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Multi-level characterization of human femoral cortices and their underlying osteocyte network reveal trends in quality of young, aged, osteoporotic and antiresorptive-treated bone

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ABSTRACT

Characterization of bone's hierarchical structure in aging, disease and treatment conditions is imperative to understand the architectural and compositional modifications to the material and its mechanical integrity. Here, cortical bone sections from 30 female proximal femurs – a frequent fracture site – were rigorously assessed to characterize the osteocyte lacunar network, osteon density and patterns of bone matrix mineralization by backscatter-electron imaging and Fourier-transform infrared spectroscopy in relation to mechanical properties obtained by reference-point indentation. We show that young, healthy bone revealed the highest resistance to mechanical loading (indentation) along with higher mineralization and preserved osteocyte-lacunar characteristics. In contrast, aging and osteoporosis significantly alter bone material properties, where impairment of the osteocyte-lacunar network was evident through accumulation of hypermineralized osteocyte lacunae with aging and even more in osteoporosis, highlighting increased osteocyte apoptosis and reduced mechanical competence. But antiresorptive treatment led to fewer mineralized lacunae and fewer but larger osteons signifying rejuvenated bone. In summary, multiple structural and compositional changes to the bone material were identified leading to decay or maintenance of bone quality in disease, health and treatment conditions. Clearly, antiresorptive treatment reflected favorable effects on the multifunctional osteocytic cells that are a prerequisite for bone's structural, metabolic and mechanosensory integrity.

1. Introduction

Bone is a complex hierarchically organized material consisting of osseous cells and a nano-composite structure composed of collagen and mineral [1,2]. Osteocytes are the most frequent occurring osseous cell type in the skeleton, which are strategically distributed throughout the entire mineralized bone. Therefore, they play a key role in the mechanosensitive behavior of bone [3] by detecting mechanical stimulation and orchestrating signal transmission between bone cells involved in bone repair and renewal. Such chemically and/or mechanically triggered responses through either intracellular calcium transients [4] or the release of nitric oxide and prostaglandin E2 [3,5–7] are critical to bone health and bone remodeling. Osteocytes reside in special cavities within the bone matrix called osteocyte lacunae and are separated from direct contact with the mineralized bone matrix through a thin layer of unmineralized tissue [8,9]. Although much of the relationship between the cell and the lacunar space is still unknown, osteocytes are believed to actively modify their surrounding environment [10,11], and cellular viability is essential for maintaining the lacunar space [12,13]. The osteocytes' characteristics are thought to

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describe the bone's matrix potential mechanosensory abilities, adaptative capacity and possibly the quality of the perilacunar tissue [2,12,14–18]. However, the death of an osteocyte within the lacuna is associated with a phenomenon termed 'micropetrosis', where the lacuna becomes completely mineralized [19]. Mineralized lacunae are thought to represent osteocytes' "fossils" as the apoptotic remnants of osteocytes have been found within the mineralized material [13]. Due to the possible negative impact of mineralized lacunae on bone health [12,20], we hypothesize that the occurrence of mineralized lacunae serves as a marker for *bone quality*. *Bone quality* is a critical aspect in maintaining bone mechanical competence as it describes the totality of features and characteristics that influence a bone's ability to resist fractures [21]. Specifically, bone quality encompasses both the composition and structure of bone through the degree of mineralization, the type of cross-linking, collagen characteristics, the presence of microdamage and non-collagenous proteins, as well as architecture/geometry patterns [1,22,23].

Previous studies have observed that the mineralization of osteocyte lacunae can be attributed to aging and the existence of skeletal diseases [12,20,24]. Thus, changes in the mineralization of osteocyte lacunae may indeed reflect bone quality and the risk of fracture in humans. However, the trends in the number and degree of mineralized lacunae and the corresponding effect on bone remodeling have not been comprehensively studied. In particular, it is of interest if age- and disease-dependent mineral occlusions of osteocyte lacunae may be prevented by bisphosphonate (BP) treatment. Indeed, anti-resorptive treatment was found to increase areal bone mineral density and decrease fracture risk [25–28]. However, associated side effects in the form of new atypical fractures related to bisphosphonate-treatment are reported as well [29,30]. Although *in vitro* and animal model studies suggested that bisphosphonates promote osteoblasts viability and express anti-apoptotic effects on osteocytes [31–33], histological findings on dogs' ribs rather showed decreased osteocyte lacunar density following long-term alendronate treatment [34]. Therefore, bisphosphonates' specific species- and site-related modes of action deserve additional attention to unravel corresponding wide-ranging effects taking place on several bone tissue hierarchical levels.

In the present study, we quantify the density and distribution of mineralized lacunae within the femoral cortical bone in young, aged, osteoporotic and alendronate-treated individuals. In addition to a rigorous assessment of osteocyte lacunar characteristics, we analyzed the bone matrix's composition and its mechanical competence in the four groups to provide a better understanding of the bone quality and osteocytes' contribution to bone health with special regard to bone remodeling characteristics in health, disease and bisphosphonate-treatment stage. The bone's composition and mechanical properties are important to understand the mechanisms that lead to fracture in human bone, teeth and other biological materials, and for providing insights on the prerequisites for designing and using new bone replacement materials and/or pharmacologic treatment options to cure bone-related diseases.

2. Materials and methods

The bone specimens used in this study were acquired during autopsy at the Department of Forensic Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, from 30 female individuals. Based on autopsy reports and clinical histories, the individuals were divided into four study groups:

1. Young female individuals without skeletal diseases/fractures (age 31.4 ± 9.5 years, n = 5)
2. Aged female individuals without skeletal diseases/fractures (age 83.7 ± 5.9 years, n = 7)
3. Female individuals with untreated osteoporosis (age 82.5 ± 5.5 years, n = 9)
4. Female individuals with osteoporosis who received bisphosphonate treatment (age 80.5 ± 7.2 years, n = 9).

These individuals did not suffer from cancer, renal diseases, primary hyperparathyroidism, or Paget's disease and did not show any other signs or symptoms of bone diseases apart from postmenopausal osteoporosis in the appropriate groups. The treated osteoporosis group received the third-generation bisphosphonate alendronate at either 10 mg per day or 70 mg per week during 6 ± 1.6 years.

The study was approved by the Ethics Committee of the Hamburg Chamber of Physicians (PV3486).

2.1. Specimen preparation

A complete horizontal cross-section of the proximal diaphysis of each femur (from all four groups of patients), as well as cross-sections of distal radius, iliac crest and the L5 vertebral body (from osteoporosis and alendronate-treated patients) were cut using a diamond belt saw (Exakt, Norderstedt, Germany) and fixed in formalin (3.5%), as reported previously [35]. The slice thickness in all specimens was 4 mm. The undecalcified specimens were dehydrated by means of an ascending ethanol series (70%, 80%, 2 × 96%, 3 × 100%) and further infiltrated with a plastic embedding medium (Technovit 7200; Heraeus/Kulzer, Wehrheim/Ts., Germany). The infiltration was performed in steps with the following volumetric ethanol/Technovit-ratios: 70:30; 50:50; 30:70; 0:100. Finally, Technovit with benzoyl-peroxide was used to continue the infiltration process for another 10 days. Ground tissue specimens were made based on Donath's grinding technique [35,36]. After the grinding and polishing processes, the specimens were carbon coated and prepared for scanning electron microscopy.

2.2. Scanning electron microscopy (SEM): evaluation of osteocyte lacunae

The femur specimens were mounted in a scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England) with a backscattered electron detector (Type 202, K.E. Developments Ltd., Cambridge, England). The microscope was operated in backscattered electron mode at 20 keV and a constant working distance, while the images were acquired with 100x magnification. The following parameters were evaluated in the femur samples: number of mineralized lacunae per bone area and total number of lacunae per bone area. Mineralized lacunae were defined following the criteria from backscattered electron imaging [12,20]. The bone area was evaluated with ImageJ software (ImageJ, 1.45q, National Institutes of Health, USA – imagej.nih.gov/ij/) using the BoneJ plugin for bone volume fraction [37].

To assess the spatial distribution of osteocyte lacunae, the femoral cortical cross-section was divided into four regions (i.e., medial, lateral, ventral, and dorsal), and each region was then subdivided into periosteal and endosteal compartments, as in previous studies [12,38]. In each of the four regions of the femoral cross-sections (i.e., medial, lateral, ventral and dorsal), a minimum of two representative images per compartment (each approximately 1 mm^2) were analyzed.

The number of Haversian systems per bone area was evaluated on the same images that were used to quantify the osteocyte lacunae. Thus, the density of osteons provides an overview (history) of the number of remodeling events [38].

2.3. Bone mineral density distribution analysis (BMDD) using quantitative backscattered electron imaging (qBEI)

We performed quantitative backscattered electron imaging (qBEI) to evaluate the bone mineral density distribution (BMDD) in cross-sections from the femora, radii, iliac crests and vertebral L5 bodies in accordance with previous studies [2,39–43]. The scanning electron microscope was operated in backscatter mode at 20 keV and 680 pA with a constant working distance of 20 mm. The beam current was controlled by use of a Faraday cup (MAC Consultants Ltd., England), and all parameters were maintained stable during imaging. The gray level was calibrated with a standard of known density, as explained in more details in previous studies, such that gray level had a linear relationship with the calcium concentration [41]. The qBEI measurements were performed on images with 50x magnification. Using a custom Matlab routine, the following parameters were automatically evaluated: mean calcium concentration (mean Ca, wt%), most frequent calcium concentration (peak Ca, wt%), standard deviation of the calcium distribution curve showing the degree of heterogeneity of BMDD distribution (width Ca, wt%), percentage of bone area that is mineralized below the 5th percentile of the reference range of the young group (Ca low, % bone area) and percentage of bone area containing Ca concentration above the 95th percentile of the reference range of the young group (Ca high, % bone area). In each individual, two images per compartment per region (each approximately 4 mm^2 in size) were considered for the qBEI evaluations.

2.4. Fourier transform infrared spectroscopy (FTIR)

To assess the quality of the bone matrix, Fourier transform infrared (FTIR) spectroscopy was performed within the medial, lateral, ventral, and dorsal regions of each femur sample. The FTIR spectra were acquired with a Universal ATR sampling accessory connected to a Frontier FTIR spectrometer (Perkin Elmer, Waltham, MA, USA). Spectra were acquired over a spectral range of 570–4000 cm^{-1} at a spectral