Appl. Phys. A 83, 229–233 (2006)
DOI: 10.1007/s00339-006-3511-6

Applied Physics A
Materials Science & Processing

A. MAZURIER
V. VOLPATO
R. MACCHIARELLI✉

Improved noninvasive microstructural analysis of fossil tissues by means of SR-microtomography

Laboratoire de Géobiologie, Biochronologie et Paléontologie Humaine, UMR 6046, Université de Poitiers, 40, av. du Recteur Pineau, 86022 Poitiers Cedex, France

Received: 5 December 2005/Accepted: 12 December 2005
Published online: 22 February 2006 • © Springer-Verlag 2006

ABSTRACT Bone is a dynamic tissue which records and partially stores a relevant amount of biological and biomechanical information. In paleobiological investigation, a satisfactory access to this hidden archive is limited by the unavoidable need to assure integrity of the fossil record. According to our experience, based on a variety of advanced nondestructive approaches in imaging fossil tissues, synchrotron radiation microtomography (SR-μCT) assures the quasi-ideal physical conditions to explore and to finely characterise at a high spatial resolution the inner structural morphology of relatively large-sized and highly mineralised fossil specimens for 2D and 3D modelling and reliable quantitative analyses.

PACS 87.19.Rr; 87.59.-e; 07.85.Qe

1 Introduction

Over the past ten years, our understanding of the genetic and molecular basis of bone development has greatly improved [1]. In particular, it has been assessed that global patterning of the human (mammalian) skeleton depends on the deployment of numerous regulatory genes [2]. At the cellular level, it has also been shown that bone cellular processes, notably involving the mechanosensitive cells osteocyts [3], allow bone to model/remodel in response to specific biomechanical conditions and requirements [4]. As a whole, advances in quantitative genetics, developmental biology, and functional morphology show that the skeleton is a 'compromise' [5]. In a functional evolutionary perspective, contrary to its only slightly mutable external appearance, mammalian bone is now viewed as a highly dynamic tissue, constantly adapting its inner conformation to the changing biomechanical environments [6].

Nonetheless, critical details of the manner in which adult bone obtains its final form are still debated [7] and osseous morphologies are alternatively seen (i) as independent features with a predominantly genetic basis largely governed by the forces of natural selection, or (ii) as the result of primarily epigenetic or nongenetic interactions with the environment. Accordingly, a number of questions concerning the 'setting' of this material still remain unresolved: how and to what extent does bone adapt? At what points in individual life should we expect bone functional adaptation to be evident in bone structure and architecture? What are the developmental, functional, and biomechanical relationships between the 'container' (i.e. the external gross morphology) and the 'contents' (i.e. the inner structural architecture)? [8]. It is likely that only a full integration among advances in quantitative genetics, developmental biology, and functional morphology will allow us to satisfactorily answer these fundamental questions.

A quantitatively and qualitatively relevant amount of biological and biomechanical information is recorded and partially stored within the bone at meso-microstructural level. Fine access to this archive, which is critical for reconstructing evolutionary pathways, adaptive strategies, age- and sex-related variation patterns, and health status, and to outline various aspects and details of individual life history, is usually granted by invasive investigative approaches [9].

In paleobiology, the traditionally poorly explored possibility of detecting the 'hidden signature' in bony tissues is objectively made more complicated by the fact that fossil specimens cannot be routinely sectioned. Accordingly, alternative technical and technological solutions to histomorphometry are necessary to extract and to decode the usually noisy fossil signal [10].

Here we provide a synthetic account of multiple research experiences and perspectives in the investigation of the inner structural morphology of mammal fossil bones by means of advanced nondestructive approaches, including synchrotron radiation microtomography (SR-μCT).

2 Exploring fossil bone structural morphology

Bone mass and trabecular orientations are adapted to the nature, intensity, and directionality of external forces. In particular, cancellous bone architecture(s) and locomotion-related mechanics are intimately dependent [11], and over 80% of the variance in cancellous bone biomechanical behaviour and patterning can be explained by measures of density and orientation [12].

From a functional standpoint, 'sandwich-like' constructions – e.g. the hip bone – provide the most effective biomechanical response to loads because they associate rather high strength properties to proportionally low-weight material. In such kinds of structures, trabecular bone lies as core material

✉ Fax: +33-(0)-5-49-45-40-17,
E-mail: roberto.macchiarelli@univ-poitiers.fr

surrounded by an outer dense shell. It offers two major adaptive solutions to the varied functional constraints: in regions of low stress, cancellous bone mostly consists of rod-like structures (average thickness $< 200\,\mu m$) connecting to form open cells, while in regions of higher stresses it forms a more closed network of plates; finally, in sites experiencing intermediate levels of stress, it results in a complex, interconnected network combination of rod- and plate-like elements [13].

When comparatively investigated in fossil mammal taxa in a morphofunctional ontogenetic perspective, the identification of the mechanisms of 'setting' and 'patterning' of the functional-related organisation of the cancellous network, and the subtle characterisation of its structural properties in homologous areas, allow the reconstruction of the paleobiomechanical dynamics most commonly experienced by an extinct organism. In this view, measures of cortical and trabecular (strut) thickness topographic variation, trabecular number, connectivity density, bone volume fraction, and site-specific degree of textural anisotropy of the cancellous network [14] can be used as proxy for assessing habitual postural/locomotor mode(s).

But how can these quantitative analyses be carried out on fossil specimens by means of noninvasive analytical approaches? And how reliable are our estimates/reconstructions?

Here we present different examples of diagnostic imaging of fossil bone based on the use of conventional radiography, computed axial tomography (CT), microtomography (μCT), and synchrotron radiation microtomography (SR-μCT) (for a detailed review of the first three analytical tools, see [10, 15]).

2.1 *Plain film radiography*

When applied to morphologies dealing with relatively simple shapes, conventional radiography is both qualitatively and quantitatively informative and comprehensive; also, it likely still represents the most practical solution to 'easily' and quickly document osteological collections. In the case of 'sandwich-like' fossil bones, radiographs (usually) provide a satisfactory compromise between spatial resolution (which is enough for imaging trabeculae) and 2D visualisation of conformation patterns [16]. Nonetheless, all structures in the path of the X-ray beam appear superimposed in the image and cannot be distinguished from each other. Moreover, in the specific case of fossil specimens, the inner trabecular bone is frequently blocked by the presence of a high(er) density sedimentary matrix resulting from postdepositional dynamics.

A rather typical example of unelaborated radiographic imaging of a 'sandwich-like' fossil bone is given in Fig. 1, which shows the supracetabular portion of the Neandertal hip bone from the Kebara 2 skeleton [17]. This is a relatively high-density region of the pelvis, where the effects of fossilisation cause the fading of the cancellous gradients and a general hazy appearance of the pattern, and the trabecular borders are rather blurred. Despite the fact that the application of specific protocols of advanced digital image processing may significantly enhance the quality of such kinds of images, thus allowing the extraction of critical (paleo)structural information [18–20], it is quite obvious that the site-specific accuracy of measures of trabecular thickness and density is affected by a number of uncontrolled diagenetic and taphonomic factors. Additionally, cortical bone topographic variation cannot be assessed at all.

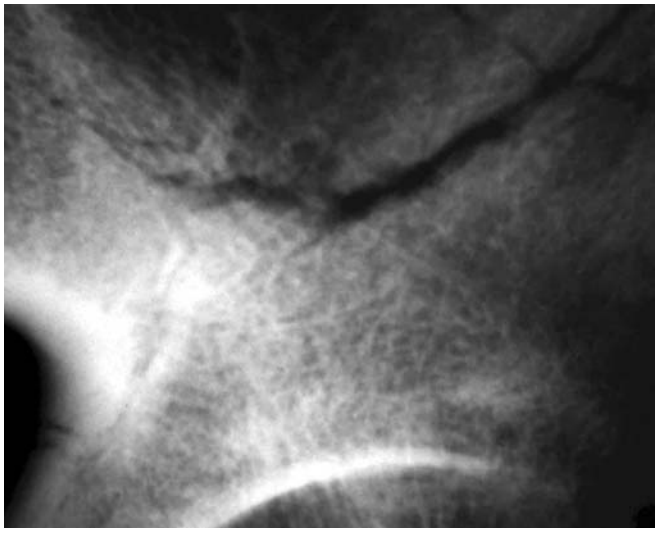

FIGURE 1 Unelaborated radiographic image of the supracetabular portion of the Neandertal hip bone from Kebara 2, Israel ($\sim$ 60 000 years)

2.2 *Computed axial tomography* (CT)

Computed axial tomography overcomes the problem of parallax distortion and superposition of structures typical of plain film radiography, because the density of the specimen is measured in multiple directions. Additionally, the extension into the third dimension dramatically enhances the possibility of quantitative and qualitative analysis of morphology [21]. Accordingly, since late 1970s, medical CT has become the most exploited analytical tool in paleobiology and paleoanthropology for 3D visualisation and study of cranial and postcranial elements, including the measure of cortical bone variation [22–25]. The development of electronic segmentation methods has granted access to otherwise unobservable features and volumes in fossil specimens of special interest [26].

Nonetheless, the best possible spatial resolution in the plane of the scan that can be obtained with current scanning systems is about 0.3–0.5 mm, thus not enough to detail individual rod-like trabeculae, to quantitatively assess local trabecular density and textural anisotropy, or to finely characterise the 'cortico-trabecular complex' in fossil specimens [27]. An example of such kinds of limits is given in Fig. 2. It shows a CT axial section across the supraorbital torus of the one-million-year-old (Ma) UA-31 human cranium from Buia (Eritrea) [28]. Despite possible local uncertainties, the resolution allows us to discriminate between fossilised bone and the internal and external concreted sediment (consisting of compacted grey silty calcareous mudstone), which mostly affects the left half of the specimen and which has almost completely filled the frontal sinus; conversely, the inner and outer compact bone tables and the intervening diploë (cancellous tissue) are indistinguishable, and no reliable measure of relative thickness may be performed.

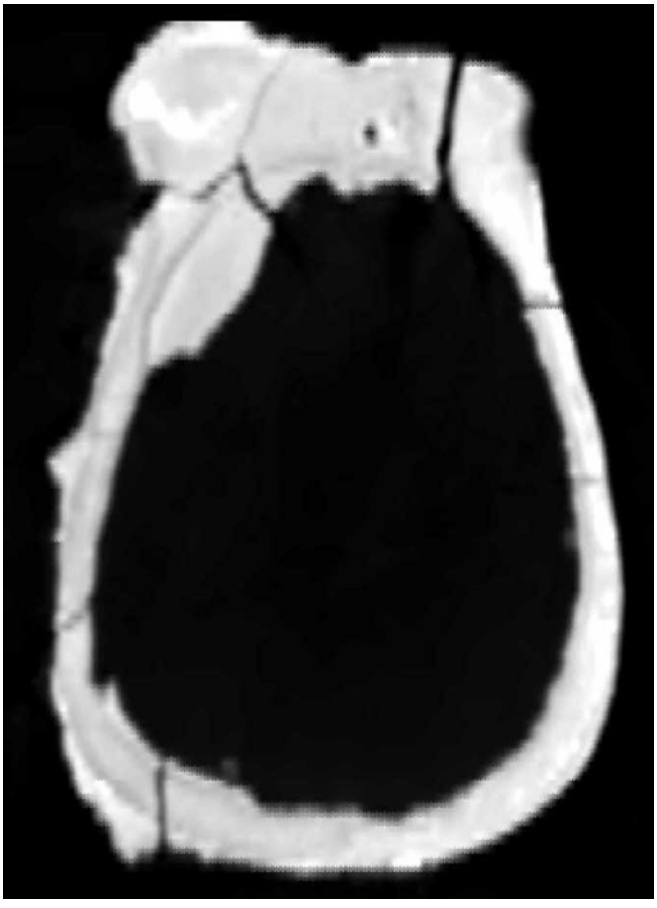

FIGURE 2 CT axial section (2 mm) across the supraorbital torus (*upper*) of the UA-31 human fossil cranium from Buia, Eritrea (∼ 1.0 Ma)

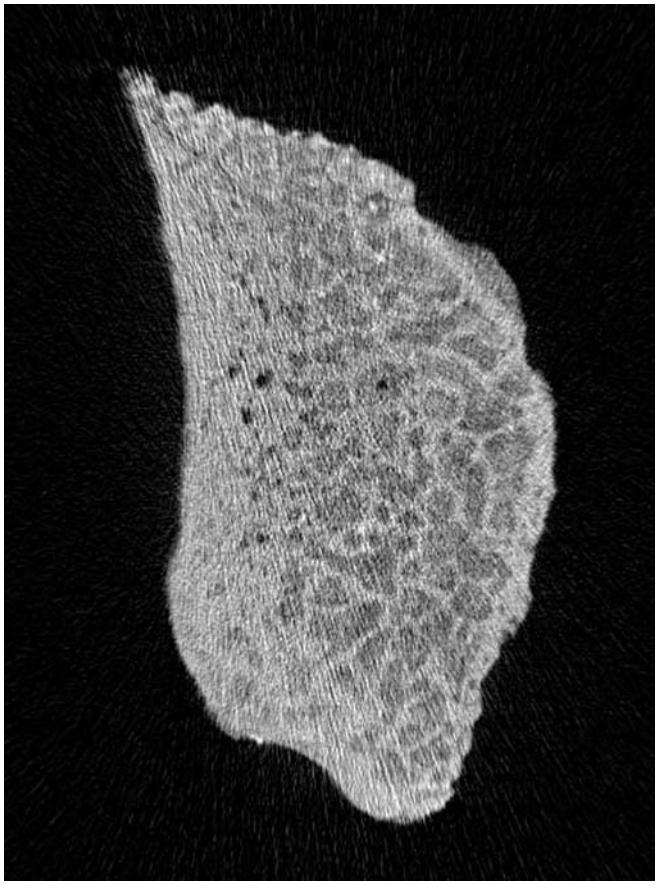

FIGURE 3 μ-CT oblique section (50 μm) across the proximal end of the KNM-ER 1476B hominin fossil tibia from East Turkana, Kenya (∼ 1.8 Ma)

2.3 *Microtomography* (μCT)

X-ray microtomography (μCT) is a miniaturised version of computed axial tomography. Nonetheless, with respect to medical CT, μCT provides images with a much higher spatial resolution and a much thinner slice thickness, of the order of a few micrometres up to about 200 μm [29]. Because of its technical characteristics, μCT is thus the most recommended tool in investigations dealing with the 2D or 3D analysis of the finest morphological and structural variation, including the bony textural one [30–32]. A test realised at a resolution of 26 μm showed that μCT measurements are highly reproducible [33]; additionally, specific tests for accuracy have shown results compatible with the histological standards [34].

Mostly developed to characterise materials for industrial use, a variety of transportable or semi-transportable μCT versions are now available for advanced research. In paleobiological research, the major limit of this analytical tool is likely represented by the only limited size of the specimen suitable for analysis, which does not usually exceed a few centimetres [35]. Also, the power of the 'transportable' version(s), which is the most appropriate for scanning fragile fossil specimens directly in their original storage site, does not necessarily assure the best contrast between matrix and mineralised bone.

Figure 3 shows an oblique μCT section across the proximal end of the Early Pleistocene KNM-ER 1476B hominin fossil tibia from East Turkana, Kenya [36], performed by means of nonindustrial transportable microtomographic equipment (a peripheral quantitative computed tomographic system), set to obtain the best ratio between image quality and acquisition time. Despite the fact that both the cortical shell and the cancellous network are visible and exploitable for quantitative assessment, similarly to the example given by Spoor et al. in their Fig. 3 [15], the high density and matrix-filled fossil tibial tissue resulted in a partial lack of detector signal causing some noise. Also, boundaries among the cancellous mesh forming the network are not easily distinguishable.

2.4 *Synchrotron radiation microtomography* (SR-μCT)

In comparison to the conventional X-ray microtomographic equipment, SR-μCT is uniquely characterised by continuous energy spectrum, high photon flux, intense monochromatic X-ray beam, parallel projections, and small angular source size. As a whole, these quasi-ideal physical conditions assure the currently highest spatial resolution (< 1 μm) and the most favourable signal-to-noise ratio, even in the analysis of bony material [37–39]. Unlike conventional X-ray tubes, a synchrotron radiation source produces very intense X-ray beams that can be easily monochromatised. Mo-

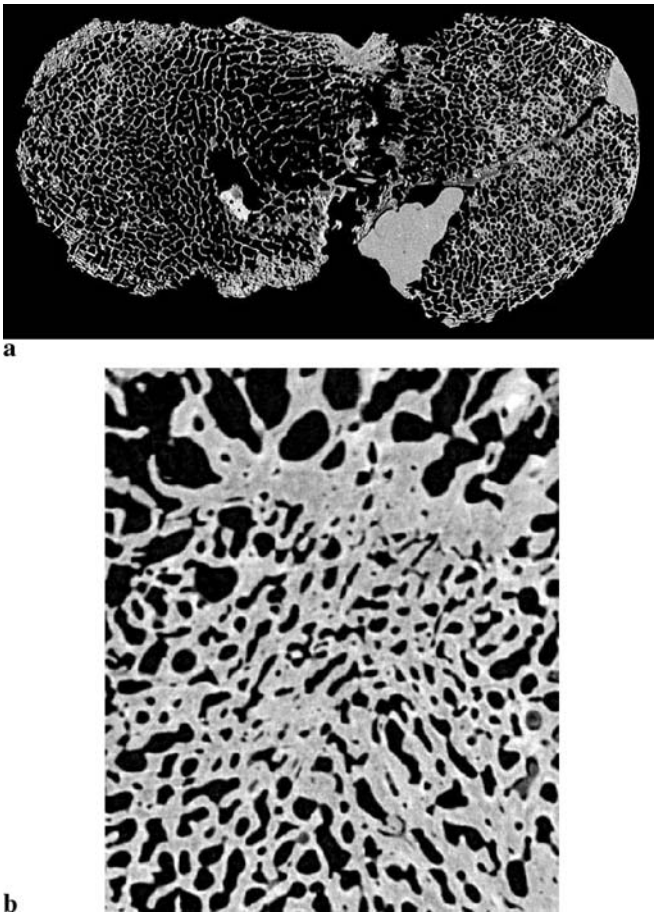

FIGURE 4 (**a**) SR-μCT transversal section (45.5 μm) through the proximal end of the Neandertal tibia from La Ferrassie 2, France (∼ 50 000 years). (**b**) Detail of a SR-μCT transversal section (45.5 μm) through the femoral head of the fossil hominoid *Oreopithecus bambolii* from Baccinello, Italy (∼ 7.0 Ma)

noenergetic X-rays beams enable absolute linear attenuation coefficients to be measured and avoid the risk of beam hardening artefacts in the reconstruction of images from dense specimens such as highly mineralised fossils.

The beamline ID 17 set at the European Synchrotron Radiation Facility of Grenoble [40] offers the unique opportunity for paleobiological research to scan relatively large-sized specimens (up to 18 cm in half-acquisition) at a final spatial resolution of 45.5 μm. For the purposes of our ongoing investigations of bony and tooth specimens [27, 41], scans are routinely performed at energies of 50–70 keV; projections are taken each 0.12–0.24° and are collected by a 2048 × 2048 CCD FRELON camera [42]. According to the current setup, the maximum sample vertical dimensions near 30 cm.

The extraordinary potentialities for paleobiology of the application of the SR-μCT technology are exemplified by the quality of the results illustrated in Fig. 4. Figure 4a shows a transversal virtual section through the proximal end of the Neandertal tibia from the La Ferrassie 2 skeleton [43]. Despite the taphonomic history of the specimen and the restoring interventions having deeply affected its inner morphology, as revealed by the high-resolution record, the distinctiveness of the preserved trabecular network allows a reliable, unequivocal quantification and subtle characterisation of its textural properties. Similarly, Fig. 4b shows a spot from a virtual slice through the femoral head of a Late Miocene hominoid, *Oreopithecus bambolii* [44]. Despite the antiquity of the specimen (IGF 11778, ∼ 7 million years old) and its high degree of mineralisation, the histology-like quality of the structural fossil signal is quite high, hardly distinguishable from that of a modern/recent great ape femur.

3 The 'BoneHomme' project

Based on our experience, synchrotron radiation microtomography constitutes the most effective nondestructive investigative tool currently available to characterise the inner structural morphology and textural properties of bone, even in highly mineralised fossil specimens. With special reference to the needs/constraints of paleobiological research, the system set at the ESRF ID 17 beamline likely represents the best compromise between spatial resolution (which is enough to carefully detail individual strut size as seen in mammals) and average size of the fossil record.

With the aim to contribute to some among the fundamental questions on bone rheology and (paleo)biology listed in the introductory section, by means of the SR-μCT-based 'BoneHomme' project we have documented a sample of 194 postcranial bones for 2D and 3D high-resolution visualisation and analysis. This sample, which likely represents the largest sample ever considered for such kinds of studies, consists of 179 normal and 15 pathological specimens (mostly hip bones ($n = 35$), femurs ($n = 32$), and tibiae ($n = 70$)) from 33 extant and fossil species (mammals and reptiles), including human beings (84 individual bones).

Specifically designed in a morphofunctional ontogenetic perspective, the currently in progress 'BoneHomme' project has been developed (i) to make precise the dynamic relationships between external growth pattern and setting of the inner structural morphology in immature bones; (ii) to comparatively document the functional/genetic relationships between biomechanical environment(s), external bony shape, general inner architecture, and site-specific fine characteristics of the cancellous network in relation to body size and positional/locomotor mode(s); (iii) to assess, in a 3D perspective, the unique locomotion-related inner architecture of the human ilium (Fig. 5) and to quantify its site-specific structural properties as related to the topographic thickness variation of the surrounding cortical bone; (iv) to estimate the structural threshold/boundary between 'normal' and 'abnormal' bony structural condition in (a) a number of human beings of both sexes and different ages-at-death known for having experienced unusual locomotion modes because of different pathological conditions, in (b) mammals recurrently adopting in the wild a quasi-vertical posture when feeding (such as Gerenuk, as opposed to Grant's gazelle), and in (c) in bipedal-trained Japanese macaques [45]; and (v) to contribute to the reconstruction of extinct locomotor modes by decoding the noisy inner structural signal coming from the mammal fossil record.

This project integrates, at high resolution and to large scale, the dynamic system notion of bone/skeleton in a biomechanical developmental perspective. In principle, our approach, which makes use of an extremely sharp analytical

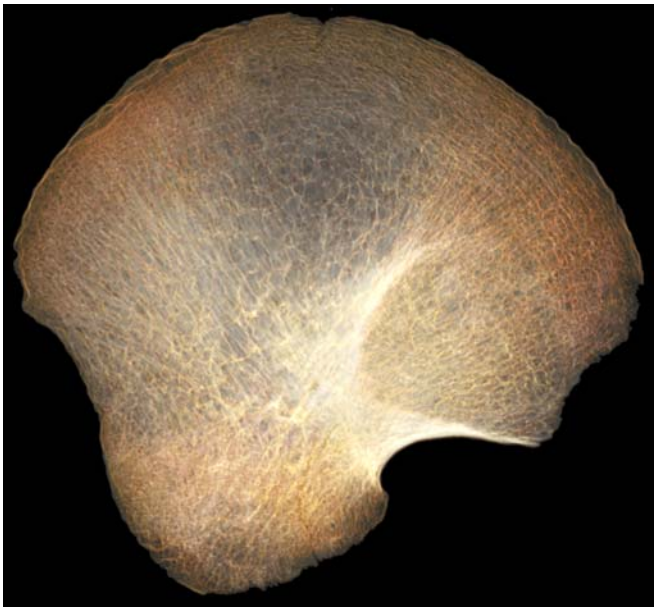

FIGURE 5 SR-μCT-based 3D virtual reconstruction (45.5 μm) of a modern juvenile (6–7 years old) human ilium (spec. SCR 749: Museum NPEP, Rome)

tool, should significantly enhance the current comprehension of the bone functional development in an evolutionary frame, and allow the shift from a two- to a three-dimensional representation, quantification, and functional interpretation of the mammalian bony inner structural morphology.

ACKNOWLEDGEMENTS The analyses at the ESRF ID 17 are developed thanks to the technical and scientific collaboration of A. Bravin, C. Nemoz, and P. Tafforeau. For their scientific contribution to the ongoing researches, we especially thank L. Bondioli, L. de Bonis, F. Delay, B. Illerhaus, G. Koufos, B. Maureille, M. Nakatsukasa, L. Rook, P. Sardini, H. Seidler, J.F. Tournepiche, and D.S. Weaver. For having granted access to fossil and/or extant reference samples in their care for μCT analyses, we are indebted to the following institutions: Kenya National Museums Nairobi, Musée de l'Homme (Paris), Musée des Antiquités Nationales (St-Germain-en-Laye), Musée des Beaux Arts (Angoulême), Musée du Périgord (Périgueux), Musée National de Préhistoire (Les Eyzies-de-Tayac), Museo Nazionale Preistorico Etnografico (Rome), Museum d'Histoire Naturelle (Lyon), Museum National d'Histoire Naturelle (Paris), Univ. of Bordeaux 1, Univ. of Florence, Univ. of Kyoto, and Univ. of Poitiers. We also acknowledge for collaboration A. Bergeret, J. Madec, and P. Mennecier. These researches are supported by ESRF, the French CNRS, Région Poitou-Charentes (to AM and VV), and Univ. of Poitiers, GDR 2152, TNT Project.

REFERENCES

1 F.V. Mariani, G.R. Martin, Nature **423**, 319 (2003)
2 D.M. Wellik, M.R. Capecchi, Science **301**, 363 (2003)
3 R. Huiskes, R. Ruimerman, G.H. van Lenthe, J.D. Janssen, Nature **405**, 704 (2000)
4 R. Ruimerman, P. Hilbers, B. van Rietbergen, R. Huiskes, J. Biomech. **38**, 931 (2005)
5 T. Skerry, in *Development, Growth and Evolution. Implications for the Study of the Hominoid Skeleton*, ed. by P. O'Higgins, M.J. Cohn (Academic Press, London, 2000), pp. 29–39
6 J.D. Currey, *Bone. Structure and Mechanics* (Princeton University Press, Princeton, 2002)
7 C.O. Lovejoy, M.J. Cohn, T.D. White, in *Development, Growth and Evolution. Implications for the Study of the Hominoid Skeleton*, ed. by P. O'Higgins, M.J. Cohn (Academic Press, London, 2000), pp. 41–55
8 R. Huiskes, J. Anat. **197**, 145 (2000)
9 M. Schultz, Yearb. Phys. Anthrop. **44**, 106 (2001)
10 W.D. Carlson, T. Rowe, R.A. Ketcham, M.W. Colbert, in *Applications of X-ray Computed Tomography in the Geosciences* (Spec. Publ. **215**), ed. by F. Mees, R. Swennen, M. van Geet, P. Jacobs (Geological Society, London, 2003), pp. 7–22
11 A. Odgaard, J. Kabel, B. Van Rietbergen, M. Dalstra, R. Huiskes, J. Biomech. **30**, 487 (1997)
12 S.A. Goldstein, R. Goulet, D. McCubbrey, Calc. Tiss. Int. **53**, 127 (1993)
13 M. Dalstra, R. Huiskes, J. Biomech. **28**, 715 (1995)
14 R.B. Martin, D.B. Burr, N.A. Sharkey, *Skeletal Tissue Mechanics* (Springer, New York, 1998)
15 F. Spoor, N. Jeffery, F. Zonneveld, in *Development, Growth and Evolution. Implications for the Study of the Hominoid Skeleton*, ed. by P. O'Higgins, M.J. Cohn (Academic Press, London, 2000), pp. 123–161
16 R. Macchiarelli, L. Rook, L. Bondioli, in *Hominoid Evolution and Climatic Change in Europe. Phylogeny of the Neogene Hominoid Primates of Eurasia*, ed. by L. de Bonis, G. Koufos, P. Andrews (Cambridge University Press, Cambridge, 2001), pp. 60–101
17 Y. Rak, B. Arensburg, Am. J. Phys. Anthrop. **73**, 227 (1987)
18 R. Macchiarelli, L. Bondioli, V. Galichon, P.V. Tobias, J. Hum. Evol. **36**, 211 (1999)
19 L. Rook, L. Bondioli, M. Köhler, S. Moyà-Solà, R. Macchiarelli, Proc. Nat. Acad. Sci. USA **96**, 8795 (1999)
20 V. Volpato, L. Bondioli, R. Macchiarelli, Am. J. Phys. Anthrop. **40**, 215 (2005)
21 G.W. Weber, Anat. Rec. **265A**, 193 (2001)
22 C.F. Spoor, F.W. Zonneveld, G.A. Macho, Am. J. Phys. Anthrop. **91**, 469 (1993)
23 J.C. Ohman, T.J. Krochta, C.O. Lovejoy, R.P. Mensforth, B. Latimer, Am. J. Phys. Anthrop. **104**, 117 (1997)
24 C.O. Lovejoy, R.S. Meindl, J.C. Ohman, K.G. Heiple, T.D. White, Am. J. Phys. Anthrop. **119**, 97 (2002)
25 K. Galik, B. Senut, M. Pickford, D. Gommery, J. Treil, A.J. Kuperavage, R.B. Eckhardt, Science **305**, 1450 (2004)
26 H. Prossinger, H. Seidler, L. Wicke, D.S. Weaver, W. Recheis, C. Stringer, G.B. Müller, Anat. Rec. **273B**, 132 (2003)
27 A. Mazurier, L. Bondioli, A. Bravin, C. Nemoz, R. Macchiarelli, Am. J. Phys. Anthrop. **40**, 147 (2005)
28 R. Macchiarelli, L. Bondioli, M. Chech, A. Coppa, I. Fiore, R. Russom, F. Vecchi, Y. Libsekal, L. Rook, Riv. Ital. Paleont. Stratigr. **110**, 133 (2004)
29 F. Mees, R. Swennen, M. van Geet, P. Jacobs (eds.), *Applications of X-ray Computed Tomography in the Geosciences* (Spec. Publ. **215**) (Geological Society, London, 2003)
30 R.J. Fajardo, R. Müller, Am. J. Phys. Anthrop. **115**, 327 (2001)
31 L. Rook, L. Bondioli, F. Casali, M. Rossi, M. Köhler, S. Moyà Solà, R. Macchiarelli, J. Hum. Evol. **46**, 347 (2004)
32 T.M. Ryan, B. van Rietbergen, Am. J. Phys. Anthrop. **126**, 82 (2005)
33 E. Nägele, V. Kuhn, H. Vogt, T.M. Link, R. Müller, E.-M. Lochmüller, F. Eckstein, Calc. Tiss. Int. **75**, 15 (2004)
34 R. Müller, H. van Campenhout, B. van Damme, G. van der Perre, J. Dequeker, T. Hildebrand, P. Rüegsegger, Bone **23**, 59 (1998)
35 M. Rossi, F. Casali, D. Romani, L. Bondioli, R. Macchiarelli, L. Rook, Nucl. Instrum. Methods Phys. Res. B **213**, 747 (2004)
36 M.H. Day, R.E.F. Leakey, A.C. Walker, B.A. Wood, Am. J. Phys. Anthrop. **45**, 369 (1976)
37 M. Salomé, F. Peyrin, P. Cloetens, C. Odet, A.M. Laval-Jeantet, J. Baruchel, P. Spanne, Med. Phys. **26**, 2194 (1999)
38 F. Peyrin, M. Salomé, S. Nuzzo, P. Cloetens, A.M. Laval-Jeantet, J. Baruchel, Cell. Mol. Biol. **46**, 1089 (2000)
39 M. Ito, S. Ejiri, H. Jinnai, J. Kono, S. Ikeda, A. Nishida, K. Uesugi, N. Yagi, M. Tanaka, K. Hayashi, J. Bone Miner. Metab. **21**, 287 (2003)
40 H. Elleaume, A.M. Charvet, P. Berkvens, G. Berruyer, T. Brochard, Y. Dabin, M.C. Dominguez, A. Draperi, S. Fiedler, G. Goujon, G. Le Duc, M. Mattenet, C. Nemoz, M. Perez, M. Renier, C. Schulze, P. Spanne, P. Suortti, W. Thomlinson, F. Esteve, B. Bertrand, J.F. Le Bas, Nucl. Instrum. Methods Phys. Res. A **428**, 513 (1999)
41 R. Macchiarelli, L. Bondioli, Am. J. Phys. Anthrop. **40**, 177 (2005)
42 A. Bravin, S. Fiedler, P. Coan, J.-C. Labiche, C. Ponchut, A. Peterzol, W. Thomlinson, Nucl. Instrum. Methods Phys. Res. A **510**, 35 (2003)
43 J.-L. Heim, Arch. Paléontol. Hum. **38**, 1 (1982)
44 W.L. Straus Jr, Science **128**, 523 (1958)
45 M. Nakatsukasa, S. Hayama, Cour. Forsch. Inst. Senckenberg **243**, 35 (2003)