

Sexual Dimorphism and Age Dependence of Osteocyte Lacunar Density for Human Vertebral Cancellous Bone

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ABSTRACT

The sexual dimorphism in age-related loss of human vertebral cancellous bone is not fully understood and could be related to dimorphism in the bone cell populations. The objective of this study was to investigate age- and gender-related differences in the osteocyte population and its relationship with bone volume fraction for human vertebral cancellous bone. Histomorphometric techniques were used to quantify osteocyte lacunae (a measure of osteocyte population) and bone volume fraction in male and female human T12 vertebrae, the most common site of vertebral fracture. Two measures of osteocyte population [number of osteocytes per bone area (OtLcDn) and number of osteocytes per total area (OtLcN/TA)] and their relationships with age and bone volume fraction were found to be sexually dimorphic. Dimorphism in osteocyte density may explain the dimorphic patterns of bone loss in human vertebrae due to the sensory and signal communication functions that osteocytes perform. © 2005 Wiley-Liss, Inc.

Key words: osteocyte; aging; dimorphism; bone volume fraction; spine; cancellous bone; bone loss

Human vertebral cancellous bone is one of the primary sites for age-related bone loss. Bone loss in women manifests itself as loss in trabecular number; men, in contrast, show a generalized thinning of trabeculae with aging (Wakamatsu and Sissons, 1969; Parfitt et al., 1983; Aaron et al., 1987; Bergot et al., 1988; Mosekilde, 1989). Consequently, the histologic basis of bone remodeling (resorption cavities, osteoid surface, lamellar width) suggests that in aging females (and in osteoporosis) bone loss occurs due to an increased rate of bone resorption while in males bone loss results from a decline in the rate of bone formation with age (Aaron et al., 1987). This sexual dimorphism in the mechanism of bone loss could be related to a dimorphism in the bone cell populations; however, no such information is available.

A number of recent studies have examined the role of osteocytes in the control of bone remodeling (Rubin and Lanyon, 1987; Marotti et al., 1990; Aarden et al., 1994; Burger et al., 1995; Mullender and Huiskes, 1995). It is now thought that the osteocyte response to an imposed mechanical or biochemical signal can lead to an anabolic

or catabolic response in bone. Osteocytes respond to changes in mechanical load by releasing prostaglandin E2 (PGE2), nitric oxide (NO), and upregulating cyclo-oxygenase (COX-2), all of which have anabolic effects on bone (Inaoka et al., 1995; Klein-Nulend et al., 1995; Forwood, 1996; Ajubi et al., 1999). Osteocytes also undergo apopto-

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sis, which is followed by bone resorption in the affected area after estrogen withdrawal (Tomkinson et al., 1998), glucocorticoid excess (Weinstein et al., 1998), and fatigue microdamage (Verborgt et al., 2000). Thus, sexual dimorphism in the mechanisms of vertebral cancellous bone loss during aging may be related to differences in the osteocyte population or response to stimuli.

The objective of this study was to investigate the age- and gender-related differences in the osteocyte population and its relationship with bone volume fraction for human vertebral cancellous bone. Histomorphometric techniques were used to quantify osteocyte lacunae (a measure of osteocyte population) and bone volume fraction in male and female human vertebral cancellous bone sections followed by analyses for gender differences in the measured and calculated variables.

MATERIALS AND METHODS

Sixty-four previously prepared human vertebral histological sections obtained from 19 white males (age range, 36–86 years; mean age, 62.2 years), 16 black males (age range, 37–96 years; mean age, 68 years), 12 white females (age range 25–89 years; mean age, 55.9 years), and 17 black females (age range, 23–91 years; mean age, 55.9 years) were used for this study. The samples were collected postmortem from a random cross-section of the population. Review of medical records revealed no known metabolic bone diseases or alcohol abuse. Causes of death included cardiopulmonary arrest, cerebral hemorrhage, malignant tachycardia arrhythmia, respiratory arrest, ventricular tachycardia, multisystem organ failure, liver failure and thrombocytopenia. Effects of these conditions on bone are various and were not controlled.

Preparation of histological sections included drilling an 8 mm diameter core in the inferosuperior direction from the most anterior-central portion of the unfixed human T12 vertebral body followed by en bloc staining in 1% basic fuchsin. The cores were embedded in PMMA and serially sectioned to 200 μm thickness. Three central sections from each core were hand-ground to 100 μm thickness and mounted onto a glass slide (Wenzel et al., 1996). The anterior-central portion of the human T12 vertebral body was chosen for this study because L1-T12 junction is the most common site for vertebral fractures (Johansson et al., 1994) and the anterior-central region of the vertebral bone is considered to be the best representative of the whole vertebral fracture properties (Cody et al., 1991).

Morphometric Analyses and Quantification of Osteocyte Lacunar Density

Based on a preliminary study conducted on 10 randomly selected slides from white females, it was decided to use blue violet light (400–440 nm excitation and 470 nm barrier filter) as it penetrates only the first few microns of the relatively thick (100 μm) bone sections and osteocyte lacunae can be readily identified due to the fluorescence of basic fuchsin present at the lacunar edges and canalicular processes. The preliminary study also determined that measuring 15 fields adequately quantified the osteocyte lacunar density of a section. The average number of osteocytes and bone area had an asymptotic variation with number of fields and stabilized before 15 fields.

One section was randomly chosen from each donor for quantification of the osteocyte lacunar density. Following

Wenzel et al. (1996), a 0.8 mm border region at the cut edges of each section was excluded from quantification to avoid any preparation artifacts. Using the upper right-hand corner below the excluded border as a reference point, 64 (8×8) fields were defined at 125 magnification, covering the majority of the remaining section. From these 64 fields, 15 were selected for quantification. Fifteen fields correspond to a section area of 7.78 mm^2 . To avoid any operator bias in the selection of a particular field for quantification, random numbers were generated between 1 and 64 using Microsoft Excel (version 5.0) and 15 fields corresponding to the random numbers were selected for each section.

Each field was viewed and quantified under blue violet light for the total number of osteocyte lacunae (direct count) and bone area (point-counting technique). Parameters obtained from each section were used to calculate the number of osteocytes per bone area [osteocyte lacunar density (OtLcDn)], number of osteocytes per total area (OtLcN/TA), and bone volume fraction (BV/TV). Consistent with previous studies (Wenzel et al., 1996; Vashishth et al., 2000), BV/TV was estimated based on a two-dimensional estimate of BA/TA (BV/TV = bone area/total area).

It is important to note that OtLcDn and OtLcN/TA provide information on two different aspects of the osteocyte network in bone. OtLcDn is the number of osteocyte lacunae per bone area and, by definition, is unable to discern whether a change in its value occurs due to a change in the total number of osteocyte lacunae or due to a change in the amount of bone matrix area. In contrast, OtLcN/TA is the number of osteocyte lacunae in the field of view and a change in its value occurs only due to a change in number of lacunae because the total area is user-defined and, unlike bone area, remains constant. Normalization of the number of osteocyte lacunae with total area allows a reader to compare results between studies. In this study, both OtLcDn and OtLcN/TA were measured to determine the cause of the age-related changes in the numerical value of OtLcDn. Furthermore, our previous work has demonstrated that the measurement of OtLcN/TA gives a unique insight into cell number-based regulation of bone matrix (Vashishth et al., 2002).

Statistical Analyses

Nonparametric methods were used for statistical analyses as not all the variables considered in this study were normally distributed in the subgroups based on race and gender. Furthermore, due to differences in the age between various groups, all comparisons were made on age-adjusted data. Mann-Whitney sign-rank tests (SigmaStat 2.0) were used to determine the influence of race (black males vs. white males; black females vs. white females) and gender (males vs. females) on the OtLcDn and bone volume fraction. The correlations between the number of osteocyte lacunae per bone area (OtLcDn) with age, the number of osteocytes per total area (OtLcN/TA) with age, and the number of osteocytes per total area (OtLcN/TA) with BV/TV were tested using Spearman's rank-order correlation test (SigmaStat 2.0). Correlations and differences between variables were considered significant for $P < 0.05$.

The coefficient of variation for the OtLcDn was calculated by dividing the standard deviation by mean of each section (represents 15 fields). The coefficient of variation for the OtLcDn was plotted with age and tested for corre-

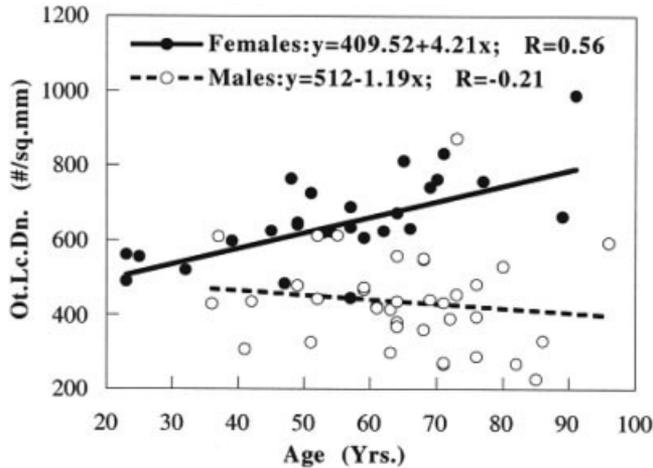


Fig. 1. Osteocyte lacunar density vs. age in male and female human vertebral cancellous bone. OtLcDn increased significantly with age in females ($P = 0.002$) but decreased nonsignificantly in males ($P = 0.23$). Age-adjusted lacunar density was significantly higher in females than in males ($P < 0.001$).

lation using Spearman's rank-order correlation test (Spearman's correlation constant r ; SigmaStat 2.0).

RESULTS

Influence of Race, Age, and Gender

Mann-Whitney sign-rank tests indicated no race-related differences in OtLcDn (blacks vs. whites, males, $P = 0.54$; blacks vs. white, females, $P = 0.94$) and BV/TV (blacks vs. whites, males, $P = 0.39$; blacks vs. white, females, $P = 0.61$). Data from blacks and whites were therefore combined for all further analyses on age and gender.

OtLcDn increased significantly with age in females ($r = 0.56$; $P = 0.002$; Fig. 1). In contrast, males showed a nonsignificant decrease in OtLcDn with age ($r = -0.21$; $P = 0.23$; Fig. 1). OtLcN/TA was also plotted with age to investigate the change in the number of osteocytes with age. Figure 2 indicates that in females, the number of osteocytes statistically declines with age ($r = -0.50$; $P = 0.006$). In males, however, the decrease in the number of osteocytes with age (Fig. 2) was not statistically significant ($r = -0.26$; $P = 0.14$). Age-adjusted OtLcN/TA was significantly higher in females than in males ($P < 0.001$).

Age-adjusted comparison of OtLcDn for gender difference indicated that females ($644 \pm 123/\text{mm}^2$) had a significantly higher OtLcDn than males ($435 \pm 130/\text{mm}^2$; $P < 0.001$). The coefficient of variation of OtLcDn decreased nonsignificantly with age in females ($r = -0.16$; $P = 0.40$) but increased significantly with age in males ($r = 0.34$; $P = 0.04$; Fig. 3).

BV/TV decreased significantly with age in females ($r = -0.65$; $P < 0.001$). In contrast, the age-related decrease in BV/TV with age was not significant in males ($r = -0.01$; $P = 0.98$; Fig. 4). Age-adjusted comparison for gender difference in BV/TV indicated that BV/TV was marginally higher in males (0.10 ± 0.05) and in females (0.09 ± 0.04) but this difference failed to reach significance ($P = 0.10$).

OtLcN/TA showed significant positive correlation with BV/TV for both males ($r = 0.706$; $P < 0.001$) and females

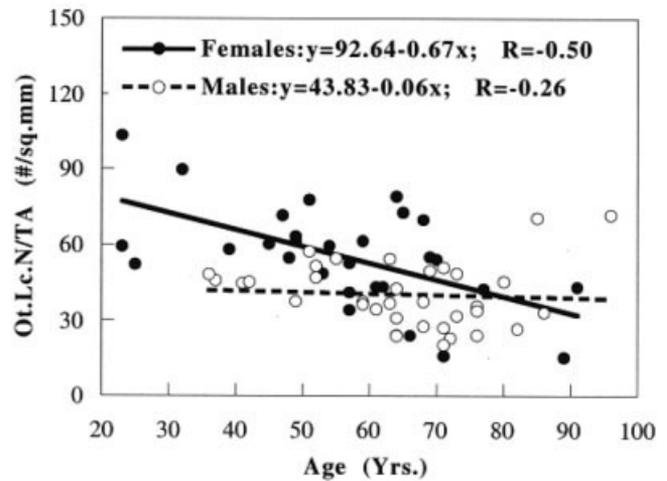


Fig. 2. Number of osteocyte lacunae per total area vs. age in male and female human vertebral cancellous bone. OtLcN/TA declined significantly and nonsignificantly with age in females ($P = 0.006$) and males ($P = 0.14$), respectively. Age-adjusted OtLcN/TA was significantly higher in females than in males ($P < 0.001$).

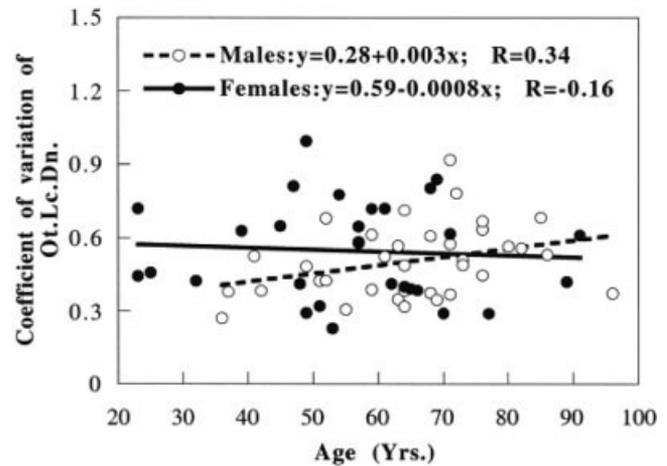


Fig. 3. The coefficient of variation (defined as standard deviation divided by mean) vs. age for males and females. The coefficient of variation significantly increased with age in males ($P = 0.04$) but decreased nonsignificantly with age in females ($P = 0.40$).

($r = 0.805$; $P < 0.001$; Fig. 5). The correlations between the males and females were significantly different ($P < 0.001$).

DISCUSSION

Sexual dimorphism in the microanatomy of human vertebral cancellous bone is considered to be related to the different age-related mechanisms of bone loss (Aaron et al., 1987; Mosekilde et al., 1989). This study has identified gender-related differences in the two measures of osteocyte population (OtLcDn and OtLcN/TA) and their relationships with age and BV/TV. This identified gender difference provides a potential cellular level explanation for the sexually dimorphic bone loss in human vertebral cancellous bone. However, being a study of ex vivo tissue, this study cannot establish the causality between the sexual

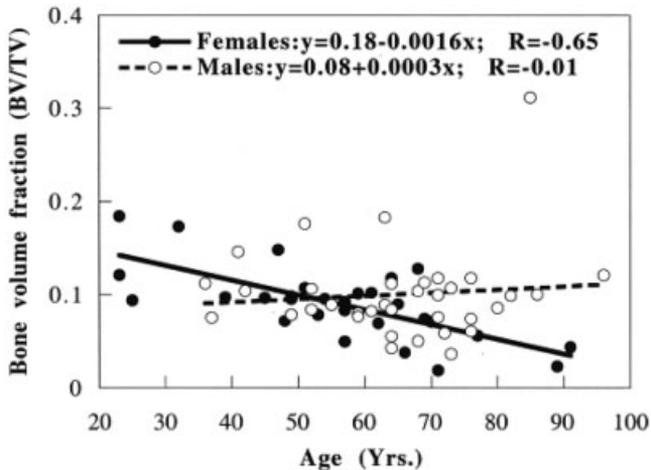


Fig. 4. Bone volume fraction vs. age in males and females. BV/TV declined significantly and nonsignificantly with age in females ($P < 0.001$) and males ($P = 0.98$), respectively. Age-adjusted BV/TV was not different between males and females ($P = 0.10$).

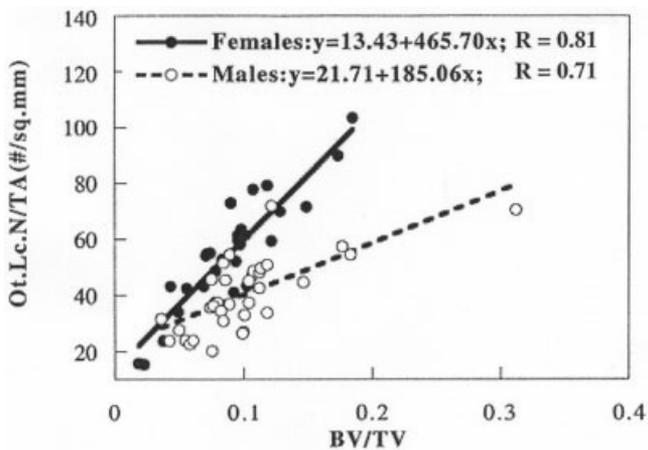


Fig. 5. The number of osteocyte lacunae per total area vs. bone volume fraction in males and females. The correlations between males and females were significantly different ($P < 0.001$).

dimorphism at cellular levels and the different mechanisms of bone loss in males and females.

In this study, no direct quantification of the osteocyte population was done and the osteocyte lacunae were considered to be a quantitative measure of the osteocyte population. Previous studies have shown that not all the lacunae contain osteocytes and that the percentage of empty lacunae in bone depends on the anatomical location and age (Dunstan et al., 1993). Human vertebral cancellous bone lacunar number is considered to represent accurately the number of osteocytes in human vertebral cancellous bone since a constant osteocyte viability of $92\% \pm 4\%$ is maintained with aging (Dunstan et al., 1993).

The present study considered only a two-dimensional measure of the osteocyte lacunar density and bone area fraction. Unlike Mullender et al. (1996b), no attempts were made to measure the lacunar area and obtain an approximation of the osteocyte lacunar density in three

dimensions. Lacunar area does not decrease significantly with age (Mullender et al., 1996b) and hence a two-dimensional approximation of osteocyte density is unlikely to affect our results. The differences in the lacunar areas between male and female lacunar areas have never been reported and previous studies, including Mullender et al. (1996b), arrived at identical conclusions on the existence of differences in the osteocyte lacunar density between their control and osteoporotic groups with two- and three-dimensional measures. Post hoc power analysis indicated that 21 male and 21 female donors were required for a 99% power of detecting a significant difference at $P < 0.05$ in vertebral cancellous OtLcDn between males and females. Our sample included 35 males and 27 females, giving the required power for valid conclusions. A two-dimensionally based estimate of osteocyte lacunar density should therefore be adequate for the purposes of this investigation.

To our knowledge, this is the first study to report gender-related difference in the osteocyte lacunar density. Age-adjusted osteocyte lacunar density was found to be higher in females than males. Previous studies on osteocyte lacunar density were either conducted on a single sex and/or for sites other than human vertebral cancellous bone, including primary and lamellar bone of mammals and reptiles (Hobdell and Howe, 1971; Mullender and Huiskes, 1995), human cortical bone from femoral mid-shaft (Vashishth et al., 2000), and cancellous bone from human femoral neck (Mori et al., 1997) and iliac crest (Mullender et al., 1996b; Qiu et al., 2002a). Studies by Mullender et al. (1996b) and Qiu et al. (2002a) found a linear and nonlinear age-related decline in the osteocyte lacunar density and osteocyte density of iliac crest cancellous bone, respectively, while Mori et al. (1997) found no change in osteocyte lacunar density in femoral head cancellous bone until 70 years, followed by a sharp decline. Mullender et al. (1996b) found no gender-related differences in the OtLcDn for either control or osteoporotic groups.

Differences in the relationships of osteocyte lacunar density with age between Mullender et al. (1996b), Mori et al. (1997), Qiu et al. (2002a), and the current study can be explained by the site-specific differences in noncollagenous proteins and bone cell phenotypes, both of which are associated with differences in bone metabolism and turnover. Aerssens et al. (1997) have recently demonstrated that cancellous bones in the iliac crest, lumbar vertebrae, and femoral head differ in the concentrations of noncollagenous proteins, including osteocalcin and insulin-like growth factor-1 (IGF-1). Compared to iliac crest, cancellous bone in the femoral head and vertebrae have higher concentrations (ng/mg of bone) of IGF-1 and osteocalcin, with vertebral bone greater than femoral. Although no direct comparisons of human bone cell phenotypes are available between iliac crest, femoral head, and lumbar vertebrae, human osteoblasts in mandible and iliac crest demonstrate site-specific differences in the level of mRNA expression for mitogenic growth factors (IGF-2, basic fibroblastic growth factor) and in the levels of TGF- β mRNA (Kasperk et al., 1995). Site-specific differences in the production of IGF binding proteins between the normal human osteoblast-like cells of calvaria, mandible, rib, vertebra, and marrow stroma have also been reported (Malpe et al., 1997). On the basis of the above evidence, it appears that human vertebral cancellous bone could be different in

its metabolism from the iliac crest or femoral head cancellous bone as noncollagenous matrix proteins are known stimulants of bone turnover. The differences in the concentration of noncollagenous matrix proteins may therefore explain the different relationships between age and osteocyte lacunar density reported in Mullender et al. (1996a), Mori et al. (1997), Qiu et al. (2002a), and the current study.

It is also possible that tissue microarchitecture can affect the relationship between osteocyte network and age. Similar to cortical bone, cancellous bone from the iliac crest, proximal femur, and calcaneus regions contains some intratrabecular osteons (Lozupone, 1985; Lozupone and Favia, 1990, 1995; Sato and Byers, 1994) and, like cortical bone (Vashishth et al., 2000), may involve different spatial distribution of osteocytes and different magnitude of age-related changes than vertebral cancellous bones. In fact, Qiu et al. (2002b) show that in iliac crest cancellous bone, the osteocyte density and lacunar number are highest at the surface and these do not decrease with age while the osteocyte density in the central regions are lowest and these undergo an exponential age-related decrease similar to cortical bone. Furthermore, iliac cancellous bone on the surface and in the deep regions (most likely to contain vascular canals and osteons) have different relationships with bone turnover/remodeling rates (Qiu et al., 2002b). Thus, the relationship between osteocyte network and age may be site-specific and not comparable across different skeletal sites.

Osteocyte lacunar density can increase due to an increase in the number of osteocytes, a decrease in the amount of matrix production, or both. Results of our study are consistent with an increase in the total number of osteocytes in the tissue. Age-adjusted osteocyte numbers, represented by number of osteocytes per total area, were greater in females than in males. The greater cell number in females was apparently not associated by an increase in matrix production as bone volume fractions were in fact lower in females than in males; however, the difference failed to reach significance. These results are consistent with TGF- β as a causal mechanism because studies on transgenic mice and Smad3 null mice have found that increased expression of TGF- β 2 or the attenuation of TGF- β -related signaling increases osteocyte number but causes no increase in the mineral apposition rate or amount of matrix production (Erlebacher et al., 1998; Borton, 2001). We propose that one cause of increased osteocyte density in older females is premature differentiation of osteoblasts into osteocytes.

Evidence exists in the literature that concentrations of the TGF- β superfamily of growth factors are higher in females than in males (Pfeilschifter et al., 1998) and are known to increase differentiation of osteoblasts into osteocytes (Erlebacher et al., 1998). It should, however, be noted that the increased levels of matrix TGF- β may not always result in increased osteocyte density and that the TGF- β -mediated mechanism can be altered in osteoarthritis causing osteocyte density to decrease (Jordan et al., 2003). Furthermore, the differentiation of osteoblast into osteocyte is a multifactorial process and may involve many unknown factors that could decrease the effective life span of an osteoblast or cause it to differentiate prematurely into an osteocyte.

A greater osteocyte lacunar density reduces the territorial matrix associated with each osteocyte. This causes a

higher slope between the number of osteocytes per total area and BV/TV in females compared to males (Fig. 5). It is suggested that the difference in the territorial matrix associated with each osteocyte could manifest itself in gender-related differences in mechanotransduction by osteocytes and/or regulation of bone matrix. Osteocytes with smaller territorial matrix are more likely to sense changes in their microenvironment and regulate their surrounding matrix more effectively. Both these effects might result in a more sensitive sensory system in females than in males. A more sensitive mechanotransduction system in females compared to males would consequently translate into a more accelerated bone loss associated with age-related disuse in females than in males. Additionally, higher cell density imposes higher metabolic and nutritional demands on the tissue and has been proposed as a contributor to osteoporosis (Hayden et al., 1995).

This study also found that the change in the coefficient of variation of osteocyte lacunar density with age was sexually dimorphic. Consistent with the age-related decline in bone formation and low bone turnover in males (Clarke et al., 1996; Fatayerji and Eastell, 1999), the coefficient of variation increased linearly with age in males, demonstrating increased variation and heterogeneity of osteocyte lacunar density with aging. In contrast, in females, the coefficient of variation decreased nonsignificantly with age, supporting the occurrence of a consistently high age-related bone turnover (Resch et al., 1994; Eastell et al., 1998).

In conclusion, the present study has identified gender-related differences in two measures of osteocyte population (OtLcDn and OtLcN/TA) and their relationship with age and BV/TV, which may provide a cellular-level explanation for the sexually dimorphic mechanisms of bone loss in human vertebrae. Some caution is necessary in interpreting our results as these samples were from sequential autopsies. Full medical information was not available due to the archival nature of the material and unknown selection biases may affect the current results. Despite this concern, further investigation of factors that modulate the osteocyte density of vertebral cancellous bone and the effect of osteocyte population density on bone loss would appear to be appropriate.

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