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Research Article

Osteocyte numbers decrease only in postcranial but not in cranial bones in humans of advanced age

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ABSTRACT

Background: Bone ageing is governed by the linked activities of short-lived osteoblasts and osteoclasts in conjunction with long-lived osteocytes present in osseous structure. Besides their maintenance function, osteogenic cells also gain specific positional information, which may potentially trigger ageing-associated cellular deviations in terminally differentiated osteocytes differently in cranial versus postcranial tissues.
Methods: We therefore investigated bone taken from deceased aged humans explanted at five distinct anatomical positions throughout the body and assessed physical and biological determinants applying radiologic and histologic measures.
Results: We were able to show that significantly more osteocytes reside in aged cortical bone at cranial positions than within axial or limb skeleton. These cellular states and conditions were not found in the corresponding trabecular bone, where osteocyte numbers remain also high at postcranial positions. Parallel comparative analyses of bone microstructure as analyzed by means of computer tomography showed no significant differences.
Conclusions: Considering differences and commonalities regarding the bone samples, such as loading, mechanisms of ossification or the surrounding stromal cell compartment, our findings indicate that positional information laid down during ontogenetic processes is instructive during the entire life thus potentially also moulding spatial-specific mechanistic distinctions of bone ageing.

1. Introduction

Bone structure is to be maintained throughout life (Boskey and Coleman, 2010). The macroscopic osseous framework is made of cortical (compact) and trabecular (woven and lamellar) structures. At the microscopic level, bone is composed of cells, matrix, and mineral. Adult bone is continuously moulded through the distinct activities of two short-lived cell types, bone-forming osteoblasts and bone-resorbing osteoclasts (Almeida and O'Brien, 2013). Yet bone also harbours a long lived cell type with a lifespan of up to 50 years, the terminally differentiated osteocyte (Manolagas and Parfitt, 2010). Osteocytes are derived from osteoblasts, and fully embedded in matrix, thereby making up approximately 90% of all cells within compact bone, and together forming the canalicular-lacunar network (Franz-Odendaal et al., 2005). Notably a specific difference in formation of mineralized bone matrix is that precursor cells undergo endochondral ossification commenced by emerging cartilaginous progenitors, or alternatively directly promote intramembranous ossification (Olsen et al., 2000). There is a strong notion in the field that cells hold on to these specific ontogenetic programmes such as to facilitate life-long osseous repair. It is further believed that stromal cells in conjunction with their mesenchymal niche bear positional information, which is for instance genetically determined through the phylogenetically conserved HOX gene code. This genetic programme specifically determines positional information along the axes of the main body as well in appendages (Leucht et al., 2008). Similarly, mesenchymal stromal cells taken from varying anatomical positions exhibit different regenerative capacities (Matsubara et al., 2004; Reichert et al., 2013). Besides their site-specific, genetically set phenotype, bones are constantly exposed to loading, which through cell-driven remodelling processes can lead to individual morphological appearances as well as to age-associated wear and tear (Metzger et al., 2017).

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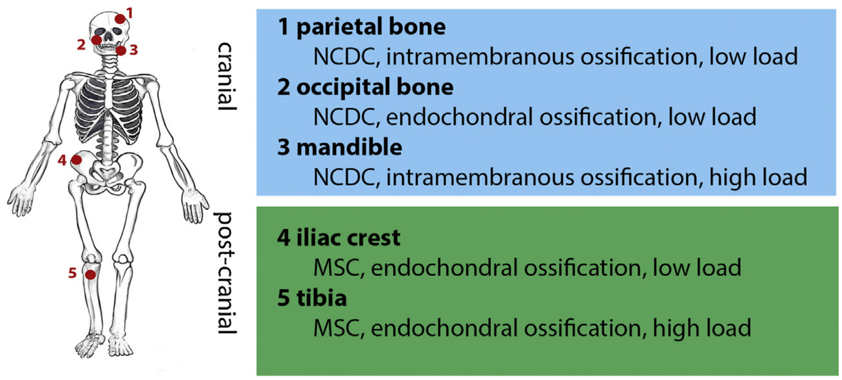

Fig. 1. Bone samples were isolated from the indicated anatomical sites. Most cranial bone is formed by neural crest-derived cells (NCDC). Postcranial bone develops from mesodermal primordia and later in life from mesenchymal stromal cells (MSC). The types of bone investigated undergo a specific mode of ossification, which is either endochondral or intramembranous, and experience different degree of load.

Within osseous hard tissue layers, osteocytes form a network interconnected through the exuberant number of dendritic processes (Noble and Reeve, 2000; Romanos, 2016). Mechanical loading results in a fluid flow and shear forces, membrane deformation, and tension in elements connecting osteocytes with the canalicular walls. These cellular interactions have been shown to trigger the release of anti-apoptotic factors (Cardoso et al., 2009). It is thus conceivable that old bone, which lacks effective mechanical stimuli, or has accumulated microfracture fatigue actually exhibits a disrupted canalicular network thus over time ensuing a microenvironment for enhanced cell death. Interestingly, osteocyte apoptosis is thought to upregulate osteoclast and osteoblast function thereby promoting local remodelling of bone tissue (Rochefort et al., 2010). Hence osteocytes, which are normally deeply buried within bone are capable of sensing microcracks and variant loading and thus are capable of guiding and instructing bone remodelling, fracture healing or osseointegration of implants (Romanos, 2016).

In ageing patients, clinical observations, in particular regarding individual regenerative capacities, suggest that cellular constitution and/or properties of the corresponding osseous niches undergo distinct changes during life. In line with this, particular differences in regenerative properties have been observed between cranial and postcranial bone. Recently, we found in aged mice that their long bones contained elevated numbers of lacunae unoccupied with osteocytes, while mandibular bone appeared uncompromised with a high density of intraosseous long-lived osteocytes well preserved into old ages (Stigler et al., 2018). Provided these results in a short-lived species, we posed the question, whether aged human bone is also prone to distinct changes, in particular deviations in osteocyte numbers. We therefore collected bone samples from different skeletal sites in aged adults. A range of specimens were examined by radiologic and histologic means in order to discern putative characteristics, which point at specific differences correlating to position in the body and thus ontogenetic mechanisms such as type of ossification, or lifelong physical loading which moulds specific distinctions of ageing bones.

2. Materials and methods

2.1. Sample harvesting and preparation

Human bone samples were explanted from human cadavers of people, who consented during lifetime the donation of their dead bodies for the use in scientific research and medical trainings. The cadaveric bodies had been preserved by means of intra-arterial perfusion with formaldehyde-phenol solution for at least one up to three months (Platzer et al., 1978). All explanted samples were additionally fixed in 4% neutral-buffered formalin shortly after isolation. After dehydration in gradually increasing alcohol concentrations, embedding in Technovit 9100 (Heraeus Kulzer, Hanau, Germany) was performed while cooling at 4 °C to avoid blistering.

Samples were taken from ten well-preserved cadaveric bodies (six males and four females). The mean age of the donors was 84.7 years (SD 10.7), the oldest was 101 years old, the youngest 64 years. Samples from the mandible, parietal bone, occipital bone and tibia were taken from all patients using a ten-millimetre trephine drill. Additional iliac crest samples could be collected from four donors. The harvesting location in the mandible was restricted to the mandibular body located at the first premolar and not to the alveolar process. The parietal region was restricted to the area two cm above the origin of the temporal muscle. At the occipital bone, the samples were taken one cm behind the foramen magnum. The iliac crest samples were explanted exactly four cm posterior to the spina iliaca anterior superior. The tibial samples were taken from the inferior region of the tuberositas tibiae (Fig. 1).

2.2. 2.2 μCT scanning

The samples were scanned with a μCT device (Viva CT 80; Scanco Medical AG, Brüttisellen, Switzerland) operated at 70 kVp, 114 μA, 8 W, 31.9 mm FOV, an integration time of 1167 ms and 2× frame averaging. The data sets were reconstructed into 3D volumes with an isotropic nominal resolution of 10.4 μm voxel size. The μCT data were analyzed with Scanco software. The following parameters were assessed: bone volume fraction (BV/TV) either in cortical and trabecular compartment, bone mineral density (in mg hydroxyapatite per mm^3) of cortical bone and trabecular bone samples, trabecular thickness (Tb.th.), trabecular number (Tb.n.) and trabecular separation (Tb.sp.). For detailed description of these parameters see Bouxsein et al. (2010).

2.3. Histological evaluation

The embedded samples were cut with a diamond saw (Exakt, Germany) such that cutting planes included both, the cortical and cancellous bone. Per human cadaver, three histological specimens per region were produced. The specimens were mounted on microscopic slides (Thermo Scientific, Menzel Gläser, Braunschweig, Germany) and consecutive sections were ground to a thickness of 20 μm as described previously (Donath and Breuner, 1982). After dissolving of Technovit 9100 with methylglycol (Merck, Germany) and consecutive rehydration with decreasing alcohol concentrations the specimen were stained with toluidine blue (Carl Roth, Karlsruhe, Germany) in order to enumerate osteocyte densi-

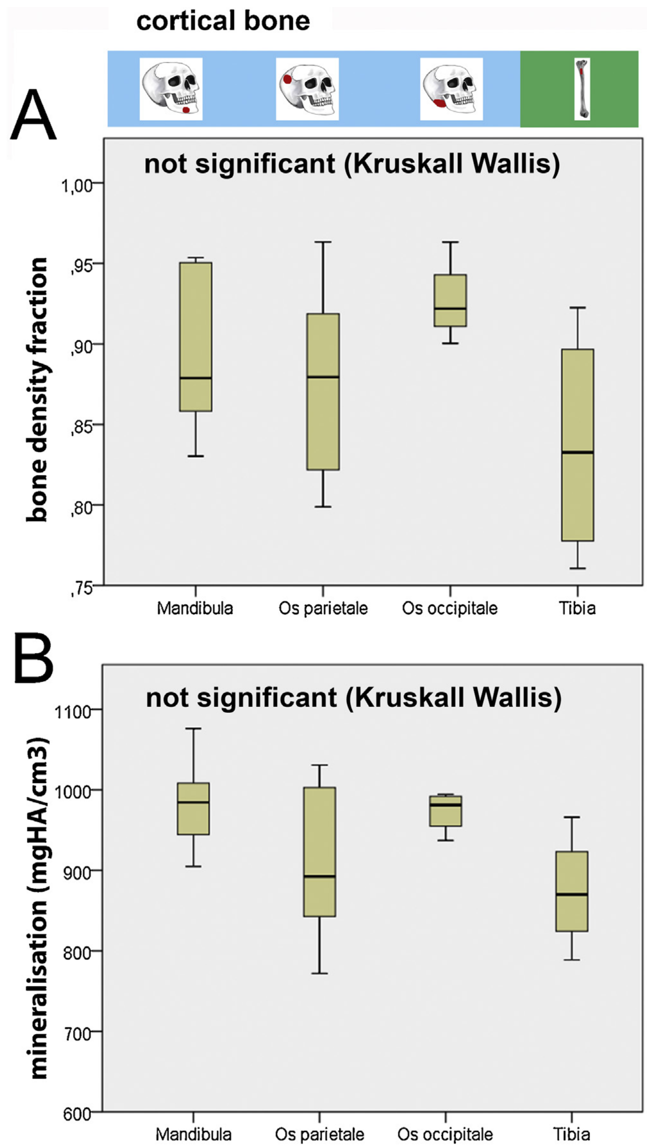

Fig. 2. Quantitative micro-computer tomographic analysis of cortical bone isolated from the mandible, the skull, the neck region and long bone of ten individuals of advanced age. Statistical evaluation revealed that the cortical bone density fraction (A) and mineralization given in mgHA/cm^3 (B) hardly differs.

ties. Nomenclature was according to the international guidelines recommended by the ASBMR histomorphometric nomenclature committee (Parfitt and Recker, 1987). Therefore, the histologic slides were separated in a cortical and a cancellous part. Within both compartments, three randomly chosen areas (ROI) were photographed at 200-fold magnification using an upright Nikon Eclipse 8001 computerized light microscope (Nikon GmbH, Austria microscope, NIS Elements BR 3.0, Nikon GmbH, Austria) which incorporated a calibrated length standard (ImageJ NIH viewer). The total osseous area (B.Ar.) was determined by carefully excluding regions of the lacunae, the bone marrow and the vessels. The osteocyte numbers were assessed (N.Oc.) within these demarcated regions and the fraction of osteocytes per bone area was calculated (N.oc./mm^2).

2.4. Statistical analysis

Statistical analyses were performed with the aid of SPSS Software (SPSS 24.0.0.1; IBM, Chicago, IL). The mean and standard deviations were calculated for descriptive purposes. In order to determine, which of the sample pairs are significantly different the Kruskall–Wallis test with Mann–Whitney-*U* post hoc testing and Bonferonni correction for multiple testing were performed, considering *p*-values below 0.05 to characterize significant differences.

3. Results

3.1. Radiologic results

Bone density (defined as bone volume per total volume) of the cortical part of the investigated bone samples was on average 88.3% (Fig. 2A). Although not reaching statistical significance, the densities of the corresponding cancellous compartments revealed a higher diversity yielding on average 38.02% osseous density, yet with a standard deviation of 14.41%. Parietal (49.34%; SD 6.6) and occipital bone (47.00%; SD 18.6) displayed higher densities than that found in mandible (29.53%; SD 11.61) or in tibia (27.44%; SD 5.98) (Fig. 3A). Also analyses regarding mineralization revealed no significant difference, also not when compared between the cortical and spongeous compartments, which were on average 1057 mm HA/cm^3 SD 51 in cortical bone, or 1040 mg HA/cm^3 SD 45 in trabecular bone (Figs. 2B and 3B). Moreover, trabecular number (Fig. 3C), trabecular thickness (Fig. 3D) and trabecular separation (Fig. 3E) did not reveal obvious and major differences in bones taken from different anatomical sites, with the exception of the mandible, for which the inter-individual differences became apparent, as these evaluations showed rather large standard deviations (Fig. 3C–E).

3.2. Osteocyte density

Osteocyte density was assessed by histological sectioning in conjunction with digital image analysis (Figs. 4 and 5). Cortical bone revealed a mean density of 4.33 cells/mm^2 in the mandible (SD 0.65) which was comparable to the parietal bone (3.99 cells/mm^2; SD 0.76) and occipital bone samples (4.05 cells/mm^2; SD 0.83). Compared to bone from the different cranial tissues, considerably less cells were found in the cortical compartments of tibia bone samples (2.47; SD 0.69; $p = 0.001$ compared to mandible, $p = 0.015$ compared to parietal bone, $p = 0.01$ compared to occipital bone) (Fig. 6A). In order to strengthen this finding, also bone samples from iliac crest were harvested from another four individuals. Osteocyte density was lower (2.07; SD 0.3) compared to cranial positions ($p = 0.001$ compared to mandible, $p = 0.007$ compared to parietal bone, $p = 0.005$ compared to occipital bone) (Fig. 6A). In contrast, trabecular bone samples revealed higher osteocyte densities (mean 6.25 cells/mm^2 (SD 1.35)). This latter measure however neither displayed significant differences between the different anatomical sites nor showed high inter-individual deviations (Fig. 6B).

4. Discussion

In humans of advancing age, the osteocyte density was significantly lower when evaluating cortical bone samples derived from axial or limb skeleton compared to head skeleton. In stark contrast, osteocyte densities in the corresponding trabeculae did not show considerable deviations. Notably, μCT-based structural analyses revealed no significant differences in cortical and cancellous bone tissues, thus pointing at comparable bone micro structures.

Osteocytes mediate homeostatic osseous adaptation to mechanical forces. Osteocytes appear to communicate with osteoclasts and osteoblasts thereby regulating cellular recruitment and activities when in need for bone regeneration, hence directing remod-

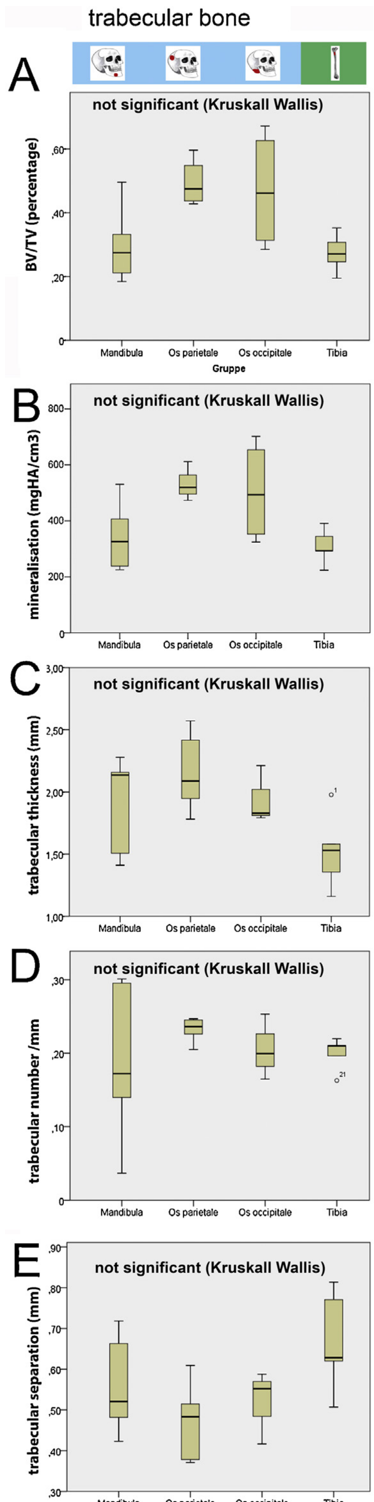

Fig. 3. Quantitative assessment of properties and features found in trabecular structures in the mandible, the skull, the neck region and in long bones of ten individuals of advanced age. Density (A), mineralization in mgHA/cm^3 (B), the trabecular number (C), thickness (D) and separation (E) were found alike.

elling processes through governing bone resorption and formation (Manolagas and Parfitt, 2013).

We investigated samples explanted from deceased humans, who had donated their bodies for research and teaching purposes to the Institute of Anatomy of the Innsbruck Medical University. Persons eventually coming down with infectious diseases are always excluded from body donation (Konschake and Brenner, 2014). Due to the constant high numbers of body donations, the current random sampling likely represents a cohort of common age-associated characteristics within a Western Society population, thus including individuals suffering from the standard variety of chronic or acute diseases, such as diabetes, osteoporosis, or hypertension. We considered a post-mortem study particular suitable as it opened the special opportunity measuring the properties of a large range of different samples taken from the exact same anatomical positions from different human bones from individuals of considerably advanced ages.

Examining these bone samples, significant differences became apparent primarily concerning osteocyte densities. Technical advancements including micro-computed tomography allow to study volumetric microstructural differences of bone biopsies, and structural changes over time also in vivo (Chapurlat, 2016). Despite, in order to discern variant osteocyte densities in aged bone, we were unable to conceive other methods than histological evaluation.

Differences between cranial and postcranial bone from the axial and limb skeleton have been reported previously, especially regarding specific mechanisms of ossification, the embryonic origin of precursor cells and regenerative capacities in older patients (Leucht et al., 2008; Matsubara et al., 2004; Olsen et al., 2000). Interestingly, we did not observe differences between occipital and parietal bone, which however greatly differ regarding ossification modes. Parietal bone develops directly from aggregating stem cells, while the interior part of occipital bone grows from chondrocytes prior to the emergence of osteocytes and ossification. In order to strictly collect endochondral ossifying bone, the occipital bone samples were taken from the occipital area directly behind the foramen magnum.

Bone remodelling is initiated by osteocytes, mostly as a result of changed loading or microcracks (Del Fattore et al., 2012). We therefore also considered the role of loading. Calvaria shows quite stable loading conditions (Iizuka et al., 2004; Mertens et al., 2013), while mandibles exhibit high loadings during chewing. Hence, significant influence of different remodelling activities should be expected. Interestingly however highly loaded tibial and mandibular bone showed no consistent difference when compared to unloaded parietal and occipital bone, or the iliac crest.

Different osteocyte numbers can be a result of different lifespan or different remodelling activities. To our knowledge, no experimental data on distinction of the lifespan of human osteocytes is available. Not-remodelling bone taken from the iliac crest has been shown previously to contain decreasing numbers, and this number was exponentially declining with age at a fractional rate of about 2.5% per year (Qiu et al., 2002). This is in line with our observation and explains the greatly unchanging osteocyte numbers residing in trabecular bone, which is being constantly remodelled. Yet this notion is in stark contrast to our finding that deep layers of aged cranial bone still contain high numbers of osteocytes. High osteocyte numbers at sites that experience both indifferent loading and ossification modes could however be due to positional information laid down during ontogenetic processes (Olsen et al., 2000). Indeed, stromal cells taken from different anatomical sites share much communality such as surface marker profiles and thus may exhibit greatly differing regenerative capacity. Osteocytes are derivatives from stromal progenitor cells. Interestingly stromal cells harvested from the mandible show poor chondrogenic or adipogenic differentiation capacity when compared to stromal cells derived from the iliac crest (Matsubara et al., 2004). It is therefore conceivable that osteocytes still bear positional information at old age, which specifically dates back to their developmental origin, representing marks, which are capable of coining cellular longevity in a site-specific fashion.

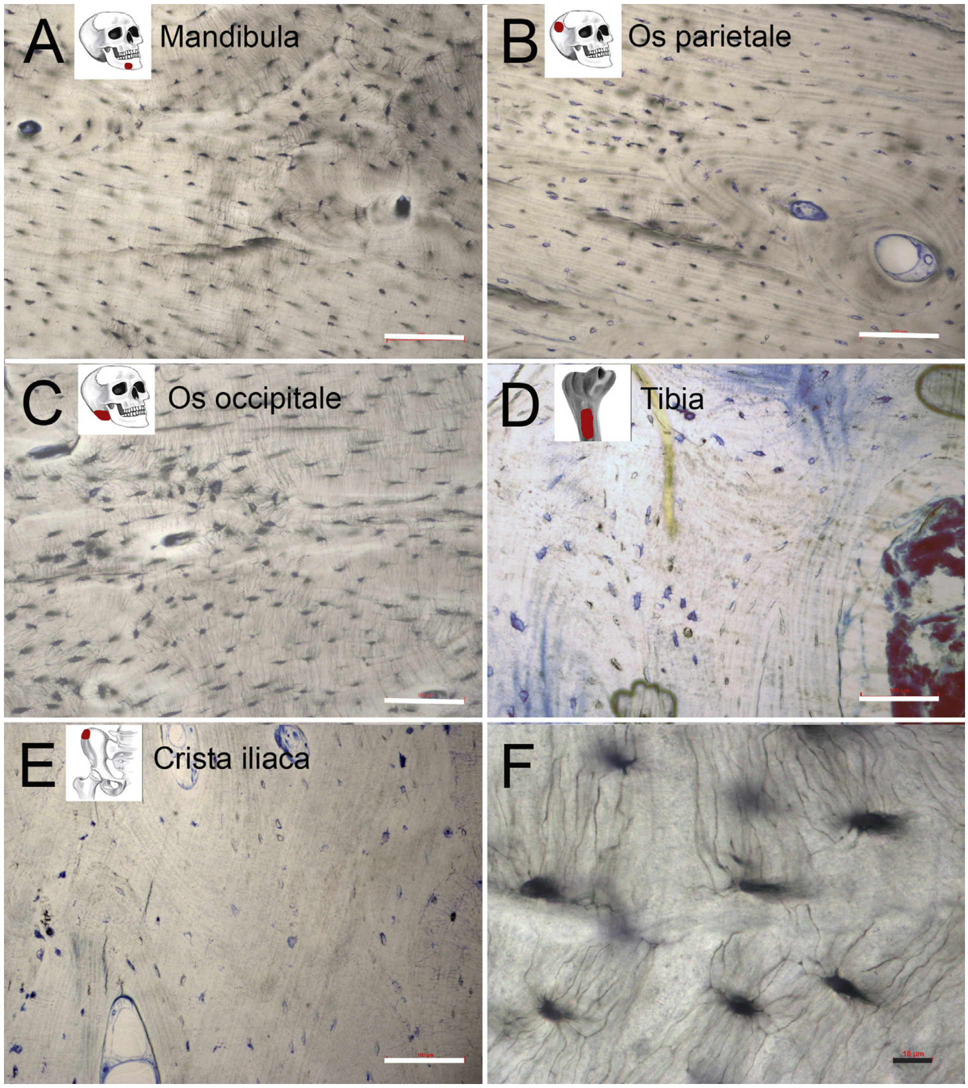

Fig. 4. Histology of cortical bone explanted from indicated sites. (A–E) Representative examples of Toluidine Blue stained sectioned bone explanted from the indicated anatomical sites. Staining revealed osteocytes residing within bone. (F) In high magnification (40×), the stellar nature of the intraosseous osteocytes can be seen (sample taken from a human mandible). Scale bars shown in (A–E) represent 100 μm, in (F) 10 μm.

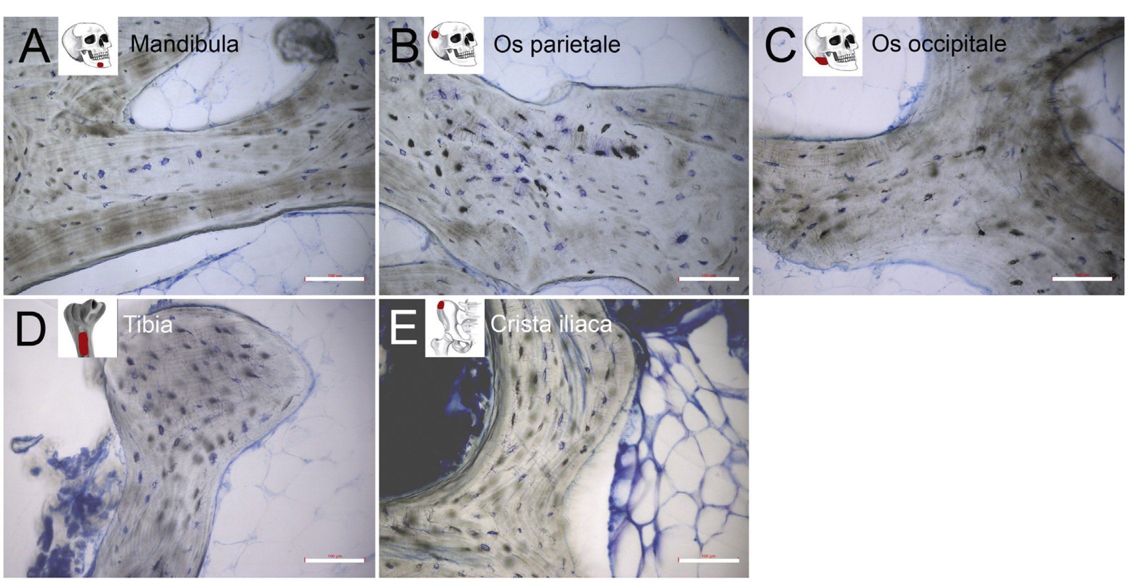

Fig. 5. Histology of characteristic trabecular structures in bone explanted from cranial trabecular bone (A–C) and peripheral bone (D and E). Sections were contrasted with Toluidine Blue in order to reveal osteocytes residing within bone. Scale bar represent 100 μm.

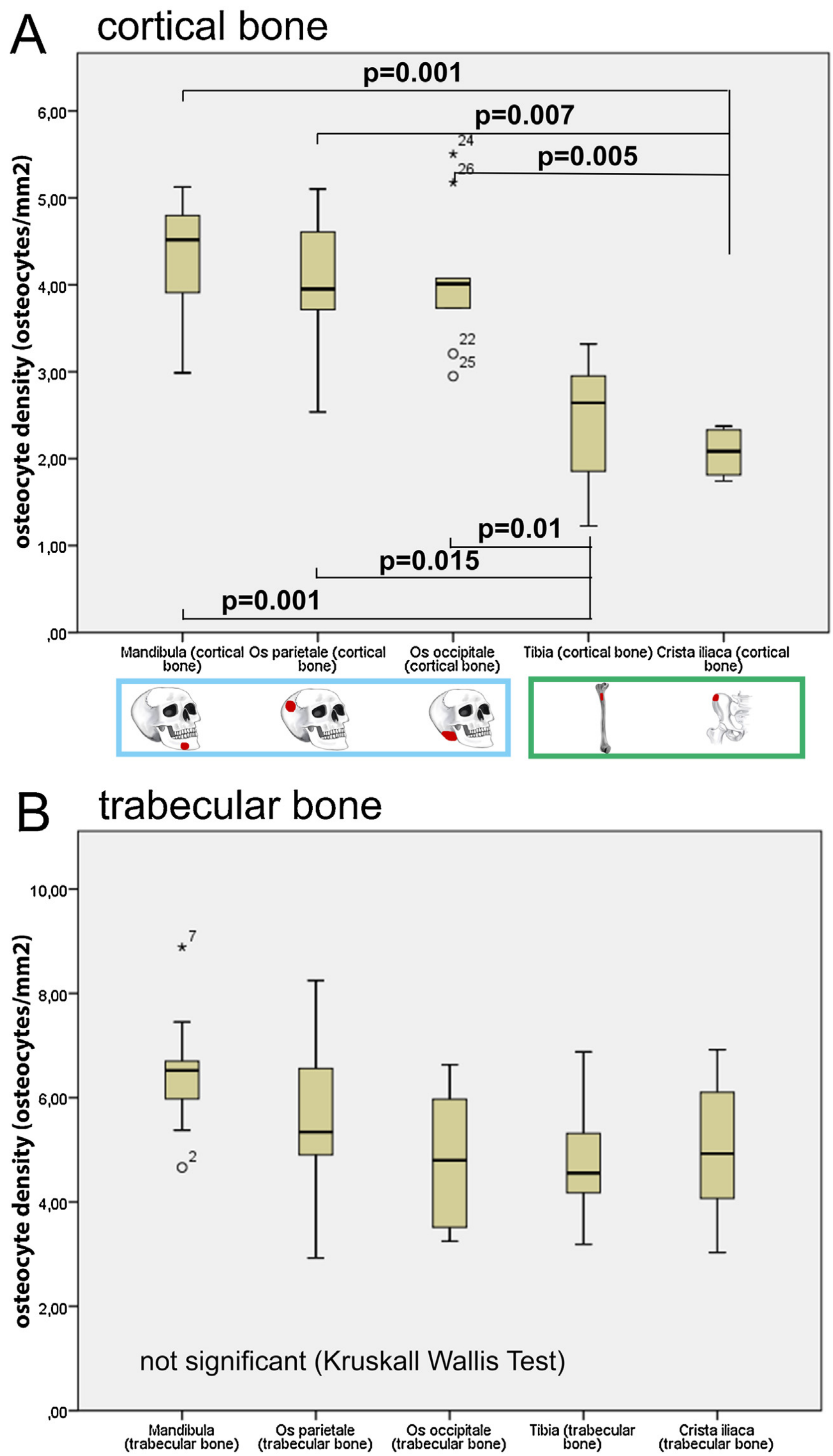

Fig. 6. The boxplots display the osteocyte densities (osteocytes per mm^2) in the cortical (A) and trabecular bone compartments (B). In the cortical bone, significant differences were found between the all cranial and post cranial bone samples. The respective *p*-values are given in the figure. In the trabecular compartments, no significant differences were detected.

Due to such increased resilience, anterior positioned osteocytes may thus also enhance osseous regeneration and tissue healing.

This study was planned and conducted once it became evident that bone augmentation within the oral cavity applying bone transplants from cranium or jaw reacts yielded more consistent and stable results compared to operations using bone from iliac crest (Deppe et al., 2012; Franz-Odendaal et al., 2005; Mertens et al., 2013). The present findings infer that grafting osseous tissue bearing high numbers of osteocytes supports healing and regeneration. This perception is further corroborated by the clinical observation that fracture healing is unimpaired in cranio-maxillofacial regions even at highly advanced ages, a process which becomes increasingly compromised over time within axial or limb skeleton (Foulke et al., 2016; Histing et al., 2013; Kloss and Gassner, 2006; Lopas et al., 2014).

5. Conclusions

Provided the constraints of the small cohort of donors with advanced age, we were able to show that significantly more osteocytes reside in cortical bone at cranial positions than within axial or limb skeleton. This cellular state and condition was not found in the corresponding trabecular bone, where osteocyte numbers remain also high at posterior positions. Parallel comparative analyses of bone microstructure as analyzed by means of μCT showed no significant differences. Considering all commonalities or differences regarding the investigated bone samples, such as loading, mechanisms of ossification or the surrounding stromal cell compartment, highlights the role of positional information reflecting ontogenetic processes. Based on this concept, regeneration experiments should be carried out in a systematic manner, which is grafting bone taken from distinct locations in conjunction with marker analysis that precisely indicate positional information.

Authors' contributions

Robert G. Stigler: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, validation, visualization, writing original draft.

Kathrin Becker: Conceptualization, data curation, visualization, methodology, writing – review & editing.

Elvin Hasanov, *Romed Hörmann and Robert Gassner*: Investigation, resources.

Günter Lepperdinger: Conceptualization, methodology, writing – review & editing.

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Conflict of interest

None declared.

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