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RAPID REPORT

Ovariectomy increases RANKL protein expression in bone marrow adipocytes of C3H/HeJ mice

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Beekman KM, Zwaagstra M, Veldhuis-Vlug AG, van Essen HW, den Heijer M, Maas M, Kerckhofs G, Parac-Vogt TN, Bisschop PH, Bravenboer N. Ovariectomy increases RANKL protein expression in bone marrow adipocytes of C3H/HeJ mice. *Am J Physiol Endocrinol Metab* 317: E1050–E1054, 2019. First published September 17, 2019; doi:10.1152/ajpendo.00142.2019.—Estrogen deficiency induces bone loss by increasing bone resorption, in part through upregulation of receptor activator of nuclear factor-κB ligand (RANKL). RANKL is secreted by osteoblasts and osteocytes, but more recently bone marrow (pre)adipocytes have also been shown to express RANKL. Estrogen deficiency increases bone marrow adipose tissue (BMAT). The aim of this study was to determine the effect of ovariectomy (OVX) on RANKL protein expression by bone marrow adipocytes in C3H/HeJ mice. Fourteen-week-old female C3H/HeJ mice ($n = 20$) were randomized to sham surgery (Sham) or OVX. After 4 wk animals were euthanized. BMAT volume fraction (BMAT volume/marrow volume) was quantified by polyoxometalate-based contrast-enhanced nano-computed tomography. The percentage of RANKL-positive bone marrow adipocytes (RANKL-positive bone marrow adipocytes/total adipocytes) and the percentage of RANKL-positive osteoblasts covering the bone surface (bone surface covered in RANKL-positive osteoblasts/total bone surface) were quantified in the distal metaphysis of immunohistochemically stained sections of the left femur. The effects of OVX were analyzed by Student's *t* test or Mann–Whitney *U* test. RANKL was detected in osteoblasts, osteocytes, and bone marrow adipocytes. OVX significantly increased mean percentage of RANKL-positive bone marrow adipocytes [mean (SD): Sham 42 (18)%; OVX 64 (12)%; $P = 0.029$] as well as BMAT volume/marrow volume [median (interquartile range): Sham 1.4 (4.9)%; OVX 7.2 (7.3)%; $P = 0.008$] compared with Sham. We show that OVX increased both the percentage of RANKL-positive bone marrow adipocytes and the total BMAT volume fraction in C3H/HeJ mice. Therefore, RANKL produced by bone marrow adipocytes could be an important contributor to OVX-induced bone loss in C3H/HeJ mice.

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bone marrow adipocytes; immunohistochemistry; ovariectomy; RANKL

INTRODUCTION

High bone marrow adipose tissue (BMAT) is associated with low bone volume, osteoporosis, and fractures, but the exact interaction between BMAT and bone metabolism remains elusive. Recent research suggests a possible role for receptor activator of nuclear factor-κB ligand (RANKL) in the bone-fat interaction, as (pre)adipocytes have been shown to express RANKL (3, 4, 6, 28).

RANKL is important for osteoclast formation (17, 20, 21) and exists as a membrane-bound and soluble protein of the tumor necrosis factor (TNF) superfamily (15, 20). Binding of RANKL to its receptor (receptor activator of nuclear factor-κB; RANK) on osteoclast precursors induces the formation and activation of osteoclasts (12, 13). RANKL can also bind to its soluble decoy receptor osteoprotegerin (OPG), which inhibits the RANKL-RANK signaling (18, 29). Therefore, RANKL-OPG binding is protective against bone loss. RANKL is mainly expressed by osteoblasts and osteocytes but also by other cell types including mesenchymal stromal cells, preadipocytes, chondrocytes, and T-cell and B-cell lymphocytes (2, 8, 9, 23, 31).

Estrogen deficiency induces bone loss by increasing bone resorption, in part through upregulation of RANKL (2, 26) and downregulation of OPG expression (5, 11, 25). Furthermore, estrogen deficiency increases BMAT (1, 19, 27), but whether these two effects are related is unknown. If estrogen deficiency not only increases the total amount of BMAT but also affects RANKL expression by BMAT, this might be an important regulatory mechanism in the bone-fat interaction. Therefore, the aim of this study was to determine the effect of ovariectomy (OVX) on RANKL expression in bone marrow adipocytes of C3H/HeJ mice. We hypothesize that RANKL ex-

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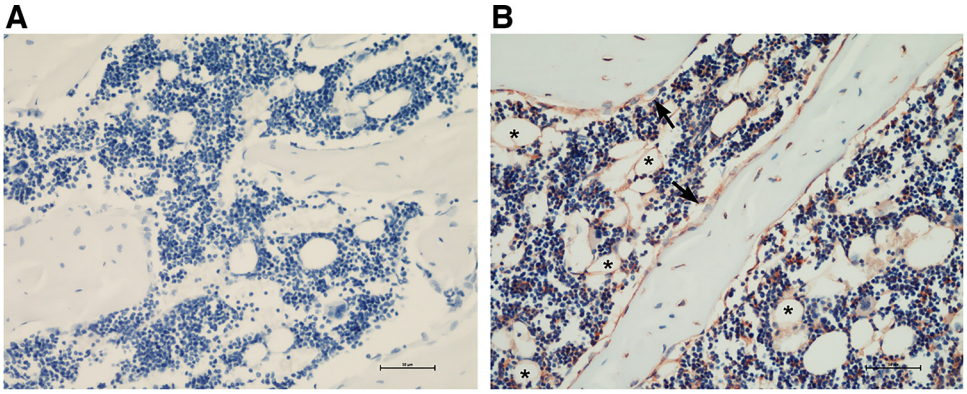


Fig. 1. *A*: negative control [×200 magnification, ovariectomy (OVX) group]. *B*: receptor activator of nuclear factor-κB ligand (RANKL)-positive bone marrow adipocytes in the bone marrow (*) and RANKL-positive osteoblasts covering the trabecular bone (arrows) (×200 magnification, OVX group). Scale bar: 50 μm.

pression by bone marrow adipocytes is increased after OVX compared with sham surgery.

MATERIALS AND METHODS

This experiment was reviewed and approved by the institutional animal care and use committee of the Amsterdam UMC, Vrije Universiteit Amsterdam (no. Endo 14-01). All procedures were performed according to local, national, and international guidelines and laws on experimental animal care. Reporting was performed according to the ARRIVE guidelines.

Twenty 12-wk-old female C3H/HeJ mice (Jackson Laboratories, Stock 000659) were randomly assigned to two treatment groups (n = 10/group). Animals were group housed, with a 12:12-h light-dark cycle at a room temperature of 21°C. Chow (2016 Teklad global 16% protein rodent diets, T.2016MI) and water were available ad libitum. Chow was formulated to exclude soybean, thus minimizing the presence of phytoestrogen in the diet. After 2 wk of acclimatization, all animals underwent surgery: 10 mice were ovariectomized (bilateral approach, isoflurane anesthesia, pain medication: buprenorphine 0.05 mg/kg subcutaneous on the day of surgery), and 10 mice underwent bilateral laparotomy (sham surgery). On *day 28* animals were euthanized via CO_2 inhalation. Uteri were weighted to confirm successful OVX. Right tibiae and left femurs were fixated in 2% phosphate-buffered paraformaldehyde (pH 6.9) at 4°C for 24 h before further processing for immunohistochemistry and contrast-enhanced nano-computed tomography (CE-CT). Analysis of the data was performed blinded for treatment group.

CE-CT image acquisition and analysis. BMAT volume fraction was quantified by polyoxometalate (POM)-based CE-CT (10). After 24 h of fixation, right tibiae were first transferred to phosphate-buffered saline (PBS) and were thereafter transferred to POM-based contrast solution (35 mg of hafnium-Wells–Dawson POM per 1 mL of PBS). Before POM staining, the distal ends of the bones were removed to allow better diffusion of the contrast agent into the bone marrow compartment. Samples were incubated in the contrast solution at 4°C, with gentle shaking, for 48 h before CE-CT imaging. Acquisition and reconstruction settings applied were similar to those described previously (10). Briefly, we used a Phoenix NanoTom M (GE Measurement and Control Solutions, Germany) at a voltage of 70 kV and a current of 60 μA, and a 0.2-mm aluminum filter was used to reduce beam hardening. The exposure time was 500 ms, and 2,400 images were acquired over 360° with the fast scan mode (frame averaging = 1; image skip = 0), resulting in a scanning time of 20 min. We scanned at a 2-μm isotropic voxel size.

BMAT volume fraction was quantified in the proximal metaphysis of the tibia by drawing volumes of interest around the trabecular bone region in the proximal metaphysis starting directly underneath the growth plate and covering a length of 1.2 mm distal to the growth plate with an in-house-developed semiautomated protocol (10). One tibia was excluded from CE-CT analysis because of damage to the proximal metaphysis. BMAT volume fraction was expressed as a percentage of the marrow volume (Ad.V/Ma.V, %).

Immunohistochemistry. RANKL expression in bone marrow adipocytes and osteoblasts was determined by immunohistochemical staining. After 24 h of fixation, left femurs were decalcified for two weeks with ethylenediaminetetraacetic acid (EDTA, 10%, pH 7.2) and subsequently embedded in paraffin. Antigen retrieval was performed with trypsin (Sigma T7168). RANKL protein expression was detected with a polyclonal rabbit anti-mouse RANKL antibody as primary antibody (LifeSpan BioSciences B1425) at a concentration of 1:900 in PBS-Tween. Thereafter, sections were incubated with EnVision+ System-horseradish peroxidase (HRP)-labeled polymer anti-rabbit (Dako K4003), containing both the second antibody and HRP. Finally, the sections were stained with AEC substrate (Sigma AEC101) to detect the bound antibody, and hematoxylin was used for counter-

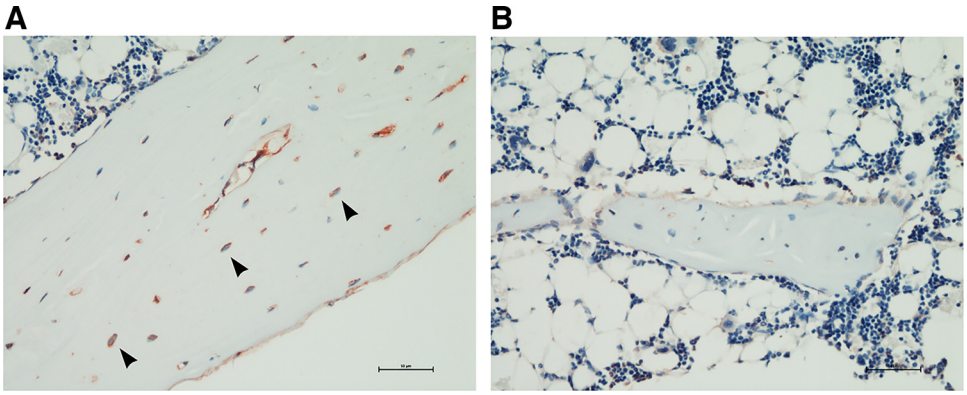


Fig. 2. *A*: abundant receptor activator of nuclear factor-κB ligand (RANKL)-positive osteocytes within the cortical bone (arrowheads) [×200 magnification, ovariectomy (OVX) group]. *B*: no RANKL immunoreactivity in osteocytes within trabecular bone (×200 magnification, OVX group). Scale bar: 50 μm.

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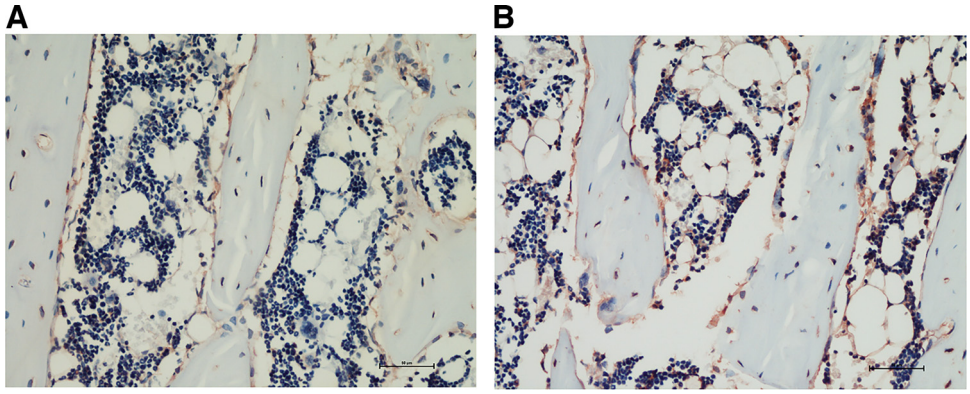


Fig. 3. Representative images of sections of a sham-operated animal that showed little receptor activator of nuclear factor-κB ligand staining in bone marrow adipocytes (*A*) and an ovariectomized animal with abundantly stained bone marrow adipocytes (*B*) (magnification ×200). Scale bar: 50 μm.

staining. A negative (no primary antibody) control staining from one mouse was included in every assay.

Measurements were performed in the secondary spongiosa of the distal metaphysis. Sections with high-quality staining and intact secondary spongiosa were used for measurements. Images were acquired with a Nikon microscope (Eclipse E 800), equipped with a DS-U1 camera (Nikon) and NIS-Elements AR software (version 2.34, Nikon, Düsseldorf, Germany). The percentage of RANKL-positive bone marrow adipocytes (RANKL.Pos.N.Ad/N.Ad) was calculated by tracing the individual adipocytes and counting the RANKL-positive bone marrow adipocytes at ×200 magnification. Per animal, all adipocytes in the secondary spongiosa were counted. If a section had <100 adipocytes in the secondary spongiosa, a second section was analyzed until at least 100 adipocytes were counted. The percentage of bone surface covered with RANKL-positive osteoblasts (RANKL.Pos.B.Pm/B.Pm) was measured by tracing the total bone surface and the bone surface covered in RANKL-positive osteoblasts. The trabecular bone perimeter covered with RANKL-positive osteoblasts was then divided by the total trabecular perimeter to determine the fraction of RANKL-positive osteoblasts. If a bone or a section was damaged during processing it was excluded from analysis. All measurements were performed at ×200 magnification.

Statistics. This study was powered to detect a difference in BMAT volume fraction of 3% with a variance of 6%. The sample size was calculated with nQuery Advisor (version 7.0), and power analysis was approved by the Ethics Committee of the Amsterdam UMC, Vrije Universiteit Amsterdam. IBM SPSS Statistics for Windows (version 22; SPSS, Chicago, IL) was used for statistical analysis. Graphs were constructed with GraphPad Prism (version 7.0 for Windows; GraphPad Software, La Jolla, CA). Means and standard deviations (SDs) or medians and interquartile ranges (IQRs) are reported, depending on the distribution of the data. The effects of OVX were analyzed by Student's *t* test or Mann-Whitney *U* test, depending on the distribution of the data. All statistical tests were two sided, and *P* values of 0.05 were considered statistically significant.

RESULTS

Expression of RANKL was detected in osteoblasts, osteocytes, and bone marrow adipocytes. Figure 1 shows a representative image of the RANKL staining in bone marrow adipocytes and osteoblasts (OVX group). Osteocytes within the cortex were stained abundantly; however, hardly any osteocytes in the trabecular bone of the secondary spongiosa were stained (Fig. 2, OVX group). Consequently, quantification of osteocytes within the trabecular bone of the secondary spongiosa was unreliable, and therefore these osteocytes were not quantified.

Figure 3 shows representative images of BMAT in a sham animal (Fig. 3*A*) and an OVX animal (Fig. 3*B*). OVX significantly increased BMAT volume fraction compared with sham surgery [median Ad.V/Ma.V (IQR): Sham 1.4 (4.9)%, OVX 7.2 (7.3)%; $P = 0.008$; Fig. 4*A*). Furthermore, OVX increased the percentage of RANKL-positive bone marrow adipocytes compared with sham surgery [mean RANKL.Pos.N.Ad/N.Ad (SD): Sham 42 (18)%; OVX: 64 (12)%; $P = 0.029$; Fig. 4*B*]. In addition, OVX increased the percentage of RANKL-positive osteoblasts covering the bone surface compared with sham surgery [mean RANKL.Pos.B.Pm/B.Pm (SD): Sham 27 (7)%; OVX 40 (11)%; $P = 0.018$; Fig. 4*C*).

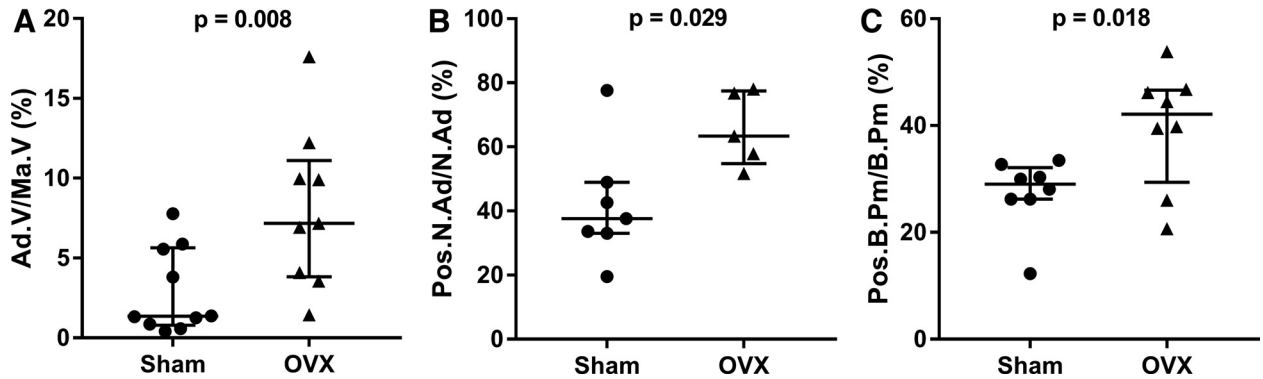


Fig. 4. *A*: ovariectomy (OVX) increased bone marrow adipose tissue volume fraction (Ad.V/Ma.V) measured by contrast-enhanced nano-computed tomography. *B*: OVX increased % of receptor activator of nuclear factor-κB ligand (RANKL)-positive bone marrow adipocytes (Pos.N.Ad/N.Ad). *C*: OVX increased the bone surface covered with RANKL-positive osteoblasts (Pos.B.Pm/B.Pm). Individual data points are shown; lines represent median and interquartile range. Sham, sham surgery.

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DISCUSSION

We show that OVX not only increases the amount of BMAT but also increases expression of RANKL by bone marrow adipocytes in C3H/HeJ mice. This could indicate that RANKL expression by BMAT could have a substantial role in postmenopausal osteoporosis.

To our knowledge, this is the first study to show RANKL expression by mature bone marrow adipocytes measured by immunohistochemical staining. Previous research showed RANKL mRNA expression by Pref-1-positive bone marrow preadipocytes in mice (3, 28) and by primary human bone marrow adipocytes (4). These studies further showed that RANKL mRNA expression in bone marrow preadipocytes increased with aging and was able to induce tartrate-resistant acid phosphatase expression when cocultured with bone marrow macrophages under adipogenic conditions (28). Furthermore, they showed that primary human bone marrow adipocytes were able to stimulate differentiation of osteoclast precursors into active resorbing osteoclasts under coculture conditions (4). Finally, they showed by immunocytochemical staining that human bone marrow adipocytes expressed RANKL protein (6). The latter results differ from our results, as they show that all bone marrow adipocytes expressed RANKL protein and we show that only a percentage of the bone marrow adipocytes express RANKL protein. Potentially the location of the bone marrow adipocytes analyzed could account for these differences, as Hozumi and coworkers (4, 6) use human bone marrow adipocytes harvested from the proximal femur during hip replacement surgery and we quantified RANKL expression in BMAT in the distal metaphysis of the femur of mice. Furthermore, this difference could be explained by the age difference of the subjects (69 yr) and animals (18 wk old), as RANKL expression by bone marrow adipocytes increases with aging (28).

In our study we used OVX as a model for estrogen deficiency; however, besides low levels of estradiol, OVX also induces high levels of gonadotrophins (LH and FSH). It is possible that the increase in BMAT following OVX could be caused by a decrease in estradiol or an increase in FSH (16) or a combination of both. Furthermore, increased levels of FSH could also affect secretion of RANKL by adipocytes (7); however, the current literature shows no effect of secreted RANKL on bone turnover and bone volume (7, 14).

We selected the C3H/HeJ mice as our model because they have high BMAT at a relatively young age. Sufficient amounts of BMAT are necessary to detect changes in BMAT/RANKL expression after ovariectomy. The C3H/HeJ mice harbor a spontaneous missense mutation in the fourth exon of the Toll-like receptor 4 (Tlr4) gene (Tlr4Lps-d) (24), causing loss of function of TLR4 pathway, which is part of the innate immune system. The TLR4 pathway increases T cells, which can also produce RANKL (30); thus inhibition of the TLR4 pathway in the C3H/HeJ mice could lead to decreased levels of RANKL (22, 30). Despite possible lower levels of RANKL in C3H/HeJ mice, we were able to detect changes in RANKL expression by bone marrow adipocytes after OVX. It would be interesting to compare changes in RANKL expression by bone marrow adipocytes in different mouse strains; however, this is beyond the scope of this study.

Our study has limitations. We did not quantify the amount of RANKL expression by BMAT. Since an amplification step is used in the immunohistochemical staining, measuring signal intensity as a parameter for the amount of expression is an unreliable method. Furthermore, because of the relatively high bone turnover in mice, we were not able to reliably quantify osteoblast number and used the percentage bone surface covered with RANKL-positive osteoblasts instead.

To conclude, we show that OVX increases both RANKL expression by bone marrow adipocytes and the total BMAT volume fraction in C3H/HeJ mice. As OVX increased not only the percentage of RANKL-positive bone marrow adipocytes but also the total volume fraction of BMAT, RANKL expression by bone marrow adipocytes could be an important contributor to postmenopausal bone loss.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.M.B., P.H.B., and N.B. conceived and designed research; K.M.B., M.Z., and H.W.v.E. performed experiments; K.M.B., G.K., and N.B. analyzed data; K.M.B., P.H.B., and N.B. interpreted results of experiments; K.M.B. prepared figures; K.M.B. drafted manuscript; K.M.B., M.Z., A.G.V.-V., H.W.v.E., M.d.H., M.M., G.K., T.N.P.-V., P.H.B., and N.B. edited and revised manuscript; K.M.B., M.Z., A.G.V.-V., H.W.v.E., M.d.H., M.M., G.K., T.N.P.-V., P.H.B., and N.B. approved final version of manuscript.

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