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Research Article

In the Search for Associations between Bone and Fat Tissues in the Control of Glucose Homeostasis: Role of Osteocalcin, Bone Resorption and Leptin, in Normoglycemic Nondiabetic Women

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ABSTRACT

Background: Osteocalcin (OCN) is involved in controlling glucose homeostasis by stimulating insulin (Ins) and adiponectin production and secretion through the under-carboxylated (uc) form of OCN. Through the sympathetic nervous system, leptin inhibits bone formation, increases RANKL production and the resortive activity of osteoclasts, regulating the bioactivation of OCN. Differences in body mass, estrogenic status, vitamin D (VD) and calcium (Ca) nutritional status might affect bone and fat interrelationship.

Study aim: The present cross-sectional study explores this relationship in normoglucemic non-diabetic pre (nM) and postmenopausal (PM) women.

Methods: Women (n=484) that met the inclusion criteria were included in the study.

Results: When only normal-weight women, were evaluated, total (t) OCN and ucOCN levels were 17.6±9.5 ng/mL and 1.0±0.8 ng/mL (0.15-4.95 ng/mL) in nM, and 20.6±8.9 ng/mL and 1.6±1.1 ng/mL (0.24-3.81 ng/mL) in PM women. In line with the fact that most circulating protein factors associated with regulatory functions are inactive, the mean percentage of ucOCN/tONC was 5% and 8% for nM and PM. When all the studied women having different BMI were included in the analysis, the mean values for tOCN showed a slight increase (26.4±9.5 ng/mL and 27.1±9.8 ng/mL in nM and PM, respectively), while ucOCN increased significantly (2.5±1.9 ng/mL and 2.9±1.7 ng/mL in nM and PM, respectively). As a result ucOCN/tOCN showed a significant increase with obesity, similar to that observed in Ins resistance, suggesting that fat mass may induce not only Ins resistance but also a certain degree of resistance to the hormonal form of OCN.

Conclusion: As obesity increases, higher circulating ucOCN related to tOCN levels are necessary to maintain glucose homeostasis. Then, obesity appears to induce a certain degree of resistance to OCN endocrine action. In ucOCN action the adequacy of Ca and VD nutritional status plays an important role.

INTRODUCTION

Bone has recently emerged as an endocrine organ that regulates glucose metabolism through the activity of osteoblast and osteoclast [1]. Osteocalcin (OCN), also named bone gamma-carboxyglutamic acid-containing protein (BGP), is one of the known protein factors involved in controlling glucose homeostasis.

OCN presents several hormonal characteristics. In this regard, OCN is synthesized by osteoblasts as a pre-pro-molecule under the control of vitamin D (VD) [2]. OCN undergoes a posttranslational modification by a vitamin K-dependent mechanism whereby three glutamic acid (glu) residues are carboxylated to γ-carboxyglutamic acid (gla) residues. Carboxylated OCN has a high affinity for the calcium (Ca) of hydroxyapatite; therefore, once secreted into circulation it is rapidly deposited in bone matrix where it is the most important non-collagen protein [3]. Bone matrix acidification performed by the osteoclast's resorbing activity induces the decarboxylation of one or more gla residues. As under-carboxylated (uc) OCN has a low affinity for hydroxyapatite, it is released into the circulation [4,5]. The ucOCN form appears to be the bioactive molecule, while the process of decarboxylation may be the mechanism by which the body controls the hormonal function of OCN [6]. As a hormone, OCN stimulates the production and secretion of insulin (Ins) by the pancreatic β-cells and adiponectin expression in adipocytes, improving both glucose uptake by the peripheral tissues and glucose sensitivity [4,7,8].

Leptin is a small polypeptide synthesized and released into the peripheral circulation by fat tissue. Through hypothalamic receptors leptin controls energetic and neuroendocrine metabolisms. Leptin affects bone metabolism through both peripheral anabolic action and central anti osteogenic effects [9]. Peripheral leptin stimulates osteoblast proliferation and osteoprotegerin (OPG) expression [10]. In contrast, through the sympathetic nervous system, leptin inhibits bone formation, increases RANKL production and the resortive activity of osteoclasts, regulating the bioactivation of OCN [8,11]. Animal studies have shown an inverse relationship between OCN and adipose tissue [12] and, in obese people, OCN levels are lower than in normal controls [13].

Estrogens are key regulators of bone metabolism via direct effects on osteoblasts and osteoclasts production and function [14]. In addition, estrogen regulates metabolism, accumulation, and distribution of adipose tissue and exerts major effects on leptin production [15]. VD not only regulates OCN production and bone metabolism but also it is negatively correlated with leptin, fasting glucose concentrations and adiposity [16–18].

All the previously mentioned mechanisms evidence the interrelationship between bone, pancreas and fat tissues in the control of glycemic homeostasis. However, these interrelationships might be affected by differences in body mass, estrogenic status and the nutritional status of Ca and VD. On these bases, we carried out a cross-sectional study to explore these relationships in normo-glucemic non-diabetic pre and postmenopausal women.

MATERIALS AND METHODS

Subjects and study design

The study design was descriptive and observational cross-sectional. The study population consisted of a cohort of 500 adult women who attended for a clinical evaluation to the Diabetes and Osteoporosis Services at the Clinical Hospital "José de San Martín", Buenos Aires University.

The present study was approved by the Human Ethics Committee of the Hospital and all the women who agreed to participate were informed verbally about the study and gave written consent. All the study protocols were carried out in accordance with the Declaration of Helsinki.

Inclusion criteria: healthy non-diabetic women (≥18 years) having serum glucose levels between 60 and 110 mg/dL and glycosylated hemoglobin A1c (HbA) lower than 5.5%.

Exclusion criteria: (1) patients who had been treated with oral hypoglycemic agents or Ins; (2) current pharmacological treatment that could affect bone metabolism, such as VD, bisphosphonates, selective estrogen receptor modulators, teriparatide, glucocorticoids, etc; (3) those women having a history of metabolic bone diseases including hyperparathyroidism, Paget's disease, or a recent history of bone fracture; (4) pathologies such us current coronary heart disease, hyperthyroidism, hypothyroidism, rheumatoid arthritis, hypercortisolism, chronic and/or acute kidney disease and cancer; (5) liver dysfunction (alanine transaminase >3 times upper limit of normal); (6) others conditions such as traumatic

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fracture during the last 6 months, prolonged immobilization, eating disorders, pregnancy, breastfeeding, serum creatinine levels >1.5 mg/dL or serum calcium (sCa) or phosphate (sPi) outside the reference range, etc.), and (7) women that did not sign the written consent.

Women were subjected to a routine clinical interview and the physicians completed a questionnaire recording age, socioeconomic status, medical history, dietary habits, and current treatment. A total of 484 women that met the inclusion criteria were included in the study.

Women were divided, according to their menopausal status [premenopausal (nM) and postmenopausal (PM)] and their Body Mass Index (BMI) degree. nM women had regular menses and none of them were taking contraceptive pills. After statistical analysis that evidenced no differences in all the asseyed parameters between pre and perimenopausal women, both women were analyzed as a nM group. PM status was defined by the absence of a menstrual period for 1 year and confirmed by measuring the circulating levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2). Women presenting FSH and LH levels above 30 IU/L, and E2 values below 10 ng/mL were considered post-menopausal at the time of the study. BMI Classification of the WHO was used to define normal weight (NW) (BMI, 18.50–24.99), overweight (OW) (BMI, 25.00–29.99), and different degrees of obesity (obesity degree I [OBI]: 30-0-34.9; degree II [OBII]: 35.0–39.9 and degree III [OBIII] >40) [19]. None of the women presented a BMI below 18.5.

Anthropometric and metabolic profile

A physical examination, including the measurement of height, weight, waist circumference (WC), blood pressure (BP), and body fat, was performed for each subject. Height and body weight (BW) were determined by standard methods. Body mass index (BMI) was calculated as weight divided by squared height as follows: $BMI=BW/height^2$ (in kilogram per square meter).

WC is considered a measure of central obesity that reflects metabolically active visceral fat [20]. WC (cm) was measured to the nearest 0.1 cm in the standing position, midway between the lower rib and the iliac crest with the abdomen relaxed after a normal expiration. The WC cutoff values for women aging <60 years was <88 cm and for those having ≥60 years was <101cm [21]. Systolic and diastolic BP values were the average of three time measurements after remaining seated for 10 min using a sphygmomanometer. Hypertension was considered for values ≥130/85 mm Hg [22].

Ca and VD nutritional status

All participants received verbal and written instructions on how to record their daily food consumption on a semi-quantitative food frequency questionnaire. All the questionnaires were checked by the same dietician to avoid inter-observer errors.

All women filled in a food-frequency questionnaire to determine Ca nutritional status. Nutrient-intake data were collected by three-day records and frequency consumption of dairy products, Ca-enriched foods and vegetables. Ca intake was calculated with a computer program containing food composition data from The National Food Composition Tables. Foods that were not listed on these tables were obtained from the CENEXA tables or the product label [23]. Ca adequacy was determined according to the Estimated Average Recommendations (EAR) as follows: adequacy: Ca intake ≥800 mg/day, insufficiency: 600< Ca intake <800 mg/day, and deficiency: Ca intake ≤600 mg/day [24, 25]. VD nutritional status was biochemically evaluated through the determination of 25OHD levels. VD nutritional status was divided according to the clinical recommendations in: sufficiency levels ≥30 ng/mL; insufficiency: levels between ≥20-29 ng/mL and deficiency: levels between <20 ng/mL [26].

Biochemical analysis

Blood samples were obtained in the morning between 8 AM and 9:30 AM after an overnight fast. Blood glucose concentrations were measured after sampling; plasma and serum specimens were immediately frozen and stored at −20°C until analyzed. HbA1c levels were measured by habitual methods. Fasting plasma concentrations of glucose, total cholesterol (tChol), HDL cholesterol (HDL Chol), triglycerides (TG), and creatinine were measured by standard techniques. tChol >200 mg/dL, was considered hypercholesterolemia; TG >150 mg/dL hypertriglyceridemia. An Abbott Clinical Chemistry Analyzer (Abbott Park, IL, USA) was used by all these biochemical analyses.

Serum Ca (sCa) concentration was determined by atomic absorption spectrophotometry. Lanthanum chloride (6500 mg/L in the final solution) was added as an interference suppressor.

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The sPi levels were evaluated following UV habitual methods using an automated analyzer (Abbott Laboratories, Abbott Park, IL, USA). Serum bone alkaline phosphatase (BAP) (IU/L) was measured using a colorimetric method that evaluates the activity of total BAP after bone isoform precipitation with wheat germ lectin. Cross-reactivity with liver isoform was less than 5%. Sensitivity was 0.7 IU/L. Intra Assay and interassay coefficient of variation (CV) were 4-5% and 6-8%, respectively. Serum CTX (ng/mL) was assessed employing ELISA (Serum Crosslaps, Osteometer, BioTech, Herlev, Denmark). The levels of 25OHD were determined by competitive protein binding assay based on the use of radiolabeled ligands (ELISA, DiaSorin, RIA). Intra-assay CV was 8.6–12.5%, and interassay CV was 8.2–11.0%. Sensitivity was ≤1.50 ng/mL. Total (t) OCN was evaluated by enzyme-linked immunosorbent assay (ELISA) (N-MID Osteocalcin ELISA, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). The intra-coefficient of variation (CV) for tOCN was <2.2. The ucOCN analysis was performed by an ELISA commercial kit (Osteometer BioTecn, USA). The intra CV for ucOCN was <8.2. The ratio between ucOCN/tOCN was calculated.

Serum Ins and leptin concentrations were measured by immunoassay (ELISA) commercial kits (Linco, St. Louis, USA). The homeostasis model assessment (HOMA) described by Matthews, based on fasting glucose and Ins measurements was used to evaluate Ins resistance (InsR) as follows: HOMA-IR= fasting Ins (μU/mL) x fasting glucose (mg/dL)/405. Healthy HOMA-IR levels range between 0.5–1.4; values above 1.9 indicates early InsR, and above 2.9 indicates significant InsR [27]. The β cell function was evaluated as follows: HOMA-β=20 x fasting Ins (uU/[fasting glucose (mmol/L) - 3.5]. The cut-off value for an abnormal β-cell function index was 73.0 and HOMA-β <73.0 indicates defective Ins secretion [28].

The TG/HDL Chol ratio as a criterion for the diagnosis of InsR and cardiovascular risk was calculated. According to bibliography, the optimal TG/HDL Chol level ranges between 0.5 and <1.9; levels between 1.9 and 2.9 indicate early InsR and above 3.0 indicates significant InsR and heart disease risk [29].

The presence of metabolic syndrome was defined as having 3 or more of the following components: (1) WC ≥94 cm or BMI >30; (2) TG ≥150 mg/dL; (3) HDL Chol <50 mg/dL and (4) BP at least 130/85 mm Hg [30].

Statistical analysis

Descriptive results for continuous variables are reported as the mean ± standard deviation (SD) for no parametric distribution as median (interquartile range [IQR] 25–75%). Before statistical analyses, Gaussian distribution and homogeneity of the variances were evaluated by the use of the Shapiro–Wilk and Levene's tests. Parameters that followed a normal distribution were compared among the groups using one-way ANOVA test, and those that were not normally distributed were compared with Kruskal–Wallis test. Bonferroni tests were used as "*a posteriori* " *test* to determine differences between groups. Unpaired Student's t test was used for comparisons between nM and PM women for normally distributed parameters in each group, while the Wilcoxon test was applied for variables with a skewed distribution. Comparison of two independent proportions (nM vs PM) were evaluated by Z test approximation. Data were analyzed using the InfoStat version 2020 (Cordoba National University, Argentine). The Spearman correlation coefficients were calculated to assess the strength of the correlations of tOCN, ucOCN and ucOCN/tOCN and parameters of adiposity, glucose, lipid and bone metabolism in nM and PM women. We also calculated Spearman correlation coefficient of tOCN, ucOCN and ucOCN/tOCN and parameters of adiposity, glucose, and lipid metabolism in women with adequacy of Ca intake and VD nutritional status (Ca intake >800 mg/d and 25OHD levels >29 ng/mL). The Spearman correlation coefficients, box plots and scatter plots were made with R software version 2021 (R Foundation for Statistical Computing, Austria). A $p<0.05$ was considered as a significant difference.

RESULTS

Clinical characteristics and biochemical analysis of PM and nM women are shown in Table 1. The median values of fat mass% ($p=0.0063$), systolic and diastolic blood pressure (SBP and DBP respectively) ($p=0.0002$ and $p<0.0001$, respectively); glucose ($p=0.0085$), tChol and HDL Chol ($p<0.0001$ and $p=0.0320$, respectively), TG ($p=0.0481$), HOMA-IR ($p=0.0157$) were significantly higher in PM as compared to nM women. The mean levels of CTX almost reached higher values

in PM than in nM women (p=0.06) and no differences in the remaining parameters were observed.

Table 1. Anthropometric and metabolic profile of premenopausal (nM) and postmenopausal (PM) women.

	nM (n= 145)	PM (n= 339)	p
Age (years)	38.0 (35.0-43.0)	62.0 (57.0-67.0)	*p< 0.0001*
BMI (Kg/cm2)	30.0 (25.1-36.8)	30.8 (28.0-34.4)	p= 0.72
WC (cm)	98.9±12.7	100.5±11.6	p= 0.45
Fat mass (%)	39.1 (34.0-45.4)	43.8 (38.7-46.5)	*p= 0.0063*
Calcium intake (mg/day)	679.6 (499.9-910.8)	722.7 (542.0-898.9)	p= 0.45
SBP (mmHg)	110.0 (100.0-120.0)	120.0 (110.0-130.0)	*p= 0.0002*
DBP (mmHg)	70.0 (60.0-70.0)	75.0 (70.0-80.0)	*p< 0.0001*
25OHD (ng/mL)	21.1±5.3	21.3±8.2	p= 0.82
Metabolic glycemic and lipid profile			
Glucose (mg/dL)	91.9±9.3	96.1±8.8	*p= 0.0085*
Total Cholesterol (mg/dL)	185.5±34.0	210.9±32.2	*p< 0.0001*
HDL Cholesterol (mg/dL)	53.6±12.3	58.3±12.8	*p= 0.0320*
TG (mg/dL)	87.0 (65.0-107.0)	105.4 (76.0-122.0)	*p= 0.0481*
TG/HDL Cholesterol ratio	1.9 (1.2-2.4)	1.6 (1.2-2.5)	p= 0.44
Insulin (µU/mL)	9.2±6.6	9.4±4.1	p= 0.88
Leptin (ng/mL)	14.5±12.3	15.5±11.7	p= 0.62
HOMA-IR	1.5 (1.2-2.1)	2.1 (1.4-2.8)	*p= 0.0157*
HOMA-β	91.9 (66.7-136.7)	94.1 (70.8-126.5)	p= 0.77
Parameters associated with phospho-calcium homeostasis and bone remodeling			
Serum Calcium (mg/dL)	9.4±0.5	9.5±0.4	p= 0.24
Serum Phosphate (mg/dL)	3.5±0.5	3.6±0.4	p= 0.18
Total OCN (ng/mL)	22.9±12.2	26.2±12.8	p= 0.16
ucOCN (ng/mL)	2.3 (1.2-3.8)	1.9 (1.0-4.0)	p= 0.67
ucOCN/total OCN ratio	0.09 (0.06-0.16)	0.07 (0.04-0.16)	p= 0.07
CTX (ng/mL)	390.0 (217.0-550.0)	520.5 (245.0-560.0)	p= 0.06
BAP (U/L)	65.5±9.8	65.0±10.5	p= 0.81

Results were expressed as mean±SD or median (IQR). Data of PM vs nM were compared by independent samples T test or Mann Whitney Wilcoxon, according to the variable distribution. Significant differences (p< 0.05) were marked in italic font. nM: premenopausal women; PM: postmenopausal women; BMI: body mass index; WC: waist circumference; SBP/DBP: systolic/diastolic blood pressure; 25OHD: 25 hydroxyvitamin D; TG: triglycerides; HOMA-IR: homeostatic model assessment of insulin resistance calculated HOMA-IR= glucose (mmol/L)x insulin (µU/mL)/22,5; HOMA-β: homeostatic model assessment of β-cell function calculated HOMA-β= 20 x insulin (µU/mL)/(glucose (mmol/L)-3.5); OCN: osteocalcin; ucOCN: undercarboxylated osteocalcin; CTX: carboxy-terminal collagen crosslinks; BAP: bone alkaline phosphatase.

Table 2 shows the percentage of women in each group with abnormal levels of the studied parameters and different risk factors. The percentage of low Ca intake was higher in nM than in PM while no difference was observed. Hypovitaminosis D was higher in nM than in the PM group while no differences in the percentage of VD deficiency was observed. PM women presented a higher percentage of abnormal SBP than nM women (37.1 % vs. 17% p=0.013) and 8.9% of PM had hiper DBP against 3.8% in the nM group however no statistical differences were observed. nM women presented a higher percentage of hypercholesterolemia (p=0.005) and a lower percentage of low HDL Chol levels (p=0.045) than PM women. In contrast, no differences were observed in the percentages of hypertriglyceridemia. Although the percentage of HOMA-IR did not show differences between both groups; PM women presented a higher percentage of early InsR and established InsR than nM women. The percentage of women who had high TG/HDL Chol ratio values indicating InsR and risk of cardiovascular disease was higher in PM (p=0.014) than nM.

Table 2. Distribution of the risk factors in premenopausal (nM) and postmenopausal (PM) women.

Risk Factors	nM	PM	p
Ca intake adequacy (≥ 800 mg/day)	32.1%	39.3%	0.471
Ca insufficiency (Ca intake 600-800 mg/day)	32.1%	32.6%	0.999
Ca intake deficiency (≤ 600 mg/day)	35.8%	28.1%	0.353
Hypovitaminosis D (25OHD 20-29 ng/dL)	60.4%	37.1%	*0.009*
Vitamin D deficiency (25OHD > 20 ng/dL)	37.7%	46.1%	0.382
Early InsR (HOMA-IR 1.4-2.9)	35.8%	48.3%	0.165
InsR (HOMA-IR ≥ 3)	18.9%	21.3%	0.831
HyperChol (> 200 mg/dL)	62.3%	37.1%	*0.005*
HyperTG (≥ 150 mg/dL)	13.2%	19.1%	0.448
Low HDL Chol (< 50 mg/dL)	54.7%	71.9%	*0.045*
Early InsR (TG/HDL 1.9-2.9)	15.1%	25.8%	0.148
InsR and heart disease risk (TG/HDL ≥ 3)	30.2%	12.4%	*0.014*
HyperSBP (≥ 130 mmHg)	17.0%	37.1%	*0.013*
HyperDBP (≥ 85 mmHg)	3.8%	8.9%	0.321
Metabolic syndrome	15.1%	22.5%	0.383

Results were expressed as a percentage. The two independent proportions of nM vs PM women were compared by the Z test approximation. Parameters with significant differences (p< 0.05) were marked in italic font. nM: premenopausal women; PM: postmenopausal women; Ca: calcium; 25OHD: 25 hydroxyvitamin D; InsR: insulin resistance; HyperChol: Hypercholesterolemia; HyperTG: Hypertriglyceridemia; Low HDL Chol: Low HDL cholesterol; TG/HDL: Triglycerides/HDL Cholesterol ratio; HyperSBP: High systolic blood pressure; HyperDBP: High diastolic blood pressure.

DIFFERENCES BY BMI

Premenopausal women

No differences in age, fat mass%, SBP and DBP between NW and OW groups were observed but Ca intake was significantly higher in the OW group (p<0.05). Fat mass% increased significantly with the increase in BMI in obese groups (p<0.05). SBP and DBP were significantly lower in NW and OW than in the 3 degrees of obesity (p<0.05). While no differences in DBP were observed between the 3 degrees of obesity, SBP was significantly lower in OBI than in OBII and OBIII (p<0.05) which did not present differences between them. The lowest significant Ca intake was observed in NW women (p<0.05); no differences were observed among the

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remaining groups. These parameters of PM and nM women are shown in Table 3.

Table 3. Characteristics of premenopausal (nM) and postmenopausal (PM) women divided by BMI.

Groups		Age (years)	BMI (Kg/cm^2)	Fat mass%	SBP (mmHg)	DBP (mmHg)	Ca Intake (mg/day)
NW	nM n=38	38.7±5.0^A	24.1±0.7^A	31.4±3.4^A	103.3±11.5^A	64.7±5.8^A	516 (326-682)^A
	PM n=49	69.1±5.4^B	23.5±1.2^A	39.8±2.1^B	110.0±9.8^B	66.2±2.4^B	637 (368-791)^B
OW	nM n=33	34.8±9.0^A	28.1±1.6^B	35.8±3.0^A	107.7±12.1^A	66.8±4.6^A	703 (612-910)^B
	PM n=106	60.0±6.1^B	28.3±1.3^B	40.3±2.5^B	117.7±12.3^B	74.6±7.4^B	663 (462-929)^B
OBI	nM n=35	35.5±7.4^A	31.9±1.6^C	42.0±2.7^B	113.3±7.8^B	69.2±6.4^B	674 (583-1404)^B
	PM n=106	61.2±7.6^B	32.4±4.4^C	44.2±2.4^C	127.6±15.7^C	75.7±9.7^B	667 (466-774)^B
OBII	nM n=14	45.4±3.2^A	38.1±1.0^D	48.5±2.2^D	133.3±5.8^C	78.0±8.4^B	583 (215-734)^B
	PM n=46	59.7±3.8^B	37.4±1.5^D	49.5±2.2^D	127.7±8.8^C	75.0±5.9^B	693 (659-808)^B
OBIII	nM n=25	38.6±7.8^A	44.1±2.5^E	57.7±1.6^E	128.3±7.5^C	77.5±7.1^B	822 (656-1087)^B
	PM n=32	62.6±6.5^B	42.8±2.1^E	60.8±3.3^E	138.3±17.2^D	80.0±0.1^C	767 (542-839)^B

Results were expressed as mean ± SD or median (IQR). Groups were divided by estrogenic status and each of them were divided by differences in BMI. One-way ANOVA test was used to compare the median of parameters that followed a normal distribution. Bonferroni tests were used as "a posteriori " test to determine differences between groups. Parameters that do not show a normal distribution were compared using the Kruskal–Wallis test. Different letters indicate $p < 0.05$. nM: premenopausal women; PM: postmenopausal women; NW: normal weight; OW: overweight; OBI: obesity degree I; OBII: obesity degree II; OBIII: obesity degree III. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; Ca: calcium; BMI: body mass index

The metabolic glycemic and lipid profile are shown in Figure 1. No differences in WC between NW and OW groups were observed. WC increased significantly with the degree of obesity ($p<0.05$), although no differences between the two higher degrees of OB were found. Leptin and HOMA-IR were significantly higher in OW than in NW women ($p<0.05$); no differences between the other parameters were observed between these groups. Glucose levels did not present differences by BMI, although these levels tended to be lower in NW than in obese groups.

The significantly highest levels of tChol and TG were observed in OBII and OBIII groups ($p<0.05$); no differences were observed among the other groups. HDLChol did not present differences by BMI. Ins levels increase with the increase in BMI reaching statistical significance between NW and OW and the remaining groups ($p<0.05$), or between OBI and II and the other obese group ($p<0.05$). Leptin levels increased significantly with the increase in BMI ($p<0.05$). HOMA-IR, HOMA-β and TG increased with the increase in BMI; the highest significant levels were observed in OBII and OBIII women ($p<0.05$).

Figure 2 showed the mean values of the different parameters related to phospho-calcium homeostasis and bone metabolism. No differences by BMI in sCa, sPi, tOCN, CTX, BAP and 25OHD levels were observed. However, ucOCN and the ucOCN/tOCN ratio increased with the increase in BMI showing statistical significance between NW and the remaining groups or between OW and OBI vs OBII and OBIII groups ($p<0.05$). The significantly highest values of ucOCN and ucOCN/tOCN ratio were observed in OBII and OBIII women ($p<0.05$). Although the levels of 25OHD did not reach statistical differences by BMI, the lowest levels were observed in the two higher degrees of obesity.

Postmenopausal women

Fat mass%, and SBP increased as BMI increases ($p<0.05$). SBP did not show differences between NW and OW or between OBI and OBII. The highest significant level of SBP was observed in OBIII ($p<0.05$). DBP only was significantly highest between OBIII and the rest of the groups ($p<0.05$) in OBIII. Ca intake levels did not show statistical differences by BMI. These parameters of PM and nM women are shown in Table 3.

The metabolic glycemic and lipid profile are shown in Figure 1. No differences in WC were observed between NW and OW women, however, the means increased significantly with the increase of obesity ($p<0.05$). Glucose levels tended to increase with the increase in BMI without reaching statistical differences. No significant differences in tChol and HDLChol levels were observed by BMI. TG levels increased with the increase in BMI showing statistical differences between NW and the other groups ($p<0.05$). Ins levels increased with the increase in BMI

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($p<0.05$) reaching statistical differences between NW, OW and OBI and the remaining groups ($p<0.05$). As expected, leptin increased significantly with the increase in BMI ($p<0.05$), only OW and OBI did not reach statistical differences. HOMA-IR, HOMA-β and TG/HDL Chol increased with the BMI and reached statistical significance in the two higher degrees of obesity ($p<0.05$).

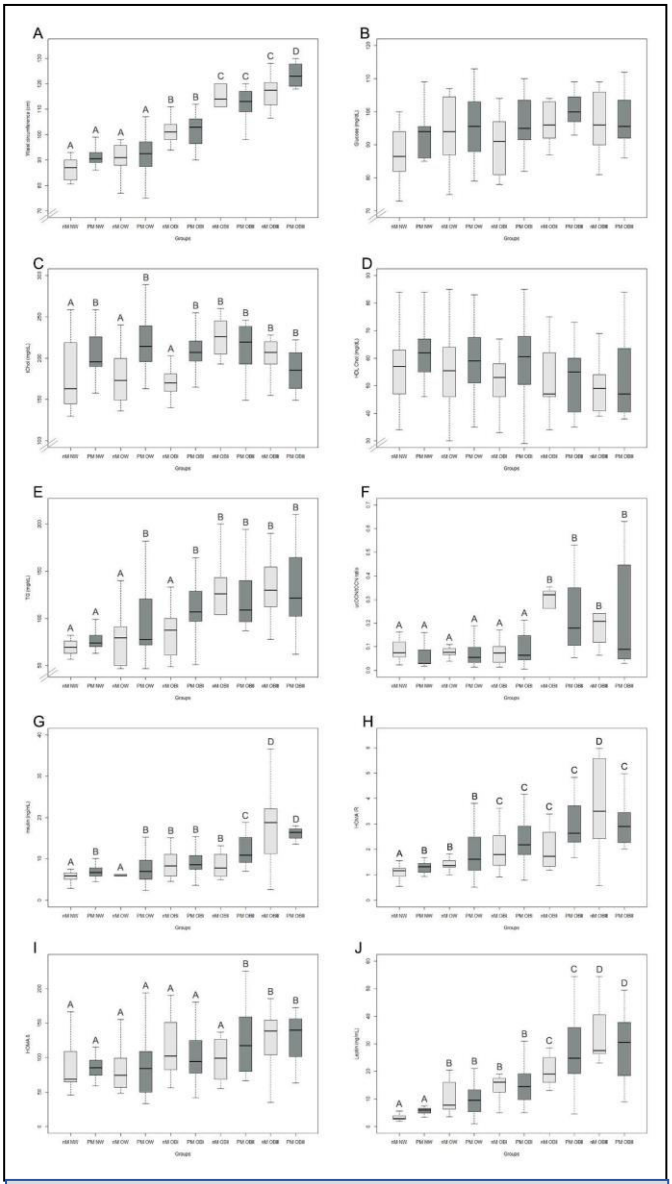

Figure 1: Box plots of metabolic glycemic and lipid profile.

A) Waist circumference (WC); the serum levels of: B) glucose; C) total cholesterol (tChol); D) serum levels of HDL cholesterol (HDL Chol) ; E) serum levels of triglycerides (TG); F) ratio TG/HDL Chol; G) insulin and H) leptin and I) homeostatic model assessment of insulin resistance (HOMA-IR) calculated as HOMA-IR= glucose (mmol/L)x Ins (µU/mL)/22,5 and J) homeostatic model assessment of β-cell function (HOMA-b) calculated as HOMA-β= 20 x Ins (µU/mL)/(glucose (mmol/L) - 3.5). Groups were divided by estrogenic status and each of them by differences in BMI. Parameters showed a normal distribution according with Shapiro–Wilk and Levene's tests. One-way ANOVA test was used to compare median values of the groups having normal distribution. Different letters indicate $p < 0.05$. nM: premenopausal women: PM: postmenopausal women; NW: normal weight; OW: overweight; OBI: obesity degree I; OBII: obesity degree II; OBIII: obesity degree III.

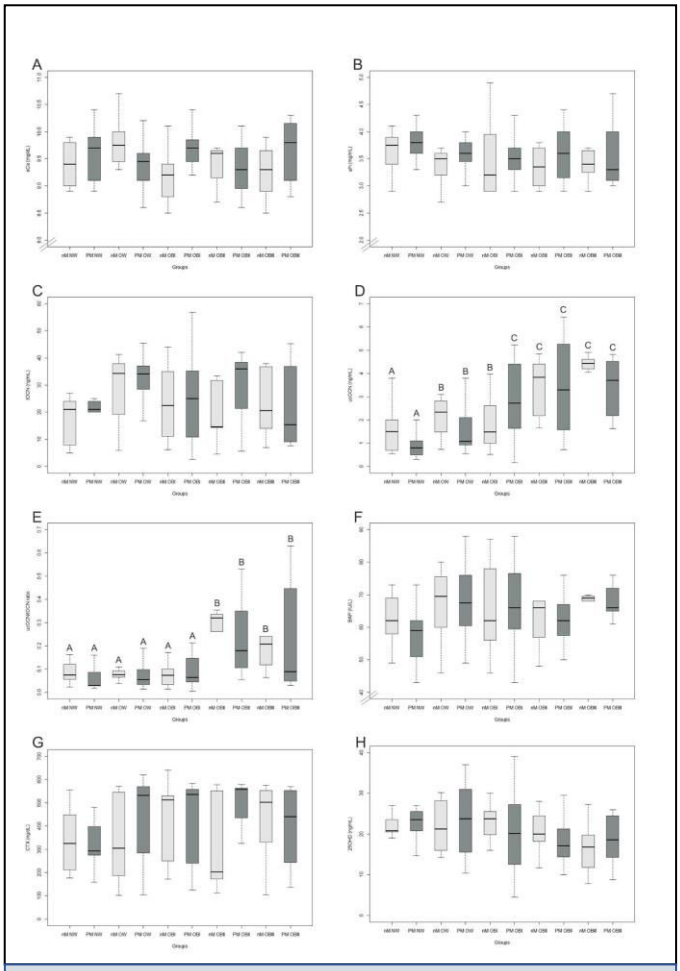

Figure 2: Serum levels of parameters associated with phospho-calcium homeostasis and with bone remodeling markers.

Results showed the box plots of serum levels of: A) calcium (sCa); B) phosphate (sPi); C) total osteocalcin (tOCN) ; D) undercarboxylated osteocalcin (ucOCN); E) ucOCN/tOCN ratio; F) bone alkaline phosphatase (BAP); G) carboxy-terminal collagen crosslinks (CTX); H) 25 hydroxyvitamin D (25OHD). Groups were divided by estrogenic status and each of them by differences in BMI. Parameters showed a normal distribution according with Shapiro–Wilk and Levene's tests. One-way ANOVA test was used to compare median values. Different letters indicate $p < 0.05$. nM: premenopausal women: PM: postmenopausal women; NW: normal weight; OW: overweight; OBI: obesity degree I; OBII: obesity degree II; OBIII: obesity degree III.

Figure 2 showed the mean values of the different parameters related to phospho-calcium homeostasis and bone metabolism. No differences by BMI sCa in sPi, tOCN, BAP and CTX levels

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were observed. Even though, ucOCN increased with the increase in BMI showing statistical significance between NW and the different degree of obesity or between OW and the other obese groups ($p<0.05$); no differences between OBI, OBII and OBIII groups were observed. The significant highest value of ucOCN/tOCN ratio was observed in the highest degrees of obesity ($p<0.05$). 25OHD levels did not show statistical differences by BMI; however, the two higher degrees of obesity presented the lowest no significant level of 25OHD.

Differences by BMI and estrogenic status

Fat mass% was significantly higher in NW, OW and OBI M vs nM groups ($p<0.0002$, $p<0.0001$ and $p<0.005$, respectively). SBP were significantly higher in NW, OW and OBI and OBII PM than in nM women ($p<0.05$, $p=0.03$; $p=0.03$ and $p<0.004$, respectively). DBP were significantly higher in NW, OW and OBIII in PM than in nM women ($p=0.01$, $p=0.002$ and $p=0.002$, respectively), Ca intake only showed statistical differences between PM and nM in the NW group ($p<0.02$) (Table 1).

WC only showed statistical differences between PM and nM in the OBIII group ($p<0.02$). Glucose, HDL Chol, TG/HDL Chol ratio and leptin levels were generally higher in PM than in nM women, however no significant differences between them were observed. tChol was higher in NW, OB and OBI PM women than in nM women ($p<0.006$; $p<0.0006$ and $p<0.0001$, respectively). TG levels were significantly higher in OW and OBI PM than in nM women ($p<0.01$ for both groups). Ins levels were significantly higher in NW, OW and OBII PM than in nM women ($p<0.04$; $p<0.05$ and $p<0.02$, respectively). HOMA-IR were significantly higher in NW, OW and OBIII PM than in nM women ($p<0.04$; $p=0.02$ and $p<0.05$, respectively). HOMA-β only showed statistical differences between PM and nM in the OBII group ($p<0.02$) (Figure 1).

The levels of sCa, sPi, tOCN, BAP, CTX and 25OHD were generally higher in PM than in nM women, however no significant differences between them were observed. The levels of ucOCN were significantly higher in PM as compared to nM only in the OBI group ($p<0.05$). The ucOCN/tOCN ratio did not show statistical differences however, in almost all degrees of obesity, the ucOCN/tOCN ratio tended to be higher in NM women (Figure 2).

CORRELATIONS

By estrogenic status

Premenopausal women

Figure 3 shows the correlations of tOCN in nM women. tOCN negatively correlated with ucOCN/tOCN ratio ($r=-0.30$; $p=0.04$) and sPi ($r=-0.33$; $p=0.03$). Showed a tendency to correlate with HOMA-IR ($r=0.21$; $p=0.09$). tOCN did not show significant correlation with the remaining studied parameters.

Figure 4 shows the correlations of ucOCN in nM women. Levels of ucOCN showed significant correlation with ucOCN/tOCN ratio ($r=0.56$; $p< 0.001$); BMI ($r=0.51$; $p<0.001$); WC ($r=0.55$; $p<0.001$); fat mass% ($r=0.41$; $p=0.007$), SBP ($r=0.33$; $p= 0.028$); TG ($r=0.51$; $p<0.001$); TG/HDL Chol ($r=0.45$; $p=0.002$); and Leptin ($r=0.49$; $p<0.001$).

Figure 5 shows the correlations of ucOCN/tOCN ratio in nM women. The ucOCN/tOCN ratio showed significant correlation with BMI ($r=0.39$; $p=0.004$); WC ($r=0.40$; $p=0.004$); fat mass% ($r=0.38$; $p=0.01$); tChol ($r=0.46$; $p<0.001$); leptin ($r=0.33$; $p=0.02$) and showed a tendency to correlate with and 25OHD ($r=-0.27$; $p=0.06$).

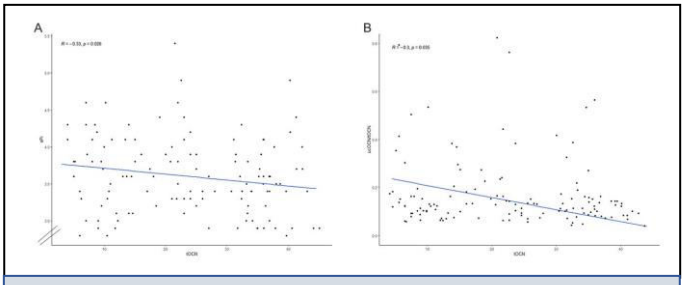

Figure 3: Correlations between total osteocalcin (tOCN) and: A) serum phosphate (sPi) and B) under-carboxylated osteocalcin

R and P values were obtained by Spearman analyses.

Postmenopausal women

Figure 6 shows the correlations of tOCN in PM women. tOCN showed a negative correlation with ucOCN ($r=-0.24$; $p=0.04$); positive correlation with DBP ($r=0.27$; $p=0.02$); and showed a tendency to correlate with ucOCN/tOCN ratio and CTX ($r=-0.20$; $p=0.07$ and $r=0.21$; $p=0.06$, respectively). tOCN did not show significant correlation with the remaining studied parameters.

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Figure 7 shows the correlations of ucOCN in PM women. Levels of ucOCN showed positive correlations with ucOCN/tOCN ratio (r= 0.43 p< 0.001), BMI (r=0.53; p< 0.001), WC (r=0.40; p<0.001), fat mass% (r=0.49; p<0.001), SBP (r= 0.32; p=0.005), and leptin (r= 0.41; p<0.001) and negative correlation with Ca intake (r=-0.3; p=0.009). Showed a

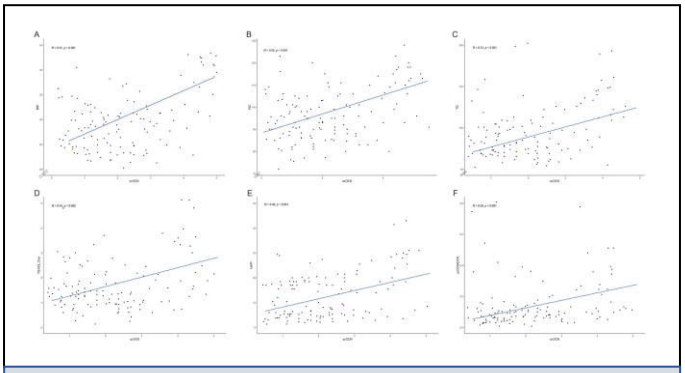

Figure 4: Correlations between under-carboxylated osteocalcin (ucOCN) and: A) Body mass index (BMI); B) waist circumference (WC); C) triglycerides (TG); D) TG/HDL Chol E) Leptin; F) ucOCN)/total osteocalcin (tOCN) ratio in Premenopausal women (nM).

R and P values were obtained by Spearman analyses.

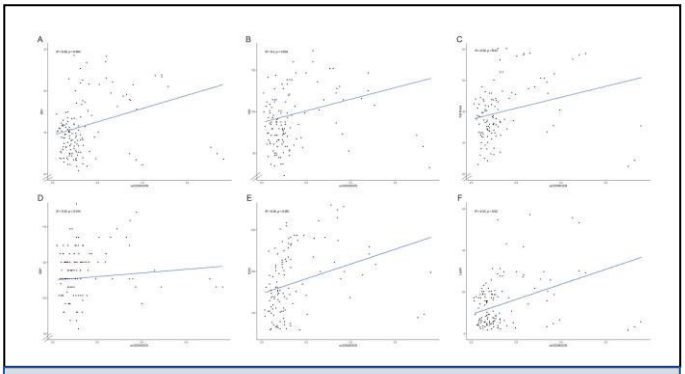

Figure 5: Correlations between under-carboxylated osteocalcin (ucOCN)/total osteocalcin (tOCN) ratio and: A) Body mass index (BMI); B) waist circumference (WC); C) Fat mass%; D) systolic blood pressure (SBP); E) total cholesterol (tChol); F) Leptin, in Premenopausal women (nM).

R and P values were obtained by Spearman analyses.

Figure 8 shows the correlations of ucOCN/tOCN ratio in PM women. The ucOCN/tOCN ratio showed similar positive correlations with BMI (r=0.37; p<0.001), WC (r=0.37; p<0.001), fat mass% (r=0.35; p=0.003); Ins (r=0.24; p=0.03) and leptin (r=0.27; p=0.014); and show a tendency to correlate with HOMA-IR (r=0.21; p=0.06).

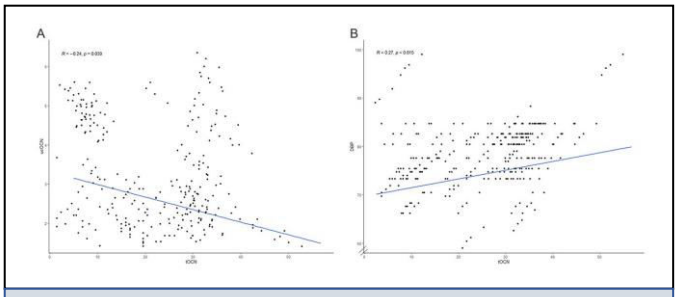

Figure 6: Correlations between total osteocalcin (tOCN) and: A) under-carboxylated osteocalcin (ucOCN) and B) Diastolic blood pressure (DBP), in Postmenopausal women (PM) .

R and P values were obtained by Spearman analyses.

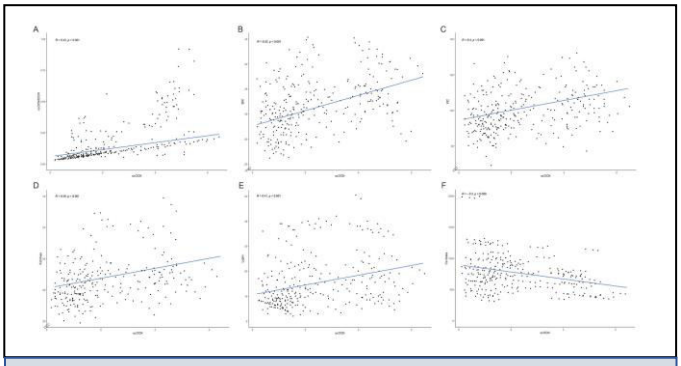

Figure 7: Correlations between under-carboxylated osteocalcin (ucOCN) and: A) ucOCN/total osteocalcin (tOCN) ratio, B) Body mass index (BMI), C) Waist circumference (WC), D) Fat mass% E) Leptin, F) Calcium (Ca) Intake, in Postmenopausal women (PM).

R and P values are from Spearman analyses.

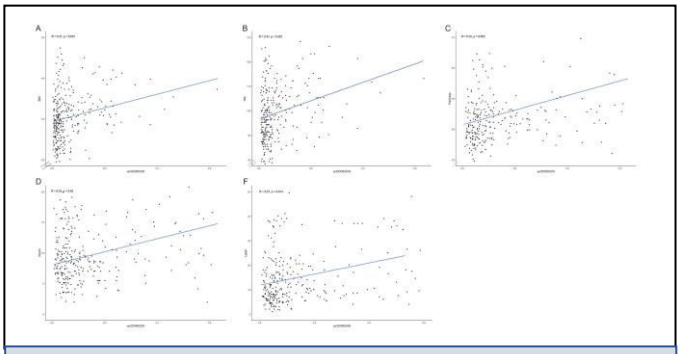

Figure 8: Correlations between under-carboxylated osteocalcin (ucOCN)/total osteocalcin (tOCN) ratio and A) Body mass index (BMI), B) Waist circumference (WC), C) Fat Mass%, D) Insulin and E) Leptin, in Postmenopausal women (PM).

R and P values were obtained by Spearman analyses.

Adequacy of Ca intake and VD nutritional status

Women with Ca intake >800 mg/d and 25OHD levels >29 ng/mL

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None of nM women had both parameters, Ca intake >800 plus 25HOD >29 ng/mL. However, 11,2% of PM women (n=38) met both requirements and could be assessed.

In PM women, the levels of tOCN only showed a positive correlation with HDL Chol (r=0.67; p=0.05) and ucOCN/tOCN (r=0.71; p=0.03) and a tendency to correlate with TG (r=-0.66; p= 0.07). Conversely, circulating levels of ucOCN showed a positive correlation with BMI as well as with leptin (r=0.77; p=0.03 and r=-0.78; p=0.02, respectively) (Figure 9).

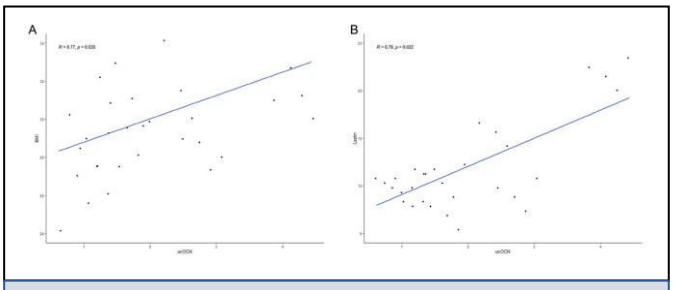

Figure 9: Correlations in PM women having Ca intake >800 mg/d and serum levels of 25OHD >29 ng/mL between ucOCN and: A) Body mass index (BMI) and B) vs leptin.

R and P values were obtained by Spearman analyses.

DISCUSSION

Circulating OCN has been associated with bone turnover and bone formation. However, besides OCN's role in bone metabolism, several studies have shown that it plays an important role in energetic metabolism regulation [31]. The effect of bone-mediated regulation of glucose homeostasis was initially suggested by the expression of Ins receptor and glucose transporter in osteoblasts [4,32]. Osteoblastic Ins signaling stimulates OCN production but also decreases OPG synthesis which stimulates osteoclast activity and the bioactivation and release of ucOCN to circulation. Endocrine undercarboxylated form of OCN regulates Ins/glucose axis and energy metabolism [33]. Fat mass also influences bone metabolism through adipocytokines secreted from adipocytes. Via the central nervous system, leptin inhibits bone formation that decreases the production of OCN by osteoblasts and favors bone resorption that increases the bioactivation of OCN and the release of ucOCN from bone, evidencing a dual effect of leptin on OCN bioactivation [10,34,35]. Adiponectin stimulates Ins-sensitivity and has two opposite influences in bone formation, favoring and inhibiting the process. In a feed-forward loop, ucOCN appears to favor the synthesis of adiponectin [32]. These interrelationships evidence a cross-talk between fat, pancreas and bone tissues. Animal studies showed that mice lacking OCN displayed a series of phenotypic abnormalities such as obesity, hyperglycemia, InsR and hypertriglyceridemia [12]. These studies also showed that ucOCN could be the active form. However, results from human studies are conflicting [2].

Estrogen loss increases bone turnover that is evidenced by an increase in the levels of bone markers. At menopause, serum OCN concentrations rise considerably, by about 50-150% [38]. However, oral contraceptives, hormone therapy, diabetes mellitus, and BMI are factors having stronger impacts on the limits of reference [36]. A previous report showed that the mean tOCN levels were 14.4 ng/mL (11.3-18.5 ng/mL) in nM, and 18.6 ng/mL (13.6-25.6 ng/mL) in PM women [37], after exclusion of all nM women using oral contraceptives or PM women receiving hormone replacement therapy, presence of diabetes or BMI < 18 or >30 kg/m^2. Our results in non-diabetic-normoglucemic women having normal body weight showed that the mean value for tOCN was 17.6±9.5 ng/mL in nM and 20.6±8.9 ng/mL in PM women. The differences in the mean value of tOCN between both studies were not so large because we evaluated tOCN by a similar assay method that detects the intact OCN polypeptide and the N-terminal-Mid OCN fragment [37]. The same study reported that the reference value of serum ucOCN concentration was <4.5 ng/ml suggesting that, theoretically, the ucOCN level should be 25% of the tOCN amount [37]. In our study, the women showed an ucOCN mean value of 1.0±0.8 ng/mL (0.15-4.95 ng/mL) in PM and 1.6±1.1 ng/mL (0.24-3.81 ng/mL) in nM women; only one PM woman presented levels higher than 4.5 ng/mL. A general rule in endocrinology determines that most circulating protein factors associated with regulatory functions are inactive. In line with this rule, the level of ucOCN would be approximately 10% of tOCN. In agreement, our NW women showed that the mean percentage of ucOCN/tONC was 5% and 8% for nM and PM.

It is important to have in mind that when all the studied women having different BMI were included in the analysis, the mean

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values for tOCN showed a slight increase (26.4±9.5 ng/mL and 27.1±9.8 ng/mL in nM and PM, respectively), while the increase was significant for ucOCN levels (2.5±1.9 ng/mL and 2.9±1.7 ng/mL in nM and PM, respectively). It is well known that the adipose tissue modulates metabolism through the release of free-fatty acids, glycerol and adipocytokines whose productions are altered in obesity leading to a low-grade of systemic inflammation, progressive InsR and other metabolic disorders [38]. In the present report Ins, leptin and InsR increased with BMI in both, PM and nM women. Despite that, and independently of the estrogenic status, tOCN levels did not have any correlations with the degree of obesity, fat mass%, levels of leptin and Ins or InsR, suggesting that the increase in pad mass may not affect OCN production in normoglycemic-non diabetic women. This finding is in disagreement with a previous report suggesting that OCN is associated to fat mass [39], measures of adiposity [40] or the lower concentration in obese people than in normal controls [13]. Conversely, ucOCN levels changed with BMI showing a positive correlation with the degree of obesity and fat mass. This finding suggests that pad mass may affect the bioactivation of OCN.

The lack of changes in tOCN along with the changes in ucOCN modified the percentage of the undercarboxylated form to the total amount of OCN. Indeed, the mean of ucOCN/tOCN ratio showed a significant increase with obesity similar to that observed in HOMA-IR, suggesting that fat mass may induce not only InsR but also a certain degree of resistance to the hormonal form of OCN. According to literature, impairments in glucose metabolism are associated with decreased serum levels of ucOCN [4], while chronic hyperglycemia (rather than InsR) plays a prominent role in reducing the total amount of circulating OCN in diabetic type II patients having poor glycemic control [39]. On these bases, it appears plausible to assume that in our normoglycemic-non diabetic women more quantity of ucOCN for the same amount of tOCN should be necessary for inducing the amount of Ins secretion that maintains glucose homeostasis. We hypothesized that the increase in the undercarboxylated form of OCN in our study may play a protective role against the development of prediabetes. This finding would be in agreement with a previous report suggesting that ucOCN could be closely related to Ins secretion and β cell proliferation, but in disagreement with others reporting that the carboxylated form of OCN is more closely related to improved Ins sensitivity [41]. In our normoglycemic-non diabetic women no correlation was observed for tOCN and InsR indexes; however, ucOCN in nM women correlated with TG/HDL Chol ratio, an alternative index of InsR. This finding is in agreement with the fact that high estradiol levels may modify Ins sensitivity in nM women while in PM women it is associated with high InsR and increased incidence of diabetes [12].

According to our results, we conclude that the undercarboxylated isoform of OCN may mediate the metabolic function of OCN in glucose homeostasis since it was reported that only this form has the ability to induce Ins secretion, and the expression of genes encoding adiponectin and Ins that improves glucose metabolism [12]. Other studies suggested that both forms of OCN increase basal and Ins-stimulated glucose transport although the effect of the carboxylated form was less robust [41]. The discrepancy among different reports may be due to differences in the design of the study, populations, and comorbidities as well as in the use of different methods to measure OCN with different accuracy that, in addition to the lack of standardized methods, makes it difficult to compare data between studies.

The levels of OCN could be affected by renal function because the different forms of OCN are cleared by the kidneys, with exponential rises reported when kidney function drops below 30–40 ml/min/1.73m^2 [42]. All the studied women had normal renal function since creatinine and urea levels higher than the reference range was used as one of the exclusion criteria of the study. The effect of OCN in energetic metabolism appears to depend on nutritional factors. OCN undergoes post-translational vitamin K-dependent modifications that could differentially affect its bioactivity and bioavailability [43]. According to the food-frequency questionnaire completed, all the studied women had vitamin K adequacy (data not shown). However, other nutritional factors may affect the relationship between ucOCN and clinical outcomes. Habitual low Ca intake and VD insufficiency increase synthesis and release of parathormone to circulation that lead to an increase in bone turnover [44], mainly bone resorption, introducing a new variable in the interrelationship between bone, pancreas and fat tissues. In the present report we wanted to evaluate the

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effect of OCN in glucose homeostasis in women having adequacy of Ca intake and 25HOD levels. However, and not surprisingly, only 10% of the PM women and none of nM women met both nutritional Ca and VD requirements. Nevertheless, analyzing the correlation in such a small group of PM women we found that tOCN only correlated with HDL Chol and ucOCN/tOCN ratio, conversely ucOCN correlated with BMI and leptin. These findings suggest that the adequate nutritional status of Ca and VD controlled the bone resorption process clarifying the role for ucOCN. Only two nM fulfilled both requirements, which made it impossible to make correlations.

The strengths of this study include the comprehensive and well-described cohort of mostly normoglycemic-non diabetic PM and nM women, with a very limited number of potential confounding factors related to comorbidities or medications that may influence glycemia or bone turnover. The present study has potential limitations, namely, a lower number of subjects with NW and OBIII related to the other degree of obesity as well as the lack of nM women with adequacy in Ca intake along with sufficiency of VD. As the number of these mentioned women was relatively low, the significance of our findings deserves further investigation increasing the number of participants.

CONCLUSION

The regulation of glucose homeostasis in mammals is a complex process based on the interplay of different factors and tissues. Data from human studies concerning the action of total and undercarboxylated forms of OCN in glucose metabolism control are rather inconsistent, and their mechanism of action is not fully understood. We evaluated the interaction between bone, pancreas and fat mass, in maintaining glucose homeostasis in normoglycemic-nondiabetic pre and postmenopausal women having differents BMI. Our results showed that tOCN levels did not change while ucOCN levels increased with obesity, leading to the raise in the percentage of the ucOCN/tOCN ratio, suggesting that the ucOCN would be the endocrine form of OCN. We speculated that higher circulating levels of the undercarboxylated form related to the total amount of OCN are required to maintain glucose homeostasis with the increase in obesity. Then, as occurs in InsR, obesity may induce a certain degree of resistance to the endocrine action of OCN in the pancreas. Furthermore, our results also showed that other nutritional factors may affect the relationship between ucOCN and clinical outcomes. In the studied women, only 10% of the PM women and none of nM met the nutritional status of Ca and VD. However, in the small number of PM women who covered both nutritional status, the levels of ucOCN correlated with BMI and leptin levels, highlighting the importance of nutrition to better clarify the role of OCN in the control of glucose homeostasis.

CONFLICT OF INTEREST

No conflict of interest was declared.

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