Aging Cell (2010) **9,** pp1065–1075 Doi: 10.1111/j.1474-9726.2010.00633.x

Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone

Björn Busse,[1,2,*] Danijela Djonic,[1,3,*] Petar Milovanovic,[1,3] Michael Hahn,[1] Klaus Püschel,[4] Robert O. Ritchie,[2] Marija Djuric[3] and Michael Amling[1]

[1]Department of Osteology & Biomechanics, University Medical Center Hamburg-Eppendorf, Lottestr. 59, D-22529 Hamburg, Germany
[2]Materials Sciences Division, Lawrence Berkeley National Laboratory, University of California – Berkeley, 1 Cyclotron Road, CA 94720, USA
[3]Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade, Dr. Subotica 4/2, 11 000 Belgrade, Serbia
[4]Institute of Legal Medicine, University Medical Center Hamburg-Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany

Summary

Aging decreases the human femur's fatigue resistance, impact energy absorption, and the ability to withstand load. Changes in the osteocyte distribution and in their elemental composition might be involved in age-related bone impairment. To address this question, we carried out a histomorphometric assessment of the osteocyte lacunar distribution in the periosteal and endosteal human femoral cortexes of 16 female and 16 male donors with regard to age- and sex-related bone remodeling. Measurements of the bone mineral density distribution by quantitative backscattered electron imaging and energy dispersive X-ray analysis were taken to evaluate the osteocyte lacunar mineral composition and characteristics. Age-dependent decreases in the total osteocyte lacunar number were measured in all of the cases. This change signifies a risk for the bone's safety. Cortical subdivision into periosteal and endosteal regions of interest emphasized that, in both sexes, primarily the endosteal cortex is affected by age-dependent reduction in number of osteocyte lacunae, whereas the periosteal compartment showed a less pronounced osteocyte lacunar deficiency. In aged bone, osteocyte lacunae showed an increased amount of hypermineralized calcium phosphate occlusions in comparison with younger cases. With respect to Frost's early delineation of micropetrosis, our microanalyses revealed that the osteocyte lacunae are subject to hypermineralization. Intralacunar hypermineralization accompanied by a decrease in total osteocyte lacunar density may contribute to failure or delayed bone repair in aging bone. A decreased osteocyte lacunar density may cause deteriorations in the canalicular fluid flow and reduce the detection of microdamage, which counteracts the bone's structural integrity, while hypermineralized osteocyte lacunae may increase bone brittleness and render the bone fragile.

Key words: Aging; apoptosis; bone histomorphometry; electron microscopy; microanalysis; osteocyte death.

Introduction

A well-regulated interplay between the bone's structural and material properties is essential to maintain bone strength and avoid fractures. Disorders on the structural level comprising the bone's size, geometry, and micro-architecture can negatively affect the bone's resistance to fracture, whereas on the material level redistributions in the collagen and mineral content are known to impair the toughness of the bone tissue (Burr, 2002; Felsenberg & Boonen, 2005; Busse *et al.*, 2009, 2010a; Djuric *et al.*, 2010). Many of these changes are based on an imbalance between bone resorption and bone formation in the physiological process of bone remodeling. Factors that modify the interaction of bone-resorbing osteoclasts and bone-forming osteoblasts are determined by the sex, occurrence of metabolic bone diseases, and, particularly, by natural aging (Parfitt, 1979; Dempster, 2003; Busse *et al.*, 2010a,b).

Once osteoblasts become entrapped in the osteoid and/or the mineralized bone matrix, they turn into another osseous cell named the osteocyte (Palumbo *et al.*, 1990; Qiu *et al.*, 2006). Osteocytes are former osteoblasts that became buried as they finish producing bone matrix, while adjacent osteoblasts continue to work (Palumbo *et al.*, 1990; Qiu *et al.*, 2006). They are considered to be nonproliferative, terminally differentiated cells of the osteoblast lineage (Noble, 2008; Teti & Zallone, 2009). Osteocytes are the most frequent cell type in bone tissue and represent approximately 90–95% of all osseous cells in the adult skeleton (Bonewald, 2007; Noble, 2008). Osteocytes intercommunicate through an extensive network via numerous cytoplasmic/dendritic processes in the canaliculi. The lacuna/canalicular system establishes an extensive molecular exchange through its

Correspondence
Björn Busse, PhD, Department of Osteology & Biomechanics, University Medical Center Hamburg-Eppendorf, Lottestr. 59, D-22529 Hamburg, Germany. Tel.: +49 40 7410 56083; fax: +49 40 7410 58010; e-mail: b.busse@uke.uni-hamburg.de
*B. Busse and D. Djonic contributed equally to this study.

Accepted for publication *7 September 2010*

Aging Cell

large surface area, which is calculated to be 400 times larger than the Haversian and Volkmann system combined and 130 times larger than the surface of trabecular rods and plates (Johnson, 1966). Such an enormous surface provides a potential mechanism to contribute substantially to bone mineral homeostasis (Noble, 2008; Teti & Zallone, 2009). Moreover, osteocytes are thought to activate osteoblasts and/or osteoclasts to initiate bone remodeling owing to mechanosensitive behavior. Hence, in response to mechanical stimuli, osteocytes play a key role in the regulation of bone remodeling (Mullender *et al.*, 2005; Bonewald, 2007; Noble, 2008; Teti & Zallone, 2009). Because of the human femur's reduced ability to withstand maximum weight and *in vivo* stresses owing to aging (Burstein *et al.*, 1976; Currey *et al.*, 1996; Busse *et al.*, 2010a), the osteocyte distribution as well as their elemental composition might be associated with age- and sex-dependent cortex reorganizations. So far, only a few studies on this issue have been published (Frost, 1960; Vashishth *et al.*, 2000; Power *et al.*, 2001, 2002), but none have focused particularly on the internal and external cortical bone with respect to their partially opposite cellular, structural and mechanical properties (Rauch *et al.*, 2007; Busse *et al.*, 2010a). To address this question, we carried out a histomorphometric assessment of the human osteocyte lacunar distribution in the periosteal and endosteal femoral cortices with regard to age- and sex-related bone remodeling. Although it was previously described that lacunar mineralization in the form of micropetrosis can be found in bone tissue (Frost, 1960; Boyde *et al.*, 1986, 1990; Vashishth *et al.*, 2000; Noble, 2008), so far there has been no quantitative assessment of this phenomenon. Therefore, 2D histomorphometry analyses of the bone mineral density distribution (BMDD) and the elemental composition were carried out. We hypothesized that combined measurements of the distribution and the elemental composition of osteocyte lacunae in human femora of various ages and sexes may help to emphasize the osteocytes' impact on the impairment of femoral material properties as well as provide a better understanding of their significance in the bone remodeling process and mineral metabolism regulation.

Results

Age-related changes in osteocyte lacunar density

Histomorphometric analyses of the total osteocyte lacunar number per bone area (Tt.L.N/B.Ar.) in the periosteal and endosteal regions of the cortex revealed age-dependent relations in both sexes (Fig. 1A,B). Hence, with increasing age, the osteocyte lacunar density decreased significantly in both the female (r_{peri} = −0.785 and r_{endo} = −0.835; $P < 0.001$) and male (r_{peri}=−0.944 and r_{endo} = −0.837; $P < 0.001$) cases (Fig. 1A,B). The decline in osteocyte lacunar density in men is more pronounced at the periosteal cortex than at the endocortical region, while in women the two regression lines are parallel (Fig. 1); however, despite such visual impression, there were no significant statistically differences between sexes. The decrease in Tt.L.N/B.Ar.

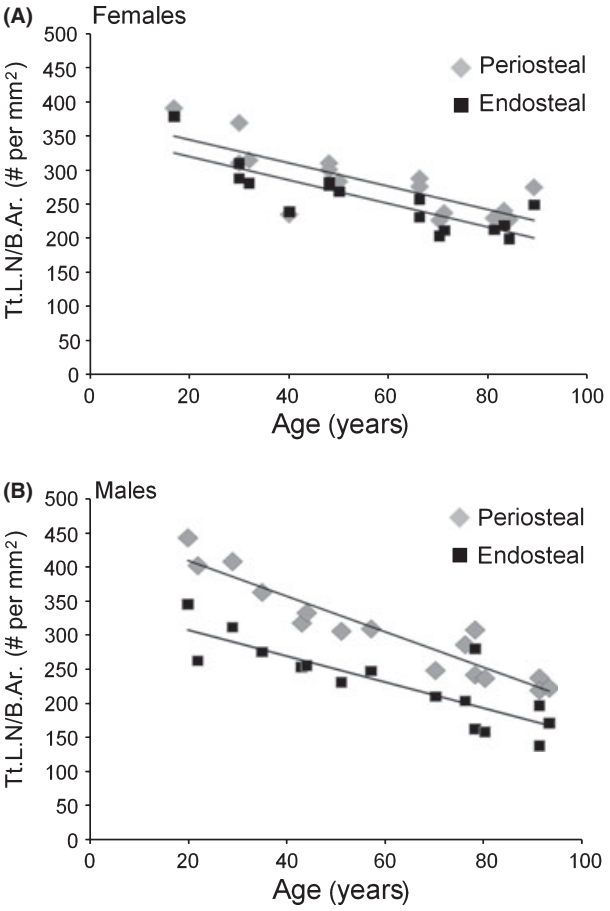

Fig. 1 Linear regression analyses. (A) In the female cases, the total number of osteocyte lacunae per bone area (Tt.L.N./B.Ar. [#/mm^2]) decreased linearly with age in the periosteal and endosteal cortexes (y_{peri} = −1.73x + 380.1; R^2 = 0.616, $P \leq 0.005$; y_{endo} = −1.74x + 355.0; R^2 = 0.697, $P \leq 0.005$). (B) In the male cases, the total number of osteocyte lacunae per bone area (Tt.L.N./B.Ar. [#/mm^2]) decreased linearly with age (y_{peri} = −2.64x + 462.8; R^2 = 0.891, $P \leq 0.005$; y_{endo} = −1.93x + 346.3; R^2 = 0.7, $P \leq 0.005$) in the periosteal and endosteal cortexes.

appeared in every field (medial, ventral, lateral, and dorsal) of the periosteal and endosteal regions (Table 1).

One-way analysis of variance, which was used to compare the osteocyte lacunar density in four age categories in women, demonstrated significant differences between the first (< 39 years) and older age groups (> 40 years) in all fields of the periosteal cortex (Fig. 2; Table 2), while the differences in the endosteal cortex were demonstrated only in the first group (< 39 years) vs. the third (60–79 years) and the fourth age group (> 80 years). In the male cases, significant increases in Tt.L.N/B.Ar. were found in the first age group in comparison with all of the other age groups (Fig. 2; Table 3). This was evident for every field in the periosteal and endosteal cortex (Table 3).

Sex-related differences in osteocyte lacunar density

A comparison of Tt.L.N/B.Ar. between the female and male groups revealed no significant differences in any of the

Table 1 Age dependency in the total number of osteocyte lacunae per bone area in female and male cortical regions expressed by Pearson's correlation coefficient

	Periosteal				Endosteal			
Sex	Medial	Ventral	Lateral	Dorsal	Medial	Ventral	Lateral	Dorsal
Female	−0.785***	−0.712**	−0.799***	−0.875***	−0.689**	−0.678**	−0.606*	−0.795***
Male	−0.950***	−0.857***	−0.898***	−0.787***	−0.897***	−0.828***	−0.929***	−0.811***

*$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$.

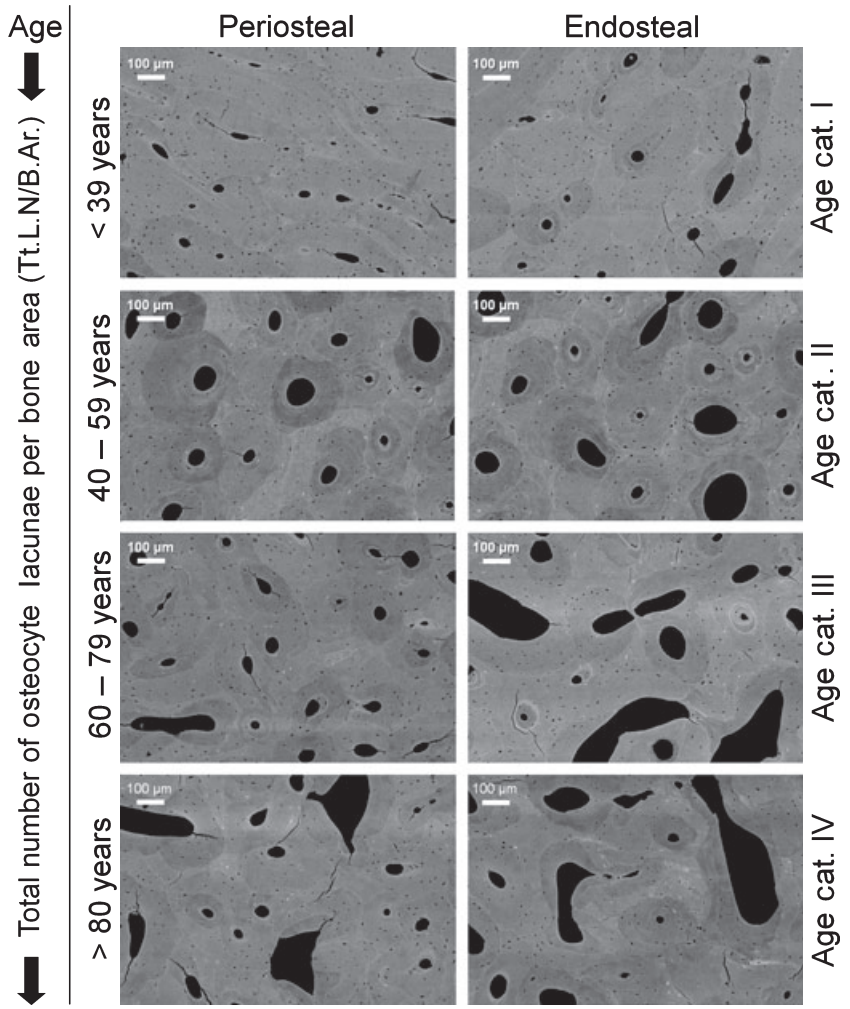

Fig. 2 Distribution of osteocyte lacunae. In the periosteal and endosteal regions of interest, the total osteocyte lacunar density per bone area (Tt.L.N./B.Ar. [#/mm^2]) declined with increasing age, whereas the cortex porosity increased with age (BSE, 100×).

evaluated cortical compartments (t_{peri} = 1.057, P = 0.300; t_{endo}=−1.394, P = 0.174). Moreover, no significant differences in osteocyte lacunar density were found between women and men in any of the evaluated age groups.

Intersite differences in osteocyte lacunar density

Analysis of the osteocyte lacunar density in each of the investigated fields (medial, ventral, lateral, and dorsal) revealed significant intersite differences between the periosteal and endosteal regions in each age category for both sexes (Fig. 3A,B).

Distribution and content of osteocyte lacunae change with age

Apart from age-related decline in total number of lacunae (Tables 1–3), analyses of the osteocyte lacunar mineral content revealed increased number of highly mineralized osteocyte

Table 2 Osteocyte lacunar density in the periosteal and endosteal cortex in aging women

Tt.L.N/B.Ar. [#/mm^2]				
	Age group I (< 39 years)	II (40–59 years)	III (60–79 years)	IV (> 80 years)
Region	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Periosteal				
Medial*	407.32 ± 22.58	299.95 ± 15.63	288.75 ± 13.07	275.05 ± 10.42
Ventral*	355.38 ± 19.35	273.90 ± 9.57	274.43 ± 6.85	255.59 ± 12.24
Lateral*	378.54 ± 16.45	284.01 ± 16.95	281.73 ± 5.18	257.48 ± 7.32
Dorsal*	357.61 ± 20.91	282.96 ± 12.49	242.99 ± 24.03	225.35 ± 10.67
Endosteal				
Medial†	302.59 ± 22.09	254.74 ± 16.92	227.62 ± 15.59	225.79 ± 9.66
Ventral†	275.95 ± 23.64	239.94 ± 11.00	208.09 ± 10.78	210.86 ± 8.27
Lateral†	278.73 ± 21.21	247.11 ± 14.87	211.07 ± 12.92	217.66 ± 9.91
Dorsal†	293.22 ± 21.57	246.49 ± 14.77	194.39 ± 14.89	190.90 ± 9.43

Values are presented as mean ± SE (standard error).
ANOVA: *Significant difference between the first and other age groups (second, third, and fourth). $P \leq 0.05$.
†Significant difference between the first group and third/fourth group. $P \leq 0.05$.

Table 3 Osteocyte lacunar density in the periosteal and endosteal cortex in aging men

Tt.L.N/B.Ar. [#/mm^2]				
	Age group I (< 39 years)	II (40–59 years)	III (60–79 years)	IV (> 80 years)
Region	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Periosteal				
Medial*	448.08 ± 14.91	334.34 ± 9.71	266.87 ± 9.70	223.90 ± 14.48
Ventral*	392.18 ± 10.95	297.59 ± 11.16	249.99 ± 12.91	231.32 ± 12.45
Lateral*	401.14 ± 10.86	323.41 ± 9.29	278.21 ± 18.63	236.73 ± 12.65
Dorsal*	377.10 ± 15.01	310.46 ± 11.00	264.84 ± 11.16	218.58 ± 11.70
Endosteal				
Medial*	316.77 ± 12.99	260.88 ± 10.86	212.72 ± 13.79	163.75 ± 10.69
Ventral*	294.89 ± 10.28	246.95 ± 5.27	198.23 ± 15.17	178.48 ± 9.01
Lateral*	291.24 ± 11.49	236.94 ± 3.77	193.19 ± 14.13	168.56 ± 14.17
Dorsal*	290.64 ± 12.29	239.86 ± 8.30	191.78 ± 13.38	167.20 ± 12.89

Values are presented as mean ± SE (standard error).
ANOVA: *Significant difference between the first and other age groups (second, third, and fourth). $P \leq 0.05$.

lacunae in old individuals (Table 4; Fig. 4A,B). A comparison of mineralized osteocyte lacunar number per bone area (Mn.L.N/B.Ar.) values for women and men did not show significant differences in the young ($t_{peri} = -1.226$, $P = 0.288$; $t_{endo} = -1.321$, $P = 0.257$) and old ($t_{peri} = -0.117$, $P = 0.913$; $t_{endo} = -0.185$, $P = 0.864$) cases. In sharp contrast, energy dispersive X-ray (EDX) microanalysis shows that an age-dependent alteration in the elemental composition of osteocyte lacunae was striking in both sexes (Fig. 4C). The young cases (< 39 years) showed a low occurrence of mineralized tissue within the lacunae (Fig. 4A,D), whereas in the old cases (> 80 years), highly mineralized osteocyte lacunae were more frequent (Fig. 4B,D). We found significant differences in the number of mineralized lacunae per bone area between the youngest and oldest groups in both the periosteal and endocortical regions (Fig. 4D). In both young and old individuals, significant differences were not observed in the density of highly mineralized osteocyte lacunae between the periosteal and endosteal regions ($t_{young} = -0.034$, $P = 0.974$; $t_{old} = -2.267$, $P = 0.073$).

Discussion

This aging study illustrates not only that the total number of osteocyte lacunae is less in human cortical bone from older cohorts but also that some of the osteocyte lacunae convert to osteocyte lacunae with hypermineralized occlusions. Both changes signify risks for the bone's structural integrity and the bone's safety at increased age. In this context, subdivision into the periosteal and endosteal regions of interest emphasized that the endosteal cortex is primarily subjected to age-dependent losses in osteocyte lacunae, whereas the periosteal compart-

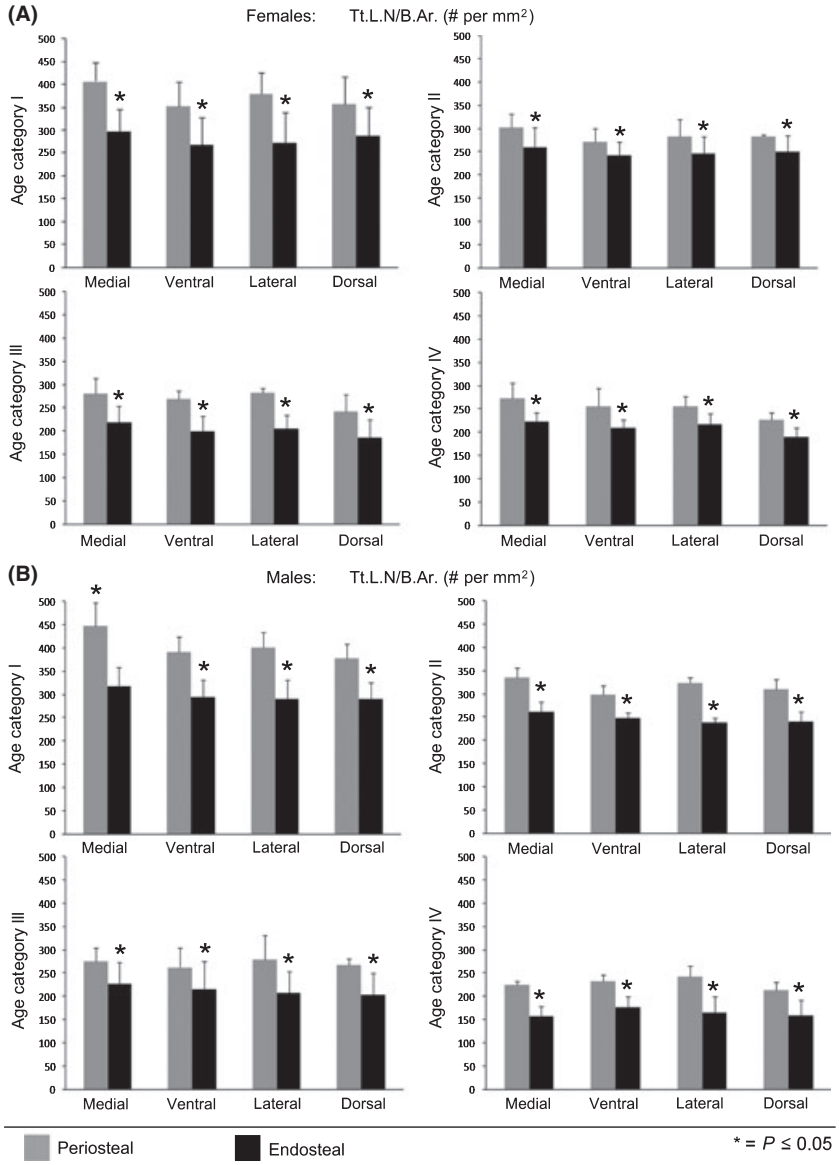

Fig. 3 Osteocyte lacunar density in anatomical landmarks. (A) Total number of osteocyte lacunae per bone area (Tt.L.N./B.Ar. [#/mm^2]) significantly decreased in the medial, ventral, lateral, and dorsal fields in the periosteal and endosteal cortex with age during lifetime in women. (B) Total number of osteocyte lacunae per bone area (Tt.L.N./B.Ar. [#/mm^2]) significantly decreased in the medial, ventral, lateral, and dorsal measuring fields in the periosteal and endosteal cortex during lifetime in men.

ment demonstrated a less pronounced osteocyte lacunar deficiency. Our findings raise the question of whether an increase in the number of mineralized osteocyte lacunae with a decrease in the total lacunar density may account for a failure or long delay in the bone repair process in femurs of aging humans.

Age-related changes in density, morphology, and osteocyte lacunar content

In humans, an adequate number of osteocytes is essential to remove bone microdamage, and the osteocyte density correlates with the biomechanical quality of bone (Ma *et al.*, 2008). We found a decrease in the osteocyte lacunar density with advancing age in all of the observed regions and both sexes, which indicates a higher likelihood of sustaining microdamage. Namely, recent studies have demonstrated that the osteocyte density correlates with the initiation and propagation of microdamage (Qiu *et al.*, 2005); in addition, the lacunae are considered to trigger microcrack initiation (Reilly, 2000), regions that dissipate energy (Vashishth, 2004) or barriers causing crack arrest (Da Costa Gómez *et al.*, 2005). Any change in osteocyte lacunar density would affect the length of cracks and the bone strength (Ma *et al.*, 2008). Moreover, a reduced number of osteocytes can result in the deterioration of canalicular fluid flow and the decreased ability to detect microdamage, which may lead to deficient repair and consequent increased bone fragility

Table 4 Mineralized occlusions of osteocyte lacunae per bone area

	Young (< 39 years)		Old (> 80 years)	
	Periosteal	Endosteal	Periosteal	Endosteal
Mn.L.N/B.Ar. [#/mm^2]	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Females	4.35 ± 0.52	4.55 ± 1.06	19.37 ± 2.07	34.44 ± 12.80
Males	2.81 ± 1.15	2.65 ± 0.97	19.12 ± 0.24	31.63 ± 8.20

Values are presented as mean ± SE (standard error).

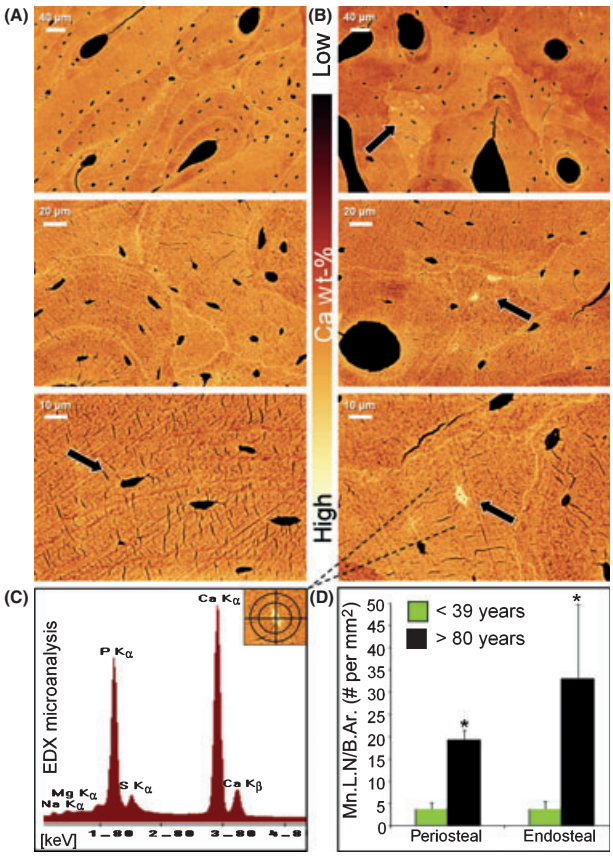

Fig. 4 Hypermineralized intralacunar occlusions. (A) Mineralized occlusions occurred highly infrequently in the osteocyte lacunae of subjects < 39 years. In the female and male cases of the first age category (< 39 years), the osteocytes showed a regular size and dimension (black arrow).(B) In the age group above 80 years, highly mineralized calcium phosphate occlusions (black arrows) were observed in both the female and male cases. (C) Energy dispersive X-ray analysis proved that the mineral contents of these occlusions are mainly calcium (Ca K alpha) and phosphorus (P K alpha). (D) In the bone tissue of women and men, the amount of highly mineralized lacunar occlusions per bone area (Mn.L.N./B.Ar. [#/mm^2]) significantly increased in comparison with the bone tissue of younger controls.

(Burger *et al.*, 2003; Qiu *et al.*, 2003; O'Brien *et al.*, 2004; Mullender *et al.*, 2005; Ma *et al.*, 2008). Qiu *et al.* (2003) reported a significantly lower number of osteocyte lacunae per bone area in patients suffering from fractures compared with healthy controls. It is suggested that the loss of osteocytes compromises the repair of microdamage and makes it more likely to accumulate into a macrofracture (Noble, 2003; Hernandez *et al.*, 2004). Although the cause of an age-related loss of osteocytes remains unclear, it is known that osteocyte apoptosis is engendered by a number of conditions including glucocorticoid excess (Kogianni *et al.*, 2004; O'Brien *et al.*, 2004; Weinstein *et al.*, 2010), microdamage (Gu *et al.*, 2005), estrogen deficiency (Tomkinson *et al.*, 1998; Lirani-Galvão *et al.*, 2009), and oxidative stress (Mann *et al.*, 2007). Because osteocytes cannot be replaced, except by remodeling (Noble, 2003), it is reasonable that cell death can possibly occur as the time spent immured in the bone matrix increases. Increased osteocyte apoptosis and decreased osteocyte density were also observed in weightlessness, disuse, and glucocorticoid-induced osteoporosis (Kogianni *et al.*, 2004; O'Brien *et al.*, 2004; Aguirre *et al.*, 2006). Qiu *et al.* (2005) reported that there were significantly fewer osteocyte lacunae in the area adjacent to microcracks than in peripheral areas (Qiu *et al.*, 2005), which indicates a spatial and possibly causal relationship between osteocyte loss and the production of microdamage. Because both the likelihood of microdamage and the frequency of osteocyte death increase with the age of the bone (Qiu *et al.*, 2005), it is still uncertain whether microdamage or osteocyte death occurs first in the process that leads to bone fragility.

One preliminary hypothesis is that initiating microdamage may lead to osteocyte death within the same region. This scenario is supported by the view that local osteocyte death is an early response to microdamage (Verborgt *et al.*, 2000) and normally followed by the formation of a new bone multicellular unit to remove the microdamaged bone (Bentolila *et al.*, 1998). As investigated in the present study, another preliminary hypothesis suggests that osteocyte death may induce lacunar hypermineralization, which increases bone brittleness and thus is a predisposing factor for microdamage (Frost, 1960; Parfitt, 1993). Also, (Boyde *et al.*, 1986, 1990) suggested that a mineralization process within osteocyte lacunae may be a possible indication of a former osteocyte death, recently termed 'living fossil' (Bell *et al.*, 2008). In general, osteocytes are harbored in lacunae, where a space exists between the cell surface and the walls of the lacunae. This space between the cell and the mineralized lacunar walls as well as the space between the dendritic processes and the walls of the canaliculi consists of a thin layer of unmineralized extracellular matrix (Jande, 1971; Sauren *et al.*, 1992; Noonan *et al.*, 1996; You *et al.*, 2004; Klein-Nulend & Bonewald, 2008; Nicolella *et al.*, 2008; Safadi *et al.*, 2009). Although osteocytes have relatively few organelles necessary for matrix production and secretion, a limited secretion of specific matrix proteins may be essential for osteocyte function and survival (Safadi *et al.*, 2009). The osteocyte's ability to alter the composition of their surrounding extracellular matrix has been reported (Aarden *et al.*, 1996; Knothe Tate, 2003; Holmbeck *et al.*, 2005; Lane *et al.*, 2006; Zhang *et al.*, 2006). Therefore, it can be stated that osteocytes are rather active inhabitants of the lacunae and inhibit the mineralization of a thin layer of matrix and so remain detached from the surrounding bone (Aarden *et al.*, 1996; Klein-Nulend & Bonewald, 2008). This nonmineral-

ized pericellular zone allows a strain-derived flow of interstitial fluid over the osteocyte's surface that is essential to keep the osteocyte healthy because it facilitates the exchange of nutrients and waste products between the haversian channel and the osteocyte network (Kufahl & Saha, 1990). These zones also facilitate the interplay between the osteocytes and the matrix molecules, which may play a necessary role in mechanotransduction (Aarden *et al.*, 1996; Klein-Nulend & Bonewald, 2008). We suggest that in the case of an osteocyte's death within the bone areas not subject to bone resorption or remodeling, the absence of osteocytes may induce mineralization within the pericellular space and a further spread into the lacuna, which ends in a consecutive deposition of mineral and thus hypermineralized lacunae as observed in our study.

Slight periosteal bone apposition, which continues during a person's life (Seeman, 2008), may explain the observation of more nonmineralized osteocyte lacunae near the periosteal surface than in the endocortical region. In addition, while in the young cases there are only a few mineralized lacunae in both the periosteal and endocortical regions, in old individuals a significant difference appears between these two regions; the endocortical region shows a higher number of hypermineralized lacunae and a lower number of total lacunae. This could indicate that the endocortical region is particularly affected by impaired bone remodeling, i.e., although it needs more repair, as the repair becomes deficient, the endocortex is debilitated with age. Mechanical loading applied to the skeleton preferentially increases periosteal bone formation in regions where mechanical stresses are the highest (Beck *et al.*, 2000; Turner, 2006). It can be speculated that the difference between the periosteal and endocortical regions observed in our study is associated with, in the case of bending loads, the endocortical surface receiving much less load than the periosteal surface (Beck *et al.*, 2000; Tanck *et al.*, 2006; LaMothe & Zernicke, 2008) with a strain gradient through the thickness of the cortex where maximum strains are reached in the periosteal region (Beck *et al.*, 2000; Tanck *et al.*, 2006).

The results of our study revealed that increasing age was correlated with a decline in osteocyte lacunar density, which may affect the bone repair processes. An impaired bone remodeling process is reflected in the accumulation of dead osteocytes and their 'fossilization' in hypermineralized lacunae. These hypermineralized lacunar occlusions appeared predominantly in small groups of two to six. Hypermineralized lacunae were found to be more frequent in the interstitial lamellae. Interstitial cortical regions have older tissue age and receive less oxygen and nutrients, and both of these factors favor osteocyte death (Frost, 1960; Noble, 2003; Qiu *et al.*, 2005). Therefore, it appears reasonable that osteocyte death represents a major contributor to lacunar hypermineralization. A failure and/or long delay in the repair process could account for the observed increase in hypermineralized lacunae together with a decrease in osteocyte lacunar density. Mineralization of lacunae should lead to a reduction in the energy absorbing/dissipating capacity of bone (Klein-Nulend & Bonewald, 2008). Indeed, local hypermineralized regions have been considered to make bones more brittle (Jee, 2001) by virtue of their presumed tendency to crack more easily, which thereby affects the ultimate mechanical properties of the whole bone tissue (Boyce & Bloebaum, 1993); in these terms, the bone might be expected to be more susceptible to fatigue damage (Jee, 2001). However, in terms of the effect of mechanical properties, the presence of hypermineralized regions, which are tens of microns in dimension and surround the osteocyte lacunae, would have two principal effects. The increased mineral content of these regions would certainly lower their toughness and make them more prone to microcracking. However, this is offset by the fact that their increased mineral content would also make these regions stiffer; thus, a growing crack within the bone would tend to avoid them because cracks always tend to follow the low modulus 'phase', which thereby increases the tortuosity of the crack path and in turn raises the toughness. We believe that the embrittlement caused by hypermineralization is likely to be the dominant effect in this case.

Because the underlying mechanism for the reported changes in the osteocyte lacunar composition in aged human cohorts is at present not well understood, beneficial approaches for further research may be based on animal models of aging. Further mechanistic insight may be gleaned by focusing on immunohistochemistry methods and the expression of typical osteocyte proteins that are involved in osteocyte homeostasis and matrix mineralization, such as DMP-1, E11, SOST, and Klotho (Bonewald, 2006; Noble, 2008). However, mineralized osteocyte lacunae have been detected in significant numbers only in humans. In rodents, Frost has observed only a slight degree of micropetrosis (i.e., mineralized lacunae), and he concluded that it is common only in human bone (Frost, 1960). This raises the question of whether the skeleton of a mouse could appropriately reflect this event because of its short life span.

Beyond the widely accepted concept that osteocyte apoptosis is a direct stimulus to initiate bone remodeling, our findings indicate that the delay and/or failure in microdamage repair may represent another key aspect in the interplay between aging and bone fragility (Fig. 5). A delay or failure in fatigue damage repair does not clinically manifest itself until the microdamage burden overpasses some critical level (Qiu *et al.*, 2003). However, bone remains a living tissue as long as osteocytes remain alive (Holmbeck *et al.*, 2005).

Experimental procedures

The right femur was taken from 32 organ donors during an autopsy at the Department of Legal Medicine, University Medical Center Hamburg-Eppendorf, Germany. The cases include 16 female and 16 male specimens with ages in the range between the 1st and 9th decade. The circumstances leading to death were sudden traumatic injuries. These individuals did not suffer from cancer, renal diseases, primary hyperparathyroidism, Paget's disease or show any other signs or symptoms of bone disease. None of the cases received medication that interfered with bone metabolism or affected the bone tissue. The femora

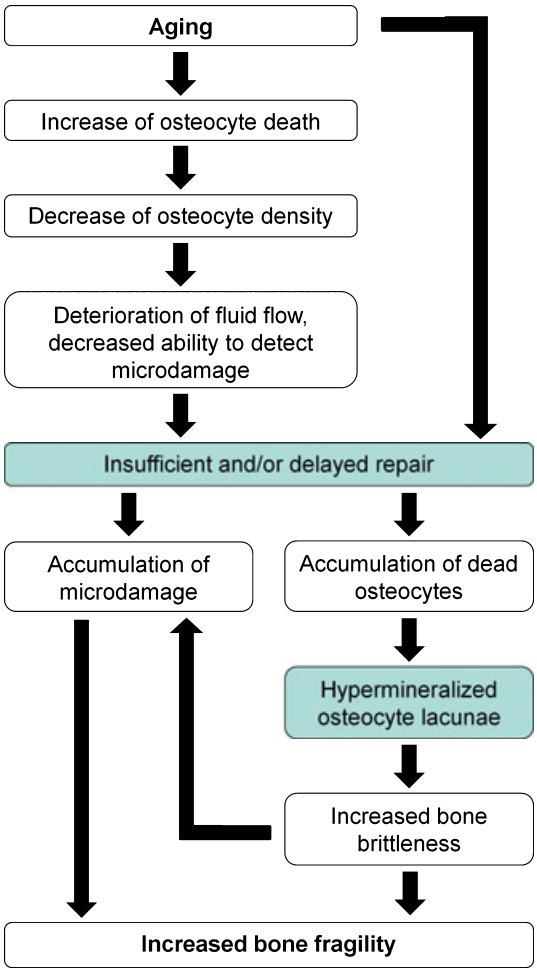

Fig. 5 Pathway: The effects of aging on bone fragility. Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveal failure and delay of the remodeling process in aged human bone.

were X-rayed to verify the bone status and the femoral geometry (Fig. S1A). According to a defined pattern (Busse *et al.*, 2008, 2010a), three 4-mm-thick horizontal sections were separated from the femora using a diamond belt saw under water cooling (EXAKT, Norderstedt, Germany) and subsequently contact X-rayed (Fig. S1A). These horizontal sections were taken beneath the trochanter minor, in each case with 4 cm length distance (Fig. S1A).

The specimen preparation in terms of fixation was accomplished in a similar manner as described in previous studies (Hahn *et al.*, 1991). Undecalcified specimens were infiltrated with successively decreasing concentrations of ethanol in a methylmethacrylate (Technovit 7200; Kulzer, Hanau, Germany) – ethanol (ET) solution with the following volumetric ratios: 30:70 (ET); 50:50 (ET); 70:30 (ET). Methylmethacrylate with benzoyl-peroxide was used to continue the infiltration process for a further 14 days under vacuum. Ten additional hours elapsed until the polymerization process was completed, and the specimens were applicable for automatic grinding (EXAKT). Donath's grinding technique was used to prepare ultra-thin grinding specimens as well as block specimens (Donath & Breuner, 1982; Hahn *et al.*, 1991). After the polishing process, the specimens were stained with von Kossa-modified solution (Donath & Breuner, 1982; Hahn *et al.*, 1991).

Two-dimensional histomorphometry of osteocyte lacunar density

The stained ground specimens were used to quantitatively evaluate the osteocyte lacunar density (total osteocyte lacunar number per bone area). In all three horizontal sections, the cortical compartment was divided centrically into a periosteal and endocortical region. Furthermore, each region was subdivided into eight fields, where the medial, lateral, posterior, and anterior areas were purposefully determined (Fig. S1B). Thus, 16 measuring fields were evaluated per horizontal section (Fig. S1B). Under microscopic magnification (100×) (Fig. S1C), total number of osteocyte lacunae per bone area was determined by evaluating the osteocyte lacunar number and bone area in five random square sections (1.51 mm^2) in each of the 16 fields. In this study, the osteocyte population was determined by evaluating osteocyte lacunar density that was considered to reflect the characteristics of the osteocyte network (Vashishth *et al.*, 2000). The numbers of total lacunae (Tt.L.N) were expressed per bone area (Tt.L.N/B.Ar.), according to the American Society of Bone and Mineral Research histomorphometry standard (Parfitt *et al.*, 1987). The mineralized osteocyte lacunae per bone area (Mn.L.N/B.Ar.) delineate a parameter that is newly introduced by the authors based on Frost's observation of micropetrosis (Frost, 1960). This parameter reflects the number of osteocyte lacunae that are filled with mineralized tissue.

Mineral content analysis of the osteocyte lacunae

For the BMDD analysis, embedded specimens were polished and carbon coated. Quantitative backscattered scanning electron microscopy was used to assess the degree of mineralization of the specimens. The application is based on the work of other groups that use quantitative backscattered electron imaging (qBEI) (Roschger *et al.*, 1995, 1998, 2008) and has been reported previously (Busse *et al.*, 2009, 2010a,b; Seitz *et al.*, 2010). The scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England) was operated at 15 kV and 665 pA at a constant working distance (BSE Detector, Type 202; K.E. Developments Ltd., Cambridge, England). The pixel size of 3 μm was determined based on the recommendations of Roschger *et al.* (2008). Synthetic hydroxyapatite (HA) samples were used to create a calibration curve. These HA samples (DOT Medical Solutions, Rostock, Germany) contained different Ca/P ratios, which were determined using energy dispersive X-ray analysis (DX-4; EDAX, Mahwah, NJ, USA) and qBEI. A highly linear relationship (r = 0.98) between the gray values of the backscattered signal intensities and the calcium content (Ca-Wt%) of each sample has been reported previously by other authors (Roschger *et al.*, 1995, 1998), which enabled

calibration of the method. The generated gray values represent the mean calcium content (mean Ca-Wt%) of the cross-sectioned bone (Fig. S1D).

Statistical analysis

The Kolmogorov–Smirnov test was used to confirm the normality of the osteocyte lacunar density distribution (Tt.L.N/B.Ar.). The age dependence of the measured parameter in the periosteal and endocortical regions was determined using a linear regression analysis separately for the men and women. In further analysis, to check for age-range specific trends, both the male and female samples were divided into four biologically relevant age categories: I (≤ 39 years), II (40–59 years), III (60–79 years), and IV (≥ 80 years). One-way analysis of variance (ANOVA) was used to examine the significance of the differences in osteocyte lacunar density between each of the age groups. For post-hoc multiple comparison procedures, we used the Bonferroni correction, which set the level of significance at 0.05 per number of comparisons. Intersex differences in osteocyte lacunar density were assessed using the *t*-test. The same test was used to compare the osteocyte lacunar density between the periosteal and endosteal regions in all of the fields and age groups. To analyze the mineralized osteocyte lacunae per bone area, a *t*-test was applied. All analyses were carried out using SPSS statistical software (version 16.0), and the results were considered statistically significant at the 0.05 level.

Acknowledgments

This study was supported by grants from the German Academic Exchange Service (DAAD) and the South-Eastern-European-Cooperation, University Medical Center Hamburg-Eppendorf under the direction of Prof. Dr. H.J. Seitz. Dr. Björn Busse is a fellow of the 'DFG – Deutsche Forschungsgemeinschaft'.

Author contributions

BB, MH, and MA designed and planned the experiments. BB, DD, PM, and MH carried out the 2D histomorphometry, energy dispersive X-ray analysis and bone mineral density distribution analysis. KP carried out full autopsies. BB, DD, and PM wrote the paper and did the statistical analysis. BB, DD, PM, MH, MD, ROR, and MA analyzed and reviewed the data as well as the conclusions.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 (A) Removal and X-ray of femoral samples. (B) Compartmentation in periosteal and endocortical measuring fields. (C) 2D histomorphometry. (D) Bone Mineral Density Distribution Analyses.

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