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## Bone Mineral Quality

Delphine Farlay<sup>1,2</sup> and Georges Boivin<sup>1,2</sup>

<sup>1</sup>INSERM, UMR 1033, F-69372 Lyon,

<sup>2</sup>Université de Lyon, F-69008 Lyon,  
France

### 1. Introduction

The main function of bone is to promote locomotion and protection of vital organs. Bone is also an important mineral ions reservoir, essential to maintain phosphocalcic homeostasis. Bone mineral is a calcium phosphate named “apatite”, which form naturally in the Earth’s crust (Wopenka & Pasteris 2005). Compared to others minerals, apatite is more “tolerant” and is very accommodating to chemical substitutions. This ability to easily absorb ions confers to bone a detoxification property, with some ions normally absent of bone and which are captured by bone mineral. But the substitutions in bone mineral change the structure of apatite, conferring to bone several properties such as solubility, morphology, hardness, strain etc.

Thanks to those remarkable properties, bone has the ability to continually adapt to changes to its mechanical environment (Bouxsein 2005). Bone is an anisotropic composite material tissue, and highly hierarchical viscoelastic (Bouxsein 2005). When a load is applied to bone, this produces energy, and as this energy can not be destroy, the bone has to absorbed it (Seeman & Delmas 2006). The elastic properties of bone allow to absorb this energy by deforming reversibly. But if the load exceeds the ability of the bone to carry this load, it can deform permanently by plastic deformation (Fig.1). This produces microcracks allowing

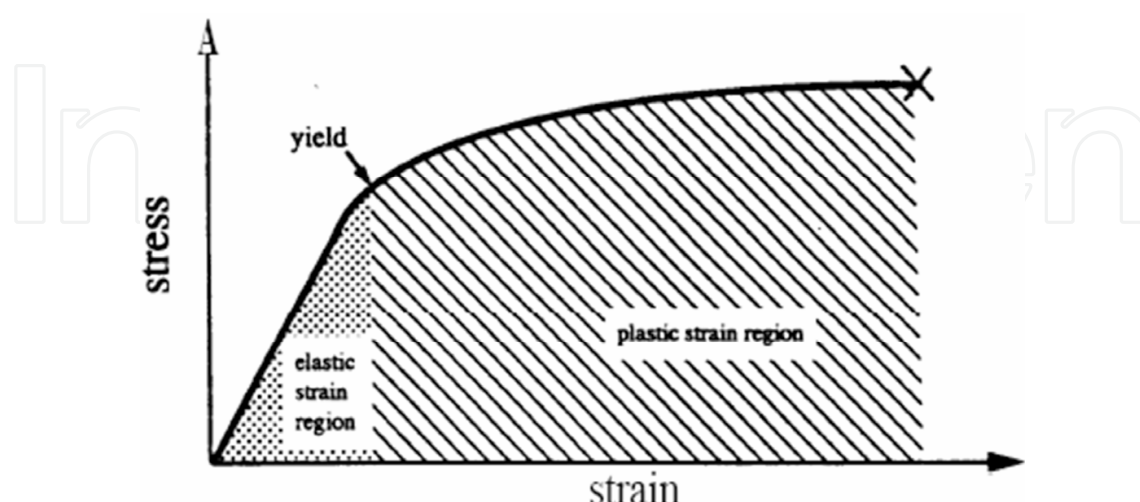


Fig. 1. Stress-strain curve divided into the elastic and plastic regions. The fracture occurs at the end of the curve (marked with X). Reprinted from Turner & Burr, 1993, with permission from Elsevier.

energy release. If the microcracks remain small, this has no impact on bone. However, if the microcracks become numerous and/or too long, the bone fractures. Thus, to resist to a fracture, the bone need to find the best compromise between *stiffness* and *flexibility* (to resist deformation) (Seeman & Delmas 2006). A high mineral content increases *stiffness* reducing *flexibility*, and if the bone is too flexible, it will deform beyond its peak strain and crack. Several studies showed that mineral part is involved in the elastic properties of bone, whereas organic part is rather involved in the plastic deformation (Bala et al., 2011b; Bouxsein 2005; Currey 2003; Follet et al., 2004).

## 2. Determinants of bone quality

Bone is constituted by three major components which are the organic matrix ( $\approx 30\%$ , mainly type I collagen), mineral ( $\approx 60\%$ , carbonated apatite) and water ( $\approx 10\%$ ). Organic matrix is essentially constituted by  $\sim 90\%$  of a network of type I collagen fibrils, and  $\sim 10\%$  of non-collagenous proteins. Type I collagen molecules are formed by three polypeptides  $\alpha$  chains [2  $\alpha 1(I)$  and 1  $\alpha 2(I)$ ] forming a tight triple helix structure with a repetition of Gly-X-Y triplets (Myllyharju & Kivirikko 2004). To provide the stability of type I collagen fibrils, several mechanisms of maturation and ageing of bone collagen occurs, including enzymatic collagen cross-linking and non-enzymatic modifications (Saito & Marumo 2010; Viguet-Carrin et al., 2006). In parallel, the organic matrix mineralizes, and the organization of type I collagen network determines the specific arrangement of mineral crystals (Höhling et al., 1990; Riggs et al., 1993). The crystals first grow in length, typically plate-like (Landis, 1995; Roschger et al., 1998), then they become thicker but stay relatively thin (Roschger et al., 1998). Concomitantly, the crystals number increases up to physiologic limits of mineralization (Bala et al., 2010; Boivin et al., 2008; Boivin & Meunier 2002).

The quantity of bone mineral (assimilated to the quantity of total bone) is usually measured by bone mineral density (BMD), using dual X-ray absorptiometry (DXA). However, about one-half of fractures occur in women having a T-Score above the World Health Organization (WHO) diagnosis threshold of osteoporosis ( $\leq -2.5$ ) (Siris et al., 2004; Sornay-Rendu et al., 2005) suggesting that other factors than bone quantity are involved in the apparition of fractures. These factors, involved in bone quality, are called intrinsic determinants. Both extrinsic determinants (including bone mass, macro/microarchitecture) and intrinsic determinants are involved in bone strength, and are directly dependent of bone remodeling activity (Fig.2).

DXA measurement gives information on bone mineral mass but not on its mineral quality. For example, when fluoride salts was used to treat post menopausal osteoporosis, an increase in bone mineral density was measured by DXA, but the bones of patients treated with fluoride salts were much more brittle than untreated patients. In fact, fluoride ions in bone mineral impaired the bone mineral quality increasing the size of bone (large crystals), and thus reducing the contact area with collagen matrix, despite a higher amount of bone mineral density.

The determinants of bone quality, called "intrinsic determinants", are thus essential in bone strength. Those determinants include mineral quality, collagen quality, and presence of microcracks. A good collagen quality is required to an optimal bone strength. For example, osteogenesis imperfecta, which is an heritable brittle-bone disease, is characterized by a type-I collagen mutation, leading to collagen fibrils abnormally thin, and to an excessive

bone fragility (Rauch & Glorieux 2004). Among these determinants, microcracks are normally present in bone, and permit to dissipate energy when bone is submitted to a load. However, the presence of too long microcracks is not good for bone. Thus, all together those extrinsic and intrinsic determinants are involved in bone strength.

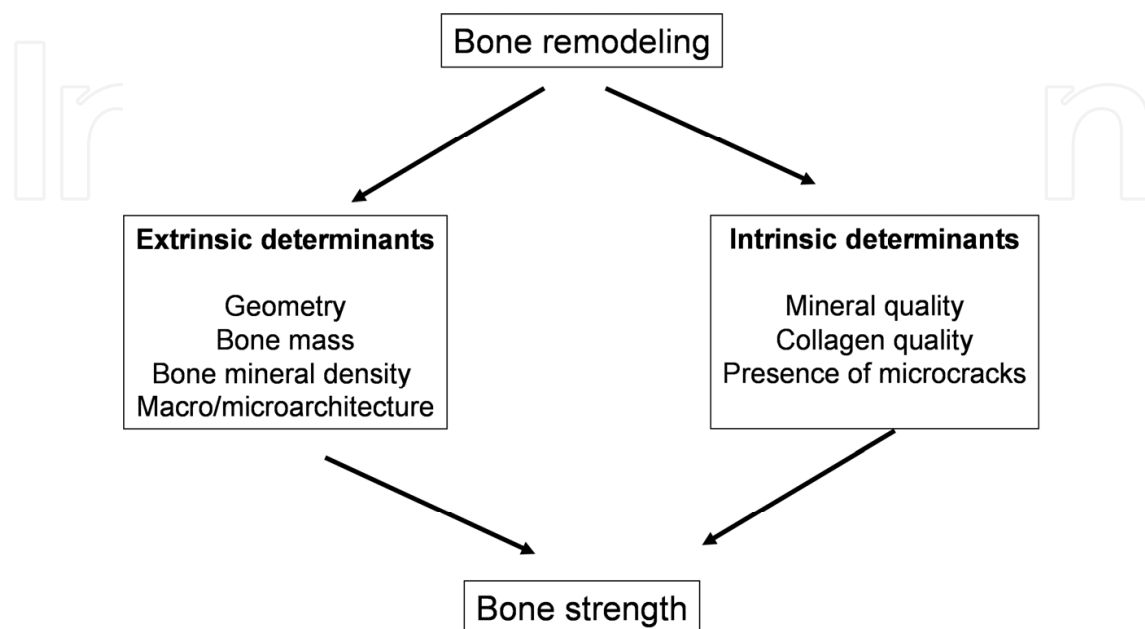


Fig. 2. Description of determinants of bone strength (INSERM UMR 1033)

Bone mineral properties are important determinants of bone strength. Those properties include material (degree of mineralization, hardness...) and crystalline (crystallinity, mineral maturity, ionic substitutions...) characteristics. The importance of the components of bone quality is thus evident and the relationships between the determinants of bone quality are essential to maintain an overall mechanical competent bone. Indeed, bone mineral is complex and knowledge of its composition is important to better understand the mechanisms of bone fragility.

The main purpose of this present chapter is to bring a best approach of bone mineral quality, mineralization process, mineral crystals and mineral composition.

### 3. Mineralization process: A dynamic process

As bone is submitted to a constant remodeling during all the adult life, old Bone Structural Units (BSUs, named the osteons in cortical bone and the trabecular packets in cancellous bone) will be resorbed by osteoclasts, and replaced by new formed bone. Thus, the recently formed BSUs will be less mineralized than the older BSUs present in interstitial bone and not already resorbed. This heterogeneity of mineralization in the different BSUs can be easily visualized on a X rays microradiograph (Fig. 3). This mineralization process related to bone remodeling can be decomposed into two steps: a primary mineralization which corresponds to a very rapid deposition of first crystals, and a secondary mineralization which is much longer, with a slow and gradual increase in size, perfection and number of crystals.

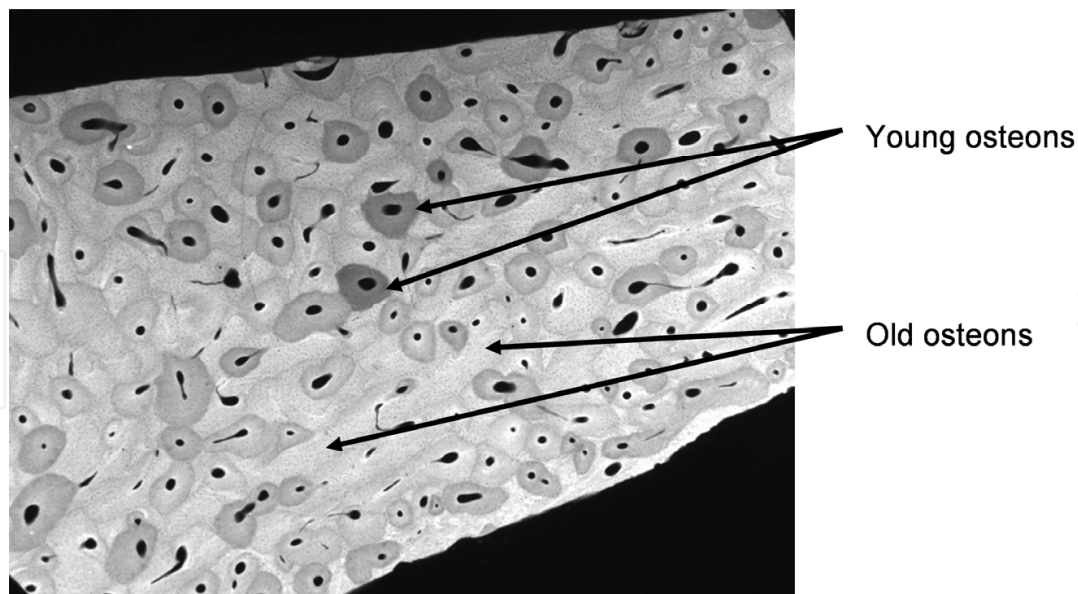


Fig. 3. Microradiograph of human femur illustrating the heterogeneity of the mineralization (cortical bone of a man, 48 year-old) (INSERM UMR 1033)

### 3.1 Primary and secondary mineralization processes

The process of primary mineralization is a very rapid process, starting in the unmineralized bone matrix (osteoid) deposited by the osteoblasts; in humans, the new matrix begins to mineralize after 5 to 10 days after the deposition of osteoid. The primary mineralization can be measured using double tetracycline labeling (Frost 1969) (Fig. 4). The double labeling involves the administration of two short courses of tetracycline which is deposited along the calcification front as two distinct lines visualized on bone sections under ultraviolet (UV) light. This allows the measurement of the mineral apposition rate (MAR). Usually, a labeling procedure is 10 mg/kg/day demethylchlortetracycline or tetracycline hydrochloride orally for 2 days, 12 days off, 4 days on. The bone biopsy is then taken 4-6 days later (Frost 1969).

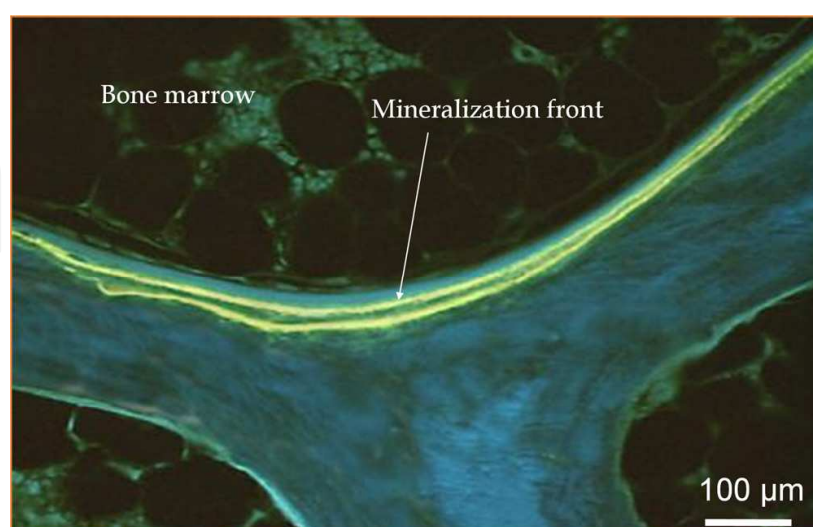


Fig. 4. Histological slide of bone tissue observed under UV light and illustrating a double tetracycline labelling (yellow lines) in trabecular bone and showing the front of mineralization (INSERM UMR 1033).



In adult, MAR varies from 0.60 to 0.80  $\mu\text{m}/\text{day}$  whatever the age and the sex (Vedi et al., 1983). MAR is slightly increased in young children, reaching 1  $\mu\text{m}/\text{day}$  (Glorieux et al., 2000). During the process of primary mineralization, the first depositions of mineral correspond to about 50 to 60 % of the maximal mineral charge in bone tissue (Meunier & Boivin 1997). This process is extremely rapid, and the first depositions of mineral are used as nucleator for the secondary mineralization. The secondary mineralization corresponds to a slow and gradual increase in both crystal size and number. This process increases until a physiological limit: once the maximum number of crystals attained in a given volume, it is not possible to exceed this limit. Thus in bone remodeling, there is no process of “hypermineralization”, because a given BSU can not contain more crystals than its physiological capacity. The duration of the secondary mineralization is unknown in humans. This duration has been reported in rabbits (Fuchs et al., 2008) and more recently in an animal model (ewes) having a remodeling activity close to the Humans (Bala et al., 2010). The chronology of secondary mineralization has been identify by injection of different fluorescent labels every six months, in order to date the “age” of the BSUs (Bala et al., 2010). In this study, it has been shown that the secondary mineralization lasts approximatively for 24 to 30 months, suggesting that after this time, no increase in degree of mineralization occurs (Fig. 5).

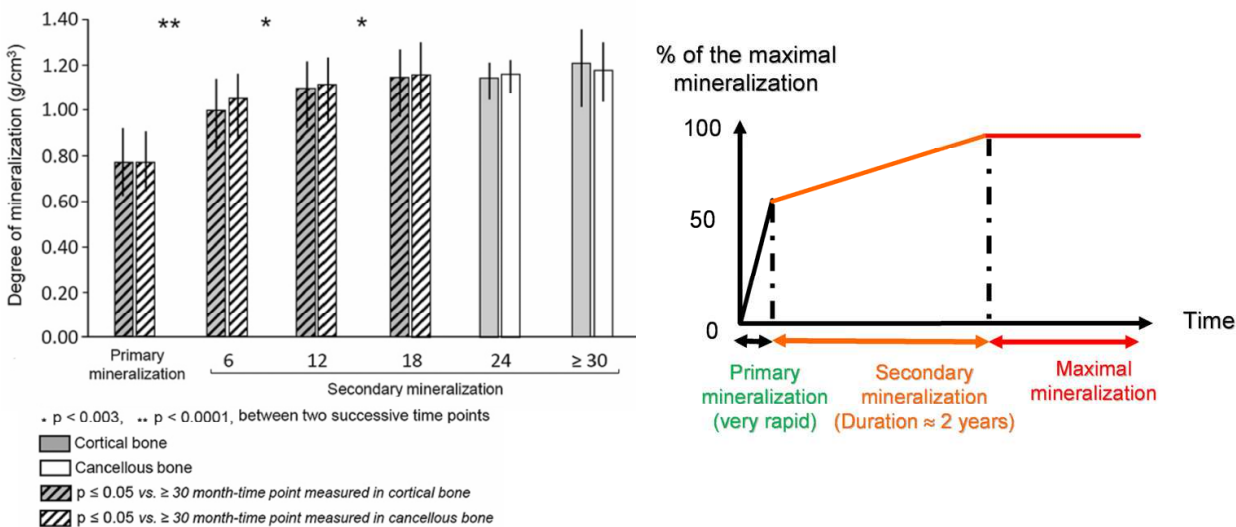


Fig. 5. Left: Degree of mineralization measured by quantitative microradiography in ewes in cortical and cancellous bone, every 6 months (on 512 BSUs; Reprinted from Bala et al., 2010, with permission from Elsevier). Right: Schematic representation of the duration of primary and secondary mineralizations.

This duration of mineralization should be taking into account into anti-resorptive treatment of post menopausal osteoporosis, because once that all the BSUs have attained their maximal mineralization, no gain in term of DMB will occurs.

### 3.2 Methods to measure degree of mineralization

Several methods are used to measure the degree of mineralization of bone. The technique used in the laboratory is the quantitative microradiography, which is a computerized microdensitometric method based on the X-rays absorption (Boivin & Meunier 2002). Others methods are used to measure the degree of mineralization, as the quantitative backscattering electron or synchrotron infrared microspectroscopy.

### 3.2.1 Quantitative microradiography

#### 3.2.1.1 Specimen preparation

Undecalcified iliac bone samples were generally used in humans, fixed in 70% alcohol for ten days or more (depending on the size of the samples), and then specimens are placed two days in absolute alcohol to complete dehydration. Alcohol baths are changed every day and specimens are substituted in methylcyclohexane again for two days, before embedding in methyl methacrylate (MMA). The latter is a transparent and hard plastic having a very low X-ray absorption power. Samples are kept for two days in MMA monomer alone, at 4°C, two days in MMA with 1% of catalyst (anhydrous dibenzoyl peroxide) and 2 days in MMA with 2% of catalyst. Then the specimens are placed in an oven (30°C) for final polymerization to obtain hard blocks. After polymerization, thick sections are cut from the embedded bone samples with a precision diamond wire saw, progressively ground to a thickness of 100 µm and polished with an alumina suspension. The thickness of the section was measured with an accuracy of 1 µm using a precision micrometer. After ultrasonic cleaning in demineralised water, the bone sections were microradiographed. If orientation of the blocks is possible before sectioning, the cutting plane perpendicular to the haversian canals of cortical bone is preferred.

#### 3.2.1.2 Measurement of degree of mineralization (DMB) (Boivin & Meunier 2002)

Soft X-rays are produced in a X-ray generator (Philips compact PW1830/40 X-ray diffraction generator, Limeil Brévannes, France), equipped with a diffraction tube PW 2273/20. A monochromatic X-ray beam is employed, i.e., nickel-filtered copper K $\alpha$  radiation with a wavelength of 1.54 Å for which the ratio of the mass- absorption coefficients of aluminium to apatite is 0.561. The distance between the X-ray source and the specimen is about 25-30 cm. In a dark room, the 100 µm-thick bone sections are placed on a photographic emulsion covered by a thin polyester (mylar) film transparent to X-rays, and placed in a specimen holder. An aluminium step-wedge is also exposed on each microradiography. Aluminium was chosen because it is convenient material having an atomic number not far from the effective atomic number of hydroxyapatite. The section is firmly pressed flat by tightening the specimen holder cap and evacuating the air situated between the mylar and the emulsion by means of a vacuum pump, thus bringing the section in direct contact with the emulsion. The specimen holder is placed in a camera perpendicular to the X-ray beam and locked into position during X-ray exposure, during 20 min at 25 kV and 25 mA.

After X-ray exposure, the film (VRP-M green sensitive emulsion from Geola, Slavich International Wholesale Office, Vilnius, Lithuania) is developed for 5 min in Kodak D19 at 20°C, rinsed and then fixed for 5 min in Ilford Hypam. The film is washed and dried, then mounted between two slides. The DMB is quantified using an automatic program for analyzing grayness levels (MorphoExpert and Mineralization, ExploraNova, La Rochelle, France). A digital camera (resolution: 1600 x 1200 pixels or 800 x 600 after binning), captures the microscopic image of the microradiograph. After calibration with the aluminium reference system, the measured regions of bone tissue are automatically selected, and the gray levels are segmented after bone thresholding. The values of the gray levels are then obtained at pixel level (for a magnification x2.5, the size of the pixel is 2.82 µm). Finally, gray-level are converted into DMB measurements with the construction of a calibration curve based on the measurements obtained on the aluminium step-wedge. DMB is finally expressed in gram of mineral over cm<sup>3</sup> of bone (g/cm<sup>3</sup>) and measured separately in cortical

and cancellous bone. The main parameters, extracted from the DMB measurements, are the mean DMB, the mean highest and most frequent DMB (DMB Freq. Max) and the mean index of heterogeneity of the distribution of DMB expressed as the mean of the widths at half-maximum measured on the individual DMB curves.

### 3.2.2 Quantitative backscattering electron imaging (qBEI)

The mineral content of bone samples has also been evaluated (Roschger et al., 1995, 1998, 2003; Ruffoni et al. 2007) by quantitative Backscattered Electron Imaging (qBEI). This method, based on the detection of electrons backscattered (BSE) on the surface of the bone specimen, is generally used on the same type of bone biopsy fixed in alcohol and embedded in MMA. As the intensity of the BSE signal is strongly related to the atomic number ( $Z$ ) of the specimen, BSE images provide information about the distribution of different elements in the sample. A calibration of the BE signal with carbon and aluminium as references was performed. Osteoid and hydroxyapatite were also employed as references to convert gray level values into calcium weight % values. In bone, the main signal is related to Ca ( $Z=20$ ) and P ( $Z=15$ ) which are the main mineral elements (Roschger et al., 1998). These authors have correlated BE gray levels of bone with calcium content (in weight percent Ca) based on the Ca  $K\alpha$ -line intensities detected from identical bone areas (Roschger et al., 1995). The intensity of the backscattered electron signal from the sample is directly proportional to the bone calcium concentration and can therefore be used for the generation of bone mineralization density distribution (BMDD). BMDDs display the frequency of certain calcium concentrations and are analyzed for the weighted mean calcium concentration (Ca mean), the most frequent calcium concentration (Ca peak) and the homogeneity of mineralization (Ca width). The BMDD of trabecular bone from healthy, adult individuals was shown to be nearly constant over several biological factors (gender, age, ethnicity, skeletal site). Technical and biological variations showed that it is a method sensitive for subtle changes in mineralization.

### 3.2.3 Synchrotron radiation microtomography (SR $\mu$ CT)

Aside from the difficulty of access to synchrotron radiation facilities, a main advantage of this technique is the use of a mono-energetic synchrotron beam, thus avoiding beam-hardening effects. Indeed, the reconstructed gray levels of tomographic images correspond directly to a map of a linear attenuation coefficient within the sample. The SR $\mu$ CT method has been tested on human bone tissue (Borah et al., 2005, 2006; Nuzzo et al., 2002) but it is still an equipment with difficulty accessible. The availability of a three-dimensional (3D) measuring technique coupled to specific image processing method opens new possibilities. SR $\mu$ CT may provide 3D images with spatial resolution as high as one micrometer. The acquisition of 3D bone samples images at high spatial resolution using SR $\mu$ CT has proved to be very accurate for quantifying human bone micro-architecture. Moreover SR $\mu$ CT is a non destructive, fast, and very precise procedure to determine the DMB in 3D, simultaneously to the micro-architecture. The calibration procedure used homogeneous phantoms of water solutions at different concentrations of  $K_2HPO_4$  (Nuzzo et al., 2002). This method was compared with the quantitative microradiography technique on the same bone samples, and showed that the values of the DMB are both in the range 0.5-1.6 g/cm<sup>3</sup> of bone, both in cortical and cancellous bone, with a mean difference around 4.7%, slightly higher in trabecular region (Nuzzo et al., 2002).



### 3.3 Bone microhardness at the tissue level

Another important characteristic of bone mineral is its hardness (Currey 2003; Nalla et al., 2003). Thanks to indentation techniques, it has been shown that microhardness of bone osteon was strongly related to its mineral content (Amprino 1958; Bala et al., 2010; Boivin et al., 2008; Carlstrom 1954; Weaver 1966). From a mechanical point of view, microhardness parameter is related to both elastic and plastic deformations, and an indentation technique has been developed to measure directly both elastic modulus ( $E$ ) and contact hardness ( $H_c$ ) on small area of bone tissue (Oliver & Pharr 1992). However, this technique has been developed for isotropic materials. While it is known that the bone tissue is complex with an anisotropic structure, the measurements of  $E$  is usually performed with a defined Poisson's ratio ( $\nu=0.3$ ). It has been shown that contact hardness was linearly interdependent with elastic modulus (Oyen 2006). Therefore, contact hardness can give an evaluation of bone stiffness which is directly related to its brittleness. Contact hardness can be evaluated at the microstructural (BSU) or nanostructural (lamellar) levels. At the microstructural level in bone, the pyramidal square-based Vickers indenter is often used (Fig. 6) (Boivin et al., 2008), and nanoindentation is rather used at the lamellar level using Berckovich indenter (Ammann & Rizzoli 2003). Very recently, it has been shown, by instrumented nanoindentation, that contact hardness was correlated both to DMB and collagen maturity (Bala et al., 2011b). Mineralization is a major determinant of microhardness, with about two-thirds of the variance, and one-third being explained by the organic matrix (Boivin et al., 2008). In human control bone, the microhardness does not vary with age and sex, in cortical and cancellous bone, as for the degree of mineralization. In 19 human control bones, the hardness in cortical bone is about  $49.30 \pm 2.16$  kg/mm<sup>2</sup> and in cancellous bone about  $48.92 \pm 1.57$  kg/mm<sup>2</sup>.

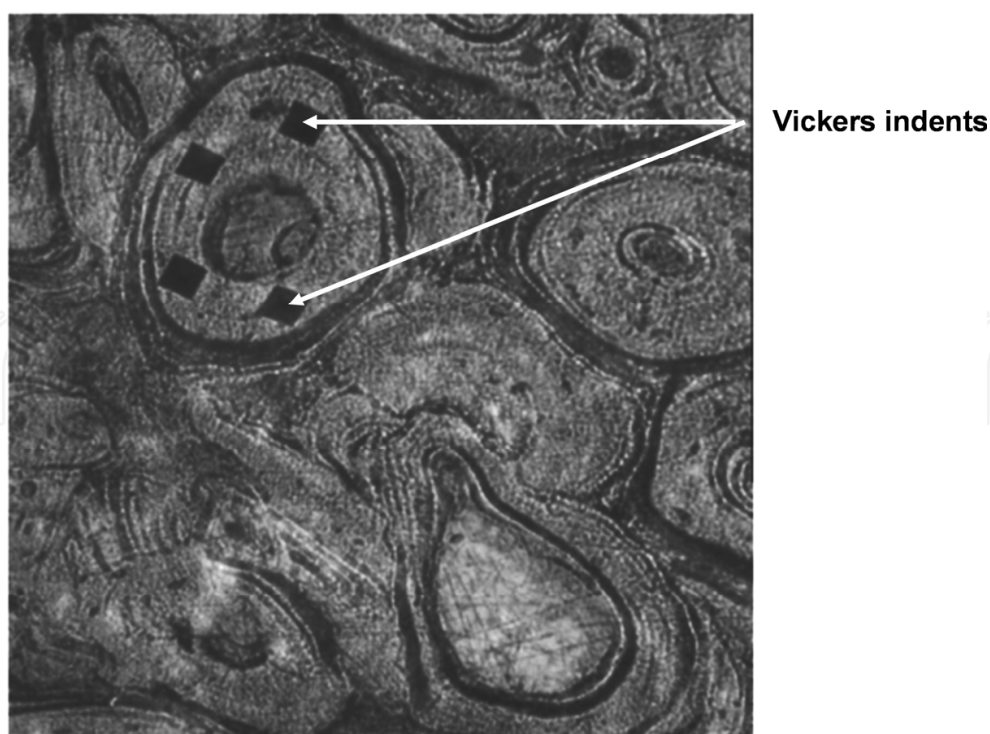


Fig. 6. Iliac bone from ewe showing a Bone Structural Units (BSU) with 4 Vickers indents (INSERM UMR 1033).

#### 4. Characteristics of bone mineral crystals

Human bone mineral is a non-stoichiometric and poorly crystallized apatite. Bone apatite structure is hexagonal with space group  $P6_3/m$ , with lattice parameters  $a=9.42\text{\AA}$  and  $c=6.88\text{\AA}$ . It is a calcium (Ca)-deficient apatite analog, contains major elements like calcium,  $[\text{Ca}^{2+}$  (40 wt %)], phosphate  $[\text{PO}_4^{3-}$  (18 wt %)], carbonates  $[\text{CO}_3^{2-}$  (6-7 wt %)], minor elements such as magnesium ( $\text{Mg}^{2+}$ ) or sodium ( $\text{Na}^{+}$ ), and trace elements (LeGeros & LeGeros 1983; LeGeros et al., 1968). Bone mineral also contains ions normally absent from body fluids (lead, fluoride, aluminium etc). Indeed, the apatite lattice is very tolerant to substitutions and vacancies. Compared to dental enamel, the bone mineral contains much more vacancies. In fact, apatite is able to incorporate itself, in its atomic structure, the half of the elements in the periodic chart (Wopenka & Pasteris 2005). Apatite lattice contains about 40 ions, and the unit cell is the smallest basic unit which is a sample of the entire lattice array (Glimcher 1998). In the apatite unit cell, four different types of crystallographic positions (or "sites") have been identified (Fig.7): (1) tetrahedral sites for six  $\text{P}^{5+}$ -ions, each in 4-fold coordination with oxygen, (2)  $\text{Ca}[I]$  sites for four of the  $\text{Ca}^{2+}$  ions, (3)  $\text{Ca}[II]$  sites for the six other  $\text{Ca}^{2+}$  ions, and (4) the channel site, occupied by two monovalent anions ( $\text{OH}^-$ ,  $\text{F}^-$  and/or  $\text{Cl}^-$ ) (Posner 1969; Wopenka & Pasteris 2005). The small ions ( $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ) are preferentially incorporated into  $\text{Ca}[I]$ , whereas bigger ions ( $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Pb}^{2+}$ ) are incorporated into  $\text{Ca}[II]$  (Fig. 7). The reason why apatite is the mineral component of vertebral skeleton is not known, but it was shown that apatite is the only calcium-phosphate mineral phase that is stable at both a neutral and basic pH (Glimcher 2006; Omelon et al., 2009). Another explanation is coming from the presence of dense granules containing polyphosphates near the mineralizing cartilage and resorbing bone (Omelon et al., 2009). Indeed, when the mineral apatite is dissolved after acidification and resorption by osteoclasts, there is no reprecipitation within the resorption pits, even the return to a neutral pH. The hypothesis of the authors was that polyphosphates formation provides a mechanism for accumulating phosphate, controlling the apatite at locations sites previously mentioned. Enzymatic action can thus control apatite supersaturation at neutral pH, directly by controlling orthophosphate ion activity (Omelon et al., 2009).

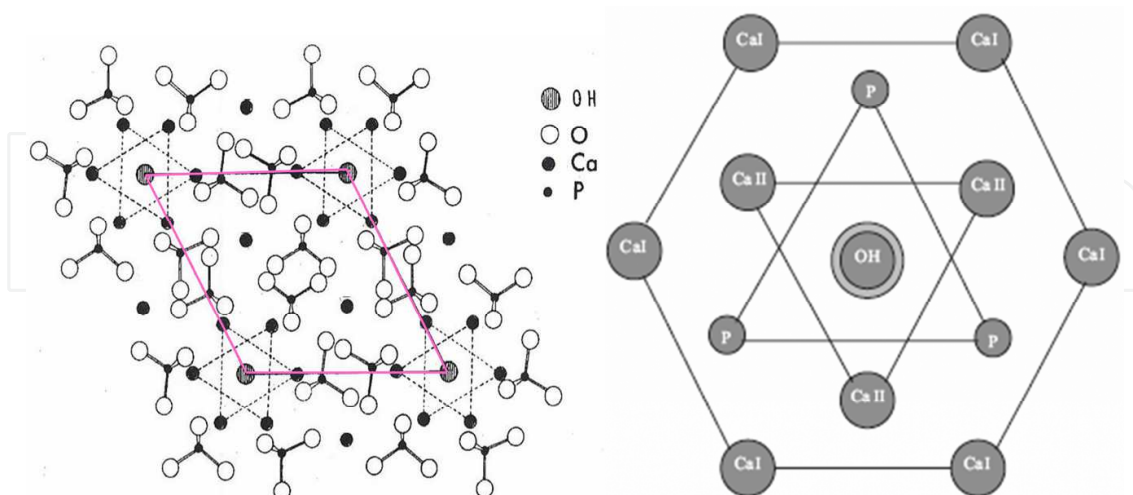


Fig. 7. (Left) Hexagonal system of apatite lattice, showing the disposition of atoms. (Right) The Ca ions occupy two crystallographic non-equivalent sites (Ca I and Ca II). From (Reprinted from Li et al., 2007, with permission from Elsevier). Small ions ( $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$ ) are preferentially incorporated into  $\text{Ca}[I]$ , whereas bigger ions ( $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Pb}^{2+}$ ) are incorporated into  $\text{Ca}[II]$ .

#### 4.1 Bone crystal size and shape

The crystal structure and morphology of bone minerals have often been controversial, mainly due to the different techniques used to characterize bone mineral. Today, with the use of more accurate method (atomic force microscopy, high resolution transmission electron microscopy), it is clear that the bone mineral crystals are very small and platelet-shaped (length  $\approx 200\text{-}600\text{ \AA}$ , width  $\approx 100\text{-}200\text{ \AA}$ , thickness  $\approx 20\text{-}50\text{ \AA}$ , Figs. 8 and 9). Compared to bone crystals, enamel crystals are needle-shape and much bigger. This small bone crystal size has several advantages. First, it permits an extended surface area ( $100\text{-}200\text{ m}^2/\text{g}$ ). Two factors are involved in the surface activity: the surface area expressed in  $\text{m}^2/\text{g}$  and the physical and chemical properties of the surface. These properties determine the type of reactions, while the surface area determines the number of reactions. The combination of both factors makes the bone mineral substance metabolically very active; consequently, crystals have a very large interface with extracellular fluids. For example, the crystals contain in a small lumbar vertebra (L1 or L2) having a dry wet of 30 g, have a specific surface comparable to that of the playing field of soccer. Bone mineral is metabolically active, various and numerous interactions between ions from the extracellular fluid and ions constituting apatite crystals, are thus possible. Second, another interest of the small crystal sizes is mechanic. Indeed, the highly ordered location and orientation of very small crystals within the collagen fibrils permits an acceptable range of flexibility without fracture or disruption of the bone substance (Glimcher 1998; Landis 1995).

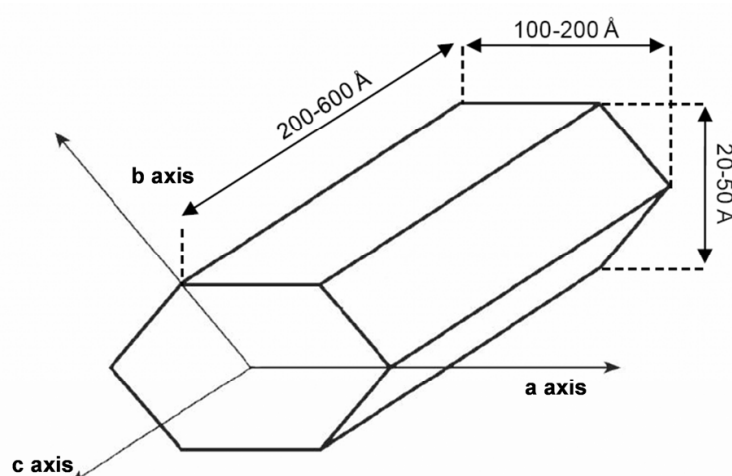


Fig. 8. Schematic representation and crystal size of bone apatite with the 3 axis (INSERM UMR 1033)

#### 4.2 Relationships between water, organic matrix and mineral

In bone, the process of crystal nucleation in bone matrix is heterogenous, and is formed within the "hole" band of type I collagen ( $670\text{ \AA}$ ) (Glimcher et al., 1957). During the process of mineralization, the apatite crystals replaced some of the molecules of water so their content is inversely proportional to that of water (Elliott & Robinson 1957; Robinson 1975). Once deposited, the mineral phase induces compaction of the collagen fibril structure. Neutron and X-ray diffraction have shown that the Bragg-spacing of collagen strongly decreases with increasing mineral content (Lees 1987). Computer modeling and SAXS confirmed the process of closer packing of the collagen molecules when clusters of mineral



crystals replaced the water within the fibrils (Fratzl et al., 1993). The expansion of mineral crystals compressed the collagen molecule packing, thus decreasing the molecular spacing. This indicated the close relationship between water and the mineral deposition process. Modification of the collagen packing probably influences secondary structure of organic matrix. More recently, in intact bovine bone, the effects of dehydration have been studied using solid-state NMR spectroscopy (Zhu et al., 2009). Interestingly, well-resolved peaks broadened with dehydration, suggesting a conformational disorder and structural changes of bone matrix. This is in agreement with other studies showing a collagen conformational change with dehydration (Naito et al, 1994 ; Saito et al, 1984, 1992).

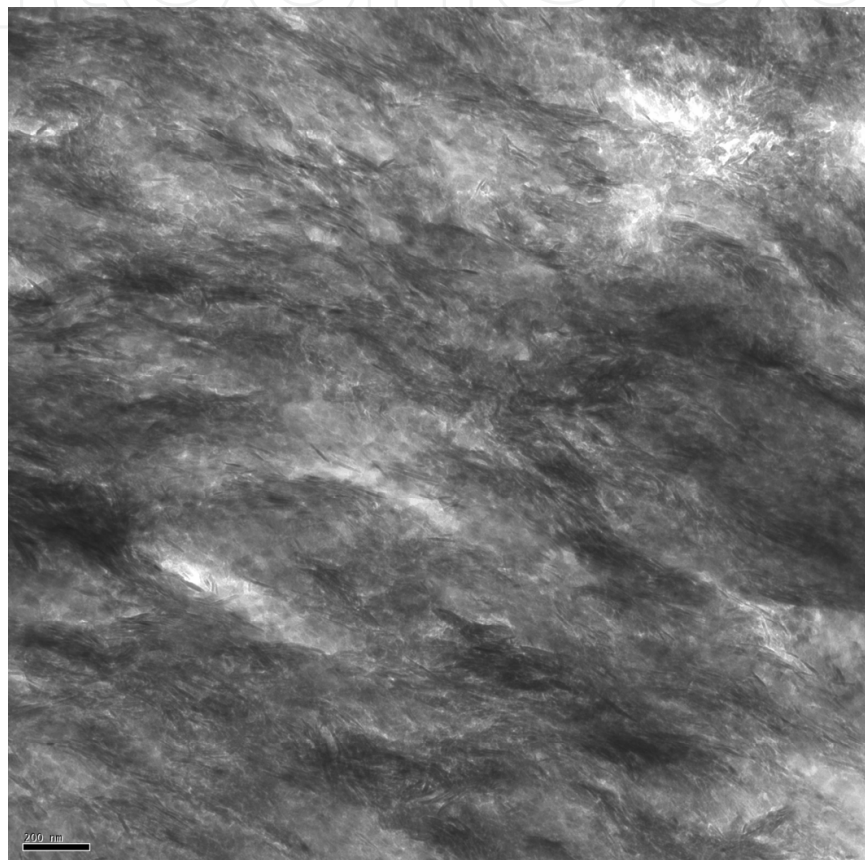


Fig. 9. Electron micrograph of human cancellous bone (woman, 80 year-old) illustrating the bone crystals within the type I collagen (INSERM UMR 1033).

It was suggested that water could play a role in the mechanical behavior of cortical bone (Nyman et al., 2006) and the removal of water alter crystallographic structure of synthetic apatites (LeGeros et al., 1978). As the water content decreases in bone with age (Jonsson et al., 1985; Mueller et al., 1966), it was suggested that water could be involved in bone fragility. Three main types of water exist in bone: the freely mobile water located into vascular lacunar canalicular spaces, the water bound to the collagen network, and the water bound to the mineral. More precisely, there is two type of water bound to the mineral: (a) the water bound to the surface of bone crystals, and (b) the water located within the apatite lattice. Water bound to the collagen fibrils provides post-yield toughness to bone and when water was removed, the strength and stiffness was increased whereas the toughness was decreased (Nyman et al., 2006). The role of the loss of water bound to the mineral on bone

strength was not clear in this study, while it was suggested that the loss of water located in apatite lattice could change the size of the bone mineral crystals (Nyman et al., 2006), since it was already observed in dehydrated enamel or precipitated apatites (LeGeros et al., 1978). Consequently, the decrease in bone strength and toughness related to age could be due to a change in water distribution.

4.3 Chemical composition of bone mineral

The composition of bone mineral was for a long time assimilated to hydroxyapatite, but it is not. Several studies showed the lack of OH<sup>-</sup>, by inelastic neutron scattering, Raman of infrared spectroscopies (Loong et al., 2000; Pasteris et al., 2004; Rey et al., 1995b). Solid state-NMR shows that the percentage of OH<sup>-</sup> does not exceed 20% of the amount expected in stoichiometric apatite in human cortical bone (Cho et al., 2003). In fact, the small crystal size could be one of the reasons for the absence of OH<sup>-</sup> ions in the bone apatite. Indeed, Wopenka and Pasteris have suggested that the small crystal size and the great the atomic disorder within the unit cells of the crystal was not energetically favourable for apatite for incorporate OH<sup>-</sup> into its channel (Pasteris et al., 2004). Another reason for the lack of OH<sup>-</sup> ions in bone apatite could be due to the type-B substitution of PO<sub>4</sub> by CO<sub>3</sub>, creating a vacancy in the channel site to maintain electrostatic equilibrium.

Nature of apatite	Chemical formula
Hydroxyapatite	Ca <sub>10</sub> (PO <sub>4</sub> ) <sub>6</sub> (OH) <sub>2</sub>
Bone mineral	Ca <sub>8.3□1.7</sub> (PO <sub>4</sub> ) <sub>4.3</sub> (HPO <sub>4</sub> ) (CO <sub>3</sub> ) <sub>1.7</sub> (OH) <sub>0.3□1.7</sub>
Dental enamel	Ca <sub>9.4□0.6</sub> (PO <sub>4</sub> ) <sub>5.4</sub> (HPO <sub>4</sub> ) (CO <sub>3</sub> ) <sub>0.6</sub> (OH) <sub>1.4□0.6</sub>

Table 1. Chemical formula of different apatites (□: vacancy) (adapted from Cazalbou et al., 2004a)

When an ion with the same electric charge is substituted within the bone apatite, no effect is produced on the structure lattice. If ions have a different electric charge (CO<sub>3</sub><sup>2-</sup> substituted for PO<sub>4</sub><sup>3-</sup>), a vacancy is created to maintained electrostatic equilibrium. Some ions can be replaced by other ions of almost identical radius. These ions induce only minor changes in shape at crystal level and do not affect the structure of the crystal. Such substitutions occur during formation of the crystal or through ionic exchanges with the existing crystals. In vitro, some interactions between mineral substance and the solution lead to the diffusion of ions within the hydrated shell, the exchanges at the crystal surface and the exchanges inside the crystal. Similar mechanisms are likely to occur in vivo during remodeling of bone tissue (Blumenthal 1990; LeGeros 1981; Posner 1985). Some cations of similar size and charge as the Ca<sup>2+</sup> (Sr<sup>2+</sup>, Na<sup>+</sup>), as well as others that cannot substitute for the Ca<sup>2+</sup> in the apatite structure (Ba<sup>2+</sup>, Ra<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>), are easily exchangeable from the solution to the surface Ca<sup>2+</sup> ions. Such substitutions lead to modifications in the *a* and *c* parameters of the apatite unit cell. Substitutions with Mg<sup>2+</sup> are only partial. In biomimetic apatite nanocrystal, the incorporation of Mg has been recently studied to analyze the effect of substitution with Ca<sup>2+</sup> on particule morphology (Bertinetti et al., 2009). Incorporation of Mg<sup>2+</sup> does not affect apatitic nature, nonetheless, a lower degree of crystallinity was observed by XRD (Bertinetti et al., 2009). Moreover, it was shown that apatites enriched with Mg<sup>2+</sup> retain more water at their surface than Mg<sup>2+</sup>-free apatites (Bertinetti et al., 2009). Calcium can be easily substituted by



significantly larger ions, but less frequently by smaller ions (Blumenthal 1990). An ion can only be substituted to another if its ionic radius is less than 10% higher than the radius of the ion replaced. The exchange of anions ( $\text{PO}_4^{3-}$ ,  $\text{F}^-$ ) around the crystal surface is also well known. The substitution of  $\text{F}^-$  with  $\text{OH}^-$  ions can not be reversed because it leads to the formation of a more stable compound:  $\text{F}^-$  ions are more similar to  $\text{Ca}^{2+}$  ions than  $\text{OH}^-$  ions and the electrostatic links between  $\text{Ca}^{2+}$  and  $\text{F}^-$  are stronger than the ones between  $\text{Ca}^{2+}$  and  $\text{OH}^-$ . The more insoluble apatite is the fluoroapatite [chemical formula:  $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$ ]. To be resorbed by osteoclasts, the bone mineral have to be more soluble than hydroxyapatite, and the vacancies present in bone mineral enable this dissolution. Carbonates can be found in apatite crystals as  $\text{CO}_3^{2-}$  ions, which can substituted for either  $\text{PO}_4^{3-}$  or  $\text{OH}^-$  ions. When the volume of  $\text{CO}_3^{2-}$  ions increases, the  $a$  parameter of the crystal unit cell decreases, while the  $c$  parameter increases.

Some foreign ions can increase or decrease the bone crystal size. In the case of  $\text{Mg}^{2+}$ , *in vitro* studies have shown that  $\text{Mg}^{2+}$  bound to the hydroxyapatite crystals retarded nucleation and growth of the crystal. *In vivo* studies show a decrease in crystal size in Mg-deficient rats, thus Mg interferes with the mineralization process (Bigi et al., 1992; Blumenthal et al., 1977; Boskey et al., 1992).

Others ions, as  $\text{Fe}^{3+}$  ions, have a direct effect on hydroxyapatite, inhibiting the growth and changing the quality of crystals (decrease in crystallinity and increase in carbonate substitution) (Guggenbuhl et al., 2008). Aluminium also affects bone mineralization, and osteomalacia renal osteodystrophy has been associated, in patients on long-term hemodialysis, with  $\text{Al}^{3+}$  accumulation in bone (Blumenthal & Posner 1984). A recent study on rats showed that a long-term  $\text{Al}^{3+}$  exposure reduces the levels of mineral and trace elements in bone (Zn, Fe, Cu, Mn, Se, B, and Sr) (Li et al., 2010b). This is accompanied by a decrease in BMD especially in cancellous bone. An high amount of ions which are normally present in small proportion in bone mineral can cause alteration of bone substance. As previously mentioned, fluoride ions at high doses cause osteomalacia and defects of mineralization (Balena et al., 1998). On the other hand, small doses of some ions can have positive effect on bone strength. For example,  $\text{Sr}^{2+}$  (Strontium ranelate is an osteoporosis treatment) reduces both the vertebral and non vertebral fractures (Meunier et al., 2004; Reginster et al., 2005). Besides the effect of  $\text{Sr}^{2+}$  on bone cells (stimulating bone formation and decreasing bone resorption) (Grynepas & Marie 1990; Marie et al., 1993), the presence of  $\text{Sr}^{2+}$  is shown in the bone mineral formed during treatment, in osteoporotic women treated with strontium ranelate for 3-5 years (Boivin et al., 2010; Doublier et al., 2011a, b). The concentration of  $\text{Sr}^{2+}$  is very low, and do not exceed in human a maximum of 0.5 ions  $\text{Sr}^{2+}$  for 10  $\text{Ca}^{2+}$  (Li et al., 2010a). Moreover, the thickness and length of the plate-shaped bone mineral crystals were not affected by the strontium ranelate treatment (Li et al., 2010a). Presence of  $\text{Sr}^{2+}$  causes no osteomalacia, no modification in the mineralization process or crystal size. However, the  $\text{Sr}^{2+}$  increases the bone strength, thus the presence of  $\text{Sr}^{2+}$  in bone mineral has certainly positive effects, but this mechanism is to date unknown.

#### 4.4 Bone apatite: A particular structure, with a hydrated layer around an apatitic core

The surface of bone crystals, formed in the water of extracellular fluid, exhibits a "hydrated layer" (Fig. 10). Ions in this layer are very labile and reactive, and constitute the non-apatitic domain, surrounding the relatively inert and more stable apatite domain of the bone crystal (Cazalbou et al., 2004; Termine et al., 1973). Newly deposited bone mineral contains many labile non-apatitic domains [ $\text{HPO}_4$ ,  $\text{PO}_4$ , and  $\text{CO}_3$ ], located in the

well-developed hydrated layer involved in the high surface reactivity of mineral (Cazalbou et al., 2004). Labile  $\text{PO}_4$  and  $\text{CO}_3$  groups are easily and reversibly exchangeable with other ions in the hydrated layer. During maturation, the decrease in labile non-apatitic environments is associated with an increase in stable apatitic environments (Cazalbou et al., 2004). A particularity of the bone mineral is its non-stoichiometry, leading to the presence of numerous vacancies in the apatite crystal. Consequently, bone crystal is mainly maintained by electrostatic cohesion, thus bone crystals are easily soluble relative to stoichiometric apatite (Barry et al., 2002). As bone becomes more mature, both the size and number of crystals increase.

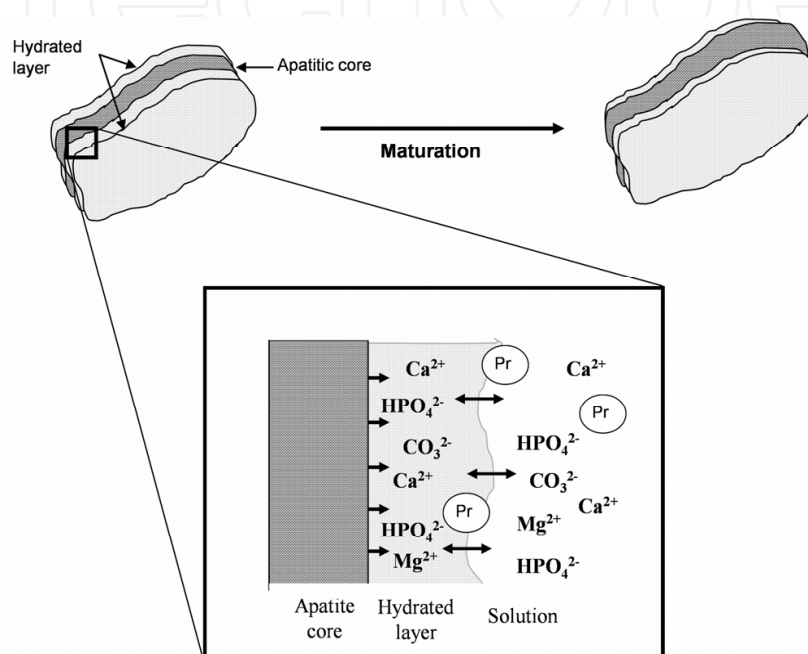


Fig. 10. Evolution of the hydrated layer and apatite core from bone crystal. During the maturation and growth of the crystal, the hydrated layer, involved in a high surface reactivity, progressively decreases and led to a stable apatitic domain. The structure of the hydrated layer constitutes a pool of loosely bound ions which can be incorporated in the growing apatite domains and can be exchanged by foreign ions in the solution and charged groups of proteins (Pr) (Adapted from Rey et al., 2009).

During mineral maturation, the hydrated layer decreases while the stable apatite domain grows, corresponding to the evolution of non-apatitic environments into apatitic environments detected by Fourier Transform InfraRed spectroscopy (FTIR). This hydrated layer, different from a hydration layer (Stern double layer), corresponds to the mode of formation of apatite crystals in physiologic conditions. The existence of these two domains (hydrated layer and apatite core) in biomimetic nanocrystals, has been recently confirmed by solid-state NMR (Jager et al., 2006). The hydrated surface layer contains loosely bound ions, which are easily exchangeable, and determine the surface properties of the nanocrystalline apatites (Cazalbou et al., 2004; Termine et al., 1973). In bone, those loosely bound ions can be also exchanged with charged groups of proteins present in collagen and non-collagenous proteins. The role of charged proteins on mineralization is well known (Boskey et al, 1989, 1998; Boskey 1989; Georges & Veis 2008; Landis et al., 1993; Malaval et al., 2008; Traub et al., 1992).

#### 4.5 Measurement of mineral crystallinity

Crystallinity is defined as the degree of structural order in a crystal. The atoms in the crystal are arranged in a regular and periodic manner. The referent method to measure *absolute crystallinity* is the X-rays diffraction, and is based on the elastic scattering of the X-rays. This technique can be used to determine the crystal structure (Le Bail & Lou er 1978; Rietveld 1969) or chemical composition of a sample through the power diffraction bank data file (Powder diffraction file). The WAXS (wide angle X-rays scattering) is based on scattering angles  $2\theta$  larger than  $5^\circ$ , and SAXS (small X-ray scattering) gives informations on angles  $2\theta$  close to  $0^\circ$ . This method gives informations on the size, strain and orientation of crystals. A peak broadening can be due the small crystal size or microstrains. The crystal size can be determined by the Scherrer equation.

Vibrational spectroscopy techniques, such as Fourier Transform InfraRed Spectroscopy (FTIRS), Synchrotron InfraRed or Raman Spectroscopy, have been extensively used to study calcified tissues (Ager et al., 2005; Akkus et al., 2003; Boskey et al., 2005; Cazalbou et al., 2004; Farlay et al., 2010b; LeGeros 1981; Miller et al., 2001; Paschalis et al., 1996; Pleshko et al., 1991; Rey et al., 1990, 1991). Spectroscopic techniques allow assessment of physicochemical modifications of mineral induced by mechanical tests (Ager et al., 2005; Akkus et al., 2003; Carden et al., 2003; Morris & Mandair 2011; Tarnowski et al., 2004), age-related modifications (Ager et al., 2005; Akkus et al., 2003; Miller et al., 2007), and pathologic or treatment-related changes (Boskey et al., 2005; Carden & Morris 2000; Fratzl 2004; Huang et al., 2003; Siris et al., 2004). The application of Fourier Transform InfraRed Microspectroscopy and Imaging (FTIRM, FTIRI) for bone allows in situ analysis of embedded bone samples at the BSU level. These techniques are based on the vibrations of the atoms of a molecule and give complementary informations. The functional groups present in the sample absorb infrared light (or scatter light for Raman) at different wavelengths. In bone, infrared and Raman spectroscopies were used to obtain information on bone mineral and organic matrix, the two major components of bone. The main advantage of these techniques is that they can be used on embedded bone biopsies, on thin sections (2 m-thick MMA sections), keeping the integrity of the bone microarchitecture. By infrared spectroscopy, different vibrations can be analyzed in bone mineral: the phosphate mode vibrations with the  $\nu_1\nu_3\text{PO}_4$  corresponding to an antisymmetric stretch, the  $\nu_4\text{PO}_4$  (bending stretch), the carbonate vibrations  $\nu_2\text{CO}_3$  and  $\nu_3\text{CO}_3$ . Organic matrix can be analyzed through the Amides I, II and III vibrations. Different parameters can be deduced from these vibrations, as the mineral maturity, crystallinity, carbonate substitution, mineral to matrix ratio, providing informations on bone mineral quality (Boskey et al., 1998, 2005; Miller et al., 2007; Paschalis et al., 1996, 1997a; 1997b; Paschalis & Mendelsohn 2000; Pleshko et al., 1991; Rey et al., 1989, 1995a). By Raman spectroscopy, crystallinity, carbonate type B substitution, and mineral to matrix ratio can be determined (Akkus et al., 2003; Morris & Mandair 2011).

Others techniques as vibrational methods have been extensively used to obtain informations on the structural and compound identification, especially on the relative crystallinity (also called crystallinity index).

#### 4.6 Maturity and crystallinity of bone crystals

As mentioned previously, with maturation, stable apatitic domains grow, whereas hydrated layer decreases. Thus, when bone crystals mature, their crystallinity also increases. These two variables can be assessed separately in infrared spectroscopy (Fig.11). Mineral maturity and mineral crystallinity are two parameters temporally linked and often well correlated in

synthetic apatite. The ratio  $1030/1020\text{ cm}^{-1}$  (apatitic phosphate over non apatitic phosphate,  $\nu_3\text{PO}_4$  vibration) was well correlated in synthetic apatites to the crystallinity measured by XRD, and it was established that the ratio  $1030/1020\text{ cm}^{-1}$  was an index of mineral maturity/crystallinity. From that, mineral maturity and crystallinity were associated in a lot of studies (Boskey et al., 2005; Paschalis et al., 1996, 1997a, 1997b). We have defined a new ratio to assess mineral maturity, the  $1030/1110\text{ cm}^{-1}$ , (apatitic phosphate over non apatitic phosphate,  $\nu_3\text{PO}_4$  vibration) equivalent to the  $1030/1020\text{ cm}^{-1}$  but more sensitive (Farlay et al., 2010b). We agree with the fact that the  $1020/1030\text{ cm}^{-1}$  ratio, which is an index of mineral maturity, evolves simultaneously with crystallinity in synthetic samples or normal bone. However, by definition, those two parameters are different, as mineral maturity corresponds to a stage of maturation, and crystallinity corresponds to the organization of the apatite lattice. We have defined a new mineral crystallinity index, measured by FTIRM on the peak  $604\text{ cm}^{-1}$  (bending vibration of phosphates) (Farlay et al., 2010b). This vibration is often inaccessible for microscopic infrared imaging, due to the cut-off of the detector. A wide band detector allows the access to this vibration and we have shown that the value of the full width at half maximum of the  $604\text{ cm}^{-1}$  peak, was inversely correlated to the crystallinity (Farlay et al., 2010b). This crystallinity index is well correlated, on the same samples, with another crystallinity index measured on the same vibration, the Shemesh ratio (Shemesh 1990) which is itself derived from the splitting factor of Termine & Posner, 1966. In human bone, it has been shown that those two parameters could evolve separately, and thus can independently affect the mineral characteristics. Indeed, mineral maturity can be affected by modification of bone remodeling (and by formative or antiresorptive treatments), whereas crystallinity can be influenced by ionic substitutions. This was verified in bone samples from patients with skeletal fluorosis (Farlay et al., 2010b). Skeletal fluorosis is a pathology caused by an excessive consumption of fluoride, and characterized by ionic substitution of hydroxyl ions by fluoride ions in bone mineral. In these samples, mineral maturity was decreased, due to a stimulation of osteoblasts on bone formation by fluoride, but crystallinity was increased due to the substitution of  $\text{OH}^-$  by  $\text{F}^-$ .

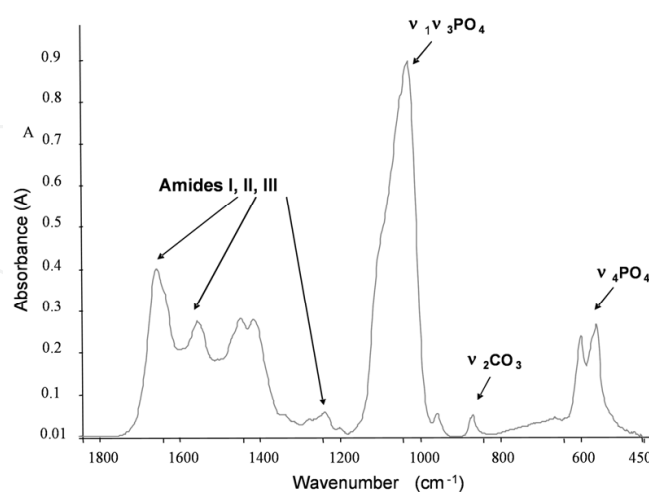


Fig. 11. Infrared spectra of human cortical bone (iliac bone) showing the mineral ( $\nu_1\nu_3\text{PO}_4$ ,  $\nu_4\text{PO}_4$ ,  $\nu_2\text{CO}_3$ ) and the organic (amides) vibrations (INSERM UMR 1033).

In the study previously mentioned for determining the chronology of secondary mineralization on ewes (Bala et al., 2010), it has been shown that kinetic of mineral maturity



was very close to the kinetic of the degree of mineralization, whereas the crystallinity index was biphasic, showing a rapid increase for the six first months, then stabilizing until 18 months, and showing another increase toward highest values after 24 months (Fig.12). In another study performed in women long term-treated by bisphosphonates, an increase in mineral maturity was observed, associated with a decrease in crystallinity, reinforcing the statement that mineral maturity and crystallinity have to be cautiously separately analyzed (Bala et al., 2011).

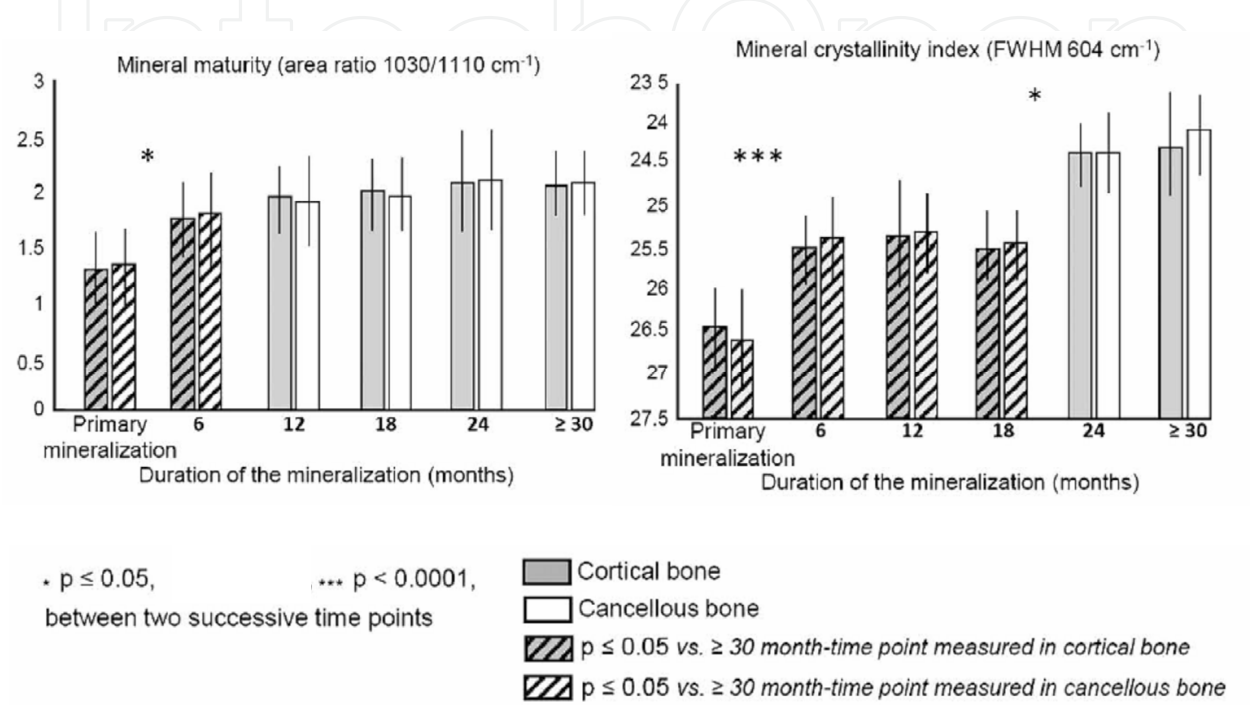


Fig. 12. Histograms showing (left) the rapid increase of mineral maturity during the 6 first months of mineralization, followed by a slowdown and stabilization; (right) the biphasic evolution of mineral crystallinity index, with an increase the 6 first months, a stabilization, and then a resumption at 18 months (Reprinted from Bala et al., 2010, with permission from Elsevier).

Finally, in a study on 53 human vertebrae, it was shown that with age of donor, crystallinity was increased and mineral maturity unchanged (Farlay et al., 2010a). This increase in crystallinity was previously shown in osteoporotic bone (Boskey, 2003), suggesting that with aging, an increase in crystal size/perfection occurs, independently of the bone remodeling (Farlay et al., 2010a).

4.7 Carbonates in bone mineral

In bone mineral, carbonates ions represent about 6-7% of the total mineral ions, thus a non negligible proportion. CO<sub>3</sub><sup>2-</sup> can be incorporated into bone mineral by substitution in the apatite lattice either to PO<sub>4</sub><sup>3-</sup> (major site, type-B carbonate) or to OH<sup>-</sup> (minor site, type-A carbonate). A third site of CO<sub>3</sub><sup>2-</sup> ions corresponds to labile carbonates which decrease with the maturation of the apatite crystal (Rey et al., 1989, 1991). The carbonates decrease the regularity of the atomic arrangement in hydroxyapatite (Blumenthal et al., 1975), thus altering the crystallinity. CO<sub>3</sub><sup>2-</sup> determine physical properties of materials, or biological behavior of cells in scaffold or ceramics. The CO<sub>3</sub><sup>2-</sup>-A/B ratio is very constant among



different species, suggesting that  $\text{CO}_3^{2-}$  incorporation in bone is a highly regulated process. Carbonates are also important for mineral dissolution (LeGeros 1981). It is well-established in literature that, in synthetic apatites,  $\text{CO}_3^{2-}$  content increases with time of maturation (Cazalbou et al., 2004b; LeGeros & LeGeros 1983; LeGeros et al., 1968; Rey et al., 1989, 1995a). However, in bone, there is a controversy with some studies showing an increase of  $\text{CO}_3/\text{PO}_4$  with mineral maturation (Petra et al., 2005) or a decrease (Farlay et al., 2006; Ou-Yang et al., 2001; Paschalis et al., 1996). In very young bone, labile carbonates and  $\text{HPO}_4$  are high, and progressively decreased with time of maturation, this being associated with an increase in mineral crystallinity (Magne et al., 2001). The amount of carbonate ions can be measured by different methods (chemical dosage, thermogravimetric analysis, vibrational spectroscopies...). In infrared spectroscopy, carbonate content is generally determined using the  $\nu_2\text{CO}_3$  line (out-of-plane bending vibration) which is free of contribution of amide vibrations, and was generally calculated as  $\text{CO}_3/\text{PO}_4$  area ratio. The role of carbonate in bone mineral is not entirely elucidated. The hypothesis of Wopenka and Pasteris is that carbonate could control bone crystal size, and that high concentration of carbonate could play an important role in constraining bone crystals to the nanometer scale (Wopenka & Pasteris 2005). Indeed, dentin, in which the amount of carbonate concentration is the same than in bone, has also a crystal size similar to bone. However, enamel crystals, which are very much larger size, have only about half the carbonate concentration as bone does.

## **5. Modifications of bone mineral characteristics with age or in post-menopausal osteoporosis**

Bone loss occurs in all individuals after middle life. This bone loss can be moderated (osteopenia), or can become more important (osteoporosis). Osteoporosis is a metabolic bone disease, leading to a decrease in the amount of mineralized bone and an increase in the risk of fracture. In post menopausal osteoporosis, the oestrogen deficiency lead to an acceleration of bone remodeling, the balance between resorption and formation is disturbed in favour of resorption. This lead to a decrease in the more mineralized bone, which is only partially replaced by young less mineralized bone, reducing material stiffness.

### **5.1 Modifications of bone mineral at tissue level**

Several studies performed in bone from osteoporotic women have shown not only that bone mass is decreased but that the bone mineral content was decreased, due to the fact the newly formed BSUs have not the time to achieve their mineralization before to be resorbed. Thus the mean age of matrix is decreased, leading to a decrease to the mean degree of mineralization.

#### **5.1.1 Degree of mineralization (DMB) in control humans (Boivin et al., 2008)**

Bone samples from persons of both sexes (sudden died and without known pathology) were studied. This control group was composed of iliac bone samples taken at necropsy from 30 women (aged  $48.4 \pm 3.7$  years; range 20-93 years) and 13 men (aged  $66.0 \pm 4.4$  years; range 43-86 years). The mean DMB expressed in g mineral/ $\text{cm}^3$  (mean  $\pm$  SEM) was  $1.082 \pm 0.017$  in cortical bone and  $1.099 \pm 0.018$  in cancellous bone (Figure 13).

In iliac crests, no significant influence of sex or age on the mean DMB was observed (Boivin et al., 2008). In cancellous bone, another study by qBEI showed the absence of influence of

sex, age, ethnicity or skeletal sites in BMDD measurements ( $\approx 22\%$  in w% Ca) (Roschger et al., 2003). A recent study on vertebral bone of 53 donors (age range: 54-95 year-old, 21 men, 27 women) showed no influence of sex and age on the DMB (Follet et al., 2010).

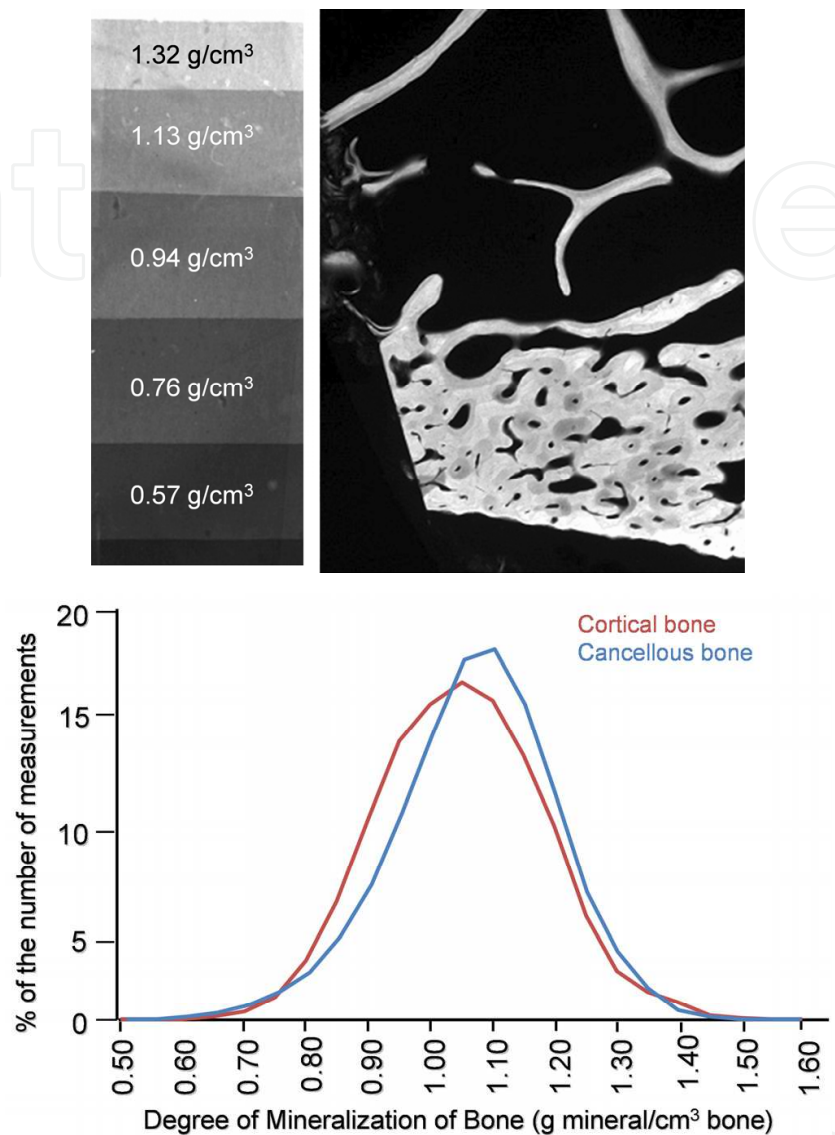


Fig. 13. Quantitative microradiography: (top) aluminium step wedge and microradiograph of bone tissue. (bottom) Distribution of the degrees of mineralization in human iliac bone (INSERM UMR 1033).

**5.1.2 Bone mineralization at tissue level in bone of post-menopausal women treated with anti-osteoporotic treatments**

In adult bone, the DMB, i.e., the bone mineral density at the tissue level depends on the rate of remodeling. Thus, agents (parathyroid hormone) or events (menopause, ovariectomy) which provoke an augmentation in the « birthrate » or activation frequency of Basic Multicellular Units (BMUs), induce a decrease of the « lifespan » of BSUs, in other words on the time available for the secondary mineralization. This leads to the fact that new BSUs are resorbed before they have fully completed their secondary mineralization, as proven by the presence of a large amount of uncompletely mineralized BSUs and a low mean DMB (Arlot

et al., 2005; Boivin & Meunier 2003; Misof et al., 2003). In a study on men and women with idiopathic osteoporosis, a decrease in DMB was observed compared to controls (-7%) (Boivin et al., 2008). However, a decrease in heterogeneity index of mineralization was observed in this study, indicating a homogeneization of the mineralization. This decrease is also accompanied, in men only, by a decrease in microhardness (-10%) (Boivin et al., 2008). However, it is important to mention that, both in control or osteoporotic bone, no variation in DMB or microhardness was correlated with age. Conversely, antiresorptive agents (bisphosphonates, calcitonin, estrogen, SERMs) which cause a marked reduction in the « birthrate » of BMUs, prolong the « lifespan » of the BSUs, allowing a more complete secondary mineralization and an increase of DMB (Bala et al., 2011a, 2011b; Boivin et al., 2000, 2003; Borah et al., 2005, 2006). A dissociating agent (Strontium Ranelate), acting both on resorption and formation, is now used in the treatment of post-menopausal osteoporosis (Meunier et al., 2004; Reginster et al., 2005). After short- and long-term treatment, the DMB was not significantly different from the physiological range (Boivin et al., 2010; Doublier et al., 2011a). Recently, also with a microradiographic method, it has been shown, in hip-fracture patients, an increase in DMB heterogeneity in both interstitial and osteons compared to controls (Bousson et al., 2011). Thus, some differences exist, considering osteoporotic or hip-fractures cases, in term of heterogeneity of mineralization.

The mineralization index measured by FTIRM, calculated as the ratio of  $\nu_1\nu_3\text{PO}_4$ /amide I vibration (Paschalis et al., 1996) is a relative index which has been correlated to ash content (Faibish et al., 2005) and DMB (Farlay et al., unpublished data). The mineralization index (or mineral to matrix ratio) has been shown to be higher in bone of post menopausal woman treated with alendronate (Boskey et al., 2009).

## 5.2 Modifications of bone crystal characteristics

Concerning crystal characteristics, it has been shown, by XRD technique that, with age, there was an increase in crystal size. In 117 iliac bone samples (range of age: 0-90 years), there was an increase, between 0-30 years, in crystallinity in c-axis, corresponding to the crystal length, and a decrease in the a axis (Hanschin & Stern 1995). After 30 years, modifications were less evident, only a slight increase in crystallinity within the basal plane was observed.

Modifications of bone crystal size impaired mechanical characteristic of bone. Indeed, it was observed that the bones of older animals or that osteoporotic bone, two cases in which bone fractures easily, contained more large crystals than normal (Boskey 2003). Conversely, young animals, which have mechanically strong bones, have a mixture of small and large crystals. In a recent study performed by Raman microspectroscopy in human femurs (age range: 52-85 years old), an overall reduction in heterogeneity of mineralization, crystallinity, and type-B carbonation was observed (Yerramshetty et al., 2006). In this study, bone became more mineralized and more highly type-B carbonated with age, whereas crystallinity was unchanged. In a study performed by FTIRM on 53 human vertebrae (range of age: 54-93 years), a significant increase in crystallinity was observed (Farlay et al., 2010a). This means that, in vertebrae, either crystal size or crystal perfection is increased. In those vertebrae, the bone volume was also significantly decreased. The reason of the increase in crystallinity is unclear, but it is possible that it corresponds to a mechanism of adaptation of bone, in order to compensate the bone loss due to age. In the same study, a decrease in  $\text{CO}_3/\text{PO}_4$  ratio was observed suggesting an alteration of the physico-chemical composition of bone mineral with age. Thus, comparing the results obtained in human femora and vertebrae in term of

physico-chemical composition, some differences appears. However, the techniques were different (FTIR and Raman microspectroscopies), and the bones were not extracted from the same donors.

## 6. Perspectives and conclusion

Different factors increase the risk of fracture, independent of bone mineral density. Among these factors, bone mineral features are important in determining some mechanical behavior, especially elastic properties. Besides the decrease in bone mineral volume occurring with age, the alteration of mineral quality plays also a major role in bone fragility. The study of bone mineral quality is a instrumental challenge, due to [1] the dimension and the nature itself of crystal apatite (nanocrystalline), [2] the fact that bone is a composite material (organic matrix intimately linked to bone mineral), and [3] because bone is a complex tissue with different levels of organization (organ, BSU, crystal).

Thus, the using of different complementary techniques (spectroscopic, diffraction, electron microscopy techniques) has been very useful in the comprehension of bone mineral structure the last 50 years. Despite very important findings, there are still lacks in the understanding of the role of certain ions (minor or trace elements) in the biological or biomechanical properties (such as carbonates, strontium ions etc.).

Bone is a fascinating tissue governed by the remodeling activity and the plasma composition. It underlies several characteristics which are interdependent. For example, carbonate ions substitutions into apatite promote the platelet-shape in bone crystals rather than needle-shape found in enamel. But the presence of carbonates, moreover, as mentioned previously, inhibits the incorporation of OH<sup>-</sup> in bone apatite. Thus, the study of correlations between different variables is still indispensable to understand the relationship between physicochemical and mechanical properties.

From the previous studies performed on bone mineral, it seems that a small crystal size associated with a heterogeneity of crystal size (nanometer size-scale) is important to an optimal mechanical strength. Indeed, the presence of too large crystal is not good for bone strength. Fluoride, as mentioned previously, induces the formation of large crystals, and the formation of a “brittle” bone, despite a high BMD. Moreover, concerning mineralization and its heterogeneity at the tissue level, values comprised between 0.8 and 1.30 g/cm<sup>3</sup> of bone could be a great deal to maintain stiffness of the bone. The preservation of mineralization heterogeneity is necessary for bone mechanical strength, allowing, for example, the stopping of microcracks propagation. No modification in degree of mineralization is observed with age or sex, but some differences exist in hip- fracture or osteoporotic cases. Thus a modification in the calcium absorption or an increase in the bone remodeling can lead to a decrease to the degree of mineralization. Differences also exist in studies in degree of mineralization due to the fact that either weight or non-weight-bearing bones are analyzed. We have an ongoing study on these different types of bone (weight or non-weight-bearing bones) in a same donor, in order to analyze the structural and mechanical bone mineral properties.

In conclusion, despite a lot of studies have permitted to understand the characteristics of bone mineral, further studies are needed to clarify the mechanisms of bone fragility at the different levels of investigation.



## 7. Acknowledgments

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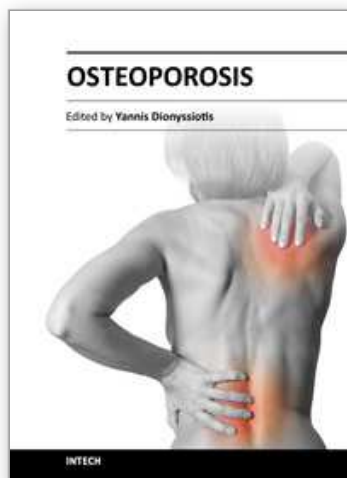
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## **Osteoporosis**

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Osteoporosis is a public health issue worldwide. During the last few years, progress has been made concerning the knowledge of the pathophysiological mechanism of the disease. Sophisticated technologies have added important information in bone mineral density measurements and, additionally, geometrical and mechanical properties of bone. New bone indices have been developed from biochemical and hormonal measurements in order to investigate bone metabolism. Although it is clear that drugs are an essential element of the therapy, beyond medication there are other interventions in the management of the disease. Prevention of osteoporosis starts in young ages and continues during aging in order to prevent fractures associated with impaired quality of life, physical decline, mortality, and high cost for the health system. A number of different specialties are holding the scientific knowledge in osteoporosis. For this reason, we have collected papers from scientific departments all over the world for this book. The book includes up-to-date information about basics of bones, epidemiological data, diagnosis and assessment of osteoporosis, secondary osteoporosis, pediatric issues, prevention and treatment strategies, and research papers from osteoporotic fields.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821



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