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 **Η λεπτίνη και αδιπονεκτίνη στο αίμα σαν διαμεσολαβητές της συσχέτισης μεταξύ της μάζας του λίπους και του BMD σε περιεμμηνοπαυσιακές γυναίκες.**

Fat mass is a predictor of BMD; however, the mechanisms involved remain uncertain. Two adipokines, leptin and adiponectin, were examined as potential mediators of this relation in 80 perimenopausal women. Adiponectin did not exert any effect on BMD, whereas leptin exerted a negative one, with insulin acting as a confounder to this relation.

**Introduction:** Fat mass is an important determinant of bone density, but the mechanism involved in this relation is uncertain. Leptin and adiponectin, as circulating peptides of adipocyte origin, are potential contributors to this relation. We investigated the role of leptin and adiponectin in mediating fat mass effects on the skeleton of perimenopausal women.

**Materials and Methods:** Twenty-five premenopausal and 55 postmenopausal, healthy women (42–68 years old) participated in our study. Lumbar spine BMD ($BMD_{L2-L4}$) and total body BMC (TBBMC) were measured with DXA, leptin levels with ELISA, and adiponectin levels with radioimmunoassay (RIA). Additionally, body composition analysis was performed, as well as measurements of several hormones.

**Results:** It was shown that serum leptin levels were negatively correlated with BMD ($\beta$=-0.005, $p$ = 0.027) and TBBMC ($\beta$=-14.32, $p$ = 0.013). The above correlation was observed only when serum insulin levels were included, as an independent variable, in the regression analysis model. Adiponectin was not significantly correlated with $BMD_{L2-L4}$ nor with TBBMC, either in the presence or absence of insulin.

**Conclusion:** Circulating adiponectin does not seem to exert any effect on bone mass. In contrast, circulating leptin showed a negative correlation with bone mass, dependent on serum insulin levels.

# Blood Leptin and Adiponectin as Possible Mediators of the Relation Between Fat Mass and BMD in Perimenopausal Women

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**ABSTRACT: Fat mass is a predictor of BMD; however, the mechanisms involved remain uncertain. Two adipokines, leptin and adiponectin, were examined as potential mediators of this relation in 80 perimenopausal women. Adiponectin did not exert any effect on BMD, whereas leptin exerted a negative one, with insulin acting as a confounder to this relation.**

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**Key words:** BMD, leptin, adiponectin, insulin, body composition

## INTRODUCTION

**B**ODY WEIGHT IS one of the strongest predictors of bone mass in both sexes and is associated with increased BMD and decreased risk of fractures.[1–4] However, the mechanisms, explaining this relation, are still not completely understood.

Obesity-induced mechanical loading is a contributing factor to the positive relationship between body weight and BMD either through body fat or through muscle-mediated mechanical effects of increased weight bearing.[5] However, this protective effect has been also observed at non–weight-bearing bone sites,[6] which implies that factors other than skeletal loading are likely important in mediating the relationship between body weight and BMD.

Body fat mass (BFM) has been proposed as a better predictor of BMD than body weight and lean mass.[7] The increased aromatization of androgen to estrogen in adipose tissue,[8] which represents the principal source of estrogens

after menopause,[9] may partly explain the positive effect of fat mass on bone in postmenopausal women. Additionally, decreased sex hormone binding globulin (SHBG) levels[10] with increased free sex steroids is also considered as possible mechanisms for the association between obesity and BMD, as well as the direct augmentation of bone formation, by increased levels of circulating insulin.[11] The latter may be caused by direct mitogenic effects of insulin on osteoblasts.[12]

Leptin, the product of *ob* gene, is a small polypeptide hormone mainly produced by adipocytes[13,14] and is strongly correlated with BFM.[15] Rarely are obese individuals leptin deficient and most of them have hyperleptinemia proportionate to body fat and are leptin resistant.[16] Apart from leptin's effects on the nervous system, it has been found that it affects a variety of endocrine axes, particularly the hypothalamic-pituitary-gonadal axis and insulin biology.[17]

In vitro and animal studies of the effects of leptin on bone metabolism are controversial. There are studies that have revealed that leptin enhances osteoblast differentiation[18,19] and inhibits osteoclast generation and bone resorption.[20–22]

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However, intracerebroventricular infusion of leptin causes bone loss in leptin-deficient mice, which are obese, hypogonadic, and have increased bone mass,[23] whereas subcutaneous administration of leptin to models of rapid bone loss prevents partially the bone loss.[20,24]

Human cross-sectional studies about the role of leptin in bone metabolism also are not conclusive. Although some authors[25–28] have reported a positive association between serum leptin levels and BMD, others[29–33] failed to find any relationship, whereas there are four studies that proposed a negative association.[34–37]

Adiponectin on the other hand, is a recently discovered adipocytokine, which is specifically and highly expressed in human adipose cells.[38] This cytokine is a collagen-like protein, and its concentrations in human plasma range from 5–30 $\mu$g/ml.[39] Adiponectin is the only adipose-specific protein known to date that is negatively regulated in obesity, because it is negatively correlated with body mass index (BMI), BFM, and fasting insulin concentrations and calculated insulin resistance.[40]

Evidence, reported so far, suggests that adiponectin possesses anti-hyperglycemic, anti-atherogenic, and anti-inflammatory properties.[41] In addition, adiponectin is present within normal bone marrow and can inhibit fat cell formation by marrow-derived stromal cells through a COX-2–dependent mechanism, suggesting a new mechanism for regulation of pre-adipocyte differentiation and possible roles for fat in hematopoietic tissue.[42] However, there are no published in vitro or in vivo studies that investigate the potential effects of adiponectin on bone mass.

In this study, we tried to elucidate the influence of leptin on bone mass by estimating most of the factors that are known to affect both bone and leptin metabolism and to explore any potential effect of the recently discovered adiponectin on bone tissue in a sample of perimenopausal women.

## MATERIALS AND METHODS

Eighty white women, 42–68 years of age, were recruited to this study through an advertisement in a local magazine. They were all in apparent good health after physical examination. Thirteen women reported use of thyroid medication, but no other established endocrine or rheumatic diseases were reported, and none of the subjects had been exposed to hormonal replacement therapy for menopause, nor had they received any contraceptives. Menopause was defined by absence of menses for more than 6 months and by elevated serum FSH levels (FSH > 40 U/l). None of the participants were receiving treatment (e.g., calcium, corticosteroids, vitamin D, calcitonin, bisphosphonates, anti-vitamin K agents, diuretics, or $\beta$-blockers) that could influence BMD. Information on dietary calcium intake through a food frequency questionnaire, as well as habitual physical activity,[43] smoking habits, and history of bone fractures were recorded. Blood samples were collected from all the volunteers after an overnight fast and between 8:00 a.m. and 9:00 a.m. and were stored at −80°C.

The study protocol was approved by the Institutional Review Board of Harokopio University.

### Anthropometry

Subjects were weighed wearing light clothes and no shoes. Body weight was measured using a Seca scale (Mod 220) to a precision of 0.5 kg. Height was measured by using a Seca stadiometer (Mod 220) to a precision of 0.5 cm. BMI was calculated as weight (kg)/height$^2$ (m$^2$).

Body composition analysis was performed for the whole sample using the bioelectrical impedance analysis (BIA) method with a single-frequency (50 kHz), four-terminal impedance plethysmograph (Model 101Q; RJL Systems, Mt Clemens, MI, USA). The prediction equation that was used for the estimation of the fat free mass (FFM) has been developed and validated in a sample of 60 women, with the same characteristics as our sample and with DXA as the reference method (unpublished observations). Fifty-eight women underwent DXA total body composition analysis (software, version 4.7e, Lunar DPX; Lunar Corp., Madison, WI, USA) and lean tissue mass (LTM) and BFM were assessed. In this last group, the BF% as predicted by the BIA equation was very strongly positively correlated with BF%, as measured with DXA (Spearman coefficient = 0.85, $p < 0.0001$).

### Bone densitometry

BMD (g/cm$^2$) was determined at the lumbar spine ($L_2$–$L_4$) for all the subjects, with a DXA total body scanner (Lunar DPX) and calculated with Lunar software (version 4.7e). Total body BMC (TBBMC; g) was also determined for 58 participants with the same absorptiometer.

### Hormone measurements

The following hormones were measured using commercially available RIAs: cortisol (DSL, Webster, TX, USA; sensitivity 0.5 $\mu$g/dl; intra-assay CV 5.3–8.4%), insulin (DSL; sensitivity 1.3 $\mu$IU/ml; intra-assay CV 8.3%); estradiol (DPC, Los Angeles, CA, USA; sensitivity 8 pg/ml; intra-assay CV 4.3–7%), testosterone (DPC; sensitivity 5 ng/dl; interassay CV 11%), free testosterone (DPC; sensitivity 0.15 pg/ml; intra-assay CV 8%), and follicle stimulating hormone (FSH; DiaSorin, Saluggia, Italy; sensitivity 0.2 U/liter). Serum adiponectin concentrations were determined with a commercial RIA (Linco Research, St Charles, MO, USA; sensitivity 2 $\mu$g/ml; intra-assay CV 1.78–6.21%) and leptin concentrations with a commercial ELISA (R&D Systems, Minneapolis, MN, USA; sensitivity 7.8 pg/ml; intra-assay CV 3.16%).

### Statistics

Descriptive statistics for the continuous variables are presented in the Results section. The Kolmogorov-Smirnov test was used to test for the normality of distributions. Two-sample $t$-tests and two-sample Mann-Whitney U-tests were used where appropriate to compare continuous variables of interest between premenopausal and postmenopausal women. Associations are given as Pearson correlation coefficients for the normal variables and as Spearman for the variables that did not follow the normal distribution or for the nominal ones. Regression analyses models and backward elimination procedures were used ($p \geq 0.1$) to test

TABLE 1. DESCRIPTIVE STATISTICS OF THE STUDY SAMPLE

| | Premenopausal (N = 25; mean ± SD) | Postmenopausal (N = 55; mean ± SD) | p Value |
|---|---|---|---|
| Age (years) | 47.80 ± 3.14 | 54.47 ± 5.36 | <0.0001‡ |
| BMI (kg/m$^2$) | 30.01 ± 5.22 | 28.98 ± 4.19 | NS§ |
| WHR | 0.78 ± 0.06 | 0.79 ± 0.05 | NS‡ |
| Body fat %* | 42.95 ± 5.79 | 42.97 ± 4.26 | NS‡ |
| Fat free mass (kg)* | 42.90 ± 4.92 | 40.36 ± 4.56 | 0.034‡ |
| Lean tissue mass (kg)† | 40.23 ± 5.70 | 37.94 ± 4.21 | NS‡ |
| BMD$_{L2–L4}$ (g/cm$^2$) | 1.23 ± 0.16 | 1.13 ± 0.18 | 0.013‡ |
| TBBMC (g) | 2789.82 ± 433.84 | 2510.29 ± 394.32 | 0.030‡ |
| Calcium intake (mg) | 785.63 ± 391.70 | 940.48 ± 369.88 | NS§ |
| Physical activity index | 7.40 ± 1.11 | 7.48 ± 1.25 | NS‡ |
| Leptin (ng/ml) | 25.66 ± 11.78 | 26.48 ± 11.33 | NS§ |
| Insulin ($\mu$IU/ml) | 7.25 ± 2.78 | 7.17 ± 4.32 | NS§ |
| Adiponectin ($\mu$g/ml) | 7.9 ± 5.81 | 11.94 ± 7.00 | 0.011§ |
| Cortisol ($\mu$g/dl) | 10.43 ± 4.59 | 10.32 ± 5.16 | NS‡ |
| Testosterone (ng/ml) | 41.26 ± 12.60 | 29.95 ± 13.45 | 0.003‡ |
| Free testosterone (pg/ml) | 1.23 ± 0.47 | 1.60 ± 1.18 | NS§ |
| Estradiol (pg/ml) | | 127.76 ± 51.42 | |
| FSH (U/liter) | | 63.31 ± 27.85 | |

* Measured with BIA.
† Measured with DXA (17 premenopausal and 41 postmenopausal).
‡ Student's $t$-test.
§ Mann-Whitney U-test.

the relationship of lumbar spine BMD (BMD$_{L2–L4}$), TBBMC, leptin, or adiponectin with several independent variables, and $R^2$ coefficients are reported. Backward logistic regression analysis was performed with outcome of interest as the membership in the normal or osteopenic/osteoporotic groups (as defined by WHO cut-off points[44]). Data were analyzed with Statistical Package for the Social Sciences (SPSS10.0) software.

## RESULTS

The characteristics of the study sample and assessments of body composition, BMD, dietary calcium, physical activity, and hormones are presented in Table 1. Twenty-five women were classified as premenopausal and 55 as postmenopausal, with mean years since menopause (YSM) of 3.3 years. None of the women reported fractures, and according to smoking habits, 70% of the subjects reported no smoking (current or past), 6.3% were ex-smokers, and 23.7% current smokers. Postmenopausal women were older ($p < 0.0001$) and had less FFM ($p = 0.034$), TBBMC ($p = 0.030$), BMD$_{L2–L4}$ ($p = 0.013$), and lower testosterone concentrations ($p = 0.003$) than premenopausal women, but higher adiponectin levels ($p = 0.011$).

In separate regression models, including menopausal variables (0 = premenopausal, 1 = postmenopausal) with each variable of interest, it was found that BMD$_{L2–L4}$ was negatively correlated with menopause ($\beta = -0.11$, $p = 0.017$), while positively correlated with BMI ($\beta = 0.013$, $p = 0.0020$), LTM ($\beta = 0.013$, $p = 0.015$), and insulin ($\beta = 0.020$, $p < 0.0001$). TBBMC was significantly correlated with age ($\beta = -22.43$, $p = 0.015$), BMI ($\beta = 49.32$, $p < 0.0001$), BF% ($\beta = 39.38$, $p < 0.0001$), LTM ($\beta = 70.11$, $p < 0.0001$), insulin ($\beta = 86.94$, $p < 0.0001$),

testosterone ($\beta = 9.52$, $p = 0.040$), and leptin ($\beta = 11.43$, $p = 0.019$), after adjustment for the menopausal status, in separate regression models. BMD$_{L2–L4}$ and TBBMC were not significantly correlated with smoking habits, calcium intake, physical activity index, serum cortisol, FSH, and free testosterone.

Leptin was significantly correlated with BMI ($r = 0.55$, $p < 0.0001$), BF% ($r = 0.56$, $p < 0.0001$), FFM ($r = 0.36$, $p = 0.001$), LTM ($r = 0.37$, $p = 0.005$), TBBMC ($r = 0.28$, $p = 0.041$), and insulin concentration ($r = 0.50$, $p < 0.0001$). The above correlations remained significant after adjustment for the menopausal status.

Adiponectin was positively correlated with menopausal status ($r = 0.32$, $p = 0.007$), with postmenopausal women having higher adiponectin concentrations ($p = 0.011$). In separate regression analyses, after adjustment for the menopausal status, adiponectin was positively correlated with age ($\beta = 0.01$, $p = 0.092$) and negatively correlated with FFM ($\beta = -0.01$, $p = 0.097$), estradiol levels ($\beta = -0.0007$, $p = 0.012$), and insulin ($\beta = -0.018$, $p = 0.010$). In multivariate analysis, adiponectin was negatively correlated with BMI ($\beta = -0.012$, $p = 0.083$), BF% ($\beta = -0.012$, $p = 0.061$), and waist-to-hip ratio (WHR; $\beta = -0.964$, $p = 0.092$) after adjustment for age and menopausal status. Finally, adiponectin was not significantly correlated with TBBMC, and it showed a negative correlation with BMD$_{L2–L4}$, which was lost after adjustment for the menopausal status.

Table 2 presents the backward multiple linear regression models fitted to data for BMD$_{L2–L4}$ and TBBMC. Several independent variables were entered in the regression models, including age, menopausal status, smoking habits, calcium intake, physical activity index, BMI, BF%, LTM, and

TABLE 2. MULTIPLE REGRESSION ANALYSIS MODELS

| Variable | $\beta$ coefficient $\pm$ SE | p Value |
|---|---|---|
| Dependent variable: | | |
| $\quad$ BMD$_{L2-L4}$ ($R^2 = 0.361$) | | |
| $\quad$ Age | $-0.008 \pm 0.003$ | 0.0060 |
| $\quad$ BMI | $0.013 \pm 0.005$ | 0.015 |
| $\quad$ Insulin | $0.017 \pm 0.005$ | 0.0030 |
| $\quad$ Leptin | $-0.005 \pm 0.002$ | 0.027 |
| Dependent variable: | | |
| $\quad$ TBBMC ($R^2 = 0.710$) | | |
| $\quad$ Age | $-16.61 \pm 6.34$ | 0.013 |
| $\quad$ Body fat % | $20.50 \pm 10.11$ | 0.050 |
| $\quad$ Lean tissue mass | $66.90 \pm 9.29$ | <0.0001 |
| $\quad$ Insulin | $22.89 \pm 11.36$ | 0.051 |
| $\quad$ Leptin | $-14.32 \pm 5.49$ | 0.013 |

serum insulin, cortisol, free testosterone, FSH, leptin, and adiponectin. The multiple regression model's variables that were significantly associated with BMD$_{L2-L4}$ in the multivariate analysis ($p < 0.1$) were age, BMI, insulin, and leptin and explained the 36.1% ($R^2 = 0.361$) of the BMD$_{L2-L4}$ variance. In the model with TBBMC as the dependent variable, the independent variables that were significantly associated with TBBMC were age, BF%, LTM, insulin, and leptin, and these explained the 71% of TBBMC variance ($R^2 = 0.71$). Leptin and age were the only variables that were negatively associated either with BMD$_{L2-L4}$ or TBBMC. In both regression models, insulin was a confounding factor, and when not included in the independent variables, leptin didn't enter the two regression models. Adiponectin did not enter the regression models either in the presence or in the absence of insulin.

In a subgroup of women ($N = 45$), in whom serum testosterone levels were also available, backward multiple linear regression analysis, with BMD$_{L2-L4}$ as the dependent variable and testosterone as an additional independent variable, showed that testosterone was also significantly associated with BMD ($\beta = 0.0046$, $p = 0.0010$), cortisol ($\beta = -0.0087$, $p = 0.056$), age, BMI, insulin, and leptin ($R^2 = 0.605$).

Finally, when we divided the subjects in normal and osteopenic/osteoporotic groups, the following differences were observed: osteopenic/osteoporotic women had lower BMI ($p = 0.032$), the duration of their postmenopausal period was longer ($p = 0.041$), and they had lower testosterone ($p < 0.0001$) and insulin levels ($p = 0.017$). Serum leptin and adiponectin levels did not differ between the two groups. When logistic regression analysis was performed with age, BMI, BF%, menopausal status, insulin, leptin, adiponectin, smoking habits, calcium intake, and physical activity as the independent variables, only insulin ($\beta = -0.33$; $p = 0.041$; exp$\beta = 0.195$; 95% CI, 0.046–0.825) and menopausal status ($\beta = 1.64$; $p = 0.026$; exp$\beta = 1.39$; 95% CI, 1.013–1.90) entered the regression model.

## DISCUSSION

Our study demonstrated that leptin had a negative correlation with bone density in perimenopausal women. In this group of individuals, this correlation was operating only if insulin levels were considered. Furthermore, it was shown that circulating adiponectin levels do not seem to exert any effect on bone mass.

Women at perimenopause have special characteristics that can be outlined as follows: changes in reproduction, stress hormones, BMC, increased fat storage, and altered body fat distribution. During perimenopause, as estrogen levels begin to decline significantly, the stress hormones decrease as well,[45] whereas the catecholamine response to stress seems to be reduced.[46] Current studies have shown that women in menopause have higher circulating levels of insulin and thus a greater propensity to store fat. The majority of women during perimenopause gain weight because of fat storage, especially in the abdominal area. This is accepted as a "normal" variant and a natural occurrence in women over 45 years of age.[47,48] Because women at perimenopause have unique characteristics, we formed a group with late premenopausal and early postmenopausal women, and their parameters were analyzed as a whole.

Premenopausal and postmenopausal women evaluated in this study did not differ statistically significantly regarding BF% and its distribution or levels of leptin, insulin, and cortisol. Postmenopausal women, however, revealed decreased bone density in the lumbar spine and increased levels of adiponectin. It is known from previous studies that serum levels of adiponectin had a negative correlation with estrogen serum levels.[49] Because adiponectin has a protective influence on vessel endothelium,[50] one could speculate that the elevated levels of adiponectin in postmenopausal women try to counterbalance the estrogen deficiency and their protective role in different tissues and cell populations.

Additionally, as shown in previous studies,[41] we also found that adiponectin has a negative correlation with insulin levels and fat mass, and our study researched, for the first time, any correlation of adiponectin to bone mass; no such correlation was found.

In relation to leptin's influence on bone mass, there are several previous studies in humans that failed to find any correlation between serum leptin concentration and bone mass.[29–33] A recently published, large study,[33] including 5815 participants from the Third U.S. National Health and Nutrition Examination Survey, failed to reveal a clear-cut correlation among leptin and BMD. In this study, however, serum hormones were not measured, and body composition was not evaluated.

In contrast, Pasco et al.[27] first reported a positive effect of serum leptin levels on bone mineralization, after adjustment for age and fat mass; however, they did not measure hormones or include menopause status in their analysis. Thomas et al.[25] have also shown that there is a correlation between fat mass and BMD, the strength of which was reduced after adjustment for leptin levels; these authors did not use multivariate analysis models. Recently, Blain et al.[29] reported that leptin was significantly correlated with whole body and femoral neck BMD in a sample of postmenopausal, nonobese women and that this association was independent of the influence that years after menopause, fat mass, creatinine clearance, calcium intake, and other hormonal factors exert on BMD. In this study, no correlation with BMD$_{L2-L4}$ was found.

On the other hand, Blum et al.[34] found that when BMD was examined as a function of percentage fat and leptin together, for a given percentage of fat, leptin seemed to be inversely associated with BMD in premenopausal women.

In vitro experiments and studies on experimental animals have shown that leptin has an inhibitory effect on new bone formation[23] and that this effect does not seem to be mediated through endocrine or paracrine action, but through the leptin's effect on the central nervous system.[23] Studies have revealed specific groups of neurons in the hypothalamus, which are clearly involved in the antiosteogenic function of leptin, and these neurons are different from the ones that regulate the energy metabolism.[23] In addition, it has been recently shown that the sympathetic nervous system is the peripheral mediator on leptin's bone action.[51]

Furthermore, leptin seems to act through the sympathetic system on other tissues such as fat, kidneys, and pancreas.[52–54] Central or systemic administration of leptin to wildtype or leptin-deficient mice was shown to reduce circulating insulin concentrations by up to 85%,[55,56] and at least one study has shown that suppression of insulin secretion after glucose administration is mediated through the sympathetic system.[57]

The above-mentioned experimental studies coupled with our results, which clearly showed that insulin has a positive effect on bone mass and that bone mass was negatively correlated with leptin, the effect of which was always influenced by insulin levels. This prompted us to hypothesize that leptin possibly acts centrally through the sympathetic system and inhibits bone formation either directly or indirectly through suppression of insulin secretion. Alternatively, other unknown factors may possibly stimulate the sympathetic system action, which in turn, may elaborate insulin and leptin resistance. This may explain why obese individuals have high leptin levels and normal bone mass for their age.[58] In our sample, the mean BMI of our subjects was $29.3 \pm 4.53$ kg/m$^2$, with 37% being overweight ($25 \leq$ BMI $< 30$ kg/m$^2$) and 30% being obese (BMI $\geq 30$ kg/m$^2$), representing an overweight population who may have high leptin concentrations caused by resistance to endogenous leptin. However, the range of leptin levels, even in the group of obese, was similar to that observed in other studies where obese individuals were excluded.[26,28]

Finally, in postmenopausal women, an activated sympathetic system may also explain the increased serum adiponectin levels. This hypothesis is supported through studies that revealed that chronic stimulation of the sympathetic system ($\beta$3-adrenergic agonists) leads to increased adiponectin serum levels.[59]

In conclusion, the adipocyte hormones leptin and adiponectin do not seem to contribute as fat mediators to increased bone mass in perimenopausal women. Adiponectin does not seem to exert any effect on bone mass, whereas the negative correlation among leptin and bone mass may be attributed to a more complicated circuit of events between hormones, sympathetic system, and fat distribution during this period of a woman's life rather than to only body fat content.

## ACKNOWLEDGMENTS

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