Osteocytic Canalicular Networks: Morphological Implications for Altered Mechanosensitivity

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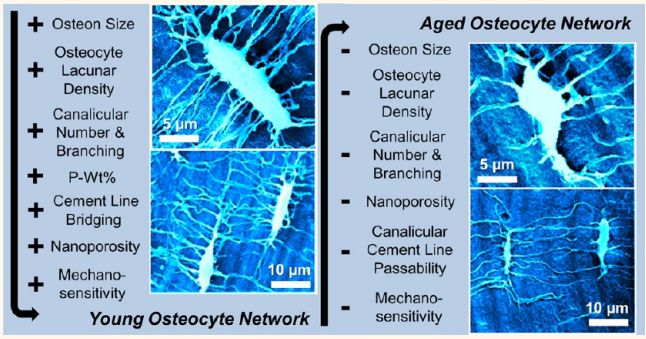
ABSTRACT Osteocytes are ramified bone cells distributed throughout the bone matrix within a network of micrometer-scale cavities (lacunae) and numerous nanometer-thick tunnels (canaliculi). The integrity of the canalicular network might influence bone quality and reflect its mechanosensory potential. In this study, we applied an acid etching technique to embedded bone specimens that allows 3D observation of the canalicular network across a 2D plane to quantitatively assess the canalicular connections in cortical bone specimens from young and aged individuals. Our results showed a nearly 30% reduction in the number of canaliculi per osteocyte lacuna in aged individuals (N.Ot.Ca/Ot.Lc: 15.92 ± 1.5 in aged *vs* 22.10 ± 2.82 in young; $p < 0.001$); moreover, canalicular number was found to be inversely related to the osteonal tissue age represented by Ca/P ratio ($p < 0.001$). We frequently observed the phenomenon that canaliculi of osteocytes located near the osteon's periphery did not end at the osteon's cement line boundary but penetrated through the cement line and spread into the surrounding bone matrix, thus establishing an "external rooting" or "connection", which might have significant relevance to bone quality. Our findings showed that not only does the aging process diminish the canalicular network within osteons, but it also significantly reduces the probability of external osteonal rooting and connections with the surrounding bone tissue. Deterioration in the canalicular network with age reduces the connectivity between osteocytes and between osteons/interstitial tissue, which affects the supply of nutrients to osteocytes, degrades their mechanosensitivity, and contributes to increased bone fragility in the elderly.



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Human cortical bone primarily formed from the basic building blocks of collagen molecules and mineral crystals[1] has a complex hierarchical structure, with distinct features existing from the nano- to millimeter length scales.[2] This hierarchical assembly provides bone with its unique mechanical properties; however, bone is not a static structure, and dynamic elements throughout the bone matrix change/regulate its characteristics and behavior. In particular, bone cells called osteocytes reside in a network of micrometer-sized pores (lacunae) connected *via* nanometer-sized channels (canaliculi); this nanonetwork is thought to play a key role in regulating and maintaining the bone's multilevel structure.[3]

The bone cells are integrated into the matrix during the continuous process of *bone remodeling*, where bone resorption and bone formation occur in a well-balanced manner to maintain the characteristic morphology of the bone material. In a cycle of bone remodeling, damaged or old bone is removed by bone resorbing cells (osteoclasts) and a new osteon (see Figure 1a) is produced by bone-forming cells (osteoblasts) through the secretion of organic matrix that is further on subject to mineral deposition, finally leading to mature mineralized bone.[4] During the course of bone formation, osteoblasts become entrapped in the newly synthesized matrix and turn into osteocytes.[5] As the largest cellular population in bone tissue,

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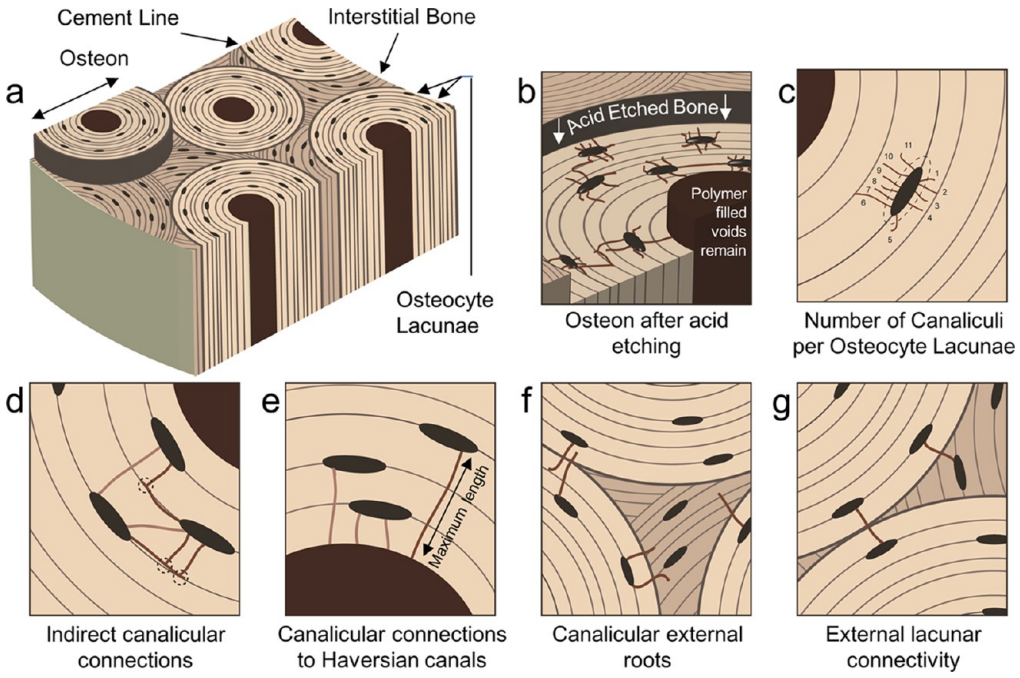

Figure 1. (a) The structure of human cortical bone is continuously being remodeled and dominantly consists of osteons, which are the basic structural units. The osteons are separated from older interstitial tissue by a hypermineralized layer called the cement line. Each osteon contains a central vascular cavity called the Haversian canal, which connects to the bone cells (called osteocytes) residing in the osteocyte lacunae *via* nanosized canaliculi. (b) Here, the lacunae and canaliculi network was morphometrically assessed through an acid etching technique, where the voids (*i.e.*, the canaliculi, lacunae, and Haversian canals) in the sample are infiltrated with polymer and then an acid solution selectively removes the bone matrix, leaving the network intact in a 3D fashion. In this analysis, we define several new variables to assess the lacuno-canalicular network's morphology. (c) The number of canaliculi per osteocyte lacuna was quantified by drawing a circle around the osteocyte lacuna and counting the number of intersections with canaliculi. (d) The proportion of osteons showing indirect (dark shade) canalicular connections to other osteocytes, which occur at junctions (enclosed by circles in the inset), was measured. (e) As the canaliculi often connect to the Haversian canal, we measured the longest connection between an osteocyte and the Haversian canal per osteon. (f) To quantify the osteon's roots to the external bone matrix, the percentage of outermost lacunae with external roots per osteon and the percentage of osteons displaying external roots were measured. (g) Additionally, canaliculi crossing the cement line were observed to connect with osteocytes in the interstitial bone or in other osteons. Here, we measured the percentage of outermost lacunae with connections across the cement line per osteon and the percentage of osteons with these connections.

osteocytes are systematically distributed throughout the bone matrix and reside in special cavities (so-called lacunae). Nanometer-sized dendritic cell processes spreading from the osteocytes' cellular bodies interconnect the osteocytes through tunnel-shaped canaliculi; this combination of cells, lacunae, and canaliculi establishes the osteocytic cellular network.[6] Such lacuno-canalicular networks extend from Haversian canals (*i.e.*, the vasculature in the bone matrix; see Figure 1a) to bone surfaces and represent a basis not only for the osteocytes' nutrition but also for their mechanosensory function.[6] Namely, fluid flow through the dynamic space between the osteocytes' soft cellular membrane and hard lacuno-canalicular walls provides a means for molecular transport from the Haversian canals to all osteocytes throughout the bone matrix.[7−9] Moreover, membrane shear stress originating from the fluid flow during mechanical loading is believed to initiate complex biochemical signaling pathways within osteocytes, reflecting their mechanosensing capabilities.[8,10−12] Osteocytes also play a key role in remodeling by detecting microdamage that occurs during habitual loading,[13] launching appropriate repair processes,[14−17] as well as molecular synthesis and modification of their surrounding matrix and structure.[14,18,19]

Age- and disease-related changes in bone quality are many times the result of imbalances in remodeling. Because the lacuno-canalicular network transports information/nutrients and signals remodeling, maintaining a proper morphology of the lacuno-canalicular network is necessary to ensure "healthy" bone quality. Indeed, studies focusing on common sites of age-associated bone fractures (such as in human femurs) revealed an evident decrease in osteocyte lacunar numbers in cortical bone from aged individuals, suggesting an impairment in bone repair abilities.[14,17] However, beyond a simple reduction in osteocyte lacunar density, the function/integrity of the present osteocytic dendrites/canaliculi additionally needs to be considered to understand the network requirements for adequate regulation of the bone remodeling process.

Imaging the lacuno-canalicular network poses a challenge due to the very small diameter of the

canaliculi (~140 nm);[20] hence, the quantification of these entities represents a serious challenge.[6] Regular high-resolution 2D/3D techniques have so far not successfully provided quantitative data; the 3D nature of the canaliculi (*i.e.*, noncoplanar spreading) cannot be observed in classical 2D electron microscopy, while high-resolution tomography requires very small specimen sizes and does not depict soft tissue.

Here, we employed an acid etching technique that allows 3D observation of the nanoscale canaliculi across a 2D plane. With this technique, we quantitatively assessed the canalicular connections in samples from young and aged individuals to provide further insight into the role of osteocytes in the age-specific pattern of bone remodeling and differential bone fragility. As quantitative data on the aging effects in canalicular networks are hitherto not available, the aim of this study was to unravel the number and distribution of canalicular connections on the nanometer level in individuals of various ages to determine if age-related differences in canalicular connections might contribute to bone quality.

RESULTS AND DISCUSSION

Acid etching of embedded bone specimens allowed rigorous observation of the bone canalicular network in cortical bone. Through this unique negative visualization of femoral osteons, we were able to perform a comprehensive quantitative assessment of the canalicular network.

Canalicular Connections within Individual Osteons (Internal Canalicular Connections). Our results showed a nearly 30% reduction in the number of canaliculi per osteocyte lacuna in aged cases with respect to a plane perpendicular to the osteonal long axis (N.Ot.Ca/Ot.Lc [#]: 15.92 ± 1.5 in aged cases *vs* 22.10 ± 2.82 in young cases; $p < 0.001$) (Figure 2a,b). Further, energy dispersive X-ray spectroscopy (EDX) microanalysis applied at specific osteons provided estimates of the tissue age as reflected in the calcium-to-phosphorus ratio (Ca/P ratio). By correlating the Ca/P ratio with the canalicular number within the same osteon, it was ascertainable that the number of canaliculi per osteocyte lacuna was inversely related to the osteonal tissue age (linear regression analysis: $R = 0.59$; $p < 0.001$) (Figure 2c–e). Representative quantitative backscattered electron imaging (qBEI) of the bone mineral density distribution on selected osteons proved the validity of the age estimation *via* the EDX measurements, as young osteons revealed lower Ca/P ratios and lower calcium weight percentages (Figure 3a,b), whereas aged osteons reflected higher Ca/P ratios and higher calcium weight percentages (Figure 3c,d)

Moreover, it was observed that osteocyte lacunae from two or more neighboring lamellae within the same osteon communicated not only through direct connections between their radially oriented canaliculi but also through indirect connections (*i.e.*, connections between the radially and transversally oriented canaliculi) (Figure 4a,b). Those indirect connections occurred in 98.41% of osteons in the young cases and only in 73.39% of osteons in the aged cases ($p = 0.013$). Apart from the connections with other intraosteonal lacunae, the lacunae were also connected with the Haversian canal (Figure 4c,d). Connections to the vasculature were observed not only for the innermost lacunae adjacent to the Haversian canal but also for the lacunae located at the outer peripheral lamellae (Figure 4d). The mean maximum length of the canalicular connections between the Haversian canal and the most peripheral located lacunae in the assessed osteons was significantly lower in aged cases when compared to the young cases (57.05 μm *vs* 78.55 μm, $p < 0.001$).

Canalicular Connections between an Osteon and the Surrounding Bone Matrix (External Connections). We frequently observed the phenomenon that canaliculi of osteocytes located near the osteon's periphery (outermost lacunae) did not end at the osteon's cement line boundary but penetrated through the cement line spreading into the surrounding bone matrix to make an "external connection" (Figure 5a). More precisely, the assessed canaliculi were connected with the peripheral lacunae of an adjacent osteon and/or with individual lacunae located in the interstitial lamellae (Figure 5a). In this context, age-specific comparisons revealed that osteons of young cases had a higher number of external connections with the surrounding bone tissue than aged individuals (Figure 5a,b) (Table 1). Considering the tissue age of osteons determined through Ca/P ratios, our study revealed that younger osteonal tissue age is related to a frequent connectivity of their outermost lacunae with the surrounding interstitial bone or the neighboring osteons (Fisher's exact probability test: $p = 0.007$).

This study provides in-depth insights into the nanosized osteocytic canalicular networks and their age-specific changes. Osteocytes are known to play a large role in maintaining and regulating the integrity of the bone tissue; thus, the diminished canalicular network and connections in aged bone highlight a possible origin for fracture susceptibility (*i.e.*, low bone quality) due to a decreased capability for mechanosensing, nutrition, and oxygen supply.

Reduction in the Canalicular Network in Advanced Age. In aged individuals, our findings showed a significant decrease in canalicular connections within individual osteons as well as a reduced connectivity between an osteon and its neighboring osteon and/or interstitial bone. Indeed, previous experiments in murine bone have shown that the molecular transport rate is directly proportional to the number of canaliculi.[21] Therefore, the reduced branching of the canalicular network observed here within osteons indicates a hampered nutrition and oxygen supply to osteocytes.

Additionally, altered canaliculi are associated with changed nutrition that may lead to increased proportions

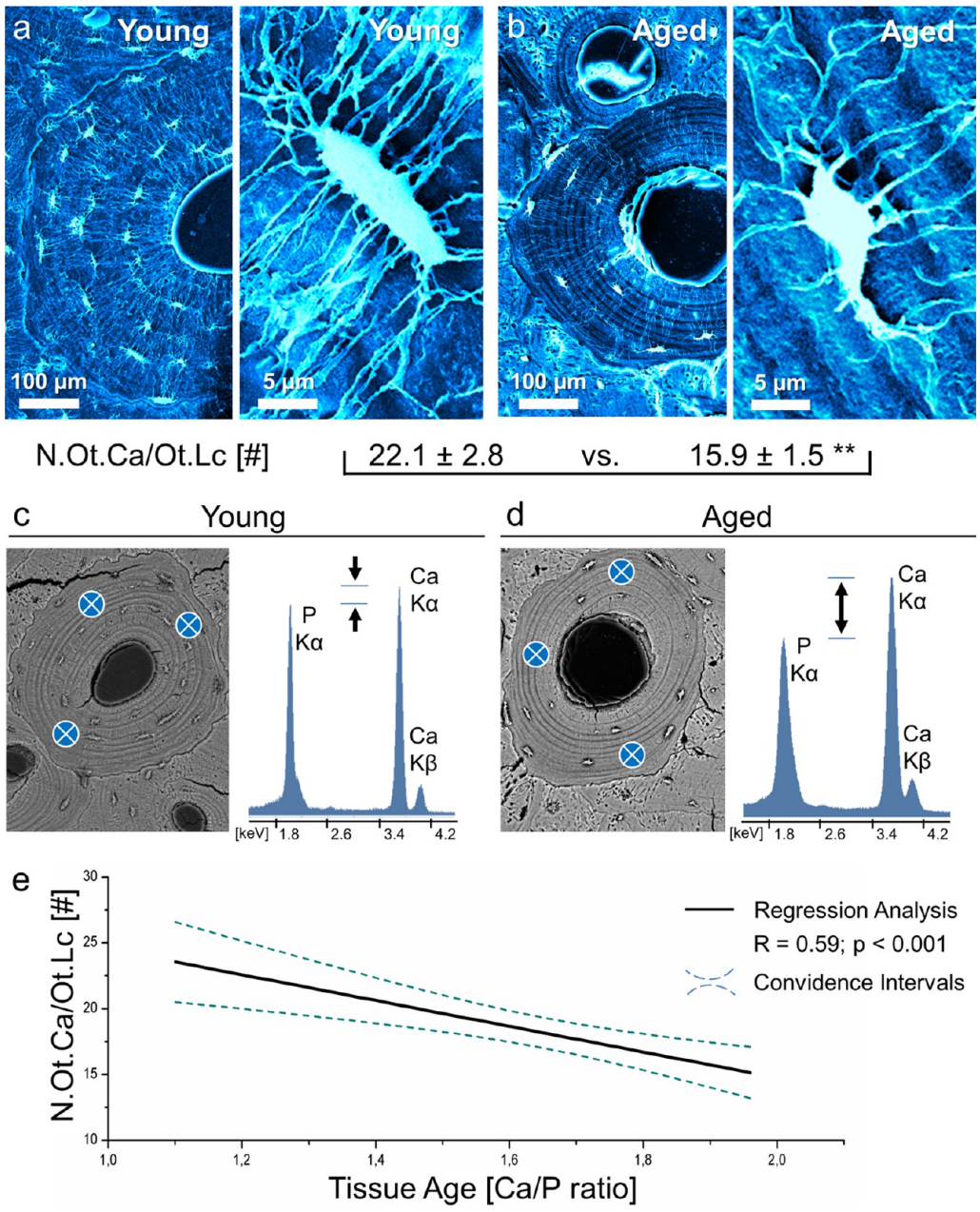

Figure 2. For (a) young and (b) aged individuals, a scanning electron micrograph of resin-embedded acid-etched osteonal bone (left) is shown with a magnified view of a single osteocyte lacuna (right). Note that the young osteon is larger, with more osteocyte lacunae that are interconnected extensively *via* numerous canaliculi. The magnified images show that more canaliculi radiate from the osteocyte lacuna in the young case (a, right) than the aged case (b, right). For (c) young and (d) aged cases, the backscattered scanning electron images (left) indicate the locations (blue circles) where the elemental content was measured using EDX; the right inset shows the EDX calcium and phosphorus contents in (c) young and (d) aged individuals, where higher Ca/P ratios indicate a higher tissue age. (e) The number of canaliculi per osteocyte lacuna (N.Ot.Ca/Ot.Lc) is inversely related to tissue age (linear regression: $R = 0.59$, $p < 0.001$).

of dead osteocytes in aged bone, as evidenced by the accumulation of hypermineralized lacunae with age mainly in interstitial bone.[14] Indeed, young cases were equipped with widespread canalicular connections that provide extensive cellular communication across cement lines and adequate transduction of mechanical stimuli to osteonal and interstitial osteocytes, which represents a prerequisite for maintaining bone quality and fracture resistance.

Higher numbers of canaliculi around a lacuna have also been computationally associated with improving osteocyte mechanosensitivity by increasing perilacunar strains;[22] furthermore, microcracks may induce remodeling by severing canaliculi like a pair of scissors and prohibiting communication between osteocytes.[13,15,23] Indeed, the osteocytic mechanosensing and/or mechanotransduction function was thought to be affected by reduced mechanical stress resulting in age-related osteoporosis.[24]

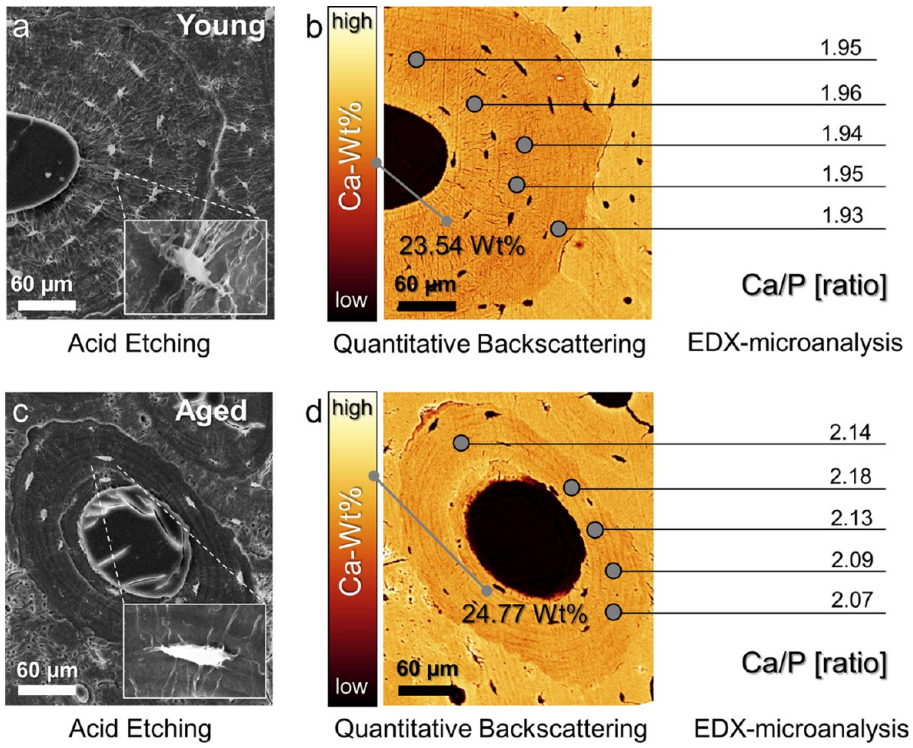

Figure 3. Representative images of acid-etched osteons with the corresponding quantitative backscattered electron imaging (qBEI) and EDX data for young (a, b) and aged (c, d) osteons. The acid-etched specimens were carefully repolished and then carbon-coated to allow qBEI measurements, confirming the validity of intergroup comparisons using EDX. The calcium weight percentages obtained from the backscatter signal intensities correlate positively with Ca/P ratios by showing lower values in young osteons and higher values in aged osteons.

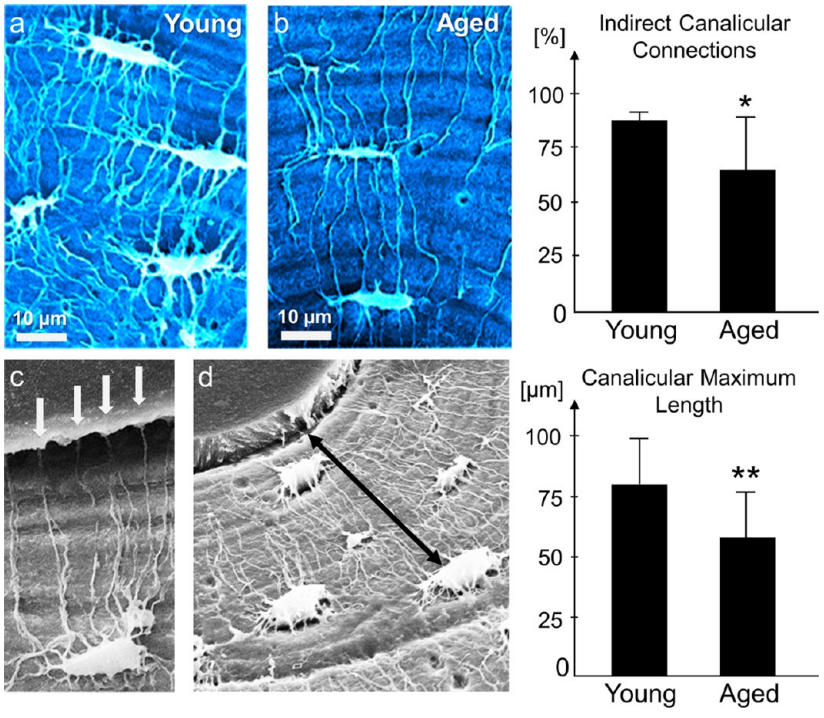

Figure 4. Canalicular connections within individual osteons (internal canalicular connections): Note that, besides direct canalicular connections, the number of indirect canalicular connections between lacunae is higher in young cases (a) than in aged cases (b). (c, d) In addition, lacunae are connected to the Haversian canal *via* canaliculi (white arrows), which frequently extend from the (d) outermost lacunae; the black line shows the distance taken as the canalicular maximum length to the Haversian canal, which was lower in aged individuals.

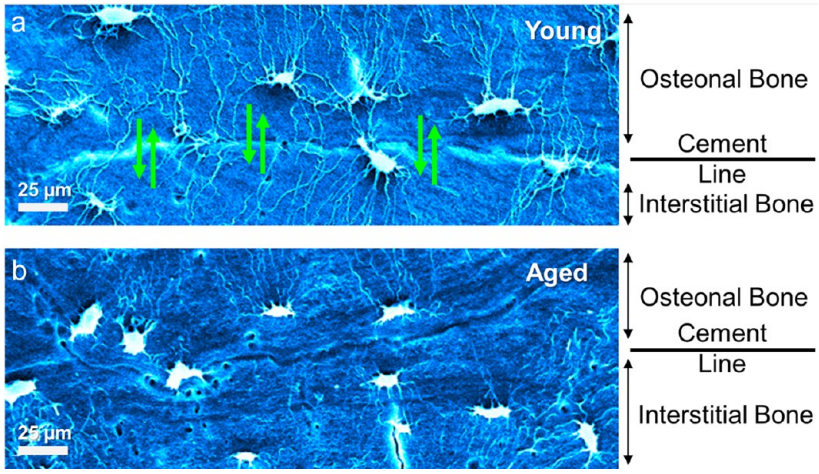

Figure 5. Canalicular connections between an osteon and the surrounding bone matrix (external connections): Evaluation of the interface between the osteon and interstitial bone reveals that in young cases (a) canaliculi of osteonal peripheral lacunae frequently cross the cement line (green arrows) of the osteon, even establishing connections with osteocyte lacunae of the surrounding bone, while aged bone shows cement lines that are almost impermeable to canaliculi (b).

TABLE 1. Age Dependence of the Osteonal External Canalicular Connections[a]

	Ext.On.Rt [%]	Ext.On.Conn [%]	Ext.Lc.Rt [%]	Ext.Lc.Conn [%]
young	100 (0)	92.49 (12.28)	66.09 (23.32)	48.96 (27.70)
aged	52.97 (28.93)	38.62 (25.44)	16.63 (18.97)	9.89 (14.47)
p (young *vs* aged)	<0.001	<0.001	<0.001	<0.001

[a] The data are expressed as a mean (SD). Ext.On.Rt: Percentage of osteons having at least one peripheral (outermost) lacuna with canaliculi crossing the cement line. Ext.On.Conn: Percentage of osteons with peripheral (outermost) lacunae (at least one) connected *via* canaliculi to a lacuna in the surrounding osteonal or interstitial bone. Ext.Lc.Rt: Percentage of peripheral (outermost) osteonal lacunae with canaliculi crossing the cement line. Ext.Lc.Conn: Percentage of peripheral (outermost) osteonal lacunae with canaliculi visibly connecting with lacunae in the surrounding osteonal or interstitial bone.

However, on the basis of the results presented here, even if mechanical loading remains the same in young and aged individuals, a reduced osteocyte population with decreased canalicular connections/branching could be responsible for a reduction of signals ensuring bone maintenance. Thus, a disturbed lacuno-canalicular network may contribute to deteriorated mechanosensing capabilities with aging, leading to insufficient response to mechanical signals and microdamage accumulation.[22]

Osteons Are Not Isolated Entities; They Show Rooting and Connections with Their Surrounding Tissue. Data concerning the connections between osteons and their surrounding interstitial or osteonal bone tissue are scarce, and the leading opinions have often contradicted. In previous papers focusing on different topics, it was occasionally stated that cement lines are a strict boundary that disrupts the canalicular network,[25,26] or that they are impermeable layers[27] almost never penetrated by osteocytic canaliculi.[28−30] However, Curtis *et al.* provided microscopic data that canaliculi can cross the cement lines.[31] In a study focusing on an individual of unknown age, connections between the osteons and interstitial bone occurred quite frequently in an ulna and rather rarely in a femur.[31] In contrast, our study revealed that canalicular connections between two or three neighboring osteons as well as between an osteon and interstitial tissue were clearly observable in human femoral cortices, and the extent of the connectivity showed a significant age-dependent reduction.

The possibility of connectivity and communication *between* osteons has not received due attention so far. Our previous studies showed that aged individuals displayed high proportions of dead osteocytes particularly within interstitial regions,[14] which may be related to their previously compromised nutrition. In that context, higher connectivity between the osteon and the lacunae of the surrounding bone tissue in young cases may provide better nutrition for the interstitial osteocytes, thus prolonging their life span. Better inter-osteonal connectivity supposes improved cellular communication and signaling, which might have a role in better coordination of osteocytic mechanical sensing/load sharing, as well. We hypothesize that the continuity of the canalicular network across an osteon's boundary and into the interstitial bone provides a framework for information exchange between osteonal osteocytes and interstitial osteocytes about their condition and requirements. This may be of paramount importance for launching targeted bone repair. In contrast, losing trans-osteonal communication may cause osteocyte apoptosis and interrupt the signaling necessary for bone remodeling of aged tissue areas (Figure 6).

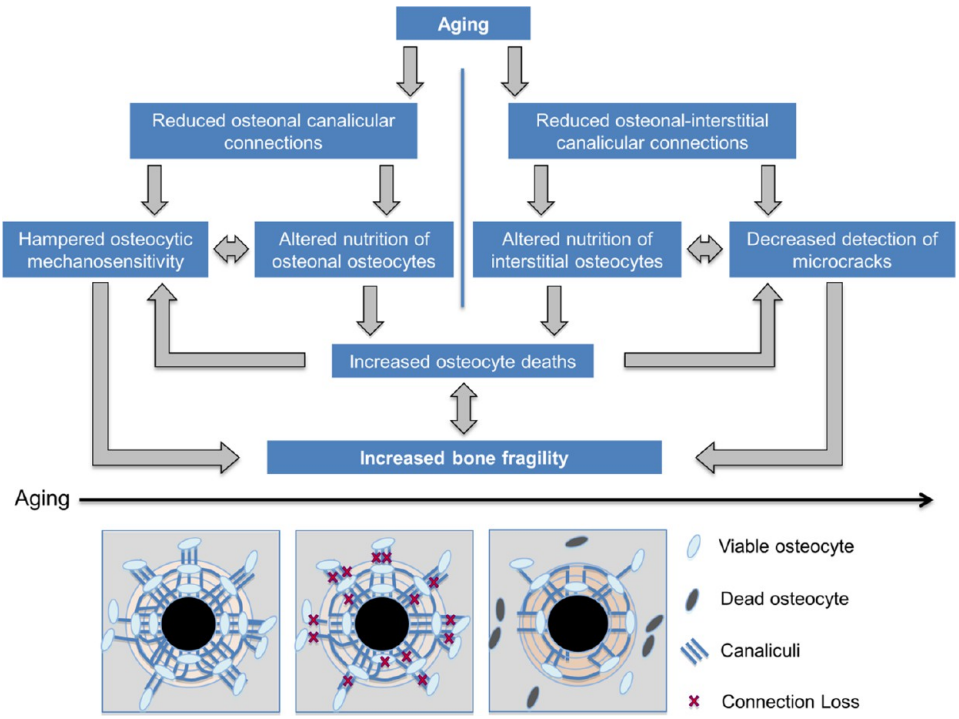

Figure 6. Pathway: The effects of aging on the canalicular network. Young osteons show an extensive internal canalicular network as well as extensive external connections with the surrounding bone tissue; aging is associated with a reduced lacunar and canalicular network within osteons, while the majority of external connections are lost; aged osteons reveal infrequent connections with the surrounding bone. Losing canalicular connections leads to hampered nutrition of interstitial osteocytes in particular, promoting osteocyte death.

Additionally, the number of canalicular connections in young cases may indicate that osteons from young individuals are better connected to the surrounding bone. Therefore, these canalicular connections may play a role in the context of fracture resistance mechanisms. Namely, canaliculi might act as nanostructural features interfering with the crack path, while load energy dissipates through the interaction with canalicular connections. This would have an influence on bone toughness, thereby representing another factor contributing to bone quality (Figure 6). However, further experimental studies are needed to explore the relationship between bone toughness and external canaliculi.

Canalicular Connections Formed in the Process of Bone Remodeling. When osteoclasts resorb bone and produce a cavity, some of the canaliculi belonging to the remnants of a previous osteon become exposed.[31] During the reversal stage of remodeling, osteoblasts arrive in the resorptive cavity and produce new bone. At this point in remodeling, the osteoblasts may establish connections with the dendrites of the remaining osteocytes (which might be crucial for their survival), but the signals involved in this attraction, recognition, and linking require further studies. Considering decreased canalicular numbers in advanced age, it is unknown whether osteoblasts initially failed to establish connections with antecedent osteocytes or whether previously formed connections were simply lost during the osteon's lifetime. However, our findings showing lower Ca/P ratios in osteons with better connectivity might suggest that a tissue-age-dependent increase of calcium in relation to phosphorus may be associated with canalicular occlusion. Because our study is based on a cross-sectional design, it cannot show canalicular occlusion as a process; therefore, such a scenario can be only inferred, given that we observed three states of the peripheral canaliculi: canaliculi crossing the cement line, canaliculi interrupted at the cement line, and complete lack of visible canaliculi on the peripheral side of the outermost lacunae in osteons.

A few limitations deserve consideration. First, a disadvantage of the acid etching method is that it does not provide a full 3D view of the lacuno-canalicular network but rather shows individual 3D cuts. Second, although the acid etching procedure acts by removing both organic and inorganic matrix, primarily through phosphoric acid binding to calcium and magnesium, essentially resulting in complete removal of the material, the underlying bone matrix might be influenced, leading to a potentially minor effect on the EDX measurements.

Due to the very small thickness of the canaliculi (~140 nm), the spatial resolution of the imaging method is critical for a proper quantification of canalicular structures. Apart from our methodological approach, serial focused ion beam (FIB) in combination with scanning electron microscopy (SEM) and X-ray microtomography could also be considered for the assessment of bone's

lacunar network.[6] Serial FIB with SEM is able to piece together impressive 3D reconstructions of the canalicular network, but does not provide in general chemical information. Moreover, it can suffer from preparation artifacts and is limited to small-scale regions of interest. While the resolution of current SEM systems can be better than 1 nm, most 3D micro-CT systems or synchrotron-radiation-based X-ray computed tomography require very small sections to achieve a resolution of 700 nm at best.[32]

CONCLUSIONS

Osteocytes play a large role in bone quality by signaling remodeling through their nanosized canalicular network; in turn, age-related changes to this network may affect bone quality. Our study showed that the aged cases had a diminished number of canaliculi per lacuna and fewer connections within the osteon. Furthermore, we found that the canaliculi traverse the cement line in human cortical bone, allowing communication with other osteons or interstitial bone; however, interosteon connections were also diminished with age. We believe that the diminished osteocyte canalicular network in aged tissue reduces the supply of nutrients and degrades the mechanosensitivity of the osteocytes, which in turn will affect bone quality.

METHODS

Specimen Selection and Preparation. The samples were taken from 20 post-mortem human subjects (young: $n = 9$, 20−40 yrs; aged: $n = 11$, 70−95 yrs). The femoral mid-diaphyseal cross sections were obtained during autopsy at the Institute of Forensic Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Based on patients' records and autopsy reports, the individuals included in the study did not suffer from any musculoskeletal disease.

Diaphyseal cross sections were cut using a water-cooled diamond band saw (Exakt, Germany) and embedded undecalcified in a methylmethacrylate medium (Technovit 7200, Kulzer, Germany) according to the preparation procedure described previously.[14,16,33−35] After dehydration with an ascending alcohol series, the specimens were infiltrated in 100% Technovit 7200. The infiltration was continued in Technovit 7200 with 1% benzoyl peroxide for an additional 10 days devoid of any light exposure. After infiltration, the polymerization was completed in a light polymerization unit (Exakt, Germany). Donath's grinding technique[35] was used to prepare flat coplanar surfaces using an automatic grinding system (Exakt, Germany) as described previously.[14,33−35]

In order to visualize the lacuno-canalicular network, polished bone samples were exposed to acid etching. As shown in Figure 1b, the acid solution removes the bone matrix at a faster rate than the polymer that had infiltrated into the bone sample's voids (*i.e.*, lacunae, canaliculi, and Haversian canals) during the embedding process. The acid etching procedure was performed following the recommendations from Kubek *et al.*[36] Namely, the specimens were immersed in 9% phosphoric acid for 20 s (with the polished side upward) followed by a short rinse in deionized water (1−2 s). Subsequently, they were exposed for 5 min to 5% sodium hypochlorite and finally rinsed in deionized water. Following the acid etching procedure, the specimens were left to naturally dry at room temperature without the use of a heating cabinet. Therefore, high temperatures were avoided to ensure a mild drying process that limited the development of vapor and surface tension.

Scanning Electron Microscopy Imaging and Quantitative Analysis of Canalicular Connections. Subsequently, in order to provide optimal conditions for acquiring high-resolution and high-magnification SEM images, the surface of the specimens was sputter coated with a gold (Au) alloy using a sputter coater (Cressington 108, Cressington Sc. Instr. Ltd., Watford, UK). Following the coating procedure, the specimens were mounted within the scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England). The microscope was operated in secondary electron mode, at 20 kV and 100 pA at a constant working distance.

In each case, bone from the midcortical compartment of the medial subregion of the cross-section was imaged. Osteons in this region were selected for quantitative and qualitative assessment of the canalicular network if they were exclusively transversally sectioned osteons with a relatively circular shape and if they contained a clearly visible Haversian canal in the center of the osteon.

The following quantitative parameters were defined by the authors and measured through digital image analysis:

1. *Number of canaliculi per osteocyte lacuna* (N.Ot.Ca/Ot.Lc): Each osteocyte lacuna was encircled by an ellipsoid, and the number of intersections between the ellipsoid and canaliculi that arise from the lacuna was counted (see Figure 1c). In this way, the number of canaliculi per osteocyte lacuna (N.Ot.Ca/Ot.Lc) was determined. The data for all lacunae were averaged for each investigated osteon.
2. *Percentage of osteons with indirect connections between its lacunae*: Indirect connections were defined as the connections between the radial canaliculi of an osteocyte and the transverse canaliculi of another osteocyte from different lamella of the same osteon (Figure 1d).
3. *Mean maximum length of canaliculi between the peripheral (outermost) lacunae and the Haversian canal* (Figure 1e).
4. *External lacunar rooting* (Ext.Lc.Rt), defined as the percentage of all peripheral (outermost) osteonal lacunae whose canaliculi cross the cement line of their osteon (Figure 1f).
5. *External osteonal rooting* (Ext.On.Rt), defined as the percentage of osteons having at least one peripheral (outermost) lacuna whose canaliculi do not end at the osteon boundary (*i.e.*, cement line) but penetrate the cement line, thus entering the surrounding bone tissue (Figure 1f).
6. *External lacunar connectivity* (Ext.Lc.Conn), depicting the percentage of the peripheral (outermost) osteonal lacunae whose canaliculi are clearly connected with the lacunae from the surrounding osteonal or interstitial bone (Figure 1g).
7. *External osteon connectivity* (Ext.On.Conn), defined as the percentage of osteons whose peripheral (outermost) lacunae (at least one) are clearly connected *via* canaliculi with the lacunae from the surrounding osteonal or interstitial bone (Figure 1g).

Quantitative data are based on evaluating nearly 1500 lacunae (approximately 800 lacunae in young subjects and 700 lacunae in aged subjects). The total number of canalicular entities investigated was approximately 18 000 in the young group and about 10 000 in the aged group.

Energy Dispersive X-ray Microanalyses. Due to the continuous renewal and resorption of human bone tissue, individual bone packets have different tissue ages.[14,33,37,38] To correlate the canalicular connections/numbers within an osteon with its chemical composition reflecting tissue age, we performed the EDX analysis directly within specific osteons. Elemental peaks reflecting the content of calcium (Ca) and phosphorus (P) in

weight percent (wt %) were evaluated *via* EDX-ZAF software provided by the manufacturer (EDAX, DX-4, Mahwah, NJ, USA) for the assessment of Ca/P ratios. In this context, Ca/P ratio primarily reflects an estimate of the mineral composition and may be regarded as an indicator of maturity of the bone tissue (*i.e.*, tissue age), where higher Ca/P ratios may be associated with increased maturation/aging of bone tissue[37,39−41] and lower Ca/P ratios to younger tissue age. EDX acquisition parameters were calibrated with Cu and Al references. The calibration process ensures that the relevant peaks are within 1 eV of the reference value. The EDX analysis was carried out with the use of the systems' internal standards (pure elements and compounds) that were obtained from reference materials. Standard acceleration voltages of 20 kV were employed. For each energy range the sensitivity of the detector is considered; also the low-energy line profiles are optimally deconvoluted. The spatial resolution of the EDX analysis is on the order of a few micrometers, allowing analysis of very small regions of interest within osteons.

Quantitative Backscattered Electron Imaging. The bone mineral density distribution was measured *via* quantitative backscattered electron imaging to confirm the tissue age measurements made with EDX. Embedded bone samples that had been previously acid etched were repolished to a coplanar finish, such that only a few micrometers of tissue were removed. The samples were then carbon coated, and the qBEI analysis was performed on the same osteon as the EDX analysis. The scanning electron microscope (LEO 435 VP, Cambridge, England) was operated at 20 kV and 580 pA at a constant working distance of 20 mm and with a backscattered electron detector (type 202, K.E. Developments Ltd., Cambridge, England). The setup parameters and histogram evaluations have been previously reported.[37,42,43]

Statistical Analysis. The Kolmogorov−Smirnov test was applied to assess the normality of the data distribution. Based on the type of data distribution, intergroup comparisons were performed using the *t*-test or the Mann−Whitney U-test for two independent samples. Linear regression analysis was used to assess the relationship between the Ca/P ratio and canalicular parameters. All the analyses were performed in SPSS (ver. 15.0) at the 0.05 level of significance.

Conflict of Interest: The authors declare no competing financial interest.

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