The generation of enlarged eroded pores upon existing intracortical canals is a major contributor to endocortical trabecularization

Andreasen, Christina Møller; Bakalova, Lydia Peteva; Brüel, Annemarie; Hauge, Ellen Margrethe; Kiil, Birgitte Jul; Delaisse, Jean-Marie; Kersh, Mariana Elizabeth; Thomsen, Jesper Skovhus; Andersen, Thomas Levin

Published in:
Bone

DOI:
10.1016/j.bone.2019.115127

Publication date:
2020

Document version:
Accepted manuscript

Document license:
CC BY-NC-ND

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use
This work is brought to you by the University of Southern Denmark.
Unless otherwise specified it has been shared according to the terms for self-archiving.
If no other license is stated, these terms apply:

• You may download this work for personal use only.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim.
Please direct all enquiries to puresupport@bib.sdu.dk

Download date: 24. Aug. 2021
The generation of enlarged eroded pores upon existing intracortical canals is a major contributor to endocortical trabecularization

Christina Møller Andreasen a,h,i*, Lydia Peteva Bakalova b, Annemarie Brüel d, Ellen Margrethe Hauge e, Birgitte Jul Kiil f, Jean-Marie Delaisse c,h,i, Mariana Elizabeth Kersh b, Jesper Skovhus Thomsen d,†, Thomas Levin Andersen c,h,i, †*

a Department of Orthopedic Surgery & Traumatology, Odense University Hospital, Odense, Denmark
b Department of Mechanical Science and Engineering, University of Illinois at Urbana-Champaign, IL, USA
c Clinical Cell Biology, Vejle Hospital – Lillebælt Hospital, University of Southern Denmark, Vejle, Denmark
d Department of Biomedicine, Aarhus University, Aarhus, Denmark
e Department of Rheumatology, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
f Department of Plastic Surgery, Aarhus University Hospital, Aarhus, Denmark
h Clinical Cell Biology, Research Unit of Pathology, Department of Clinical Research, University of Southern Denmark and Department of Pathology, Odense University Hospital, Odense, Denmark
i Department of Molecular Medicine, University of Southern Denmark, Odense, Denmark

* Corresponding authors
† Shared last authorship

Authors e-mail address ORCID Phone
Christina M. Andreasen: cmmandreasen@health.sdu.dk 0000-0002-2624-5677 (+45) 5160 5534
Postal address: J. B. Winsløwsvej 25, 1st floor, DK-5000 Odense C
Lydia Peteva Bakalova: bakalov2@illinois.edu
Annemarie Brüel: mb@biomed.au.dk 0000-0003-4833-0888
Ellen Margrethe Hauge: ellen.hauge@clin.au.dk 0000-0003-2562-9174
Birgitte Jul Kiil: birgkiil@rm.dk
Jean-Marie Delaisse: Jean-marie.delaisse@rsyd.dk 0000-0001-7370-1500
Mariana Elizabeth Kersh: mkersh@illinois.edu
Jesper Skovhus Thomsen: jst@biomed.au.dk 0000-0001-9386-6679
Thomas L. Andersen: thomas.levin.andersen@rsyd.dk 0000-0002-6981-7276 (+45) 2228 4772
Postal address: J. B. Winsløwsvej 25, 1st floor, DK-5000 Odense C

One supplemental table is included.
Grant supporters: The VELUX Foundation for funding support (VELUX34368) and donation of the μCT scanner (VELUX26922), Aase and Ejnar Danielsen Foundation (10-001584) and the Danish Southern Region Research Grant (15/24851).

Disclosures

The authors have no disclosures to report.
Abstract

The gradual conversion of cortical bone into trabecular bone on the endocortical surface contributes substantially to thinning of the cortical bone. The purpose of the present study was to characterize the intracortical canals (3D) and pores (2D) in human fibular bone, to identify the intracortical remodeling events leading to this endocortical trabecularization. The analysis was conducted in fibular diaphyseal bone specimens obtained from 20 patients (6 women and 14 men, age range 41–75 years). µCT revealed that endosteal bone had a higher cortical porosity ($p < 0.05$) and canals with a larger diameter ($p < 0.05$) than periosteal bone, while the canal spacing and number were similar in the endosteal and periosteal half. Histological analysis showed that the endosteal half versus the periosteal half: (i) had a higher likelihood of being non-quiescent type 2 pores (i.e. remodeling of existing pores) than other pore types (OR = 1.6, $p < 0.01$); (ii) that the non-quiescent type 2 pores contributed to a higher porosity ($p < 0.001$); and that (iii) amongst these pores especially eroded type 2 pores contributed to the elevated cortical porosity ($p < 0.001$).

In conclusion, we propose that endocortical trabecularization results from the accumulation of eroded cavities upon existing intracortical canals, favored by delayed initiation of bone formation.

**Key words:** Cortical porosity, bone remodeling, bone resorption, bone formation, trabecularization
**Introduction**

Aging induces loss of cortical bone, causing it to thin and become increasingly porous (1-4), making the bone more fragile (5-9). The process by which the cortical bone becomes thinner is classically referred to as “trabecularization”, as cortical bone appears to be converted into trabecular bone (10, 11). Currently, trabecularization is not thought to be the result of extensive resorption at the endosteal or periosteal surface, but the result of increased cortical porosity and larger pores adjacent to the endocortical surface, which gradually transforms the endosteal part of the cortex into trabecular bone (12-14). The concept of trabecularization is not new. In 1964–1965, Atkinson reported a gradual increase in porosity along the radial axis from the periosteal to the endosteal cortical surface, and that this gradual increase in porosity from a macroscopic perspective became more pronounced with age (15, 16). Atkinson observed the first signs of increased endosteal porosity in some bone specimens from individuals in their thirties, while cortical thinning appeared in specimens from individuals in their forties (16). Similar observations were reported in both women and men by Arnold, who also found that the increased endosteal porosity was the result of enlarged intracortical cavities (17). Furthermore, in a radioactive tracer study of small animals, Weidmann showed in 1956 that the endosteal half of the cortex had a higher remodeling activity than the periosteal half (18). In some studies, the endosteal trabecularization zone is referred to as the transition zone, defined as the zone in between cortical bone with a high BV/TV and trabecular bone with a low BV/TV (11, 13). Collectively, these studies emphasize the importance of increased endosteal porosity. However, the biological mechanism responsible for this endosteal porosity and trabecularization remains to be identified.

Intracortical pores are in reality 3D canals forming a network, which is generated and modified by intracortical bone remodeling. Under physiological conditions this remodeling renews the cortical bone matrix and alters the cortical bone structure to sustain its mechanical properties (19-21). Bone remodeling is conducted by microscopic basic multicellular units (BMUs), which replace
small packages of old bone with new bone (22). The intracortical BMUs generating new canals (observed as type 1 pores) were recently demonstrated to include three sequential phases: (i) an initial resorption phase, in which penetrative resorption by osteoclasts generates a canal; (ii) a reversal-resorption phase, where resorption by scattered osteoclasts widens the generated canal, intermixed with reversal cells i.e. osteoprogenitor cells that are recruited to the eroded surfaces and gradually mature into bone-forming osteoblasts; and (iii) a formation phase, where osteoblasts refill the canal until only a narrow canal remains (23, 24). Importantly, the intracortical BMUs have been shown not only to generate new canals, but also to remodel existing canals (25-28). The remodeling of existing canals (observed as type 2 pores) has been demonstrated to be the predominant remodeling type with age in human iliac bone (28, 29). Moreover, these type 2 remodeling events have been shown to accumulate at the reversal-resorptive phase with age, likely due to a delayed or absent initiation of the subsequent bone formation phase (28). This causes the widened pores of these events to coalesce into even larger eroded cavities, thus being the major contributor to age-induced cortical porosity (28). The question is whether type 2 remodeling events are contributors to endosteal porosity, which is believed to be a key step in endosteal trabecularization.

The objectives of the present study were: i) to characterize the 3D microstructure of the endocortical canals in endosteal and periosteal bone using micro computed tomography (µCT), and ii) to identify the remodeling events leading to endosteal porosity and trabecularization using histology. The study was performed using human fibular bone. The histological analysis included a detailed classification of the intracortical pores and the remodeling events generating or modifying these pores. This histological classification takes remodeling type and stage, and whether the pores result from coalescence of existing pores into consideration (9, 28).
Materials and Methods

Bone samples

This cross-sectional study was conducted on fibular diaphyseal bone samples obtained from 6 women (aged 41–73 years) and 14 men (aged 43–75 years) undergoing mandibular and maxilla reconstructive surgery at the Department of Plastic Surgery, Aarhus University Hospital (9). The bone specimens were approximately 12-mm-long bone autografts obtained from the fibular diaphysis 7–12 cm proximal to either the medial or lateral malleolus. Immediately after surgery the lateral half of the bone samples were fixated in formalin and later transferred to 70% ethanol until the subsequent µCT scan. The study was approved by the Danish National Committee on Biomedical Research Ethics (project ID# S-20130149).

µCT

The bone samples were scanned in 70% ethanol in a desktop µCT scanner (µCT35, Scanco Medical AG, Brüttisellen, Switzerland) in high-resolution mode (1000 projections per 180°) with an isotropic voxel size of 6 μm, an X-ray tube voltage of 70 kVp, an X-ray tube current of 114 μA, and an integration time of 3200 ms. The µCT 35 was equipped with a fixed 0.5 mm Aluminum filter. During scanning the fibular length axis was oriented perpendicular to the scanning plane. Volumes of Interests (VOIs) were delineated interactively using the standard software supplied with the µCT scanner and consisted of either the periosteal half or the endosteal half separated by the cortical mid-line. The 3D data sets were low-pass filtered with a Gaussian filter (σ = 0.8, support = 1) and segmented with a fixed threshold filter (508.4 mg HA/cm³). The threshold was found as the minimum point between the peaks in the attenuation histogram representing bone and canals. The images were then inverted and cortical porosity (Ct.Po), the canal degree of anisotropy (Ca.DA) and connectivity density (Ca.Conn.D) were determined using the µCT scanner software. In addition, the
canal diameter (Ca.Dm), spacing (Ca.Sp), and number (Ca.N) were determined (1, 30) using the direct method without parallel plate assumptions (31). The endosteal and periosteal VOIs were analyzed separately in each bone sample. The distribution of the canal diameter and canal spacing was characterized by their probability density functions, which were found as the histograms provided by the scanner software, normalized so that the area under the curve was 1 (32, 33). The µCT-derived data were volume weighted, i.e. each voxel have equal weight, which means that a canal with a large diameter containing many voxels will have a larger weight in the mean values than a tiny canal containing only a few voxels. This also means that e.g. the Ca.Dm is determined for each voxel and the mean Ca.Dm is found as the average over the Ca.Dm values determined by all voxels in the VOI.

The 3D image stack overlaid with the VOIs was exported from the scanner as DICOM files for later 2D analysis. 3D data was visualized using Amira (version 5.6, FEI Visualization Science Group, Mérignac, France) with the canals in solid white and the bone in semitransparent red.

Sectioning and alignment with the 3D µCT
After µCT, the bone samples were embedded undecalcified in methyl methacrylate and cut in 7-µm-thick sections on a Jung model K microtome. From each embedded fibula specimen one section was stained with Masson-Goldner trichrome and used to localize the 2D section in the 3D µCT image stack (Figure 1). This made it possible to link the 3D structures of intracortical canals with their 2D appearance as pores (Figure 1).

2D µCT-based pore analysis
The 2D µCT images matching the corresponding histological sections were used to identify and label each pore (Figure 1). First, the images were processed with custom software (Matlab R2015-
Academic, Mathworks, Natick, MA, USA) to remove very small pores (connected regions containing less than 20 voxels). Bone fragments that were not connected to the main cortical structure and pores that partly opened into the marrow cavity were removed. Then, the pore area and the distance from the bone edges were quantified. The endosteal and periosteal bone surfaces were identified in the images, and the relative Euclidian distance from the centroid of each pore to the respective bone surface was calculated (Figure 1D). The pore diameter was estimated by measuring the diameter of the largest circle that could be inscribed into the pore (Figure 1D) using the “Maximum Inscribed Circle using Distance Transform” function for Matlab.

**Histomorphometry**

From each bone specimen, one section that matched with the 2D µCT images underwent a detailed histomorphometric analysis. Within that section a subregion of the cortex was outlined and herein approximately 50 pores were defined in order to have a comprehensively, but manageable, number of pores for each biopsy. The subregion was chosen as the best representative region based on the quality of the section. In order to match the 2D µCT-based pore analysis, pores with a pore diameter below 15 µm were excluded from the analysis. For all analyzed pores, the pore diameter was measured, and each pore was characterized according to its remodeling type, stage, and location using the classification scheme described by Andreasen et al. (28, 29). In brief, the pores were classified according to whether they reflected the generation of a new canal (type 1 pore), or the remodeling of an existing canal (type 2 pore) as illustrated in Figure 2 and suppl. Figure 1. Then, the pores were classified according to their remodeling stage. Here, type E pores with eroded surfaces reflected cross-sections of BMUs in the reversal-resorption phase or to a less extent BMUs in the initial resorption phase. Type F pores with osteoid surfaces reflected cross-sections of BMUs completely in the formation phase, while type EF pores reflected cross-sections of BMUs in both
the reversal-resorption and formation phase. These three pore types are also referred to as non-quiescent pores. Type Q pores with quiescent surfaces reflected cross-sections of BMUs with a completed bone remodeling, i.e. pores of quiescent osteons (Figure 2, Suppl. Figure 1).

Type 2 pores were also classified according to the location of their resorptive area relative to existing osteons (Figure 2). Here, the pores were classified as intraosteonal type 2 pores (type 2IN pores) when the pores resorption area was within the cement line of an existing osteon; cement-line breaking type 2 pores (type 2BK pores) when the pores resorption area broke through the cement line of the existing parent osteon; or osteon coalescing type 2 pores (type 2CO pores) when the resorption area overlapped with the pore of two or more existing osteons (Figure 2, Suppl. Figure 1) (28, 29).

Pores classified as quiescent (reflecting sites with a terminated remodeling) had their osteon diameter measured and their wall thickness calculated (Figure 1). Cortical porosity was estimated based on µCT. Moreover, the contribution of specific pore types to the cortical porosity was calculated by multiplying the overall cortical porosity with the percentage of the porosity reflecting the pore type.

Statistical analysis

Endosteal and periosteal µCT-derived data were compared using a Mann-Whitney test. Statistical significance of the prevalence of different pore types was assessed by logistic regression adjusted for a clustering effect from the individual patients and any significant differences were reported as odds ratios. Evaluation of statistically significant differences between the pore types contribution to the cortical porosity and selected remodeling parameters for the endosteal and periosteal quiescent pores were calculated by a Mann-Whitney test. Linear regression and Pearson’s correlation coefficient ($r$) was used for comparison of the overall remodeling balance.
In all comparison $p < 0.05$ was defined as statistically significant. The D’Agostino-Pearson omnibus normality test was used to address whether the percentages of the different pore types were normally distributed. The statistical analyses were performed in GraphPad Prism, version 6 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

The 3D µCT analyses were based on all tissue inside either the endosteal or the periosteal VOI of the 20 cortical fibular bone specimens. The 2D µCT analyses conducted in a single plane of each specimen encompassed a total of 2787 pores corresponding to 53–257 pores per specimen depending on size of biopsy. The histological analysis conducted in a subregion corresponded to a total of 948 pores with approximately 50 pores per biopsy (range 31–77). The analyzed bone samples had a mean Ct.Th of $2.15 \pm 0.73$ mm (Suppl. Table 2).

_A larger canal diameter and higher porosity in the endosteal versus periosteal cortex_

Scans of the fibular bone sample from two patients are shown for the entire bone sample in Supplementary Figure 2A and for the endosteal and periosteal VOI in Figure 3. The canal diameters were distributed over a wider range in the endosteal half than in the periosteal half (Suppl. Figure 2B), indicating that the canal diameters in the periosteal half were more homogeneously distributed than the canal diameters in the endosteal half (Suppl. Figure 2B). In contrast, the distribution of the canal spacing was very similar for the periosteal and endosteal fibular halves (Suppl. Figure 2C).

Similar results were seen when canal diameter distributions were averaged over all bone samples (Figure 3B–C). The average canal diameter was significantly larger in the endosteal half than in the periosteal half (Figure 3B, E, Table 1). In contrast, the spacing between the canals did not differ between the two halves (Figure 3C, F), Table 1) neither did the number of canals (Table 1). The
larger canals in the endosteal half resulted in a significantly higher porosity in the endosteal half than in the periosteal half (Table 1). Furthermore, the connectivity density – an indicator of how well connected the canal system is – was also significantly higher for the endosteal half than for the periosteal half (Table 1). The cortical porosity estimated from the histological sections reflected the cortical porosity measured by µCT (r = 0.85) (data not shown).

The 2D analysis of the pores radial location showed a significantly higher frequency of pores with a diameter above 100 µm in the endosteal half (p < 0.0001), while pores with diameter of 0–50 µm and 50–100 µm were significantly more abundant in the periosteal half (p < 0.0001, Figure 3H). Clustered logistic regression analysis showed that the endosteal half had a 3-fold higher likelihood of having larger pores (Po.Dm > 100 µm) than smaller pores (Po.Dm < 100 µm) than the periosteal half (OR = 3.6; 95% CI, p < 0.0001, Figure 3I). Though the prevalence of large pores was rather low they contributed to 2.1% and 11.0% of the periosteal and endosteal porosity, respectively (Figure 3J).

**Classification of intracortical pores/sites in terms of remodeling type, stage and location**

In total, 819 of the 948 pores in the histological regions of interest (ROI) were classified according to their remodeling type, stage, and location (Figure 2). The remaining pores were not possible to classify due to unclear lamellar structure or small folds in the cross-sections.

Most pores, 68.2% of the periosteal pores and 78.9% of the endosteal pores, were classified as type 2 pores (Figure 4A).

**Increased endosteal porosity is mainly the result of cumulative eroded type 2 pores**

The endosteal half showed a 1.6-fold higher prevalence of non-quiescent (type E, EF, or F) type 2 pores versus other pore types (OR = 1.6; 95% CI, 1.2–2.1, p < 0.01, Figure 4A) compared to the
periosteal half. Moreover, this pore type was more abundant among the larger endosteal pores (Po.Dm > 100 µm) showing a 7-fold higher probability of being non-quiescent type 2 pores versus other pore types (OR = 7.2; 95% CI, 3.8–13.6, p < 0.0001, Figure 4A) compared to smaller endosteal pores (Po.Dm < 50 µm). The non-quiescent type 2 pores contributed significantly more to the porosity in the endosteal half than in the periosteal half (p < 0.0001, Figure 4B).

Overall, only very few of the identified pores in the fibular specimens showed any signs of bone formation. Instead, the majority of the non-quiescent type 2 pores were eroded (Figure 4C). Considering all the pore types in general, a mean ratio of 6.1 was found between the number of eroded pores (type E-EF) relative to the number of formative pores (type F) (Supp. Table 2).

With regards to the abundance of eroded type 2 pores there was no difference between the endosteal half and the periosteal half. However, among the large pores (Po.Dm > 100 µm), eroded type 2 pores were 5-fold more prevalent than any other pore compared to the smaller pores (OR = 5.5; 95% CI, 2.9–10.3, p < 0.001, Figure 4C). Additionally, type eroded 2 pores contributed significantly more to the porosity in the endosteal half than in the periosteal half (p < 0.001, Figure 4D).

Enlarged pores reflect to great extent pore coalescence

The endosteal and periosteal halves had an equivalent prevalence of coalescing type 2 pores. However, the prevalence of coalescing type 2 pores versus breaking and intraosteonal type 2 pores were 7-fold higher among the large endosteal pores (Po.Dm > 100 µm) than among smaller endosteal pores (Po.Dm 0–50 µm) (OR = 7.1; 95% CI, 2.6–19.7, p < 0.001, Figure 5A). Also, coalescing type 2 pores contributed statistical significantly more to the porosity in the endosteal half than in the periosteal half (Figure 5B, p < 0.05). However, the differences arise from a limited number of biopsies (n = 3) having only a few pores with an extremely extended pore area.
The pore diameter, osteon diameter and wall thicknesses of quiescent type 1 and type 2 osteons

The quiescent type 1 and 2 osteons in the endosteal half had a significantly higher pore diameter than the quiescent osteons in the periosteal half although the magnitudes were not that different ($p < 0.0001$, Figure 6A). The osteon diameter and wall thickness did not differ significantly between endosteal and periosteal quiescent osteons (Figures 6B–C). However, quiescent type 2 osteons had a significantly smaller osteon diameter and wall thickness than quiescent type 1 osteons, irrespective of their intracortical location (Figures 6B–C).

The relationship between wall thickness and osteonal diameter was slightly different for periosteal and endosteal osteons (Figure 6D). The relationship between osteon diameter and wall thickness differed between quiescent type 1 and type 2 osteons ($p < 0.0001$, Figures 6E), as previously shown in iliac specimens (28).
Discussion

Several studies have reported a gradual conversion of the endosteal cortex into what topologically resembles trabecular bone, as a process that considerably contributes to the thinning of cortex and reduces bone strength (5, 34). In the present study, we histomorphometrically investigated the underlaying cellular mechanism responsible for this trabecularization process, which has until now largely remained unknown. Firstly, our study demonstrated that the endosteal half of fibular bone has a higher porosity, associated with an enlargement of the intracortical canals, which gradually trabecularize the endosteal cortex. Secondly, our histomorphometric analysis revealed that the enlarged endosteal canals identified by µCT mainly reflected cumulative eroded type 2 pores. This suggests that a prolongation of the reversal-resorption phase within BMUs remodeling existing canals, observable as eroded type 2 pores in the endosteal half, is a major contributor to endosteal porosity and trabecularization. A prolongation of the reversal-resorption phase could be the consequence of a reduced recruitment of osteoprogenitor cells to the eroded surfaces, causing a delayed initiation of the bone formation. The critical steps contributing to this gradual transformation of the endosteal cortex has been summarized in Figure 7.

Enlarged endosteal pores likely reflect the gradual trabecularization of the endosteal cortex

Our 3D analysis showed that the cortical porosity was much higher in the endosteal half than in the periosteal half. Higher endosteal porosity is strongly associated with an overall decline in bone strength (5, 35). Similar to the present study, others have described a gradual increase in pore size from the periosteal surface towards the endosteal surface at the femoral neck (14, 36-38), tibia (39), rib (40), as well as femur and distal radius (15, 41). Ours and others studies (12, 13, 42-44) highlight that elevated endosteal cortical porosity, and subsequent trabecularization, is associated with an enlarged canal diameter, not a higher canal density.
A prolonged reversal-resorption phase leads to enlarged eroded endosteal type 2 pores

Most previous studies investigating endosteal porosity and trabecularization have used radiological imaging such as µCT or high-resolution pQCT (13, 39, 41, 45-47). Although imaging tools provide a unique and non-destructive investigation of the 3D microstructure, the histomorphometric analysis employed in the present study provides a unique insight into the critical cellular activities responsible for this endosteal porosity.

We have shown that the large endosteal pores (Po.Dm >100 µm) predominantly were pores reflecting remodeling of existing canals (type 2 pores) rather than generation of new canals (type 1 pores). These type 2 pores were recently shown to be increasingly prevalent with age at the iliac crest in women (28). The higher frequency of canals experiencing repeated cycles of remodeling is in line with the 3D µCT data, showing that the endosteal and periosteal halves had similar values for canal spacing and number, but different values for canal diameter indicating that the increased endosteal porosity mostly results from remodeling and enlargement of existing canals rather than creation of new canals. In the endosteum, the majority of the large pores were eroded type 2 pores, while only 2.5% of these pores had formative surfaces and indicates a lowered activation frequency of bone formation. This is supported by a mean ration of 6.1 between the prevalence of eroded (type E-EF) and formative pores (type F) considering all non-quiescent pores throughout the cortex. This ratio is far from what is expected from previous studies, which suggest that the intermixed resorption and reversal phase is expected to last for weeks, while the formation period should take months giving an estimated ratio of approximately 0.25 (21, 48). The present study, together with a previous study of iliac specimens (28), indicate that the reversal-resorption phase lasts longer than first anticipated, supporting the notion that cortical bone loss is caused by a marked prolongation or arrest of the reversal-resorption phase, resulting in a significant delay in the initiation of bone formation. Here, we demonstrate that this prolongation plays an important role in the endocortical
trabecularization. This confirms the previous notion that the trabecularization is the result of an extended resorption phase, as previous hypothesized (14, 16, 49-52).

A possible reason for this prolonged reversal-resorption phase could be due to a deficient supply of osteoprogenitor cells, which is crucial to end the reversal-resorption phase and thus initiate bone formation (21, 23, 53). If the amount of osteoprogenitor cells does not reach the required critical density necessary to initiate bone formation, the BMU remains in the reversal-resorption phase, rendering it susceptible to further secondary resorption and widening of the pore (23). One can further speculate whether this insufficient supply of osteoprogenitor cells is related to an inadequate vascularization, since capillaries are strongly believed to be a prerequisite for bone formation (54-56) supplying the pericytes that ultimately differentiate into mature osteoblasts (57-59). Moreover, the prolonged reversal-resorption phase could also be a consequence of senescence within the reversal cells, which have been shown to be osteoblast-lineage cells (21, 53, 60). However, further analysis of the cells essential for the initiation and termination of the remodeling cycle needs to be performed to clarify the mechanisms involved.

*Large pores often lead to coalescence of more pores into one larger cavity*

The distinctive occurrence of enlarged pores at the endosteal surface has been speculated to be the result of fusion of two or more adjacent pores (41, 50, 51, 61). Our recent studies have linked the generation of enlarged pores during aging to the coalescence of existing pores, due to a prolonged reversal-resorption phase during the remodeling of existing canals (28). This favors an extended secondary resorption widening the canals until they coalesce with other canals (28). The pore coalescence and generation of so-called ‘giant canals or super-osteons’ have also been suggested to be the result of remodeling cluster, which are more prevalent in endosteal cortex, that have an excessive resorption depth with age (49, 62). In the present study, we show that the enlarged
endosteal pores are more prevalent coalescent type 2 pores, but not exclusively coalescent pores. Still, they contribute to 73% of the porosity in the endosteal half. These findings are in agreement with the original suggestion by Arnold, who proposed that the endocortical trabecularization is due to a focal resorption in local pores leading to pore coalescence (51). This again implies that not all pores are necessarily affected.

Pore coalescence may obviously reduce the number of endosteal pores, as proposed by others (41, 49, 61, 62), but may also increase the connectivity between the remaining pores. Here, our μCT results show an increased connectivity density of canals in the endosteal versus the periosteal half, indicating that the endosteal canal-network is more branched than the periosteal canal-network. The coalescence of pores is, however, not only occurring in the endosteal half as shown by a similar level of coalescent type 2 pores in the endosteal and periosteal halves. Similarly, Bell and co-workers reported the presence of giant pores (Po.Dm > 385 µm) in both the mid-cortex and periosteal cortex (49).

*The BMU balance of quiescent osteons is influenced by their remodeling type and radial location*

Considering the quiescent osteons, representing the result of a complete remodeling cycle, some differences exist depending on the pores radial location. Quiescent osteons located in the endosteal half had a slightly, although significantly, larger pore diameter than the quiescent osteons in the periosteal half indicating an altered bone remodeling balance in the endosteal half. Interestingly, in fibular bone the quiescent type 2 osteons versus quiescent type 1 osteons had a smaller osteon diameter and wall thickness than the quiescent type 1 osteons. The smaller osteon diameter and wall thickness in the quiescent type 2 osteons could be related to the higher prevalence of intraosteonal remodeling, whereas in iliac bone quiescent breaking type 2 pores were more abundant (29).
In general, the relationship between wall thickness and osteon diameter differs between endosteal and periosteal quiescent pores, and between quiescent type 1 and type 2 pores. This suggests that the balance between the magnitude of the resorption and formation is influenced by the radial location and the pore type. These differences may reflect an altered rate, duration, and starting point of the resorption and formation.

One may speculate whether the largest, quiescent osteons may have a resorbed area that exceeds a critical size at which it is impossible to refill the pore (29). Nevertheless, the total number of extremely enlarged quiescent osteons with a pore diameter above 300 µm was less than nine, consequently the quiescent osteons are not contributing significantly to the cortical porosity in fibular bone, as we have previously shown to be the case for iliac bone (29).

Limitations of the study

One limitation is the voxel size (6 µm) used for the 3D µCT analysis making the smallest resolvable object 12 µm according to the Nyquist sampling theorem. Moreover, intracortical cavities less than 20 connected voxel were removed from the 2D analysis, in order to prevent the inclusion of any osteocyte lacunae. However, this also means that small canals with a spotted appearance were removed from the 2D µCT analysis and the histological analysis. In Figure 6A, it is apparent that pores of quiescent osteons with a diameter below 15 µm were excluded from the histological analysis. Moreover, the histological analysis was limited to a ROI with approximately 50 pores, in order to limit the demanding and highly time-consuming histological analysis. The inclusion of all small canals and a larger ROI in the histological analysis may have improved the significance of the findings, but it is questionable whether it would have changed the main conclusion of the study. One also needs to bear in mind that the endosteal cortex is likely under sampled as this highly trabecularized part may no longer be recognized as cortex, a general problem emphasized in many
studies of cortical bone (1, 3, 13). It was not possible to perform dynamic histomorphometry with fluorochrome labeling due to the specimen’s collection procedure. Finally, the present study was limited to fibular bone specimens from both women and men, but without a sufficient number to address the influence of age and sex. Thus, a detailed histological investigation of the intracortical pores from other skeletal sites, different ages, and a comparison between the two sexes is warranted in order to give a more complete picture of the critical intracortical remodeling mechanisms contributing to the enhanced endosteal porosity.

Future perspectives

Future studies of pathological specimens might reveal how recruitment and proliferation of osteoprogenitor cells is critical for initiation of the bone formation – discoveries that could potentially influence the development of future therapies not only focusing on the resorptive but also on the reversal-resorption phase and the initiation of the bone formation.

Conclusion

The present study shows that the endocortical trabecularization of fibular bone results from accumulation of enlarged eroded cavities upon existing canals, which reflects BMUs with a prolonged reversal-resorption phase, favored by a delayed initiation of bone formation. The resorptive area of observable eroded pores often expands, ultimately merge, and create enlarged coalescent cavities. This process is the main contributor of increased endocortical porosity and to the gradual endocortical trabecularization – at least in fibular bone.
Acknowledgements

We would like to thank Gete Ester Toft Eschen at Aarhus University Hospital for collecting the analyzed bone specimens. Further we would like to acknowledge Jette Barlach, Kaja Søndergaard Laursen, and Birgit MacDonald for their excellent technical assistance in the laboratory.

We want to acknowledge the Region of Southern Denmark Research Fund (MD, J. no. 15/24851), Aase & Ejnar Danielsen Foundation (10-001584) and VELUX Foundation (VELUX34368) for funding the study. We also want to thank VELUX Foundation for kindly donating the μCT scanner (VELUX26922).

Author roles: TLA, JST, CMA, JMD, and MEK designed the study. All bone specimens were obtained by EMH, BJK, and GTE. JST and AB conducted the μCT analysis; LPB and MEK conducted the 2D structural analysis, while all histological measurements and subtyping’s were performed by CMA and validated by TLA. The manuscript was drafted by CMA, JST, and TLA and revised by all authors before final approval.
References


locations as determined by synchrotron micro-computed tomography images. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2013;24(3):1023-33. doi:10.1007/s00198-012-2044-4


44. Thompson DD. Age changes in bone mineralization, cortical thickness, and haversian canal area. Calcified tissue international. 1980;31(1):5-11.


Figure legends

Figure 1
Analysis of the intracortical bone remodeling was conducted on bone specimens from the fibular diaphysis obtained from 6 women and 14 men. The specimens were µCT-scanned and plotted in 3D with the canals shown in white and the bone in semi-transparent red (A). One µCT image from each specimen had all pores labeled generating a pore map (B). The corresponding Masson-Goldner trichrome stained section was used for histological investigation (C). The distance of the centroid of each pore to the periosteum ($d_1$, blue hashed line) and endosteum ($d_2$, orange hashed line) as well as the radial location, pore diameter/perimeter, wall thickness and area of the pores were determined (D). The rectangular frames in figures 1B and C illustrate the magnified area in 1D, right. In figure 1D the pores are outlined with encircled red lines, while the green circles represent the largest possible circle inscribed into the pore; the ellipse shows the largest is in osteon #75.

Figure 2
Examples of new intracortical remodeling sites and their appearance in histological cross-sections. Type 1 and type 2 pores reflecting either the generation of a new pore or the remodeling of an existing pore, respectively, as they appear in histological cross-sections. All pores were classified according to their remodeling stage (Figure 2). Eroded pores (type E pores) have eroded surfaces without any osteoid; mixed eroded and formative pores (type EF pores) have both eroded and formative surface; formative pores (type F pores) have only osteoid surfaces; and finally quiescent pores with a terminated remodeling (type Q pores) show no signs of erosion or osteoid. Additionally, the pores were further subdivided based on their position in relation to their parent osteon, thus type 2 pores were classified as either; intra-osteonal type 2 pores (type 2IN) reflecting pores with a resorptive area inside the cement line of the existing parent osteon; breaking type 2 pores (type 2BK) reflecting pores with a resorptive area breaking through the cement line of the existing parent osteon, or coalescing type 2 pores (type 2CO) reflecting pores with a resorptive area that overlap the pores of two or more existing parent osteons. The pores are illustrated as they appear in histological cross-sections through a remodeling process, which is initiated by a resorptive phase followed by a reversal-resorption phase eventually leading to a formation phase and eventually enter a quiescent phase where the remodeling is resting. The white dotted line illustrates that type 1 pores could develop into a type 2 pore if the resorbed area covers the area of another osteon.
**Figure 3**
Structural properties and radial location of all the analyzed intracortical canals/pores. 3D plots of µCT scans of the endosteal and periosteal half of one bone sample (A). Probability density functions of canal diameter (B) and canal spacing (C) for all samples combined. The canal diameter was positively correlated with cortical porosity for both endosteal and periosteal canals, whereas the canal spacing was negatively correlated with cortical porosity (D). Canals in the endosteal half had a larger canal diameter (E), were spaced similarly to the periosteal half (F) and resulted in a higher cortical porosity (G). Each dot represents the mean in a given specimen/individual (E, F, G), the red lines represent the means (B, C), the horizontal lines indicate the median for each type (E–G), while bars indicate maximum and minimum values (H). 2D analysis showed that large pores were more abundant in the endosteal half than the periosteal half (H, I) showing a higher frequency of these large pores in the endosteal half than in the periosteal half (I) contributing to a higher endosteal porosity (J).
Statistically significant differences between the parameters in the endosteal and periosteal halves were calculated by a Students t-test (E–G) or a Mann-Whitney test (J), while differences in the pores radical location were calculated by a Kruskal-Wallis test followed by Dunn’s multiple comparison test (H). Clustered logistic regression analysis was used for the calculation of odds ratios (I): *p < 0.05, ****p < 0.0001.

**Figure 4**
The relation between pore types and their radial location. Among the large endosteal pores (pore diameter >100 µm) there were a 7–fold higher likelihood of non-quiescent pore types (2E–2F) (A), and they contributed to a higher endosteal porosity (B). Especially eroded type 2 pores are abundant (C) and contribute to the enhanced endosteal porosity (D). Clustered logistic regression analysis was used for calculation of odds ratios (A, C), while the difference in contribution of non-quiescent type 2 pores at periosteal and endosteal sides was assessed using a Mann-Whitney test (B, D): **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 5
The radial location of type 2 pores in different positions. The overall difference in the prevalence of coalescing type 2 pores comparing endosteal with periosteal, and comparison in between pore sizes for endosteal pores alone (A) and their contribution to the overall cortical porosity (B). Odds ratios were calculated by clustered logistic regression analysis (A), while the differences in contribution of coalescing type 2 pores at periosteal and endosteal halves was assessed by using a Mann-Whitney test (B): *p < 0.05, ***p < 0.001.

Figure 6
Comparison of the remodeling balance in periosteal and endosteal located type 1Q and 2Q osteons. The pore diameter (A), osteon diameter (B), and wall thickness (C) for periosteal and endosteal quiescent osteons. Additionally, the relationships between the wall thickness and osteon diameter comparing periosteal and endosteal pores (D) and quiescent type 1 and type 2 pores (E). Horizontal lines indicate the median of each group and statistically significant differences between periosteal and endosteal bone were calculated by a Mann-Whitney test (A–C), while linear regression analysis was used to establish the relationship between the osteon diameter and wall thickness for periosteal and endosteal pores (D) and for quiescent type 1 and type 2 pores (E): **p < 0.01, ****p < 0.0001.

Figure 7
Model illustrating that the gradual trabecularization of the cortex mainly located in the endosteal half also have the highest intracortical porosity. In the endosteal half the pores generally have a larger diameter and type 2 remodeling events (remodeling of existing canals) are more frequent. Type 2 pores, and especially eroded type 2 pores, possibly with an extended reversal-resorption phase and a delayed or absent bone formation phase ultimately leads to the coalescence of these pores into enlarged coalescing type 2 pores. This merging of two or more pores create larger resorptive cavities is a major contributor to the ongoing endosteal trabecularization gradually reducing the cortical thickness.

Supplementary table 1
The pore type as well as their remodeling stage and position were determined based on the specified characteristics.
Supplementary table 2
Overview of the sex and age-distribution among the analyzed specimens as well as the ratio between type EF and type F pores in the entire bone specimen and subdivided into endosteal and periosteal bone. Furthermore, the estimated cortical thickness of the analyzed bone samples. Statistically significant differences between the ratios were calculated by a Students $t$-test.

Supplementary figure 1
Examples of new intracortical remodeling sites and their appearance in histological cross-sections. Type 1 and type 2 pores at the four remodeling stages: E (eroded), EF (eroded + formative), and F (formative) reflecting non-quiescent pores with a non-terminated remodeling, while Q (quiescent) pores reflect the pores with a terminated remodeling.

Supplementary figure 2
3D plots of $\mu$CT scans of the entire bone sample (A). Probability density functions for the canal diameter distributions for the endosteal and periosteal half (B). The large peak in the canal diameter probability density function for patient 2 (endosteal side) is caused by the two large canals seen to the left in the endosteal half of the bone as the distribution is volume weighted. The blue line represents the median and the red line represents the mean. Probability density functions for the canal spacing for the endosteal and periosteal halves (C).
### Measurements of pores:

- Pore diameter (Po.Dm)
- Osteon diameter (O.Dm)
- Wall thickness ($W$.Th) = \( \frac{O.Dm - Po.Dm}{2} \)
- Radial location = \( \frac{d_1}{d_1 + d_2} \)
Characterization of pores:

Type 1 pore (Type 1):
(penetrative resorption generating a new osteon)

Intra-osteonal type 2 pore (Type 2a):
(within the cement line of existing parent osteon)

Breaking type 2 pore (Type 2b):
(breaking the cement line of existing parent osteon)

Coalescing type 2 pore (Type 2c):
(overlapping with the pore two or more existing parent osteons)

Eroded (E)
Eroded & Formative (EF)
Formative (F)
Quiscence (Q)

Osteoclast
Osteoblast
Reversal cell
Bone lining cell
Resorbed area
Cement line of existing osteon
Outlining of original pore/canal

Figure 2:
Figure 3:

A. Periosteal vs. Endosteal images.

B. Probability density of canal diameters for Periosteal and Endosteal regions.

C. Probability density of canal spacings for Periosteal and Endosteal regions.

D. Correlation between canal diameter and spacing for Periosteal and Endosteal regions.

E. Scatter plot showing canal diameter distribution for Periosteal vs. Endosteal regions.

F. Scatter plot showing canal spacing distribution for Periosteal vs. Endosteal regions.

G. Scatter plot showing cortical porosity distribution for Periosteal vs. Endosteal regions.

H. Box plot of radial location for different ranges of Po.Dm (µm) and location (Peri vs. Endo).

I. Heatmap showing % of all pores for different ranges of Po.Dm (µm) and location (Peri vs. Endo).

J. Bar graph showing cortical porosity for different ranges of Po.Dm (µm) and location (Peri vs. Endo).
Figure 4:

A) Graph showing the percentage of different types of pores: 2 Non-Q, 2 Q, 1 Non-Q, 1 Q. The bars are divided into Po.Dm (μm): All Peri, All Endo, 0-50, 51-100, >100.

B) Graph showing cortical porosity (%). The bars are divided into Part: All Peri, All Endo.

C) Graph showing the percentage of all type 2 pores. The bars are divided into Po.Dm (μm): All Peri, All Endo, 0-50, 51-100, >100.

D) Graph showing cortical porosity (%). The bars are divided into Part: All Peri, All Endo.

OR: 5.5***
OR: 1.3
OR: 7.2****
OR: 1.7

% of all type 2 pores

2 Non-Q
2 Q
1 Non-Q
1 Q
2E
2EF
2F
2Q
Figure 5:

A

% of all type 2 pores

Or: 1.5
Or: 1.7
Or: 7.1***

Po.Dm (μm): All Peri All Endo 0-50 51-100 >100

B

Cortical porosity (%)

Po.Dm (μm): All Peri All Endo

*
Type 1Q: $r = 0.83$, slope 0.41

Type 2Q: $r = 0.72$, slope 0.31

$p < 0.0001$

Endo: $r = 0.70$, slope 0.33

Peri: $r = 0.81$, slope 0.38

$p < 0.001$

$n = 3$, $p < 0.01$
Figure 7:

<table>
<thead>
<tr>
<th></th>
<th>Peri</th>
<th>Endo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical porosity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pore diameter:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2 pores:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2E pores:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Enlarged eroded pores
- Coalescent pores
- Endocortical trabecularization
Supplementary figure 1:

Type 1 pore (Type 1): (penetrative resorption generating a new osteon)
Intra-osteonal type 2 pore (Type 2_{in}): (within the cement line of existing parent osteon)
Breaking type 2 pore (Type 2_{br}): (breaking the cement line of existing parent osteon)
Coalescing type 2 pore (Type 2_{co}): (overlapping with the pore of two or more existing parent osteons)

Eroded (E)
Eroded & Formative (EF)
Formative (F)
Quiscence (Q)

Osteoclast
Osteoblast
Reversal cell
Bone lining cell
Eroded surface/cement line lining resorption area
Cement line of existing osteon
Quiescent surface (QS)
Osteoid
Supplementary figure 2:

A

Patient 1

Cortical

Patient 2

Cortical

Periosteal

Endosteal

Periosteal

Endosteal

B

C

D

Canal diameter (µm)

Canal diameter (µm)

Canal spacing (µm)

Canal spacing (µm)

Probability density (µm⁻¹)

Probability density (µm⁻¹)

Probability density (µm⁻¹)

Probability density (µm⁻¹)
### Tables

**Table 1:** Results of the 3D µCT-based histomorphometrical evaluation. Mean ± SD. *p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Endosteal</th>
<th>Periosteal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct.Po (%)</td>
<td>14.4 ± 10.5</td>
<td>4.23 ± 2.4*</td>
</tr>
<tr>
<td>Ca.Dm (µm)</td>
<td>213 ± 120</td>
<td>73.3 ± 34.4*</td>
</tr>
<tr>
<td>Ca.Sp (µm)</td>
<td>377 ± 97.3</td>
<td>365 ± 60.7</td>
</tr>
<tr>
<td>Ca.N (mm⁻¹)</td>
<td>2.72 ± 0.47</td>
<td>2.78 ± 0.42</td>
</tr>
<tr>
<td>Ca.Conn.D (mm⁻³)</td>
<td>34.3 ± 20.3</td>
<td>20.0 ± 14.7*</td>
</tr>
<tr>
<td>Ca.DA</td>
<td>2.83 ± 0.41</td>
<td>2.82 ± 0.48</td>
</tr>
</tbody>
</table>
Supplementary table 1

Categorization of pores:

<table>
<thead>
<tr>
<th>Remodeling type:</th>
<th>Characteristics:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>The resorptive area does not overlap with a pore of an existing osteon</td>
</tr>
<tr>
<td></td>
<td><em>(Reflecting the generation of a new pore)</em></td>
</tr>
<tr>
<td>Type 2</td>
<td>The resorptive area overlap with a pore of an existing osteon</td>
</tr>
<tr>
<td></td>
<td><em>(Reflecting the remodeling of an existing pore)</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Remodeling stages:</th>
<th>Characteristics of the bone surface of the pores:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage E</td>
<td>Eroded surface and no osteoid</td>
</tr>
<tr>
<td>Stage EF</td>
<td>Both eroded and formative surface</td>
</tr>
<tr>
<td>Stage F</td>
<td>Only formative surface</td>
</tr>
<tr>
<td>Stage Q</td>
<td>Quiescent surface <em>(Terminated bone remodeling)</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spatial location:</th>
<th>Location of resorptive area relative to pores of existing osteons:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position IN</td>
<td>Within the cement line</td>
</tr>
<tr>
<td>Position BK</td>
<td>Breaking through the cement line</td>
</tr>
<tr>
<td>Position CO</td>
<td>Overlapping with the pore of two or more parent osteons</td>
</tr>
<tr>
<td>Patient</td>
<td>Sex</td>
</tr>
<tr>
<td>---------</td>
<td>-----</td>
</tr>
<tr>
<td>1</td>
<td>Man</td>
</tr>
<tr>
<td>2</td>
<td>Man</td>
</tr>
<tr>
<td>3</td>
<td>Man</td>
</tr>
<tr>
<td>4</td>
<td>Man</td>
</tr>
<tr>
<td>5</td>
<td>Man</td>
</tr>
<tr>
<td>6</td>
<td>Man</td>
</tr>
<tr>
<td>7</td>
<td>Man</td>
</tr>
<tr>
<td>8</td>
<td>Man</td>
</tr>
<tr>
<td>9</td>
<td>Man</td>
</tr>
<tr>
<td>10</td>
<td>Man</td>
</tr>
<tr>
<td>11</td>
<td>Man</td>
</tr>
<tr>
<td>12</td>
<td>Man</td>
</tr>
<tr>
<td>13</td>
<td>Man</td>
</tr>
<tr>
<td>14</td>
<td>Man</td>
</tr>
<tr>
<td>15</td>
<td>Woman</td>
</tr>
<tr>
<td>16</td>
<td>Woman</td>
</tr>
<tr>
<td>17</td>
<td>Woman</td>
</tr>
<tr>
<td>18</td>
<td>Woman</td>
</tr>
<tr>
<td>19</td>
<td>Woman</td>
</tr>
<tr>
<td>20</td>
<td>Woman</td>
</tr>
<tr>
<td>MEAN</td>
<td>-</td>
</tr>
<tr>
<td>SD</td>
<td>-</td>
</tr>
<tr>
<td>p</td>
<td>-</td>
</tr>
</tbody>
</table>