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Architecture and mineralization of developing cortical and trabecular bone of the mandible

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Abstract Ossification of the presumptive trabecular bone in the mandibular condyle and the presumptive cortical bone in the mandibular corpus of the pig mandible was investigated during development, using micro-computed tomography (microCT). Three-dimensional architecture and mineralization characteristics were assessed from ten pigs of different developmental ages. In the condyle, increases in trabecular thickness and separation and a decrease in the trabecular number, led to an unchanged bone volume fraction. A conversion from rod-like into plate-like trabeculae was observed. Bone volume and trabecular thickness were always higher in the corpus, where an increase in bone volume fraction was caused by an increase in the trabecular thickness and a decrease in separation. A transition from a plate-like structure into a more compact structure took place. The average degree of mineralization in the condyle and the corpus increased with age. In the corpus, the degrees of mineralization were higher than in the condyle. The differences between the condyle and corpus and the changes with age could be explained by differences in the distribution of mineralization within the trabecular elements. Generally, the degrees of mineralization increased from the surface toward the centers of the trabecular elements, indicating growth of the trabecular elements by the surface apposition of new mineral.

Keywords Bone histomorphometry · Degree of mineralization · Quantitative microCT · Mandible · Development

Introduction

The ossification of bone during prenatal development comprises two different mechanisms. Chondral ossification is a process in which an initial mesenchymal condensation converts into bone through an embryonic cartilage intermediate. During desmal ossification the mesenchyme is directly transformed into bone. Both processes are present in the developing mandible; ossification of the condyle and symphyseal region of the mandible takes place through both endochondral ossification and ossification originating from the perichondrium, while desmal ossification is responsible for the development of the corpus. Regardless of its origin, all bone develops from an initial open structure into either a dense (compact) cortical bone structure or a trabecular bone structure (Leeson and Leeson 1970; Cadet et al. 2003). It is presumed that the regulation mechanisms of trabecular and cortical bone development are similar (Tanck et al. 2004).

The mandible is among the first bones in the body to ossify during fetal development (Hodges 1953), thus providing the opportunity to study the development of the bone at early fetal stages. The gross development of bony regions of the mandible during fetal life has been subject to investigation (Goret-Nicaise and Dhem 1984; Lee et al. 2001; Radlanski et al. 2003). These studies, however, have described mostly qualitative observations and did not treat the quantitative description of the developing bone structure in terms of architecture and mineralization. Moreover, they have not differentiated between the cortical and trabecular bone structure. Therefore, the resemblance of their developmental pathways remains unknown and is, thus, the object of the present study. Knowledge of the development of these early bone structures, and their similarities and

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differences, augments the basic understanding of both normal cortical and trabecular bone formation.

Recently, micro-computed tomography (microCT) has been established as an accurate and powerful tool for determining three-dimensional architectural parameters of young and adult trabecular bone in a non-destructive manner (Rüegsegger et al. 1996; Müller et al. 1998). It has been proven applicable to investigate changes in trabecular architecture during postnatal development and aging (Ding 2000; Nafei et al. 2000; Tanck et al. 2001). Furthermore, it has been recently demonstrated that commercial microCT systems are capable of not only describing the architectural, but also the physical properties of bone, such as the degree and distribution of mineralization, right down to the level of individual trabeculae (Mulder et al. 2004, 2005). Therefore, in the present study, microCT was used to analyze mandibles from ten pig specimens of different developmental ages. It was applied to investigate the concurrent architectural and mineralization properties of developing trabecular (condyle) and cortical bone (corpus).

Materials and methods

Materials

The mandibles from ten pigs (standard Dutch commercial hybrid race) of different developmental ages were used in this study. Included were eight fetuses with an estimated age of 40–45, 45–50, 50–55, 55–60, 65–70, 70–75, 82–87, and 95–100 days of gestation, obtained from sows in a commercial slaughterhouse. The fetal age was estimated from the mean weight of the litter, using growth curves (Evans and Sack 1973). Furthermore, one newborn (112–115 days postconception) and one 2 weeks old (130 days postconception) piglet, obtained from the experimental farm of the Faculty of Veterinary Medicine in Utrecht, The Netherlands, were used. They were euthanized by an intravenous overdose of ketamine (Narcetan) after premedication. The specimens were obtained from other experiments that were approved by the Committee for Animal Experimentation of the Faculty of Veterinary Medicine, Utrecht, The Netherlands. They were stored at -20°C prior to assessment.

The mandibles were harvested by dissection and cut in half at the symphyseal region. No attempt was made at removing all the soft tissue. The older specimens were divided into smaller sections in order to be able to analyze all specimens with the same resolution, which was limited by the diameter of the microCT specimen holders.

Micro-computed tomography

Three-dimensional, high resolution reconstructions of the trabecular bone of the specimens were obtained,

using a microCT system (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland). The hemimandibles were mounted in cylindrical specimen holders (Polyetherimide, 20 mm outer diameter, wall thickness, 1.5 mm) and secured with synthetic foam. The mandibular specimens were completely submerged in 70% ethanol. The scans yielded an isotropic spatial resolution of 10 μm . A 45 kV peak voltage X-ray beam was used, which corresponds to an effective energy of approximately 24 keV. The microCT system was equipped with an aluminum filter and a correction algorithm, which reduced the beam hardening artifacts sufficiently to enable quantitative measurements of the degree and distribution of mineralization of developing bone (Mulder et al. 2004). The computed linear attenuation coefficient of the X-ray beam in each volume element (voxel) was stored in an attenuation map and represented by a gray value in a three-dimensional reconstruction. This attenuation coefficient can be considered to be proportional to the local degree of mineralization (Nuzzo et al. 2002).

Architecture

The architecture and degree of mineralization of the bone specimens were determined in volumes of interest that were built up out of $10 \times 10 \times 10 \mu\text{m}^3$ voxels and segmented using an adaptive threshold, which was visually checked. In a segmented reconstruction, every voxel with a linear attenuation value below the threshold (assumably representing soft tissue or background) was made transparent and voxels above this threshold (representing bone) were made opaque.

The volumes of interest were chosen at six different locations in the mandibular corpus; three regions, from anterior to posterior on the buccal side, and three more on the lingual side. They were chosen in regions of presumptive cortical bone. In the condyle, four volumes of interest were chosen that were located anteroinferiorly, anterosuperiorly, posteroinferiorly, and posterosuperiorly. The data from the selected regions of the corpus and condyle were averaged to obtain values representative for the entire corpus and condyle.

To quantify changes in the architecture of the bone during development, several bone architectural parameters (BV/TV: bone volume fraction, Tb.N: trabecular number, Tb.Th: trabecular thickness, Tb.Sp: trabecular separation, Conn.D: connectivity density, SMI: structure model index, DA: degree of anisotropy) were calculated (Software Revision 3.2, Scanco Medical AG).

Degree and distribution of mineralization

The degree of mineralization was estimated from the attenuation values in the portion of the reconstructed samples that were characterized as bone. The previously determined threshold, used to separate bone from background, was applied. For this analysis, the voxels

exceeding the threshold kept their original gray value. The outermost voxel layer, characterized as bone, was disregarded since this layer is likely to be corrupted by partial volume effects. The degree of mineralization was estimated by comparing the linear attenuation coefficient with reference to the measurements obtained from a series of solutions with different concentration of the mineral K_2HPO_4 (Mulder et al. 2004). The distribution of mineralization within the structural elements of the bone in the condyle and corpus was determined using a so-called peeling-algorithm. By determining the degree of mineralization in voxel layers that were consecutively peeled from the surface of the reconstructed bone structure, a relationship between the degree of mineralization and the distance from the trabecular surface was established. The average degree of mineralization was estimated for the consecutive layers, using the method mentioned.

Statistics

Regression analysis was applied by the best fit of the obtained results for architectural and mineralization parameters. This yielded, for the condyle, a linear regression and, for the curvilinear results of the corpus, a second-degree polynomial regression. Statistical analysis was performed in SPSS (11.5.1 software SPSS Inc., Chicago, IL). A *P*-value of less than 0.05 was considered statistically significant.

Results

When assessing the gross anatomical changes that occurred during the development of the pig mandible

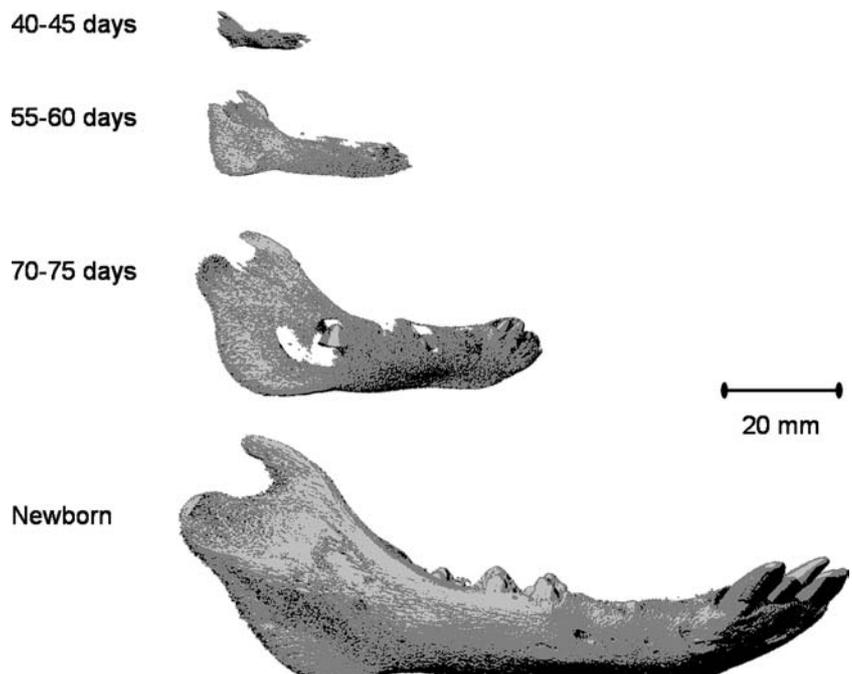
(Fig. 1), it was calculated that the fetal pig mandible roughly increased 0.8 mm in length per day. During this growth a porous type of cortex developed in the area of the mandibular corpus. In other regions, for instance the condyle, the development of apparent cortical bone was not observed.

Architecture

During the development of the mandibular condyle, there was a marked increase in the trabecular thickness and an increase in trabecular separation, in the case of the presumptive trabecular bone (Fig. 2). Also, in the mandibular corpus, the presumptive cortical bone underwent trabecular thickening, which ultimately led to the coalescence of trabecular elements into a highly porous compact-like bone (Fig. 2).

Quantitative changes in the presumptive trabecular (condyle) and cortical bone (corpus) have been summarized in Fig. 3. The amount of trabecular bone in the mandibular condyle, expressed by the bone volume fraction (BV/TV), did not significantly increase over the investigated age range. On the other hand, there was a significant drop in the trabecular number (Tb.N) ($r = -0.86$, $P < 0.05$) and a significant increase in the trabecular thickness (Tb.Th) ($r = 0.92$, $P < 0.01$) and trabecular separation (Tb.Sp) ($r = 0.90$, $P < 0.05$). A change from rod-like into plate-like trabeculae was expressed by a significant drop in the structure model index (SMI) during development ($r = -0.92$, $P < 0.01$). Furthermore, the number of connections (Conn.D) between trabecular elements in the condyle decreased significantly ($r = -0.94$, $P < 0.01$). The presumptive trabecular bone in the condyle was highly oriented, with

Fig. 1 Three-dimensional reconstruction of the right mandible of pigs of different gestational ages. The specimens are 40–45, 55–60, 70–75 days of age, and a newborn (approximately 115 days) specimen. Note the absence of mineralized trabecular tissue in the mandibular condyle of the younger stages, which is, thus, not visible in these reconstructions



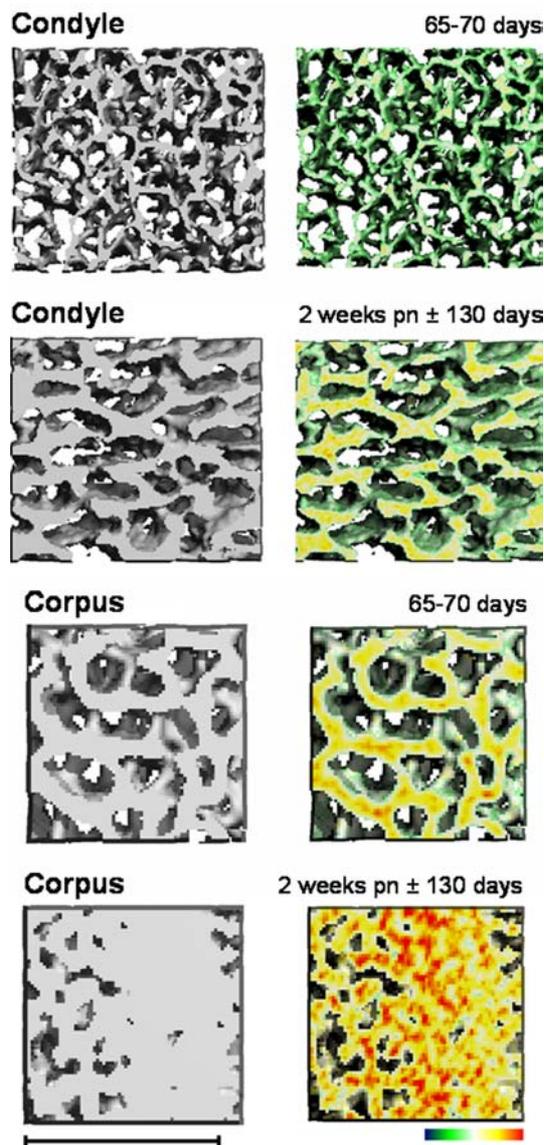


Fig. 2 *Left* a number of reconstructed volumes of interest from the corpus (middle region on buccal side) and condyle (posterior), from a 65- to 70-day-old specimen and from a 2-week-old specimen to qualitatively illustrate development of the architecture with age. Increasing bone volume fraction could be clearly observed in both the corpus and condyle, although there was no significant increase in the condyle (Fig. 3). Note the coalesced trabecular elements in the corpus. *Right* the same volumes of interest, but now the original attenuation coefficients of the trabecular elements remained. An increase in the global degree of mineralization could be qualitatively observed as well as a spatial inhomogeneous distribution of the mineralization, with the centers of the trabecular elements being more mineralized than their surfaces. *Bar* 1.0 mm; *color-scale* increasing degree of mineralization from blue to red

degrees of anisotropy (DA) generally above two that did not change over the investigated age range.

As opposed to the presumptive trabecular bone in the condyle, the presumptive cortical bone in the corpus did show a significant increase in BV/TV ($r=0.95$, $P<0.001$). On the other hand, no changes were observed concerning the Tb.N. There was, however, a significant

increase in the Tb.Th ($r=0.97$, $P<0.001$) and a decrease in Tb.Sp ($r=0.92$, $P<0.01$). The average trabecular thickness increased by 1.1 μm per day. The SMI showed a significant decrease ($r=0.99$, $P<0.001$) with developmental age, with values reaching well below zero. In the early developmental stages more connections were established between trabecular elements. Later on in the development, Conn.D decreased again ($r=0.91$, $P<0.01$). Just like the presumptive trabecular bone of the condyle, the presumptive cortical bone of the corpus showed a high orientation throughout the developmental period examined, but no change.

Degree and distribution of mineralization

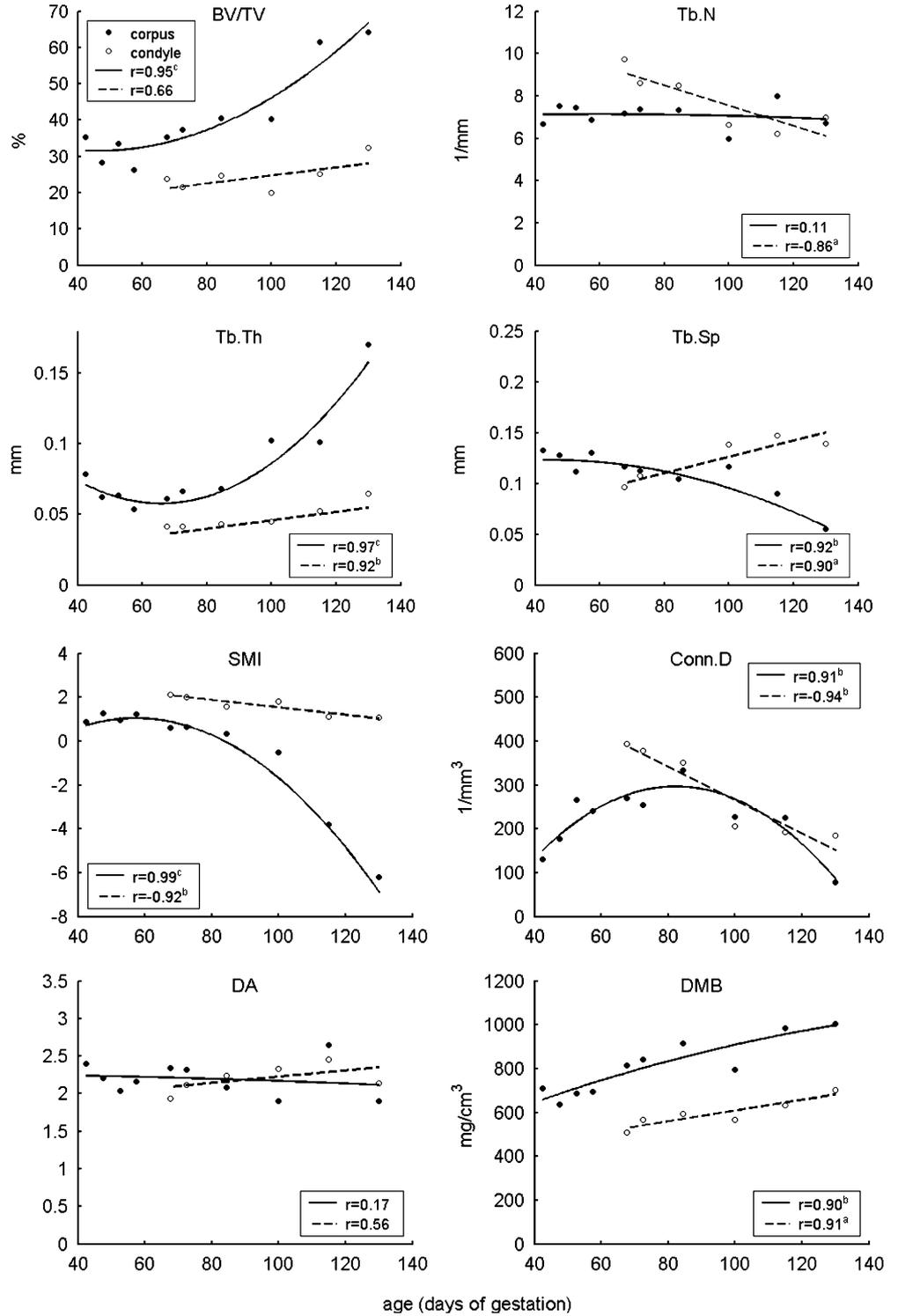
A clear global increase in the degree of mineralization of the trabecular elements with age, in the mandibular condyle, was evident (Fig. 2). Furthermore, there is a marked gradient in mineralization, increasing from the surface of the trabecular elements toward their centers. The average degree of mineralization (DMB) of both the condyle and corpus increased significantly with developmental age (condyle: $r=0.91$, $P<0.05$; corpus: $r=0.90$, $P<0.01$) (Fig. 3; bottom right panel). In the developing corpus, the degree of mineralization was higher than in the condyle.

In the trabecular elements the degree of mineralization increased from their surfaces toward their centers. This was the case for both the condyle and the corpus (Fig. 4). The degree of mineralization in the center of the trabecular elements of the corpus was higher than in the trabecular elements of the condyle. The degree of mineralization in the surface of the trabecular elements was similar in both regions. With increasing developmental age the gradient in the degree of mineralization in the condyle became steeper. Besides this, a relatively larger constant region of higher mineralized bone material was present in the centers of the trabecular elements of the condyle of older specimens.

Discussion

Although both the presumptive cortical bone in the corpus and the presumptive trabecular bone in the condyle initially had a similar trabecular appearance during development, the current study shows that there is a considerable difference in development between the two. Knowledge of the development of these early bone structures and their similarities and differences, augments the understanding of normal cortical and trabecular bone formation. It may provide baseline data on healthy developing bones. For instance, bone diseases such as osteoporosis and osteogenesis imperfecta and the influences of pathogenic drugs or noxious environmental conditions can be traced back to the fetal development of bones (Cooper et al. 2002; Javaid and Cooper 2002). Furthermore, as initial bone regeneration

Fig. 3 Results of all the investigated parameters in this study plotted against age. *Closed circles* values for the corpus. *Open circles* values for the condyle. *BV/TV* bone volume fraction, *Tb.N* trabecular number, *Tb.Th* trabecular thickness, *Tb.Sp* trabecular separation, *SMI* structure model index, *Conn.D* connectivity density, *DA* degree of anisotropy, *DMB* degree of mineralization of bone. *Solid line* second-degree polynomial regression for corpus data points. *Dashed line* linear regression for condyle data points. The significance of the *r*-values of the regression is indicated as follows: ^a*P* < 0.05; ^b*P* < 0.01; ^c*P* < 0.001. Values for parameters of the condyles of the youngest specimens are absent, due to absence of a mineralized trabecular structure in these specimens



closely resembles fetal bone formation (Ferguson et al. 1999), it may be possible to provide insights into the mechanisms involved in fracture healing or bone formation during surgical distraction methods.

The bone volume fraction of the corpus increased significantly with age, with values up to approximately 70%. It is known that the bone volume fraction in adult compact bone can reach values as high as 95%; often

referred to as a porosity of 5% (Wachter et al. 2001; Cooper et al. 2004). In the condylar bone, where no significant increase with age was observed, the bone volume fraction was much less. Below, changes in bone volume fraction of both regions, with age, will be explored and possibly explained by changes in other architectural parameters. Subsequently, changes in mineralization will also be discussed.

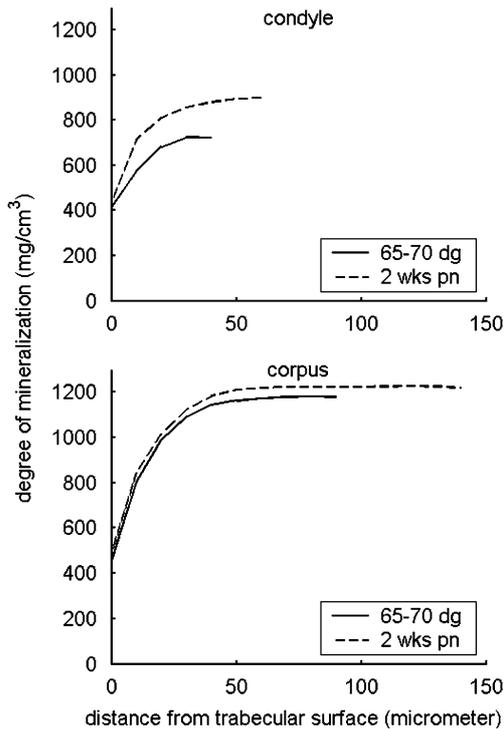


Fig. 4 Distribution of the degree of mineralization, within trabecular elements, from a 65- to 70-day-old specimen and a 2-week-old specimen, as a function of the distance from the surface of the trabecular elements. With increasing developmental age a steeper gradient in the degree of mineralization from the surface to the center was observed in the condyle of older specimens, as compared to younger ones (*top figure*). Besides this, a relatively larger “constant” region of higher mineralized bone was present in the centers of the trabecular elements of the condyle of older specimens. These two phenomena cause the increase in the average degree of mineralization with developmental age. For the corpus, only the latter was present (*bottom figure*)

Architecture

The increase in bone volume fraction in the corpus could be mainly attributed to an increasing trabecular thickness and to a decrease in the trabecular separation, while there was no change in the trabecular number (Fig. 3). Despite the increase in trabecular thickness with age, in the condyle, no change in bone volume fraction was observed. This could be explained by the decrease in trabecular number and the increase in their separation, which counteracts the effects of increasing trabecular thickness.

The presumptive trabecular bone in the mandibular condyle displayed an ongoing change, from a rod-like structure toward a more plate-like one, and was characterized by a significant decrease in the structure model index. The presumptive cortical bone in the corpus showed a more plate-like structure from the beginning. With age, the structure model index for this region decreased sharply toward negative values, indicating a compact bone structure. Normally, the structure model index varies between the values 0, for perfect plates, and 3, for perfect rods. Negative values can come from

isolated marrow spaces (Hildebrand and Rüegsegger 1997). When the bone is getting more compact, these spaces might increase in number.

The considerable decrease in connectivity density, of the presumptive trabecular bone in the condyle, is most probably caused by the decreasing number of trabecular elements. During the earlier developmental stages in the corpus, an increase in the number of connections was established while, in the later stages, a decrease was observed. This decrease could be caused by the fusion of rod-like trabecular elements into more plate-like elements and the filling up of perforations in plates. The degree of anisotropy in both investigated structures remained unchanged, but was relatively high (generally values above 2) when compared to juvenile pigs (Teng and Herring 1995). It was suggested (Teng and Herring 1995) that the orientation of the trabecular elements was merely a reflection of growth in the juvenile pigs, which is also the most probable explanation for the strongly oriented trabecular structure in the regions of the mandible investigated in the present study. Both in the condyle and the corpus, the main orientation of the trabecular elements was anteroposteriorly. This coincides with the direction of condylar growth in fetal pigs (Wissmer 1927). In the corpus, where ossification starts in a single ossification centre located near the future mental foramen, bone grows in an anterior, upward, and posterior direction (Radlanski et al. 2003). These orientations are, during later development and adulthood, still reflected in the orientations of the Haversian canal system in the mandibular corpus (van Eijden 2000).

It has been demonstrated that, during adulthood, the architecture of the human mandibular condyle is optimized to resist applied mechanical loading (Giesen and van Eijden 2000; van Ruijven et al. 2002). The development toward such an optimized structure presumably starts early, as has been demonstrated for bone structures in utero (Goret-Nicaise 1981; Burger et al. 1991). The onset of the influence of mechanical loading of the mandible is most probably reflected in the curves of the bone volume fraction, trabecular thickness, structure model index, connectivity density, and, to a lesser extent, the trabecular separation of the presumptive cortical bone in the mandibular corpus. During early development, little change was found in these parameters while, at ages around 70 days of gestation, they exhibited sharp increases or decreases. This is also the age at which the presumptive trabecular bone in the mandibular condyle starts to develop. It has been shown that at the corresponding developmental stage in human development, repetitive jaw movements as well as suckling and swallowing reflexes appear, suggesting functional loading of the mandible by developing muscles (de Vries et al. 1985).

Degree and distribution of mineralization

The degree of mineralization was quantified by comparing linear attenuation values, found in bone speci-

mens, with that of homogeneous K_2HPO_4 solutions. K_2HPO_4 has exactly the same absorption properties as hydroxyapatite (Nuzzo et al. 2002), the main constituent of mineralized bone in adults and which is also already abundantly present in fetal bone (Meneghini et al. 2003; Nuzzo et al. 2003). An increasing degree of mineralization with developmental age, observed in both the presumptive trabecular bone of the condyle and the presumptive cortical bone of the corpus (Fig. 3), might be based on several phenomena. Firstly, in the condyle, minerals in the trabecular centers apparently continue to mature, showing an increasing degree of mineralization (Fig. 4, top panel). This more mineralized central region also gets larger with age as the average trabecular thickness increases (Figs. 3, 4), thus contributing to a higher average value of the degree of mineralization of the structure. This latter phenomenon seems to be the dominant contributor to the observed increase in average degree of mineralization observed in the corpus since, in the centers of younger and older specimens, no difference in the degree of mineralization was found (Fig. 4, bottom panel). Secondly, the bone surface to bone volume ratio decreases with developmental age in both the condyle and corpus (not shown). Therefore, poorly mineralized tissue at the surface of bone elements contributes less to the overall degree of mineralization at later stages. Similarly, a higher degree of mineralization in the corpus, when compared to the condyle, is most probably caused by higher degrees of mineralization in the centers of the trabecular elements as well as by a markedly larger center region and a lower bone surface to bone volume ratio in the corpus.

The values for the average degree of mineralization found in this study were low when compared to values found in earlier studies that focused on healthy and osteoporotic adult bone (Meunier and Boivin 1997; Boivin and Meunier 2002; Follet et al. 2004). In the papers mentioned, the average values for the degree of mineralization were generally higher than $1,100 \text{ mg/cm}^3$, with maximum values going beyond $1,600 \text{ mg/cm}^3$. This could indicate that the mineralized tissue in the developing skeletal structures in the fetus was fairly young mineral tissue that still had to undergo maturation. The lower degrees of mineralization could also suggest that the bone in these developing structures is subjected to extensive remodeling. Therefore, the mineralized tissue is not long-lasting and has to be constantly renewed and replaced by younger, less mineralized tissue.

It can be concluded from this study that marked changes in architectural as well as in mineralization properties of bone occur during its development in the mandible of pigs. Moreover, differences between different bone structures within the mandible were evident. Bone in the condyle develops into a spongy trabecular structure whereas the bone in the corpus starts out as a trabecular-like structure in which, gradually, trabecular elements coalesce to transform into compact bone. Considerable changes in architectural parameters of the bone in the corpus, at the age of approximately 70 days

of gestation, and the appearance of trabecular bone in the condyle, at this age, are assumed to relate to the onset of the functional loading of the mandible by developing masticatory muscles. Considerable changes in the pace of mineralization, at this age, were not found. It seems reasonable to conclude that the increase in dimensions of the trabecular elements occurs via the apposition of new bone material at their surfaces, which is reflected in the differences in the degree of mineralization observed between their surface and their centers.

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