fractures of varying severity and 569 patients with low back pain without

fractures. The authors found no correlation between vitamin D levels. They measured BMD in the lower back but reported significantly lower vitamin D levels in patients with vertebral fractures. They concluded that there was a significant association between serum vitamin D levels and the incidence and severity of vertebral fractures only in the age group between 60 and 80 years. Similar results were reported by Ying et al., who studied a group of 80 women with postmenopausal OP divided into a group with vertebral fractures and a group without fractures. They found statistically significantly lower vitamin D levels in the patients with fractures than those without fractures and a negative correlation between vitamin D and vertebral fractures.

Vitamin D is involved in bone growth and remodeling by osteoblasts and osteoclasts, and levels below 50 nmol/l accelerate bone turnover, bone loss, and osteoporotic fractures. Although there is still a lack of consensus on optimal vitamin levels for fracture prevention today, combined calcium and vitamin D supplementation is believed to be associated with significant reductions in total and femur fractures among various populations. It is now accepted that serum vitamin D concentrations above 75 nmol/l have the greatest benefits in maintaining BMD and normal function of the musculoskeletal system and lower limbs. The percentage of postmenopausal women with OPs with serum vitamin D concentrations below 75 nmol/l in winter approaches 90 – 100% in Europe, 80% in Canada and the United States, and 34.3% in Brazil. Adopting the cut-off value of 75 nmol/l would mean that almost 80% of postmenopausal women with OP worldwide should be treated for hypovitaminosis D in winter and up to 75% in summer. We found that 90% of menopausal women had vitamin D concentrations below 75 nmol/l, which is particularly worrying, so assessing vitamin D levels is essential.

Osteoporosis and arterial calcification are major health problems in modern societies. Concurrent arterial calcification and OP have been termed the “calcium paradox” and occur frequently in postmenopausal women. However, study results show that women who receive calcium supplements are at higher risk of cardiovascular disease, atherosclerosis, angina pectoris and myocardial infarction. The increased risk of death from heart disease associated with calcium supplementation negates any bone health benefit. A growing number of reports from recent studies view postmenopausal OP as a risk factor for cardiovascular disease, just like other traditional risk factors such as hypertension, dyslipidemia, and diabetes. This is a paradigm shift in terms of the outlook for OP. The relationship between OPs and cardiovascular disease can be partly explained by

the fact that they share common risk factors such as diabetes, dyslipidemia, smoking, excessive alcohol consumption, and hypodynamia. Calcification processes in atheromatous plaques are similar to those observed in bone remodeling. Both processes involve common regulators such as osteoprotegerin, and proteins such as osteonectin, osteopontin, and type I collagen are found in the bone matrix and atheromatous plaques, suggesting that a single mechanism may cause both diseases. There is emerging evidence that individuals with OP are at increased risk of coronary artery disease and stroke even after controlling for other risk factors. This necessitates a change in the perception that OP is a metabolic disease. It should be accepted as a cardio-metabolic disorder, thus highlighting the need for enhanced disease prevention strategies.

Reducing cardiovascular risk in patients with OP can be achieved with a balance of calcium, vitamin D, and vitamin K2 intake, which will optimize the prevention and treatment of the disease. Evidence supports the benefit of supplementing both vitamin K2 in therapeutic regimens in OP patients. Most clinical trials have examined the effect of vitamin D and K2 supplementation on BMD in postmenopausal women. Published data support the idea that their concomitant use may be more effective for bone and cardiovascular health than taking them alone. Vitamin K2 is officially registered in Japan to prevent and treat postmenopausal OP. In European countries, there is still no consensus on its use in prevention and treatment strategies. The potential beneficial effect of vitamin K2-containing supplements for the prevention and therapy of various diseases has focused the efforts of the scientific community on expanding research to study the extrahepatic effects of vitamin K2 and establishing standardized methodologies for testing vitamin levels.

Results from various clinical studies have shown that in modern diets, vitamin K consumption decreases gradually, and even a well-balanced diet cannot provide vitamin K in amounts sufficient to meet the body’s needs. Furthermore, due to modern production processes, the vitamin K content, particularly the vitamin K2 content, of foods today has been significantly reduced, making vitamin K2 supplementation a more reliable way of ensuring adequate intake. There is currently no consensus on plasma vitamin K levels indicating deficiency or insufficiency.

The study of dietary intake of K vitamins is based on the Food Frequency Questionnaire of the National Health and Nutrition Examination Survey. The questionnaire is a limited checklist of foods and beverages with a frequency of

response section, and subjects indicate how often each item was consumed over a specified period.

The main dietary intake in the US, Europe, and most Western countries is phylloquinone, while in Japan, it is mainly menaquinone. In the Netherlands and Germany, for example, the intake of menaquinones is only between 10 – 25% of the dietary intake of vitamin K. Different types of cheese are the most important sources of long-chain menaquinones in the Western diet. However, the actual content of menaquinones varies considerably and depends on the type of cheese, ripening time, fat content, and the geographical area where it is produced. In general, hard cheeses contain more menaquinones than soft cheeses. Other important sources of menaquinones, mainly menaquinone-4 in the Western diet, are meat, liver, and egg yolk. There are no studies on the dietary intake of vitamin K2 in Bulgaria because there is still no adapted and validated questionnaire for use. Another major problem in studies is the lack of data on the vitamin K2 content of regional dairy products and other foods traditionally consumed in our country. There are also currently no norms for daily vitamin K2 intake. The regulation of the Ministry of Health of January 2018 on the physiological norms for the nutrition of the population sets only a norm for vitamin K1 intake. The recommended daily intake for adults is 70 – 80 μg.

Age, sex, and diet are thought to be major determinants of circulating vitamin K levels. Daily intakes of K1 and K2 are higher in younger people of both sexes than in older people. This is particularly true for dietary vitamin K2 intake. The latter accounts for only 10.7% of total vitamin K intake in adults and 11.5% of that in middle-aged women. Evidence shows that vitamin K2 requirements increase with age, and the mechanism is not precisely established. It is probably related to the reduced number of osteoblasts or decreased enzymatic activity of γ-glutamyl carboxylase with advancing age. This also determines the difference in recommended daily intake in adult and young healthy women. In human clinical studies, a commonly used dosage of vitamin K2 is 45 mg/day, and its administration may be an interesting strategy to improve bone and blood vessel health, especially in women with postmenopausal OP. With advancing age and reduced dietary intake, the need for greater amounts of vitamin K2 suggests an existing deficiency in adults. Decreased γ-glutamyl carboxylase activity results in incomplete carboxylation of OC. UcOC cannot be deposited in the bone matrix and enters circulation. Elevated ucOC concentrations are an indirect indicator of vitamin K2 deficiency, and there is scant published evidence that they are associated with poor bone status and increased risk of fractures. Bone ucOC

content adversely affects bone strength with a greater effect on bone quality than on BMD.

Interpretation of published data on ucOC concentrations is hampered by the lack of sufficient studies in OP patients. Also, as yet, there are no defined reference ranges for ucOC defining poor vitamin K2 status for different populations of individuals. There is evidence that postmenopausal women with a serum ucOC level ≥ 4.0 ng/ml exhibit lower serum vitamin K concentrations, higher markers of bone resorption, and an increased incidence of vertebral fractures. To facilitate discussion of guidelines for vitamin K deficiency, ucOC values measured by different assay systems will need to be standardized as there is considerable variation. For the Japanese OP patient population, a cut-off value of less than 4.5 ng/ml is assumed, calculated using the concentrations assumed for vitamin K deficiency. Results from various studies show concentrations of OC and ucOC within a wide range, probably due to the different methodologies used to determine them. Comparisons between different countries show differences in ucOC concentrations in different ethnic groups as well. This again highlights the need for randomized clinical trials with well-defined groups from different geographical regions. Standardizing these indicators will make them useful and more widely used in clinical practice. Our results show high concentrations of ucOC in both groups studied, with no significant difference, which may be associated with low dietary intake and poor vitamin K2 status. However, our country still lacks defined reference ranges for the indicators for different groups of individuals.

Vitamin K2 status can be measured indirectly by the ucOC/OC ratio, and values above 20% are assumed to be dietary vitamin K2 deficiency. In the scientific literature, there is insufficient data to assess vitamin K2 levels in postmenopausal women and those with OP. In a study of dietary intake of the vitamin, McKeown et al. examined 837 men and women of various ages and found poor vitamin K status in 44% of men and 54% of women. Population-based studies of vitamin K status conducted among Caucasians showed poor vitamin K status in 25 – 33% of individuals studied. Our results show a very high ucOC/OC ratio. In the working and control groups, the ucOC/OC values were 77.36 and 83.63%, respectively, with no statistically significant differences. A gradual increase in ucOC/OC was observed in both groups, but the differences were statistically significant only in the control group. The lowest values of ucOC/OC in both groups were above the accepted norm, which defines poor vitamin K2 status and its deterioration with advancing age in our studied women. Similar

results were reported by Theuwissen et al., who examined the vitamin K2 status of 22 healthy Belgian women aged 18 – 45 years and found a high ucOC/OC ratio of 95%. After Menaquinone-7 supplementation, the ratio decreased significantly only in the group receiving 90 μg/day, which was considered a high dose. A study published by Shea et al. on vitamin K status in a multiethnic group of 438 adults (60 – 80 yrs, 59% women) in the USA showed deficiency or insufficiency in 97% of the subjects.

We can only speculate about the main reasons for the poor vitamin K2 status in the group of women we studied because we could not study dietary intake and the unclear composition and quality of food in Bulgaria. The data we obtained are alarming and need to be confirmed by studying larger groups of individuals to generate the development of prevention and treatment strategies, which is important for at-risk groups, especially at older ages. Today, widespread vitamin K2 deficiency may be considered possible. The reasons for this are many and varied: the bioavailability of vitamin K1 and K2 in foods is poor, circulating blood levels are very low compared with other fat-soluble vitamins, they are rapidly metabolised and excreted, and they have low tissue stores. The synthesis of vitamin K2 by the intestinal colonic microflora in sufficient amounts has also been questioned. Studies have demonstrated poor bioavailability of bacterial menaquinones because of their tight binding to the bacterial wall and the lack of bile salts required for their emulsification and resorption. The widespread use of antibiotics killing gut bacteria may further exacerbate an individual's risk of vitamin K2 deficiency.

Vitamin K2 is a versatile vitamin that has focused much attention on its efficacy in improving bone turnover. It promotes bone formation by stimulating osteoblast differentiation and increasing levels of certain markers of bone formation, such as ALP. It regulates extracellular matrix mineralization via γ-glutamyl carboxylation of OC. In addition, vitamin K2 reduces bone resorption through its anticatabolic effects, namely reduction of osteoclast differentiation and inhibition of osteoblast apoptosis. These effects are thought to have been confirmed in animal models and cell cultures but have yet to be confirmed in randomized clinical trials and in humans. Vitamin K2 deficiency may adversely affect bone quality more than bone mass. Our study found a significantly stronger negative correlation between the ucOC/OC ratio and vitamin K2 and alkaline phosphatase, respectively, in women of the study group but not in controls.

Furthermore, the ucOC/OC ratio showed a high negative correlation with OC in both study groups. High levels of ucOC/OC associated with vitamin K2

deficiency lower alkaline phosphatase and carboxylated OS, adversely affecting osteoblast function and bone formation. This disrupts the balance between bone formation and bone resorption, in favour of resorption, impairs the quality of the bone formed, and may increase fracture risk, independent of BMD.

Some new publications link the ucOC/OC ratio and vitamin K2, respectively, in humans to muscle strength, physical function, and risk of falls, but so far, the data are limited. This fact highlights the need to identify potential clinical markers that will be able to identify individuals at risk of reduced muscle function and falls so that appropriate prevention strategies can be put in place. The carboxylated fraction of OC is thought to be found primarily in bone due to its high binding capacity to hydroxyapatite in vitro. In contrast, ucOC has been reported by some authors to function in a paracrine and endocrine manner, being involved in glucose metabolism and influencing muscle mass and strength. The ucOC/OC ratio has been shown to be higher in older compared to younger people, partly due to reduced vitamin K intake, and the correlations we found between ucOC/OC and age also support this in the Bulgarian women we studied. An interesting retrospective study published in 2020 demonstrated a correlation between elevated ucOC/OC ratio levels, reduced muscle strength, increased risk of falls, and associated hospitalizations. The authors tested the hypothesis that a higher ucOC/OC ratio is associated with reduced muscle function and increased long-term risk of fall-related hospitalizations in a large cohort of 1261 Australian Caucasian women with a mean age of 75.2 ± 2.7 y. Their measured OC concentrations of 25.05 ± 10.28 ng/ml were similar, but ucOC concentrations of 11.99 ± 5.34 ng/ml and ucOC/OC ratios of 49 ± 12% were lower than we found. The authors reported that women with a higher ucOC/OC ratio had poorer physical function, reduced mobility, greater fear of falling, and reduced dietary vitamin K intake. They found positive correlations between ucOC/OC and BMI and between ucOC/OC and BMI with reduced physical function with increased long-term risk of traumatic falls associated with hospitalizations. The authors highlighted the importance of identifying high-risk women early and developing prevention and intervention strategies. In our study of women with OP, we also confirmed the positive correlation between increasing ucOC/OC and increasing BMI. A high BMI is clearly not associated with adequate dietary vitamin K2 intake. At the same time, it impairs muscle strength and may increase the risk of falls and fractures, but in our country, there is still no data on the correlation between ucOC/OC and fracture incidence. In our study group of women with OP, ucOC/OC levels increased gradually in women without fractures, with forearm

fractures and vertebral fractures, but the differences were not statistically significant.

In adults, bones are continuously remodeled to repair micro-damage that has occurred, to maintain its mechanical strength and durability, and to maintain calcium homeostasis. Bone turnover biochemical markers provide dynamic information regarding skeletal status independent of BMD and are recommended to accompany and complement its measurements. However, there are still problems with establishing reference ranges and harmonizing the assays used today. Recently, efforts have focused on standardizing their use, which should facilitate their comparison and use in clinical practice.

In Bulgaria, BTM testing has not yet routinely entered clinical practice, and there is no experience of their use in women with postmenopausal OP, and published data on their levels to date are scarce. In our country, automated laboratory assays, which are rapid, accurate, and user-friendly, are used. Manufacturers have developed their reference values, but normal marker levels for different countries and even regions still need to be determined and compared. Our study examined serum concentrations of alkaline phosphatase, one of the oldest but available markers of bone formation, osteocalcin, which is accepted as a marker of osteoblast function and bone quality, and beta CrossLaps, a marker of bone resorption.

Alkaline phosphatase plays an important role in osteoid formation and bone mineralization and was the first biochemical marker of bone formation used for scientific and clinical purposes. The serum concentration of ALP increases with increased bone remodeling. Sex-based differences in metabolism are associated with menopause, which is strongly associated with bone loss in women. There are data confirming elevated levels in postmenopausal women and those with OP compared with premenopausal women, with the greatest increase seen in the first 5 years of menopause due to increased bone turnover. Mukaiyama et al. studied 626 women with OP and reported significantly higher ALP levels at the age of 80 years compared with those over 60 years of age. Information on ALP levels in healthy postmenopausal women compared with those with OP is scarce. Several studies conducted among a small number of women have shown no significant difference in concentrations of the enzyme in healthy postmenopausal women and women with OP. We found ALP levels in reference values and no significant difference in the two groups. The enzyme levels in the OP group were significantly elevated within reference values compared with those in the control group only at ages younger than 59 years, confirming the increased bone turnover

in women with OP in the first years after the onset of menopause. The unexplained, at times, variations in ALP concentrations and the equivocal data are reasons why the marker should not be used in diagnosing OP. However, it may be useful in the evaluation of bone turnover.

Osteocalcin is used today as a marker of bone quality. Osteoblast-specific expression of OC is controlled at the transcriptional level by vitamin D. Osteocalcin plays an important role in metabolic regulation, bone mineralization, and calcium ion homeostasis and is considered a specific marker of osteoblast function as its levels have been shown to correlate with the rate of bone formation. It is secreted into the bone microenvironment and undergoes vitamin K2-dependent γ-carboxylation, which facilitates calcium binding in hydroxyapatite crystals and improves bone quality. At present, the physiological effects of OC are not fully understood, but its synthesis is known to be dependent on vitamin D, and its concentration increases with increased bone formation.

In women with postmenopausal OC, calcium and phosphate deficiency may lead to decreased formation of hydroxyapatite crystals, and, with decreased bone mineralization rate, OC enters the circulation, which may explain the increased plasma OC concentrations in these women. Elevated OC levels may be more effective for early detection of individuals with rapid bone turnover.

Several laboratory methods available for OC testing differ, including their recognizing the various circulating protein fragments. This leads to different results, and for their correct interpretation, it is critical to define method-specific reference intervals. In addition, OC concentrations show significant ethnicity-related variations, and the reference values of available laboratory kits do not reflect regional and ethnic differences. After menopause, OC increases as a marker of increased bone turnover with increased bone formation and bone resorption, but at present, the question regarding changes in OC levels in women with postmenopausal OC compared with those in controls remains controversial, and cut-off values for normal and increased bone turnover are still lacking. In a meta-analysis, Lui et al. summarized the results of 10 studies of serum OC levels in European and Asian patients with OP and postmenopausal women without OP. Each study evaluated and compared serum OC concentrations in OP patients and controls. Six studies reported significantly elevated OC levels in postmenopausal OP patients. Three studies reported the same OC levels in women with OP and controls, and only one study reported a decreased OC level in female OP patients. Results from separate studies also showed increased OC levels in women with postmenopausal OP and osteopenia. Previously published data on OC

concentrations measured with the laboratory assay we used in women with postmenopausal OP are scarce. We measured OC levels of 23.41 ± 10.08 ng/ml in the study group and 23.09 ± 6.94 ng/ml in the control group, respectively, which fell within the reference range of the laboratory kit used. Similar results were also published by Meceska-Jovcevska et al., who studied 100 women with postmenopausal OC from Northern Macedonia aged 59.35 ± 5 years. Their study found concentrations similar to those we found but slightly higher concentrations of 26.52 ± 8.63 ng/ml, with the mean age of their study group being younger.

We found no statistically significant differences in OC concentrations between the study and control groups, with statistically significant differences in BMD and no correlations with spinal BMD in either group. Similar results were published by Naeem et al. and Kerschan-Schindl et al. They found no statistically significant differences in serum OC levels in OP patients and healthy postmenopausal women, and their concentrations were within reference ranges. In women with OP, serum OC was slightly elevated, but the difference was not statistically significant. Today, there is a consensus that BTMs cannot be used to diagnose OP because of their low sensitivity and specificity, and our data confirm the currently accepted position. In the currently accepted European guidelines for the diagnosis and treatment of OP in postmenopausal women, OC is not recommended as a marker for diagnosis and bone formation.

Osteocalcin is not involved in regulating BMD but is essential for the arrangement of hydroxyapatite crystals and optimal bone strength and quality. The OC content of the bone matrix increases until age 45, then reaches a plateau and a relatively stable level for the next decade of human life. A decrease in OC occurs after the age of 55. Given that OC reflects the activity of osteoblasts, the significant decline in its level reflects reduced bone formation. In our study, analysis in the working and control groups stratified by age showed statistically significantly higher OC concentrations in the age group up to 59 years compared with the other age groups only in the OC group but not in the control group. We also found a positive correlation between OC and ALP levels in the OP group. Osteoblasts produce and secrete large amounts of collagen, which forms a fibrillar network, together with other non-collagenous proteins that play a role in the subsequent deposition of minerals as hydroxyapatite crystals. The main stages of osteoblastogenesis-proliferation, matrix maturation, and mineralization-are characterized by consistently distinctive osteoblast markers. Early differentiation of osteoblasts is defined by the expression of high levels of ALP, while the expression of osteocalcin and osteopontin characterizes late differentiation.

Higher concentrations of OC and ALP in women with OP by age 59 years and a positive correlation between the two markers reflect increased osteoblast activity and increased bone turnover associated with accelerated trabecular bone loss in the perimenopausal period. Our results are similar to those published by other authors. Atalay et al. investigated the diagnostic value of OC, ucOC, and ALP in 40 premenopausal and 42 postmenopausal Turkish women. The authors reported statistically significantly elevated OC values in women in the first 5 years after the onset of menopause. They concluded that serum OC levels, with or without ucOC and ALP, may be useful for monitoring bone changes that cannot be assessed by BMD measurement and diagnosing OC in the femoral neck and lumbar spine at L1 – L4. Statistically higher OC concentrations in women in the first 5 – 10 years after the onset of menopause were also reported by Park et al., who examined the age-associated dynamics of BMD in 78 Korean postmenopausal women.

Existing data on correlations between serum OC levels and age are conflicting. Published clinical study results show both positive and negative correlations between OC levels and age. Kalaiselvi et al. and Singh et al. reported a positive correlation. The increase in OC with advancing age has also been well documented by Hannemann et al. in a study to determine reference ranges of OC in a large group of healthy European pre- and postmenopausal men and women. Results from other studies reject this positive correlation. Diemar et al. investigated the relationship between OC, age, and sex in a large group of Danish men and women of different ages. They found a negative correlation between OC and age and defined three clinical reference ranges for women based on age and menopausal status. The authors note that establishing valid reference ranges is important before the full potential of BTM can be used in clinical practice. Our data showed a moderately strong negative correlation between decreasing serum OC levels and increasing age in the OP group but not in the control group. The decrease in OC with advancing age is a marker of decreased osteoblast function with decreased bone formation and deterioration in bone quality.

The study results of the relationship between OC and BMI are also inconclusive, and in women with postmenopausal OC, they are scarce. Osteocalcin is considered a bone-derived hormone that influences body fat distribution and BMI. The relationship between OC and BMI has been confirmed by multiple studies in different populations. A meta-analysis of 28 cross-sectional studies reported a significant negative correlation between serum OC and BMI in healthy adults, especially in patients with metabolic syndrome. Results from

studies on postmenopausal women showed OC concentrations in obese and overweight women lower than those of normal controls. We found a moderately strong negative correlation between OC and BMI in the OP group. A similar strength of negative correlation was reported by Hendrijantini et al. in a group of 54 postmenopausal Indonesian women divided into three groups according to BMI. The authors associated lower body weight and BMI with increased bone turnover and vice versa.

Despite the obvious correlation between vitamin D and OC and the great interest of researchers, very few results from randomized clinical trials in humans prove it, especially in women with postmenopausal OC. A positive correlation between vitamin D and OC was reported by Buranasinsup et al. in a group of healthy adult men and women. Guney et al. reported a significant positive correlation between vitamin D and OC levels in healthy postmenopausal women. OC synthesis requires the involvement of vitamin D and vitamin K2. Vitamin D stimulates gene expression and synthesis of the immature and functionally inactive form, and vitamin K2 is required to transform ucOC into mature functionally active carboxylated OC. The notion that ucOC is a sensitive marker for determining vitamin K2 status is now questioned because ucOC levels also depend on vitamin D levels. Correlations between vitamin K2 or vitamin D with OC or ucOC indicate that both vitamins have a relationship with OC, which means that each can affect protein synthesis, and correlations between the two vitamins indicate their synergistic function concerning bone metabolism. The postmenopausal women we studied were not supplemented with vitamin D, K2, or calcium. Vitamin D levels showed insufficiency without a statistically significant difference in the OP or control group. In the OP group, OC concentrations were statistically significantly higher in the group with normal vitamin D levels compared with the deficient group. We found a positive correlation between vitamin D and OC levels in the OP and control groups. In the OP group, vitamin D levels also showed a moderate positive correlation with ucOC concentrations. The correlation between vitamin D levels, ucOC, and ucOC/OC is inconclusive and requires confirmation in randomized clinical trials. There is a paucity of data in the literature, and positive and negative correlations are reported mainly in volunteers of different age groups. Negative correlations and reciprocal seasonal variations in vitamin D and ucOC levels were reported by Szulc et al. in a group of 195 French institutionalized women aged between 70 and 101 years. Negative correlations were also reported by Saadi et al. in a group of 259 female volunteers from the United Arab Emirates. Buranasinsup et al. and

Bunyaratavej et al. reported positive correlations between vitamin D levels and ucOC in healthy adult volunteers.

The IOF and IFCC recommend using beta CrossLaps, now measured by standardized laboratory assays, as a reference marker for bone resorption in obesity and interventional studies to extend the international experience of its application in clinical medicine. Serum beta CrossLaps concentrations show variations associated with circadian rhythm and food intake, which is avoided by blood sampling in the morning after a 12-hour fasting. Reference values of the marker in healthy postmenopausal women and OP patients in different countries have not yet been standardized due to the lack of sufficient data because most studies have focused on the study of healthy premenopausal women. Reference values of beta CrossLaps for cohorts of premenopausal women in several European countries, the USA, Australia, and Saudi Arabia have been determined by Roche automated analyses so far, but the results obtained show some variation. This suggests that reference values may not be universal, and reference intervals need to be established for different geographical areas and ethnicities and different commercial clinical assays. There are currently insufficient data on levels in postmenopausal women and OP patients. In the study group of women with OP, we found reference values for beta CrossLaps of 0.589 ± 0.266 ng/ml. Serum levels of beta CrossLaps measured with the same laboratory diagnostic kit showed significant variations in healthy postmenopausal women and OP patients. Meceska-Jovcevska et al., previously cited, found concentrations of 0.48 ± 0.12ng/ml in 100 women with postmenopausal OP. Garnero et al. and Boudou et al. reported values of 0.556 ± 0.226 ng/ml and 0.13 – 0.60 ng/ml in healthy French postmenopausal women, respectively. Adami et al. reported 0.26 ± 0.13 ng/ml values in healthy premenopausal Caucasian women aged 20 to 50.

In the Camargo Cohort study, Martines et al. reported values of 0.387 ± 0.197 ng/ml in postmenopausal Spanish women. Trento et al. found values of 0.45 ± 0.10 ng/ml and 0.47 ± 0.12 ng/ml in 200 Italian postmenopausal women (54.6 ± 6.1 years) with normal and osteopenic BMD values, respectively. In the TRIO study, only 20% of women diagnosed with OP by DXA had serum beta CrossLaps concentrations above the upper limit of normal for healthy postmenopausal women. Another study conducted among Pakistani women showed no significant differences in serum concentrations in healthy, osteopenic, and osteoporotic postmenopausal women.

Our measured concentrations of beta CrossLaps in women with OP were similar to those published for healthy French postmenopausal women but higher

than those reported for Italian and Spanish postmenopausal women without OP and for northern Macedonian women with OP. This confirms geographical variations and the need to set reference values according to geographical regions.

The correlations between BTM and BMD have been actively studied over the last 20 years, and the quantitative relationship between them has not yet been definitely established. The correlations between OC and BMD are inconsistent, and in postmenopausal women and with OC are insufficient. Publications have reported a negative correlation between OC and BMD in postmenopausal women and patients with OP. Other studies do not confirm such correlations. Our study showed no significant correlations of OC with BMD in the spine in either group.

We found a weak negative correlation between beta CrossLaps and BMD measured at the spine in women with OP. This is consistent with the sparse data published by Gurban et al. in 149 untreated Romanian women with OP, Kerschan-Schindl et al. in 40 German women with OP and 40 healthy menopausal women, and Wei et al. in 1055 Chinese women with postmenopausal OP.

Bone mineral density alone cannot explain the risk of fractures. Osteoporotic fractures are the result of a complex interaction between bone strength (assessed in vivo as BMD) and the loading of the skeleton during daily activities or trauma, especially falls. Today, it is accepted that BMD cannot identify individuals who will develop a fracture in the future. Although low BMD is a determinant of osteoporotic fractures, some fractures occur at higher BMD, indicating that it is not a sufficient indicator to predict fracture risk. High bone turnover over time is considered an additional factor that increases the likelihood of future fractures. With the aging of the world population, the prevalence of osteoporotic fractures is increasing significantly, and existing prevention strategies are not sufficiently effective. Although altered bone and mineral metabolism is considered one of the most important and modifiable risk factors for fractures, the diagnostic and prognostic value of BTM is still disputed. Recently, numerous studies have attempted to investigate BTM levels in relation to osteoporotic fractures. Some of these studies have found a positive association between bone turnover and fracture incidence. The IOF proposes using BTMs as powerful BMD-independent tools for predicting fracture risk. Combined BTM and BMD testing may be a better predictor of fracture than BMD testing alone. Osteocalcin, as a marker of bone turnover, can be used as an independent predictor of fracture risk.

High bone turnover may disrupt trabecular architecture by increasing the incidence of trabecular perforation and distortion, thereby decreasing bone strength without necessarily significantly affecting BMD. There are reports that

OC may be useful in this regard in the elderly, especially in women. Results from a study of 90 postmenopausal Egyptian women demonstrated the benefits of OC testing in identifying individuals at high fracture risk. In the group of women with OP, we found serum OC and vitamin D values statistically significantly lower in women with vertebral fractures than those without fractures. Vitamin D insufficiency was observed in women with vertebral fractures, which may explain the reduced OC formation. We also found a moderate strength negative correlation between serum OC concentration and the presence of fractures. Low OC concentrations are associated with suppression of osteoblast function, reduced bone formation, and impaired bone repair processes against a background of enhanced bone resorption in compromised BMD. In the control group, OC concentrations were higher in women with fractures than women without fractures. Although the differences did not reach statistical significance, this reflected normal osteoblast function and preserved bone formation capacity. Statistically significant lower OC concentrations in women with postmenopausal OP and vertebral fractures compared with women with OP without fractures were reported by Mohammed et al. in a group of 58 Syrian women. Similar results were reported by Feng et al., who studied 120 women with postmenopausal OP. The authors found no statistically significant difference in spine BMD. However, they reported that vitamin D and OC levels were statistically significantly lower in the group of women with spinal fractures compared with those without fractures and noted the need for further studies with larger numbers of participants to determine the threshold of each marker in assessing fracture risk. Low OC levels indicate suppressed osteoblast function, reduced bone formation, impaired bone quality, and increased fragility. The results of our study suggest that OC may be an independent predictor of fracture occurrence in women with postmenopausal OP. Currently, there is no data on using OC to predict fractures in Bulgaria.

Bone loss in postmenopausal women is due to accelerated bone turnover and deterioration in bone quality. Thus, increased bone turnover is a risk factor for rapid bone loss, and its suppression with antiresorptive medications may maintain BMD after menopause. Some authors recommend BTM as a useful tool in treatment and medication selection decision-making. Patients with high bone turnover may benefit from antiresorptive therapy, whereas those with low bone turnover should be treated with anabolic agents. It is important that individuals with high bone turnover and an increased risk of fracture can be identified and treated promptly. However, there is currently no international consensus on the absolute characteristics of normal, high, or low bone turnover. In light of the

dearth of studies investigating bone turnover variants, Fisher et al. attempted to develop and introduce a practical classification model based on the simultaneous investigation of bone formation and resorption markers and their correlation. Following the IOF and IFCC recommendations, the authors used beta CrossLaps as a marker for resorption with the clarification that there was still no consensus on normal reference intervals. They chose cut-off concentrations of 0.250 ng/ml recommended by most experts. Serum betaCrossLaps concentrations ≤ 0.250 ng/ml are associated with normal and betaCrossLaps concentrations ≥ 0.250 ng/ml with increased bone resorption. The marker concentrations we found in the group of women with OP point to increased bone resorption and increased bone turnover. Dimitrova et al. proposed a threshold cut-off value of beta CrossLaps ≥ 0.44 ng/ml that can differentiate OP patients from healthy controls with 50% accuracy. The authors associated values above 0.44 ng/ml with decreased BMD.

It is clear from the preceding that adequate nutrition is extremely important for preventing OP, reducing fracture risk, and as an element of the non-pharmacological treatment of OP and fractures. Evidence is accumulating that modern diets and food processing do not supply adequate amounts of calcium, vitamin D, and vitamin K2, particularly in the elderly, and the provision of adequate amounts is increasingly likely to be achieved by supplementation with the listed nutrients. Correction of deficiencies may reduce individual risk factors for OP and fractures. Combined vitamin D and calcium supplementation increases spinal BMD in healthy postmenopausal women, and vitamin D is critical in the processes of calcium ion mobilization in bone tissue. Vitamin D and calcium supplementation in women with OP alone cannot restore lost bone tissue and impaired microarchitectonics, and long-chain menaquinones can partially inhibit bone resorption induced by inflammation, hypovitaminosis D, and subsequent PTH production. Today, the synergistic effect of vitamins D and K2 on bone and blood vessels is considered undisputable because of their crucial role in calcium metabolism. Studies in women with OP have shown a significant increase in BMD in individuals taking vitamin K2 supplements, which correlates with an increase in osteogenic activity and provides evidence that the risk of osteoporotic fractures can be reduced by dietary supplementation.

We studied Bulgarian women who did not take calcium, vitamin D, and K2 supplements and had not been treated for osteoporosis. Our data confirmed that poor vitamin D status in postmenopausal and OP women could be expected. The evidence of possible vitamin K2 deficiency is very worrying, highlighting the importance of investigating them in these fracture risk groups. We found no

correlations between vitamin D and K2 levels with BMD in the lumbar spine but confirmed that both vitamins affect bone turnover by several interrelated mechanisms. Vitamin D depletion leads to increased PTH levels with adverse effects on bone resorption, as well as reduced OC formation and slowing of bone turnover with suppression of osteoblast function. Although not used as a marker of bone formation OC, its low levels indicate impaired bone quality and increased fragility. Our results also confirm the assumption that low vitamin D and OC levels in OP patients are associated with poorer bone quality and increased incidence of vertebral fractures. Vitamin K2 showed a negative correlation with OC and ALP. High levels of ucOC/OC associated with vitamin K2 deficiency decrease ALP and carboxylated OC, adversely affecting osteoblast function and bone formation. This disturbs the balance between bone formation and bone resorption, in favour of resorption, also impairs the quality of bone formed and increases fracture risk, independent of BMD. Osteoporosis is a debilitating disease, and the high morbidity associated with fractures increases healthcare costs. Evidence that calcium, vitamin D, and vitamin K2 supplementation benefit bone health and fracture prevention in postmenopausal women and those with OP for our country needs to be confirmed with randomized clinical trials because it may underlie preventive and therapeutic strategies.

The treatment for OP lasts for years. Modern antiresorptive drugs increase BMD and reduce new fractures and associated morbidity. However, the effect on BMD occurs slowly and is difficult to measure with reference methods in the first few years after treatment initiation. Measurement of BMD cannot be used as the sole tool for therapeutic monitoring because changes are slow and initially minimal, and changes in bone mass and density in response to antiresorptive therapy cannot fully account for the reduction in fracture risk. The recommended interval for repeat measurement of BMD at the spine or femoral neck is 1 to 3 years after initiation of treatment.

Prolonged treatment and adherence is a major challenge for many patients, especially asymptomatic patients and those starting oral bisphosphonates. This can significantly reduce compliance and lead to premature discontinuation. Patients not responding to initial treatment pose another problem: they need to be identified early for a change in therapeutic strategies. In contrast to BMD measurements, BTM levels show a significantly rapid response to changes in bone metabolism, making them potentially useful for early monitoring of therapeutic response and motivating their use in clinical practice. Biochemical bone markers seem to be a useful tool in the management of OP, but their widespread clinical

use still faces some difficulties. In order to exploit their clinical potential, the definition of reference ranges for BTM is crucial. Today, the interpretation of BTM concentrations and reference values remains a major challenge, and data generated from their measurements are currently best applied when serial measurements of a single patient are possible. This can help clinicians decide whether the response to treatment is adequate, identify patients who should undergo other therapy in the event of a suboptimal response, and confirm patient compliance with the therapeutic regimen.

Results from clinical studies have shown that total AF can be used as an indicator of the effectiveness of drug treatment of OP with bisphosphonates, which are the most commonly prescribed oral antiresorptive drugs. The use of beta CrossLaps in monitoring the treatment of OPs with oral bisphosphonates is recommended in several guidelines worldwide. In Bulgaria, ALP is not recommended for monitoring the treatment of OPs. The use of beta CrossLaps has not widely entered clinical practice, and data on its use are lacking. According to the current consensus, measurement of beta CrossLaps is recommended before initiation of pharmacological treatment and again between the third and sixth month of treatment, and therapeutic success of bisphosphonate treatment is assumed when marker concentrations decrease by more than 56%. Our study followed the dynamics of ALP and beta CrossLaps before and six months after Denosumab administration.

Denosumab is one of the newest and most potent pharmacological antiresorptive agents for treating OP. It is a human monoclonal antibody against RANKL, a key mediator of the resorptive phase of bone remodeling. The drug is administered at a dose of 60 mg subcutaneously once every six months. Such a therapeutic regimen is easy to follow. It significantly improves patient adherence, making Denosumab one of the preferred drugs for the treatment of OP in recent years in women without contraindications.

Data from several clinical trials demonstrate an early reduction in serum levels of beta CrossLaps after initiation of antiresorptive treatment. This allows us to suggest that the marker may be useful in managing OP much earlier than BMD measurement, and its measurement in a blood sample is simple, non-invasive, and relatively inexpensive. In addition, morning blood samples from fasting patients are easily obtained in clinical settings, as this practice is used for many other common laboratory measurements. This should be the preferred way to assess early therapeutic response. Arslan et al. compared changes in beta CrossLaps and BMD levels in women with osteoporosis before and after six months of treatment.

They reported no change in BMD, but serum beta CrossLaps levels decreased by 70% in all cases. The results of our study were similar. Our results showed pre-treatment beta CrossLaps levels within the reference range for the commercial kit. However, after six months of treatment with Denosumab, serum beta CrossLaps concentrations showed a more than 70% reduction from the baseline. The reduction in the level of beta CrossLaps after the start of treatment indicates the presence of pharmacological effects on bone cells, confirms patient adherence and suggests treatment success. This is particularly important during the first year when an obvious change in BMD is indistinguishable by DXA scanning. A reliable tool to confirm therapeutic effects can motivate patients to adhere to treatment. Today, the lack of sufficient data on beta CrossLaps levels in patients with postmenopausal OP should not discourage clinicians because there is still no consensus on universal reference values, making it difficult to define bone turnover levels as low, normal, or high. Currently, the possibility of performing serial studies of serum beta CrossLaps concentrations on a single patient is more useful and indicative

Most publications on BTM dynamics with Denosumab treatment report results from a bone-specific ALP study. We evaluated the dynamics in total concentrations, although the enzyme is not recommended for monitoring in Bulgaria because the assay is simple, non-invasive, and relatively inexpensive. In addition, examining plasma concentrations of ALP calcium, phosphorus, PTH, and vitamin D is important in the initial evaluation of patients with OP. ALP concentrations before and after the six-month treatment with Denosumab showed a significant decrease. In the scientific literature, data on changes in ALP and the usefulness of testing the enzyme in early monitoring of OP treatment are scarce. A reduction in serum ALP concentrations was shown by the results of a study of the effect of Denosumab in increasing BMD and modulating some BTMs after prior treatment with bisphosphonates in a group of 429 Italian OP patients.

Based on the results obtained, we can conclude that tracking the dynamics of beta CrossLaps and ALP concentrations may have a potential role in the early monitoring of the therapeutic effect of Denosumab treatment, but more data needs to be accumulated.

CONCLUSIONS

1. Bone mineral density, BMI and fracture risk:

- Bone mineral density in women with postmenopausal osteoporosis was significantly lower compared with controls. A strong negative correlation between fracture risk and bone mineral density was found in both study groups.
- In patients with osteoporosis, BMI was within the normal body weight range and was statistically significantly lower compared to controls whose BMI was within the overweight range.
- We found a positive correlation of BMI with BMD and fracture risk – FRAX-MO and FRAX-Hfr.
- The ten-year risk FRAX-MO and FRAX-Hfr was statistically significantly higher in patients with osteoporosis compared with controls.

2. Indicators of calcium-phosphorus metabolism and bone turnover markers:

- The mean concentrations of calcium, phosphorus, ALP, and OC were within reference ranges and showed no statistically significant differences in either study group.
- The concentrations of OC and ALP in women with osteoporosis up to the age of 59 years were statistically significantly higher compared with other age groups.
- In the osteoporosis group, PTH concentrations were elevated.
- Beta CrossLaps concentrations in osteoporosis group were within reference values.

3. Vitamin D:

- A total of 58% of postmenopausal women studied had poor vitamin D status.
- The mean age of women with vitamin D deficiency was statistically significantly higher compared to women with normal levels and insufficiency in both groups studied.
- PTH concentrations at normal vitamin D levels were statistically significantly lower than PTH at vitamin D deficiency in both groups.

- Vitamin D levels in women with osteoporosis and vertebral fractures were statistically significantly lower than vitamin D levels with forearm fractures.

4. Vitamin K2:

- Vitamin K2 status as determined indirectly by the ucOC/OC ratio, was poor in both group studied. All women studied showed values above the accepted norm.

5. Correlations between vitamin D and vitamin K2 levels calcium-phosphorus metabolism, bone turnover and bone mineral density:

- Vitamin D levels showed a negative correlation with age, PTH, and FRAX-Hfr and a positive correlation with OC in both study groups.
- Only in the osteoporosis group was there a moderate positive correlation between vitamin D concentrations and ucOC, a significant positive correlation in strength between the ucOC/OC ratio and BMI, and a negative correlation between ucOC/OC and OC and ALP.
- In women with osteoporosis, serum OC showed a moderate strength negative correlation with the number of fractures and a positive correlation with ALP.
- Beta CrossLaps concentrations in women with osteoporosis showed a moderate negative correlation with BMD.

6. Early monitoring of the treatment effect:

- Beta CrossLaps and ALP concentrations measured after six months of treatment with denosumab showed a statistically significant decrease.
- Determination of beta CrossLaps and ALP, although in reference values, is recommended for serial measurements in the same patient because their dynamics may be a useful tool in early monitoring of the effect of denosumab treatment.

CONTRIBUTIONS

Original contributions

1. For the first time in Bulgaria, data on vitamin K2 status were obtained in postmenopausal women with osteoporosis who were not taking medications and supplements affecting bone mineral density.

2. For the first time in our country, the relationship between vitamin D and vitamin K2, as well as some biochemical markers of bone turnover, bone mineral density, and fracture risk in women with postmenopausal osteoporosis, was studied.

3. For the first time in our country, the potential role of monitoring the dynamics of beta Cross Laps and alkaline phosphatase concentrations for early monitoring of the effect of Denosumab treatment was evaluated.

4. The correlation with BMD and vitamin D and K2 levels was evaluated according to the data obtained for the ten-year fracture risk in postmenopausal OP patients and postmenopausal women using the FRAX web-based calculator validated in our country.

Contributions of a confirmatory nature

1. Data obtained on vitamin D levels in postmenopausal women confirm poor vitamin D status.

2. Our data confirm poor vitamin K2 status in postmenopausal women and those with postmenopausal osteoporosis.

3. Low vitamin D levels are an independent risk factor for vertebral fractures independent of bone mineral density.

4. Fracture risk depends not only on BMD but on several other factors and can be predicted by BTM. Low levels of OC may be an independent predictor of fracture occurrence in women with postmenopausal OP.

PUBLICATIONS AND PARTICIPATIONS

Publications in the scientific press:

In journals with an impact factor (IF) in Web of Science and/or with an impact rank (SJR) in Scopus:

1. Simeonova, T., Stefanova, K., Himcheva, I., Yordanova-Laleva, P. and Dimitrova, A. Vitamin D status, bone mineral density and body mass index in patients with newly diagnosed postmenopausal osteoporosis and healthy menopausal women. Revmatologiia (Bulgaria). 27, 3 (Oct. 2019), 18-28 ISSN: 13100505 (Q4; SJR – 0.11)

2. Simeonova T, Stefanova K, Himcheva I, Yordanova-Laleva P, Ruseva B, Dimitrova A. Assessment of vitamin D status and calcium-phosphorus metabolism in postmenopausal women with osteoporosis. J of IMAB. 2020 Jan-Mar; 26(1):3011-3014. ISSN: 1312 773X (Q4; SJR – 0.225)

3. Simeonova T, Stefanova K, Yordanova-Laleva P, Ruseva B, Dimitrova A. Bone resorption marker Beta-CrossLaps for early monitoring of osteoporosis treatment with Denosumab - J of IMAB. 2022 Oct-Dec;28(4):4731-4734; ISSN 1312-773X (Q4; SJR – 0.225)

Reports and scientific communications:

1. Simeonova, T., Stefanova, K., Himcheva, I., Yordanova-Laleva, P., Ruseva, B. and Dimitrova, A. "Assessment of vitamin D status and calcium-phosphorus metabolism in postmenopausal women with osteoporosis" – 29th IMAB Annual Assembly, 9 - 12 May 2019, Varna

2. Tatiana Simeonova, Krasimira Stefanova, Ivelina Himcheva, Pavlina Yordanova-Laleva, Boryana Ruseva, Anelia Dimitrova. "Vitamin D levels in patients with postmenopausal osteoporosis" – 10th National Jubilee Conference on Nutrition with international participation. 30 May - 1 June 2019, Varna

3. Simeonova, T., Stefanova, K., Himcheva, I., Yordanova-Laleva, P.,Ruseva, B. "Vitamin D status of patients with newly diagnosed postmenopausal osteoporosis and menopausal healthy women" – 45th Jubilee Scientific Conference – MU Pleven, 31.10. - 02.11.2019, Pleven. JBCR. Vol.12 Number 1, Supplement 2, 2019

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5. Tatyana Simeonova, Krasimira Stefanova, Boryana Ruseva. "Assessment of Bone Formation Marker Osteocalcin and Vitamin D Levels in Women with Postmenopausal Osteoporosis and Healthy Women in Menopause" – 4th International European Conference on Interdisciplinary Scientific Research, August 8-9, 2021 Warsaw, Poland, ABSTRACT BOOK, ISBN: 978-1-955094-13-9

6. Tatyana Simeonova, Krasimira Stefanova, Ivelina Himcheva, Pavlina Yordanova-Laleva, Boryana Ruseva, Aneliya Dimitrova. "Dynamics of serum β-Cross Laps levels in early monitoring of osteoporosis treatment with Denosumab" – World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases, August 26-28, 2021, London, UK In Osteoporosis International (Vol. 32, No. SUPPL 1, pp. S199-S199), (Q1, SJR 2021 – 1.11; IF 2021 – 5.071).

7. Tatyana Simeonova, Krasimira Stefanova, Ivelina Himcheva, Petya Dragomirova, Pavlina Yordanova-Laleva, Sergey Kostadinov, Zdravka Radionova, Boryana Ruseva. "Assessment of vitamin K2 and vitamin D status in Bulgarian women with postmenopausal osteoporosis" – World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases, March 24-26, Berlin, Germany, 2022. Aging Clin Exp Res 34 (Suppl 1), 35-474, 2022. (Q2; SJR 2022 – 0.982; IF 2022 – 4.481)

8. Tatyana Simeonova, Krasimira Stefanova, Petya Dragomirova, Pavlina Yordanova-Laleva, Zdravka Radionova, Boryana Ruseva. "Vitamin K2 status in Bulgarian postmenopausal women" – 32-st Annual Assembly of International Medical Association Bulgaria, 20-23 October, 2022, Trakia University - Stara Zagora

9. Tatyana Simeonova, Krasimira Stefanova, Ivelina Himcheva, Petya Dragomirova, Pavlina Yordanova-Laleva, Sergey Kostadinov, Zdravka Radionova, Boryana Ruseva. "Comparative evaluation of osteocalcin and vitamin D serum levels in women with postmenopausal osteoporosis and healthy women in menopause" – National Congress of the Bulgarian Physiological Society from 30 - 1.11.2022, Stara Zagora Mineral Baths