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Circadian concentrations of free testosterone, selected markers of bone metabolism, osteoprotegerin and its ligand sRANKL in obese postmenopausal women

Okołodobowe stężenia wolnego testosteronu, wybranych markerów metabolizmu kostnego, osteoprotegeryny i jej ligandu sRANKL u otyłych kobiet po menopauzie

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Summary

Background: It has been suggested that increased testosterone secretion in postmenopausal obese women might have some protective effect on bone tissue; the association might be significantly influenced by the RANKL/RANK/OPG system.

Aim: The aim of the study was to determine whether postmenopausal obese women showed any relationship between the pattern of adipose tissue distribution, circadian free testosterone (FT) concentrations and bone metabolism (as assessed based on circadian osteocalcin [OC] and C-terminal telopeptide [CTx] levels), and to establish whether osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) might play a role in the relationship.

Material/Methods: FT, OC, CTx, OPG and soluble RANKL (sRANKL) levels were determined by ELISA in serum samples collected every three hours for 24 hours from 47 postmenopausal women (12 with gynoid obesity [GO], 17 with android obesity [AO], and 18 healthy individuals).

Results: Obese women demonstrated an adipose tissue distribution-dependent increase in mean circadian FT levels and a decrease in mean circadian OC, CTx, OPG and sRANKL compared to control participants. In GO subjects, these changes were accompanied by smaller FT amplitudes, suppression of the circadian rhythms of bone markers and OPG, and a shift of sRANKL rhythm acrophase, whereas AO subjects showed a decrease in bone marker amplitudes and suppression of OPG and sRANKL rhythms. In comparison with the controls, significant adipose tissue distribution-dependent changes were found in the correlations between FT and bone markers, FT and OPG, OC and CTx, OPG and sRANKL, CTx and OPG, and CTx and sRANKL. Compared to GO participants, those with AO had higher coefficients of correlations between mean circadian FT and OC as well as between OC and CTx, and lower in the case of FT and sRANKL as well as CTx and OPG and CTx and sRANKL.

Discussion/ Conclusions: Postmenopausal obesity results in adipose tissue distribution-dependent alterations in circadian FT levels accompanied by suppression of bone metabolism and a decline in circadian variations

of the osteokines under investigation, especially sRANKL. Increased FT secretion in postmenopausal women might exert a protective effect on bone tissue, most likely via a shift in the OPG/RANKL ratio that tilts the balance toward a functional excess of OPG.

Key words: free testosterone • bone metabolism • OPG • sRANKL • obesity • menopause

Streszczenie

Wstęp: Istnieją sugestie, że zwiększone wytwarzanie testosteronu u otyłych kobiet po menopauzie może wpływać ochronnie na tkankę kostną, a istotną rolę w mechanizmie tych zależności pełni najprawdopodobniej system RANKL/RANK/OPG.

Cel: Celem pracy było wykazanie, czy u otyłych kobiet po menopauzie istnieje związek między typem rozmieszczenia tkanki tłuszczowej a okołodobowymi stężeniami wolnego testosteronu (FT) i metabolizmem kostnym (ocenianym na podstawie okołodobowych stężeń OC i CTx) oraz ustalenie, czy OPG i RANKL mogą mieć znaczenie w mechanizmie tych zależności.

Materiał/Metody: U 47 kobiet po menopauzie (12 z otyłością gynoidalną – OG, 17 z otyłością androidalną – OA i 18 zdrowych z prawidłową masą ciała) oznaczono metodą ELISA stężenia FT, OC, CTx, OPG i sRANKL w surowicy krwi pobieranej w odstępach trzygodzinnych w ciągu doby.

Wyniki: U otyłych kobiet wykazano zależny od rozmieszczenia tkanki tłuszczowej wzrost średniodobowych stężeń FT oraz obniżenie średniodobowych stężeń OC, CTx, OPG i sRANKL w porównaniu z kontrolą. Zmianom tym u kobiet z OG towarzyszyło obniżenie amplitudy rytmu FT, stłumienie rytmu markerów kostnych i OPG oraz przesunięcie akrofazy rytmu sRANKL, a u kobiet z OA zmniejszenie amplitudy rytmu markerów kostnych oraz stłumienie rytmu OPG i sRANKL. U otyłych kobiet po menopauzie stwierdzono istotne, zależne od rozmieszczenia tkanki tłuszczowej zmiany powiązań średniodobowych stężeń: FT z markerami kostnymi i OPG, OC z CTx, OPG i sRANKL oraz CTx z OPG i sRANKL w odniesieniu do grupy kontrolnej. U kobiet z OA uzyskano wyższe wartości współczynników korelacji niż u kobiet z OG w przypadku zależności średniodobowych stężeń: FT z OC oraz OC z CTx, a niższe w przypadku zależności FT z sRANKL oraz CTx z OPG i sRANKL.

Dyskusja/Wnioski: Otyłość u kobiet po menopauzie wywołuje zależne od rozmieszczenia tkanki tłuszczowej zmiany w okołodobowych stężeniach FT, którym towarzyszy supresja metabolizmu kostnego i okołodobowych oscylacji badanych osteokin, zwłaszcza sRANKL. Zwiększone wytwarzanie FT u pomenopauzalnych otyłych kobiet może wpływać ochronnie na tkankę kostną, najprawdopodobniej poprzez przesunięcie relacji OPG do sRANKL na korzyść OPG.

Słowa kluczowe: wolny testosteron • metabolizm kostny • OPG • sRANKL • otyłość menopauza

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Background

Clinical studies have indicated that an increase in obesity extent in postmenopausal women is associated with increased levels of circulating androgens, and not only those of adrenal origin [1,13,47]. A significant correlation was found between body mass and body mass index (BMI), and circadian levels of total and free testosterone (T and FT) and/or bone metabolism in obese postmenopausal women [1,26,28,33,37,41]. A relationship was also observed between the general pattern of adipose tissue distribution in women and bone mineral density (BMD); the latter was proved higher in android obesity [12,39]. Android obesity in women is believed to reduce sex hormone-binding globulin (SHBG) thus increasing the amount of FT [35,36,43,44], which, in turn, may affect bone metabolism. *In vitro* studies have indicated that osteokines of the receptor activator of nuclear factor-κB ligand/receptor activator of the nuclear

factor-κB/osteoprotegerin system (RANKL/RANK/OPG), an essential signaling pathway by which osteoblasts control the pool of active osteoclasts and thereby bone resorption, might be involved in the effect of testosterone on the bone [5,7,13,22]. Testosterone has been reported to inhibit, through specific receptors, parathyroid hormone (PTH) stimulated osteoclast differentiation in cultured murine bone cells [7]. It also increases the OPG/RANKL ratio (in mouse bone-cell cultures and the osteoblastic cell line MC3T3-E1) through stimulation of OPG mRNA expression, but does not affect RANKL mRNA expression [5]. Other authors believe that testosterone increases the OPG/RANKL ratio via the inhibition of RANKL mRNA expression [7,19]. The above-mentioned data seem to suggest that the FT increase observed in obese women, and especially those with android obesity, might act as a protective factor against postmenopausal bone loss.

It should be emphasized that the investigations into factors affecting bone status, previously performed in obese pre- and postmenopausal women, mainly relied on single determinations thereof made in the morning hours [28]. Since the levels of FT, OPG, sRANKL and bone markers are subject to circadian variations [28,29,30,31,32,33], it seemed well justified to examine the chronobiological aspects of their rhythmicity.

The aim of the study was to determine whether postmenopausal obese women demonstrated any relationship between the pattern of adipose tissue distribution, circadian FT concentrations and bone metabolism (as assessed based on circadian osteocalcin [OC] and C-terminal telopeptide of type I collagen alpha 1 chain [CTx]), and to establish whether OPG and RANKL might play a role in the relationship.

Material and Methods

The investigations were carried out in 29 postmenopausal obese women hospitalized in the Endocrinology Division, Department of Pathophysiology and Endocrinology of the Medical University of Silesia in Zabrze. After exclusion of hormonal causes, the patients were diagnosed with simple obesity. The study group comprised women with a gynoid (lower body) distribution of body fat (GO; waist to hip ratio circumference [WHR] <0.8, n=12) and android (upper body) distribution of body fat (AO; WHR ≥0.8, n=17) whose baseline biochemical parameters were not suggestive of insulin resistance, abnormal lipid profile or glucose tolerance test. The following exclusion criteria were applied: liver and kidney failure, coronary heart disease, arterial hypertension, diabetes, systemic connective tissue disorders, neoplastic and autoimmune disease, thyroid hormone or steroid therapy (estrogen/progestin therapy) and nonsteroidal anti-inflammatory agents. Women receiving antiresorptive or immunosuppressive therapy were also excluded. Age of onset of obesity was ≥30 years. The control group consisted of 18 postmenopausal women with normal body weight.

All study participants had an intravenous cannula inserted into the cubital vein. 5-ml blood samples were collected every three hours for 24 hours (at 08.00, 11.00, 14.00, 17.00, 20.00, 23.00, 02.00 and 05.00 hours). Serum samples obtained by centrifugation were frozen and stored at –75°C until analysis.

Serum FT (DRG Instruments GmbH, Germany), OC (DSL Inc., USA), CTx (Serum CrossLaps – IDS Inc., USA), OPG and sRANKL (BIOMEDICA, Austria) were determined by ELISA. Fasting serum samples were also assayed for 17β estradiol (E2) using RIA (ORION DIAGNOSTICA, Finland), and follicle-stimulating hormone (FSH) using IRMA test kits (ORION DIAGNOSTICA, Finland). The method sensitivity and intra- and interassay errors were as follows: FT 0.007 pmol/l, 6.4 and 8%; OC 0.05 μmol/l, 5.8 and 7.3%; CTx 0.08 nmol/l, 5.2 and 6.7%; OPG 0.14 pmol/l, 7 and 7.5%; sRANKL 0.04 pmol/l, 4.0 and 7.5%, E2 5 pmol/l, 2.8 and 5.8%; FSH 1 U/l, 2.3 and 4.2%.

All subjects gave their informed consent to participate in the study; the research adhered to the tenets of the Declaration of Helsinki.

The project was approved by the Bioethics Committee of the Medical University of Silesia (NN-013-240/03 and NN-013-24/I/03/04).

The obtained results of FT, OC, CTx, OPG and sRANKL determinations were subjected to routine statistical analysis; the level of significance was set at $p \leq 0.05$. Circadian rhythms were assessed using the cosinor method of Halberg et al. [14].

Results

Significant differences in body mass, BMI and WHR were found between postmenopausal obese women and control subjects with normal body weight (Table 1).

Figures 1-5 present the results of circadian FT, bone markers and osteokines of the RANKL/RANK/OPG system determinations in the sera of postmenopausal women with gynoid obesity (GO), android obesity (AO) and age-matched healthy controls (C).

Cosinor analysis revealed a circadian rhythm for FT, with acrophases at 9.09, 11.13 and 10.43 hours in the control, GO, and AO subjects, respectively. AO subjects exhibited a significant increase of FT mesor (mean circadian level) when compared to GO and control participants as well as a significant decline in circadian FT rhythm amplitude compared to the control. GO participants had greater differences in the amplitude of FT circadian rhythmicity than those with AO in comparison with the control group (Fig. 1).

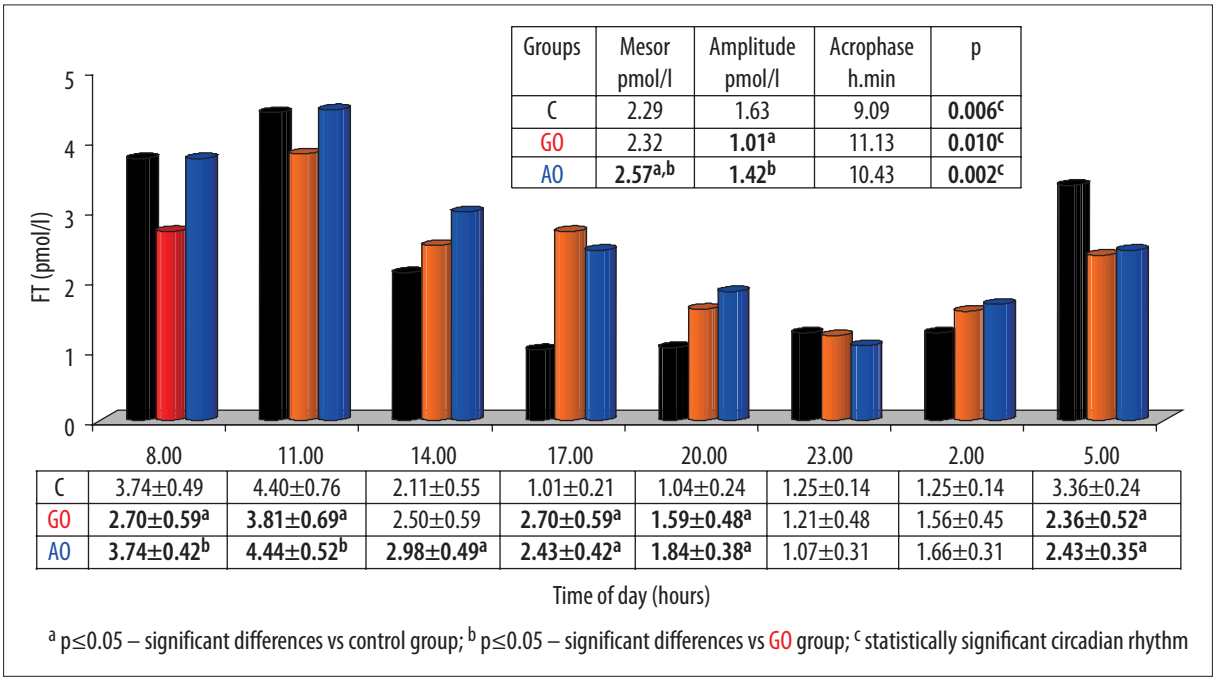
The analysis also indicated a circadian rhythm for OC in control and AO participants, with peaks at 3.28 and 1.31 hours, respectively, whereas GO subjects showed suppression of circadian OC rhythm. Mean circadian OC levels as well as amplitude of OC circadian rhythmicity were significantly decreased in both obese subgroups compared to the control. GO participants had greater differences in the amplitude of OC circadian rhythmicity than those with AO in comparison with control subjects (Fig. 2).

The cosinor method validated a significant circadian rhythm of CTx in AO and control participants with acrophases

Table 1. Basic clinical and anthropometric data in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8) and in postmenopausal healthy women with normal body weight (control group – C). The table gives value ranges and arithmetic mean ±SD

Variables	Postmenopausal obese women (n–29)		Control group
	GO: WHR <0.8 (n-12)	AO: WHR ≥0.8 (n-17)	C: WHR 0.69–0.78 (n-18)
Age (years)	52–65 57.00±7.00	53–63 58.44±4.27	57–62 59.50±3.54
Height (m)	1.52–1.76 1.61±0.12	1.5–1.68 1.59±0.05	1.55–1.68 1.62±0.09
Body mass (kg)	75.60–102.00 87.87±13.30[a]	69.40–110.70 91.33±12.88[a,b]	57.00–73.20 65.10±11.46
Body mass index – BMI (kg/m^2)	31,11–37,22 33.74±3.14[a]	31.79–40.80 36.16±3.08[a,b]	23.75–25.96 24.86±1.56
WHR	0.730.78 0.75±0.03	0.80–0.98 0.87±0.05[a,b]	0.69–0.78 0.74±0.06
Last menstrual period (age in years)	51–55 53.00±2.82	48–56 51.58±2.11	50–54 52.00±2.82
Time since physiological menopause (years)	4–7 5.50±2.12	3–10 6.50±2.31	4–8 6.00±2.83
Duration of obesity (years)	14–24 19.00±7.07	14–26 18.00±5.66	–

[a] p≤0.05 – significant difference vs control group (C); [b] p≤0.05 – significant difference vs GO group.

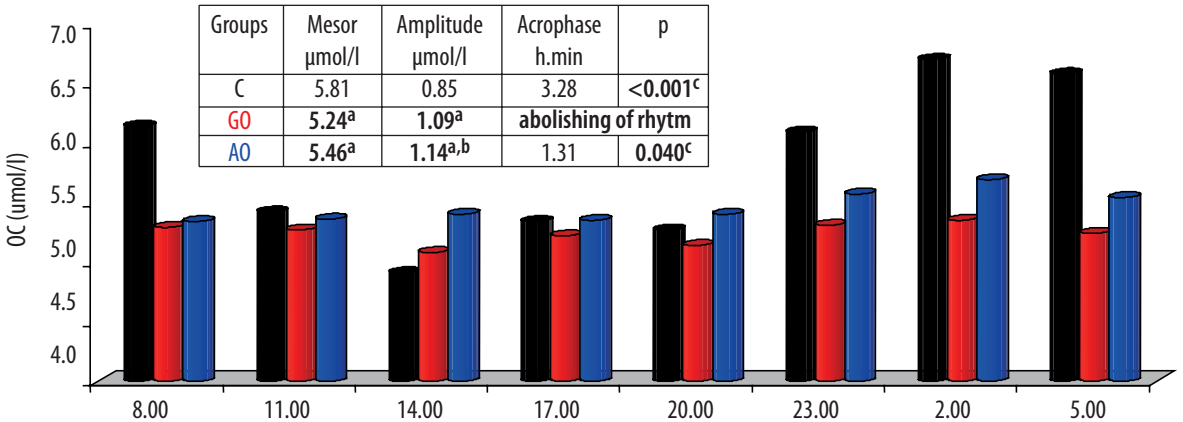


[a] p≤0.05 – significant differences vs control group; [b] p≤0.05 – significant differences vs GO group; [c] statistically significant circadian rhythm

Fig. 1. Circadian oscillations of mean serum concentrations of free testosterone (FT; pmol/l ±SD) and chronobiological parameters of FT circadian rhythm in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8)

at 3.27 and 1.42 hours, respectively. Compared to the control, both obese subgroups showed similar decreases in circadian CTx levels and variations of the marker amplitudes in comparison with the control group. GO subjects also showed suppression of circadian CTx rhythm (Fig. 3).

A statistically significant circadian OPG rhythm with acrophase at 4.36 was only revealed in the control group. Both subgroups of obese women had a significant decrease and of similar magnitude of mean circadian levels of the osteokine compared to the control. The amplitudes of circadian OPG variations were also smaller in both obese



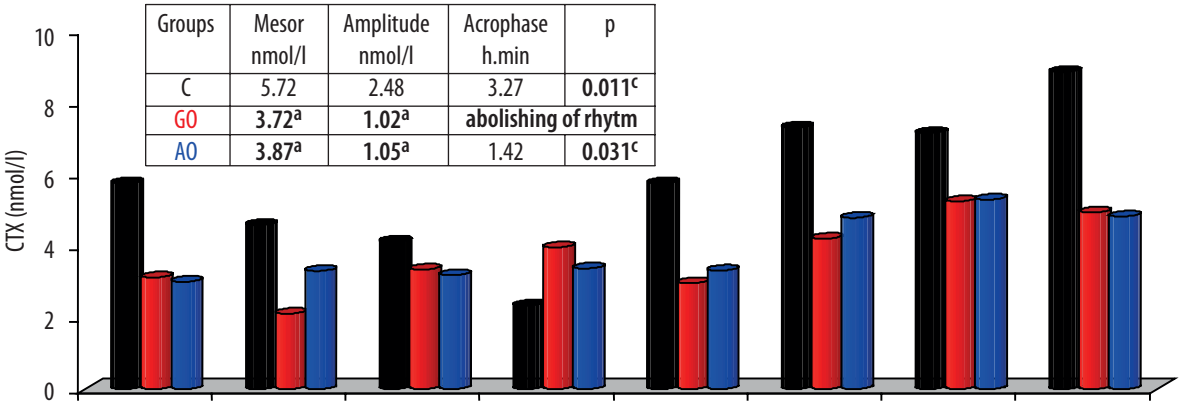
Groups	Mesor µmol/l	Amplitude µmol/l	Acrophase h.min	p
C	5.81	0.85	3.28	**<0.001[c]**
GO	**5.24[a]**	**1.09[a]**	**abolishing of rhytm**	
AO	**5.46[a]**	**1.14[a,b]**	1.31	**0.040[c]**

	8.00	11.00	14.00	17.00	20.00	23.00	2.00	5.00
C	6.14±0.71	5.42±0.68	4.91±0.52	5.34±0.69	5.27±0.60	6.09±0.64	6.70±0.70	3.36±0.24
GO	**5.29±0.58[a]**	5.27±0.41	5.08±0.47	5.22±0.44	5.14±0.40	**5.31±0.60[a]**	**5.35±0.52[a]**	**5.24±0.48[a]**
AO	**5.34±0.45[b]**	5.36±0.49	**5.40±0.41[a]**	5.35±0.48	5.40±0.52	**5.57±0.61[a]**	**5.69±0.64[a]**	**5.54±0.64[a]**

Time of day (hours)

[a] p≤0.05 – significant differences vs control group; [b] p≤0.05 – significant differences vs GO group; [c] statistically significant circadian rhythm

Fig. 2. Circadian oscillations of mean serum concentrations of osteocalcin (OC; µmol/l ±SD) in serum and chronobiological parameters of OC circadian rhythm in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8)



Groups	Mesor nmol/l	Amplitude nmol/l	Acrophase h.min	p
C	5.72	2.48	3.27	**0.011[c]**
GO	**3.72[a]**	**1.02[a]**	**abolishing of rhytm**	
AO	**3.87[a]**	**1.05[a]**	1.42	**0.031[c]**

	8.00	11.00	14.00	17.00	20.00	23.00	2.00	5.00
C	5.74±1.98	4.57±2.00	4.11±1.23	2.33±1.19	5.74±1.35	7.29±1.89	7.13±2.01	8.84±2.54
GO	**3.11±1.53[a]**	**2.10±1.53[a]**	3.33±0.44	**3.95±0.56[a]**	**2.95±1.21[a]**	**4.19±0.77[a]**	**5.23±0.38[a]**	4.92±0.49
AO	**2.99±1.21[a]**	**3.29±1.96[a,b]**	3.17±1.80	3.36±2.11	**3.30±2.04[a]**	**4.77±2.60[a]**	**5.28±2.87[a]**	4.81±2.80

Time of day (hours)

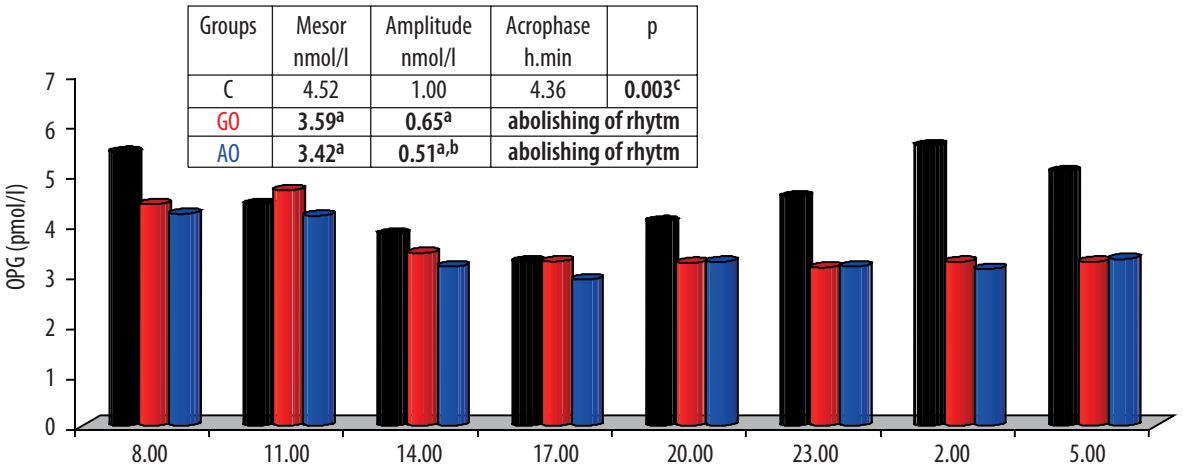
[a] p≤0.05 – significant differences vs control group; [b] p≤0.05 – significant differences vs GO group; [c] statistically significant circadian rhythm

Fig. 3. Circadian oscillations of mean serum concentrations of C-terminal telopeptide of type I collagen (CTx; nmol/l ±SD) and chronobiological parameters of CTx circadian rhythm in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8)

subgroups; however, the difference was more marked between AO women and the control than in the case of the GO subgroup (Fig. 4).

The analysis revealed a circadian rhythm for sRANKL both in the control and GO participants, with acrophases at 6.54 and 11.13 hours, respectively. Circadian variations of sRANKL levels were disturbed in the obese women. The GO subgroup showed a decline in the sRANKL circadian mesor, amplitude increase, and acrophase shift towards later hours. The AO subgroup demonstrated sRANKL rhythm suppression compared to the control; an increase was also observed in the mean circadian sRANKL level and a decrease in circadian variations amplitude compared to GO participants (Fig. 5).

Table 2 presents correlations between mean circadian FT levels, bone markers and osteokines of the



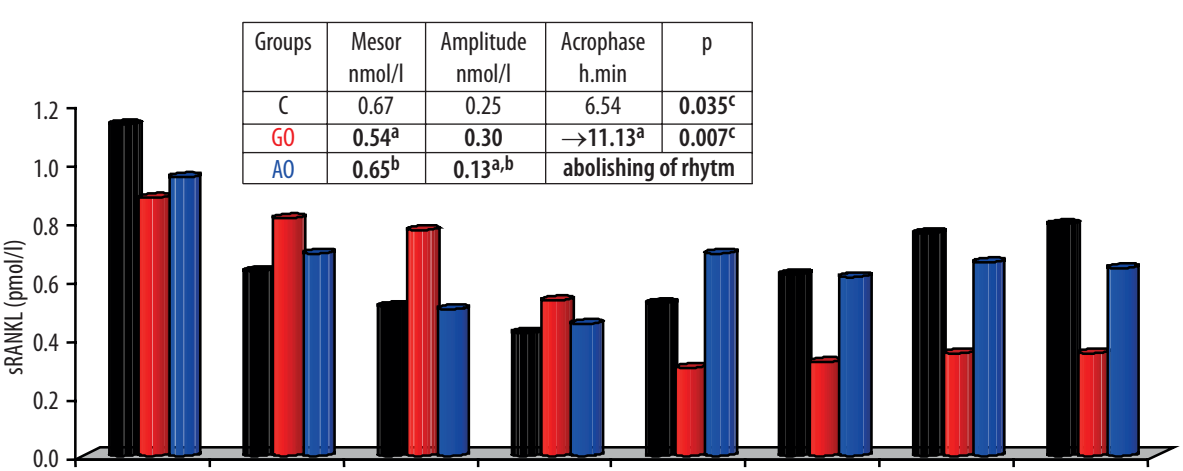
Groups	Mesor nmol/l	Amplitude nmol/l	Acrophase h.min	p
C	4.52	1.00	4.36	**0.003[c]**
GO	**3.59[a]**	**0.65[a]**	**abolishing of rhytm**	
AO	**3.42[a]**	**0.51[a,b]**	**abolishing of rhytm**	

	8.00	11.00	14.00	17.00	20.00	23.00	2.00	5.00
C	5.45±0.89	4.41±1.27	3.82±1.54	3.27±0.88	4.07±0.95	4.56±1.02	5.58±1.32	5.06±0.89
GO	**4.41±1.93[a]**	4.69±0.16	3.44±0.70	3.27±0.62	**3.24±0.34**	**3.15±0.11[a]**	**3.26±0.13[a]**	**3.26±0.57[a]**
AO	**4.22±0.70[a]**	4.18±1.13	3.17±1.48	2.91±0.93	**3.26±0.89**	**3.17±1.00[a]**	**3.12±0.83[a]**	**3.32±0.74[a]**

Time of day (hours)

[a] p≤0.05 – significant differences vs control group; [b] p≤0.05 – significant differences vs GO group; [c] statistically significant circadian rhythm

Fig. 4. Circadian oscillations of mean serum concentrations of osteoprotegerin (OPG; pmol/l ±SD) and chronobiological parameters of OPG circadian rhythm in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8)\



Groups	Mesor nmol/l	Amplitude nmol/l	Acrophase h.min	p
C	0.67	0.25	6.54	**0.035[c]**
GO	**0.54[a]**	**0.30**	**→11.13[a]**	**0.007[c]**
AO	**0.65[b]**	**0.13[a,b]**	**abolishing of rhytm**	

	8.00	11.00	14.00	17.00	20.00	23.00	2.00	5.00
C	1.13±0.052	0.63±0.048	0.51±0.071	0.42±0.064	0.52±0.083	0.63±0.069	0.76±0.040	0.79±0.080
GO	**0.88±0.016[a]**	**0.81±0.021[a]**	**0.77±0.122[a]**	0.53±0.083	**0.30±0.34[a]**	**0.32±0.029[a]**	**0.35±0.024[a]**	**0.35±0.012[a]**
AO	**0.95±0.105[a]**	**0.69±0.071[a]**	**0.50±0.031[b]**	0.45±0.032	**0.69±0.148[a,b]**	**0.61±0.129[b]**	**0.66±0.137[b]**	**0.64±0.128[a,b]**

Time of day (hours)

[a] p≤0.05 – significant differences vs control group; [b] p≤0.05 – significant differences vs GO group; [c] statistically significant circadian rhythm

Fig. 5. Circadian oscillations of mean serum concentrations of soluble receptor activator of nuclear factor-κB ligand (sRANKL; pmol/l ±SD) and chronobiological parameters of sRANKL circadian rhythm in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8)

RANKL/RANK/OPG system in the gynoid and android obesity subgroups and controls.

Normal weight postmenopausal women had a significant positive correlation between mean circadian FT and sRANKL as well as OC and CTx. OC and CTx showed a significant positive correlation with OPG and sRANKL. OPG correlated positively with sRANKL. The GO subgroup had a significant negative correlation between mean circadian FT levels and mean circadian CTx. FT also correlated positively with OPG and sRANKL but the FT/OPG correlation coefficient was higher. AO study participants demonstrated

Table 2. Correlation between mean daily serum concentrations of free testosterone (FT), osteocalcin (OC), C-terminal telopeptide of type I collagen (CTx), osteoprotegerin (OPG) and soluble receptor activator of nuclear factor-κB ligand (sRANKL) in postmenopausal obese women with gynoid (GO: WHR <0.8) and android distribution of body fat (AO: WHR ≥0.8) and in postmenopausal healthy women with normal body weight (control group – C)

Variables		Postmenopausal obese women		Control group
		GO WHR <0.8 (n-12)	AO WHR ≥0.8 (n-17)	C WHR 0.69–0.78 (n-8)
FT (pmol/l)	OC (µmol/l)	NS	–0.679[a] ↑	NS
	CTx (nmol/l)	–0.600[a]	–0.655[a]	NS
	OPG (pmol/l)	0.790[a]	0.801[a]	NS
	sRANKL (pmol/l)	0.786[a]	NS ↓	0.560[a]
OC (µmol/l)	CTx (nmol/l)	NS	0.967a ↑	0.799[a]
	OPG (pmol/l)	NS	NS	0.854[a]
	sRANKL(pmol/l)	NS	NS	0.648[a]
CTx (nmol/l)	OPG (pmol/l)	–0.706[a]	NS ↓	0.754[a]
	sRANKL(pmol/l)	–0.650[a]	NS ↓	0.492[a]
OPG (pmol/l)	sRANKL (pmol/l)	0.824[a]	0.789[a]	0.859[a]

[a] $p \leq 0.05$ – statistically significant values of correlation coefficients; ↓ or ↑ correlation vs GO group.

a significant negative correlation between mean circadian FT levels and mean circadian levels of both bone markers. FT also correlated positively with OPG. Compared to the GO subgroup, a higher correlation coefficient was obtained between FT and OC and lower between FT and sRANKL. AO women also showed a significant positive correlation between mean circadian OC levels and mean circadian CTx, whereas GO participants had significant positive correlations between mean circadian CTx levels and OPG as well as between CTx and sRANKL. In both obese subgroups a significant positive correlation was observed between mean circadian OPG and sRANKL levels.

Discussion

Obesity is a growing health and social problem, which contributes to the development of serious or even life-threatening diseases including type 2 diabetes, dyslipidemia, arterial hypertension, coronary heart disease, gallstones, sleep apnea syndrome, bone and articular diseases and some forms of neoplastic disease [43].

Obesity is associated with several endocrine disorders including insulin resistance with hyperinsulinemia, suppression of the hypothalamic-pituitary-thyroid axis and somatotropic axis, hyperactivity of the hypothalamo-pituitary-adrenocortical axis, enhanced testosterone secretion and functional androgen excess [35,36]. The disturbances are predominant in women with android obesity [15,35,36,43].

On the other hand, due to the boost in load on the bone tissue as well as adipose tissue acting as an endocrine gland, obesity is more and more frequently considered as protective against osteoporosis, and especially in the postmenopausal period [6,9,18,21,28,37,38,42]. Healthy but postmenopausal obese women have higher BMD of the L_2-L_4 region, proximal femoral epiphysis and the radius compared to their age-matched slim counterparts [9,10,12,21,28,37,38,39]. Most authors emphasize a positive correlation between BMI and BMD of the L_2-L_4 region and proximal femoral epiphysis [9,21,28,37]. Postmenopausal obese women also demonstrate changes in bone turnover rate compared to control subjects. Several determinations of biochemical bone formation and resorption markers carried out in the 1980s [21,28] indicated that obese women might be characterized by increased bone formation and/or decreased bone resorption which could lead to bone turnover rate decrease, and, consequently, to BMD increase in later life. However, recent investigations using modern, highly sensitive and specific bone markers have revealed that an increase in obesity extent is associated with inhibition rather than enhancement of bone formation and concomitant suppression of bone resorption [6,34,37]. Our chronobiological studies seem to confirm the above conclusions. We not only found a decrease in mean circadian OC and CTx levels in both obese subgroups compared to the control group, but also statistically significant suppression of the markers' circadian variations. A positive correlation between mean circadian OC and CTx levels in postmenopausal women was also revealed; the correlation proved significant in AO subjects. Adipose tissue distribution-dependent alterations in circadian bone marker concentrations seen in our obese subjects might have been related to changes in osteotropic agents (mainly hormones) frequently observed in obese females [35,36]. It has been assumed that the mechanisms actively participating in the maintenance of bone mineral density in obese individuals, and, in particular, postmenopausal women, include hormones, i.e., estrogens, adrenal androgens (dehydroepiandrosterone [DHEA] and its sulfate-bound form [DHEAS]), leptin and melatonin, and changes in calcium

and vitamin D_3 metabolism as well as in growth hormone (GH)/insulin-like growth factor-I (IGF-I) relationships [18,21,28,29,30,31,32]. However, the potentially protective role of testosterone level changes in reducing postmenopausal bone loss has not been unequivocally accounted for.

Similar to our previous investigations [33], we demonstrated adipose tissue distribution-dependent differences in mean circadian FT concentrations and/or circadian amplitude variations. It is well established that the peri- and postmenopausal tendency to deposit a disproportionate amount of fat in the trunk (abdominal) region is usually associated with increased androgen production [4]. A decline in sex hormone-binding globulin (SHBG), characteristic of postmenopausal AO women, results in an increase of FT levels; the latter, in turn, exerts a stimulating effect on androgen receptors in visceral fat, thus increasing the tendency to develop visceral obesity and insulin resistance [35,36,43,44]. Our postmenopausal AO subjects demonstrated significant elevation of mean circadian FT levels compared to GO and control participants. Both obese subgroups showed a decrease in circadian FT amplitude compared to the control group, although considerably larger differences in the amplitude were found in the GO than in the AO subgroup. This influenced circadian OC amplitude variations in our obese subjects compared to the control, since the differences in circadian OC variations were also larger in the GO than in the AO subgroup. Mean circadian FT levels in women with gynoid obesity correlated significantly and negatively only with CTx (although no significant correlation was found between OC and CTx), whereas AO subjects showed a significant negative correlation between FT and OC as well as FT and CTx (there was a significant and positive correlation between OC and CTx). Compared to GO participants, those with AO had higher correlation coefficients. The obtained results seem to confirm that adipose tissue distribution-dependent changes in circadian FT levels of postmenopausal women might play a role in altering the balance between bone resorption and bone formation.

Although *in vitro* studies involving bone cell cultures of different species have revealed that both testosterone and 5α-dihydrotestosterone (DHT) directly stimulate the proliferation of osteoblast precursor cells [7], the effect of the hormones on osteoblast differentiation has not been fully elucidated. They may have no influence on or, alternatively, stimulate or inhibit the expression of alkaline phosphatase, type I collagen and OC by osteoblasts, and bone matrix mineralization [7]. Most authors believe, however, that androgens stimulate osteoblast differentiation and decrease osteoblast and osteocyte apoptosis [7]. Androgens are capable of exerting both direct and indirect effects on bone tissue via the regulation of growth factor and cytokine expression [7,13,22]. DHT has been shown to reduce OPG levels, which could potentially stimulate osteoclast activity [16]. Osteoclasts derived from the bone marrow colony-forming unit-granulocyte macrophage hematopoietic cell lineage undergo proliferation after orchidectomy, presumably due to androgen deficiency. Osteoclast differentiation requires contact with stromal cells of the osteoblastic lineage in the bone marrow microenvironment, and stimulation by RANKL expressed and secreted by osteoblastic cells, which binds to RANK on osteoclasts [3,17,46]. The effect of RANKL on osteoclasts is regulated by OPG, which acts as a specific decoy receptor for RANKL, and inhibits the RANKL-RANK pathway through competitive binding to RANKL. Thereby, ultimate stages of osteoclastogenesis are inhibited and osteoclast activation reduced. Consequently, the pool of active osteoclasts becomes limited and bone resorption diminished [3,17,46]. Chen et al. [5] demonstrated that testosterone increased OPG mRNA expression in both mouse bone-cell cultures and MC3T3-E1 cells, whereas 10^{-8} M PTH^{-} (1-34) as well as 10^{-8} M $1,25(OH)_2D_3$ inhibited OPG mRNA expression in mouse bone cells. 10^{-8} M testosterone antagonized OPG mRNA expression inhibited by 10^{-8} M PTH^{-} (1-34), but failed to affect OPG mRNA expression inhibited by 10^{-8} M $1,25(OH)_2D_3$. 10^{-8} M α-DHT increased OPG mRNA expression. On the other hand, testosterone did not affect RANKL mRNA expression in MC3T3-E1 or mouse bone cells. The results indicated that testosterone increased OPG mRNA expression in mouse bone-cell cultures and the osteoblastic cell line. These effects are likely to occur through the androgen receptor [5]. Other authors suggest that testosterone might suppress osteoclast formation through the inhibition of RANKL and macrophage-colony stimulating factor (M-CSF) [19], which mediates the initial stage of osteoclast differentiation. During this stage, monocyte-macrophage precursor cells acquire sensitivity to RANKL [13,22]. *In vitro* data indicate that androgens appear to exert their bone protective effects, in part directly and in part indirectly, through osteoblastic cells.

In vivo studies concerning relationships between OPG and RANKL concentrations, bone metabolism and/or BMD and testosterone are scarce and fairly ambiguous. The inconsistency of results is largely due to the influence of age on OPG levels. Khosla et al. [24] suggest that, *in vivo*, testosterone decreases OPG levels in normal elderly men, whereas estrogen tends to have the opposite effect. These differential effects of estrogen versus testosterone on OPG production may explain, at least partially, why testosterone has weaker effects than estrogen on inhibiting bone resorption *in vivo* in humans. Khosla et al. [23] found no relationship between serum OPG and testosterone in postmenopausal women; however, a weak correlation was observed in men below the age of 50. Serum OPG increased with age in both men and women. Significantly higher serum levels of the osteokine were observed in premenopausal women compared to men below the age of 50; no difference was seen in postmenopausal women compared with men over 50 years of age. Our results also did not confirm an association between mean circadian FT and OPG in non-obese postmenopausal participants, whereas we did find a significant correlation between mean circadian FT levels and sRANKL. Kudlacek et al. [25] observed a sharp increase of serum OPG in females after 60 years and in males after 70 years of age; a significant correlation was found between OPG levels in postmenopausal women, and especially those after the age of 65. Other authors [11,20,45] confirmed a significant increase of OPG with age in healthy females and males; Trofimov et al. [45] demonstrated a clear positive correlation with age in both sexes, but after the age of 40.

Only a few researchers have investigated the relationship between OPG and bone metabolism in healthy women. Some of them did not find any correlation between OPG levels and bone markers [11,23,45] as well as between bone markers and BMD [23,45], while some others [20] did observe

a correlation between OPG and bone formation markers but not between OPG and BMD. Moreover, their female subjects demonstrated a negative correlation between serum OPG and tartrate-resistant acid phosphatase. Our chronobiological studies yielded similar results regarding the relationships between OPG and bone markers in postmenopausal women with normal body weight. Mean circadian OPG levels correlated positively and significantly with mean circadian OC and CTx. A significant positive correlation was also seen between sRANKL and mean circadian OC and CTx levels, although the associations were weaker compared to those between OPG and the above-mentioned bone markers. Untreated patients with established osteoporosis evaluated by Fahrleitner-Pammer et al. [11] showed a strong relationship between serum OPG and bone markers, i.e., OC and CTx. The concentrations of bone resorption markers decreased after subcutaneous OPG injections in postmenopausal women and patients with Paget's disease [2,8].

The lack of detailed multidirectional *in vivo* studies on the associations between testosterone and OPG and RANKL concentrations in postmenopausal obese and normal weight women urged us to take a closer look into the issue. Our results have revealed that adipose tissue distribution--dependent increase in mean circadian FT and suppression of OC and CTx in obese postmenopausal women were associated with 1) a decrease in mean circadian OPG levels and 2) a decrease of mean circadian sRANKL (larger in the AO compared to GO subgroup). GO participants demonstrated a shift of sRANKL rhythm acrophase towards later hours, whereas AO subjects showed suppression of OPG and sRANKL rhythms. Compared to the control group, our obese postmenopausal subjects also had significant adipose tissue distribution-dependent changes in the correlations between 1) mean circadian FT and bone markers; mean circadian FT and OPG, 2) mean circadian OC and CTx, 3) mean circadian OPG and sRANKL, and 4) mean circadian CTx and OPG; mean circadian CTx and sRANKL. Compared to GO participants, those with AO had higher coefficients of correlations between mean circadian FT and OC as well as between OC and CTx, and lower in the case of FT and sRANKL as well as between CTx and OPG and CTx and sRANKL.

Based on our findings and literature reports, it seems that testosterone may have a positive, partly direct and partly indirect, effect on bone tissue by stimulating OPG and/or RANKL expression in osteoblasts and bone marrow stromal cells. The indirect effect might be mediated through osteoblast-specific receptors as well as via production of cytokines and growth factors, which modulate OPG and/or RANKL expression. Some of these factors can also be secreted by adipose tissue [13,22]. It should be noted that testosterone can be metabolized via the cytochrome P450 aromatase enzyme complex into 17β-estradiol [7], which has been shown to regulate the balance of the RANKL/RANK/OPG system via specific receptors. Estradiol was found to stimulate OPG expression in human osteoblastic cells via estrogen receptor α [40]. It can also indirectly modulate RANKL and/or OPG expression through the inhibition of proresorptive cytokines such as IL-1, IL-6, TNF-α, M-CSF and PGE_2, and stimulation of transforming growth factor beta (TGF-β) and IGF-I production [13,22,27,40]. Greater changes in circadian OC variations and mean circadian osteokine concentrations in the GO compared to AO subgroup might be partially accounted for by increased estrogen production in gynoid obesity.

To sum up, adipose tissue distribution-dependent increase of testosterone secretion in obese women might be among the factors of the mechanism protecting against bone loss in postmenopausal years. The hormone might exert its effects through the interaction of the above-mentioned mechanisms.

Conclusions

1. Postmenopausal obesity results in adipose tissue distribution-dependent alterations in circadian free testosterone levels accompanied by suppression of bone metabolism and a decline in circadian variations of osteokines under investigation, especially sRANKL.

2. Increased FT secretion in postmenopausal women might exert a protective effect on bone tissue, most likely via a shift in the OPG/RANKL ratio that tilts the balance toward a functional excess of OPG.

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