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Association between circulatory levels of adipokines and bone mineral density in postmenopausal women

--Manuscript Draft--

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Abstract:	Objective: Growing evidence indicate that fat excess may be beneficial for the bone health with a protective effect on postmenopausal osteoporosis onset; moreover, experimental data suggest that this link might be due to a direct effect of adipokines on the bone tissue. However, evidences confirming this association are still limited. Methods: We evaluated the levels of interleukin (IL) -6, IL-8, IL-1β, adipsin, lipocalin-2/NGAL, tumor necrosis factor alpha, monocyte chemotactic protein-1, plasminogen activator inhibitor-1, hepatocyte growth factor, resistin, leptin and adiponectin in a sample (n = 127) including postmenopausal women with osteoporosis, osteopenia and normal bone mass density (BMD). Results: At univariate analysis, only leptin and adiponectin were significantly correlated with the BMD. In particular, leptin was positively associated with the BMD of spine ($r = 0.22$, $p < 0.05$), femoral neck ($r = 0.23$, $p < 0.05$), trochanter ($r = 0.20$, $p < 0.05$) and total hip ($r = 0.27$, $p < 0.01$), while adiponectin was inversely correlated with the BMD of trochanter ($r = −0.21$, $p < 0.05$). Adjustment for waist circumference and age led to the disappearance of all correlations. Conversely, the stratification of the sample according to IL-6 levels revealed that adiponectin was still significantly associated with the BMD regardless of confounders ($β = −0.29$, $p < 0.05$; $r2 = 0.198$) in the subgroup of participants with low

	IL-6 levels. Conclusions: Our data suggests that circulating adiponectin might be inversely associated with markers of bone health in postmenopausal women, with an interaction apparently influenced by IL-6.

Ferrara, December 19, 2015

Isaac Schiff, MD
Editor-in-Chief
MENOPAUSE - The Journal of The North American Menopause Society

Re: MENO-D-15-00319

Dear Professor Schiff,

Please, find enclosed our revised manuscript entitled "**Association between circulatory levels of adipokines and bone mineral density in postmenopausal women**" by Cervellati et al., which has been carefully revised in accordance with all Editorial Office comments and Reviewers' comments and suggestions, as detailed in the "Point-to-point response".

We would like to inform you that we have followed your last recommendation regarding the need of reviewing and editing the manuscript for correct English. Indeed, our paper has been edited by the service afforded by "Editage" (also suggested by Menopause") as witnessed by a certificate (which we can send in case it will be requested).

All authors have read and approved the submission of the revised manuscript; the manuscript has not been published previously and is not being considered for publication elsewhere. There are no financial and personal conflicts of interest.

Hoping you will find our revised manuscript acceptable for publication in *Menopause*, I look forward to hearing from you at your earliest convenience.

Sincerely,

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MENO-D-15-00319

Point-to-point response to the Editorial Office and Reviewers

Response to the Editorial Office

In response to the Editorial Office comments, we have modified the manuscript as follow:

1. The highest academic degree for Arianna Gonelli has been added on the title page [Title page, line 6];
2. As requested, the words "*subjects or patients*" have been changed with the words "*participant or women*" throughout the manuscript [Abstract, Material and Methods, Results and Discussion sections]
3. The sentence "*Hormone replace therapy*" has been substituted with the sentence "*Hormone therapy*" to be consistent with the journal preferences [Material and Methods, first paragraph of page 6, line 15]
4. We apologize for the mistake of uploading the Supplementary Table 2 that was prepared during data analysis but excluded from the final version of the manuscript since it was redundant and it included data not relevant to the main aim of the study. Table 2 has been now removed from the submitted revised version of the manuscript
5. The list of Supplemental Digital Content (SDC) has been added accordingly with the request of Editorial Office.
6. We apologize for the grammar mistakes, the manuscript has been now corrected and edited by English speaking employer of the editing company **Editage**.
7. Dr Arianna Gonelli, Prof Pantaleo Greco and Dr Carlo Bergamini have already confirmed their authorship and have completed the copyright questionnaire.

Response to the Reviewer 1

We thank the Reviewer for his/her positive comments on our study "*Association between circulatory levels of adipokines and bone mineral density in postmenopausal women is of interest*".

- We agree with the reviewer's observation *"Association does not mean causation and in this cross sectional study better to say associated rather than might affect bone health"*. In order to have a more "prudent" approach in interpreting our results we have

modified the Abstract including a more cautious sentence [Abstract, page 3, lines 17 and 18]. Moreover, we have emphasized the intrinsic limitation of the cross-sectional design of our investigation at the end of the Discussion section [Discussion, second paragraph of page 16]. Nevertheless, in light of the current literature on the subject, our idea is that adiponectin might be causative of modification in bone homeostasis, but not *vice versa*. As implicitly suggested by the reviewer, a longitudinal approach represents the only way to draw a definite conclusion about the issue [Discussion, second paragraph of page 16].

- We have now provided information about the precision of the assay and the quality control measures for the biological markers assessed We have in particular listed the details including Intra- and inter-assay precision and Internal Quality Controls [Material and methods, first and second paragraphs of page 7]
- As pointed out by the reviewer, we are aware of the statistical weakness of some R2 values, and we have added a comment on the manuscript on this issue [Discussion, second paragraph of page 16]. However, we would like to underlie that the correlations "leptin *vs* bone markers" and "adiponectin *vs* bone markers" showed similar strength to those obtained in larger studies (as reported in the meta-analysis by Biver et al. Influence of adipokines and ghrelin on bone mineral density and fracture risk: a systematic review and meta-analysis. J Clin Endocrinol Metab 2011;96:2703–13). In this light, we intentionally did not emphasize the clinical significance of these associations, particularly because they all disappeared after adjustment for abdominal circumference. On the other hand, the regression coefficient for the link between adiponectin and BMD in the LOW IL6 group is relatively strong (and independent of WC), even if it has been obtained in a small subset of women.

  Finally, in our opinion also negative results can be valuable, in particular when they bring some novelties and when they add new insight into the current literature. Indeed, as stressed in the manuscript, our work is the first to explore the relationship between MCP-1, HGF, adipsin and PAI-1 and bone markers in humans. Moreover, with the exception of leptin, adiponectin and IL-6, only few data are available regarding the other adipokines considered.
- With regard to the reviewer's comment "*Without quality control measures of the assessment weak correlations of this magnitude seem to be of questionable clinical significant effect size"*, as previously outlined, we have now mentioned in the

manuscript that the correlation coefficients of some of the associations are weak and the sample size is low [Discussion, second paragraph of page 16].

- As correctly observed by the reviewer, "*there are a number of other determinants of marker elevation that may be important*" such as a number of biochemical, physiological and pathological conditions and factors that can strongly contribute to marker elevation. In this line, we have added a paragraph in the Discussion section to comment this remark [Discussion, end of page 15 and first paragraph of page 16].
- With regard to reviewer's observation about possible ongoing inflammatory conditions "*Unclear how inflammation ruled out or how many had inflammatory states*", unfortunately, we are not able to have information on the presence of inflammatory states at the time of the inclusion of the subjects in the study. The best way to "make a diagnosis" of an ongoing inflammatory condition (especially when subclinical and unrelated with symptoms and full-blown diseases), is the measurement of hs-CRP, which is a reliable marker for this purpose (Pradhan et al. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286:327–334). We recognize that this might represent a limitation of the study, and, thus, we have added a comment regarding the role of "low grade inflammation" as potential, undetected, determinant of markers variation [Discussion, first paragraph of page 16].
- *The question raised by reviewer "Could adiponectin merely reflect fat mass differences?"* is an interesting point. As excellently reviewed by Fain (Fain, Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. Mediators Inflamm 2010;2010:513948) and by Shehzad et al (Shehzad et al., Adiponectin: regulation of its production and its role in human diseases. Horm 2012;11:8–20), within the adipokines evaluated in our study, adiponectin, along with leptin, is the only one that is mostly synthetized by adipocytes of WAT. However, the release of adiponectin in the general circulation accounts for only the 42% of the total release (Fain, Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. Mediators Inflamm 2010;2010:513948; Shehzad et al., Adiponectin: regulation of its production and its role in human diseases. Horm 2012;11:8–20; Fain JN et al., Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004;145:2273–82) regardless of the high ratio for mRNA

expression in fat cells with respect to "non-fat cells". Therefore, fat mass is not the only determinant for serum adiponectin levels. Accordingly, we found a weak correlation between adiponectin and BMI/WC, and the relationship shown in Table 5 (adiponectin *vs* BMD trochanter in the Low IL-6 group) was independent of WC. More precisely, in the final multiple regression model, the standardized regression coefficients were as follows: Adiponectin: $\beta = -0.29$, $p<0.05$; WC= $\beta = -0.07$, $p>0.05$; Age= $\beta = -0.29$, $p<0.05$.

Response to the Reviewer 2

We thank the Reviewer for his/her positive comments on our study "*This is an interesting manuscript analyzing the association between circulatory levels of adipokines and bone mineral density in postmenopausal women*".

As suggested, we have used paragraph breaks throughout the manuscript. Moreover, we have modified the manuscript in response to the specific comments/suggestions as detailed below:

- We agree with the reviewer that the potential influence of confounding variables could be a limitation. Therefore we have now clearly stated this issue [Discussion, end of page 15 and first paragraph of page 16]
- We have corrected the sentence "*osteoclasts activity*" with the suggested sentence "*osteoclastic activity*" [Introduction, page 4, line 3]
- The word "*leaded*" has been corrected with "*led*" [Introduction, page 4, line 5 from the bottom]
- The word "*modifications*" has been corrected [Introduction, page 5, line 1]
- The abbreviation "*HRT*" has been changed with the sentence "*Hormonal Therapy*" [Material and methods, Page 6, line 9 from the bottom]
- The sentence "…*women resulted eligible and were enrolled…*" has been modified with the sentence "…*women were eligible and enrolled...*" [Material and methods, Page 6, line 8 from the bottom]
- As correctly suggested the word "*adipokine*" has been changed with the word "*leptin*" [Results, Page 11, line 9]
- On page 12, the sentence has been corrected into "*…from osteopenic or osteoporotic…"*

- We are not sure to have correctly understood the point raised by the reviewer "*You should discuss why this might be different from what was seen in mice. i.e., see your data in the intro, page 4, lines 16-19*". We suppose that the reviewer intended to point out the important issue of the general discrepancy between animal and human *data* in relation to the link between adipokines and osteoporosis. In this light, the most common animal model for osteoporosis is a rodent model both for type I (using OvX mice) and II with the mouse model as the most used for genetic manipulation pourposes (Turner S. Animal models of osteoporosis-necessity and limitations. Eur Cell Mater 2001;1:66–81). In the sentences highlighted by the reviewer (see Introduction section), we referred to a report from Ducy et al. (Ducy P et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 2000;100:197–207) where the effects of leptin were studied by using leptin-deficient and leptin receptor–deficient mice (which were obese and hypogonadic). The evidence that both mutants showed increased high bone mass suggested that leptin might act as inhibitor of bone formation. In our view, the interpretation of the results should be more cautious, because it should be also considered the variety of hormones, cytokines and other biological factors that could be altered in terms of production and activity when leptin is down-regulated. For example, our *data* suggest that IL-6 may interfere with the interaction between leptin or adiponectin and bone. The origin of the discrepancy rising by the different experimental setting considered (in vitro cell culture models, *in vivo* animal models or humans) have been emphasized at the end of the revised discussion
- The extra period has been removed
- The circulating levels of IL-1β were below the detection range and therefore this interleukin has not been included in Table 2. This consideration has now been added in the manuscript [Results, second paragraph of page 9]

Association between circulatory levels of adipokines and bone mineral density in postmenopausal women

Running title: IL-6 and adiponectin in osteoporosis

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Conflict of interest: the authors declare no conflicts of interest/financial disclosure. The authors alone are responsible for the content and writing of the paper.

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Abstract

Objective: Growing evidence indicate that fat excess may be beneficial for the bone health with a protective effect on postmenopausal osteoporosis onset; moreover, experimental data suggest that this link might be due to a direct effect of adipokines on the bone tissue. However, evidences confirming this association are still limited. **Methods:** We evaluated the levels of interleukin (IL) -6, IL-8, IL-1β, adipsin, lipocalin-2/NGAL, tumor necrosis factor alpha, monocyte chemotactic protein-1, plasminogen activator inhibitor-1, hepatocyte growth factor, resistin, leptin and adiponectin in a sample (n = 127) including postmenopausal women with osteoporosis, osteopenia and normal bone mass density (BMD). **Results:** At univariate analysis, only leptin and adiponectin were significantly correlated with the BMD. In particular, leptin was positively associated with the BMD of spine ($r = 0.22$, $p < 0.05$), femoral neck ($r = 0.23$, $p < 0.05$), trochanter ($r = 0.20$, $p < 0.05$) and total hip ($r = 0.27$, $p < 0.01$), while adiponectin was inversely correlated with the BMD of trochanter ($r = -0.21$, $p < 0.05$). Adjustment for waist circumference and age led to the disappearance of all correlations. Conversely, the stratification of the sample according to IL-6 levels revealed that adiponectin was still significantly associated with the BMD regardless of confounders ($\beta = -0.29$, $p < 0.05$; $r^2 = 0.198$) in the subgroup of participants with low IL-6 levels. **Conclusions**: Our data suggests that circulating adiponectin might be inversely associated with markers of bone health in postmenopausal women, with an interaction apparently influenced by IL-6.

Key words: osteoporosis; postmenopausal women; fat accumulation; adipokines; inflammation; adiponectin; interleukins

Introduction

Menopause is associated with increased incidence of osteoporosis and risk of bone fractures, mainly because of a predominance of osteoclastic activity leading to bone resorption, compared to bone formation performed by osteoblasts [1]. Estrogen decline and aging appear to be the main triggering factors of the detrimental bone turnover, leading to the onset of postmenopausal osteoporosis (PO) [1]. However, owing to the complex network that regulates bone biology in both physiological and pathological conditions, many other factors can contribute in either protecting or damaging this tissue.

Extensive epidemiological data show that high body mass index (BMI), especially when coupled with accumulation of central fat, is closely related to high bone mass density (BMD), and that a reduction in body weight may contribute to the bone loss [2,3]. One of the proposed mechanisms to explain this interplay relies on the role of adipokines, bioactive molecules released by the adipose tissue, during bone metabolism [4,5]. Several in vitro/in vivo lines of evidence suggest that some adipokines such as lipocain-2, resistin, adipsin, and in particular leptin, might have direct or indirect protective effects on the bone resorption by inducing survival, differentiation and proliferation of the osteoblasts [6–9]. Nevertheless, there are also several in vivo studies that report conflicting results on this positive interaction between obesity and the bone health [10]. These contradictions also emerge from some reports exploring the impact of adipokines on bone biology in in vivo preclinical models. In particular, Ducy et al. showed that transgenic mice down-expressing leptin increased the bone formation, and intra-cerebroventricular leptin administration led to bone loss in both the leptin-deficient and the wild-type mice [11]. In a similar approach, Kajimura et al. showed a dual role of adiponectin in mice where it was able to induce an increase in the bone mass and promoted the osteoblasts apoptosis [12]. Moreover, in different experimental models obesity onset was closely associated with an increase of cytokines able to mediate bone resorption [13–15]. Accumulation of

abdominal subcutaneous and visceral fat is accompanied by a marked modification in the secretory pattern of adipose tissue, mainly due to alterations in the number and localization of infiltrating cells of the immune, vascular and stromal components [16]. These cells, along with adipocytes, lead to an increased expression of potent pro-inflammatory cytokines, including interleukin 6 (IL-6), interleukin 1 (IL-1), interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α), responsible for the low-grade chronic inflammation linking obesity to metabolic dysfunctions such as insulin resistance, dyslipidemia, and other associated diseases [16]. Most likely, this mechanism might also involve a modulation of the interaction between the receptor activator of nuclear factor-kB (RANK) and its natural ligand receptor activator of nuclear factor-kB ligand (RANKL), two molecular mediators known for their ability in promoting the bone resorbing activity of the osteoclasts and in prolonging their survival [17–19]. Moreover, of note is the evidence that the expression and release of such cytokines in the systemic circulation, as well as the activation of the RANK-RANKL axis, are down-regulated by estrogens [18,20,21].

In this light, the main aim of the present study is to explore potential interconnections between the central fat, adipokines and PO. Therefore, we evaluated a large panel of circulating adipokines in a cohort of osteopenic and osteoporotic postmenopausal women as compared to healthy controls.

Materials and methods

Study participants

The study population consisted of a randomly selected sample of women attending the Menopause and Osteoporosis Centre (MOC) of the University of Ferrara (Ferrara, Italy). This study was designed and performed following The Code of Ethics of the World Medical Association (Declaration of Helsinki), and was conducted according to the guidelines for Good Clinical Practice (European Medicines Agency). Written informed consent was obtained from each participant before inclusion in the study. Inclusion criteria was postmenopausal women, defined as cessation of menses for at least one year, in accordance with the recent ReSTAGE modification of the Stages of Reproductive Aging Workshop (STRAW) staging criteria [22,23]. According to a priori defined criteria, the exclusion criteria were: women using antioxidant supplements (such as vitamins E and C) or following vegan diet; women with pathological conditions such as cancer, diabetes, malabsorption, and cardiovascular diseases; women under pharmacological treatment (including thyroid hormones and diuretics) in the month prior to blood withdrawal; and women undergoing hormone therapy. One hundred and twenty-seven postmenopausal women were eligible and thus enrolled in the study. Each participant underwent measurement of anthropometric parameters such as weight, standing height and waist circumference (WC) by trained personnel. Peripheral blood samples were collected into vacutainer tubes devoid of anticoagulant, by venipuncture after an overnight fast. After 30 min of incubation at room temperature, blood samples were centrifuged at 4.650 ×*g* for 20 min and the obtained sera were stored at −80°C.

Biochemical assays

Frozen serum samples were thawed before performing the MILLIPLEX MAP Human Adipokine Panels HADK1MAG-61K and HADK2MAG-61K (Merck Millipore, Billerica, MA),

bead-based multiplex immunoassays. Simultaneous quantification of the following human adipokines was carried out: resistin (intra-assay coefficient of variation (CV) = 3%; inter-assay (CV) = 14%), lipocalin-2/NGAL (intra-assay CV = 4%; inter-assay CV = 12%), plasminogen activator inhibitor-1 (PAI-1) total (intra-assay CV = 5%; inter-assay CV = 14%), adipsin (intra-assay CV = 2%; inter-assay CV = 6%), adiponectin (intra-assay CV = 4%; inter-assay CV = 10%), IL-8 (intra-assay CV = 3%; inter-assay CV = 14%), TNF-α (intra-assay CV = 3%; inter-assay CV = 16%), IL-6 (intra-assay CV = 2%; inter-assay CV = 10%), monocyte chemotactic protein-1 (MCP-1) (intra-assay CV = 2%; inter-assay CV = 11%), IL-1β (intra-assay CV = 7%; inter-assay CV = 12%), hepatocyte growth factor (HGF) (intra-assay CV = 3%; inter-assay CV = 11%), leptin (intra-assay CV = 5%; inter-assay CV = 13%). Samples were processed in duplicate following the manufacturer's recommended protocols and read on a MAGPIX instrument equipped with the MILLIPLEX Analyst software using five-parameters non-linear regression formula, to compute sample concentrations from the standard curves [24]. Quality controls provided with the multiplex kits, were used to qualify assay performance.

The measurement of bone-specific alkaline phosphatase (BAP) and C-terminal telopeptide of type I collagen (CTX-1) levels was performed separately by using OCTEIA Ostase BAP immunoenzymometric assay and ß Cross-Laps Siero (CTx I) respectively, (both kits were from Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK) according to the manufacturer's guidelines. Two quality controls, provided within each kit, were used to qualify assay performance. The intra-assay CV was 2.2 % (inter-assay CV = 7.7 %) and 4.1 % (inter-assay CV = 7.7 %) for CTX-1 and BAP respectively. Each serum sample was assessed for BAP and CTX-1 in duplicate.

Bone densitometry assessment

Areal bone density was assessed at lumbar spine, hip and total body by Discovery dual energy X-ray absorptiometry scanner (Hologic Inc., Bedford, MA). PO was diagnosed when the

BMD T-score (the number of standard deviations below the average for a young adult at peak bone density) was lower than 2.5 standard deviations from the BMD peak at either femoral neck or lumbar spine, according to World Health Organization (WHO) guidelines. In accordance with these criteria, women with T-score between −2.5 and −1.0 were classified as osteopenic and those with a value higher than −1.0 were considered as normal.

Statistical analysis

Data was analyzed using The Statistical Package for the Social Sciences (SPSS) version 18.0 for Windows (IBM, Chicago, IL., USA). Continuous variables were first analyzed for the normal distribution by the Kolmogorov-Smirnov and the Shapiro-Wilkinson tests. Comparisons between groups were performed by using one-way analysis of variance (ANOVA) implemented with post-hoc for linear trend, or Kruskal-Wallis if the variables considered were normally (after base-10 logarithm transformation) or not-normally distributed respectively. Chi-square test was used to compare differences in categorical variables. Simple correlation analysis was performed by the Pearson's or the Spearman's test when the variables of interest were normally or not-normally distributed respectively. Finally, multiple regression analysis was performed to determine if the associations were independent of potential confounding factors. Preliminary multiple regression analyses were performed to check if there was multi-collinearity among variables to include into multivariate analyses. Values of variance inflation factor (VIF) above 2.5 were considered indicative of the presence of this statistical problem. A two-tailed probability value < 0.05 was considered statistically significant.

Results

Study population and clinical assessment

[Insert Table 1]

The main clinical characteristics of the enrolled women's cohort are summarized in **Table 1**. Participants were normal controls, osteopenic and osteoporotic as defined in the criteria reported in the Material and Methods section. Women with PO were significantly ($p < 0.01$) older and showed lower BMI ($p < 0.05$) and WC ($p < 0.01$) compared with the two other groups ($p < 0.05$ in both cases). Consistently, there was a trend towards a lower incidence of women with central obesity (classified according to WHO criteria) in the normal control group as compared to the osteopenic and the osteoporotic groups. Women with hypertension were more frequent ($p < 0.05$) among the normal controls, whereas the prevalence of smokers did not change across the three groups (**Table 1**). By definition, lumbar spine, neck, total hip and trochanter BMD, and the correspondent T-score values, were significantly ($p < 0.01$) higher in the normal controls with respect to the osteopenic and the osteoporotic women. Finally, the levels of CTX-1 and BAP did not differ between the three groups analyzed (**Table 1**).

Correlations between circulating adipokines and bone markers

[Insert Table 2]

As summarized in **Table 2**, among the adipokines analyzed by multiplex analyses, only IL-1β results were undetectable in more than the 80% of the samples (therefore, the data regarding this interleukin are not shown), while IL-8, TNF-α, IL-6, MCP-1, resistin, adiponectin, HGF, adipsin, leptin and lipocalin-2/NGAL were detectable at different levels (**Table 2**). Although the statistical analysis showed that there were no significant differences in the serum levels of the detectable adipokines between the three study groups, ANOVA post hoc test revealed a statistically significant

linear trend for both adiponectin (r^2 = 0.04, $p < 0.05$) and leptin (r^2 = 0.04, $p < 0.05$), which progressively increased and decreased from normal to osteoporotic subsets respectively (**Table 2**).

[Insert Table 3]

Potential associations between adipokines levels and the BMD at all sites or between adipokines levels and serum bone markers were first evaluated by univariate analysis. As shown in **Table 3**, we observed a negative correlation between adiponectin and the BMD at trochanter (r^2 = 0.05, $p < 0.05$). On the contrary, leptin levels were found to be positively associated with all the BMD parameters considered, showing in particular a strong (r^2 = 0.06, $p < 0.01$) positive association with the total hip BMD (**Table 3**).

IL-6 may be a mediating factor in the leptin/adiponectin and BMD relationship

[Insert Table 4]

Multiple regression analyses were performed to check whether the associations that emerged in univariate analyses were independent of age and/or WC. Other potential covariates such as hypertension, time from menopause onset, and smoking were not considered in the regression models due to the absence of significant correlations with the variables of interest. BMI was also excluded from multiple regression analyses due to its collinearity with WC and its weaker correlation with the variables of interest (**see supplementary Table 1**). As shown in **Table 4**, leptin and adiponectin remained associated with the BMD even after adjusting for age (**Table 4, Model 1**). On the contrary, the inclusion of WC in the multivariate models led to a marked decrease of the strength of the regression coefficients and to the loss of significance for all the associations involving the adipokines (**Table 4, Model 2**). Interestingly, WC and age remained independent predictors of either increase or decrease in the BMD at every site respectively.

[Insert Table 5]

[Insert Figure 1]

Finally, since IL-6 is considered a reliable marker of systemic inflammation and it appears to act as a key mediator of accelerated bone loss during menopause [18], we investigated if IL-6 levels might act as an influencing factor for the emerging relationships between leptin/adiponectin and the BMD. To this end, we stratified the sample into two subgroups defined as Low IL-6 and High IL-6, by using the entire sample's IL-6 median value as cut off. As shown in **Table 5**, in the Low IL-6 subgroup, leptin was significantly correlated with the BMD at femoral neck ($r^2 = 0.04$, $p < 0.05$), trochanter ($r^2 = 0.05$, $p < 0.05$) and total hip ($r^2 = 0.07$, $p < 0.05$). The total hip BMD was the only one significantly correlated with leptin ($r^2 = 0.04$, $p < 0.05$) in the High IL-6 subgroup. The same trend across the two subgroups was observed for the relationship between adiponectin and the BMD at trochanter ($r^2 = 0.12$, $p < 0.01$; **Figure 1**). After adjustment for the WC and age, the correlations did not reach significance in all cases, with the sole exception of the correlation between adiponectin and the BMD at trochanter (multiple regression model: $r^2 = 0.198$, $p < 0.05$) in the Low IL-6 subgroup (**Table 5**). Moreover, it is worthy to underlie that, with a few exceptions in the Low Il-6 subsample (leptin versus the BMD at lumbar spine/ total hip; adiponectin versus the BMD at trochanter) the multiple regression coefficients were of opposite directions in one group compared to the other one.

Discussion

It is well-recognized that some marrow adipocytes originate from the same mesenchymal stem cells that can differentiate into osteoblasts and adipocytes [25]. Moreover, there is abundant experimental evidence suggesting that the human adipose tissue can influence bone metabolism through the biochemical mediation of adipokines, which can also play a role in the osteoporosis pathophysiology [4,5]. However, whether systemic levels of these factors might influence the in vivo bone biology is an issue that still needs to be clarified. Therefore, we investigated the potential interplay between a selected group of adipokines and the BMD/bone markers in a sample of postmenopausal women stratified according to their osteopenic/osteoporotic status. In particular, we have considered adipose-derived cytokines and hormone-like factors that appear to be potential regulators of the bone resorption/formation homeostasis processes.

The main findings of our work can be summarized as follows: (*i)* none of the examined adipokines could be used to differentiate the normal from the osteopenic or osteoporotic women, or were correlated with bone turnover serum markers; (*ii)* only leptin and adiponectin showed a significant positive and inverse association respectively, with the BMD at some skeleton sites; (*iii)* the univariate correlations considered were no longer significant after adjustment for WC; (*iv)* the circulating levels of IL-6 appeared to affect the interaction between the leptin/adiponectin and the bone density.

One of the most striking novelties of our work lays in the evaluation of the circulating levels of some adipokines, in particular MCP-1, HGF, adipsin and PAI-1, in relation to osteoporosis incidence and bone markers. Indeed, although other experimental evidences have suggested a role for these adipokines in the stimulation (or perhaps a role in suppression for adipsin [9,26]) of osteoclasts-mediated bone resorbing activity [27–29], the present work is the first exploring this potential relationship in humans. The study revealed the lack of significant correlations for TNFα,

IL-8, resistin, and lipocalin molecules that have already been considered in handful of clinical studies on osteoporosis [5,8,30–33]. In agreement with our results, a previous report showed that serum lipocalin-2/NGAL was not correlated with the BMD in a large cohort of postmenopausal women, although it still is an independent predictor of future non traumatic fractures [8]. Likewise, despite the potential role of resistin in bone remodeling as reported by Thommesen et al. in in vitro models [34], two independent epidemiological studies on men reported the lack of correlation between this adipokine and the BMD at lumbar spine [32,33]. Converging in vitro and in vivo preclinical data suggested that IL-8 and mostly TNFα, i.e., two cytokines directly involved in inflammatory processes, may play a role in the osteoporosis pathogenesis [35,36]. However, evidences on a similar role of these factors in bone loss in humans are still limited. This consideration is firstly referred to IL-8 that contrary to our results, had demonstrated the ability to differentiate PO status (as results of correlation BMD vs. IL-8), although this evidence has been reported only by one study [30]. Moreover, to our knowledge Al Daghiri et al. were the only one to report an association between TNFα and PO, while two other larger studies showed results in line with our findings. Indeed, Kim et al. could not demonstrate an association between TNFα and the BMD at all sites [37], and Ding et al. [31] reported that baseline, as well as longitudinal change in TNFα levels, were not related to changes in the BMD at multivariate analysis.

On the contrary, majority of the current literature dealing with the potential involvement of adipokines in bone metabolism had focused on leptin, adiponectin and IL-6 [4,5,15,31,38–41]. Our results showing a positive association between leptin and the BMD at all sites, are consistent with reports of several studies performed on older men and postmenopausal women [4,32,42–45]. Of note, our observation of adiponectin, that unlike leptin decreases with the abdominal fat accumulation, showing an inverse correlation with the BMD at trochanter, is a finding in line with other published epidemiological data [33,41]. Less frequently, it has been explored if these associations might be influenced by potential confounding factors such as fat mass variables. In most cases, and in

agreement with our results, when fat mass parameters were included in multivariate models, the association between leptin and the BMD disappeared and the regression coefficient reversed its direction [4,42,45]. Thus, our results show that the epidemiological positive effect of fat on bone density might not be leptin-mediated, rather might be due to other mechanisms such as estrogen secretion by adipocytes [46] or, more simply, the physiological adaptation of the skeleton to the increased load. Only one study focused on postmenopausal women (n = 61) considered this statistical approach on adiponectin, showing an inverse correlation that still conserved statistical significance after adjustment [47]. Larger observational studies are needed to clarify whether fat might be a confounding factor in the relationship between adiponectin and BMD, as it appears in the case of leptin.

Further analyses of the potential interconnections between the considered adipokines, did not draw a definitive conclusion about possible interactions between leptin/adiponectin and bone biology. The idea of comparing the strengths of the associations in women stratified for different levels of IL-6, comes from the experimental and clinical evidences suggesting that chronic low grade inflammation contributes to the etiopathogenesis of PO [15,18,31,38,39]. In particular, IL-6 is considered a mediating factor in the accelerated bone loss during menopause [38,39] and converging in vitro evidences [13,48] and in vivo animal [14] and human [38] findings, suggest that estrogen withdrawal upregulates synthesis and release of pro-inflammatory cytokines, including IL-6. The most evident result obtained from the comparison of adjusted (for age and WC) regression coefficients between the two subsamples of women characterized by Low and, in particular, High circulating levels of IL-6, was the inversion of the sign of almost all regression coefficients **(Table 5)**. To translate this clinical/statistical evidence into a biological explanation/hypothesis is very complicated due to the novelty of the data and the complexity (still partially unknown) of the molecular network that regulates adipokines biology in the context of bone metabolism. However, we can speculate that IL-6 might be one of the factors implicated in the modulation of leptin/adiponectin effects on the

osteoclast/osteoblasts. Indeed, these two adipokines appear to influence the bone cells cycle and activity in different ways. In one report, leptin was observed to lead to bone genesis suppression and bone erosion intensification via RANKL [49], while in another report it induced osteoblasts activity and mineralization [6]. In the same fashion, adiponectin was shown to stimulate in vitro osteoblasts proliferation [50], while the results of two separate transgenic animals studies showed that adiponectin-deficiency resulted in a protective effect against osteoporosis [51,52]. We can theoretically speculate that the multifunctional nature of IL-6, or perhaps the local inflammatory status, might have a role in inducing a shift on the target cells and on the effects of these two adipokines, in particular with regard to adiponectin. Indeed, the relationship between this molecule and the BMD at trochanter was the only one to exhibit independence from the central fat mass. Therefore, it can be speculated and hypothesized that the observed apparent ability of IL-6 interference with bone responsiveness to adiponectin might occur through the interaction with RANK/RANKL system, a signaling pathway that plays a key-role in the osteoporosis pathogenesis [17,20]. In particular, IL-6 acts as an inducer of the RANKL expression on the osteoblasts surface [53]. The binding of RANKL with its receptor RANK, localized in the osteoclast progenitors, leads to maturation and contextual activation of these cells [20]. Since adiponectin also seems to promote the RANKL expression [54], we hypothesize that the impact of this adipokine on RANKL and on its pro-resorption function, might become evident and dominant only when the other modulator is at low concentration (**Figure 2**). Hence, the effect at bone level might be the overall balance between the anti-inflammatory/pro-resorption effects of adiponectin, together with the pro-inflammatory/pro-resorption effects of IL-6 (**Figure 2**). As a result, in postmenopausal women with high abdominal fat deposition, low/normal levels of IL-6 can result in a beneficial effect on the bone health, with a better control of bone resorption.

IL-6 is defined as an upstream inflammatory pleiotropic cytokine, a key player in propagating the downstream inflammatory response [55]. This definition suggests that additional

factors influencing this interleukin's release might be the true determinants of the observed association linking IL-6, adiponectin and bone markers. Indeed, our results might be confounded by the presence of additional undetected physiopathological conditions such as low grade inflammatory states, oxidative stress or insulin resistance syndrome, which are strictly associated with IL-6 increase [56–58]. Moreover, IL-6 plays a central role in the regulation of a plethora of other chemokines expressions (e.g., some members of beta chemokines (CC), and alpha chemokines (CXC) family) and other pro-and anti-inflammatory cytokines, that might directly or indirectly affect the bone metabolism [58]. In addition, further determinants of IL-6 and adiponectin effects on bone are their specific receptor expression level [58,59], the biological targets relative concentration, i.e., the RANK and RANKL axis together with additional RANK/RANKL modulators such as osteoprotegerin [60].

[Insert Figure 2]

We acknowledge that our study has some limitations. First, the small sample size may have affected the reliability and the clinical significance of the observed associations. Nevertheless, the strength of the correlation between leptin and adiponectin found in the whole sample (**Table 2**) were comparable to those reported in larger studies [5]. Secondly, the cross-sectional design of the study did not allow us to draw a definitive conclusion on the cause-effect relationship between adipokines and bone markers. Nevertheless, we believe that our findings may provide a conceptual basis for longitudinal investigations, involving examination of IL-6/adiponectin levels and BMD in postmenopausal women on several follow-up visits. Third, we cannot obviously exclude any other bias or confounding factors that might have limited the reliability of our findings.

To overcome these limitations, future studies should include evaluation of the following factors: (*i)* hormones such as estrogen and insulin, that might potentially influence both the bone health and adipokines release; (*ii)* nutrients such as vitamin D and calcium that play a role in bone metabolism; and (*iii)* distribution and composition of the adipose tissue, especially in abdominal depot, where

visceral fat contributes more significantly than subcutaneous fat in the production of adipokines. Notably, it must be clear that circulating adipokines levels may only partially reflect the local levels of these proteins in the bone microenvironment [5,61]. This is an important issue to consider since the adipokines present in the bone marrow, exert a direct influence on the osteoclasts and osteoblasts activity. This expected difference between peripheral and local microenvironment could actually explain the discrepancies between in vitro data (where adipokines added to cell culture medium can directly interact with bone cells) and the epidemiological studies results on this topic.

Conclusion

In our opinion, this study has outlined the complex network of interactions linking different adipokines, which might influence bone health during the postmenopausal phase of women's life. In particular, our finding of an inverse association between adiponectin and the BMD suggests that abdominal fat accumulation, leading to decreased adipokine levels, might improve bone health. Of note, this apparent effect might be elicited only in a condition of low circulating IL-6 levels, thus when fat mass increase is not accompanied by an exacerbation of the inflammatory processes.

Although we are aware that this preliminary study might be limited by the relatively small sample size, the reported findings can provide the conceptual basis for larger investigations on this challenging topic, perhaps including longitudinal approaches to determine and dissect the real nature of this biological link.

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Figure Legends

Figure 1. Scatter plots of the relationship between adiponectin and BMD at trochanter in both at low and high IL-6 subgroups.

Figure 2. Schematic representation of the "dominant" pro-resorption stimulus at bone level. The picture highlight the key factors leading to bone resorption *via* RANK/RANKL pathway depending on the levels of IL-6. Pre-osteoclasts differentiation into activated mature osteoclasts is driven by IL-6 when this cytokine is at high levels (**left panel**) or by adiponectin when IL-6 is at low levels (**right panel**). Abbreviations: Adne: adiponectin.

List of Supplemental Digital Content (SDC)

1. Supplementary Table 1: table in word file

Table 1. Main characteristics of normal, osteopenic and osteoporotic postmenopausal women

Clinical parameters	Normal BMD (n= 31)	Osteopenia (n= 53)	Osteoporosis (n= 43)	P[a]	P Linear trend
Age, years	55.3 ± 0.7	56.7 ± 0.6	58.5 ± 0.4	<0.01	<0.05
Years since menopause, years	40	78	90	NS	-
BMI, kg/m^2	26.0 ± 0.7	24.4 ± 0.6	23.6 ± 0.4	<0.05	<0.05
WC, cm	90.0 ± 2.2	81.4 ± 1.1	84.3 ± 0.9	<0.01	<0.05
Smoking , %	12.9	6	9	NS	-
Hypertension,%	16.1	2.2	3.7	<0.05	-
DXA-assessed parameters					
Lumbar spine BMD, g/cm^2	0.99 ± 0.04	0.89 ± 0.01	0.77 ± 0.07	<0.01	<0.01
Lumbar spine T-score	0.1 (-0.6; 0.4)	-1.6 (-2.0; -0.9)	-2.6 (-2.9; -2.1)	<0.01	<0.01
Femoral neck BMD, g/cm^2	0.81 ± 0.01	0.67 ± 0.01	0.62 ± 0.02	<0.01	<0.01
Femoral neck T-score	-0.4 (-0.6; 0.0)	-1.7 (-2.0; -1.2)	-2.1 (-2.5; -1.4)	<0.01	<0.01
Total hip BMD, g/cm^2	0.91± 0.02	0.81 ± 0.02	0.72 ± 0.03	<0.01	<0.01
Total hip T-score	-0.1 (-0.4; 0.4)	-1.1 (-1.5; -0.8)	-1.8 (-2.2; -1.4)	<0.01	<0.01
Trochanter BMD, g/cm^2	0.70 ± 0.02	0.59 ± 0.01	0.54 ± 0.02	<0.01	<0.01
Trochanter T-score	0 (-0.4; 0.2)	-1.1 (-1.3; -0.6)	-1.7 (1.9; 1.2)	<0.01	<0.01
Bone markers					
CTX-1, ng/mL	0.52 ± 0.04	0.47± 0.06	0.62 ± 0.04	NS	<0.01
BAP, µg/L	30.4 ± 3.2	26.6 ± 1.2	32.1 ± 2.3	NS	<0.01

Data presented are expressed as: % within the group for categorical variables; mean ± standard error of the mean (SEM) for normal continuous variables; median (interquartile range) for non-normal continuous variables .

[a] ANOVA or Kruskall-Wallis; NS: not significant

Abbreviations: BMI, body mass index; BMD, bone mass density; WC, waist circumference; CTX-1, C-terminal telopeptide of type I collagen; BAP, bone-specific alkaline phosphatase

Table 2. Serum concentration of adipokines and insulin in normal, osteopenic and osteoporotic postmenopausal women

Adipokines	Normal BMD (n= 31)	Osteopenia (n= 53)	Osteoporosis (n= 43)	P [a]	P Linear trend
IL-8 (pg/mL)	3.2 ± 0.6	2.9 ± 0.2	2.2 ± 0.1	NS	NS
TNFα (pg/mL)	1.9 (1.3; 2.4)	1.9 (1.4; 2.4)	1.8 (1.2; 3.0)	NS	NS
IL-6 (pg/mL)	0.7 (0.4; 1.4)	0.4 (0.3; 1.1)	0.4 (0.3; 0.8)	NS	NS
MCP-1 (pg/mL)	225.4 ± 100.1	214 ± 128.2	253.1 ± 116.2	NS	NS
Resistin (pg/mL)	12574 ± 1022	13079 ± 870	11678 ± 1031	NS	NS
Adiponectin (µg/mL)	75.1 ± 12.6	92.2 ± 10.2	118.2 ± 13.9	NS	<0.05
HGF (pg/mL)	330 (217;479)	285 (151; 435)	258 (106; 398)	NS	NS
Adipsin (µg/mL)	4.5 ± 5.1	5.3 ± 3.1	4.9 ± 3.0	NS	NS
Leptin (ng/mL)	22.6 ± 1.4	14.5 ± 1.4	16.1 ± 1.5	NS	<0.05
Lipocalin-2/NGAL (ng/mL)	154 (113; 192)	138 (98; 164)	127 (89; 170)	NS	NS
PAI-1 total (ng/mL)	57.9 ± 5.1	53.1 ± 3.1	49.3 ± 3.0	NS	NS

Data presented are expressed as mean ± standard error of the mean (SEM) or median (interquartile range) for normal or non-normal variables, respectively .

[a] ANOVA or Kruskall-Wallis; NS: not significant ($p > 0.05$)

Abbreviations: IL (interleukin); PAI-1 total, plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein; HGF, hepatocyte growth factor

Table 3. Simple correlation between BMD at various sites and adipokines

Adipokines	BMD lumbar spine	BMD femoral neck	BMD trochanter	BMD total hip	CTX-1	BAP
IL-8[d]	0.04	-0.01	0.08	-0.03	-0.01	0.02
TNFα[c]	-0.02	0.02	-0.04	-0.08	-0.04	0.05
IL-6[c]	0.18	0.14	0.05	0.08	0.02	0.08
MCP-1[d]	0.05	0.06	0.06	0.06	-0.03	0.10
Resistin[d]	0.11	0.14	0.04	0.11	-0.09	-0.02
Adiponectin[d]	-0.11	-0.16	-0.21[a]	-0.15	0.07	0.08
HGF[c]	0.13	0.11	0.19	0.15	-0.02	0.03
Adipsin[d]	0.06	0.03	0.03	0.06	-0.13	0.08
Leptin[d]	0.22[a]	0.23[a]	0.20[a]	0.27[b]	0.18	0.14
lipocalin-2/ngal[c]	0.07	0.16	0.05	0.15	-0.08	0.04
PAI-1 total[d]	0.13	0.10	0.05	0.09	-0.11	0.08

[a] $p<0.05$ [b]; $p<0.01$; [c] Spearman's analysis;[d] Pearson's analysis

Abbreviations: BMD, bone mass density; CTX-1, C-terminal telopeptide of type I collage; IL (interleukin); PAI-1 total, plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein; HGF, hepatocyte growth factor

Table 4. Effects of age and waist circumference on the relationship between selected indexes of BMD and leptin, adiponectin as assessed by multiple regression analysis

	Model 1		**Model 2**	
Dependent variable	Predictors	β[c]	Predictors	β
BMD lumbar spine	**Leptin**	0.22[a]	**Leptin**	-0.08
	age	-0.30[b]	Age	-0.35[b]
			WC	0.45[b]
BMD femoral neck	**Leptin**	0.23[b]	**Leptin**	-0.01
	age	-0.33[b]	Age	-0.26[a]
			WC	0.31 [b]
BMD trochanter	**Leptin**	0.19[a]	**Leptin**	-0.03
	age	-0.24[b]	Age	-0.27[b]
			WC	0.37 [b]
BMD total hip	**Leptin**	0.27[b]	**Leptin**	0.07
	age	-0.19[a]	Age	-0.23[a]
			WC	0.31 [b]
BMD trochanter	**Adiponectin**	-0.171[a]	**Adiponectin**	-0.05
	age	-0.214[a]	Age	-0.27[b]
			WC	0.32[b]

Model 1: age, cytokine
Model 2: age, cytokine, waist circumference
[a] $p<0.05$; [b] $p<0.01$ [c]standardized regression coefficient
Abbreviations: BMD, bone mineral density; WC, waist circumference

Table 5. Simple and multiple regression coefficients for the relationship Leptin/Adiponectin and selected BMD sites according to IL-6 levels stratification

		Low IL-6[c]		High IL-6	
Adipokines	**BMD sites**	**$\beta^{\#}$**	**$\beta_{adjusted}$**	**$\beta^{\#}$**	**$\beta_{adjusted}$**
Leptin	Lumbar Spine	0.18	-0.05	0.24	-0.09
	Femoral neck	0.26[a]	0.17	0.19	-0.15
	Trochanter	0.28[a]	-0.11	0.20	-0.10
	Total hip	0.33[b]	0.17	0.27 [a]	-0.04
Adiponectin	Trochanter	-0.35[b]	-0.29[a]	-0.16	0.04

[a] $p<0.05$; [b] $p<0.01$

[c]Lower (or higher) than 0.45 pg/mL (median value)

[d]standardized regression coefficient; $\beta_{adjusted}$, standardized regression coefficient after adjustement for age and waist circumference

Figure

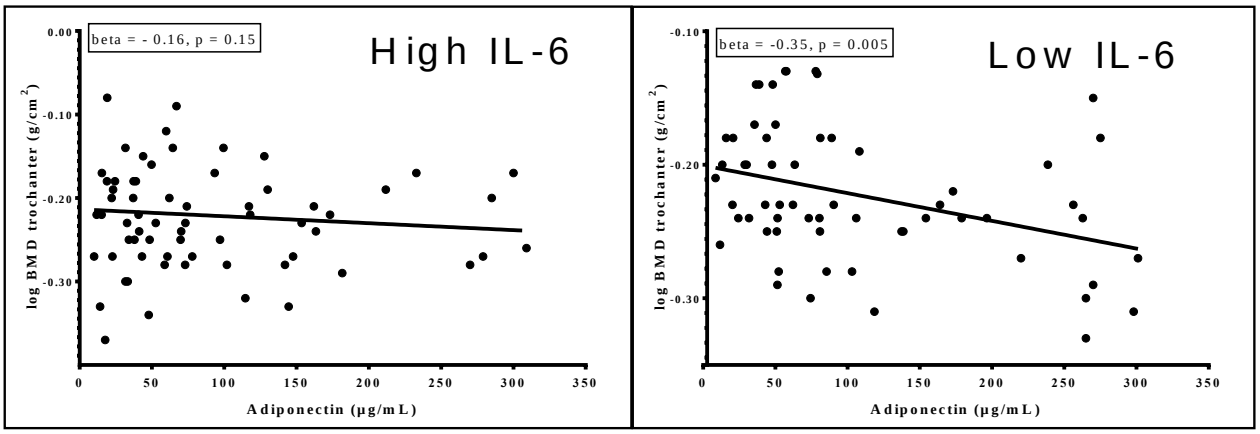

Figure. 1

Figure 2