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## Analysis of the influence of hormone replacement therapy on TNF-alpha serum levels in menopausal women

Analiza wpływu hormonalnej terapii zastępczej na poziom TNF-alfa w surowicy kobiet w wieku menopauzalnym

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### Summary

**Objective:** The aim of the study was to investigate and compare levels of TNF- $\alpha$  in serum of menopausal women treated and not treated with hormone replacement therapy (HRT).

**Design:** The study was designed to verify whether there is a correlation between the concentrations of this cytokine and bone mineral density (BMD).

**Material/Methods:** The study was carried out on a group of 60 women during menopause – 30 untreated (control group) and 30 treated with HRT (study group). Half of the patients were after natural menopause and the other half were after ovariectomy. Blood samples were collected. Densitometry was conducted on the vertebral column. To evaluate the results of densitometric examination the T-score index was calculated.

**Results:** The T-score index of the control group reached values below  $-2$ . T-score results for the study group were significantly higher than in the control group. Hormone replacement therapy used by women from the study group caused a decrease in the TNF content in serum, compared with the control group.

**Conclusions:** Beneficial effects of HRT on bone tissue may be exerted through decreased concentration of TNF- $\alpha$  in serum. The use of HRT allows constant bone mineral density to be maintained, which leads to prevention of osteoporotic changes.

**Key words:** TNF-alpha • osteoporosis • menopause • hormone replacement therapy

### Streszczenie

**Cel:** Celem pracy było zbadanie i porównanie poziomu TNF- $\alpha$  w surowicy krwi kobiet w wieku menopauzalnym, leczonych i nieleczonych za pomocą hormonalnej terapii zastępczej (HTZ).

**Projekt pracy:** Badanie zaprojektowano w sposób umożliwiający sprawdzenie istnienia korelacji pomiędzy stężeniami tej cytokiny a gęstością mineralną kości (BMD).

**Materiał/metody:** Badania przeprowadzono w grupie 60 kobiet w okresie menopauzy, 30 nieleczonych (grupa kontrolna) i 30 leczonych HTZ (grupa badawcza). U połowy pacjentek menopauza została wywołana przez usunięcie jajników. Od pacjentek pobierano próbki krwi. Badania densytometryczne przeprowadzono na kościach kręgosłupa. Aby ocenić wyniki badania densytometrycznego dla każdej z pacjentek określono wartość wskaźnika T-score.



**Wyniki:** Indeks T-score w grupie kontrolnej osiągnął wartości poniżej (–2). Wyniki T-score w grupie badawczej były istotnie wyższe niż w grupie kontrolnej. Hormonalna terapia zastępcza stosowana u kobiet z grupy badawczej powodowała zmniejszenie stężenia TNF- $\alpha$  w surowicy, w porównaniu z grupą kontrolną.

**Wnioski:** Korzystny wpływ HTZ na tkankę kostną może być wywierany m.in. przez zmniejszenie stężenia TNF- $\alpha$  w surowicy. HTZ umożliwia utrzymanie stałej gęstości mineralnej kości, co prowadzi do prewencji rozwoju osteoporozy.

**Słowa kluczowe:** TNF-alfa • osteoporoza • menopauza • Hormonalna Terapia Zastępcza

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## INTRODUCTION

Menopause is defined as termination of the menstrual cycle due to lack of ovarian hormonal function (natural menopause), or surgical removal of ovaries (surgically induced menopause). Currently one third of women's lifespan is the postmenopausal period [3]. The loss of sex hormones results in the development of a range of ailments, called "climacteric syndrome". Climacteric syndrome occurs in all women, but its intensity varies and depends on many factors, including hormonal, socio-economic, psychological and genetic factors.

Female sex hormones have a protective effect on the connective tissue. One of the major results of estrogen deficiency is an adverse effect on bone tissue. The deficit of estrogen results in disorder of homeostasis of the skeleton and the emergence of pathological lesions. Imbalance between bone formation and the process of bone resorption leads to bone loss, impaired bone microarchitecture and the development of systemic metabolic disease of bone tissue – postmenopausal osteoporosis.

The mechanism of this process is complex and multifactorial. Recent studies indicated the possibility of linkages between metabolic processes occurring in the skeleton and the immune system. Estrogen deficiency leads to increased production of proinflammatory cytokines (IL-1, IL-6, IL-8, IL-15, TNF- $\alpha$ , TGF) [11]. Cytokines are produced mainly by immune cells: lymphocytes, macrophages and fibroblasts [10]. Cytokines have a biological effect through the influence on target cells via receptors on the surface of these cells. Cytokines take part in the development of inflammation, stimulate the rise of body temperature, regulate cell morphogenesis and have a cytotoxic effect.

Cytokines can be divided according to their functions and structure. One of the major groups is the superfamily of TNF (tumor necrosis factor), which includes more than 20 protein molecules of similar structure. One of the most important cytokines is TNF- $\alpha$  (tumor necrosis factor alpha,

cachectin), which is secreted by activated macrophages and monocytes, osteoblasts, NK cells, and T and B lymphocytes. Cachectin has antitumor and immunomodulating properties. It is involved in the physiological immune response and in inflammatory processes. TNF- $\alpha$  also has a significant impact on bone tissue by stimulating osteoclastogenesis [7]. TNF- $\alpha$  increases the metabolism of connective tissue by activating transcription of proteases, which degrade unmineralized coat protein, facilitating the access of osteoclasts to the mineralized tissue and slowing down the maturation of the extracellular matrix formed by osteoblasts. Increased levels of TNF- $\alpha$  have been found in such diseases as rheumatoid arthritis or Crohn's disease.

There are various standards of therapy used to avoid development of climacteric syndrome and its consequences, such as the destruction of the skeletal system. One of the most effective methods of treatment is hormone replacement therapy (HRT). HRT may be used either as estrogen monotherapy or estrogen-progestagen (combination therapy). The use of HRT is aimed at preventing disorders associated with hormone deficiency. Supplementary hormone therapy improves the quality of life of women's life by preventing long-term effects of menopause, among which some of the most significant are osteoporotic changes. The present study was aimed to investigate the effect of HRT on the osseous system in menopausal patients. The study was designed to demonstrate the possible relation between the state of bone tissue and the level of TNF- $\alpha$  in serum.

## MATERIALS AND METHODS

The study was conducted on a group of 30 postmenopausal women undergoing HRT for at least 6 months (age range 49–59 years, mean age 53.0 years) (study group). The control group consisted of 30 postmenopausal women, at least 12 months after the last menstruation (age range 53–59 years, mean age 55.4 years). Patients in the control group did not receive HRT. Examined patients were treated in the out-patient gynecological clinic of Public Hospital No. 4 in Lublin. All patients gave their consent to the examination

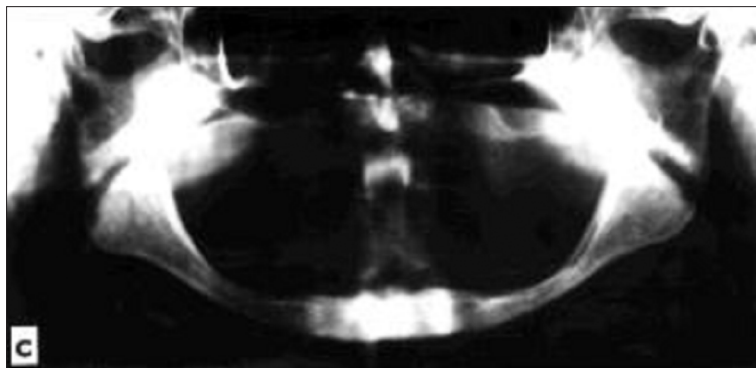


Fig. 1. Radiographic image showing bone resorption of corpus of mandible

and research protocol. Testing procedures received approval of the Local Ethics Committee in Lublin, no of decision: KE-0254/140/2005 and KE-0254/47/2010. The study was carried out in accordance with the ethical principles contained in the Declaration of Helsinki.

Patients were divided into four subgroups according to established guidelines: M – a group of menopausal women; OV – a group of women after surgical removal of ovaries; OV + HRT – a group of women after surgical removal of ovaries, using HRT; M + HRT – a group of menopausal women using HRT. Patients from groups OV and OV + HRT (mean age 54.2 years) underwent surgery at least 3 years before the study was conducted. Surgical procedures were performed as treatment of diseases of the reproductive system (neoplasms, prolapse of the uterus, endometriosis). In the case of patients with malignant tumors no metastases were detected. None of the patients received chemo- or radiotherapy at least for 2 years before the study. Women from groups M + HRT and OV + HRT were supplemented with estrogen and progesterone in combination (combination therapy). Patients were administered Femoston in tablets (2 mg of estradiol hemihydrate and 10 mg of dydrogesterone). Estrogen was taken on a daily basis and progesterone was added for the last 14 days of each course (a course lasted 28 days).

An individual anamnesis chart was developed in order to obtain exact information about health of patients. The questions in the survey included age, occupation, social conditions, date of last menstrual period, number of births, duration of HRT, addictions, physical activity, medications and surgeries. Women qualified for the study did not suffer from any severe general diseases. Patients had no addictions and have not been taking any medications continuously. During the examination of the oral cavity attention was paid to the condition of teeth and oral mucosa, periodontal status, the presence of dentures and the time of their use and other possible problems occurring from the oral cavity. Patients who were selected for further tests did not have any aggressive inflammations. Patients with periodontal sockets deeper than 5 mm were excluded from the study. Patients did not need any extractions of teeth and had average or good oral hygiene. After examination unstimulated saliva and venous blood were collected from women in the fasting state. Blood and saliva samples were collected in morning hours (7–9 a.m.). Chewing gum was prohibited for 2 hours before collection of diagnostic material. Before collection of saliva patients were asked to rinse the mouth with distilled water and relax for 5 minutes. After

this time, patients were asked to lean their head forward and keep their mouth open for 5 minutes in order to allow saliva to drain into the testing tube. At the end of the time of collection, patients were asked to spit remaining saliva into the tube. Venous blood was collected from a cubital vein. After centrifugation of the material, obtained serum (blood) was stored until biochemical tests at a temperature of  $-70^{\circ}\text{C}$ . The concentration of  $\text{TNF-}\alpha$  in serum was determined by enzyme-linked immunosorbent assay (ELISA) using an ELISA Kit from BD Biosciences Pharmingen. The examination was performed according to procedures specified by the manufacturer.

The study of bone mineral density (BMD) of the vertebral column was performed in the Densitometric Laboratory of the Institute of Agricultural Medicine in Lublin, by means of the DPX-A Luna equipment and absorptiometry of X-ray beams of two energies. Bone density was specified in  $\text{g}/\text{cm}^2$ . To evaluate the results of densitometric examination the T-score index was calculated. T-score is the ratio of BMD of the examined patient to the average bone density of young people. T-score values characterizing bone quality are defined as follows:

- healthy bone – T-score higher than  $-1$  (bone density higher than  $833 \text{ mg}/\text{cm}^2$ ),
- osteopenia – T-score between  $-1$  and  $-2.5$  (bone density between  $833$  and  $648 \text{ mg}/\text{cm}^2$ ),
- osteoporosis – T-score less than  $-2.5$  (bone density below  $648 \text{ mg}/\text{cm}^2$ ).

Obtained results were statistically analyzed. The arithmetic mean (M) and standard deviation (SD) were calculated. The significance of differences between groups is based on confidence intervals (NIR) determined from the analysis of variance (ANOVA). The interdependence between selected traits was expressed by Pearson's correlation coefficient. The paired t-test was used in order to determine whether results from 2005 differ significantly from those in 2010. Results were considered significant if the p-value was equal to or less than 0.05.

## RESULTS

Results of BMD examination conducted on the vertebral column in 2005 are shown in table 1. The data obtained from control and study groups differed slightly. The lowest value of BMD occurred in the OV group ( $0.969 \text{ g}/\text{cm}^2$ ) and the highest in the OV + HRT group ( $1.06 \text{ g}/\text{cm}^2$ ). The results of densitometric examination performed on the same patients after 5 years revealed a significant decrease

Table 1. BMD levels in groups of women

Study group	Sample quantity	BMD of vertebral column (2005) g/cm <sup>2</sup> (M ± SD)	Control group	Sample quantity	BMD of vertebral column (2005) g/cm <sup>2</sup> (M ± SD)	Statistically significant differences between results in control and study group (p<0.05)
M+HRT	15	1.03±0.16	M	15	1.05±0.14	Yes
OV+HRT	15	1.06±0.11	OV	15	0.97±0.09	Yes
Study group	Sample quantity	BMD of vertebral column (2010) g/cm <sup>2</sup> (M ± SD)	Control group	Sample quantity	BMD of vertebral column (2010) g/cm <sup>2</sup> (M ± SD)	Statistically significant differences between results in control and study group (p<0.05)
M+HRT	15	1.11±0.02	M	15	0.95±0.04	Yes
OV+HRT	15	1.09±0.04	OV	15	0.92±0.08	Yes

BMD – bone mineral density; M – group of menopausal women; OV – group of women after surgical removal of ovaries; M + HRT – group of menopausal women using HRT; OV + HRT – group of women after surgical removal of ovaries and using HRT.

Table 2. Correlation between T-score values in 2005 and 2010

Group	Sample quantity	T-score for vertebral column (2005) (M ± SD)	T-score for vertebral column (2010) (M ± SD)	Significant differences between results in 2005 and 2010 (p<0.05)
M	15	-1.25±1.17	-2.06±0.36	Yes
OV	15	-1.92±0.72	-2.29±0.61	Yes
M+HRT	15	-1.17±0.90	-0.89±0.18	No
OV+HRT	15	-1.41±1.36	-1.03±0.31	No

M – group of menopausal women; OV – group of women after surgical removal of ovaries; M + HRT – group of menopausal women using HRT; OV + HRT – group of women after surgical removal of ovaries and using HRT.

Table 3. Level of TNF-α in blood serum in 2010

Study group	Sample quantity	Level of TNF-α in blood serum (2010) [pg/ml] (M ± SD)	Control group	Sample quantity	Level of TNF-α in blood serum (2010) [pg/ml] (M ± SD)	Statistically significant differences between results in control and study group (p<0.05)
M+HRT	15	3.20±0.86	M	15	4.14±1.36	No
OV+HRT	15	3.08±1.40	OV	15	3.76±1.25	No

M – group of menopausal women; OV – group of women after surgical removal of ovaries; M + HRT – group of menopausal women using HRT; OV + HRT – group of women after surgical removal of ovaries and using HRT.

in vertebral column BMD in groups not receiving HRT. In group M, the BMD was on average 0.95 g/cm<sup>2</sup>, while in group OV the average value was 0.92 g/cm<sup>2</sup>. BMD in the study group had the following values: in group M + HRT 1.11 g/cm<sup>2</sup> and in group OV + HRT 1.09 g/cm<sup>2</sup>. The differences between study and control groups were statistically significant.

T-score results are presented in table 2. Data obtained in 2005 showed no statistically significant differences between the groups. The T-score index in 2010 significantly decreased in the control groups M and OV, compared to levels from 2005, and reached values below -2. In groups

M + HRT and OV + HRT there was no significant change in the index, compared to results from 2005.

The results for TNF-α level in serum are presented in table 3. TNF-α level in serum of women from group M had an average value of 4.14 pg/ml and in group OV 3.76 pg/ml (the difference was statistically insignificant). Hormone replacement therapy used by women from group M + HRT and group OV + HRT was associated with a decrease of TNF-α content in serum, compared with control groups. The differences in the levels of this cytokine between groups M and M + HRT, and OV and OV + HRT were significant, but not high enough to be statistically relevant.

## DISCUSSION

Hormone replacement therapy is regarded as one of the most effective methods of preventing the emergence of effects of sex hormone deficiency. The results obtained in this study allow us to draw the conclusion that HRT has a beneficial effect on BMD and allows a high level of bone mineral density to be maintained during menopause.

Administration of combined HRT resulted in maintenance of a constant value of the T-score index in the study group, whereas there was a statistically significant decrease in the level of this index within 5 years between densitometric examinations in patients from the control group. If this process would continue, in future years patients from the control group would develop osteoporosis. These results are consistent with the results obtained by Stanosz and coworkers, who studied the effects of HRT on hormone levels and BMD of women with diagnosed osteopenia [14]. Their results supported the conclusion that appropriate hormonal supplementation in women with hormone deficiency leads to normalization of estrogen levels and increase in BMD. Similar results were obtained by Miller and associates, who analyzed the impact of HRT on markers of bone metabolism [8]. Mitwally and colleagues studied the effectiveness of combined estrogen-progestagen therapy. Their work showed that HRT has a beneficial effect on bone status and patient comfort [9].

The analysis of mechanisms occurring in bone tissue associated with bone resorption and osteoporosis have been the subject of numerous studies in recent years. Knowledge of the role of cytokines in cell interactions is still not complete. Too high production of these protein substances may be important in the etiology of certain diseases, including diseases of the skeleton. Groblewska and colleagues described in their work increased levels of proinflammatory cytokines in such disorders as Paget's disease, rheumatoid arthritis and hyperparathyroidism [4]. Knowledge of concentrations of various cytokines in a state of homeostasis and certain pathological processes may be used to reveal their biological importance. Analysis of cytokine levels in the human organism may be an additional diagnostic parameter. It was found that the diseases causing the loss of bone mass have a common pathogenesis at the molecular level. Researchers are still analyzing the effects of cytokines on the occurrence of pathological processes, poor healing or lack of osseointegration of implants [15]. The subject of many studies is correlation between the decrease in estrogen levels and increased production of cytokines such as TNF- $\alpha$ , IL-1, and IL-6 [1,2]. Results obtained in the present study indicate that TNF- $\alpha$  levels in serum of women receiving HRT were lower than in women from the control group.

The obtained data indicate a negative correlation between TNF- $\alpha$  serum concentration and the level of BMD of the vertebral column. Based on the test results it can also be

concluded that hormone supplementation may have an osteoprotective effect through cytokine TNF- $\alpha$ . Lack of hormonal supplementation results in increased levels of cachectin and reduced BMD. This statement is consistent with the viewpoint of Kastelan and colleagues [5], who found that patients with low levels of estrogen have elevated levels of tumor necrosis factor. These conclusions are also in accordance with data presented by Warenik-Szymankiewicz. These authors reported that patients with low levels of sex hormones have significantly higher levels of bioactive TNF in serum compared with the contents of this cytokine in serum of women from the control group [17]. Studies determining the extent of bone resorption showed that activity of TNF- $\alpha$  is one hundred times higher in the presence of IL-1. It seems that both cytokines synergistically determine bone destruction. It would be worth carrying out future studies to examine a possible link between these cytokines in patients with low levels of sex hormones [6,16].

Drugs which lead to neutralization of TNF- $\alpha$  activity (etanercept, infliximab, adalimumab) are currently used in such diseases as psoriasis, arthropathies and Crohn's disease. These medicaments affect the inhibition of elevated activity of cachectin. The results of the present study allow us to advance the hypothesis that these drugs could also be effective in the prevention of osteoporotic changes resulting from estrogen deficiency. Studies carried out on animals by Saidenberg-Kermanach have indicated the effectiveness of systemic administration of osteoprotegerin (OPG) and anti-TNF-alpha [12]. Their results proved the effectiveness of these compounds in osteoprotection in inflammatory diseases. The positive impact of compounds neutralizing activity of cachectin on the state of the skeleton was also confirmed by a study conducted by Seriole and colleagues [13]. The researchers analyzed the effect of TNF blockers in 30 patients suffering from rheumatoid arthritis. Six-month therapy with anti-TNF drugs led to a slight increase in the level of bone formation and reduction of bone resorption. Further studies carried out on a larger group of menopausal women could allow a clear therapeutic effect of TNF blockers to be achieved in the treatment of osteoporosis.

## CONCLUSIONS

The results obtained in this study clearly show that the use of hormone replacement therapy in women with estrogen deficiency has a beneficial effect on the skeleton. The prophylactic effect of sex hormones is multidirectional. Hormone supplementation leads, inter alia, to the reduction of concentration of TNF- $\alpha$  in serum, and thus has a protective effect on BMD. The use of HRT allows constant BMD to be maintained, which leads to the prevention of osteoporotic changes. What is more, administration of agents neutralizing cachectin seems to be a promising way of preventing the development of osteoporosis. Further studies should be conducted on the effect of TNF on the skeleton, performed on a broader group of patients, to clearly confirm these observations.

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