REVIEW

Phenomenon of osteocyte lacunar mineralization: indicator of former osteocyte death and a novel marker of impaired bone quality?

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Abstract

An increasing number of patients worldwide suffer from bone fractures that occur after low intensity trauma. Such fragility fractures are usually associated with advanced age and osteoporosis but also with long-term immobilization, corticosteroid therapy, diabetes mellitus, and other endocrine disorders. It is important to understand the skeletal origins of increased bone fragility in these conditions for preventive and therapeutic strategies to combat one of the most common health problems of the aged population. This review summarizes current knowledge pertaining to the phenomenon of micropetrosis (osteocyte lacunar mineralization). As an indicator of former osteocyte death, micropetrosis is more common in aged bone and osteoporotic bone. Considering that the number of mineralized osteocyte lacunae per bone area can distinguish healthy, untreated osteoporotic and bisphosphonate-treated osteoporotic patients, it could be regarded as a novel structural marker of impaired bone quality. Further research is needed to clarify the mechanism of lacunar mineralization and to explore whether it could be an additional target for preventing or treating bone fragility related to aging and various endocrine diseases.

Introduction

From a medical materials science viewpoint, bone is a remarkably hierarchically organized nanostructured material with a peculiar organization of mineral and organic phases (1, 2, 3). Bone is subject to various mechanical demands and must withstand loads, while bone also shields organs and serves as a means for mineral homeostasis (4, 5). Bone is also a living and dynamic tissue, and the bone matrix houses numerous living bone cells (6, 7). Therefore, in contrast to most materials, human bone has a remarkable ability to adapt its volume and structure to the local (8, 9) and global needs of the organism (10). Moreover, human bone has the ability to repair damage that was induced during repetitive loading by renewing packets of bone tissue (11). Osteocytes, the most numerous bone cells that are strategically distributed through entire bone

matrix (7) exhibit extraordinary behavior in bone renewal processes.

During aging and in some diseases, bone loses its ability to successfully withstand mechanical loads (12), exhibiting diminished ability to successfully adapt to sudden and repetitive mechanical situations (demands) (13, 14). Its capacity for damage repair also reduces, and targeted bone renewal drastically declines (15, 16). The latter might contribute to increased bone fragility and, subsequently, fracture risk increases underpinning major problems in the elderly population (17). In the year 2000 alone, 9 million osteoporotic fractures occurred worldwide, and epidemiological trends suggest further increases in bone fracture burden in aging populations (18, 19). However, a deeper understanding of the origins and mechanisms of increased bone fragility is essential for

the development of preventive and therapeutic strategies to combat one of the most common health problems in aged individuals worldwide.

Role of osteocytes in bone adaptation

Currently, many bone properties are considered to be controlled or mediated by osteocytes (20). Osteocytes are primarily considered sensors of mechanical stress, which is generally based on: (i) their strategic distribution throughout the bone matrix, a network that provides intercellular communication, and (ii) their ability to respond to mechanical stimuli *in vitro* by producing biochemical signals that are capable of affecting other bone cells (21, 22, 23, 24). Although osteoblasts can sense and respond to loads, the osteocyte network is a necessary 'antenna' to detect signals and bone mechanosensory potential (25). In addition, *in vitro* studies have demonstrated that osteocytes are the most mechanoresponsive bone cells, particularly to fluid flow (21, 26). There is growing data on mechanotransduction pathways (for a review see: (27)), for example, on how bone (osteocytes) detects mechanical signals and converts these signals into biochemical signals that can affect neighboring cells. The relationship between osteocytes and other bone cells is intriguing, given that the same osteocytes can exert both positive and negative regulation on osteoblasts and osteoclasts (28). However, since an osteocyte receives complex information (not only mechanical but also biochemical via various cytokines and signaling molecules), the sum of the stimuli determines whether osteocytes stimulate or inhibit bone formation or resorption (28). In the case of loading, increased fluid flow stimulates osteocytes to produce biochemical signals that inhibit osteoclast formation and resorption (29, 30) and promote osteoblast proliferation and differentiation (26), with a net bone-forming effect. Nitric oxide (NO) is one of the biochemical markers of the osteocyte response to mechanical loading (22, 24, 31) that is known to induce bone formation (32, 33) and promote osteocyte survival (34). In cases of insufficient loading, the lack of NO production leads to osteocyte apoptosis, and bone resorption is initiated, which adapts the bone structure to low-load conditions.

Osteocyte death

The lifetime of osteocytes is variable, but unlike other bone cells, osteocytes are capable of exceptional longevity;

if a bone region remains unaffected by remodeling long enough, osteocytes can even live for several decades (35). Since the initial report by Frost (36), numerous studies visualized or quantified osteocyte death in various conditions (37, 38, 39, 40, 41, 42, 43, 44). Osteocyte death is, in general, dependent on patient age and tissue age, but premature osteocyte death also occurs due to hormonal reasons, such as estrogen deficiency or corticosteroid excess (38, 39, 45). Moreover, osteocyte death may be caused by mechanical factors (36, 46), but the relationship between mechanical loading and osteocyte survival is actually biphasic (47). Namely, for survival, osteocytes need constant 'stimulation', for example, a certain level of strain and/or fluid flow is necessary. For example, Noble *et al.* (47) reported that physiological strain decreased the number of apoptotic cells and even increased the number of young osteocytes. It has been shown that load-induced fluid flow inhibits osteocyte apoptosis via NO production (34). Strain and fluid flow promote osteocyte viability possibly by facilitating antiapoptotic pathways in osteocytes (34, 47), and fluid flow enhances molecular transport and nutrient supply to keep osteocytes healthy (48). In addition to insufficient strain, such as during weightlessness (44) or long-term immobilization (49), too much strain is also dangerous and osteocytes can be considered to 'overdose'. Specifically, excessive strain was found to increase the number of apoptotic osteocytes (47). It is not exactly clear why osteocytes die under high strain conditions; however, Noble *et al.* (47) suggested that either pathologically high strain launches apoptosis machinery within osteocytes or matrix damage is responsible for cell death.

Mineralization of osteocyte lacunae

Based on basic fuchsin-stained bone samples observed under bright field microscopy, Frost noticed unstained lacunae and canaliculi and suggested that they had been filled with mineral, for which he coined the term 'micropetrosis' (46).

Boyde and colleagues used backscattered electron microscopy and observed mineralized lacunae in human mandibles, especially in aged individuals, but also in calvarial bones, long bones near metal implants, and grafted bone (50, 51, 52). They showed that such lacunae can express various degrees of mineralization and may occupy extensive bone areas with higher mineralization levels than the neighboring osteons (51). An ultrastructural study that applied scanning and transmission electron

microscopy of mineralized lacunae showed apoptotic remnants of osteocytes within the mineral matter (53) and labeled such lacunae as 'living fossils' that preserve the fragments of a previous cell (53).

The first quantitative analysis of mineralized lacunae (Fig. 1) in cortical bone from human femoral diaphysis in young (20–40 years) and aged (>80 years) individuals of both sexes was based on backscattered electron microscopy and showed a marked age-related increase in mineralized lacunae per bone area (54) along with a notable reduction in normal appearing osteocyte lacunae (54). While these findings confirmed that with aging many osteocytes die, we suggest that if the bone areas containing dead osteocytes are not remodeled, such lacunae would become occluded with a high mineral content, as verified via energy dispersive X ray spectroscopy (54). The endocortical compartment tended to have a higher number of mineralized lacunae per bone area than the pericortical compartment in aged individuals (1, 54). Considering the concomitant lower overall mineralization of the pericortical compartment (1), such findings may be explained by the increased apposition of fresh bone to the periosteal surface during a person's lifetime (55, 56).

Our backscattered electron microscopy data on femoral cortical bone in young females, healthy aged females, females with untreated osteoporosis, and females with bisphosphonate-treated (BP) osteoporosis showed that in addition to the observed age-related increase in the number of mineralized lacunae per bone area, lacunar occlusion further increased due to osteoporosis (1).

Namely, aged females with untreated osteoporosis displayed nearly twice as many mineralized lacunae per bone area as age-matched healthy women (1). However, the osteoporosis patients who underwent long-term bisphosphonate treatment exhibited a reduced number of lacunar occlusions, which was comparable to the prevalence in healthy aged individuals (1). Obviously, there is a consistent positive influence of bisphosphonates on osteocyte viability, reflected by a reduced number of mineralized lacunae, compared with individuals with untreated osteoporosis (1). This finding is compatible with recent cell culture and animal studies revealing osteocyte antiapoptotic effects of bisphosphonates (BPs) (57, 58).

It is not yet clear whether there is any difference in terms of mineralization starting point, for example, whether mineral occlusion starts in lacunae or in canaliculi. Indeed, canaliculi can be mineralized even before the lacunae are occluded with mineral substance (51), which is an important sign of aged bone (6). Namely, our study on acid-etched resin embedded mid-cortical bone from the proximal femoral diaphysis showed a significant reduction in the number of canaliculi per osteocyte lacunae in aged individuals compared with young individuals (6). As mid-cortical bone is difficult to access during remodeling due to its large distance from bone surfaces (59, 60), it bears signs of aged tissue, especially in aged individuals, evidenced by a higher calcium to phosphorus ratio according to EDX and increased calcium concentration according to quantitative backscattered electron imaging (6). Consequently, the decrease in the number of canaliculi leads to a prominent
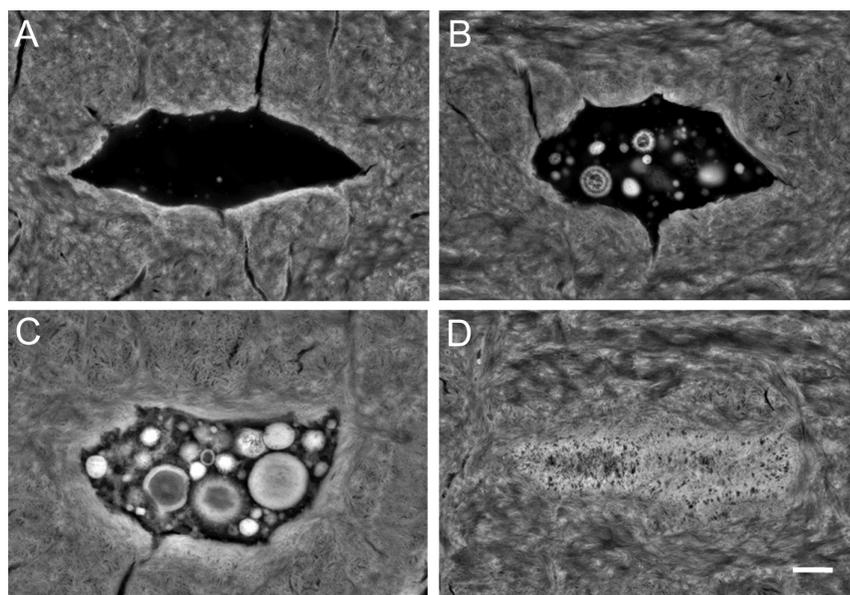

Figure 1
The process of lacunar mineralization (micropetrosis) passes through different stages from normal appearing osteocyte lacuna (A) via partly filled lacuna with clearly identifiable calcified nanospherites (B and C) and finally complete occlusion of the lacuna with mineral (D). (Backscattered electron microscopy images, Scale bar 1 μm).

reduction in connections between osteocyte lacunae within mid-cortical osteons (6). Moreover, we have observed that, in young bone, canaliculi frequently cross the cement lines and often have connections with interstitial bone osteocyte lacunae; we believe that these connections may be essential for the viability of interstitial osteocytes by providing them with nutrients through canalicular fluid flow and by providing appropriate mechanical stimulation for their survival (6). Thus, interstitial osteocytes can still be part of a dynamic and mechanosensitive lacunocanalicular system (6). However, in aged individuals, we found a dramatic reduction in canalicular connections between osteonal and interstitial lacunae, which could explain why interstitial regions are particularly rich in dead osteocytes and represent a preferential location for the development of mineralized lacunae (1, 54). Indeed, one of the reasons for the lower number of canaliculi is their mineralization, while there is a possibility that even new osteocytes initially form fewer dendrites in aged bone. In future studies, it would be interesting to compare the osteocytes in the bone packets of similar tissue age in young and aged individuals to clarify whether individual's age or tissue age has a decisive role in loss of dendrites. The results from a recent animal study on osteocytes isolated from young and aged mice showed lower number of dendrites in aged osteocytes (61), while study using confocal microscopy reported a reduced dendritic number in aged mice (62).

Despite the observations that mineralized nanospherites (Fig. 1) appear at both lacunae and canaliculi (63), there is some evidence that canaliculi are first to be occluded by minerals. It is unknown whether this is always a part of the same phenomenon of lacunar mineralization, although canalicular reduction is associated with tissue age-related increases in calcium content in the surrounding bone (6). Likewise, the number of mineralized osteocyte lacunae per osteon bone area is positively correlated with both osteonal calcium content and cement line calcium content, two indicators of tissue age (64). Our acid-etching study showed three states of the peripheral canaliculi in mid-cortical osteons: preserved canaliculi that fully cross the cement line to establish an external 'routing' of osteons; canaliculi interrupted at the cement line; and a complete lack of visible canaliculi on the peripheral side of the outermost osteonal lacunae, as the mineral that had filled them was removed by acid while the lacuna remained patent as it was filled with polymethylmethacrylate (6). Canalicular mineralization certainly indicates a loss of dendritic processes,

while dendrite numbers were shown to be inversely related to age (61, 62).

Mineralized osteocyte lacunae as indicators of impaired bone quality

Our quantitative data on osteocyte lacunae revealed that the amount of mineralized osteocyte lacunae is associated with differential bone quality, as bone with different mechanical properties (young, aged, untreated osteoporosis, BP-treated osteoporosis) can be distinguished based on the number of mineralized lacunae per bone area (1). Namely, mineralized lacunae are related to the age of the individual, and the occurrence and density of mineralized lacunae are significantly higher in untreated osteoporotic patients than in healthy aged individuals and BP-treated osteoporotic patients (1).

We recently demonstrated a decreased osteocyte lacunar number per bone area of the femoral neck's periosteal cortex in aged females who sustained hip fracture compared with aged-matched healthy controls (65). However, while there is a general agreement regarding osteocyte depletion with aging (66, 67, 68, 69, 70, 71, 72, 73, 74), data in the literature on osteocyte lacunar density in iliac, vertebral, and femoral trabecular bone compartments of osteoporotic patients are inconsistent and sometimes contradictory (75, 76, 77, 78, 79). Our study on femoral diaphysis did not show a significant difference in lacunar density between three different groups of elderly women (healthy aged, untreated osteoporosis, BP-treated osteoporosis), while young cases presented with higher osteocyte lacunar density, although without reaching significance level (1). Therefore, taken together, our findings suggest that differences in mineralized lacunae are better than osteocyte lacunar density alone in discriminating among different groups of individuals.

Currently, the main mechanisms by which mineralized lacunae may promote bone fragility can be considered as follows (Fig. 2):

– Mineralized lacunae are 'plugs' in the osteocyte lacunocanalicular network that cause localized dysfunction of the network (63) in terms of blocked fluid flow:

Certainly, this would block nutrient supply to other osteocytes, especially interstitial osteocytes, favoring more cell death. As fluid flow is also essential for mechanosensitivity (48, 80, 81), it is apparent that obstacles to fluid flow compromise the ability of bone
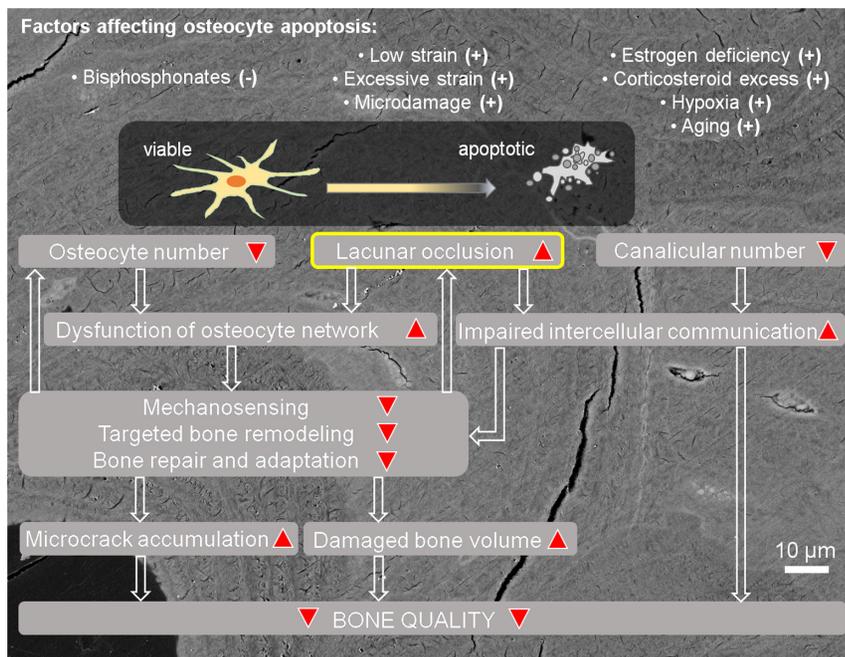
Figure 2
Schematic representation of the effects of accelerated osteocyte death and micropetrosis on bone quality.

to sense and appropriately respond to mechanical signals. Localized dysfunction of the osteocyte network may cause local mechanical imbalances, making these areas particularly vulnerable to mechanical loading.

– A lack of viable osteocytes hampers bone mechanosensitivity and delays or reduces the repair of microdamage:

Osteocytes are crucial regulators of bone remodeling. In particular, osteocytes are the main source of signals needed for targeted repair of damaged areas. Here, osteocyte death may result in a loss of antiresorptive signals or the secretion of proresorptive signals (11, 82). However, it is likely that a certain density of osteocytes is needed to detect damage and launch remodeling processes (16); in this sense, extensive loss of viable osteocytes in a certain area would leave significant amounts of bone tissue with unprocessed damage which further accumulates and hampers bone quality.

Considering the accumulation of such mineral-rich spots and delayed/hampered remodeling that leads to increased calcium content (increased tissue age), these particular tissue areas increase their brittleness and are more prone to microcracking (54, 83). More advanced tissue age is associated with increased size of mineral crystals (13, 65, 84, 85, 86, 87), mostly via crystals' coalescence, which decreases interfibrillar sliding and thereby reduces toughness at the level of mineralized collagen fibrils (65, 88, 89, 90, 91, 92). Older tissue presents altered collagen characteristics as well,

which may be another important hallmark of increased bone fragility (83, 90, 93, 94). Although detrimental in skeletal sites subject to load bearing, excessive and early micropetrosis occurring in the auditory ossicles (sites with low mechanical loads) could be an adaptive response conserving the architecture of the auditory ossicles and ensuring stable sound transmission from early childhood throughout life (95).

Mechanisms of lacunar mineralization

There is a general agreement that lacunar mineralization is associated with osteocyte apoptosis (52, 54, 77). However, understanding the precise mechanism of lacunar mineralization remains of particular importance to the bone research community. Lacunar occlusion can be regarded as a passive phenomenon, an active mineralization phenomenon, or a combination of the two.

The theory behind the *passive* (*spontaneous*) *mineralization mechanism* is based on the current belief that osteocytes are capable of preserving an unmineralized pericellular space by inhibiting mineralization (96, 97) to allow fluid flow-based mechanosensitivity and nutrient transfer (98). For this goal, osteocytes produce crystallization and mineralization inhibitors (SIBLING proteins such as osteopontin and MEPE, fetuin-A or tethering elements' component - perlecan) as well as enzymes for active digestion of their direct surroundings

(such as matrix metalloproteinases) (96, 99, 100, 101) to prevent spontaneous calcium and phosphorus precipitation around the cell (102, 103) and are even able to dissolve bone minerals, as observed in lactation (10). Additional molecules involved in the process may be locally produced FGF23 and osteopontin, as animal studies suggested that increased local production of FGF23 by osteocytes reduces activity of alkaline phosphatase, leading to increased levels of pyrophosphate, a known mineralization inhibitor (104). However, following osteocyte death, the lack of crystallization inhibitors allows spontaneous mineralization of the lacuna and canaliculi, given the normally available calcium in the extracellular space. Our rigorous characterization of mineralized lacunae revealed that its mineral composition significantly differs from that of normal bone matrix (63). The higher mineral-to-matrix ratio and the markedly reduced collagen content suggest that there is no organic deposition in the lacuna that is being mineralized, emphasizing how lacunar mineralization processes differ from the process of normal bone matrix formation, suggesting the existence of a passive mineralization process (63).

However, we believe that calcification is not just a passive phenomenon but rather an active biological solution that is employed when dying cells cannot be taken up by phagocytosis, as in the case of mineralized tissue. It should also be noted that some other initially noncalcified tissues (such as vascular smooth muscle, valves, and cartilage (105, 106, 107)) may show mineralization processes that are analogous to lacunar mineralization, suggesting that mineral deposition in osteocyte lacunae is just one example of a more widely ranging type of biological mineralization.

Namely, our recent study revealed that mineralized lacunae consist of a various number (up to more than a hundred) of mineralized spherical entities that we labeled *nanospherites*, based on their average diameter of 655±448 nm (63). Considering the shape and size of the nanospherites, as well as the fact that apoptotic osteocyte material rarely disappears from the lacuna, the nanospherites may originate from apoptotic bodies; however, they can also originate from matrix vesicles released from still living osteocytes (63). The cellular origin of the nanospherites is also corroborated by the higher magnesium content in nanospherites than in bone matrix (63, 108), as magnesium is an important intracellular ion (109). Therefore, the question regarding the active components of lacunar mineralization remains unanswered. Previous studies of cartilage and vascular smooth muscle have suggested that both matrix vesicles and apoptotic bodies have the specific ability to actively concentrate calcium (106, 107, 110, 111) owing to the presence of several calcium-binding molecules, such as phosphatidyl-serine at the surface of the membrane (106, 112). In this context, osteocytes may be initiators of lacunar mineralization, which is complemented also by spontaneous precipitation of calcium between the nanospherites. Finally, our data suggest that mineral nanospherites grow by adding new minerals and eventually fuse with each other, thus leading to complete mineral occlusion of the lacuna (63).

Hormones and osteocyte viability

Postmenopausal osteoporosis is associated with estrogen deficiency. Our previous studies in human bone showed

Table 1 Factors affecting osteocyte death and viability.

Factors affecting osteocyte death and viability	References
Factors favoring osteocyte death	
Aging	1, 16, 54
Low mechanical strain (e.g. immobilization or weightlessness)	44, 47, 49
Excessive mechanical strain, microcracking	16, 47
Estrogen deficiency (e.g. postmenopause or treatment with GnRH)	38, 39
Glucocorticoid excess (endogenous or exogenous)	42, 45, 113
Vitamin D deficiency	83, 118
Vitamin D receptor deficiency	118
Factors promoting osteocytes viability	
Normal mechanical loading (load-induced fluid flow in lacunocanalicular network)	34, 47
Intermittent administration of PTH(1–34)	115, 116, 117
Bisphosphonates treatment	1, 57, 58
Nitric oxide (NO)	34

higher number of mineralized lacunae in postmenopausal compared to premenopausal women (1, 54). While aging itself likely also plays a role, estrogen deficiency is certainly an important contributor to increased osteocyte apoptosis. Experimental data supporting the role of estrogen in osteocyte viability are available from studies on human bone (e.g. estrogen withdrawal due to gonadotropin-releasing hormone administration was linked to increased osteocyte apoptosis in iliac crest biopsies (38)) as well as from animal studies (ovariectomy increases osteocyte apoptosis in rats, which can be rescued by administration of estrogen (39)).

Apart from postmenopausal estrogen deficiency, there is no direct data on the amounts of mineralized lacunae in conditions with a changed hormonal status. Nevertheless, a number of observational or experimental studies showed the effects of hormones on osteocyte apoptosis and viability (Table 1). Numerous articles reported that glucocorticoid excess increases osteocyte apoptosis both in human bone and animal models (42, 45, 113). However, proapoptotic effects are not the only negative impact that glucocorticoids have on osteocytes, since there is experimental data showing that glucocorticoids suppress osteocytic expression of matrix metalloproteinases and consequently impair perilacunar remodeling with degeneration of the lacunocanalicular network and matrix hypermineralization (114). Unlike glucocorticoids, PTH(1–34) is generally linked with prosurvival signals for osteocytes (115), and it was shown that intermittent administration of PTH can prevent osteocyte apoptosis caused by glucocorticoid excess (116) or radiotherapy (117). Vitamin D was also found to affect the viability and characteristics of osteocytes. Namely, vitamin D deficiency was accompanied by reduced osteocyte numbers, increased osteocyte apoptosis, and decreased osteocytic connectivity; while vitamin D receptor deficiency also reduced viable osteocyte numbers (118).

Conclusion

Mineral nanospherites should be investigated more closely to determine their impact on mineral homeostasis. In particular, experimental studies would be beneficial for further clarification of the process of lacunar mineralization, including the conditions of endogenous or exogenous hormonal excess or deficiencies. A better understanding of the lacunar mineralization phenomenon may potentially be explored as another target for preventing or treating bone fragility.

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